THE THERAPEUTIC POTENTIAL OF TARGETING HEPATIC MCT1 IN NON-ALCOHOLIC STEATOHEPATITIS

A Dissertation Presented

by

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This work was undertaken in the Graduate School of Biomedical Sciences Interdisciplinary Graduate Program

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DEDICATION

My beloved family, a source of unwavering love and support,
my esteemed mentors, a source of life wisdom and guidance,
and my dear colleagues, a source of constant inspiration and passion
- this thesis is dedicated to them.
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ABSTRACT

Emerging evidence supports substrate overload to the liver as an initial driver of non-alcoholic steatohepatitis (NASH) progression. Metabolic disease patients have increased plasma lactate levels. Circulating lactate is a fuel source for liver metabolism but may exacerbate metabolic diseases. Indeed, monocarboxylate transporter 1 (MCT1) haploinsufficiency in mice causes resistance to diet-induced obesity, steatosis, and hepatic inflammation. However, little is known about the therapeutic potential of hepatic MCT1 and its contribution to NASH fibrogenesis. In this study, we developed novel adeno-associated virus (AAV) lecithin-retinol acyltransferase (Lrat)-Cre vectors that selectively target hepatic stellate cells, as well as two types of hepatic MCT1-targeting fully chemically modified siRNA compounds with distinct cell delivery capabilities: Tetra-ethyleneglycol-cholesterol (Chol)-conjugated siRNAs, which enter all hepatic cell types, and hepatocyte-selective tri-N-acetyl galactosamine (GN)-conjugated siRNAs. We demonstrated that stellate cell MCT1KO (AAV-Lrat-Cre) attenuated liver type 1 collagen protein expression and caused a downward trend in trichrome staining. Similarly, MCT1 silencing by Chol-siRNA decreased liver collagen 1 level in mice, and decreased LX2 stellate cell collagen expression. Interestingly, however, hepatocyte-selective MCT1 depletion by AAV-TBG-Cre or by GN-siRNA unexpectedly increased collagen 1 and total fibrosis, without alleviating triglyceride accumulation. These findings demonstrate that stellate cell lactate transporter MCT1 significantly
contributes to liver fibrosis through increased collagen 1 protein expression in vitro and in vivo, while hepatocyte MCT1 does not appear to be an attractive therapeutic target for NASH. Furthermore, combining our previously developed chemically modified siRNA compound (DGAT2-1473) with Chol-MCT1-siRNA was more effective than Chol-MCT1-siRNA alone in preventing NASH fibrosis, indicating that a dual targeting approach may be most effective for alleviating NASH.
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Chapter I. Introduction

1.1 Nonalcoholic Fatty Liver Disease and Non-alcoholic Steatohepatitis

Nonalcoholic fatty liver disease (NAFLD) is the most common chronic liver disease, afflicting over a quarter of the world’s population\(^1\). It describes a spectrum of liver diseases ranging from simple steatosis to nonalcoholic steatohepatitis (NASH)\(^1,2\). Simple steatosis is considered relatively benign as lifestyle modifications can reverse fatty liver to a healthy condition. On the other hand, NASH is characterized by severe steatosis, inflammation, and fibrosis. Severe fibrotic stages of NASH can develop into permanent liver damage, and the disease can progress to cirrhosis and hepatoma. Currently, NASH is a leading cause of liver transplantation, and there is no FDA-approved therapeutic for NASH\(^2-5\).

1.1.1 Diagnostics and Epidemiology

There are multiple diagnostic techniques for NAFLD: 1) imaging techniques including ultrasound, computed tomography scan, and magnetic resonance imaging, 2) liver biopsy, and 3) blood test monitoring fatty liver index and liver enzymes\(^1\). Diagnosis of NAFLD or simple steatosis requires evidence of triglyceride accumulation in hepatocytes in the absence of excessive alcohol consumption (40g/day) by imaging or histological methods\(^6\). NASH is diagnosed when a microscope examination confirms steatosis in hepatocytes along with an accumulation of inflammatory cells, liver cell injury/death, and liver fibrosis\(^4,6\).
According to the recent systematic review and meta-analysis performed by Riazi et al. (2022) in Lancet\(^7\), the overall estimated prevalence of NAFLD is 32.6\% (95\% CI 29.9 – 34.9, publication years ranged from 2002 to 2021). Considering that it used to be 24.3\% (95\% CI 17.8 – 30.8, publication years ranged from 2022 to 2005) but has risen to 38.2\% (95\% CI 33.2 - 43.1, publication years ranged from 2016 to 2021), both the prevalence and incidence of NAFLD have been rising globally, in parallel with the increasing number of obese and type 2 diabetic patients\(^1,7,8\). As NAFLD is the leading cause of liver-related morbidity and mortality, it could raise serious global health problems, followed by an elevated economic burden in return. NASH prevalence among NAFLD patients is presumably more than a quarter\(^4\). Depending on the stratification methods, wide ranges of NASH prevalence were reported. With an indication for biopsy, 59.10\% of NAFLD patients are estimated to have NASH, but without the indication for biopsy, 6.67\% and 29.85\% of NAFLD patients had NASH in Asia and North America, respectively\(^1\).
Figure 1.1 Non-alcoholic fatty liver disease is the most common chronic liver disease.
Non-alcoholic fatty liver disease is the most common chronic liver disease affecting more than a quarter of the world's population. One-fourth of the population develops simple steatosis, and more than one-fifth of the subjects progress to non-alcoholic steatohepatitis (NASH). In the US, individuals with simple steatosis are estimated to be over 60 million, and individuals with NASH are estimated to be more than 20 million. NASH is characterized by three major pathological features; severe steatosis, inflammation, and fibrosis. As NASH can progress to cirrhosis and hepatocellular carcinoma that necessitates liver transplant as the only way to cure the disease, NASH prevention and alleviation are critical (Cartoon created in Biorender).
1.1.2 Pathogenesis

1.1.2.1 Metabolic syndromes

Individuals with metabolic syndrome are more likely to develop NAFLD, and the association between metabolic syndrome and NALFD is bidirectional. Metabolic syndrome includes obesity, diabetes, hyperglycemia, dyslipidemia, and systemic hypertension\(^9\). Up to 50%-90% of patients with metabolic conditions such as obesity and type 2 diabetes also have NAFLD\(^1,10\). It is estimated that 51.34% of NAFLD patients and 81.83% of NASH patients are obese, and 22.51% of NAFLD patients and 43.63% of NASH patients are estimated to have type 2 diabetes\(^1,7,8\). Additionally, nearly, 70% of NALFD patients are hyperlipidemia/dyslipidemia. NAFLD patient has a higher risk for type 2 diabetes (2.2 - 2.7-fold) and cardiovascular events (1.5-fold) as comorbidities\(^5,7,11\). Moreover, up to 50% of patients with hypertension have NAFLD\(^12\). Arterial stiffness, myocardial remodeling, and heart failure have shown a strong association with NAFLD\(^13,14\). In addition to the fact that hypertension is highly associated with fibrosis progression\(^15\), there are multiple reports suggesting that alleviating systemic hypertension improves NASH and hepatic fibrosis\(^16-18\). These facts support the notion that NAFLD/NASH is not limited to a liver-autonomous disease, but rather a systemic metabolic syndrome.

1.1.2.2 Substrate overload-derived liver injury
In general, NAFLD is caused by chronic overnutrition, which drives metabolic disorders\textsuperscript{1,5,7}. The most well-known substrate associated with NAFLD/NASH condition is fatty acids. According to Donnelly et al, there are three major sources of fatty acids\textsuperscript{19}; 59.0\% ± 9.9\% of triacylglycerols arise from circulating non-esterified free fatty acids, 26.1\% ± 6.7\% from hepatic de novo lipogenesis, and 14.9\% ± 7.0\% from the diet in NAFLD patients. As most of the non-esterified free fatty acids originate from adipose tissue lipolysis, excessive delivery of circulating non-esterified free fatty acids in the disease condition is highly associated with adipose tissue insulin resistance and dysregulated lipolysis\textsuperscript{20}.

Due to an overabundance of upstream metabolites, NAFLD and NASH patients display hyperactive liver tricarboxylic acid (TCA) cycle flux\textsuperscript{21,22}. Accumulated hepatic free fatty acids are processed by lipid breakdown via mitochondrial and peroxisomal β-oxidation or by lipid disposal via VLDL release\textsuperscript{23,24}. However, during chronic overnutrition, the liver can no longer manage overloaded substrates. As a result, reactive oxygen species (ROS) and lipids start to accumulate and turn into lipotoxic species such as diacylglycerols, ceramides, lysophosphatidic choline, and free cholesterol etc\textsuperscript{25-29}. Consequentially, this provokes ER stress and hepatocyte injury and induces hepatic steatosis\textsuperscript{30}. Accumulated lipotoxic species damage the liver thus activating immune cells and fibrogenic stellate cells, which further accumulate in the extracellular matrix\textsuperscript{31,32}. This steatotic, inflamed, fibrotic liver is called NASH and is often accompanied by insulin resistance, inflammation, and increased circulating free fatty acids\textsuperscript{5,33,34}. These pathogenic pathways
suggest that lowering substrate influx to the liver is a promising strategy to prevent and possibly alleviate steatosis and NASH.

1.1.2.3 Genetic Variants in lipid metabolism pathways

Recent human genome-wide association studies (GWASs) strongly support the idea that NASH develops in the context of lipotoxicity and hepatic steatosis\(^1,35\), as most of the variants found in NASH patients were highly associated with lipid metabolism pathways. For example, single nucleotide polymorphism (SNP) of phospholipase domain-3 (\textit{PNPLA3})\(^{36,37}\), transmembrane 6 superfamily member 2 (\textit{TM6SF2}), and hydroxysteroid 17-beta dehydrogenase 13 (\textit{HSD17B13})\(^{38-40}\) are not only associated with steatosis, but are also clinically relevant with NASH and cirrhosis\(^41\).

\textit{PNPLA3} is a lipid droplet-associated protein. An allele in \textit{PNPLA3} (rs738409), most common in Hispanics, was associated with increased hepatic fat levels and hepatic inflammation\(^36\). However, another allele in \textit{PNPLA3} (rs6006460) in African Americans was associated with lower hepatic fat contents\(^36\). A point mutation of \textit{TM6SF2}, rs58542926, which was common in East Asians, was associated with elevated liver triglycerides, and increased alanine aminotransferase (ALT)\(^{42-44}\). Its association with NAFLD was also confirmed, as patients with rs58542926 had reduced secretion of very-low-density lipoproteins (VLDLs)\(^45\). Another lipid droplet-associated protein, \textit{HSD17B13}, acts upstream of lipid biosynthetic process. Decreased risk of steatosis and NASH has been reported upon a loss of function
SNP, rs72613567⁴¹,⁴⁶. rs62305723 was significantly associated with less hepatocyte ballooning and inflammation¹⁷. However, higher serum liver enzymes were shown associated with an allele in HSD17B13 (rs6834314), reflecting liver fat or injury⁴⁷. These GWASs support the idea that dysfunction in lipid metabolism drives NASH inflammation and fibrosis. Thereby, targeting lipid metabolism could be a valid therapeutic strategy.

1.1.2.4 Inflammation

Fatty acid and lipotoxic species induce hepatocellular injury and activate inflammasomes, multiprotein cytoplasmic complex, via danger-associated molecular patterns (DAMPs) and pathogen-associated molecular protein (PAMPs). DAMPs include saturated fatty acids such as palmitate (C16:0) and stearate (C18:0), lipotoxic species⁴⁸, while PAMPs include gut microbiota products. As activated inflammasomes accelerate the release of danger signals and stimulate immune cells⁵, hepatocyte inflammasome activation is considered one of the linkages between the primary metabolic stress and stimulation of fibrogenesis in NASH³². Activated inflammasomes lead to proinflammatory cytokines maturation, such as interleukin-1β and interleukin-18, and enhance the expression of apoptosis via caspase-1 activation and nod-like receptor protein, as well as mitochondrial dysfunction that enhances reactive oxygen species and ATP leakage⁶,⁴⁸-⁵⁰. Other than inflammasome, Toll-like receptors are another intracellular pattern recognition receptors that sense PAMPs. The most well-
studied TLR families in NASH are TLR4, which recognizes bacterial components, lipopolysaccharide, and TLR9, which recognizes bacterial DNA\textsuperscript{51}.

Hepatic resident macrophages, Kupffer cells, are involved in the NASH inflammatory response. They are not only the major source of reactive oxygen species in the liver, but also secrete inflammatory chemokines (interleukin-1β, interleukin-18, tumor necrosis factor-α, transforming growth factor-β, etc.) and cytokines (C-C motif chemokines 2, C-C motif chemokines 5, CXC-chemokine ligand 1, CXC-chemokine ligand 2, CXC-chemokine ligand 9, CXC-chemokine ligand 10, CXC-chemokine ligand 11, etc.) upon DAMPs signals\textsuperscript{48,52-54}. Multiple studies have revealed that Kupffer cells and monocytes cooperatively regulate NASH inflammation by Kupffer cells initiating the inflammatory response through chemokines production and blood monocytes promoting infiltration during NASH progression\textsuperscript{55-57}.

1.1.2.5 Fibrosis, fibrogenic hepatic stellate cells, and collagen production

Major organ fibrosis is directly correlated with morbidity and mortality, contributing up to 45% of deaths in developed countries\textsuperscript{58}. NASH is a critical clinical interphase, as advanced liver fibrosis in NASH implicates a defective regeneration of healthy liver architecture, increasing the risk of advanced disease conditions such as cirrhosis, hepatocellular carcinoma, and further end-stage liver failure necessitating liver transplantation\textsuperscript{5,59-61}. Liver fibrosis advances on a scale ranging from F0 (no fibrosis), to finally F4 stage (cirrhosis), with stages F1 (portal fibrosis),
F2 (portal fibrosis with few septa), and F3 (bridging fibrosis between central and portal veins) in between\textsuperscript{1,4}. Reportedly, it takes an average of 7 years for NASH patients to progress one stage of liver fibrosis, even though it varies among patients and may not be linear over time\textsuperscript{15,62}.

The aforementioned DAMPs signal, derived from lipid-stressed hepatocytes or cytokines and chemokines secreted from activated Kupffer cells, can also stimulate fibrogenic hepatic stellate cells\textsuperscript{48}. While hepatic stellate cells account for only 5-10\% of the hepatic cell population, they are the major cell type contributing to hepatic fibrogenesis\textsuperscript{63, 64}. Fate-tracing studies have revealed that 82-96\% of myofibroblasts are derived from hepatic stellate cells, which are liver-specific pericytes\textsuperscript{64}. Hepatic stellate cells are localized in the interposed area between sinusoidal endothelial cells and hepatocytes, called the subendothelial space of Disse\textsuperscript{63, 65}. Recent transcriptome profiles have characterized distinguishable markers of hepatic stellate cells, including the enzyme lecithin retinol acyltransferase (Lrat), the cell surface protein platelet-derived growth factor receptor-\(\beta\) (PDGFR\(\beta\)), the cytoskeletal protein desmin, and glial fibrillary acidic protein (GFAP)\textsuperscript{64, 66-69}. In healthy normal liver conditions, hepatic stellate cells maintain a quiescent phenotype. However, during NASH progression, multiple liver injury signals stimulate the transition of vitamin A-storing quiescent hepatic stellate cells into fibrogenic, proliferative myofibroblasts that produce and secrete collagen fibers\textsuperscript{65, 70-72}. As a result, healthy hepatic parenchyma is replaced with a collagen-rich extracellular matrix, turning the liver into a hardened and scarred
tissue\textsuperscript{73}. Thus, targeting activated hepatic stellate cells has become a major strategy in NASH therapeutics development\textsuperscript{5,65}.

