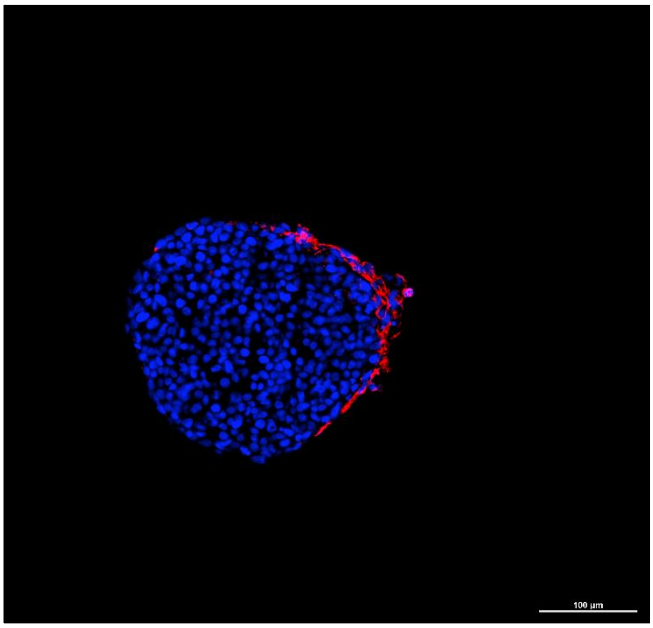
UNIVERSITY OF GOTHENBURG

DEPARTMENT OF BIOLOGICAL AND ENVIRONMENTAL SCIENCES

# LIVER FIBROSIS: 2D EXPRESSION ANALYSES AND 3D SPHEROID MODELING SUGGEST A POTENTIAL INVOLVEMENT OF THE GENE TM4SF4 IN FIBROTIC SIGNALING AND COLLAGEN DEPOSITION

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## Abstract

Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD) is a growing global health concern that can progress to advanced liver fibrosis and cirrhosis, and it is currently lacking effective pharmacological treatment. The progression of fibrosis is characterized by the sustained activation of hepatic stellate cells (HSCs) and the excessive deposition of collagen, primarily driven by the TGFβ-1 signaling pathway. Identifying novel molecular targets for fibrosis is therefore crucial for developing therapeutic strategies to stop or reverse it. This study investigated the pro-fibrotic function of *TM4SF4* and its relation to the TGFβ-1 pathway. Different hepatocyte cell lines (Hep3B2, HuH7 and HepG2) were used for initial analysis in a 2D culture. This was followed by investigation into a 3D di-lineage spheroid model, consisting of hepatocytes and hepatic stellate cells. We employed small interfering RNA (siRNA) to achieve specific knockdown of *TM4SF4* in 3D and *TGFBR2* in 2D. Our findings first demonstrated that *TM4SF4* is differentially expressed across different hepatocyte cell lines and HSCs. Moreover, *TM4SF4* is significantly downregulated upon TGFβ-1 incubation in the 2D model. Mechanistically, *TM4SF4* expression was found to be inversely correlated with the downregulation of *TGFBR2* (TGF-β1 receptor Type 2), suggesting an interaction in the signaling cascade. Most importantly, siRNA-mediated *TM4SF4* knockdown in the 3D di-lineage spheroid model resulted in a marked and consistent decrease in the levels of major fibrosis markers (*COL1A1*). In conclusion, this study suggests that TM4SF4 may play a key role as a regulator of liver fibrosis, given its correlation with the TGF-β1 pathway and inverse relationship with TGFBR2. These findings offer novel insights into the biology of a poorly characterized gene and propose *TM4SF4* as a potential modulator in liver fibrosis.

# 1. Introduction

Chronic liver disease represents a major and growing global health concern, progressing from metabolic and inflammatory insults toward fibrosis, cirrhosis, and ultimately liver failure. Central to this progression are changes in hepatocyte function and intercellular signaling within the hepatic microenvironment, which together drive chronic extracellular matrix deposition and tissue remodeling. While key pro-fibrotic pathways, such as transforming growth factor-β1 (TGF-β1) signaling have been extensively studied, the contribution of less well-characterized hepatocyte-associated factors remains incompletely understood. In particular, the identification of novel modulators of hepatocyte responses to metabolic and fibrotic stress is essential for improving our understanding of liver disease mechanisms and for identifying potential therapeutic targets.

## 1.1 The Liver

Inside the human body the liver plays a fundamental role. Its main functions are: the production of bile acids for the digestion of dietary triglycerides, the metabolism of xenobiotics (such as drugs and alcohol), and the regulation of blood glucose levels[1]. In addition, it synthesizes albumin and other transport proteins involved in hormone distribution[1].

The liver is located in the upper right abdomen, partially protected by the rib cage. It has two main surfaces: a smooth and convex diaphragmatic surface that is in contact with the diaphragm; and the visceral surface that faces downward and backward to accommodate surrounding organs[2]. The liver can be classified anatomically and functionally. Anatomically, it is divided into four lobes[1]. Functionally, the liver is divided into two lobes, right and left, based on the internal organization of the hepatic blood vessels and bile ducts[2]. The hepatic artery supplies oxygen-rich blood, while the portal vein delivers nutrient-rich blood from the gastrointestinal tract[1]. After the nutrients and xenobiotics have been processed, blood exits the liver through the hepatic veins, which drain directly into the inferior vena cava. This dual circulation is crucial for the metabolism of xenobiotics[1,2].

As previously mentioned, the liver contributes to digestion through the production of bile. Bile is essential for lipid digestion; although it does not directly digest lipids, it facilitates the action of pancreatic enzymes. Following hydrolysis, bile salts surround the lipid products and form micelles. Micelles act as transport carriers, bringing lipids close to intestinal epithelial cells for absorption. Bile is produced by hepatocytes and transported through small bile ducts[1,2]. The liver is responsible for multiple metabolic processes, including nutrient absorption, storage, and detoxification. It functions as both an endocrine and an exocrine organ[1]. It functions as an exocrine organ by synthetizing bile salts, conjugation of bilirubin and bilirubine excretion. Its endocrine functions are gluconeogenesis and glycogenesis, which are regulated by insulin and glucagon secreted by the pancreas, as well as protein and amino acid synthesis. Triglycerides are supplied to peripheral tissues by hepatic lipoproteins[1].

At microscopic level, the liver is organized into repeated structural and functional units known as lobules. Lobules are composed of hepatocytes arranged in a hexagonal shape (Fig. 1). Each lobule presents central vein and it is surrounded by portal triads, which consist of branches of the portal vein, hepatic artery, and bile duct[1,2] (Fig. 1). Hepatocytes are arranged in cords, and between these cords there are vascular spaces surrounded by a fenestrated endothelium. These spaces form the liver sinusoids, which are contained by a discontinuous basement membrane[1,2] (Fig. 1).

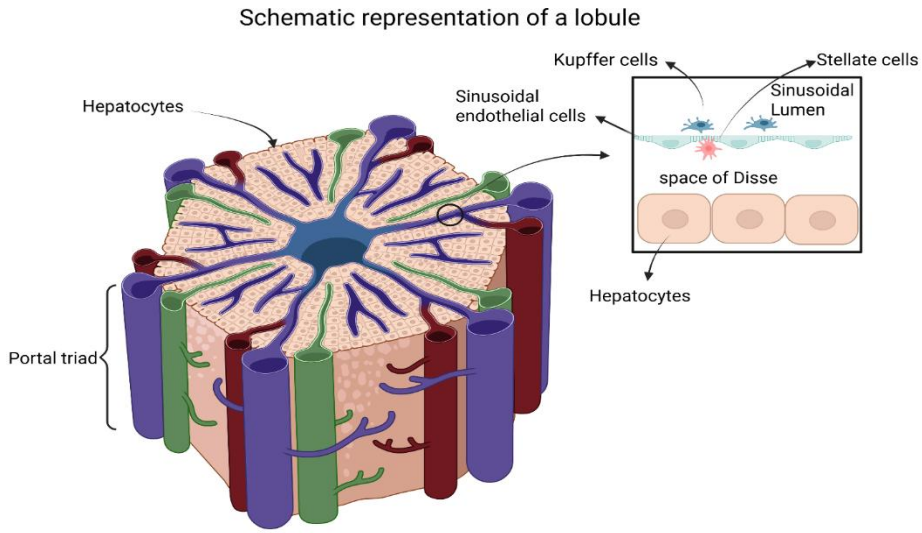


**Figure 1.** The liver structural and functional unit is the lobule. It is formed mainly by hepatocytes arranged in a hexagonal shape. It is surrounded by portal triads (vein, hepatic artery and bile duct). In the center is located the central vein (CV). Between each cord of hepatocytes is present a vascular space made of fenestrate endothelium, the liver sinusoid. The liver sinusoid is composed of liver sinusoidal endothelial cells, and thanks to its properties it allows the molecules to pass through itself, from the sinusoidal lumen to the space of Disse. Inside the space of Disse reside stellate cells. Kupffer cells, instead, reside inside the sinusoidal lumen. The image was created with BioRender.com.

