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ORIGINAL ARTICLE

Thyroid hormone receptor alpha modulates fibrogenesis in hepatic stellate cells

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Abstract

Objective: Progressive hepatic fibrosis can be considered the final stage of chronic liver disease. Hepatic stellate cells (HSC) play a central role in liver fibrogenesis. Thyroid hormones (TH, e.g. thyroxine; T4 and triiodothyronine; T3) significantly affect development, growth, cell differentiation and metabolism through activation of TH receptor α and/or β (TRα/β). Here, we evaluated the influence of TH in hepatic fibrogenesis.

Abbreviations: ACTA2, alpha-smooth muscle actin; ADRB2, Adrenoceptor Beta 2; BCL3, B-cell lymphoma 3-encoded protein; BDL, bile duct ligation; CCl_4, carbon tetrachloride; CLD, chronic liver disease; COL1A1, alpha-1 type I collagen; Dio, deiodinase; FBS, foetal bovine serum; MCD diet, methionine-choline deficient diet; MEF, mouse embryonic fibroblasts; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; PBC, primary biliary cholangitis; PDGF, platelet-derived growth factor; PDGFrb, platelet-derived growth factor receptor beta; phH, primary human hepatocytes; phHSC, primary human hepatic stellate cells; phKC, primary human Kupffer cells; phLSEC, liver sinusoidal endothelial cells; T3, triiodothyronine; T4, thyroxine; TH, thyroid hormone; TRα/β, thyroid hormone receptor alpha/beta; WT, wild type; αSMA, alpha-smooth muscle actin.

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Design: Human liver tissue was obtained from explanted livers following transplantation. TRα-deficient (TRα-KO) and wild-type (WT) mice were fed a control or a profibrogenic methionine-choline deficient (MCD) diet. Liver tissue was assessed by qRT-PCR for fibrogenic gene expression. In vitro, HSC were treated with TGFβ in the presence or absence of T3. HSC with stable TRα knockdown and TRα deficient mouse embryonic fibroblasts (MEF) were used to determine receptor-specific function. Activation of HSC and MEF was assessed using the wound healing assay, Western blotting, and qRT-PCR.

Results: TRα and TRβ expression is downregulated in the liver during hepatic fibrogenesis in humans and mice. TRα represents the dominant isoform in HSC. In vitro, T3 blunted TGFβ-induced expression of fibrogenic genes in HSC and abrogated wound healing by modulating TGFβ signalling, which depended on TRα presence. In vivo, TRα-KO enhanced MCD diet-induced liver fibrogenesis.

Conclusion: These observations indicate that TH action in non-parenchymal cells is highly relevant. The interaction of TRα with TH regulates the phenotype of HSC via the TGFβ signalling pathway. Thus, the TH–TR axis may be a valuable target for future therapy of liver fibrosis.

KEYWORDS
HSC, liver fibrosis, TGFβ, thyroid hormone receptor α, thyroid hormones

1 | INTRODUCTION

The development of fibrosis in chronic liver disease (CLD) presents a vast unmet clinical challenge. At present, there is no effective anti-fibrotic treatment that halts or reverses the progression of liver fibrosis in patients with CLD.[1] Therefore, individuals with liver fibrosis are at risk of developing cirrhosis and complications such as liver cancer and liver failure, for which the only potential treatment is a liver transplantation.[2,3] Furthermore, the prevalence of CLD is predicted to increase in the coming decades due to the global epidemic of major risk factors for non-alcoholic fatty liver disease (NAFLD), including obesity, metabolic syndrome, and type 2 diabetes mellitus.[4]

Several human studies have recently reported that hypothyroidism, both subclinical and overt, is associated with severity of liver fibrosis.[5–7] Among individuals with non-alcoholic steatohepatitis (NASH), the inflammation-associated and progressive stage of NAFLD, liver fibrosis progression (i.e. early fibrosis vs. advanced fibrosis), negatively correlates with triiodothyronine (T3) serum concentration and liver expression of thyroid hormone (TH)-regulated genes.[8,9] These studies suggest that TH is an essential regulator of CLD outcomes and that modulating TH-signalling in CLD may prevent or reverse fibrosis.

Chronic liver injury triggers a liver repair response. Fibrosis occurs when this repair becomes deregulated. In the liver, fibrosis and cirrhosis development occur when hepatic stellate cells (HSC) become activated into collagen-producing myofibroblasts.[2] To our knowledge, the role of TH in HSC function has not been directly evaluated. However, a recent study showed that fibrosis progression in NASH is associated with altered expression of deiodinases (hepatic decrease of deiodinase [Dio] 1 and stromal increase of Dio3), which led to an intracellular reduction of T3 (i.e. a state of intrahepatic hypothyroidism).[10] Another study reported that mice deficient in TH receptors (TR) develop liver fibrosis, while treatment with T3 inhibited chemically induced fibrosis.[11]

Key points

Thyroid hormones play a central role in the development and regulation of our bodies. Scarring in the liver is an essential part of the progression of chronic liver diseases. This work shows that thyroid hormones can regulate important cells involved in liver scarring (hepatic stellate cells).