Multiple pathways regulating hepatic stellate cell activation have been reported\textsuperscript{19}. One of the most potent fibrogenic and proliferative cytokines is transforming growth factor-1β (Tgf-1β), which is released by various hepatic cell types. Its binding to type I receptor induces downstream SMAD3 protein phosphorylation (pSMAD3). The active pSMAD3 then promotes transcription of type I and III collagen\textsuperscript{74-76}. Tgf-1β can also activate mitogen-activated protein kinase (MAPK) signaling pathway\textsuperscript{77,78}. Other than Tgf-1β, various cytokines such as platelet-derived growth factor\textsuperscript{79,80}, vascular endothelial growth factor\textsuperscript{81,82}, and connective tissue growth factor\textsuperscript{83} have been known to promote hepatic stellate cell activation.

To note, there are a total of 28 types of collagens that exist\textsuperscript{84}. The majority of collagen in the human body is composed of type I, II, and III, with type I collagen being the most common, making up 80-90\% of the collagen\textsuperscript{85}. Type I and III are two major types of collagens related to NASH, while type IV, V, and IV are also present in the liver. In the normal adult liver, the proportion of type I, III, IV, and V collagen is approximately 43\%, 40\%, 7\%, and 11\% respectively\textsuperscript{86}. These proportions can vary depending on the disease state. In cirrhosis or end-stage alcoholic livers, type I collagen becomes dominant, reaching around 54-59\%, while type III, IV, and V collagen account for 25-31\%, 5-6\%, and 9-10\%, respectively\textsuperscript{86,87}. In rodent systems, particularly in rats, type I collagen is even more dominant.
According to Friedman et al type I collagen accounts for up to 80%, while type III and IV collagens make up 5% and 20%, respectively. The study also demonstrated that hepatic stellate cells are the primary producers of collagen, contributing 10 times and 20 times more collagen than hepatocytes or endothelial cells, respectively, in rats.
Figure 1.2 Substrate overload liver injury model for NASH progression. According to this model, hepatic free fatty acids come from three major sources; circulating non-esterified free fatty acids, hepatic de novo lipogenesis, and direct deposit of dietary fats. During chronic overnutrition, fat breakdown via mitochondrial β-oxidation and fat disposal via VLDL release pathways are saturated, not being able to process overloaded substrates in the liver. Consequentially, remaining free fatty acids turn into lipotoxic species such as diacylglycerols, ceramides, lysophosphatidic choline, and free cholesterol, inducing hepatocellular injury and cell death. These damage signals accelerate inflammatory cells activation and finally stimulate fibrogenic hepatic stellate cells that produce collagen fibers (cartoon modified from Friedman et al., 2018, Nature medicine review article)
1.1.3 Current status of NASH therapeutic development

As pathogenic drivers are highly heterogenous and not identical among patients\textsuperscript{89}, the development of a single therapy for NASH has not been successful. Dozens of clinical trials for NASH have failed. While some candidates such as thyroid hormone receptor-\(\beta\) (THR-\(\beta\)) agonists or farnesoid X receptor (FXR) agonists seem close to winning FDA approval, multiple pharmaceutical companies started combination therapy\textsuperscript{4,5,90}. Here, I will introduce the current status of NASH therapeutic development in multiple aspects.

1.1.3.1 Metabolic homeostasis

Peroxisome proliferator-activated receptor (PPAR) family (PPAR \(\alpha\), \(\gamma\), and \(\delta\)) has been a therapeutic target for various metabolic diseases, such as diabetes, dyslipidemia, and cardiovascular disease\textsuperscript{91,92}. As nuclear receptors and transcription factors, PPAR family is not only a key regulator of glucose and lipid metabolism, but it also plays a role in inflammation and fibrosis in NASH\textsuperscript{93}. PPAR\(\gamma\) agonists, such as rosiglitazone and pioglitazone, have shown improved steatosis, and NASH phenotypes including hepatocellular ballooning, inflammation, and fibrosis, even though there were some reports on weight gain and increased fracture risk that need to be addressed\textsuperscript{94-96}. Recently, PPAR \(\alpha\) and \(\gamma\) dual targeting Saroglitazar was accepted for NASH treatment in India. It lowered liver enzymes and lipid values in NAFLD patients and showed histological improvement of NASH
after 52 weeks of treatment (Clinical trial; EVIDENCES I, II). Now, it has completed the Phase 2 trial (EVIDENCES IV) in the United States\textsuperscript{90,97}.

Another nuclear receptor superfamily, Farnesoid X receptor (FXR), is involved in glucose, lipid, and bile acid metabolism. FXR agonists have been shown to improve systemic insulin sensitivity and NASH phenotypes\textsuperscript{98}. Semisynthetic bile acid analog obeticholic acid (OCA) acts as an FXR agonist. Intercept Pharmaceuticals’ Phase 3 clinical trial (REGENERATE) has reported improvement in serum liver enzymes, liver stiffness, and fibrosis among NASH patients with stage F2, F3 liver fibrosis after 18 months of treatment\textsuperscript{99-101}. However, potential liver toxicity and lipoprotein profile issues need to be addressed in ongoing Phase 3 clinical trials\textsuperscript{102}. As FXR enhances the intestinal release of fibroblast growth factor 19 (FGF-19), the clinical value of FGF-19 analog is under investigation for NASH therapeutics as well. FGF-21 is another growth factor family but is mostly expressed in hepatocytes. It not only regulates glucose homeostasis and insulin sensitivity in response to circulating free fatty acids or glucose levels but also exhibits anti-fibrotic properties\textsuperscript{103,104}.

Glucagon-like peptide (GLP)-1 is an intestinal hormone that acts as an insulin sensitizer and promotes metabolic improvement. GLP-1 receptor agonists liraglutide and semaglutide have been reported to stimulate insulin release, lowered blood glucose level, decreased hepatic fat contents, and improved
histological resolution of NASH\textsuperscript{105-108}. Multiple clinical trials in combination with glucagon inhibitory peptide are underway\textsuperscript{5}.

Thyroid hormone regulates systemic energy metabolism, glucose homeostasis, and lipid utilization. Various thyroid hormone receptor-β (THR-β) agonists are developed. Recently, Phase 3 clinical trial of Resmetirom was successfully done, reporting a safe and effective reduction of haptic fat content, inflammation, and NASH after 52 weeks of treatment (MAESTRO-NASH)\textsuperscript{109}. In the study, 966 randomized patients’ baselines were characterized. In detail, 26\% (p<0.0001) of individuals who received resmetirom 80mg exhibited more than a two-point reduction in NAS score with no worsening of fibrosis, and 24\% (p<0.0002) showed more than 1- stage improvement in fibrosis with no worsening of NAS score. Meanwhile, 30\% (p<0.0001) of individuals who took 100mg resmetirom exhibited more than a two-point reduction in NAS score with no worsening of fibrosis, and 26\% (p<0.0001) showed more than 1- stage improvement in fibrosis with no worsening of NAS score. In both dosages, there was a clinically meaningful decrease in LDL levels as well. Based on these results, FDA granted Madrigal Pharmaceuticals, Inc. a fast-track designation in April 2023.

1.1.3.2 Hepatic steatosis

Acetyl-CoA carboxylase (ACC) catalyzes the condensation of acetyl CoA to form malonyl-CoA. The effect of inhibiting ACC on decreasing hepatic fat content and ameliorating fibrosis was significant compared to the placebo group\textsuperscript{110,111}. 
However, ACC inhibitor firsocostat was withdrawn from Phase 3 clinical due to safety concerns regarding liver toxicity including increased serum liver enzymes and increased plasma triglycerides in 2022\textsuperscript{112-114}. Recently, combination therapy with DGAT2 inhibitors has been investigated.

As a multi-enzyme protein, fatty acid synthase (FASN) catalyzes long-chain fatty acid synthesis by performing condensation of acetyl CoA and malonyl CoA to produce palmitate. FASN inhibitor, denifanstat (TVB-2640), has reduced hepatic de novo lipogenesis and hepatic fat contents in obese men with metabolic abnormalities\textsuperscript{115}. Phase 2a, FASCINATE-1, clinical trial improved pro-inflammatory and fibrotic markers at the highest dose (50mg)\textsuperscript{116}. Currently, Phase 2b clinical trial (FASCINATE-2) is underway.

Stearoyl-coenzyme A desaturase-1 (SCD1) plays an important role in fatty acid biosynthesis by catalyzing the conversion of saturated fatty acids into monounsaturated fatty acids\textsuperscript{117}. Aramchol, developed by Galmed Pharmaceuticals, has been granted fast-track designation, as previous clinical trials (ARREST) have revealed a significant effect on hepatic fat reduction, and fibrosis resolution\textsuperscript{118}. Based on the results, Phase 3/4 clinical trial (ARMOR) is underway.

Diacylglycerol O-acyltransferase (DGAT) catalyzes triglyceride synthesis. DGAT2 KO mice were lethal losing more than 90% of triglyceride storage, while DGAT1 KO mice were viable with reduced fat contents, suggesting the dominant role of DGAT2 in fat storage of triglyceride\textsuperscript{119}. Multiple big pharmaceutical companies
such as Pfizer, Novartis, and Ionis Pharmaceuticals have been targeting DGAT2. Pfizer’s selective DGAT2 inhibitor (PF-06427878) remarkably reduced hepatic and plasma triglyceride concentrations in rodent models as well as in healthy adult participants\textsuperscript{120}. In Phase 2 clinical trials that screened 174 people, Ionis Pharmaceuticals’ antisense oligonucleotide compounds (IONIS-DGAT2\textsubscript{Rx}) reduced more than 5% of baseline fat content with no side effect of hyperlipidemia, serum liver enzyme increase, and body weight changes after 13 weeks of treatment\textsuperscript{121}. Moreover, the recent development of therapeutic siRNA (DGAT2-1473) tested in a humanized mouse model provided a practical long-lasting DGAT2 silencer that can be used as a potential therapeutics\textsuperscript{122}.

\textbf{1.1.3.3 Inflammation and Fibrosis}

C-C motif chemokine receptor 2 (CCR2) and CCR5 mediates liver innate immune response to accelerate liver inflammation and fibrosis. Small molecule inhibitor, cenicriviroc (CVC) has received a fast-track designation in NASH as it improved liver fibrosis without worsening NASH in Phase 2b clinical trial (CENTAUR) and Phase 3 clinical trial (AURORA)\textsuperscript{123,124}. However, AURORA trial was terminated due to lack of efficiency in 2021. Recently, CVC has been applied in combination with FXR agonist, tropifexor in Phase 2b TANDEM study for a 48-week treatment\textsuperscript{125}.

Activation of apoptosis signaling kinase-1 (ASK-1) upon oxidative stress can stimulate p38 mitogen-activated protein kinase (MAPK) and c-Jun N-terminal
kinase (JNK) signaling pathways, enhancing a stress response, hepatic inflammation, and fibrosis\textsuperscript{126}. ASK-1 inhibitor, Selonsertib’s Phase 3 clinical trials (STELLAR-3, STELLAR-4) were one of the biggest disappointments in the last few years, as in the previous short-term clinical trials some patients reported improved NASH fibrosis\textsuperscript{126}. Both studies failed to achieve the primary endpoints, not being able to reduce liver biochemistry and improve fibrosis without worsening NASH in patients with NASH F3 bridging fibrosis or F4 cirrhosis after 48 weeks treatment\textsuperscript{127}.

Galectin-3’s roles in apoptosis, immune response, and fibrosis have been well-known in multiple organs and disease conditions including NASH\textsuperscript{128-131}. However, Phase 2b clinical trials (NASH-CX) with Galectin-3 inhibitor belapectin treatment for 52 weeks did not show significant improvement in portal hypertension or fibrosis in NASH patients\textsuperscript{132}. Another Phase 2b/3 clinical trial (NAVIGATE) is underway.

While most of the fibrogenic targets are developed to prevent fibrogenesis, there is another approach to reduce fibrosis; enhancing fibrolysis or fibrosis degradation. For example, LOXL2 is an amine oxidase and enhances fibrosis by promoting covalent crosslinking of collagen fibers\textsuperscript{132,133}. Simtuzumab, monoclonal antibodies were developed to direct against lysyl oxidase-2 (LOXL2). Unfortunately, however, the Phase 2 clinical trial was terminated after 96 weeks of simtuzumab treatment due to a lack of efficacy in advanced NASH patients\textsuperscript{134}.

1.1.3.4 Challenges in NASH mono-therapy development
Overall, the aforementioned clinical trials exhibited limitations of monotherapy. In most cases, each monotherapy was only effective for less than 40% of enrolled patients, suggesting that combination therapies that target multiple NASH pathways could benefit NASH patients with heterogenous pathogenic backgrounds.\textsuperscript{5,89,135} Thus, the field is recognizing the necessity of multi-targeting therapeutic development in NASH.\textsuperscript{136} In general, most combination therapies include metabolic targets or anti-steatosis agents combined with either anti-inflammatory or anti-fibrogenic agents. For example, multiple big pharmaceutical companies’ combination therapies are being tested in clinical trials; Pfizer (DGAT2/GLP-1R, and DGAT2/ACC), Gilead (ACC/GLP-1R/FXR, and ACC/ASK1/FXR), Novartis (FXR/SGLT1,2, and FXR/CCR2,5), etc.\textsuperscript{5,90}

1.2 Lactate and its major transporter monocarboxylate transporter 1

Human subjects with metabolic diseases condition such as type 2 diabetes and obesity reportedly have increased plasma lactate levels.\textsuperscript{137-144} Lactate is produced and released into circulation when cellular glycolytic flux surpasses mitochondrial oxidative capacity. Once considered to be simply a metabolic waste product, lactate is now recognized as a primary fuel for the TCA cycle in liver and thus an essential energy source.\textsuperscript{145,146} Additionally, it is a critical regulator that contributes to whole-body energy homeostasis.\textsuperscript{147,148} Cellular lactate levels are tightly controlled by monocarboxylate transporters (MCTs).

1.2.1 Role of lactate in metabolic homeostasis
More than half of the caloric intake in humans depends on carbohydrate sources. Carbohydrates are broken down into glucose, and two molecules of pyruvate, two ATP, and two NADH are produced via glycolysis. Lactate dehydrogenase catalyzes the conversion of pyruvate into lactate in the presence of NADH. Glucose and lactate are the two essential carbon sources in mammals. According to Hui et al., glucose feeds tissues indirectly via circulating lactate. The circulatory turnover flux of lactate was 1.1-fold or 2.5-fold higher than that of glucose in fed or fasting mice, respectively, suggesting lactate is the primary fuel for the TCA cycle in most tissues and thus energy, except the brain.