The liver is composed of multiple cell types that cooperate to maintain physiological function (Fig. 1).

Hepatocytes account for approximately 80% of liver cells and are arranged in single-cell-thick hexagonal plates[3]. They regulate liver homeostasis through metabolic, detoxification, and immune-related functions[3].

Kupffer cells are the macrophages of the liver. They are located within the sinusoids and are important for the immune surveillance. They perform their function by removing pathogens, waste products, and cellular debris[3].

Sinusoidal endothelial cells form the liver sinusoids, a thin and fenestrated endothelium [3]. This structure allows for the efficient exchange of nutrients, metabolites, and other substances between the blood and hepatocytes[3].

When dormant, hepatic stellate cells (HSCs) store vitamin A[3]. Upon liver injury, these cells become active and transdifferentiate into myofibroblast-like cells[3]. Stimulated myofibroblasts contribute to the fibrotic response by producing extracellular matrix components, leading to scar tissue deposition[3].

Cholangiocytes form the epithelial coating of the bile ducts and are responsible for bile transport[4]. They can also modify the bile composition through water, electrolytes and bicarbonate secretion[4]. Although they represent a small portion of liver cells, cholangiocytes can actively respond to injury and inflammation, contributing to liver homeostasis and defense mechanisms[4].

### 1.2 Liver -related Diseases

Liver diseases comprise conditions with diverse etiologies, including metabolic, inflammatory, infectious, and toxic causes. Despite diverse causes, many liver diseases share common pathological features and progress from hepatocellular stress to inflammation, fibrosis, and loss of function. Fibrosis represents a central pathological process in chronic liver disease and is a major determinant of disease severity and clinical outcome.

In recent years, among the different liver diseases, Metabolic dysfunction-associated steatotic liver disease (MASLD) has become a global health concern[5]. This condition is characterized by triglyceride accumulation in the liver, known as steatosis, and it is associated with obesity, insulin resistance and metabolic syndrome. Initially, MASLD can be asymptomatic, sometimes for decades; however, if left untreated, it can progress to severe conditions such as steatohepatitis, end-stage liver disease, and hepatocellular carcinoma[6].

MASLD was previously referred to as nonalcoholic fatty liver disease (NAFLD)[7]. The revision of the terminology for NAFLD and nonalcoholic steatohepatitis (NASH) became necessary because these terms do not adequately reflect the metabolic causes of the disease. In addition, the term "nonalcoholic" does not accurately describe its pathophysiology. For these reasons, a more precise and inclusive nomenclature has been adopted[8].MASLD is classified within the broader category of steatotic liver diseases (SLD)[8]. Unlike the previous definition of NAFLD, the diagnosis of MASLD requires the presence of at least one cardiometabolic risk factor[8]. This updated classification provides a clearer understanding of the connection between disease and metabolic risk factors.

MASLD represents a disease spectrum (Fig. 2), that begins with liver steatosis. This early stage is often reversible and asymptomatic. Persistent triglycerides accumulation in the liver promotes inflammation and the deposition of collagen and fibrotic tissue, which may progress to cirrhosis and hepatocellular carcinoma, both of which are life-threatening conditions. Once cirrhosis develops, this condition becomes irreversible (Fig. 2).

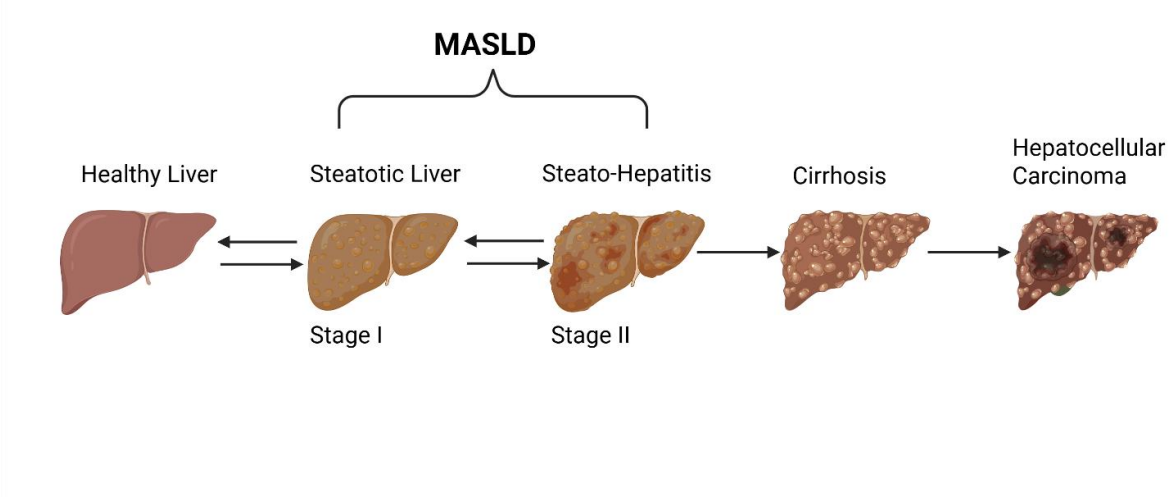


**Figure 2.** Within MASLD there are different progressive stages of liver disease spectrum. The progression begins with a healthy liver, which can develop into a steatotic liver (Stage I), characterized by fat accumulation in hepatocytes (steatosis) without significant inflammation or damage. This condition can still be reversible with lifestyle modifications. If left untreated, the disease may advance to steatohepatitis (Stage II), also referred to as MASH, where fat accumulation is accompanied by liver inflammation and hepatocellular injury. Importantly, MASH is still considered a reversible stage if addressed early with appropriate interventions. If MASH is left untreated it leads to cirrhosis, a stage marked by widespread scarring and loss of normal liver architecture, which significantly impairs liver function. Finally, in some individuals, the disease may culminate in hepatocellular carcinoma, a primary form of liver cancer. The image was created with BioRender.com.

### 1.3 Diagnostic Criteria and Risk factors for MASLD

The diagnosis of MASLD and other types of SLD follows a structured approach (Fig. 3). First, hepatic steatosis must be identified using imaging or biopsy. Hepatic steatosis is defined by neutral lipid accumulation in >5% of hepatocytes. Thresholds for steatosis detection vary among diagnostic methods[9] (Fig. 3). For MASLD the presence of at least one cardiometabolic risk factor is required[10] (Fig. 3). MASLD is linked to metabolic comorbidities, primarily obesity and type 2 diabetes (T2D).

Notably, the prevalence of MASLD and MASH in people with T2D is 55% and 37%, respectively[11]. Obesity promotes insulin resistance and ectopic fat accumulation, driving MASLD development and progression[12]. Both obesity and T2D increase fibrosis risk in MASLD, especially

in younger adults[13]. These conditions cause metabolic dysregulation, including dyslipidemia, that worsens liver injury[12]. Both are strongly linked with lifestyle-related factors, such as excessive caloric intake, physical inactivity, and alcohol consumption. Lifestyle acts as both a driver of MASLD and a key therapeutic target. Caloric restriction, physical activity, and reduced alcohol intake are among the most effective therapies[14].

