Original Article

DGAT1-deficiency affects the cellular distribution of hepatic retinoid and attenuates the progression of CCl_4-induced liver fibrosis

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Background: Diacylglycerol O-acyltransferase 1 (DGAT1) catalyzes the final step of triglyceride synthesis, transferring an acyl group from acyl-CoA to diacylglycerol. DGAT1 also catalyzes the acyl-CoA-dependent formation of retinyl esters *in vitro* and in mouse intestine and skin. Although DGAT1 is expressed in both hepatocytes and hepatic stellate cells (HSCs), we reported genetic and nutritional studies that established that DGAT1 does not contribute to retinyl ester formation in the liver.

Methods: We now have explored in more depth the role(s) of DGAT1 in hepatic retinoid metabolism and storage.

Results: Our data show that DGAT1 affects the cellular distribution between hepatocytes and HSCs of stored and newly absorbed dietary retinol. For livers of *Dgat1*-deficient mice, a greater percentage of stored retinyl ester is present in HSCs at the expense of hepatocytes. This is also true for newly absorbed oral [^{3}H]retinol. These differences are associated with significantly increased expression, by 2.8-fold, of cellular retinol-binding protein, type I (RBP1) in freshly isolated HSCs from *Dgat1*-deficient mice, raising the possibility that RBP1, which contributes to retinol uptake into cells and retinyl ester synthesis, accounts for the differences. We further show that the retinyl ester-containing lipid droplets in HSCs are affected in *Dgat1*-null mice, being fewer in number but, on average, larger than in wild type (WT) HSCs. Finally, we demonstrate that DGAT1 affects experimentally induced HSC activation *in vivo* but that this effect is independent of altered retinoic acid availability or effects on gene expression.

Conclusions: Our studies establish that DGAT1 has a role in hepatic retinoid storage and metabolism, but this does not involve direct actions of DGAT1 in retinyl ester synthesis.

Keywords: Hepatic stellate cell (HSC); diacylglycerol acyltransferase 1 (DGAT1); hepatic fibrosis; lipid droplet; retinyl ester; retinoic acid

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Introduction

Retinoids (vitamin A and its natural metabolites and synthetic analogs) are potent transcriptional regulators (1-3) affecting expression levels of more than 500 genes (3). The all-*trans*- and 9-*cis*-isomers of retinoic acid are transcriptionally active retinoid metabolites, regulating transcription upon binding to one of 3 distinct retinoic acid receptors (RARs) or 3 distinct retinoid X receptors (RXRs) (1-3). There is growing understanding that retinoids also act within cells to regulate signal transduction pathways independent of their interactions with RARs and RXRs (4,5). Retinoids participate in regulating normal cellular processes including differentiation, proliferation, and apoptosis (6,7) and are therefore needed for maintaining immunity, male and female reproduction, pre- and post-natal development, barrier function, and vision (6,7). There is also considerable interest in the study of retinoid actions in both the prevention and the development of metabolic disease including obesity, insulin resistance, liver disease and cardiovascular disease (8-14).

Although the isomers of retinoic acid represent the functionally important retinoid species, the most abundant retinoids in the body are retinyl esters, followed quantitatively by retinol (15). Collectively, retinyl esters and retinol account for greater than 95% of all the retinoid present in the body. It has been estimated that 80-90% of the retinoid in the body of healthy, well-nourished mammals is stored in the liver. The majority of this retinoid is stored as retinyl ester within the lipid droplets of hepatic stellate cells (HSCs), with the remainder of hepatic retinyl ester and retinol found in hepatocytes (16,17). It is well established that, upon hepatic injury, HSC retinyl ester stores are rapidly lost as the HSCs become activated and assume the characteristics of fibrogenic, proliferative myofibroblasts (17). At present, it remains unclear if this concomitant loss of retinyl ester during HSC activation contributes to the progression of fibrogenesis, although the absence of HSC retinyl ester stores in mice lacking the enzyme lecithin:retinol acyltransferase (LRAT) does not affect the development of CCl_4-induced hepatic fibrosis (18).

LRAT catalyzes the transesterification of the sn-1 acyl moiety of membrane phosphatidyl choline to retinol in the synthesis of retinyl ester (19). Studies of induced mutant mice have established that LRAT is responsible for almost all retinyl ester formation within the body (20-22). The older literature indicates that an acyl CoA-dependent enzyme termed acyl CoA:retinol acyltransferase (ARAT) also contributes to retinyl ester formation in liver and intestine (17,23). Diacylglycerol O-acyltransferase 1 (DGAT1) is one of two mammalian enzymes, the other being diacylglycerol O-acyltransferase 2 (DGAT2), that catalyze acyl CoA-dependent triglyceride formation from diacylglycerol (24). DGAT1, but not DGAT2, possesses ARAT activity *in vitro* (21,25,26) and can act *in vivo* as an ARAT in small intestine and skin (27,28). Although DGAT1 is expressed in both HSCs and hepatocytes, based on data obtained from *Lrat*-deficient mice, DGAT1 does not catalyze retinyl ester formation in either HSCs or hepatocytes, since these mice completely lack hepatic retinyl esters and HSC lipid droplets (21). This is true even when the animals were fed very high levels of dietary retinoid (29). These findings are surprising since DGAT1 catalyzes retinyl ester formation in some tissues and, based on the studies of whole liver homogenates, may be able to catalyze acyl CoA-dependent retinyl ester synthesis in liver (21,25-28). It has been reported by Yamaguchi *et al*. (30) that the inhibition of DGAT1 in primary rat HSCs with anti-sense oligonucleotides reduced mRNA expression levels of markers for HSC activation. These investigators further reported elevated cellular retinol-binding protein, type 1 (*Rbp1*) and *Lrat* mRNA expression in anti-sense treated HSCs and proposed that the loss of DGAT1, as an important ARAT activity in the liver, leaves more retinol available for oxidation to retinoic acid (30). The present studies explore in greater depth the role of DGAT1 in hepatic retinoid metabolism and HSC activation.

Materials and methods

Animals, animal husbandry and diets

Mice employed in this study were on the C57BL/6J genetic background. Diet-matched male *Dgat1*$^{-/-}$ and WT mice were 6 months of age at the time of experiments. Throughout life, mice had *ad libitum* access to water and a standard, nutritionally complete rodent chow diet containing 15 IU retinol/g diet (W. F. Fisher and Sons, Inc., Somerville, USA). The mice were maintained on a 12-h dark-light cycle in a conventional barrier facility with the period of darkness between 7:00 p.m. and 7:00 a.m. All experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals [National Research Council 2011. Guide for the Care and Use of Laboratory Animals (8th ed), Natl Acad Press, Washington, USA], and were approved by the Columbia University Institutional Animal Care and Use Committee.

Isolation of hepatocytes

For primary hepatocyte isolations, livers were perfused *in situ* with 1 mM EDTA for 5 min followed by 0.5 mg/mL collagenase D (Roche Diagnostics, Indianapolis, USA) for 13 min. Liver digests were filtered through a 100 µm nylon cell strainer (BD Biosciences) to remove undigested material and centrifuged at 20 ×g for 5 min to sediment hepatocytes. Non-parenchymal cells and debris were removed with the supernatant and the hepatocytes subsequently were washed 4 times with Gey's Balanced Salt Solution (GBSS, Sigma, St. Louis, MO), centrifuging at 20 ×g for 5 min, 50 ×g for 10 min, and 2 times at 20 ×g for 5 min. Purified hepatocytes were flash frozen in liquid N_2 and stored as pellets at –80 ℃ until further analysis.