The lactate shuttle hypothesis has proposed the role of lactate as gluconeogenic or lipogenic precursors, oxidative substrates, and cellular signaling molecules. It describes that lactates produced in the cells with high energy sources can be transported to the other energy-demanding cells. Lactate can be used as a precursor for gluconeogenesis and lipogenesis. The lactate/pyruvate ratio regulates redox homeostasis, as it reflects the NADH/NAD ratio, which buffers redox stresses in various conditions. Moreover, lactate serves as a signaling molecule by activating lactate-specific receptor G protein-coupled receptor 81 (GPR81). GPR81’s role in various metabolism, including cancer angiogenesis, immune responses, lipid metabolism, neuronal development has been well demonstrated. Additionally, lactates’ role in regulating gene expression by histone lactylation has been recently established. For example, upon hypoxia or bacterial exposure, enhanced glycolysis results in increased lactate levels, which
can stimulate histone lactylation on 28 lysine residues, shifting M1-like macrophage into M2-like macrophage polarization\textsuperscript{160}. Altogether, these findings support the notion that lactate is no longer a waste product, but essential in metabolic homeostasis.

1.2.2 Monocarboxylate transporter family

MCTs are members of the solute carrier 16A (SLC16A) family. Among 14 MCT isoforms, only four human MCTs, MCT1 (SLC16A1), MCT2 (SLC16A7), MCT3 (SLC16A8), and MCT4 (SLC16A3), transport monocarboxylate molecules such as lactate, pyruvate, short-chain fatty acids, and ketone bodies\textsuperscript{161}. Recently, MCT7 (SLC16A6) was reported to participate in ketone body transportation in Zebrafish\textsuperscript{162}. MCT8 (SLC16A2), MCT9 (SLC16A9), and MCT10 (SLC16A10/TAT1) transport thyroid hormone, carnitine, and aromatic amino acids, respectively\textsuperscript{163-165}. The function of other isoforms is not well studied and needs more investigation.

MCT1-4 have distinct expression profiles, substrate affinity, and properties. Notably, MCT1/SLC16A1 is expressed in the broadest range of tissues, such as brain, heart, muscle, kidney, liver, intestine, testis, breast, placenta, and skin. Among many naturally occurring substrates, such as L-lactate, pyruvate, D-β-hydroxybutyrate, acetoacetate, acetate, and butyrate, L-lactate is quantitatively the most important metabolites to MCT1\textsuperscript{166}. Thus, MCT1 is considered a primary lactate transporter, widely distributed in various metabolic tissues, with a high affinity for lactate. This allows maintenance of basal cellular homeostasis.
according to transmembrane lactate gradients\textsuperscript{148,166}. MCT2 has considerable species differences and exhibits relatively low expression in human tissues, such as brain, liver, kidney, and testis\textsuperscript{167-169}. The substrate specificity of MCT2 is similar to that of MCT1, but MCT2 has a higher affinity for most of the substrates (Km for L-lactate: 0.74 mmol/L, Km for pyruvate: 0.1 mml/L) than MCT1 (Km for L-lactate: 3.5 mmol/L, Km for pyruvate: 1 mmol/L)\textsuperscript{166}. MCT3 is limitedly expressed in retinal pigment epithelium\textsuperscript{170,171}. MCT4 is expressed in multiple tissues that depend on glycolysis activity for energy metabolism, such as skeletal muscle, brain, white blood cells, etc. MCT4 has a low affinity for most of the substrates (Km for lactate: 28 mmol/L, Km for pyruvate: 153 mmol/L)\textsuperscript{172}

1.2.3 Role of MCT1 in metabolism

1.2.3.1 Diet-induced obesity-resistant MCT1 haploinsufficient mice

To investigate the role of MCT1 in overall metabolism, mice with MCT1 haploinsufficiency were first generated by the Pellerin group in 2013\textsuperscript{173}. Different from MCT1 whole-body KO mice that were developmentally lethal, mice with MCT1 haploinsufficiency were viable, grew healthy, and surprisingly were resistant to a high-fat diet or high-fat high-sucrose diet-induced obesity, insulin resistance, liver steatosis, and inflammation\textsuperscript{173-175}. There were significant reductions of plasma biochemical profiles in mice with MCT1 haploinsufficiency fed a high-fat diet for 12-16 weeks. Their serum ALT level, insulin level, and homeostasis model assessment (HOMA) index were reduced, suggesting improved metabolic health.
with MCT1 depletion in major tissues. Liver steatosis, monitored by histological H&E staining and accumulated liver triglycerides, was remarkably improved, and the cleavage of a major transcription factor regulating De novo lipogenesis, SREBP1 was prevented in mice with MCT1 haploinsufficiency under a high-fat diet.

1.2.3.2 Role of MCT1 in other metabolic tissues

MCT1 protein levels were reduced by nearly half in major metabolic tissues such as liver, brain, and white adipose tissues when the mice were resistant to diet-induced obesity, insulin resistance, liver steatosis, and inflammation\textsuperscript{173-175}. However, the question of which tissue or tissues are responsible for the phenotype of whole-body MCT1 haploinsufficiency is not solved.

The role of MCT1 in hypothalamus in these phenotypes was ruled out, as selective MCT1 depletion in those tissues increased food intake and body weight\textsuperscript{176}. MCT1 is highly expressed in tanycytes, highly polarized glial cells that border the basal third ventricle\textsuperscript{177}. Tanycytes facilitate lactate secretion to hypothalamic arcuate nucleus neurons\textsuperscript{178}. As a master regulator of neuronal and nutritional signals, arcuate nucleus neurons are considered a major hypothalamic glucose sensor\textsuperscript{179,180}. Recently, the communication between tanycytes and arcuate nucleus neurons via lactate is thought to be one of the pathways contributing to feeding behavior\textsuperscript{181,182}. Elizondo-Vegato et al. have shown that the injection of adenoviral shRNA targeting MCT1 into the third ventricle of rat in vivo successfully inhibits MCT1 in hypothalamic tanycytes\textsuperscript{176}. The MCT1 ablation significantly
impaired both orexigenic and anorexigenic neuropeptides expression, as well as increased food intake and body weight\textsuperscript{176}. Adversely, intracerebroventricular injection of 5mM lactate has been reported to lower the food intake and body weight in rats\textsuperscript{183,184}. These data suggested that lactate and MCT1 play an important role in nutritional sensing and food intake regulation, confirming that hypothalamic MCT1 KO does not reproduce the phenotypes shown in mice with MCT1 haploinsufficiency.

As selective MCT1 depletion in adipose tissues (MCT1 f/f X aP2 Cre) enhanced systemic inflammation and insulin resistance\textsuperscript{185}, the role of MCT1 in adipose tissues in beneficial phenotypes of MCT1 haploinsufficiency was ruled out as well. Lin et al. have demonstrated that selective MCT1 KO in adipose tissues blocks lactate release, thereby increasing intracellular lactate levels and decreasing circulating lactate under a 14-week of high-fat diet. The accumulated intracellular lactate in adipocytes initiates apoptosis and enhances cytokine release, recruiting macrophages. These processes amplify systemic inflammation and induce insulin systemic resistance. Additionally, \textit{in vitro} co-culture studies utilizing RAW264.7 macrophage cell lines and MCT1-deleted differentiated 3T3-L1 adipocytes confirmed that loss of MCT1 in adipocytes promoted migration of macrophage and enhanced pro-inflammatory markers. Interestingly, the treatment of apoptosis inhibitors successfully prevented inflammation caused by loss of MCT1 in adipose tissue. This data supports the idea that adipocyte MCT1 depletion enhances
systemic inflammation via apoptosis and assures that adipose tissue MCT1 KO cannot reproduce the phenotypes shown in MCT1 haploinsufficiency.

The above considerations suggest the possibility that MCT1KO in one or more liver cell types may explain the effects of MCT1 haploinsufficiency in mice. Since hepatocytes account for the majority of liver cells and have high rates of lipogenesis and triglyceride accumulation, lactate levels governed by hepatocyte MCT1 could be involved in regulating steatosis. Moreover, the role of MCT1 in hepatic stellate cells activation or fibrogenesis has not been investigated.

1.3 RNA interference (RNAi)

RNAi is the cellular phenomenon in which post-transcriptional gene silencing is caused by complementary sequences of double-stranded RNA\(^{186}\). First discovered in the \textit{C. elegans} in 1998, RNAi has since expanded its application and is now being used in therapeutic development\(^{187,188}\). Mechanistically, RNAi leads to enzymatic cleavage of target mRNA, reducing the corresponding protein translation. As it takes advantage of the complementary sequence of target mRNA, RNAi technology has high specificity and potent application overcoming the challenges that traditional pharmaceuticals approaches had. Development of synthetic small interfering RNAs (siRNAs) enables the new paradigm of drug development.

1.3.1 Small interfering RNAs (siRNAs)
siRNA is the product of RNase Dicer's enzymatic activity. As a part of the RNase III family, Dicer cleaves long double-stranded RNA into 21-25 nucleotide small fragments of double-stranded RNA (siRNA). This activates RNA-induced silencing complex (RISC), which embraces siRNA\textsuperscript{189}. Upon RISC activation, the sense (non-guided) strand is separated and discarded from the complex\textsuperscript{190}. The RISC containing the remaining antisense (guide) strand assists to find and bind to the target mRNA which has complementary sequences. The Argonaute 2 protein in RISC cleaves the mRNA, leading to mRNA degradation\textsuperscript{191}.

### 1.3.2 Development of RNAi-based therapeutics

Therapeutics based on RNA interference (RNAi) render a remarkable advantage over traditional pharmaceutical approaches\textsuperscript{192,193}. Not only is it applicable to target all classes of molecules which have difficulty in selective modulation by small molecules, but it can also ramp up the speed of highly potent and specific drug candidate identification.

The biggest hurdle in utilizing RNAi as therapeutics was the effectiveness of \textit{in vivo} delivery strategy including its stability and longevity against systemic nucleases. Recent development in siRNA duplexes' chemical modification strategies such as 2-Fluoro, 2-O-Methyl ribose, and phosphorothioate backbone replacement overcomes these challenges\textsuperscript{194}. Furthermore, as different conjugation to siRNA can direct its tissue or cell-type-specific biodistribution, various conjugates have been developed and tested in the field. For example, highly hydrophobic
cholesterol conjugates mostly distribute in the liver\textsuperscript{195}, but within the liver, it non-selectively targets multiple hepatic cell types as their uptake is highly dependent on membrane hydrophobic interaction. On the other hand, N-Acetyl-galactosamine conjugation is highly selective to hepatocytes, as it binds to the asialoglycoprotein receptor (ASGPR) which is primarily expressed in hepatocytes\textsuperscript{196}.

To date, five RNAi based therapeutic agents (patisiran, givosiran, lumasiran, inclisiran, and vutrisiran) have received FDA approvals for multiple disease areas including polyneuropathy, acute hepatic porphyria, primary hyperoxaluria type 1, and atherosclerotic cardiovascular disease, reinforcing the potential of RNAi therapeutics.

1.4 Study aims

The aim of the present studies was to investigate the role of hepatic lactate transport via MCT1 in lipid metabolism and fibrogenesis in NASH, and to determine its potential suitability as a therapeutic target. Two key unanswered questions were of particular interest: 1) is hepatocyte-specific MCT1 depletion that protects mice with MCT1 haploinsufficiency from liver lactate overload and NAFLD and 2) does liver stellate cell MCT1 promote hepatic fibrogenesis that occurs in NASH? We tested the possible enhancement of lipogenesis and fat accumulation via MCT1 function specifically in hepatocytes using adeno-associated virus (AAV) mediated thyroxin binding globulin (TBG)-Cre MCT1KO in MCT1\textsuperscript{fl/fl} mice, and in other experiments by silencing hepatocyte MCT1 with tri-N-acetyl galactosamine (GN)-
conjugated siRNA. These experiments showed hepatocyte MCT1 loss decreased expression of enzymes in the *de novo* lipogenesis (DNL) pathway but did not diminish overall steatosis. Surprisingly, hepatocyte MCT1KO increased liver fibrosis in two mouse models of NASH. In contrast, hepatic stellate cell-selective MCT1KO, achieved by injection of AAV9-lecithin-retinol acyltransferase (Lrat)-Cre into MCT1<sup>fl/fl</sup> mice, did attenuate collagen production and fibrosis. Our findings underscore the critical importance of implementing cell type-specific targeting strategies to diminish NASH fibrogenesis.
Chapter II. Method and Material

2.1 Oligonucleotide synthesis

The 15-20 or 18-20 sequence oligonucleotides were synthesized by phosphoramidite solid-phase synthesis on a Dr Oligo 48 (Biolytic, Fremont, CA), or MerMade12 (Biosearch Technologies, Novato, CA) using 2'-F and 2'-O-Me phosphoramidites with standard protecting groups (Chemgenes, Wilmington, MA).

For the 5’-VP coupling, 5’-(E)-Vinyl tetraphosphonate (pivaloyloxymethyl) 2'-O-methyl-uridine 3'-CE phosphoramidite was used (Hongene Biotech, Union City, CA). Phosphoramidites were prepared at 0.1 M in anhydrous acetonitrile (ACN), except for 2'-O-methyl-uridine phosphoramidite dissolved in anhydrous ACN containing 15% dimethylformamide. 5-(Benzylthio)-1H-tetrazole (BTT) was used as the activator at 0.25 M, coupling time for all phosphoramidites was 4 min. Detritylations were performed using 3% trichloroacetic acid in dichloromethane. Capping reagents used were CAP A (20% n-methylimidazole in ACN) and CAP B (20% acetic anhydride and 30% 2,6-lutidine in ACN). Phosphite oxidation to convert to phosphate or phosphorothioate was performed with 0.05 M iodine in pyridine-H2O (9:1, v/v) or 0.1 M solution of 3-[(dimethylaminomethylene)amino]-3H-1,2,4-dithiazole-5-thione (DDTT) in pyridine respectively. All synthesis reagents were purchased from Chemgenes. Unconjugated oligonucleotides were synthesized on 500Å long-chain alkyl amine (LCAA) controlled pore glass (CPG) functionalized with Unylinker terminus (ChemGenes). Chol conjugated
oligonucleotides were synthesized on a 500Å LCAA-CPG support, functionalized with a tetra-ethyleneglycol cholesterol moiety bound through a succinate linker (Chemgenes). GN conjugated oligonucleotides were grown on a 500Å LCAA-CPG functionalized with an aminopropanediol-based trivalent GalNAc cluster (Hongene).

2.2 Deprotection and purification of oligonucleotides for screening of sequences

Prior to the deprotection, synthesis columns containing oligonucleotides were treated with 10% diethylamine (DEA) in ACN to deprotect cyanoethyl groups. Synthesis columns containing the oligonucleotides covalently attached to the solid supports were cleaved and deprotected for 1 hour at room temperature with anhydrous Mono-Methylamine gas (Airgas). Columns with deprotected oligonucleotides were washed with 1mL of 0.1M sodium acetate in 85% ethanol aqueous solution, followed by rinse with an 85% ethanol aqueous solution. The excess ethanol was dried from the column on a vacuum manifold. Finally, the oligonucleotides were eluted off the columns with MilliQ water.