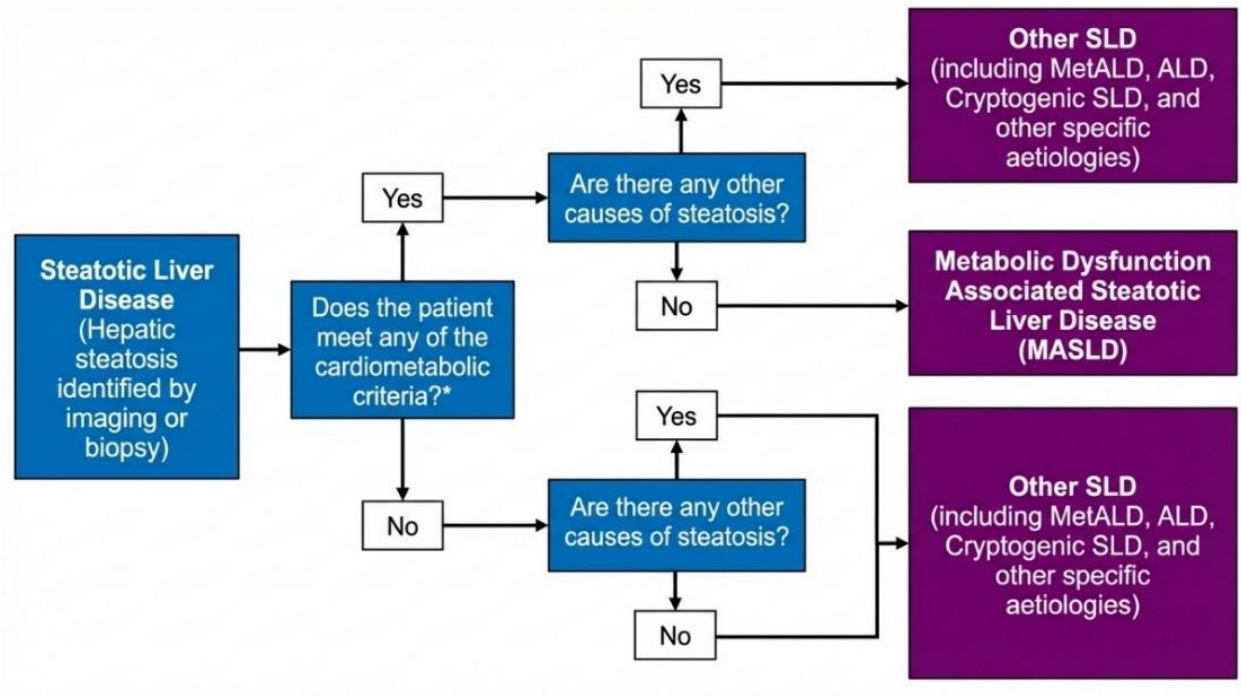


**Figure 3.** Diagnosis begins with the identification of hepatic steatosis through imaging or biopsy. The next step is to assess whether the patient meets any of the cardiometabolic criteria. If none of the criteria are met, the evaluation continues by checking for other causes of steatosis. If no alternative causes are identified, the condition is classified as Cryptogenic Steatotic Liver Disease (SLD). If other causes are found (e.g., drug-induced, monogenic, or alcohol-related), the diagnosis is another specific aetiology SLD. On the other hand, if the patient meets at least one cardiometabolic criterion, two diagnostic options are possible depending on the presence of other causes. If no other causes are present, the diagnosis is Metabolic Dysfunction-Associated Steatotic Liver Disease (MASLD). If other causes are found, especially involving alcohol, the condition is classified as MetALD or ALD, depending on the extent of alcohol use or mixed aetiology. Figure based on the diagnostic framework described by Rinella, M. E. et al.[10].

Caloric restriction and changes in food intake have been associated with improvements in MASLD in most studies, and it is mainly connected to the loss of weight. Lean MASLD occurs in some ethnicities despite BMI <25 kg/m², highlighting the importance of metabolic health over weight[14]. Exercise reduces liver triglyceride content by 20–30% independently of weight loss[14]. Most studies combine exercise with dietary intervention rather than assessing exercise alone[14]. Exercise improves the peripheral insulin activity, and the guidelines suggest at least 150 minutes of exercise per week, both endurance and resistance training, spread over 3-5 sessions[14].

In adults, MASLD is more common in men than in women[5]. Men are also at a higher risk of developing severe forms of the disease, such as MASH and liver fibrosis. Additionally, MASH-related hepatocellular carcinoma (HCC) is two to four times more frequently diagnosed in men, although HCC-related mortality rates are similar between sexes[15]. The prevalence of MASLD varies by sex and age. In men, MASLD is most common during young adulthood to middle age, with a regression after the age of 50–60. In contrast, women experience a rise in MASLD after the age of 50, which is likely linked with menopause and related metabolic changes, followed by a drop after the age of 70[16].

These sex-specific trends suggest that hormonal and metabolic differences have a significant role in the development and progression of MASLD. Sex hormones can shape the development and progression of MASLD[17]. Estrogens exert hepatoprotective effects through metabolic and anti-inflammatory mechanisms[17]. As a result, young women with impaired estrogen secretion have a higher prevalence of MASLD compared to healthy women with normal estrogen levels[18]. In

contrast, in males, androgens may influence liver functions, disrupting metabolism and signaling pathways, contributing to the development of SLD[19]. Sex chromosomes may also influence MASLD, as the X chromosome has been linked to increased food intake, adipose tissue mass, and higher body weight[20,21].MASLD has a strong genetic component that contributes to its onset and progression to advanced liver disease.

Liver fibrosis is part of the body's wound-healing response. It begins with liver injury, triggering inflammation and immune activation. Fibrosis is a physiological process that enhances liver resilience to injury. Additionally, type I collagen, a key scar tissue component, shields hepatocytes from toxic stimuli[22]. Thus, HSCs are widely recognized as the main performer in liver fibrosis.

However, hepatocytes also contribute to matrix turnover and remodeling[23]. Upon occurring injuries, hepatocytes interact with multiple cell types, particularly HSCs. Cellular crosstalk promotes reactive oxygen species (ROS) release, inflammatory signaling, and repair pathway activation[24]. However, chronic activation of these signals can disrupt the normal repair mechanisms, leading to a pathogenic fibrotic response. Growing evidence indicates that lipotoxicity promotes necroinflammation and fibrosis by impairing hepatic lipid handling, due to excess fatty acid flux, de novo lipogenesis, and free cholesterol accumulation[25].

Liver fibrosis is considered the most important prognostic feature of MASLD and is the only feature that predicts liver disease mortality[26]. In the liver, transforming growth factor-beta 1 (TGF-β1) is recognized as one of the most potent pro-fibrotic mediators. During chronic injury, multiple liver cell types release TGF-β1, activating HSCs and fibrogenesis[27,28]. The canonical TGF-β1 signaling pathway proceeds through the TGF-β receptors II and I. Binding of TGF-β1 to TGF-βRII recruits and activates TGF-βRI, which phosphorylates the intracellular mediators Smad2 and Smad3. These associate with Smad4 and translocate into the nucleus, where they drive the transcription of pro-fibrotic genes, including collagens and α-SMA[29,30]. TGF-β1 also activates several non-Smad pathways, which further promote HSCs activation and fibrogenesis[31].

### 1.4 Present and Future Therapies

Currently, several therapeutic approaches are available for MASLD. Therapies range from lifestyle interventions to pharmacological and novel strategies[14]. MASLD drug development has progressed slowly over the past decade[21]. Recently, phase III trials showed incretin mimetics reduce liver fibrosis by histological assessment[32]. Thyromimetics have also shown positive outcomes in improving MASLD or MASH[33]. Genetic therapy represents another emerging treatment approach[33].

Resmetirom is a thyromimetic that selectively binds thyroid hormone receptor β1 in the liver[33]. Its primary mechanism is increased hepatocyte β-oxidation. Additional effects include reduced lipogenesis and enhanced mitochondrial biogenesis[34]. A Phase III clinical trial showed its efficacy in reversing liver fibrosis. It has been approved by the FDA and the EMA for MASLD treatment in 2024 and 2025[33].

Incretins regulate glucose homeostasis by enhancing insulin secretion and suppressing glucagon[33]. They are secreted by the gut in response to food intake.[33] Glucagon-like peptide-1 (GLP1) and glucose-dependent insulinotropic polypeptide (GIP) are the primary incretins secreted. Glucagon-like peptide-1 receptor agonists (GLP1Ras) act as an incretin mimetic. GLP1Ras are approved for T2D and obesity[35,36]. Fibrosis improvement is largely attributed to weight loss[36]. Recently, semaglutide, a GLP-1 receptor agonist, has been approved by the FDA as a treatment for MASLD and MASH. Efficacy was measured in combination with lifestyle interventions[37].