Isolation and culture of HSCs

Primary HSCs were isolated from WT and *Dgat1*$^{-/-}$ mice according to established protocols (31-33). Livers were perfused sequentially *in situ* with 0.5 mM EGTA for 5 min, pronase E (0.4 mg/mL, EMD Chemicals Inc., Gibbstown, USA) for 5 min, and collagenase D (0.5 mg/mL, Roche Diagnostics) for 8 min, all at a flow rate of 5 mL/min. The resulting liver digest was filtered through a 100 µm nylon cell strainer (BD Biosciences) and washed twice with GBSS containing DNase I (2 mg/mL, Roche Diagnostics) and by centrifugation at 580 ×g for 10 min. HSCs were purified by flotation through 9% (w/v) Nycodenz (Axis-Shield PoC AS, Oslo, Norway) in GBSS lacking NaCl by centrifugation at 1,380 ×g for 15 min. In order to obtain pure samples, all of the primary HSC isolates were subjected to a second flotation step in 9% Nycodenz. The HSCs were then washed with GBSS and either pelleted, flash frozen in liquid N_2, and saved in –80 ℃ for further analysis or resuspended in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Grand Island, USA) and placed in culture.

For culture, freshly isolated HSCs were plated onto 35 mm plastic dishes at a seeding density of 2.5×10^5 cells/well. The cells were maintained in DMEM containing 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (final concentration 100 units penicillin and 0.1 mg streptomycin/mL) (Sigma Aldrich, St. Louis, USA). Plates were incubated at 37 ℃ in a humidified atmosphere containing 5% CO_2. After 4 h of incubation, the medium was changed to remove dead cells and debris. The next day, the medium was changed again and every 2 days afterwards. For microscopy, freshly isolated HSCs were seeded in 35 mm glass dishes (MatTek Co., Ashland, USA) and images were captured using a FSX100 microscope (Olympus America Inc., Center Valley, USA).

High performance liquid chromatography (HPLC) analysis of retinoids

Tissue and cell retinol and retinyl ester levels were determined as previously described (21). Retinoid extracted in hexane was resuspended in benzene and separated by reversed-phase HPLC using acetonitrile:methanol:methylene chloride (70:15:15, v/v) as running solvent. Retinoid was detected at 325 nm and quantitated by comparing integrated peak areas against a known amount of a purified standard.

Triglyceride analysis

Triglyceride was extracted with chloroform:methanol (2:1, v/v), dried under nitrogen gas, and resuspended in 1% Triton X-100. Triglyceride concentrations were measured using a colorimetric assay kit from Thermo Scientific according to the manufacturer's instructions.

DGAT enzymatic assay

Total DGAT activity was measured as described in Liu *et al*. (34) using diacylglycerol and [^{14}C]palmitoyl-CoA as substrates in a first order reaction with respect to protein and reaction time. The triglyceride synthesized was separated by thin layer chromatography on silica plates (Whatman, Maidstone, UK) using hexane:diethyl ether:acetic acid (80:20:1, v/v/v) as solvent and the corresponding band scraped for radioactivity measurement using a liquid scintillation counter (Beckman Coulter, Jersey City, USA).

Hepatic clearance of an oral retinol challenge

The *in vivo* uptake of recently ingested oral retinol into hepatic cell fractions was assessed using 1 µCi [^{3}H]retinol as radiolabeled tracer, administered in a single gavage dose containing 6 µg of retinol dissolved in 100 µL of corn oil. Hepatocytes and HSCs were isolated 24 h after gavage, as described above. Immediately prior to perfusion of the liver, a small lobe was ligated and excised for whole liver measurement of ^{3}H-cpm. Total retinoids were extracted from the liver and from the isolated cell fractions into chloroform:methanol (2:1, v/v), dried under nitrogen, and measured for radioactivity using a liquid scintillation counter (Beckman Coulter). ^{3}H-cpm detected for the

individual cell fractions were normalized to cell number and expressed as a percent of total liver ^{3}H-cpm. These calculations were based on 135 million hepatocytes (35) and 10 million HSCs per gram of mouse liver (36-38).

CCl_4 treatment

Two studies were carried out which differed in the doses and durations of CCl_4 treatment. For study 1, mice were given an intraperitoneal (IP) injection, every 48 h, of either 1 µL CCl_4 per gram of body weight dissolved in corn oil (1:4, v/v) or an equal volume of pure corn oil as control, for a total of four injections. The mice were then euthanized 24 h following the last injection, and liver samples were collected for histology, analysis of mRNA expression and retinoid measurements. For study 2, mice were given IP injections, every 7 days, of either 0.5 µL CCl_4 per gram of body weight dissolved in corn oil (1:4, v/v) or an equal volume of corn coil as control, for a total of four injections. Treated mice were euthanized 7 days after the last injection for liver tissues.

Histological analysis

Liver was fixed overnight in 10% formalin and embedded in paraffin for sectioning and Masson's Trichrome staining. Histology was performed by the Pathology Core Facility at Columbia University Medical Center, and images were captured with a FSX100 biological microscope (Olympus America Inc., Center Valley, USA).

RNA isolation, reverse transcription, and qualitative real-time PCR (qRT-PCR)

Total RNA was extracted with TRIzol (Invitrogen, Carlsbad, USA) according to the manufacturer's instructions and treated with DNAse using RNeasy columns (Qiagen, Valencia, USA). cDNA was synthesized with a high-capacity reverse transcription kit (Applied Biosystems, Carlsbad, USA) and subjected to qRT-PCR using SYBR Green PCR master mix from Roche Diagnostics. mRNA levels were quantitated against a standard curve generated by serial dilution of tissue/cell type appropriate cDNA and normalized to *18S* rRNA levels.

Statistical analyses

All data are presented as mean ± standard deviation. Statistical differences were determined by student's *t*-test, with a P value <0.05 indicating statistical significance.

Results

DGAT1-deficiency results in an altered cellular distribution of hepatic retinoid

Primary HSCs were isolated from 6-month-old, male *Dgat1*$^{-/-}$ and WT mice and analyzed for retinoid content by reversed-phase HPLC. Cellular levels of retinyl esters were significantly elevated in *Dgat1*$^{-/-}$ compared to WT HSCs (*Figure 1A*). This was unexpected since total hepatic retinoid levels are not different for *Dgat1*$^{-/-}$ and WT mice (27). When we analyzed freshly isolated hepatocytes, we found a correspondingly lower retinyl ester content in *Dgat1*$^{-/-}$ hepatocytes (*Figure 1B*). Cellular levels of retinol were 2-fold greater in *Dgat1*$^{-/-}$ compared to WT HSCs (*Figure 1C*) but were not different between *Dgat1*$^{-/-}$ and WT hepatocytes (*Figure 1D*). A similar cellular difference in retinoid distribution was observed 24 h after administration of an oral dose of 6 µg of retinol and 1 µCi [^{3}H]retinol, dissolved in 100 µL of corn oil. HSCs isolated from the *Dgat1*$^{-/-}$ mice contained significantly more of the radiolabeled dose than WT HSCs (*Figure 1E*), whereas hepatocytes isolated from *Dgat1*$^{-/-}$ mice contained less of the dose than WT hepatocytes (*Figure 1F*). Collectively, these data suggest that, in the absence of DGAT1, retinol is preferentially accumulated by HSCs at the expense of the hepatocyte.