2.3 Deprotection and purification of oligonucleotides for in vivo experiments

Chol- or GN-conjugated oligonucleotides were cleaved and deprotected with 28-30% ammonium hydroxide and 40% aq. Methylamine in a 1:1 ratio for 2h at room temperature. VP-containing oligonucleotides were cleaved and deprotected as
described previously. Briefly, CPG with VP-oligonucleotides was treated with a solution of 3% diethylamine in 28–30% ammonium hydroxide for 20 hours at 35°C. The cleaved oligonucleotide solutions were filtered to remove CPG and dried under a vacuum. The pellets were resuspended in 5% ACN in water and purified on an Agilent 1290 Infinity II HPLC system. VP and GN-conjugated oligonucleotides were purified using a custom 20 x 150mm column packed with Source 15Q anion exchange resin (Cytiva, Marlborough, MA). Run conditions were the following. Eluent A: 20 mM sodium acetate in 10% ACN in water. Eluent B: 1 M sodium bromide in 10% ACN in water. Linear gradient 10–35% B 20 min at 40°C. Chol-conjugated oligonucleotides were purified using 21.2 x 150mm PRP-C18 column (Hamilton Co, Reno, NV). Run conditions were the following: Eluent A, 50 mM sodium acetate in 5% ACN in water; Eluent B: 100% ACN. Linear gradient, 40–60% B 20 min at 60°C. Flow used was 40mL/min for both systems. Peaks were monitored at 260 nm. Fractions collected were analyzed by liquid chromatography-mass spectrometry (LC-MS). Pure fractions were combined and dried under a vacuum and resuspended in 5% ACN. Oligonucleotides were desalted by size exclusion on a 50 x 250mm custom column packed with Sephadex G-25 media (Cytiva, Marlborough, MA), and lyophilized. Reagents for deprotection and purification were purchased from Fisher Scientific, Sigma-Aldrich, and Oakwood Chemicals.
2.4 LC-MS analysis of oligonucleotides

The identity of oligonucleotides is verified by LC–MS analysis on an Agilent 6530 accurate mass Q-TOF using the following conditions: buffer A: 100 mM 1,1,1,3,3,3-hexafluoroisopropanol (HFIP) (Oakwood Chemicals) and 9 mM triethylamine (TEA) (Fisher Scientific) in LC–MS grade water (Fisher Scientific); buffer B: 100 mM HFIP and 9 mM TEA in LC–MS grade methanol (Fisher Scientific); column, Agilent AdvanceBio oligonucleotides C18; linear gradient 0–35% B 5min was used for unconjugated and GN-conjugated oligonucleotides; linear gradient 50–100% B 5min was used for Cholesterol conjugated oligonucleotides; temperature, 60°C; flow rate, 0.85 ml/min. LC peaks are monitored at 260nm. MS parameters: Source, electrospray ionization; ion polarity, negative mode; range, 100–3,200 m/z; scan rate, 2 spectra/s; capillary voltage, 4,000; fragmentor, 200 V; gas temp, 325°C.

2.5 LX2 human hepatic stellate cell studies

Human hepatic stellate cell line, LX2, was purchased from Millipore Sigma, cat SCC064. LX2 cells were cultured in DMEM/high glucose media (Gibco, cat 11995-065 and Fisher, cat 11965092) with 10% FBS. To test the preventative effect of MCT1 depletion in Tgf1β-stimulated hepatic stellate cell conditions, LX2 cells were plated in 6 well plates (300k cells/well) or 12 well plates (150k cells/well) in DMEM/high glucose media with 2% FBS. The next day, cells were transfected with either NTC-siRNA or MCT1-siRNA (IDT, cat 308915476) using lipofectamine RNAi Max (Thermofisher, cat 13778075) for 6 hours in less serum optiMEM media.
(Thermofisher, cat31985062). Then, cells were maintained in serum-starved media with or without 10ng/ml of recombinant human Tgf1β (R&D Systems, cat 240-B/CF) for 48 hours and harvested. As a housekeeping gene, β-actin (ACTB) was used.

2.6 Human HepG2 hepatoma cell studies

Human hepatoma cell line, HepG2, was purchased from ATCC, cat HB-8065. Cells were cultured in RPMI media (Gibco, cat 11875-093) with 10% FBS. To test the effect of MCT1 depletion, cells were plated in 6 well plates (300k cells/well) or 12 well plates (150k cells/well). The next day, cells were transfected with either NTC-siRNA or MCT1-siRNA using lipofectamine RNAi Max (Thermofisher, cat 13778075) for 6 hours in less serum optiMEM media (Thermofisher, cat31985062). After 48 hours, HepG2 cells were harvested. The media were saved to further test for secreted factors that may affect hepatic stellate cell activation. LX2 cells were incubated with the conditioned media (40% conditioned media + 60% fresh media), and cells were harvested after 48 hours.

2.7 In vitro screening of chemically modified siRNAs

Mouse hepatocyte cell line FL83B was purchased from (ATCC, CRL-2390). FL83B cells were plated in 12 well plates (150k cells/well) in F-12K medium with 3% FBS. Then, 1.5uM of each Chol-MCT1-siRNA candidate compound was added and Chol-NTC-siRNA was used as a control. Then, 72 hours after the treatment, cells
were harvested, and the Mct1 mRNA silencing potency was monitored. To further evaluate the half maximal inhibitory concentration (IC50) values, the dose-dependent silencing effect of the compounds was calculated upon six different concentrations (1.5uM, 0.75uM, 0.38uM, 0.19uM, 0.05uM, and 0uM). As a housekeeping gene, β-2-microglobulin (B2m) was used.

2.8 Generation and Validation of hepatic stellate cell-specific AAV-Lrat-Cre

Lrat-Cre-mediated KO mice have been widely utilized in the field to delete genes in hepatic stellate cells. We newly synthesized AAV9-Lrat-Cre to establish an inducible hepatic stellate cell KO system in collaboration with Vector Biolabs. Proximal mouse Lrat promoter region from -1166bp, including the putative transcriptional start site (TSS), to +262 bp downstream sequence was chosen. A 1428b Lrat promoter was synthesized and cloned into Vector Biolabs' AAV-CMV-Cre vector to replace CMV promoter with Lrat promoter. The AAV-Lrat-Cre was then packaged into AAV9 virus. As a control, AAV-Lrat-null constructs were used.

2.9 Animal studies

All animal procedures were performed in accordance with animal care ethics approval and guidelines of University of Massachusetts Chan Medical School Institutional Animal Care and Use Committee (IACUC, protocol number A-1600-19). All wild-type C57BL6/J male mice (JAX, #Cat: 000664), and genetically obese
ob/ob male mice (JAX, #Cat: 000632) were obtained from Jackson Laboratory. MCT1 f/f mice were generated in the Rothstein lab\textsuperscript{198}. Mice were group-housed on a 12-hour light/dark cycle and had \textit{ad libitum} access to water and food. \textbf{For obese NASH model studies}, 10 week old genetically obese ob/ob male mice (n=6) were subcutaneously injected with 10mg/kg of siRNAs accordingly (Chol-NTC-siRNA, Chol-MCT1-siRNA, GN-NTC-siRNA, and GN-MCT1-siRNA), every 10-12 days. Mice were fed the GAN diet (Research diets, cat D09100310) for 3 weeks. Food intake and body weight were monitored. Mice were sacrificed with CO\textsubscript{2}, and double-killed with cervical dislocation. \textbf{For CDAHFD-induced NASH model studies}, 8 week old male MCT1 f/f mice (n=10) were intravenously injected with 2X10\textsuperscript{11}gc of AAV-TBG-Cre or AAV-Lrat-Cre or both. As a control, the same amount of AAV-TBG-null or AAV-Lrat-null control was used. A week after the injection, mice were fed a CDHFD (Research diets, cat A06071302i) for 8 weeks and sacrificed.

\textbf{2.10 Primary mouse cell isolation}

Male C57BL/6 wild-type mice 16-18 week old (n=4) were subcutaneously injected with 10mg/kg of siRNAs accordingly (Chol-NTC-siRNA, Chol-MCT1-siRNA, GN-NTC-siRNA, and GN-MCT1-siRNA), twice within 15 days. Mice were put on a chow diet (LabDiet, cat 5P76) and sacrificed on day 15. Primary hepatocytes, hepatic stellate cells, and Kupffer cells were isolated from the livers using the modified perfusion method previously described\textsuperscript{199,200}. Briefly, livers were digested
in situ with 14mg pronase (Sigma-Aldrich, cat P5147) and 3.7U collagenase D (Roche, cat 11 088 882 001) via inferior vena cava. Digested livers were isolated and minced with 0.5mg/ml pronase, 0.088U/ml collagenase, and 0.02mg/ml DNase I (Roche, cat 10 104 159 001). After centrifuging cells for 3min at 50g at 4C, primary hepatocytes were obtained in the pellet. The remaining supernatant was collected and centrifuged for 10 min at 580g at 4C and the pellet was saved for further hepatic stellate cell separation using Nycodenz (Accurate Chemical, cat 1002424) gradient solution. Lastly, Kupffer cells were isolated from the remaining cells using a Percoll (Sigma, cat P1644) gradient solution. Separation of hepatocytes, hepatic stellate cells, and Kupffer cells was validated using representative mRNA markers of each cell type such as Alb, Des, and Clec4f, respectively, by rt-qPCR.

2.11 Serum analysis

Retro-orbital bleeding was performed prior to sacrificing mice. Blood was collected in heparinized capillary tubes and centrifuged 10min at 7000 rpm at 4C. Supernatant plasma was saved for further serum analysis. Plasma lactate level was measured using a specific apparatus, Lactate Plus meter (Nova biomedical, cat 62624). ALT level was determined using ALT Colorimetric Activity Assay Kit (Cayman, cat 700260). Absorbances were detected using a Tecan safire2 microplate reader.
2.12 Triglyceride assay

Frozen mouse livers samples (50mg) were homogenized in NP40 lysis buffer with 1:100 ratio of phosphatase and protease inhibitor cocktail (Sigma-Aldrich) using the Qiagen TissueLyser II. Triglyceride levels were determined using the Triglyceride Colorimetric Assay Kit (Cayman, cat 10010303) which involved a series of enzymatic hydrolysis steps by lipase, glycerol kinase, glycerol phosphate oxidase, and peroxidase. The absorbances were detected using a Tecan safire2 microplate reader.

2.13 Glucose tolerance test

GTT was performed after 16 hours of fasting. Basal glucose level was measured using a glucometer (Contour NEXT ONE glucose Meter), then mice were Intraperitoneally injected with 1g/kg body weight D-glucose dissolved in sterile saline. Blood glucose was measured with a single drop of tail blood at 15, 30, 45, 90, and 120 minutes after the glucose injection.

2.14 Shear wave elastography

Mouse liver stiffness was monitored by Vega robotic ultrasound imager, SonoEQ 1.14.0 (SonoVol), as described in a previous study\textsuperscript{201}. Before SWE measurement, mice had their abdomen hair shaved and the residual hair was removed using chemical depilation cream (Nair). After being anesthetized with isoflurane, mice were located in prone position on the fluid chamber through an acoustically
transmissive membrane with ultrasound transducer imaging from below. During the imaging, wide-field B-mode was captured, a 3D volume was reconstructed, liver was visualized, and fiducial markers in 3D space indicating the position of the desired SWE capture were placed. Liver stiffness was monitored by Young’s modulus.

2.15 RNA isolation and rt-qPCR

Frozen mouse livers samples (25mg) or in vitro cell samples were homogenized in Trizol (Ambion) using Qiagen TissueLyser II. Chloroform was added and centrifuged for 15 min at maximum speed at 4C. The supernatant was collected and 100% isopropanol was added. After another 10 min centrifugation at maximum speed at 4C, the pellet was saved and washed with 70% ethanol with 5 min centrifugation at maximum speed at 4C. The pellet was dried briefly and resuspended with ultrapure distilled water (Invitrogen). cDNA was synthesized using 1ug of total RNA using iScript cDNA synthesis Kit (Biorad) on Bio-Rad T100 thermocycler. Real-time quantitative PCR was performed using iQ SybrGreen Supermix on CFX96 1000 thermocycler (Biorad) and analyzed as described. Primer sequences used for rt-qPCR were listed in Table 3.
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### Human primers:

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**Table 2.1. List of primers used for rt-qPCR**
2.16 Immunoblotting

Frozen mouse livers samples (50mg) or in vitro cell samples were homogenized in a sucrose lysis buffer (250mM sucrose, 50mM Tris-Cl pH7.4) with 1:100 phosphatase and protease inhibitor cocktail (Sigma-Aldrich) using Qiagen TissueLyser II. Protein concentration was determined by BCA assay. Immunoblotting loading samples were prepared after adjusting the protein concentration using 5X SDS (Sigma-Aldrich) and denatured by boiling. Proteins were separated in 4–15% sodium dodecyl sulfate/polyacrylamide gel electrophoresis gel (Bio-Rad) and transferred to nitrocellulose membranes. The membranes were blocked with Tris-buffered saline with Tween (TBST) with 5% skim milk or 5% bovine serum albumin. Membranes were incubated with primary antibodies overnight at 4C, washed in TBST for 30 mins, then incubated with secondary antibodies for an hour at room temperature, and washed for 30 min in TBST. Antibodies used in the studies were listed in Table 4. ECL (Perkin Elmer) was added to the membranes and the protein signals were visualized with ChemiDox XRS+ image-forming system.
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Table 2.2 List of antibodies used in this study
2.17 Immunofluorescence

FL83B cells were seeded in coverslips placed in 12 well plates (150k cells/well). Then, 1.5uM of either NTC-siRNA or MCT1-siRNA at final concentration were added for 72 hours. To stain mitochondria membranes, cells were incubated with Mitotracker at 37C (Thermofisher, cat M7512) for 45 min in serum-free media. Then, cells were fixed with 4% paraformaldehyde at room temperature for 30 min. Fixed cells were blocked by fresh permeabilization buffer (0.5% Triton, 1% FBS in PBS) at room temperature for 30 min and incubated with 1:100 Anti-MCT1 (Proteintech, cat 20139-1-AP) overnight at 4C. As a secondary antibody, 1:1000 goat-anti-Rabbit-488 was used, while cells were protected from light. Coverslips were mounted on Prolong Gold antifade Mountant with Dapi (Invitrogen, cat P35934). Images were acquired using an Olympus IX81 microscope (Central Valley, PA) with dual Andor Zyla sCMOS 4.2 cameras (Belfast, UK). Images were quantified using ImageJ software.

2.18 Histological analysis

For the immunohistochemistry (IHC), half of the biggest lobe of each mouse liver was fixed in 4% paraformaldehyde and embedded in paraffin. Sectioned slides were stained where indicated with H&E, Trichrome, Sirius Red, and anti-MCT1 (Proteintech, cat 20139-1-AP) at the University of Massachusetts Chan Medical School Morphology Core. The whole stained slides were scanned with ZEISS Axio Scan Z1. Images were analyzed by ZEN 3.0 and ImageJ software.
2.19 H&E lipid droplet analysis

To quantify the mean size and mean number of lipid droplets, H&E images further underwent thorough image analysis. The 2D RGB images (8 bits per channel) were read into the Fiji version\textsuperscript{203} based on ImageJ\textsuperscript{204}. An ImageJ macro language program was then written to analyze each image. First, the Labkit plugin\textsuperscript{205} was used to classify pixels as either lipid or background. The classifier used was trained on a few short line segments drawn in either lipid or background regions. The binary objects created then had their holes filled and were culled using "Analyze Particles" to eliminate objects larger than 10,000 pixels (typically veins). Then the "watershed" algorithm was used to separate touching lipid droplets, and "Analyze Particles" was used again, this time to keep objects with a circularity of 0.5-1 and a size of 40-5000 pixels. Pixel size was converted to um using a 0.47um/pixel width conversion ratio (so a 0.22 um\textsuperscript{2}/pixel conversion factor).