Advances in molecular biology make it now possible to identify the genes responsible for various pathological conditions (e.g., genetic disorders, cancer). Modulating their expression is a promising strategy for improving human health. This approach make use of small interfering RNA (siRNA),

a powerful tool that utilize the RNA interference (RNAi) mechanism to specifically suppress gene expression[38]. siRNA is a double-stranded RNA composed of approximately 21–23 nucleotides, which mediates post-transcriptional gene silencing by binding to the complementary messenger RNA (mRNA) and it leads to the mRNA degradation through the RNA-induced silencing complex (RISC)[39] (Fig. 4). Due to its high sequence specificity, siRNA therapy may be a suitable candidate for targeting disease-associated genes. The RNAi pathway, present in eukaryotic cells, regulates gene expression and defends against viral infections[38]. This targeted degradation prevents the translation of the corresponding protein. Diseases such as viral infections (e.g., hepatitis B, HIV) and cancers that rely on the expression of specific genes for their progression are primary targets for siRNA-based therapies[40].

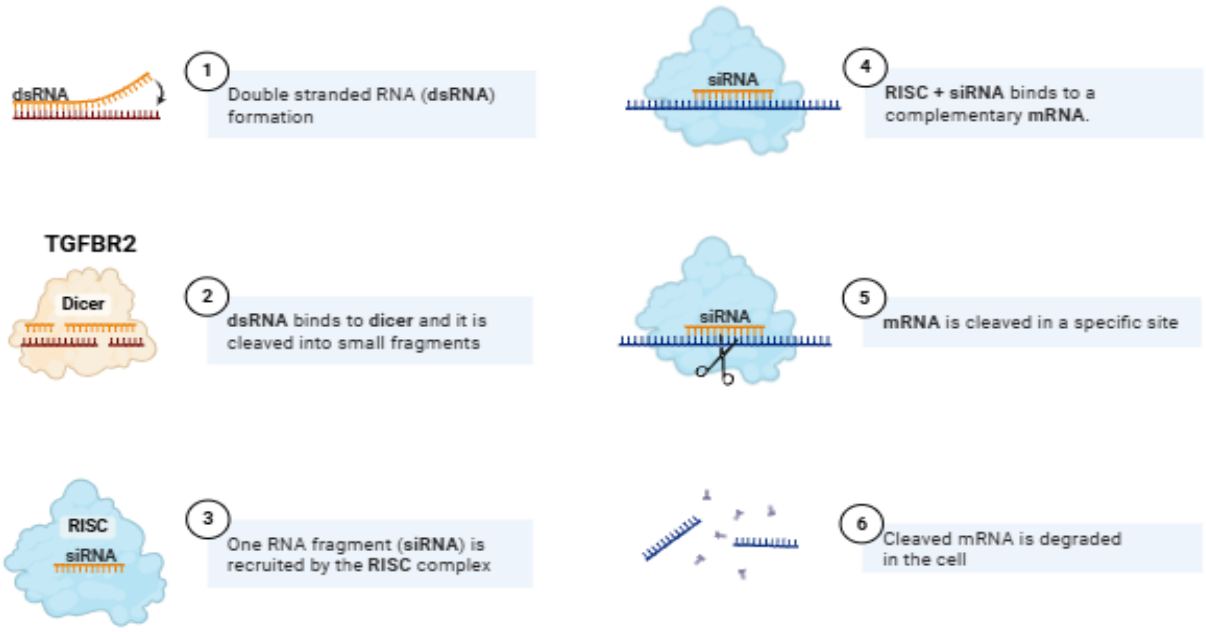


**Figure 4.** Schematic representation of siRNA-mediated gene silencing via the RNA-induced silencing complex (RISC). Double-stranded RNA (dsRNA) is processed by the Dicer enzyme into small interfering RNA (siRNA) fragments. One siRNA strand is then incorporated into the RISC complex, which guides the complex to a complementary target mRNA. Upon binding, the target mRNA is cleaved at a specific site and subsequently degraded, resulting in post-transcriptional gene silencing. Created with Biorender.com.

Despite its potential, many challenges must be addressed before the adoption of siRNA therapy in clinical practice. One of these challenges is the intrinsic instability of siRNA, which in the bloodstream and tissues is prone to degradation with a short half-life (minutes to hours)[41]. To improve siRNA stability, strengthen nuclease resistance, and reduce immune activation, different modifications of its chemical structure can be added. Substitutions at the sugar or phosphate groups protect against nuclease degradation and enhance overall therapeutic performance[41]. A second major challenge is the correct delivery.

Effective carrier systems are needed to protect siRNA molecules from enzymatic degradation and ensure they reach their target cells. Strategies such as lipid nanoparticles (LNPs), polymer-based carriers, and viral vectors have been explored to improve siRNA delivery, and more recently, conjugates like GalNAc have shown promise for hepatocyte targeting[42]. Taken together, stabilization and advanced delivery technologies work synergistically to increase siRNA stability, minimize immunogenicity, and optimize efficacy in vivo[41,42].

Off-target effects represent a major challenge that must be addressed when using siRNA. These effects occur when siRNA molecules silence untargeted genes, causing cytotoxicity and adverse effects[43]. Computational bioinformatics is being used to optimize siRNA design and reduce unwanted interactions, enhancing specificity and therapeutic efficacy[44] .

Genome-wide association studies (GWAS)[32] have identified several genetic variants that might be future target for siRNA therapy, including those in *PNPLA3*, *TM6SF2*, and *MBOAT7*, which affect hepatocyte triglyceride homeostasis, causing an increase in triglyceride content[45–47]. The interaction between genetic predisposition and environmental factors, such as diet and physical activity, amplifies the impact of these genetic variants, accelerating the progression of MASLD.

The *PNPLA3* I148M variant is the strongest genetic risk factor for MASLD and impairs triglyceride hydrolysis within hepatocyte lipid droplets, leading to intracellular lipid accumulation and progressive liver damage[48]. As a result, triglycerides accumulate intracellularly, predisposing individuals to steatosis and progressive liver damage. *PNPLA3* is expressed in the liver, with the highest levels observed in hepatocytes, hepatic stellate cells, and the retina[49]. PNPLA3, at the subcellular level, is localized to the endoplasmic reticulum and the membranes of lipid droplets[50].

The *TM6SF2* E167K variant reduces the secretion of very-low-density lipoproteins (VLDL) from hepatocytes. With reduced export of triglycerides into circulation, lipids are retained within the liver, exacerbating steatosis. *TM6SF2* primarily disrupts intracellular lipid trafficking and export[48]. The protein is predicted to reside in the endoplasmic reticulum (ER) and in the endoplasmic reticulum Golgi intermediate compartment (ERGIC)[51].

A variant in the *MBOAT7* locus (membrane-bound O-acyltransferase domain–containing 7) lowers arachidonoyl-phosphatidylinositol levels, resulting in a futile cycle that increases a non-canonical pathway of triglyceride synthesis. *MBOAT7* is expressed ubiquitously; its RNA is detected in all human tissues. MBOAT7 is a membrane protein that is associated with intracellular membranes, mainly the endoplasmic reticulum, but also mitochondria-associated membranes and lipid droplets[52].

Another recent multi-ancestry genome-wide association study on liver cirrhosis and related phenotypes, alanine aminotransferase (ALT) and γ-glutamyl transferase was conducted by Ghouse et al. 2024[53]. They identified several novel genetic associations with liver cirrhosis. An intronic variant (rs12633863) located in the Transmembrane 4 superfamily member 4 (*TM4SF4*) gene was found to be associated with liver cirrhosis[53]. Few studies suggested that *TM4SF4* is associated with hepatocellular carcinoma and liver fibrosis[54,55].

However, the role of this gene in liver fibrogenesis and its potential therapeutic in liver fibrosis is still unclear. *TM4SF4* encodes a protein that is a member of the transmembrane 4 superfamily, also known as the tetraspanin family. This family is primarily composed of cell-surface proteins, each containing four hydrophobic domains. According to the Human Protein Atlas, *TM4SF4* shows elevated expression in the liver, gallbladder, and small intestine, with particularly strong levels in hepatocytes and cholangiocytes. Its RNA expression pattern associates it with liver and intestine-enriched genes involved in metabolic processes. At the protein level, *TM4SF4* is mainly localized into the plasma membrane, with additional staining in the cytosol, consistent with its classification as a transmembrane protein with roles in epithelial cell function.

## 2. Aims

This study aims to clarify the role of *TM4SF4* in hepatocytes and hepatic stellate cells in relation to fibrogenesis. Specifically, we investigated whether this gene is expressed in different hepatocyte cell lines. We then measured gene expression *in vitro* to assess the effect of fibrotic conditions on this gene's RNA levels. Using 3D di-lineage cell culture models, we then assessed the protein levels of COL1A1, marker for collagen deposition, and neutral lipid levels after *TM4SF4* downregulation. This study provides valuable insights into the cellular role of *TM4SF4* in liver fibrosis and MASLD, as well as its potential as a therapeutic target.