In order to rule out the possibility that the ARAT activity of DGAT1 might contribute to retinol esterification and hence the storage of retinyl ester in hepatocytes, we compared the ratios of the major products of LRAT activity, retinyl palmitate and retinyl stearate, to the major products of ARAT activity, retinyl linoleate and retinyl oleate (27). Identical ratios of these products were observed for *Dgat1*$^{-/-}$ and WT HSCs (*Figure 1G*) and hepatocytes (*Figure 1H*). This rules out DGAT1 as a significant ARAT in these two hepatic cell types. Interestingly, despite the role of DGAT1 in triglyceride synthesis, triglyceride levels were not different in *Dgat1*$^{-/-}$ HSCs (*Figure 1I*). This was despite a significant reduction, by approximately 40%, of the total DGAT activity measured in whole *Dgat1*$^{-/-}$ HSC lysates (*Figure 1J*). The residual total DGAT activity present in *Dgat1*$^{-/-}$ HSCs must reflect DGAT2 activity. We observed no differences in *Dgat2* mRNA expression between *Dgat1*$^{-/-}$ and WT HSCs (*Table 1*).

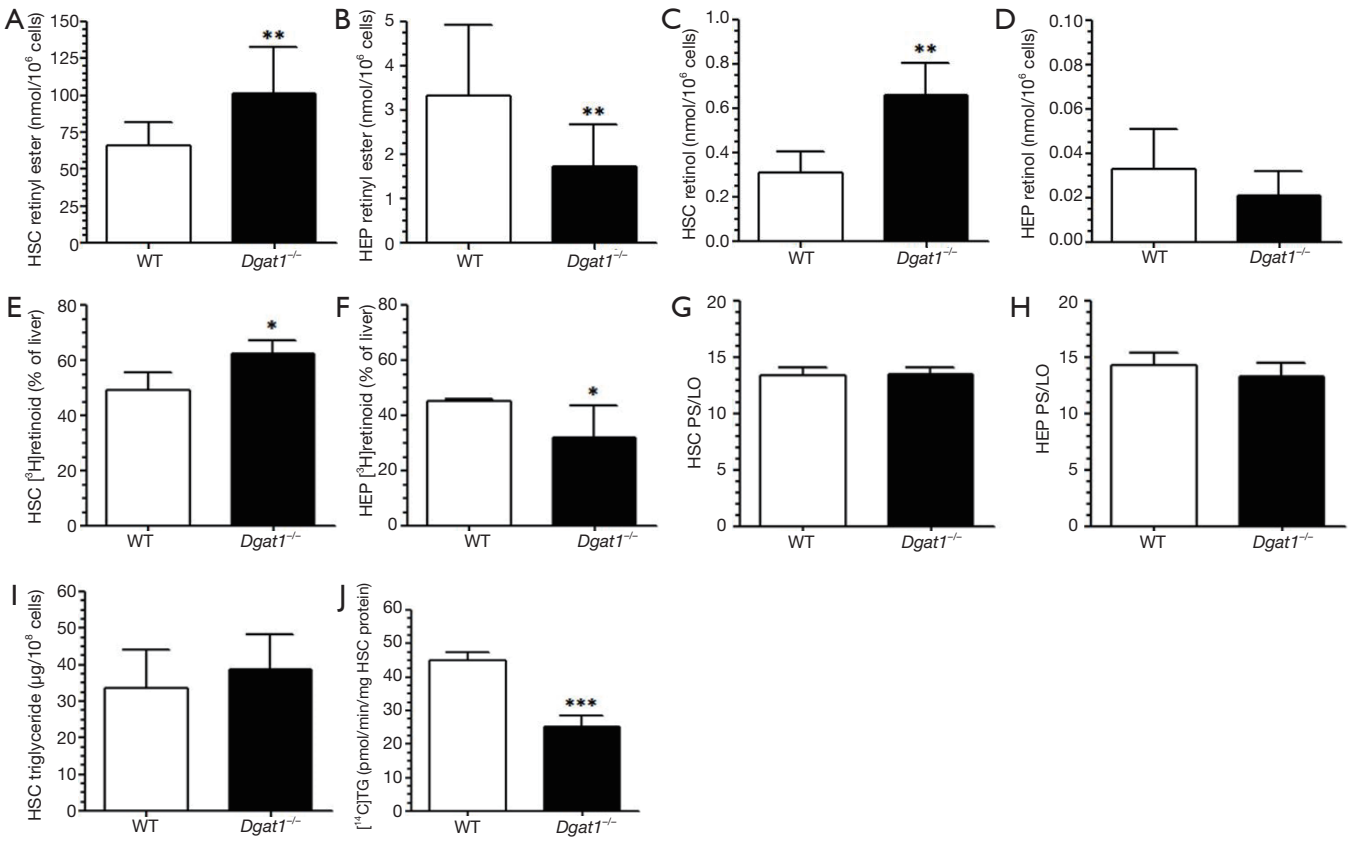

Figure 1 Cellular distribution of stored and newly absorbed hepatic retinoid. Cellular retinyl ester levels measured in freshly isolated HSCs from *Dgat1*$^{-/-}$ (n=10) and WT mice (n=10) show significantly more retinyl ester for *Dgat1*$^{-/-}$ compared to WT HSCs (A). Retinyl ester levels measured in freshly isolated hepatocytes from *Dgat1*$^{-/-}$ mice (n=10) were significantly lower than those of freshly isolated hepatocytes from WT mice (n=13) (B). Cellular retinol levels were significantly elevated in HSCs from *Dgat1*$^{-/-}$ (n=10) versus WT mice (n=10) (C) but not different in hepatocytes from *Dgat1*$^{-/-}$ (n=10) and WT mice (n=13) (D). *In vivo* accumulation of [^{3}H]retinoid (retinol + retinyl ester) by HSCs 24 h after administration of an oral dose consisting of 1 μCi [^{3}H]retinol in 6 μg of retinol dissolved in 100 μL of corn oil for *Dgat1*$^{-/-}$ (n=4) and WT (n=4) mice (E). *In vivo* accumulation of [^{3}H]retinoid (retinol + retinyl ester) by hepatocytes 24 h after administration of the same oral dose of [^{3}H]retinol in corn oil by *Dgat1*$^{-/-}$ (n=6) and WT (n=4) mice (F). To assess LRAT versus ARAT involvement in retinyl ester synthesis in hepatocytes and HSCs, the ratios of different retinyl ester acyl species were compared for the same cell isolates (27). Specifically the ratios of retinyl palmitate + retinyl stearate versus retinyl linoleate + retinyl oleate (PS/LO) were not different between *Dgat1*$^{-/-}$ and WT for either HSCs (G) or hepatocytes (H). Triglyceride levels measured for the same cell samples were not different for HSCs obtained from *Dgat1*$^{-/-}$ and WT livers (I). Total DGAT specific activity measured in whole cell lysates is significantly lower in *Dgat1*$^{-/-}$ compared to WT HSCs (J). All values are given as mean ± SD. Statistical significance: *, P<0.05; **, P<0.01.