2.20 Trichrome and Sirius Red Image analysis for fibrosis

To quantify the % of fibrotic regions, 2D RGB images (8 bits per channel) of Sirius Red and Trichrome (without the hematoxylin stain) were read into the Fiji version\textsuperscript{203} based on ImageJ\textsuperscript{204}. Analyze Particles was used to threshold the Sirius Red images (20X magnification) in the green channel, keeping pixels with an intensity < 100 and object size > 100 pixels as fibrotic regions. Pixels in the Trichrome images (2.5X magnification) in the red channel with intensity < 60 and object size > 0.0005 pixels were considered as fibrotic regions.
2.21 Quantification and statistical analysis

All statistical analyses were calculated using GraphPad Prism 9 (GraphPad Software). A two-sided unpaired Student t-test was used for the analysis of the statistical significance between the two groups. For more than three groups, One-way ANOVA was used for the analysis of statistical significance. Data were presented as mean ± SD or otherwise noted. Differences were considered significant when p < 0.05 (*: p < 0.05, **: p < 0.005, and ***: p < 0.0005). Data were excluded only when a technical error occurred in sample preparation. Sample sizes were decided based on previous publications.
Chapter III. Results

3.1 MCT1 depletion prevents Tgf1β-stimulated type 1 collagen production in cultured human LX2 stellate cells.

MCT1 haploinsufficient mice exhibited resistance to a high-fat diet or high-fat high fructose diet-induced obesity, attenuated steatosis, and hepatic inflammation\textsuperscript{173,174}. However, the effect of MCT1 depletion on NASH fibrogenesis has not been investigated. As fate-tracing studies have revealed that 82-96\% of myofibroblasts are derived from hepatic stellate cells\textsuperscript{64}, we employed a simple \textit{in vitro} system utilizing LX2 human hepatic stellate cells to investigate effects of MCT1 silencing on expression of type 1 collagen, a major component of fibrosis. 6 hours after the transfection with lipofectamine and native MCT1-targeting siRNA (MCT1-siRNA) or nontargeted control (NTC)-siRNA, cells were treated with the most potent fibrogenic inducer Tgf1b (10ug/ml) for 48 hours. MCT1-siRNA diminished \textit{MCT1} expression by about 80\% (Figure 3.1A). As expected, Tgf1β stimulated expression of \textit{ACTA2} and collagen 1 isoform, \textit{COL1A1}, by several folds (Figure 3.1B, C). \textit{MCT1} silencing significantly inhibited Tgf1β-stimulated \textit{ACTA2} mRNA expression as well as collagen 1 protein production (Figure 3.1B, C), indicating cell-autonomous functions of MCT1 in hepatic stellate cells. Interestingly, siMCT1 does not reduce \textit{COL1A1} mRNA levels as significantly as collagen type1 protein level, suggesting more translational regulation of collagen production than transcriptional.
Figure 3.1 MCT1 depletion attenuates TGF-β-stimulated collagen 1 production in human hepatic stellate cell lines, LX2.

Cells were transfected with either NTC-siRNA or MCT1-siRNA for 6 hours. Then, TGF-β was treated to induce collagen production. 48 hours after the TGF-β treatment, cells were harvested and processed for rt-qPCR or Western blotting. (A) Mct1 mRNA expression levels. (B) Collagen 1 protein levels. Quantification was added below. (C) Representative fibrogenic marker genes, Acta2, and Col1a1 expression levels were monitored. (t-test, One way ANOVA, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)
3.2 Identification of potent chemically modified MCT1-targeting siRNA in vitro

Given the therapeutic potential of MCT1 silencing in preventing fibrogenesis (Figure 3.1), we aimed to develop MCT1-siRNA compounds, chemically modified for stability, potency and delivery in vivo for use in this research and potentially for therapeutic advancement. Our asymmetrical siRNA compounds used here for in vitro screening are composed of 15 double-stranded nucleotides with 5 nucleotides short overhanging single-strand (15-20) that promotes cellular uptake. To enhance the stability of the constructs, the 2′-OH of each ribose was modified to either 2′-O-methyl or 2′-fluoro. In addition, phosphorothioate linkage backbone modifications were applied to avoid exonuclease degradation. Tetra-ethylenglycol-cholesterol linker (Chol) was conjugated to the 3′ end-sense strand to enhance stability and cellular uptake of candidate compounds. Each Chol-conjugated, fully chemically modified MCT1-siRNA (Chol-MCT1-siRNA) candidate construct's sequence and targeting region on the Mct1 transcript is described in Table 3.1 and Figure 3.2A.

We performed in vitro screening to select the most potent Chol-MCT1-siRNA compounds that were initially synthesized (Figure 3.2A, B). Each Chol-MCT1-siRNA compound candidate was transfected into mouse hepatocyte FL83B cells. As opposed to native siRNA, our Chol-MCT1-siRNA does not require transfection reagents as it is fully chemically modified. The silencing effect on Mct1 mRNA was
monitored after 72 hours (Figure 3.2B). Several compounds elicited a silencing effect greater than 80% compared to the NTC-siRNA. The two most potent Chol-MCT1-siRNA, Chol-MCT1-2060 (IC50: 59.6nM, KD%: 87.2) and Chol-MCT1-3160 (IC50: 32.4nM, KD%: 87.7) were evaluated for their inhibitory effect on MCT1 protein levels (Figure 3.2D, E). Based on its IC50 value and silencing potency, Chol-MCT1-3160 construct was chosen for further studies in vivo (Table 3.2).
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</table>

**Table 3.1 Sequences of chemically modified siRNA candidates targeting MCT1 used in in vitro screening.**

siRNAs utilized in in vitro screening were a double-strand oligonucleotide comprised of 15 sense and 20 antisense nucleotides. The sequences of each candidate’s antisense and sense strands were listed. (P: 5’-Phosphate, #: phosphorothioate, m:2’-O- methyl, f: 2’-fluoro, Chol: tetra-ethylenglycol-cholesterol conjugate)
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Sense strands:

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</tr>
<tr>
<td>GN-MCT1-3160</td>
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</tr>
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</table>

Table 3.2 Sequences of the selected final chemically modified siRNA candidates targeting MCT1 used for in vivo studies
MCT1-3160 was selected for the final construct for in vivo studies. MCT1-siRNAs utilized in in vivo study was a double-strand oligonucleotide comprised of 18 sense and 20 antisense nucleotides. To sense strands, either Chol- or GN- was attached. (VP: 5’-(E)-vinyl phosphonate, #: phosphorothioate, m:2′-O- methyl, f: 2′-fluoro, Chol: tetra-ethylenglycol-cholesterol conjugate, GN: tri-N-Acetyl-galactosamine)
Figure 3.2 Screening of chemically modified Chol-MCT1-siRNA in vitro.
(A) Targeted regions of multiple Chol-MCT1-siRNA candidates on Mct1 transcript. (B) Silencing efficacy of each Chol-MCT1-siRNA candidate (1.5uM) on Mct1 mRNA expression levels was monitored 72 hours after the transfection in mouse hepatocyte cell lines, FL83B in vitro. Chol-NTC-siRNA was used as a control. (C) Dose-response potency
test was performed to identify the most potent Chol-MCT1-siRNA compound. IC50 values were determined using six serially diluted concentrations of each compound starting from 1.5uM. IC50 values and knockdown % of the two most potent compounds were shown in the table below. (D) 72 hours after the transfection of either Chol-MCT1-2060 or Chol-MCT1-3160 compounds (1.5uM), their silencing efficacy on MCT1 protein expression levels was examined by Western blotting. (E) MCT1 protein expression levels upon MCT1-siRNA transfection were visually monitored by immunofluorescence.
3.3 Distinctive hepatic cellular biodistribution of Chol- and GN-conjugated-siRNA

For in vivo studies, further chemical modifications were applied (Figure 3.3A). MCT1-siRNAs utilized in in vivo studies are double-strand oligonucleotides comprised of 18 sense and 20 antisense nucleotides. At the 5’-end of the antisense strand, a 5’-(E)-Vinyl-phosphonate modification was added to prevent phosphatase-induced degradation, enhancing in vivo stability and promoting its accumulation in target cells. Either a hydrophobic Chol or a hepatocyte-targeting GN was attached to the 3’-end of sense strands of MCT1-siRNAs to direct different hepatic cellular biodistribution.

To validate the biodistribution of siRNAs with these two conjugates, 10mg/kg of each siRNA was subcutaneously injected into 16-18 weeks-old male C57BL/6 wild-type mice twice within a 15 day period. On day 15, mice were sacrificed and the livers were perfused to isolate multiple hepatic cell types, including hepatocytes, stellate cells, and Kupffer cells. Isolation of each hepatic cell type was validated for enrichment (Figure 3.4A-F). As expected, GN-conjugated, fully chemically modified MCT1-siRNA (GN-MCT1-siRNA) silenced Mct1 mRNA only in the hepatocyte fraction (Figure 3.3B-D), as GN binds to the asialoglycoprotein receptor primarily expressed in hepatocytes. On the other hand, Chol-MCT1-siRNA silenced Mct1 mRNA levels in all hepatic cell types (Figure 3.3E-G), as its cellular uptake is highly dependent on the non-specific, hydrophobic interaction between
cholesterol and plasma membranes. Notably, the hepatic stellate cell fraction distinguishes GN-MCT1-siRNA from Chol-MCT1-siRNA in biodistribution, as only the latter silences Mct1 in stellate cells (Figure 3.3C vs F). We also confirmed that both GN- MCT1-siRNA and Chol-MCT1-siRNA do not affect MCT1 levels in other major metabolic tissues (Figure 3.4G-J).
Figure 3.3 Biodistribution of Chol- and GN-MCT1-siRNA in the liver.
Male C57BL/6 wild-type mice (16-18 wk, n=4) were subcutaneously injected with 10mg/kg of each siRNA, twice within 15 days, while fed a chow diet. Mice were sacrificed on day 15. (A) Chemical structure of the fully chemically modified siRNA that was used for further in vivo studies; Chol-MCT1-siRNA and GN-MCT1-siRNA. (B, E) Primary hepatocytes, (C, F) stellate cells, and (D, G) Kupffer cells were isolated from each mouse using different gravity centrifugations and gradient solutions after the liver perfusion. Mct1 mRNA expression levels in each cell type fraction were measured. (t-test, *: p<0.05, **: p<0.01)
Male C57BL/6 wild-type mice (16-18 wk, n=4) were subcutaneously injected with 10mg/kg of siRNAs twice within 15 days. Livers were perfused through inferior vena cava and multiple liver cells were isolated using different gravity centrifugations and gradient
solutions. (A, D) Purity of isolated hepatocytes, (B, E) hepatic stellate cells, (C, F) and Kupffer cells was validated with representative marker genes, Alb, Des, and Clec4f expression, respectively. (G, H) MCT1 protein expression levels in multiple fat tissues (inguinal white adipose tissue, gonadal white adipose tissue, and brown adipose tissue) were monitored by immunohistochemistry. % MCT1 positive areas were quantified. (H, J) MCT1 protein expression levels in multiple tissues (heart, lung, kidney, spleen, and intestine) were monitored by immunohistochemistry. % MCT1 positive areas were quantified. (t-test, One way ANOVA, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)
3.4 Subcutaneous injection of Chol-MCT1-siRNA and GN-MCT1-siRNA successfully silenced hepatic MCT1 in a genetically obese mouse model

We next investigated the effect of Chol-MCT1-siRNA on reversing severe steatosis in the genetically obese ob/ob mouse on a NASH-inducing Gubra Amylin NASH (GAN)122 diet. As ¼ of the world population develops steatosis, and ¼ of them progress into the detrimental effects of NASH, it is the patient with severe steatosis but not yet developed fibrosis who needs the proactive medication the most. Our genetically obese NASH mouse model fits well for this purpose, as these mice normally develop severe steatosis from an early age but hardly develop fibrosis until fed the GAN diet.

Each siRNA (10mg/kg) was subcutaneously injected once every 10 days and mice were fed a GAN diet for 3 weeks before sacrifice (Figure 3.5A). Hepatic MCT1 protein levels were visually monitored by MCT1-positive staining immunohistochemistry (Figure 3.5B, C), showing more than 70% MCT1 protein depletion (Chol-MCT1-siRNA: 77.99% & GN-MCT1-siRNA: 71.35% silencing). Similar silencing was observed when Mct1 mRNA levels were measured by rt-qPCR (Figure 3.5D). Importantly, there was no surge in plasma lactate level (Figure 3.5E), addressing the concern of potential lactic acidosis after MCT1 depletion in the liver, the major lactate-consuming tissue. We also monitored food intake and body weight over time (Figure 3.6A, B), as there was a report of decreased food anticipation activity upon hepatic MCT1 deletion followed by
reduced plasma β-hydroxybutyrate levels\textsuperscript{207}. Intriguingly, the GN-MCT1-siRNA administration led to a decrease in both food intake and body weight, while the Chol-MCT1-siRNA did not. Neither Chol-MCT1-siRNA administration nor hepatocyte-specific MCT1KO improved glucose tolerance on the genetically obese NASH mouse model or a 12 week HFD-induced NAFLD model, respectively (Figure 3.7A, B).
Figure 3.5 Hepatic MCT1 depletion did not resolve steatosis in a genetically obese NASH mouse model.

(A) Male ob/ob mice (10 wk, n=6) were subcutaneously injected with 10mg/kg of siRNA once every 10 days. Mice were fed a GAN diet for 3 weeks and sacrificed. (B) Livers were stained with MCT1 antibody and the representative images of each group are shown. (C) % of MCT1 positive area shown in immunohistochemistry images were quantified. (D) Hepatic Mct1 mRNA level was measured by rt-qPCR upon MCT1-siRNA administration. (E) Plasma lactate levels were monitored. (F) Mean size of lipid droplets was quantified from H&E images (mean, sem). (G) Mean number of lipid droplets was quantified from H&E images (mean, sem). (H) Liver TG levels were examined in each group.
Figure 3.6 GN-MCT1-siRNA decreased food intake and body weight.
Male ob/ob mice (10 wk, n=6) were subcutaneously injected with 10mg/kg of siRNA. Mice were fed a GAN diet for 3 weeks and sacrificed. (A) Accumulative food intake and (B) body weight were monitored during the study. (t-test, *: p<0.05)
Figure 3.7 Neither Chol-MCT1-siRNA administration nor hepatocyte-specific MCT1KO improved glucose tolerance.

(A) Male ob/ob mice (8 wk, n=4) were subcutaneously injected with 10mg/kg of either Chol-NTC-siRNA or Chol-MCT1-siRNA once every 10 days. Mice were fed a GAN diet for 3 weeks. Then, a GTT was performed after 16 hours of fasting.

(B) Male MCT1 fl/fl mice (n=6) were intravenously injected with 1x10^{11}gc of either AAV-TBG-Cre or AAV-Lrat-Cre. Mice were fed a high-fat diet for 12 weeks. Then, a GTT was performed after 16 hours of fasting.
3.5 Hepatic MCT1 depletion downregulates lipogenic genes but not steatosis in the ob/ob NASH diet mouse model

In order to fully analyze steatosis in NASH, lipid droplet morphology and total hepatic TG were assessed (Figure 3.5F-H). The results showed that neither GN-MCT1-siRNA nor Chol-MCT1-siRNA decreased total hepatic TG levels (Figure 3.5H), although quantitative analysis of H&E images showed a small decrease in mean lipid droplet size and increased number of lipid droplets upon MCT1 silencing (Figure 3.5F, G). These data suggest the possibility that hepatic MCT1 depletion either 1) inhibits formation or fusion of lipid droplets, or 2) enhances lipolysis to diminish lipid droplet size.