# 3. Materials and methods

## 3.1 2D Cell Culture

Cryopreserved immortalized human hepatocyte cell lines (HepG2 and Hep3B2) and immortalized human hepatic stellate cells (LX-2) were cultured and seeded as previously described[56] with slight modifications. The HuH7 cell line was cultured in DMEM Low-glucose Dulbecco's Modified Eagle Medium (HyClone Laboratories) containing 10% FBS, 100 units/mL penicillin, and 100 µg/mL streptomycin.

## 3.2 Di-lineage Spheroids

Di-lineage spheroids, a three-dimensional cell culture composed of two different cell types grown together, allowing them to interact and self-organize in a spheroidal structure, were generated using two immortalized cell lines: HepG2 and LX-2. A total of 2,000 viable cells were seeded per well into 96-well round-bottom ultra-low attachment plates. The HepG2/LX-2 cell ratio was maintained at 24:1. Spheroids were cultured in Minimum Essential Medium (MEM) supplemented as described[56] for 2D culture conditions. Spheroids were incubated at 37°C in a humidified atmosphere containing 5% $CO_2$ for a total of 96 hours. Spheroid volume was calculated using the formula$4/3\ \pi\ r^3$, where "r" represents the average of the long and short diameters of each spheroid divided by two as described[56].

## 3.3 RNA Extraction, cDNA and Quantitative PCR

Total RNA was extracted following the protocol previously described[57]. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Complementary DNA (cDNA) was synthesized from the extracted mRNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific, 4368813). Gene expression analysis was performed on the resulting cDNA using real-time quantitative PCR (qPCR) with three technical replicates. Reactions were prepared with TaqMan Gene Expression Master Mix (Applied Biosystems by Thermo Fisher) and included TaqMan probes targeting human *ACTB* (Thermo Fisher Scientific ID Hs01060665_g1) to detect β-actin, used as the endogenous control, along with probes for the genes of interest. The probes for the genes of interest that have been employed were: *TM4SF4* (Thermo Fisher Scientific ID Hs00270335 m1), *COL1A1* (Thermo Fisher Scientific ID Hs00164004 m1), and *TGFBR2* (Thermo Fisher Scientific ID Hs00559661 m1).

## 3.4 Fibrogenic and Steatotic model

Palmitic acid, oleic acid, PDGF, and BSA were purchased from Sigma-Aldrich (St. Louis, MO, USA), while TGF-β1 was obtained from R&D Systems. To investigate whether *TM4SF4* expression in hepatocytes is influenced under fibrotic conditions, three hepatocyte cell lines (HepG2, HuH7, and Hep3B2) were used. Four experimental conditions were applied: 1% BSA (control), combination of TGF-β1 (10 ng/mL) and fatty acids (oleic acid 333 µM and palmitic acid 167 µM; total 500 µM), combination of PDGF (10 ng/mL) and 500 µM fatty acids, and only fatty acids (oleic acid 333 µM and palmitic acid 167 µM; total 500 µM). This protocol was adapted from a previous study[56].

Cells were seeded into 6-well plates and incubated for 24 hours. Afterward, the treatments were applied, and cells were incubated for an additional 48 hours. Following incubation, cell pellets were collected for RNA extraction and qPCR analysis.

A second experiment with a similar setup was conducted to focus specifically on the role of TGF-β1 in regulating *TM4SF4* expression. Four experimental conditions were applied: 1% BSA (control), TGF-β1 (10 ng/mL), a combination of TGF-β1 (10 ng/mL) fatty acids (oleic acid 333 μM and palmitic acid 167 μM; total 500 μM, and only fatty acids (oleic acid 333 μM and palmitic acid 167 μM; total 500 μM). However, in this case, *TGFBR2* was silenced using si*TGFBR2* to partially block TGF-β1 signaling. Four 6-well plates were used in total, two transfected with siSCR (scrambled control) and two with si*TGFBR2*. Before applying treatments, cells were pre-incubated for 2 hours with either siSCR or siTGFBR2.

### 3.5 siRNA Transient Transfection

HuH-7, Hep3B2 or HepG2 cells were trypsinized and seeded at 70% confluency. After 24 h, the cells were transiently transfected with SCR siRNA (AM4611; Thermo Fisher Scientific) and *TGFBR2* siRNA at 20 nM using Lipofectamine 3000 transfection reagent for 48 h, and cells were collected. The same protocol was applied for transient transfection for *TM4SF4* using *TM4SF4* siRNA (Thermo Fisher Scientific siRNA ID s14211) in di-lineage spheroids after the cells were seeded in 96-well round bottom ultra-low attachment plates. The protocol was adapted from precedent work[57].

### 3.6 Oil-Red-O (ORO) staining and COL1A1 immunofluorescence

To assess intracellular neutral fat, spheroids were stained with Oil Red O (ORO; Sigma-Aldrich, St. Louis, MO, USA) before and after transient knockdown of *TM4SF4* through si*TM4SF4*. Spheroids were fixed in 10% w/v paraformaldehyde (PFA) (Sigma-Aldrich) for 2 h. Following fixation, they were incubated overnight at 4°C in a 20% sucrose solution prepared in PBS. The di-lineage spheroids were then embedded in OCT cryomount (Tissue-Tek OCT compound). Next, 8-μm-thick sections of di-lineage spheroids were sectioned using a cryostat (Leica, Wetzlar, Germany) and transferred onto glass slides. After lipid staining, nuclear counterstaining was performed with DAPI (Sigma-Aldrich; 1:8000 dilution in PBS) for 5 minutes. Slides were then mounted using a fluorescence mounting medium (Dako). Quantification of ORO staining was normalized to the number of DAPI-positive nuclei. For immunofluorescent detection of collagen type I (COL1A1), tissue sections were first blocked in 5% (w/v) Bovine Serum Albumin (BSA; Sigma-Aldrich) in PBS for 1 hour. This was followed by overnight incubation at 4°C with a primary anti-COL1A1 antibody (Sigma-Aldrich, HPA011795, 1:500). The next day, sections were incubated with an AlexaFluor594-conjugated anti-rabbit secondary antibody (Invitrogen, 1:2000 dilution) for 1 hour at room temperature. Nuclei were again counterstained with DAPI (1:8000 in PBS) for 5 minutes and mounted using Dako mounting medium. Microscopy images were acquired using a Nikon microscope equipped with NIS-Element software (version 5.30.04; Bergman Labora, Gothenburg, Sweden) at 20x magnification. Quantitative analysis of ORO and COL1A1 staining, normalized to DAPI-stained nuclei, was performed using a custom macro developed in ImageJ (v1.52h; NIH)[56].

### 3.7 Statistical Analyses

Statistical analyses were performed using GraphPad Prism software version 10.4.2. P values were calculated by unpaired t-test (2D *in vitro* experiments) and the Mann-Whitney non-parametric test (ORO and COL1A1 Staining). Data are presented as mean values ± standard deviation (SD) of at least three technical replicates unless specified otherwise.

## 4. Results

The experimental findings obtained during this study aimed to investigate the expression and regulation of *TM4SF4* in hepatocytes under fibrotic, steatotic (fatty liver) and mixed (steatotic in combination with a fibrotic model) models. Therefore, several different *in vitro* experiments have been carried out.

### 4.1 Expression of *TM4SF4* Across Different Hepatocyte Cell Lines.

To assess baseline expression of TM4SF4 in hepatocytes, quantitative real-time PCR (qPCR) was performed on three human hepatocyte cell lines (HepG2, HuH7, and Hep3B2). Total RNA was extracted, reverse-transcribed into cDNA, and analyzed using TaqMan probes specific to *TM4SF4*, with *ACTB* as the housekeeping gene control. Among the tested cell lines, Hep3B2 showed the highest *TM4SF4* expression (Ct = 23.38) (Fig. 5A). HuH7 also expressed *TM4SF4* (Ct = 31.07), though at lower levels relative to *ACTB* (Ct = 21.14) (Fig. 5A). In contrast, HepG2 revealed very low *TM4SF4* expression (Ct = 33.16) compared to its *ACTB* (Ct = 17.56) (Fig. 5A). The human hepatic stellate cell line LX-2 was included for compatibility with the 3D di-lineage culture model (Fig. 5A). Overall, these results demonstrate endogenous *TM4SF4* expression under standard culture conditions, with the highest levels in Hep3B2, moderate levels in HuH7, and low expression in HepG2 and LX-2. All cell lines were then used for further experiments.