Dgat1$^{-/-}$ *HSCs have fewer but larger lipid droplets*

Overnight cultures of freshly isolated HSCs from *Dgat1*$^{-/-}$ and WT mice show visible differences in cell morphology associated with their characteristic lipid droplets. As seen in *Figure 2A*, the lipid droplets present in *Dgat1*$^{-/-}$ HSCs appear to be fewer in number but larger than those of WT HSCs. This is also evident in the bottom panels of *Figure 2A*, which show this same difference in the autofluorescence of endogenous retinoid emitted upon excitation at 330 nm. These morphological differences were analyzed quantitatively by counting the number of lipid droplets present per HSC (*Figure 2B*) and by measuring the diameter of each of the lipid droplets (*Figure 2C*). More than 50 cells from each genotype were selected at random from HSC cultures prepared from 4 *Dgat1*$^{-/-}$ and 5 WT mice. On average, *Dgat1*$^{-/-}$ HSCs had 36 lipid droplets per cell, while WT HSCs had 49. The WT lipid droplets had an average diameter of 1.4±0.1 μm,

Table 1 Relative gene expression levels in freshly isolated and activated HSCs from WT and *Dgat1*$^{-/-}$ mice

Gene	Freshly isolated		Culture activated (5d)	
	WT	*Dgat1*$^{-/-}$	WT	*Dgat1*$^{-/-}$
Triglyceride synthesis				
Dgat1	1.0±0.5	ND	2.3±0.2$^{\#}$	ND
Dgat2	1.0±0.8	1.0±0.8	1.2±0.2	1.1±0.3
HSC activation and proliferation				
Tgfb1	1.0±0.2	1.7±0.7	8.9±2.8$^{\#\#}$	8.1±3.0$^{\#}$
Ki67	1.0±0.5	2.1±1.4	113.5±40.8$^{\#}$	94.6±38.6$^{\#}$
PAT proteins				
Plin1	ND	ND	ND	ND
Plin2	1.0±0.4	1.7±0.7	17.8±5.1$^{\#\#}$	15.4±4.5$^{\#\#}$
Plin3	1.0±0.4	1.8±0.7	21.5±4.6$^{\#\#}$	20.0±3.0$^{\#\#\#}$
Plin4	1.0±0.4	0.7±0.3	5.4±1.0$^{\#\#}$	5.2±0.8$^{\#\#\#}$
Plin5	1.0±0.2	1.4±0.6	0.2±0.1$^{\#}$	0.1±0.0
Retinoid metabolism				
Lrat	1.0±0.8	1.1±0.4	2.2±2.1	2.4±2.3
Rbp1	1.0±0.7	2.8±0.6*	10.6±2.8	12.2±3.1
Rarb	1.0±0.4	1.0±0.5	12.4±2.3$^{\#\#}$	10.9±1.4$^{\#\#\#}$
Rdh10	1.0±0.5	1.4±0.8	4.7±1.8$^{\#}$	4.0±1.3$^{\#}$
Dhrs3	1.0±0.5	0.9±0.3	12.4±3.6$^{\#\#}$	12.3±6.4$^{\#}$
Raldh1	1.0±0.5	1.3±0.6	28.6±12.6$^{\#}$	31.5±10.0$^{\#}$
Cyp26b1	1.0±0.8	0.6±0.4	0.9±0.5	0.8±0.4
Lipid metabolism				
Ppara	1.0±0.5	1.5±0.7	1.2±0.5	1.2±0.4
Ppard	1.0±0.5	1.3±0.6	7.9±1.6$^{\#\#}$	7.6±2.0$^{\#\#}$
Pparg	1.0±0.6	1.0±0.8	0.5±0.0	0.7±0.2
Srebp1c	1.0±0.7	1.0±0.8	0.8±0.1	0.7±0.1
Cd36	1.0±0.1	2.1±1.2	1.5±0.7	3.7±1.3*

All the data shown are normalized to 18S rRNA expression and given relative to levels in freshly isolated WT HSCs. *, P<0.05 for comparison between genotypes. $^{\#}$, P<0.05, $^{\#\#}$, P<0.01, $^{\#\#\#}$, P<0.001 for comparison between freshly isolated and culture activated HSCs within the same genotype.

while *Dgat1*$^{-/-}$ lipid droplets were significantly bigger with an average diameter of 1.7±0.1 μm. These values indicate that the total lipid droplet volume is 32% greater in *Dgat1*$^{-/-}$ compared to WT HSCs, which is quantitatively consistent with the difference in HSC retinyl ester levels observed for the two genotypes (see *Figure 1A*). When the lipid droplets were grouped according to size, it is clear that there is a shift in the size distribution of the *Dgat1*$^{-/-}$ lipid droplets (*Figure 2D*). Compared to WT, in *Dgat1*$^{-/-}$ HSCs, the larger lipid droplets >2.25 μm in diameter constitute a significantly greater percentage of the total lipid droplets present (21% *vs.* 11%), whereas the smaller lipid droplets <0.75 μm in diameter make up a significantly smaller percentage (12% *vs.* 20%).

To understand the basis for this difference, we assessed by qRT-PCR for possible alterations in gene expression profiles for freshly isolated HSCs from matched WT and *Dgat1*$^{-/-}$ mice. Expression of mRNA was measured for the lipid droplet-related PAT protein genes (*Plin1*, *Plin2*, *Plin3*, *Plin4* and *Plin5*), for genes involved in retinoid metabolism and actions (*Lrat*, *Rbp1*, *Rarb*, *Rdh10*, *Dhrs3*, *Raldh1* and *Cyp26b1*), and genes encoding transcription factors that regulate lipid metabolism (*Ppara*, *Ppard*, and *Pparg*, and *Srebp1c*). The results from this survey are provided in *Table 1*. We observed no significant differences in expression levels between WT and *Dgat1*$^{-/-}$ HSCs either for genes associated with lipid droplet formation or for genes involved in the transcriptional regulation of lipid metabolism. Only one of the genes associated with retinoid metabolism and actions was significantly different between the 2 genotypes. Expression of *Rbp1* mRNA was significantly elevated (P<0.05) by 2.8-fold in *Dgat1*$^{-/-}$ HSCs compared to HSCs from WT mice.

Dgat1$^{-/-}$ *HSCs are not protected against activation by culture on plastic dishes*

Yamaguchi *et al*. reported that DGAT1 has a role in HSC activation, reporting that the downregulation of DGAT1 in primary WT HSCs following treatment with antisense oligonucleotides reduced expression levels of genes associated with fibrogenesis (30). We were unable to reproduce this finding in primary HSCs isolated from *Dgat1*$^{-/-}$ mice. After 5 days of culture, there were no morphological differences in activated *Dgat1*$^{-/-}$ HSCs compared with WT. The extent of lipid droplet dissolution and dispersal in *Dgat1*$^{-/-}$ HSCs was visually indistinguishable from that in WT HSCs (*Figure 3A*). Markers for HSC activation also show that *Dgat1*$^{-/-}$ HSCs were not less susceptible to activation in culture; *Col1a1* and *Acta2* mRNA levels were not different in either freshly isolated or cultured HSCs (*Figure 3B*,*C*). Cellular retinyl ester levels were not different upon 5 days of culture (data not shown), and mRNA levels for retinoid related genes provide no evidence for elevated retinoic acid or increased retinoic acid signaling in *Dgat1*$^{-/-}$ HSCs (*Table 1*). Expression levels of genes involved in the interconversion