To investigate the underlying mechanism by which lipid droplet morphological dynamics change, we monitored the effect of hepatic MCT1 depletion on DNL-related gene expression. Both GN-MCT1-siRNA and Chol-MCT1-siRNA strongly decreased the mRNA and protein levels related to representative DNL genes (Figure 3.8A-D). Intriguingly, both modes of hepatic MCT1 depletion also inhibited expression of the upstream regulatory transcription factors SREBP1 and ChREBP. Because phosphorylated AMPK (pAMPK), an active form of AMPK, is known to inhibit SREBP nuclear translocation\textsuperscript{208} as well as the DNA binding activity of ChREBP\textsuperscript{209}, we evaluated pAMPK levels. As a result, there was a significant increase in pAMPK levels and pAMPK/AMPK ratio in both GN-MCT1-siRNA and Chol-MCT1-siRNA injected groups (Figure 3.8E, F).
Figure 3.8 Both Chol-MCT1-siRNA and GN-MCT1-siRNA significantly decreased hepatic DNL gene expression.

Representative DNL gene expression levels were measured in (A, B) mRNA and (C, D) protein upon Chol-siRNA or GN-siRNA administration, respectively. Protein expression levels were quantified. (E, F) pAMPK and AMPK protein levels and their expression ratio were quantified. (t-test, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001).
3.6 Opposite effects of Chol-MCT1-siRNA versus GN-MCT1-siRNA on fibrotic collagen expression

We next monitored fibrosis, a tissue damaging phenotype that is associated with NASH. Surprisingly, there were opposite phenotypes on fibrogenic gene expression upon GN-MCT1-siRNA versus Chol-MCT1-siRNA administration. Hepatocyte-specific GN-MCT1-siRNA actually enhanced the expression of type 1 collagen protein in these experiments (Figure 3.9A, B), and this effect was also apparent at the mRNA expression level (Figure 3.9C). An opposite phenotype on collagen 1 protein expression was observed in response to administration of Chol-MCT1-siRNA (Figure 3.9D, E). Consistent with the results in LX2 stellate cells (Figure 3.1), Chol-MCT1-siRNA administration to ob/ob mice on GAN diet significantly reduced liver collagen 1 protein levels (Figure 3.9D, E). This result could be attributable to the fact that subcutaneous injection of the Chol-MCT1-siRNA compound is able to silence genes in hepatic stellate cells, the predominant cell type that produces collagens. Interestingly, decreases in mRNA encoding collagen 1 isoforms were not detected by rt-qPCR analysis (Figure 3.9F), indicating possible effects at the level of translation or protein turnover.

Overall fibrosis as detected by Sirius Red was also analyzed in these experiments, as this staining detects all types of collagen fibers that are involved in hepatic fibrosis, such as III, IV, V, and VI. In line with collagen 1 mRNA and protein levels, GN-MCT1-siRNA significantly enhanced Sirius red positive areas in the images,
as shown in Figure 3.9G, H. Despite its inhibitory effect on collagen 1 production levels, Chol-MCT1-siRNA did not reduce Sirius Red positive areas, suggesting that either reduction of major type 1 collagen might is not the major contributor to fibrosis in this model or that compensatory effects are occurring.
Figure 3.9 Opposite effects of Chol-MCT1-siRNA versus GN-MCT1-siRNA on fibrotic type 1 collagen expression.
Representative fibrogenic gene expression levels were measured for (A, B) mRNA and (C, D) protein. (E, F) Protein expression levels were quantified. (G) Livers were stained with Sirius Red and the representative images of each group are shown. (H) % of Sirius Red positive areas were quantified. (t-test, *: p<0.05, **: p<0.01, ***: p<0.001)
3.7 Our AAV-Lrat-Cre constructs selectively target HSC in vivo

The results presented above suggested that Chol-MCT1-siRNA downregulates type 1 collagen protein by depleting MCT1 in hepatic stellate cells, which hepatocyte-specific GN-MCT1-siRNA cannot target. To test this hypothesis, we developed and validated AAV9-Lrat-Cre constructs to generate hepatic stellate cell-specific MCT1 knockout mice. Male MCT1$^{fl/fl}$ mice were intravenously injected with AAV9-Lrat-Cre (1X$10^{11}$gc) and sacrificed 3 weeks later (Figure 3.10A). Isolation of hepatocytes and hepatic stellate cells were validated with their representative marker genes encoding albumin and desmin, respectively (Figure 3.10B, C). Successful depletion of hepatic stellate cell selective Mct1 mRNA was confirmed in the MCT1$^{fl/fl}$ mice injected with the AAV9-Lrat-Cre construct. Mct1 mRNA levels in the hepatocytes, which accounts for up to 70% of total liver cell types, were intact (Figure 3.10D, E). No change in MCT1 protein level was observed in other metabolic tissues (Figure 3.10F, G).
Figure 3.10 Intravenous injection of AAV9-Lrat-Cre in MCT1 fl/fl mice specifically targets hepatic stellate cells.

(A) Male mice (9-10wk, n=4) were intravenously injected with 1×10^{11}gc of either AAV9-Lrat-null or AAV9-Lrat-Cre. Mice were fed a chow diet for 3 weeks and sacrificed. Isolation of either (B) primary hepatocytes or (C) stellate cells was validated with each cell type's representative marker, albumin and desmin, respectively. Mct1 mRNA expression levels in (D) hepatocyte or (E) stellate cell fractions were examined. (F) MCT1 protein expression levels in multiple fat tissues (inguinal white adipose tissue, gonadal white adipose tissue, and brown adipose tissue) were monitored by immunohistochemistry. % MCT1 positive areas were quantified. (G) MCT1 protein expression levels in multiple tissues (heart, lung, kidney, spleen, and intestine) were monitored by immunohistochemistry. % MCT1 positive areas were quantified. (t-test, *: p<0.05, ****: p<0.0001)
3.8 MCT1 depletion did not resolve steatosis in the choline-deficient high-fat diet-induced NASH model in vivo

To test the hypothesis that Chol-MCT1-siRNA prevents fibrosis by depleting MCT1 in hepatic stellate cells, which hepatocyte-specific GN-MCT1-siRNA cannot target, we compared the role of MCT1 in hepatocytes and stellate cells utilizing AAV-TBG-Cre and AAV-Lrat-Cre, respectively. In this experiment, another NASH model, CDHFD-induced NASH, was employed. CDHFD induces severe steatosis due to inhibited VLDL secretion and β-oxidation, and thereby exacerbates NASH fibrosis in a relatively short time\textsuperscript{210,211}.

MCT1\textsuperscript{fl/fl} mice were intravenously injected with 2X10\textsuperscript{11}gc of AAV8-TBG-Cre or AAV9-Lrat-Cre or both (Figure 3.11A). A week after the injections, mice were fed with a CDHFD for 8 weeks to induce NASH. Depletion of hepatic Mct1 mRNA in each group was confirmed (Figure 3.11B). There was no food intake or body weight difference between the groups (Figure 3.11C, D). Similar to the results we obtained by MCT1 silencing with siRNAs (Figure 3.2), MCT1 deletion in either hepatocytes or in hepatic stellate cells did not resolve steatosis (Figure 3.11E, F).
Figure 3.11 MCT1 depletion did not resolve steatosis in the CDHFD-induced NASH model.

(A) Male MCT1 f/f mice (8 wk, n=10) were intravenously injected with 2X10^{11}gc of AAV-TBG-Cre or AAV-Lrat-Cre or both. The same amount of AAV-TBG-null or AAV-Lrat-null was used as a control. A week after the injection, mice were fed a CDHFD for 8 weeks and sacrificed. (B) Mct1 mRNA expression levels in whole livers were examined. (C) Food intake and (D) body weights were monitored. (E) CDHFD-induced steatosis was monitored by H&E. (F) % of lipid droplet areas was quantified. (t-test, One way ANOVA, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)
3.9 Hepatocyte-specific MCT1KO accelerated fibrosis, while hepatic stellate cell-specific MCT1KO decreased it

The effects of MCT1 depletion on fibrogenesis were then monitored. Consistent with results obtained by hepatocyte selective MCT1 silencing with GN-MCT1-siRNA (Figure 3.9), hepatocyte-specific knockout of MCT1 (Hep KO) enhanced the collagen 1 level compared to the control group (Figure 3.12A) as expected. In contrast, hepatic stellate cell-specific MCT1 knockout (HSC KO) prevented CDHFD-induced collagen 1 protein levels (Figure 3.12B). MCT1KO in combined hepatocytes and hepatic stellate cells blunted the effect shown in each single KO (Figure 3.12C).

Overall liver fibrosis detected by trichrome staining again confirmed the acceleration of fibrosis in the Hep KO group and a downward trend in the HSC KO group (Figure 3.12D, E). Dual MCT1KO in hepatocytes plus hepatic stellate cells showed no change in overall fibrosis, similar to the Chol-MCT1-siRNA results (Figure 3.9G, H). Additionally, liver stiffness was monitored via ultrasound-based shear wave elastography (SWE) in a noninvasive diagnostic mode for liver disease\textsuperscript{201,212}. After 8 weeks of CDHFD, all groups had the same level of increased liver stiffness above what the control mice showed at 4 weeks; however, Hep KO mice exhibited elevated liver stiffness over all other groups at 4 weeks of the diet (Figure 3.12F, G). There was no change in plasma alanine transaminase (ALT) levels among the groups (Figure 3.12H). It reassured the opposite roles of
hepatocyte MCT1 versus hepatic stellate cell MCT1 in multiple NASH models, reinforcing the importance of considering cell type specificity while developing a therapeutic target for NASH fibrosis.
Figure 3.12 Hepatocyte-specific MCT1KO accelerated fibrosis, while hepatic stellate cell-specific MCT1KO decreased it.

Male MCT1 f/f mice (6 wk, n=10) were intravenously injected with 2X10^{11}gc of AAV-TBG-Cre or AAV-Lrat-Cre or both. The same amount of AAV-TBG-null or AAV-Lrat-null was used as a control. A week after the injection, mice were fed a CDHFD for 8 weeks and sacrificed. (A) Collagen 1 protein levels were compared between the control and the hepatocyte MCT1KO groups. (B) Collagen 1 protein levels were compared between the control and the hepatic stellate cell MCT1KO groups. (C) Collagen 1 protein levels were compared between the control and the hepatocyte and hepatic stellate cell MCT1KO groups. (D) Trichrome staining was used to visualize fibrosis. (E) The percentage of trichrome area was quantified. (F, G) Stiffness measured by SWE (4wk and 8wk CDHFD). (H) Alanine Transaminase (ALT) activity.
control and the hepatic stellate cell MCT1KO groups. (C) Collagen 1 protein levels were compared between the control group and MCT1KO in both hepatocyte and hepatic stellate cell groups. (D) Livers were stained with Trichrome and the representative images of each group were shown. (E) Trichrome staining images were quantified. (F) Liver stiffness was monitored 4 weeks after CDHFD feeding via SWE. (G) Liver stiffness was monitored 8 weeks after CDHFD feeding via SWE. (H) ALT levels were measured in every CDHFD-fed group. (t-test, One way ANOVA, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)
3.10. MCT1 depletion enhanced fibrogenic gene expression levels in human hepatoma cell lines, HepG2

To investigate if the detrimental effect of hepatocyte MCT1-targeting translates in human cells, we assessed the effect of MCT1 depletion on fibrogenic gene expression levels in a human hepatoma cell line HepG2. A day after seeding the HepG2 cells, cells were transfected with either siNTC or siMCT1 for 6 hours. After 48 hours, HepG2 cells were harvested, and media were saved. LX2 cells were provided with the conditioned media (40% conditioned media + 60% fresh media). After 48 hours, LX2 cells were harvested. siMCT1 successfully depleted MCT1 mRNA levels (Figure 3.13A) and it significantly enhances fibrogenic gene markers ACTA2, COL1A1 (Figure 3.13B). HepG2-derived conditioned media-treated LX2 cells did not show any change in fibrogenic genes (Figure 3.13C). It suggested that enhanced fibrogenesis that has been shown in vivo studies after GN-MCT1-siRNA treatment or Hep KO would be attributed to the direct effect of hepatocyte MCT1 at least primarily.
Figure 3.13 MCT1 silencing enhanced fibrogenic gene expression levels in human hepatoma cell lines, HepG2.

Cells were transfected with either NTC-siRNA or MCT1-siRNA for 6 hours. 48 hours after, cells were harvested, and media were collected. LX2 cells were provided with the conditioned media (40% conditioned media + 60% fresh media) and harvested in 48 hours. (A) MCT1 mRNA expression levels were measured in HepG2 cells upon MCT1-siRNA treatment. (B) Fibrogenic gene expression levels were measured in HepG2 cells upon MCT1-siRNA treatment. (C) Fibrogenic gene expression levels were measured in LX2 cells upon conditioned media treatment. (t-test, *: p<0.05, **: p<0.01)
3.11 A combination therapy targeting both hepatic MCT1 and DGAT2 has no synergistic effect on alleviating steatosis in an obese NASH mouse model

Even though we repeatedly observed an obvious reduction in collagen type 1 protein levels in both chol-MCT1-siRNA and MCT1 HSC KO groups, it was not enough to resolve overall fibrosis detected in Sirius red or Trichrome staining. Since both Chol-MCT1-siRNA and MCT1 KO in stellate cells were not able to attenuate severe steatosis assessed in NASH models, we next investigated if reducing hepatic lipotoxicity and steatosis could assist MCT1 depletion in stellate cells to resolve overall liver fibrosis. To alleviate lipotoxicity and steatosis, we utilized GN-DGAT2-siRNA (DGAT2-1473), which our lab has previously reported to have an effect on resolving hepatic steatosis but not hepatic fibrosis in the rapid NASH model\textsuperscript{122}. We designed a dual targeting study that employed both Chol-MCT1-siRNA and GN-DGAT2-siRNA.