**A**

| | Ct *TM4SF4* | Ct *ACTB* |
|---|---|---|
| HepG2 | 33.16 | 17.44 |
| Hep3B2 | 23.38 | 17.57 |
| HuH7 | 31.07 | 21.14 |
| Lx-2 | 31.97 | 16.41 |

**B**

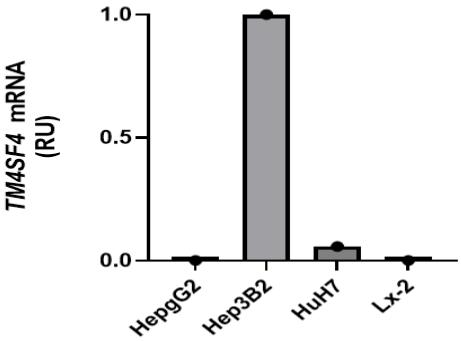


**Figure 5. Baseline expression of *TM4SF4* in hepatocyte and hepatic stellate cell lines.** ***(A)*** Ct values of *TM4SF4* and *ACTB* obtained by qPCR in HepG2, Hep3B2, HuH7, and LX-2 cells. ***(B)*** Relative *TM4SF4* mRNA expression normalized to *ACTB*.

### 4.2 Assessment of *TM4SF4* Expression Under Different Experimental Conditions.

After confirming *TM4SF4* expression in HepG2, HuH7, and Hep3B2 cells, the cells were exposed to different settings, mimicking steatotic (only FA) and fibrotic (TGF-β1 + FA) conditions. Incubation with TGF-β1 + FA downregulated *TM4SF4* expression in all three cell lines, with reductions in Hep3B2 ($p = 0.0101$), HuH7 ($p = 0.0026$), and HepG2 ($p < 0.0001$) compared with bovine serum albumin (BSA) controls (Fig. 6A). In HepG2, fatty acid exposure alone also reduced *TM4SF4* expression ($p < 0.0001$) (Fig. 6A). These results suggest an inverse relationship between TGF-β1 activity and *TM4SF4* expression under fibrotic conditions. As a positive control, *COL1A1*

expression was strongly induced by TGF-β1 in all cell lines, confirming the pro-fibrotic response (Fig. 6B).

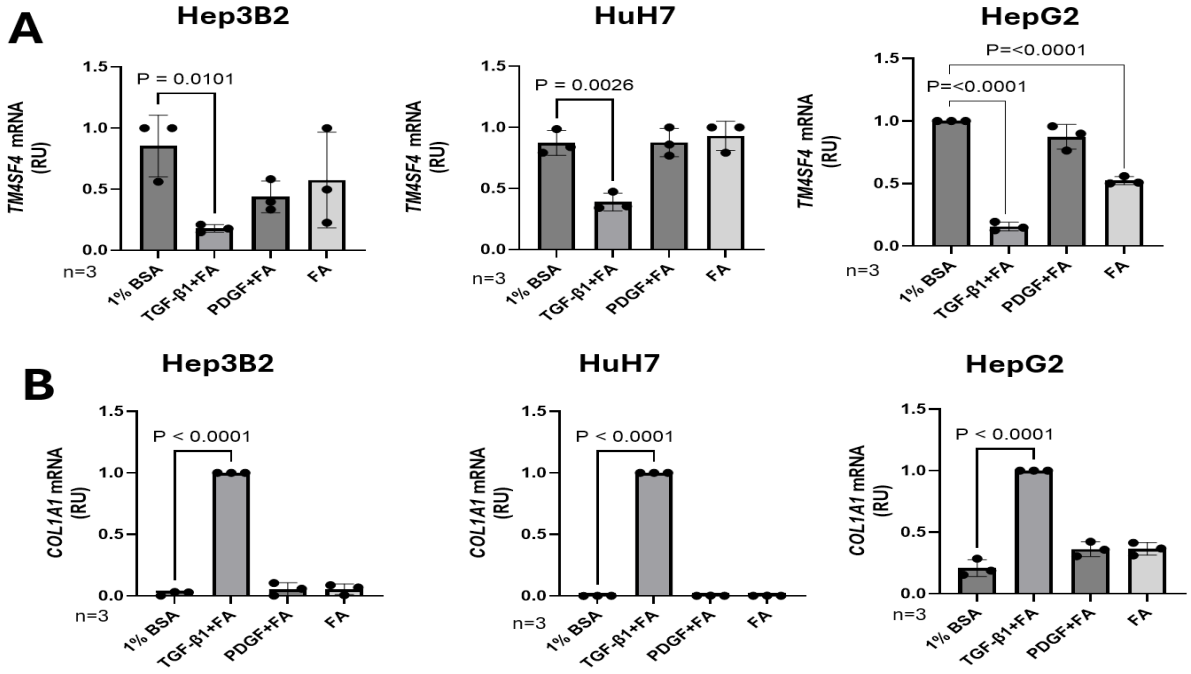


**Figure 6. Regulation of *TM4SF4* and *COL1A1* expression in hepatocyte cell lines under fibrotic and steatotic conditions.** ***(A)*** *TM4SF4* mRNA levels in Hep3B2, HuH7, and HepG2 cells following treatment with 1% BSA (control), TGF-β1 + fatty acids (FA), PDGF + FA, or FA alone. ***(B)*** *COL1A1* mRNA expression under the same conditions. As expected, TGF-β1 robustly induced *COL1A1* expression in all three cell lines ($p < 0.0001$), confirming the activation of a pro-fibrotic response. Data are presented as relative units (RU), mean ± SD, n = 3.

### 4.3 Effect of *TGFBR2* silencing on *TM4SF4* expression in Hepatocytes.

To investigate whether *TM4SF4* expression was regulated through TGF-β1 signaling, the main TGF-β1 receptor (*TGFBR2*) was silenced by siRNA in Hep3B2, HuH7, and HepG2 cells. A marked reduction in TGFBR2 transcript levels confirmed knockdown efficiency (Fig. 7A). In Hep3B2 cells, *TM4SF4* expression was consistently elevated under all experimental conditions following *TGFBR2* silencing except FA alone, while *COL1A1* induction by TGF-β1 or TGF-β1 + FA was completely abolished (Fig. 7B, 7C). In HuH7 cells, *TM4SF4* upregulation was observed specifically under TGF-β1 and TGF-β1 + FA treatments, followed by a reduction in *COL1A1* expression (Fig. 7B, 7C). In HepG2 cells, *TM4SF4* expressions increased under most conditions after *TGFBR2* knockdown, except FA alone (Fig. 7B). In this cell line, *COL1A1* expression decreased under TGF-β1 stimulation but was unchanged by TGF-β1 + FA (Fig. 7B). Together, these results indicate that *TM4SF4* expression is negatively regulated by TGF-β1 signaling through TGFBR2, while *COL1A1* serves as a reliable readout of pro-fibrotic activity (Fig. 7C).