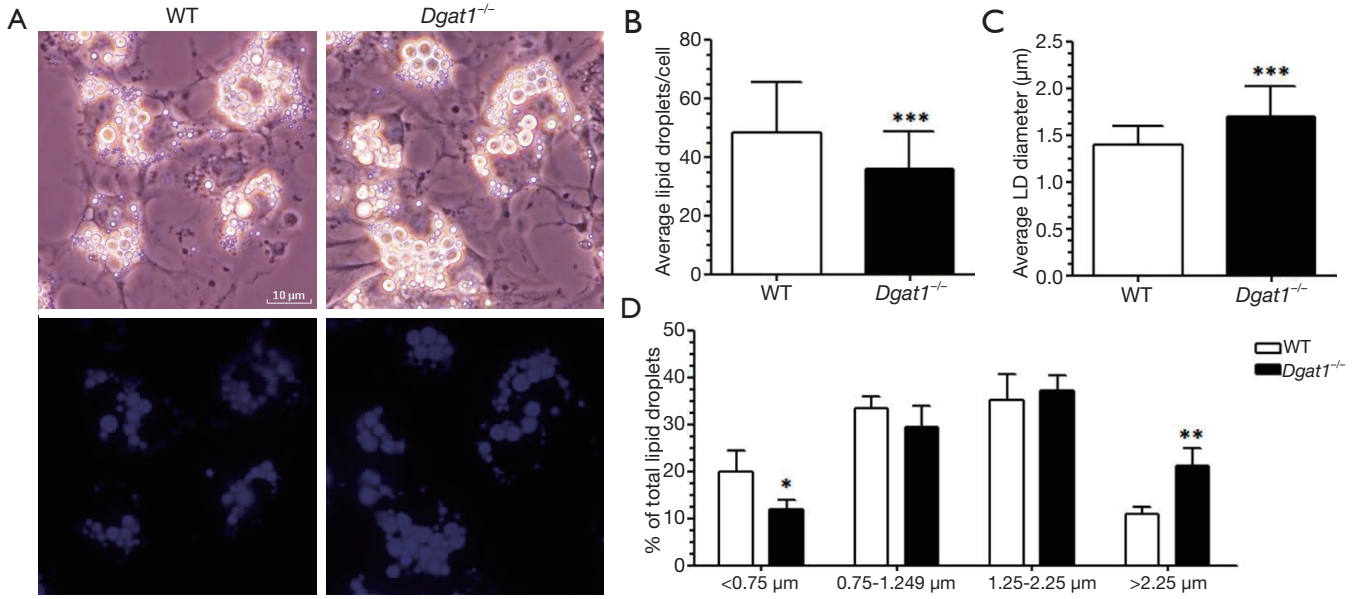

Figure 2 HSC lipid droplets are larger but less numerous in the absence of DGAT1. In primary culture, 24 h after isolation, *Dgat1*$^{-/-}$ HSCs have larger lipid droplets than WT HSCs (A). The lower portion of this panel shows the retinoid autofluorescence emitted upon excitation at 330 nm. The numbers present and diameters of individual lipid droplets were assessed for a total of 83 *Dgat1*$^{-/-}$ and 52 WT HSCs randomly selected from cells in culture for 24 h prepared from 5 *Dgat1*$^{-/-}$ and 4 WT mice. The *Dgat1*$^{-/-}$ HSCs had, on average, fewer lipid droplets per cell (B) but these were larger than those present in WT HSCs (C). The size distribution of the lipid droplets shows that the *Dgat1*$^{-/-}$ HSCs, compared to WT HSCs, had significantly fewer of the smaller lipid droplets (under 0.75 µm in diameter) and significantly more of the larger lipid droplets (over 2.25 µm in diameter) (D). All values are given as mean ± SD. Statistical significance: *, P<0.05; **, P<0.01; ***, P<0.001.

of retinol and retinaldehyde, *Rdh10* and *Dhrs3*, and in retinoic acid synthesis, *Raldh1*, as well as the retinoic acid responsive genes, *Lrat*, *Cyp26b1* and *Rarb*, were all not different between the genotypes for either freshly isolated or activated HSCs (*Table 1*). However, expression levels of the retinoid-related genes *Lrat*, *Rbp1*, *Rarb*, *Rdh10*, *Dhrs3*, and *Ralh1* were all significantly elevated in the activated HSCs as compared to freshly isolated HSCs. Similarly, expression levels for *Plin2*, *Plin3* and *Plin4* were significantly elevated in culture activated HSCs, although the magnitude of these elevations was identical for WT and *Dgat1*$^{-/-}$ HSCs. Only expression of *Ppard* was different (elevated) in the culture activated HSCs but the same degree of elevation was observed for both WT and *Dgat1*$^{-/-}$ HSCs.

Dgat1$^{-/-}$ *livers are protected against* CCl_4*-induced hepatic fibrosis*

Although *Dgat1*-deficiency does not influence culture-induced HSC activation, DGAT1-deficiency may have an influence on HSC activation *in situ* within the liver. Hence, we investigated the susceptibility of the *Dgat1*$^{-/-}$ liver to CCl_4-induced fibrosis in two studies, which differed by dose and duration of treatment. In Study 1, *Dgat1*$^{-/-}$ and matching WT mice were given IP injections of 1 µL CCl_4 per gram of body weight dissolved in corn oil (1:4, v/v) or an equal volume of the corn oil vehicle every 48 h, for a total of four injections, and were euthanized 24 h after the last injection. Trichrome staining of the liver samples shows that *Dgat1*-deficiency indeed protects against CCl_4-induced fibrosis; the amount of staining clearly reveals less production of extracellular matrix and less inflammatory cell infiltration in the *Dgat1*$^{-/-}$ liver (*Figure 4A*). Consistent with this histological analysis, mRNA expression of markers of fibrogenesis, *Col1a1* and *Acta2*, were not different in vehicle-injected livers, but were significantly lower in *Dgat1*$^{-/-}$ livers when exposed to CCl_4 (*Figure 4B,C*). Following CCl_4 treatment, the *Dgat1*$^{-/-}$ livers had significantly higher retinyl ester levels compared to WT livers (*Figure 4D*). Compared to vehicle-treated livers, hepatic retinyl ester levels were significantly lower in WT livers after CCl_4 treatment; however, CCl_4 treatment did not significantly alter retinyl

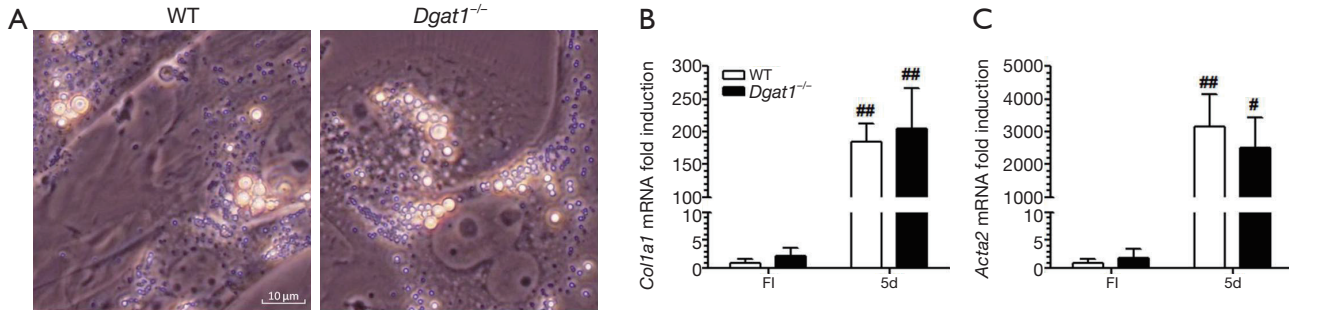

Figure 3 *Dgat1*-deficiency does not influence HSC activation upon culture on plastic dishes. Upon 5 days in primary culture, no morphological differences were observed between *Dgat1*$^{-/-}$ and WT HSCs (A). For freshly isolated (FI) HSCs, mRNA expression of markers for HSC activation, *Col1a1* and *Acta2*, were not significantly different between *Dgat1*$^{-/-}$ (n=3) and WT HSCs (n=3) (B,C). Expression is normalized to 18S rRNA and shown as fold induction relative to levels in freshly isolated WT HSCs. After 5 days (5d) in culture, mRNA levels of these genes were both elevated but not significantly different between *Dgat1*$^{-/-}$ (n=4) and WT HSCs (n=4). All values are given as mean ± SD. Statistical significance between freshly isolated and 5-day cultured HSCs: $^{\#}$, P<0.05; and $^{\#\#}$, P<0.01.