9-week-old male ob/ob mice (n=6) were divided into four groups: Control, Chol-MCT1-siRNA, GN-DGAT2-siRNA, and Combination group. Mice were subcutaneously injected with 10mg/kg of siRNA as indicated. Either Chol-NTC-siRNA or Chol-MCT1-siRNA was injected every 10 days. Either Chol-NTC-siRNA or Chol-MCT1-siRNA was injected every 10 days. GN-NTC-siRNA or GN-DGAT2-siRNA was injected only once at the beginning of the study, as we previously reported that the silencing potency of GN-DGAT2-siRNA lasted more than 12 weeks after a single injection\textsuperscript{122}. One week after the first injection of each siRNA,
mice were fed a GAN diet for 4 weeks and sacrificed (Figure 3.14A). Both Chol-MCT1-siRNA and GN-DGAT2-siRNA successfully silenced Mct1 and Dgat2. Interestingly, Dgat2 silencing enhanced Mct1 mRNA levels (Figure 3.14B). As expected, GN-DGAT2-siRNA significantly decreased liver-to-body weight ratio, and the same effect remained in combination group as well (Figure 3.14C). We then monitored if there was an additive or synergistic effect on resolving hepatic steatosis. Chol-MCT1-siRNA could not resolve steatosis, which is consistent with our previous findings. Unexpectedly, however, GN-DGAT2-siRNA did not alleviate steatosis in the obese NASH model under the conditions of these experiments. Additionally, the combination group did not decrease steatosis as monitored in H&E staining and hepatic triglyceride levels (Figure 3.14D, E).
Figure 3.14 A combination therapy targeting both hepatic MCT1 and DGAT2 has no synergistic effect on alleviating steatosis in an obese NASH mouse model. (A) 9 weeks old male ob/ob mice (n=6) were divided into four different groups and subcutaneously injected with 10mg/kg of siRNA as indicated. Either Chol-NTC-siRNA or Chol-MCT1-siRNA was injected every 10 days. GN-NTC-siRNA or GN-DGAT2-siRNA were injected only once at the beginning of the study. One week after the first injection of siRNA, mice were fed a GAN diet for 4 weeks and sacrificed. (B) Mct1 and Dgat2 mRNA levels in whole livers were measured by rt-qPCR. (C) Liver to body weight ratio was calculated. (D) Steatosis was monitored by H&E. (E) Liver TG levels were examined in each group. (One-way ANOVA, *: p<0.05, **: p<0.01, ***: p<0.001, ****: p<0.0001)
3.12 Synergistic effect of MCT1 and DGAT2 dual targeting strategy on NASH fibrogenesis

We then tested if there was a synergistic effect on collagen production and fibrogenesis. The results of the study showed that the combination strategy targeting both hepatic MCT1 and DGAT2 has a remarkable synergistic effect on alleviating fibrosis. Not only type 1 collagen but also type 3 collagen levels were significantly reduced in the combination group (Figure 3.15A, B). Immunohistochemistry of type 1 collagen staining examined in the whole liver exhibited the same trend (Figure 3.15C). Additionally, liver stiffness was monitored via ultrasound-based shear wave elastography (SWE) in a noninvasive diagnostic mode for liver disease. It confirmed the softening of the liver in the combination group (Figure 3.15D). Altogether, the study suggests that targeting both MCT1 and DGAT2 in liver may be a promising therapeutic strategy for alleviating NASH fibrosis.
Figure 3.15 Synergistic effect of MCT1 and DGAT2 dual targeting strategy on NASH fibrogenesis.

(A, B) Type 1 and 3 collagen protein levels were monitored by Western blotting, and the expression levels were quantified. (C) Livers were stained with Type 1 collagen and the representative images of each group are shown. % of COL1 positive area shown in immunohistochemistry images were quantified. (D) Liver stiffness was measured by shear wave elastography. (One-way ANOVA, *: p<0.05, **: p<0.01).
Chapter IV. Discussion

4.1 Significance of the study

In our study, we applied two different siRNA conjugate molecules that have distinct biodistribution in multiple hepatic cell types, Chol- and GN-siRNAs, and a novel AAV9-Lrat-Cre construct to elucidate the roles of hepatic MCT1 in NASH progression. Here we demonstrated that 1) hepatic MCT1 regulates the expression of SREBP1 and ChREBP, the major transcription factors regulating lipid metabolism, as well as their target DNL genes, and 2) MCT1 in hepatocytes and fibrogenic hepatic stellate cells has opposite roles in liver collagen production and fibrogenesis.

To understand the underlying mechanism by which hepatic MCT1 depletion drives the downregulation of DNL gene expression, we investigated AMPK activation (Figure 3.8). Phosphorylated AMPK has a negative regulatory effect on SREBP1 and ChREBP activation\textsuperscript{208,209}. We showed that hepatic MCT1 silencing enhanced AMPK phosphorylation, consistent with the previous MCT1 haploinsufficient mice study \textsuperscript{174}. This was in line with another study’s data in which MCT1 inhibition reduced ATP production and activated AMPK, thus deactivating SREBP1c and lowering levels its target SCD1\textsuperscript{213}. It remains to be examined whether other mechanisms are also at play that connect MCT1 function in liver to DNL gene regulation.
In our genetically obese NASH model study, despite significantly decreasing DNL gene expressions after hepatic MCT1 depletion, there remains unresolved liver steatosis. Although a slight decrease in mean lipid droplet size was observed (Figure 3.5), this could be attributed to adipose tissue lipolysis as a remaining hepatic fatty acid source, as DNL only accounts for 25% of total hepatic fatty acid accumulation, while adipose tissue lipolysis is up to 65%\(^{19}\).

However, given that the previous MCT1 haploinsufficiency study showed cleared HFD-induced hepatic steatosis, the discrepancies of the NAFLD models should be considered as well. Our genetically obese NASH model exhibited a different level of disease severity with a genetic and diet-combined underlying pathogenesis. At 10 weeks old when we started MCT1-siRNA administrations, the mice had already developed severe steatosis. Thus, our hepatic MCT1 silencing studies focused on reversing existing severe steatosis rather than characterizing the prevention of high-fat diet-induced steatosis. Moreover, our MCT1-siRNA were liver-specific and did not affect MCT1 expression in major tissues including iWAT, gWAT, BAT, intestine, heart, lung, kidney, and spleen (Figure 3.4). We also did not rule out the possibility of compensatory effects from other MCT isoforms. Although MCT1 is the primary lactate transporter in the liver, there remains a possibility that lactate flux was partially compensated by other transporters, preventing steatosis to be fully alleviated. Lastly, hepatic MCT1 silencing did not suppress certain genes that mediate lipid metabolism, in contrast to the haploid-insufficiency studies. For example, the triglyceride synthesis enzyme *Dgat2* was not decreased in the MCT1-
siRNA study. Major lipogenic transcription factors *Pparg* and *Cd36* and *Fatp2* (Slc27a2), which are involved in hepatic fatty acid uptake were even enhanced, (data not shown). This could be also attributed to the discrepancy between the two studies. Overall, it is clear from our studies that steatosis is not much affected by hepatic MCT1 depletion at least in two NASH mice models (ob/ob + GAN diet or CDHFD-induced NASH model)

The major finding of this study is that MCT1 function in hepatic stellate cells promotes collagen 1 expression, as MCT1 depletion in this cell type, either *in vitro* in cell culture (Figure 3.1) or *in vivo* in mice (Figure 3.12), attenuates fibrotic collagen 1 protein production. Surprisingly, however, MCT1KO in hepatocytes, both *in vitro* (Figure 3.13) and *in vivo* (Figure 3.12), evoked the opposite effect: a robust upregulation of collagen 1 expression and fibrosis. Our finding of the opposite role of MCT1 in hepatocytes and stellate cells provides new insight into previously unknown functions of MCT1 in fibrogenesis and reinforces the importance of cell-type-specific targeting strategies in therapeutic development. This may be in part a cell-autonomous effect in hepatocytes, as we observed increased collagen 1 expression in Hep2G cells in culture upon silencing *MCT1* (Figure 3.13).

Our conclusion was first obtained by utilizing chemically stabilized siRNA. Chemically stabilized siRNA takes advantage of RNA modifications 2-Fluoro, 2-
Methyl ribose, and phosphorothioate backbone replacement to block siRNA degradation and immune responses as well as enhance in vivo delivery effectiveness and silencing longevity\textsuperscript{194,206}. To note, that injection of our chemically modified Chol- and GN-MCT1-siRNA did not affect MCT1 expression in other major tissues including iWAT, gWAT, BAT, intestine, heart, lung, kidney, and spleen (Figure 3.4). The only difference was in hepatic biodistribution. Chol-MCT1-siRNA generally targets major hepatic cell types including hepatocyte and stellate cells. On the other hand, conjugation of such siRNA compounds with GN promotes binding to the asialoglycoprotein receptor that is primarily expressed in hepatocytes at high levels, and we confirmed hepatocyte selective gene silencing with such constructs.

Interestingly, GN-MCT1-siRNA significantly upregulated Col1 production, while Chol-MCT1-siRNA surprisingly prevented it in a genetically obese NASH model (Figure 3.9). To delineate the underlying mechanism that derived the counter-effect on fibrogenesis, we tested if Chol-MCT1-siRNA prevents fibrosis by depleting MCT1 in HSCs that hepatocyte-specific GN-MCT1-siRNA cannot target, by employing novel AAV-Lrat-Cre constructs (Figure 3.10). As a result, we successfully showed that HSC-MCT1 is the key regulator of Col1 production and further fibrogenesis in NASH (Figure 3.12). Collectively, we postulate that depletion of hepatocyte MCT1 leads to the secretion of fibrogenic inducers that activate HSC, but HSC-MCT1 depletion prevents these HSC activation pathways.
Further hepatocyte-derived secretome analysis and HSC activation pathway analysis will be necessary to prove this idea.

Since the discovery of HSCs, the importance of HSC activation during NASH progression has been well established\textsuperscript{65,70-72}. Targeting-activated HSCs have become a fascinating strategy in NASH therapeutics development\textsuperscript{5}. In this regard, the generation of Lrat-Cre transgenic mice from the Schwabe lab has enabled the generation of a constitutive knockout gene of interest selectively in HSCs in vivo\textsuperscript{64}. Here, we went one step forward and developed and validated the hepatic stellate cell-specific AAV-Lrat-Cre construct for the first time in the field (Figure 3.10). Our AAV-Lrat-Cre construct will allow the use of a highly selective method to generate an inducible knockout model, cutting down on labor-consuming and time-demanding mice crossing/breeding processes.

We applied this inducible cell-type selective system to further investigate the roles of HSC and hepatocytes MCT1. Another NASH mouse disease model, CDHFD-induced NASH, has been employed. As expected, HSC-MCT1 KO significantly reduced collagen type 1 protein level compared to the control group, while Hep-MCT1 KO (AAV-TBG-Cre) upregulated its expression (Figure 3.12). Interestingly, fibrogenic gene expression in MCT1 HSC-KO does not follow their protein expression level, reinforcing the idea that MCT1 regulates fibrogenesis in a translational manner, rather than transcriptional levels. The data from siMCT1 treatment to Tgf1b-stimulated LX2, a human HSC cell line, \textit{in vitro} revealed its
inhibitory effect on collagen production as well. Thus, we presented the therapeutic potential of targeting HSC-MCT1, as depletion of HSC-MCT1 significantly decreased collagen production in both \textit{in vitro} (Figure 3.1) and \textit{in vivo} (Figure 3.12). Notably, our HSC-MCT1 KO exhibited MCT1 silencing in HSC was around 60%, and it decreased overall fibrosis by half. It suggested the possibility that more depletion of the gene could have a better reduction in overall fibrosis.

In summary, the data presented here highlight hepatic stellate cell MCT1 as a potential therapeutic target to prevent NASH fibrogenesis related to collagen 1 production. Its utility as a therapeutic target is complicated by our finding that MCT1 depletion in hepatocytes actually increases fibrosis. This work highlights the importance of contemplating cell-type specificity when developing therapeutic strategies, especially in systems of complex cellular landscapes such as NASH.
Figure 4.1 Graphical abstract.
Hepatocyte MCT1KO enhances fibrosis, while stellate cell MCT1KO decreases it.
4.2 Limitations of the study and future directions

The major finding of this study is that MCT1 function in hepatic stellate cells promotes collagen 1 expression. However, MCT1 depletion in stellate cells was not enough to resolve steatosis (Figure 3.11). It led us to consider a combination therapy to fulfill the overall resolution of both steatosis and fibrosis in NASH, as a multi-targeting approach is increasingly applied for addressing pathologically complex diseases\textsuperscript{136}. A possible future strategy for NASH therapeutics may be to combine the depletion of MCT1 in stellate cells to decrease fibrosis with a potent anti-steatosis target such as DGAT2\textsuperscript{121,122,214}, which we and others have shown is effective in reducing steatosis when depleted. To summarize our previous study, we demonstrated that our GN-DGAT2-siRNA (Dgat2-1473) effectively resolved hepatic steatosis but was insufficient in preventing fibrosis in NASH\textsuperscript{122}. In the study, we screened and developed chemically modified RNAi compounds targeting Dgat2, the final enzyme in triglyceride synthesis, with potential therapeutic value. A single subcutaneous injection of our GN-conjugated siRNA compound, Dgat2-1473, exhibited remarkable silencing strength (80%) in the liver, remaining effective for at least 12 weeks post-injection. Moreover, Dgat2-1473 successfully targeted both human and mouse Dgat2 mRNA, as demonstrated in NSG-PiZ "humanized" mice with engrafted human hepatocytes. In a genetically obese NASH mouse model (ob/ob + GAN diet), administration of Dgat2-1473 significantly reversed and prevented triglyceride accumulation in the liver. However, there was no reduction in inflammation and fibrosis, indicating that targeting multiple factors
may be necessary to alleviate NASH. Based on these findings, we hypothesized that the fibrosis-reducing Chol-MCT1-siRNA and steatosis-resolving GN-DGAT2-siRNA would have a synergistic effect in preventing NASH.

For this purpose, we tested a combination therapy in a genetically obese NASH model that we previously optimized (Yenilmez et al., 2022, Mol Ther) but with a slightly revised study design. To our surprise, GN-DGAT2-siRNA or combination group did not reduce steatosis (Figure 3.14). The compensatory increase in Mct1 mRNA level could not be the reason for the phenotypes since Mct1 mRNA level was significantly depleted in the combination group as well. To note, there were certain technical differences in this experiment compared to our previous reports, which could account for the unexpected results. This study marked the first instance of combining Chol-conjugated siRNA and GN-conjugated siRNA together, and there might have been unknown toxicity issues that were not monitored in each of the individual targeting studies conducted previously. Additionally, in terms of study design, we administered the first injections of each siRNA a week earlier than starting the GAN diet, and steatosis was monitored 5 weeks after the first siRNA administration, which is 2 weeks longer than our previous report. Additionally, Chol-NTC-siRNA was repeatedly injected every 10 days in between.

As a future direction, we are testing the potential toxicity of combining Chol- and GN-conjugates and optimizing the siRNA chemical modification to enhance the potency and longevity of GN-DGAT2-siRNA, while repeating this dual targeting strategy with our original protocol (ob/ob + 3 weeks GAN diet). On the other hand,
there was a synergistic inhibitory effect on collagen production and overall hepatic fibrosis upon dual targeting (Figure 3.15). This effect was confirmed through multiple different methods, including Western blotting, immunohistochemistry, and ultrasound imaging that measures liver stiffness. Thus, the MCT1 and DGAT2 combination targeting strategy has therapeutic potential to prevent NASH fibrogenesis.

Targeting hepatic MCT1’s utility as a therapeutic target is complicated by our finding that MCT1 depletion in hepatocytes actually increases fibrosis. To avoid potential side effects associated with hepatocyte MCT1 targeting, we aim to develop a hepatic stellate cell-specific ligand-conjugated siRNA in future studies. Although a selective receptor for hepatic stellate cells is not currently available\textsuperscript{215}, ongoing research in the field may identify promising receptor candidates such as M6P-polyethylene glycol (M6P-PEG)\textsuperscript{216} and IGF2R-specific peptide coupled nanocomplex\textsuperscript{217}. Several other receptors including type VI collagen receptor, PDGF-b-receptor, synaptophysin receptor, and vitamin A receptor have also been investigated as potential targets\textsuperscript{218}. The safety, stability, and selectivity of utilizing these receptors as conjugators will need to be carefully assessed.