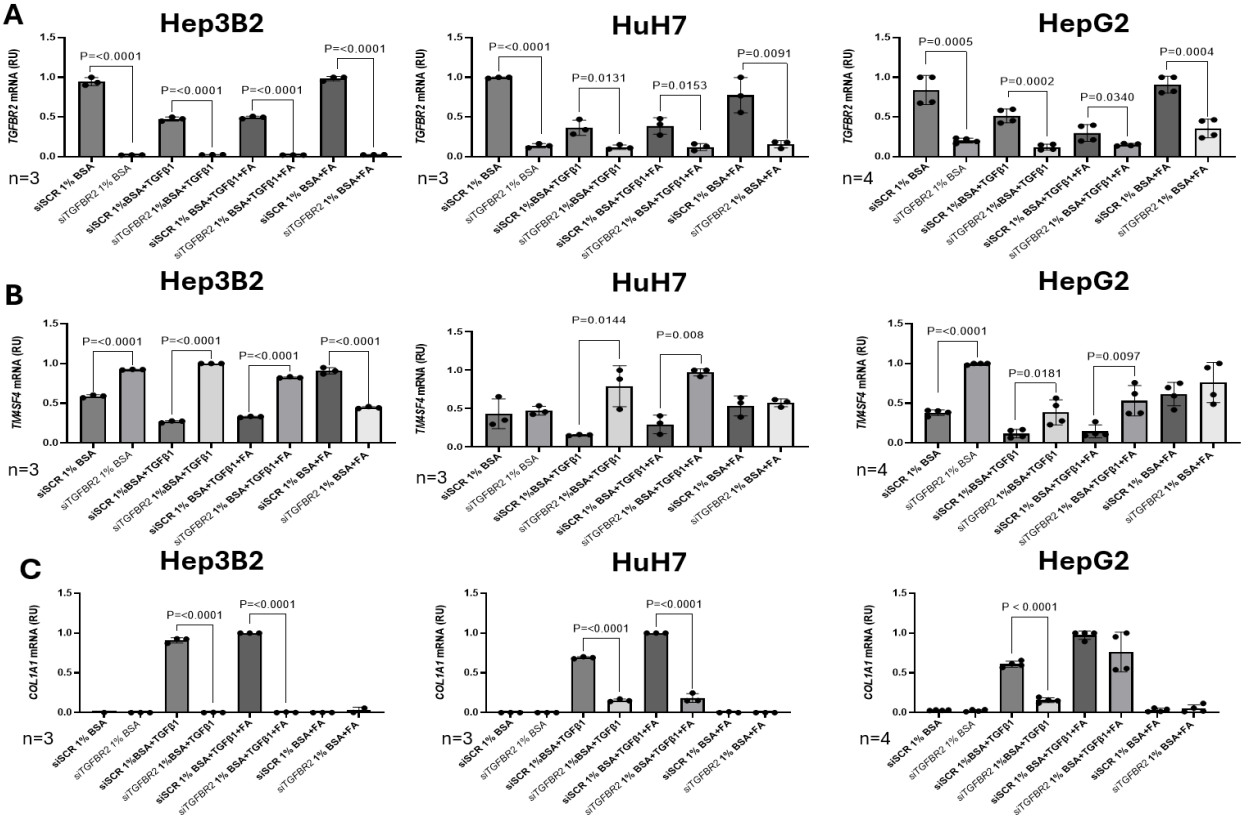


**Figure 7. Effect of *TGFBR2* silencing on *TM4SF4* and *COL1A1* expression in hepatocyte cell lines.** ***(A)*** Validation of *TGFBR2* knockdown by siRNA (siTGFBR2) compared with scrambled control (siSCR) in Hep3B2, HuH7, and HepG2 cells. ***(B)*** *TM4SF4* mRNA expression following *TGFBR2* silencing under control (1% BSA), TGF-β1, TGF-β1 + FA, PDGF + FA, or FA treatments. ***(C)*** *COL1A1* expression under the same treatments. Silencing TGFBR2 abolished COL1A1 induction by TGF-β1 and TGF-β1 + FA in Hep3B2 and HuH7. In HepG2, *COL1A1* expression was reduced under TGF-β1 stimulation ($p < 0.0001$) but remained unchanged with TGF-β1 + FA. Data are shown as relative units (RU), mean ± SD, with n = 3–4 technical replicates.

## 4.4 *COL1A1* and ORO Staining before and after *TM4SF4* knockdown in 3D di-lineage spheroid cultures.

Immunofluorescence staining of type I collagen (COL1A1) was used to determine if *TM4SF4* downregulation affects fibrosis. The staining was performed on spheroids consisting of a 24:1 ratio of immortalized hepatocytes to hepatic stellate cells. Immunofluorescence staining of type I collagen (COL1A1) revealed a significant reduction in collagen deposition in spheroids incubated with si*TM4SF4* compared to siSCR (control), revealing that *TM4SF4* knockdown reduces fibrotic activity (Fig. 8). Quantitative analysis showed a significant decrease in COL1A1-positive area per nucleus ($p = 0.0345$), supporting the role of *TM4SF4* in promoting collagen accumulation (Fig. 8). To evaluate neutral lipid levels, spheroids were stained with Oil Red O (ORO), which did not show a significant difference between si*TM4SF4* and siSCR conditions ($p = 0.1126$), suggesting that *TM4SF4* knockdown does not alter lipid storage in this model (Fig. 8). These findings collectively

imply that *TM4SF4* specifically contributes to collagen deposition without markedly affecting lipid accumulation.

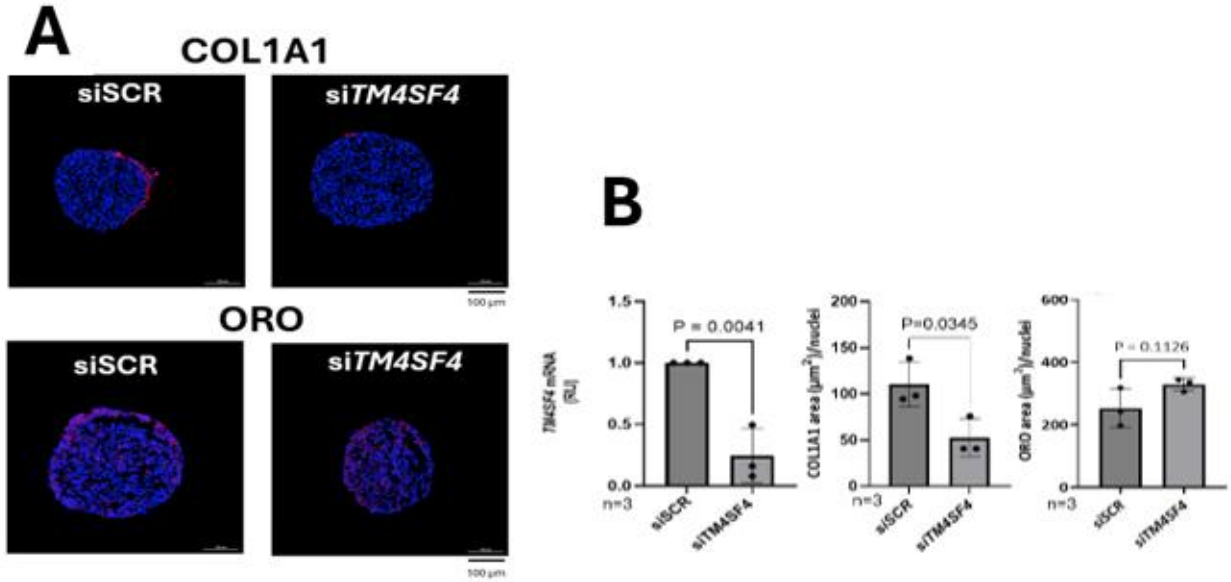


**Figure 8. *TM4SF4* knockdown reduces type I collagen deposition without significantly affecting lipid accumulation in hepatic spheroids. A)** Representative immunofluorescence images show COL1A1 (top row, red) and Oil Red O (ORO; bottom row, red) staining in spheroids composed of immortalized hepatocytes and hepatic stellate cells (24:1 ratio) treated with either siSCR or si*TM4SF4*. Nuclei are stained with DAPI (blue). **B)** Quantification of *TM4SF4* mRNA confirms effective knockdown (left graph). COL1A1 area per nucleus is significantly reduced following *TM4SF4* silencing (middle graph), whereas ORO staining area per nucleus shows no significant change (right graph). Data represent mean ± SEM (n = 3); statistical significance determined by unpaired t-test. Scale bars = 100 µm.

## 5. Discussion

In this study, the expression and regulation of *TM4SF4* were investigated in human hepatocyte-derived cell lines (Hep3B2, HuH7, and HepG2) as well as in the hepatic stellate cell line LX-2, as these cell lines are an established model for preliminary liver studies in humans[58,59]. At baseline, *TM4SF4* expression was the highest in Hep3B2, low in HuH7, and negligible in HepG2 and LX-2, indicating heterogeneity across hepatocyte models. After exposure to steatotic and fibrotic stimuli, *TM4SF4* expression showed a consistent downregulation across all hepatocyte cell lines after incubation with TGF-β1, despite *TM4SF4*'s low basal expression. Furthermore, treatment with fatty acids alone reduced *TM4SF4* expression in HepG2 cells.

As expected, *COL1A1* was robustly induced by TGF-β1, validating the pro-fibrotic conditions of the model. It is important to note that collagen expression in hepatocytes serves only as an indicator of the incubation with TGF-β1 and, therefore, its expression (mRNA level) can be used as a proof of concept. Based on this, the work proceeded with the 3D model containing hepatocytes and hepatic stellate cells, which are the actual sources of collagen deposition in the liver[60]. Furthermore, silencing of *TGFBR2* abolished *COL1A1* induction and relieved the downregulation of *TM4SF4*, demonstrating that TGF-β1 suppresses *TM4SF4* expression through canonical TGF-βRII signaling.