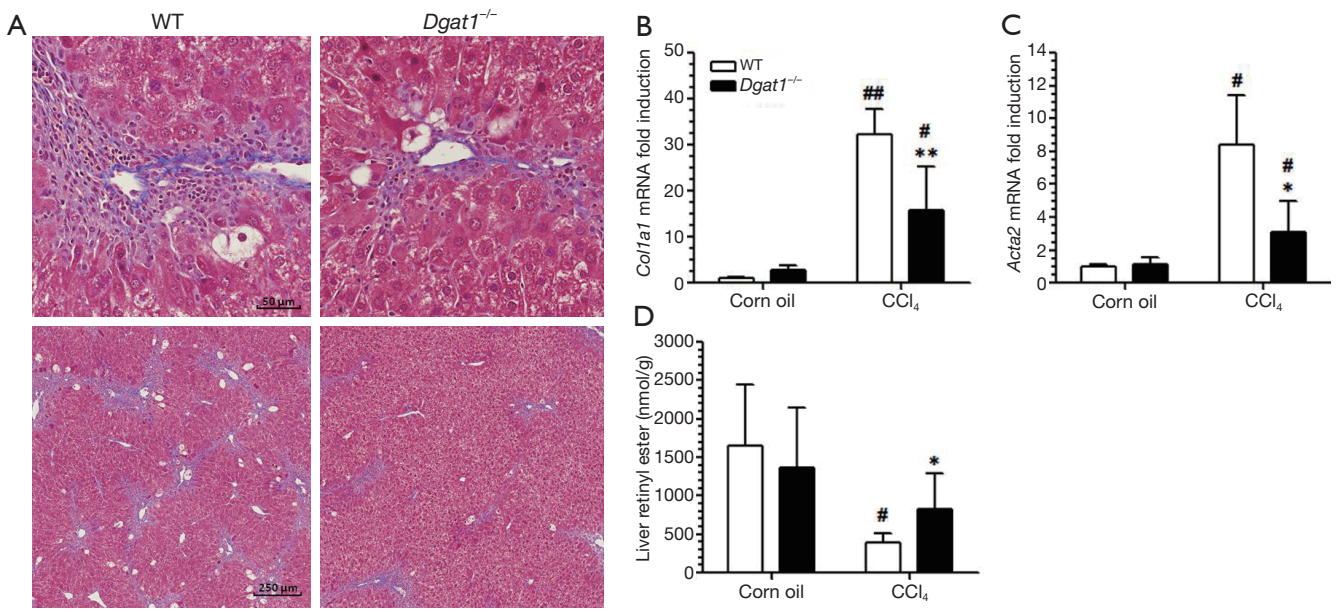

Figure 4 *Dgat1*-deficiency lessens fibrosis development in mice acutely treated with CCl_4. Trichrome staining of liver tissue sections from *Dgat1*$^{-/-}$ and WT mice given IP injections of 1 µL of CCl_4 per gram of body weight [administered in corn oil, 1:4 (v/v)] 4 times at 48 h intervals (A). More collagen staining and more inflammatory cell infiltration was observed for WT compared to *Dgat1*$^{-/-}$ liver. Although mRNA levels for markers for fibrogenesis, *Col1a1* and *Acta2*, were not different between *Dgat1*$^{-/-}$ (n=7) and WT mice (n=4) given four injections of corn oil vehicle, after CCl_4 treatment, expression levels of *Col1a1* and *Acta2* were significantly lower in *Dgat1*$^{-/-}$ compared to WT livers (B,C). Hepatic retinyl ester levels were not different between the genotypes after corn oil treatment, but after CCl_4 injections, retinyl ester levels were significantly higher by more than 2-fold in *Dgat1*$^{-/-}$ compared to WT livers (D). Compared with the corn oil treatment, CCl_4 treatment did not significantly reduce retinyl ester levels in the *Dgat1*$^{-/-}$ livers, while retinyl ester levels in the livers of WT mice given CCl_4 were reduced to 24% of the levels in the livers of mice given only corn oil. All values are given as mean ± SD. Statistical significance between genotypes: *, P<0.05; **, P<0.01. Statistical significance between corn oil and CCl_4 treatments: $^{\#}$, P<0.05; $^{\#\#}$, P<0.01.

Table 2 Relative hepatic gene expression levels in WT and *Dgat1*$^{-/-}$ mice after corn oil and CCl_4 treatment

Gene	Corn Oil		CCl_4	
	WT	*Dgat1*$^{-/-}$	WT	*Dgat1*$^{-/-}$
Study 1				
Lrat	1.0±0.2	0.9±0.2	0.9±0.1	1.0±0.4
Rbp1	1.0±0.3	3.4±1.8*	5.5±1.4$^{\#\#}$	7.3±2.0$^{\#}$
Rarb	1.0±0.1	0.9±0.2	1.3±0.2	1.4±0.3
Rdh10	1.0±0.2	0.9±0.1	0.4±0.1$^{\#\#}$	0.6±0.1$^{\#\#}$
Dhrs3	1.0±0.1	0.9±0.2	0.4±0.1$^{\#\#\#}$	0.4±0.2$^{\#}$
Raldh1	1.0±0.1	0.6±0.1**	0.2±0.0$^{\#\#\#}$	0.2±0.1$^{\#\#}$
Cyp26a1	1.0±0.4	0.4±0.3	0.1±0.0$^{\#}$	0.3±0.2*
Study 2				
Lrat	1.0±0.4	1.0±0.1	1.5±0.4	0.8±0.2
Rbp1	1.0±0.4	3.3±1.2*	4.7±1.9$^{\#}$	5.6±2.9
Rarb	1.0±0.2	1.2±0.1	2.3±0.7$^{\#}$	1.7±0.3$^{\#}$
Rdh10	1.0±0.3	0.5±0.1	0.8±0.1	0.6±0.0
Dhrs3	1.0±0.3	0.8±0.1	0.8±0.2	0.6±0.1
Raldh1	1.0±0.1	0.5±0.2*	0.6±0.1$^{\#\#}$	0.4±0.1
Cyp26a1	1.0±0.1	1.6±1.0	5.8±1.7$^{\#}$	6.0±1.5$^{\#\#}$

All the data shown are normalized to 18S rRNA expression and given relative to levels in WT mice given injections of pure corn oil. *, P<0.05; **, P<0.01 for comparison between genotypes. $^{\#}$, P<0.05, $^{\#\#}$, P<0.01, $^{\#\#\#}$, P<0.001 for comparison between corn oil and CCl_4 treatments in mice of the same genotype.

ester levels in the *Dgat1*$^{-/-}$ livers (*Figure 4D*).