The potential underlying pathway of how MCT1 depletion in hepatocytes accelerates NASH-induced collagen production, while MCT1 depletion in hepatic stellate cells prevents it, needs to be addressed in future studies. Our hypothesis
is that the lactate transporter MCT1 in hepatic stellate cells promotes the expression of fibrotic collagens by regulating lactate flux. Furthermore, we propose that in the case of MCT1 depletion in hepatocytes, lactate present in the hepatic blood flow that has not been taken up by hepatocytes is redirected to other hepatic cell types, including hepatic stellate cells. This assumption explains why depletion of MCT1 in hepatocytes enhances NASH-induced collagen production and overall fibrosis, as it leads to an increased supply of lactate to stellate cells within the system. Conversely, depletion of MCT1 in hepatic stellate cells blocks this pathway, thereby preventing collagen production.

Currently, there is a limited approach available due to some limitations in both biological and technical aspects. RNA sequencing or single-cell RNA sequencing would not be an appropriate technique to probe the mechanism of MCT1 in stellate cells, as the inhibitory effect of stellate cells MCT1 KO on NASH fibrogenesis was rather transcriptional regulation in both in vitro and in vivo. Neither metabolomics nor phosphor-proteomics seem to be alternative techniques that we could have employed to hepatic stellate cells specifically due to technical limitations. During primary liver cell isolation, the liver goes through lengthy perfusion followed by enzymatic and physical digestion in the presence of pronase and collagenase. Despite the use of a balanced salt solution, which helps maintain the structural and physiological integrity of cells, it would not be the same metabolically homeostatic condition as in vivo NASH condition. Furthermore, cells are further processed in the harsh conditions of these enzymatic and physical digestive processes and
unfortunately, isolated stellate cells would lose their authentic function before analyzing intracellular metabolites or protein phosphorylation. Thus, it leaves the *in vitro* LX2 system as the most practicable study setting for now.

To test our hypothesis that the lactate transporter MCT1 promotes fibrotic collagen expression by regulating lactate flux in hepatic stellate cells, we examined whether lactate itself acts as a fibrogenic inducer, activating hepatic stellate cells to produce collagens. LX2 cells were treated with increasing doses of either sodium lactate or L-lactate (0, 2.5, 5, 10, 20, 40, 80 mM) for 48 hours and then harvested. However, lactate had no effect on collagen protein levels (data not shown). We observed slight increases in mRNA levels of other fibrogenic markers, such as ACTA2 and TGF-β1, but these effects were only observed at the 40 or 80 mM lactate treatment conditions (data not shown). Considering that the physiological level of lactate ranges from 1-2 mM and can reach a maximum of 10 mM during exercise, disease conditions, or lactic acidosis, these results suggest that lactate itself is not a fibrogenic inducer.

We then investigated whether lactate assists in collagen production in the presence of other potent fibrogenic inducers, such as TGF-β1. LX2 cells were treated with three different conditions for 48 hours: (1) TGF-β1 only, (2) TGF-β1 with sodium pyruvate (1 mM), and (3) the combination of sodium pyruvate (1 mM) and sodium lactate (10 mM) along with TGF-β1. Interestingly, both protein and mRNA levels of collagen expression were further increased in the condition of
sodium pyruvate (1 mM), sodium lactate (10 mM), and TGF-β1, compared to the TGF-β1 only condition (data not shown). Taken together, these findings suggest that although lactate itself is not a fibrogenic inducer, it can assist in TGF-β1-induced collagen production.

The underlying pathway by which lactate exerts a synergistic effect on TGF-β1-induced collagen production needs to be addressed in future studies. Our hypothesis is that the supply of lactate into hepatic stellate cells can serve as a precursor for the production of glycine, proline, and hydroxyproline, which are the three major amino acids that comprise collagen proteins. It's important to note that collagen is a triple helical structure composed of three polypeptide chains, each containing repetitive patterns of glycine, proline, and hydroxyproline$^{219}$. Glutamine metabolism is a well-established pathway to produce these three amino acids$^{220-222}$. The association of glutamine metabolism with MCT1 or lactate in fibrogenic hepatic stellate cells has not been thoroughly investigated. However, there have been reports in other systems, such as cancer or pulmonary fibrosis, suggesting a potential pathway involving lactate uptake to enhance glutamine uptake and subsequently facilitate glycine and proline production, which can serve as a source of collagen. Perez-Escuredo et al$^{223}$ demonstrated that lactate uptake via MCT1 promotes c-MYC activation, which in turn triggers the expression of the glutamine transporter ASCT2 and glutaminase GLS1, leading to enhanced glutamine uptake and catabolism. This finding was observed in SiHa and HeLa cancer cell lines in vitro. Furthermore, in the context of idiopathic pulmonary fibrosis, which shares a
high TGF-β1 dependency with hepatic fibrogenesis, glutamine metabolism, including glutaminase GLS1, phosphoserine aminotransferase PSAT1, and de novo proline synthesis via P5CS, has been demonstrated as a requirement for collagen protein synthesis. Although not extensively studied, if our hypothesis regarding lactate’s contribution to proline and glycine production through glutamine metabolism for collagen synthesis is correct, it could potentially explain why MCT1 depletion in stellate cells has a greater translational effect on collagen expression rather than transcriptional regulation.

We also examined the canonical pathway of TGF-β1-induced collagen production, as MCT1’s regulatory role in collagen production may involve multiple pathways. In the present study, we have shown that MCT1-siRNA successfully prevented Tgf1β-induced collagen production in human hepatic stellate LX2 cells in vitro (Figure 3.1). As SMAD3 is an essential downstream mediator of the Tgf1β canonical pathway, we hypothesized that stellate cell MCT1 depletion prevented Tgf1β-induced activation and phosphorylation of SMAD3. Tgf1β signals through SMAD proteins complex activation, predominantly SMAD3, to produce collagens. Tgf1β binding to the type I and type II receptor complex that has intracellular kinase domain results in phosphorylation of the SMAD2 and SMAD3 protein complex, and subsequently recruits SMAD4. The SMAD complex is translocated into the nucleus, enhancing the transcription of fibrogenic genes. As a result, we observed that MCT1-siRNA significantly decreased pSMAD3 protein level in the absence of Tgf1b treatment, implying the potential regulatory
effect of MCT1 on Tgf1β-SMAD3 axis (data not shown). Interestingly, however, in the presence of Tgf1β pretreatment, in which MCT1-siRNA prevented collagen 1 production, there was a Tgf1β does-dependent effect on pSmad3/Smad3 expression ratio. MCT1-siRNA significantly reduced the pSMAD3/SMAD ratio upon low concentrations of Tgf1β (1, 2.5ng/ml) pretreatment, but it could not reduce pSMAD3/SMAD ratio at the higher concentrations of Tgf1β (5, 10ng/ml) (data not shown). This data suggests that both SMAD3-dependent and independent MCT1 regulation could exist in the Tgf1β-induced collagen production in which MAPK, NF-kB, and PI3K are potential mediators in SMAD3-independent Tgf1β pathways. However, the mechanism of these pathways and biological consequences are still not fully understood226,227.

AMPK activation in hepatic stellate cells could be another possible underlying mechanism that regulates Tgf1β-induced collagen production. AMPK is often called an “energy sensor” or “metabolic master switch”, as it senses nutrient status by monitoring ATP and AMP levels and further regulates energy homeostasis. There is a report supporting that AMPK activation alleviates PDGF-stimulated cell proliferation and reduces MCP1 and type 1 pro-collagen secretion in human hepatic stellate cells228. Additionally, the potential role of AMPK on Tgf1β-induced fibrogenesis has been demonstrated by Lim et al229. Upon Tgf1β stimulation, the SMAD complex is translocated into the nucleus, binds to DNA, and initiates the transcription of fibrogenic genes. As a transcriptional coactivator, CBP/p300 binds to the SMAD complex in the nucleus, induces SMAD3 acetylation, and further
enhances the transcription of fibrogenic genes. Lim et al have demonstrated that pharmacological AMPK activation induced by 5-aminoimidazole-4-carboxamide-1-beta-4-ribofuranoside (AICAR), metformin, or adiponectin decreased collagen production, followed by increased proteasomal degradation of transcriptional coactivator p300. This suggests a decreased interaction between SMAD3 and p300. The effect of AMPK activation on collagen production was not associated with the phosphorylation, nucleus localization, and DNA-binding activity of the SMAD2/3 complex.

As mentioned previously, there is cumulative evidence supporting MCT1 depletion-induced AMPK activation in hepatocytes. Zhao et al. (2020) have demonstrated that MCT1 inhibition prevents lactate uptake into hepatocellular carcinoma cells HepG2 and Huh-7 in vitro, and decreases ATP production. This results in the activation/phosphorylation of AMPK, as increased AMP/ATP ratio is the cue for AMPK activation. In line with this, the present study and Carneiro et al. (2017) have shown the activation of AMPK in total liver samples upon hepatic MCT1 depletion in two different NAFLD in vivo mice models. However, the relationship among MCT1, AMPK, and p300 has not been well investigated in hepatic stellate cells. Thus, we hypothesized that MCT1 depletion in hepatic stellate cells prevents Tgf1β-induced collagen production via activation of AMPK that enhances p300 degradation. However, we, unfortunately, could not get a consistent result on AMPK activation or p300 degradation on MCT1-siRNA.
transfected LX2 cells upon Tgf1β-stimulation (data not shown), leaving the potential pathway not conclusive.

Interestingly, a classic histone acetyltransferase p300 was the first mediator discovered to exerts lactylation\textsuperscript{155,231}. The role of p300 in histone lactylation was confirmed by overexpressing or knocking down p300 in HEK293T cells or mouse bone marrow-derived macrophages\textsuperscript{155,232}. Since Zhang et al. (2019) discovered that lactate can directly modulate gene expression by histone lactylation, the epigenetic contribution of lactate via lactylation has started to be appreciated\textsuperscript{160,233,234}. The role of histone lactylation in activating profibrogenic macrophages via p300 has been reported. For example, Zhang et al. have demonstrated that histone lactylation via lactyl-CoA intermediate drives the swift of M1 macrophages into profibrogenic M2-like macrophage polarization upon bacterial exposure\textsuperscript{155}. Similarly, Cui et al. have shown that histone lactylation promotes macrophage profibrotic activity via p300 in lung myofibroblasts\textsuperscript{232}. Interestingly, there is a preliminary short article that claims targeting MCT1-mediated lactate flux attenuates pulmonary fibrosis by preventing macrophage profibrotic polarization\textsuperscript{235}. These findings suggest the possibility that reduced histone lactylation in macrophages, indirectly affected by MCT1 depletion in the stellate cells, prevents fibrogenesis, and inhibits pro-fibrogenic M2 macrophage polarization. Although we have not measured histone lactylation on macrophage upon MCT1 depletion in hepatic stellate cells, we have monitored M1/M2 macrophage polarization. However, there was no switch in M1/M2 macrophage
polarization upon hepatic MCT1 depletion when GN-MCT1-siRNA or Chol-MCT1-siRNA was treated in a rapid NASH model (data not shown).

Lactylation is not limited to histones. The discovery of non-histone protein lactylation has been reported in multiple systems, including the mammalian brain (neural excitement)\textsuperscript{236}, tumor microenvironment (immunosuppressive capacity)\textsuperscript{237}, grains\textsuperscript{238}, and plant-fungal pathogen\textsuperscript{239}. Gaffney et al. introduced another lactate-derived post-translational modification, lactoylation via lactoglutathione intermediate. They demonstrated that glycolytic enzymes are the major targets of lactoylation in HEK293T cells, decreasing glycolytic activity\textsuperscript{234}. Yang et al. reported that lactate promotes lactylation of high mobility group box-1 (HMGB1) protein via p300 and enhances the release of lactylated HMGB1 from macrophage through exosome secretion. These results suggest that MCT1-mediated protein lactylation may play a role in hepatic stellate cell fibrogenesis, independent of histone lactylation. More research is needed to confirm this finding.

I would like to reiterate the fundamental concept that MCT1 mediates the transport of monocarboxylates. As MCT1 is the major lactate transporter, the present study mainly focused on MCT1-mediated lactate metabolism in hepatocytes or hepatic stellate cells in NASH. However, there are other undervalued substrates that can be transported by MCT1, such as pyruvate, short-chain fatty acids, and ketone bodies\textsuperscript{161}. Unfortunately, due to the aforementioned biological and technical
constraints, we were unable to conduct a metabolomic analysis. However, we were able to gather some insights from a study of MCT1 chaperone protein Basigin/CD147 knockout (KO) mice in a high-fat diet-hepatic steatosis model\textsuperscript{240}. Basigin serves as an auxiliary protein for MCT1, and its loss prevents proper localization and stabilization of MCT1, thereby mimicking a condition of MCT1 deficiency. In this state, hepatic lactate levels were reduced by half, and other metabolites such as pyruvate, citrate, α-ketoglutarate, fumarate, and malate were significantly decreased. Although caution must be exercised in applying these findings to our MCT1 study, they suggest that multiple metabolites, particularly pyruvate, may play an important role in the context of MCT1 deficiency.

In terms of pyruvate metabolism, lactate can indeed be converted to pyruvate through the action of lactate dehydrogenase (LDH). Additionally, MCT1 and other monocarboxylate transporters (MCTs) are responsible for the transport of pyruvate, along with the mitochondrial pyruvate carrier (MPC). The Finck group’s series of publications have demonstrated the significance of MPC in various studies. They have shown that inhibiting MPC using a small molecule inhibitor\textsuperscript{241}, or MPC2 depletion utilizing albumin-Cre (Hep-KO)\textsuperscript{241} or Lrat-Cre mice (HSC-KO)\textsuperscript{242} resulted in attenuated NASH fibrosis in vivo. Although the fibrotic phenotype observed in the mice with hepatocytes MCT1 depletion is opposite to the mice lacking hepatocyte MPC2, we cannot rule out the possibility that the MCT1 depletion in stellate cells could be either directly or indirectly associated with pyruvate. This is supported by the fact that stellate cell knockout of both MCT1 and MPC has
exhibited similar phenotypes. Further investigation is needed to fully understand the mechanism. Moreover, recently, succinate, a dicarboxylate, has been demonstrated as a substrate of MCTs, as it can exist in the monocarboxylate form at a pH of 4.5-7.5\textsuperscript{243,244,245}. According to the studies conducted by the Cho group, succinate has been shown to induce fibrosis through the succinate receptor (GPR91/SUCNR1)\textsuperscript{246,247}. Since elevated levels of succinate in plasma or liver extracellular fluid have been reported in metabolic diseases, which can lead to hepatic inflammation and fibrogenesis through the same succinate receptor\textsuperscript{248}, we cannot disregard the potential involvement of succinate via MCT1 in NASH pathogenesis. Given that the function of MCT1 should be the sum of each substrate’s biological action, metabolomics-based further studies will allow a deeper understanding of MCT1’s role in NASH.

Lastly, this study was conducted solely on male mice. Historically, male rodents have been predominantly used in animal studies\textsuperscript{249}. Males are preferred in the early stages of metabolic studies to minimize the physiological variations exhibited by females due to the estrous hormonal cycle. However, this approach significantly limits the applicability and interpretation of the findings to females, as noticeable differences between sexes are not uncommon. Therefore, in future studies, it is crucial to replicate the major phenotype observed in the present study using female mice.
Chapter V. Bibliography


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