### 5.1 *TM4SF4* in Liver Fibrosis and Hepatocellular Carcinoma

The role of *TM4SF4* in liver fibrosis has been studied only sparsely. Prior reports indicate that *TM4SF4* is expressed in normal hepatocytes and upregulated in acute liver injury models, such as $CCl_4$-induced damage in rats, where overexpression was associated with worsened hepatocellular injury[55]. This observation differs from our findings, in which *TM4SF4* expression was suppressed

under pro-fibrotic stress in vitro. Such discrepancies may arise from differences between acute injury models in vivo, and from the fact that hepatocytes are not the cells synthesizing collagen in the liver.

On the other hand, the fact that overexpression increases liver fibrosis in rats is consistent with our model, where silencing of the gene reduces collagen levels in 3D spheroids. Current evidence on *TM4SF4* derives primarily from studies on hepatocellular carcinoma (HCC) rather than liver fibrosis. Two comprehensive multi-omics investigations demonstrated that *TM4SF4* is significantly upregulated in HCC compared to normal liver tissue[61]. Mechanistically, *TM4SF4* has been shown to associate with mitochondrial components and oxidative phosphorylation pathways, suggesting a role in metabolic reprogramming during tumor progression[61]. Furthermore, *TM4SF4* promotes epithelial–mesenchymal transition (EMT) by regulating the expression of key markers such as MMP9, N-cadherin, vimentin, and E-cadherin, and supports the maintenance of cancer stem cell properties[62]. Functional validation studies confirmed these oncogenic properties, as siRNA-mediated knockdown of *TM4SF4* in HepG2 and HuH7 cells significantly reduced cell proliferation, sphere formation, migration, and invasion[62].

Despite these findings, existing studies have largely focused on the role of *TM4SF4* in established HCC, while its involvement in earlier pathological stages, such as liver fibrosis, remains largely unexplored. These functional studies did not assess whether modulation of *TM4SF4* affects collagen deposition, a central marker of fibrogenesis. The use of a 3D di-lineage spheroid model in the present study provides a relevant model to address this knowledge gap.

### 5.2 *TM4SF4* as a Regulator of Fibrogenesis and Hepatocyte–HSC Crosstalk

By incorporating hepatic stellate cells, the main drivers of collagen production during fibrosis, the di-lineage 3D model allows the investigation of *TM4SF4* function in a more physiologically similar microenvironment and enables assessment of its potential role in fibrotic progression.

Overall, the present study provides evidence supporting a role for *TM4SF4* in earlier stages of liver disease. Our findings also complement a mechanistic insights study where was demonstrated that tetraspanins of the TM4SF family are highly expressed on the surface of hepatic stellate cells and that their functional blockade significantly inhibits HSC migration, a critical event in fibrosis progression[63].

Taken together, these observations suggest that *TM4SF4*, similarly to other TM4SF family members, may coordinate multiple aspects of the fibrotic response, including HSC recruitment to sites of liver injury and subsequent extracellular matrix deposition[60]. Consistently, *TM4SF4* downregulation in our 3D co-culture model resulted in reduced COL1A1 deposition, COL1A1 is potential biomarker for liver fibrosis, providing functional evidence that *TM4SF4* actively contributes to fibrogenesis[64]. The involvement of both hepatocytes and HSCs further indicates that *TM4SF4*-mediated effects are not confined to a single cellular compartment but instead involve intercellular crosstalk. This dual role supports the potential of *TM4SF4* as a promising therapeutic target for interventions aimed at modulating liver fibrosis.

### 5.3 Limitations

While our analyses were limited to transcript levels, the consistent results across three cell lines support the robustness of our findings and encourage further studies at the protein level. Hepatoma-derived cell lines and LX-2 stellate cells cannot fully represent the cellular diversity and signaling complexity of the whole liver, where immune cells, endothelial cells, and stellate cells interact with each other.

While the use of cancer-derived cell lines does not fully reflect the heterogeneity of primary cells, they provide a convenient, reproducible, and well-characterized model that allows initial screening before moving to more complex systems. Only one signaling pathway, TGF-βRII, was examined, and it remains possible that other pathways also regulate *TM4SF4*.

A critical limitation of this study is the number of replicates, as only one biological replicate (performed as technical triplicates) was included. To increase the reliability and robustness of the results, at least two additional biological replicates, each in triplicate, would be required, as generally recommended by scientific standards.

Nevertheless, despite the limited number of biological replicates in this study, the observed results were consistent across all three cell lines, suggesting that the findings reflect a genuine biological effect. While additional replicates would strengthen statistical robustness, the current data still provide valuable insight and a solid foundation for further investigation.

### 5.4 Future directions

Future work should address these limitations to confirm our observations at the protein level. Western blotting should be performed to determine whether the protein level reflects the changes seen in the mRNA levels. It will also be important to repeat the experiments in primary hepatocytes and hepatic stellate cells, ideally using samples from multiple donors.

As highlighted earlier, the current cell line-based experiments cannot fully capture the biological diversity of the human liver. Functional studies, including overexpression and knockout approaches, will be critical to define whether *TM4SF4* plays a protective or pathogenic role in hepatocyte biology. Additionally, exploring the downstream pathways affected by *TM4SF4* and its interaction with extracellular matrix remodeling or lipid metabolism could provide mechanistic insights. After evaluating responses in primary hepatocytes, the next essential step would be to use an *in vivo* model to investigate how the liver responds to the same stimuli and conditions.

### 5.5 Conclusion

In conclusion, this work demonstrates that *TM4SF4* is differentially expressed in hepatocyte models, suppressed under fibrotic stress, and regulated through TGF-βRII-dependent signaling. These findings add new insights into the biology of a poorly characterized gene and suggest that *TM4SF4* may be a key regulator in the cellular response to liver fibrosis. Although further research is required, this study provides a foundation for considering *TM4SF4* as a candidate regulator, biomarker, or therapeutic target in liver disease.

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## Appendices

**Appendix 1** Popular Science Summary

# A Little-Known Gene Could Help Fight Liver Scarring and Disease

*Did you know your liver can heal itself... until it can't anymore?*

*Globally, liver diseases, especially* ***Metabolic dysfunction-associated steatotic liver disease (MASLD),*** *are on the rise. MASLD has a worldwide prevalence of 30-35% and it is closely linked to obesity and type 2 diabetes. MASLD is a complex disease with separate phases:* ***liver Steatosis, steato-hepatitis (MASH)*** *(start of fibrosis),* ***cirrhosis and hepatocellular carcinoma (HCC).*** *Only the first two are reversible and often asymptomatic. Cirrhosis and HCC are irreversible and lead to death.* ***Liver fibrosis*** *is like the body's attempt to patch a wound that never closes. Over time, those "patches" harden into permanent scars, disrupting the liver's ability to clean your blood, store energy, and digest fats. When chronic damage pushes it past its limit, scar tissue starts to replace healthy cells. Nevertheless, there is still* ***no effective drug*** *to stop fibrosis once it begins. As we have understood now, this process, called* ***fibrosis****, is at the heart of many liver diseases that affect millions of people worldwide, and in my research, I explored a little-known gene that could be part of the puzzle:* ***TM4SF4****.*

## *A Hidden Player: TM4SF4*

*TM4SF4* might not be a famous name, but it's active in the liver cells that keep us alive. Earlier studies hinted that it might influence how the liver responds to damage, but nobody really knew how. That is where my project came in. I used human liver cell models, both traditional 2D cultures and 3D spheroids, to investigate what happens when we turn this gene **on or off**. The goal was to find out if *TM4SF4* helps or harms when the liver starts scarring.

## *What We Found*

Here's the surprising part: when I silenced *TM4SF4* using a genetic tool called **siRNA (small interfering RNA)**, the liver models produced **less collagen,** the main component of scar tissue. At the same time, *TM4SF4* activity dropped when liver cells were exposed **to a pro-fibrotic molecule**. This suggests that the gene and the pro-fibrotic molecule may be connected in the same signaling pathway. In simple terms: **reducing *TM4SF4* slowed down the scarring process.**

## *Why TM4SF4 Matters*

If future research confirms these results in animal and human studies, *TM4SF4* could become a **new therapeutic target**. That means scientists might one day design drugs to block its activity and prevent fibrosis from progressing.
This is especially promising since liver diseases are often silent for decades, and patients are usually diagnosed when damage is already irreversible.

## *Future Insight*

My work is an early step, but it helps map out how our liver's own genes influence its repair process. Understanding these molecular players brings us closer to developing **precision therapies,** treatments that work with the body's natural mechanisms instead of against them. The hope is simple but powerful: keeping the liver's healing process under control, so it repairs itself and does not damage itself.