Despite these differences in fibrogenesis, there was no indication of elevated retinoic acid signaling in the *Dgat1*$^{-/-}$ liver. For both genotypes, CCl_4 exposure did not alter expression levels of the retinoic acid-responsive *Lrat* and *Rarb* genes (*Table 2*). Expression levels of *Rbp1* were elevated in both WT and *Dgat1*$^{-/-}$ CCl_4-treated livers. However, mRNA levels of *Rdh10*, *Dhrs3*, and *Raldh1* were all significantly lower for both WT and *Dgat1*$^{-/-}$ livers compared to vehicle-treated livers (*Table 2*). CCl_4 treatment also significantly lowered *Cyp26a1* expression in WT livers, but not in *Dgat1*$^{-/-}$ livers.

In Study 2, *Dgat1*$^{-/-}$ and WT mice were given IP injections of either 0.5 μL of CCl_4 per gram of body weight dissolved in corn oil (1:4, v/v) or an equal volume of corn oil vehicle every 7 days, for a total of four injections, and euthanized 7 days after the last injection. Trichrome staining of the liver sections shows a more moderate difference in collagen deposition between the genotypes compared to that in Study 1. As seen in *Figure 5A*, CCl_4 treatment induced more extracellular matrix production and more infiltration of inflammatory cells in the WT liver than in the *Dgat1*$^{-/-}$ liver. Levels of mRNA of *Col1a1* were significantly lower in the CCl_4-treated *Dgat1*$^{-/-}$ liver compared to the CCl_4-treated WT liver (*Figure 5B*). Expression levels of *Acta2* mRNA were elevated 2-fold with CCl_4 treatment in the WT liver, although this upregulation is modest compared to the 8-fold increase seen in Study 1 (*Figure 5C*). In the *Dgat1*$^{-/-}$ liver, *Acta2* expression was not statistically different after CCl_4 treatment compared to vehicle treatment (*Figure 5C*). Following CCl_4 treatment, retinyl ester levels were significantly higher in *Dgat1*$^{-/-}$ livers compared to WT livers (*Figure 5D*).

After exposure to CCl_4, we observed no differences in hepatic mRNA expression for any of the retinoid related genes between the WT and *Dgat1*$^{-/-}$ mice (*Table 2*). For both genotypes, mRNA levels of *Lrat*, *Rdh10* and *Dhrs3* were not affected by CCl_4 treatment. In contrast to Study 1, hepatic mRNA levels of *Rarb* and *Cyp26a1* were significantly elevated upon CCl_4 treatment for both WT and *Dgat1*$^{-/-}$ mice (*Table 2*).

Discussion

The goal of our investigations was to answer two questions regarding potential actions of DGAT1 in the liver. First, we wished to understand whether DGAT1, which in some tissues and *in vitro* catalyzes retinyl ester formation, has a role in retinol/retinyl ester metabolism and storage in the liver. And second, we wanted to understand whether, as proposed by Yamaguchi *et al.*, DGAT1 influences HSC activation through effects on retinoid metabolism and actions (30).

Although we earlier reported that hepatic total retinol (retinol + retinyl ester) levels are not different for age-, gender- and genetic background-matched chow fed WT and *Dgat1*$^{-/-}$ mice (27,29), in the present study we unexpectedly observed differences in the cellular distribution of retinyl ester stores between hepatocytes and HSCs in the livers of WT and *Dgat1*$^{-/-}$ mice. While hepatic total retinol levels are not different for WT versus *Dgat1*$^{-/-}$ liver, agreeing with our earlier work, significantly more retinyl ester is found in *Dgat1*$^{-/-}$ HSCs compared to WT HSCs, while significantly more retinyl ester is found in WT hepatocytes compared to *Dgat1*$^{-/-}$ hepatocytes. It is unlikely that this involves the ARAT activity of DGAT1. We were unable to show that DGAT1 catalyzes any retinyl ester formation in the liver,

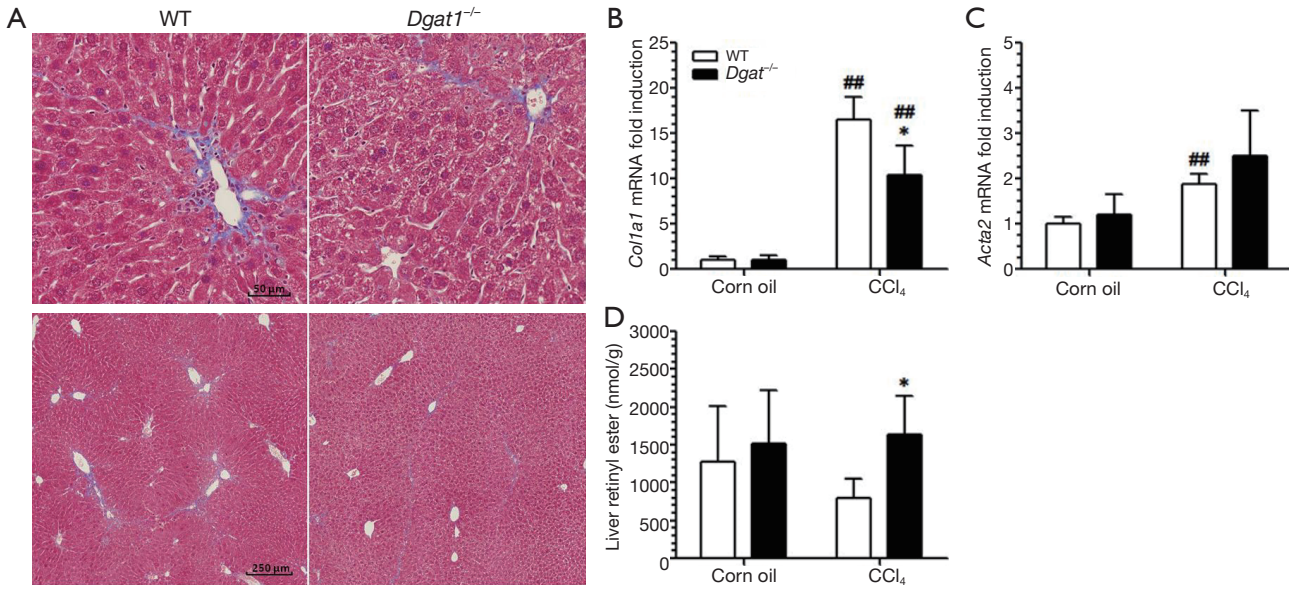

Figure 5 *Dgat1*-deficiency lessens fibrosis development in mice chronically treated with CCl_4. Trichrome staining of liver tissue sections from *Dgat1*$^{-/-}$ and WT mice given IP injections of 0.5 µL of CCl_4 per gram of body weight [administered in corn oil 1:4 (v/v)] 4 times at 7 day intervals (A). More collagen staining and more infiltration of inflammatory cells were observed in the WT liver compared to the *Dgat1*$^{-/-}$ liver. Following CCl_4 treatment, *Col1a1* expression was significantly elevated in the livers of both the WT (n=4) and *Dgat1*$^{-/-}$ mice (n=4), but these levels were significantly lower in the *Dgat1*$^{-/-}$ compared to the WT liver (B). mRNA expression of *Acta2* were significantly elevated in the WT liver after CCl_4 exposure, but not statistically different in the *Dgat1*$^{-/-}$ liver (C). After CCl_4 treatment, hepatic retinyl ester levels were significantly higher in the *Dgat1*$^{-/-}$ compared to WT livers (D). Statistical significance between genotypes: *, P<0.05. Statistical significance between corn oil and CCl_4 treatments: $^{\#}$, P<0.05; $^{\#\#}$, P<0.01.

even in the absence of *Lrat*, or the combined absence of *Lrat* and *Rbp1*, or the combined absence of *Lrat* and *Rbp1* combined further with chronic administration of a 25-fold excess of dietary retinol (over that present in a standard chow diet) (27,29). Consequently, there is no basis for attributing this observation to DGAT1-catalyzed retinyl ester formation. It is well established that dietary retinoid is taken up by hepatocytes and then transferred to HSCs for storage (16,17) and that retinol stored in HSCs must be transferred to hepatocytes, the cellular site of retinol-binding protein 4 (RBP4) synthesis within the liver (16), before it can be mobilized in the circulation bound to RBP4. Thus, there are dynamic interactions between these two hepatic cells types that are required for hepatic retinoid uptake, storage and mobilization to occur. We propose that DGAT1 has a role in these processes that is disrupted in *Dgat1*-deficiency. *Rbp1* mRNA levels are elevated by 2.8-fold in HSCs of *Dgat1*$^{-/-}$ mice (see *Table 1*). RBP1 is known to have a role in the cellular uptake of retinol and its delivery to LRAT for esterification (39,40). Since RBP1 is highly expressed in HSCs (16,31) and LRAT is almost entirely localized to this cell type within the liver (41), it seems likely to us that the absence of DGAT1 increases the capacity of HSCs for retinyl ester formation and storage. This will disrupt the normal equilibrium in the bidirectional movement of retinol between HSCs and hepatocytes, resulting in increased accrual of retinyl ester by the HSCs. This conclusion is further supported by the data from the studies involving oral administration of [^{3}H]retinol to WT and *Dgat1*$^{-/-}$ mice (*Figure 1E,F*). The molecular basis for how *Dgat1*$^{-/-}$ deficiency gives rise to elevated *Rbp1* expression in the HSC will need to be determined in future studies.

The lipid droplets present in HSCs are unique in that they contain large amounts of retinyl ester (approximately 40% of the total lipid) that exceed the concentrations of triglyceride (approximately 33% of total lipid) (42). This is unlike the much more extensively studied lipid droplets that are present in adipocytes whose lipid composition consists predominantly of triglyceride (43). From genetic studies, it has been established that either DGAT1 or DGAT2 can synthesize the triglyceride needed to maintain adipocyte lipid droplets, since only the absence of both *Dgat1* and *Dgat2* gives rise to the

complete absence of lipid droplets in adipocytes (44). DGAT1 is also known to have a role in lipid droplet formation in the hepatocyte (45-47). As an endoplasmic reticulum (ER)-associated enzyme with a dual membrane topology (46,48), DGAT1 is thought to be the DGAT activity responsible for triglyceride synthesis within the ER lumen of hepatocytes (47), and has been proposed to contribute to a population of small lipid droplets, 0.1-0.3 μm in size, destined for the cytosol (49). If these lipid droplets contain retinoids, then the capacity for retinoid accumulation in hepatocytes may also be compromised in the absence of DGAT1, leaving more retinol to be transferred to HSCs for storage.

The lipid droplets present in *Dgat1*-deficient HSCs are fewer in number but, on average, larger than those of WT HSCs (*Figure 2*). This observation is consistent with the proposed role of DGAT1 in the formation of small lipid droplets (49). We were not able to attribute these differences in lipid droplet size and number to other parameters measured. We did not observe differences in the HSC levels of triglyceride between WT and *Dgat1*$^{-/-}$ HSCs (see *Figure 1I*) indicating that the differences are not simply due to impairments in HSC triglyceride synthesis/accumulation. It is well established in the literature that many different proteins can be associated with lipid droplets and that many of these can affect lipid droplet formation and size (50-52). These include Plin1-5 and enzymes involved in the synthesis and degradation of neutral lipids and phospholipids (50-52). Although we did not carry out an exhaustive study of these, we obtained no evidence that changes in expression levels of the Plin proteins contribute to the differences we observed. Unlike LRAT, which is absolutely required for lipid droplet formation in HSCs (21), DGAT1 appears to have a role in the synthesis of smaller and more numerous HSC lipid droplets (49).

Yamaguchi *et al.* reported that knockdown of *Dgat1* using anti-sense oligonucleotides reduced mRNA expression levels of markers for HSC activation both in mice fed a methionine choline-deficient diet and in cultured, plastic activated HSCs obtained from healthy rats (30). These investigators further reported that treated livers/HSCs displayed elevated *Rbp1* and *Lrat* mRNA expression and concluded that the loss of DGAT1, which they maintained is an important ARAT in liver, caused more retinol to be available for oxidation to retinoic acid, hence suppressing HSC activation (30). Since the only role for DGAT1 in hepatic/HSC retinoid metabolism that can be identified from our studies is an effect on the distribution of retinyl ester in HSCs [present study and (27,29)], we investigated how the complete absence of DGAT1 from the liver affects HSC activation upon culture on plastic dishes and upon CCl_4 exposure *in vivo*, two experimental protocols commonly used to activate HSCs (53). For plastic activation (see *Figure 3* and *Table 1*), the absence of DGAT1 had no effect on HSC activation. Markers of HSC activation were identical for WT and *Dgat1*-deficient HSCs cultured for 5 days on plastic. Moreover, as noted above, the only difference in gene expression we did observe was for *Rbp1*, which was elevated by 2.8-fold in freshly isolated *Dgat1*-deficient HSCs. There were no differences in expression of the canonical retinoic acid-responsive *Lrat* and *Rarb* genes. Upon CCl_4 treatment, for both study 1 and study 2, and in agreement with the report from Yamaguchi *et al.*, we observed less fibrosis development in the *Dgat1*$^{-/-}$ mice as assessed both by trichrome staining and by marker gene expression. We did not, however, observe differences between WT and *Dgat1*$^{-/-}$ livers with regards to expression levels of the retinoic acid-responsive *Lrat*, *Rarb* and *Cyp26a1* genes. Expression of these genes would be expected to be elevated if retinoic acid was contributing to the lessened fibrosis. Since the literature indicates that different experimental protocols used to activate HSCs (culture on plastic versus CCl_4 treatment versus bile duct ligation) each involves different patterns of gene expression (53), it may not be too surprising that we observed different effects for *Dgat1*-deficiency on fibrogenesis for the culture versus CCl_4 experiments. However, we did not obtain evidence from either experiment that supports the notion that differences in retinoic acid synthesis or transcriptional regulatory activity account for the diminished activation observed for *Dgat1*$^{-/-}$ HSCs.

In summary, our studies establish that DGAT1 has a role in hepatic retinol/retinyl ester metabolism and storage, albeit one that does not involve the ARAT activity of DGAT1. Rather, our data point to a role that involves the movement of retinol between hepatocytes and HSCs, probably directly involving the actions of RBP1 in facilitating retinol uptake and/or LRAT catalyzed esterification in the HSC. Moreover, DGAT1 has a role in modulating the number and size of lipid droplets in HSCs, which is consistent with its proposed actions on triglyceride incorporation into lipid droplets (49). Finally, *Dgat1*-deficient livers appear to be more resistant to experimentally induced HSC activation and fibrosis. However, we obtained no evidence that this results from differences in retinoid metabolism or retinoic acid synthesis and actions between WT and *Dgat1*$^{-/-}$ mice as proposed by Yamaguchi *et al.* (30).

Acknowledgements

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Disclosure: The authors declare no conflict of interest.

Ethical Statement: All experiments were conducted in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals, and were approved by the Columbia University Institutional Animal Care and Use Committee.

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