Of note, there are currently two ongoing phase 2/3 (NCT03900429 and NCT04173065) clinical trials with TRβ-agonists in patients with NASH and fibrosis.[12,13] Those studies' rationale is the metabolic impact of TRβ agonism as a dominant receptor in hepatocytes while having little systemic side effects due to TRβ isoform specificity and, for Resmetirom (MGL-3196), hepatocyte specificity.[14] However, there is only rare information about the relevant TR isoform in HSCs and its role in HSC activation.

In aggregate, these studies suggest that TH signalling is an essential regulator of NAFLD and CLD outcomes and that modulating TH signalling in CLD may prevent or reverse liver fibrosis. How TH

signalling modulates fibrogenic cell-specific responses during liver injury in HSCs is unknown. As HSCs are the principal collagen-producing cells in the liver, we examined the role of TH and its receptors in CLD.

2 | METHODS

2.1 | Ethics Approval Statement

All investigations into human material and the use of patients' data were approved, whether by the Ethics Committee of the University of Birmingham or the University Hospital Essen. Study protocols follow the ethical guidelines of the Declaration of Helsinki, and all patients provided written documentation of informed consent. Liver samples from primary biliary cholangitis (PBC) and NASH patients were acquired from the Liver and Hepatobiliary Unit in Birmingham in accordance with local ethical approval (REC 06/Q2702/61 University of Birmingham RG-06235 / Trust Ref RRK3181). The liver specimens for hepatic stellate cell isolation were obtained from fresh tumour resections at the University Hospital Essen (reference number: 12-5232-BO).

2.2 | Cell culture and treatments

The clonally derived mouse myofibroblastic HSC (HSC/GRX)[15,16] were cultured in DMEM media supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, .1 mg/mL streptomycin as described earlier.[17] Mouse embryonic fibroblasts (MEF) were isolated from embryos at E13.5 from the pregnant females as described earlier.[18] MEF were also cultured in DMEM medial and supplemented with 10% FBS, 100 U/mL penicillin, .1 mg/mL streptomycin, and .1 mM Non-Essential Amino Acids. LX-2cells, a human HSC line, was cultured in DMEM medium containing 1% FBS, 100 U/mL penicillin, and .1 mg/mL streptomycin.[16,19] Primary human HSC (phHSC) were isolated from liver tissue samples of three different patients undergoing liver surgery for metastases as described previously.[20] Cells were cultivated on uncoated plates in a stellate cell medium (ScienCell, Carlsbad, CA, USA). All cells were plated on a 10 cm^2 dish and incubated at 37 °C in the presence of 5% CO_2. For the experimental part, thyroid-depleted serum was used.[21] To mimic the activation of the cells and recognize TH's effects on this activation, cells were treated in triplicate with TGFβ, T3 or a combination of TGFβ and T3. The control and TGFβ groups were incubated with the same medium used for the starvation of the cells. The T3 and double treatment groups were treated with the same medium containing 10 nM T3 (Sigma-Aldrich St. Louis, Missouri, USA). After 1 h of incubation, recombinant mouse TGFβ1 (R&D systems, Minneapolis, Minnesota, USA) was added to (a) the TGFβ and (b) the TGFβ + T3 treatment group yielding an end concentration of 5 ng/mL. Unless otherwise indicated, all cell culture reagents and supplements were obtained from Thermo Fisher Scientific (Waltham, MA, USA).

To examine the effect of agonism and antagonism of the TR, LX-2 and phHSC (primary human hepatic stellate cells) were treated with the agonist GC-1 (Tocris Cookson, Bristol, United Kingdom) and the antagonist 1-850 (Cayman Chemicals, Ann Arbor, Michigan, USA). GC-1 is a thyromimetic, high-affinity agonist at TRβ and TRα receptors (KD values are 67 and 440 pM, respectively). According to the supplier's information, it displays 5- and 100-fold greater potency than the endogenous agonist T3 in vitro at TRα1 and TRβ1 receptors, respectively. 1-850 is a cell-permeable hydrazinylcarboxamide compound that acts as a selective and high-affinity TR antagonist (IC50 = 1.5 μM in HeLa cells). It is shown to block T3- (L-triiodothyronine) mediated interaction of TRα with nuclear receptor coactivator (NRC) and prevent the stimulation of gene expression. According to manufacturers' information, it does not affect the retinoic acid receptor α (RARα) activity. We performed a dose–response curve for LX-2 and phHSC to assess the substances' optimal treatment concentration. Cells were treated with GC-1 in the concentrations 1, 10 and 20 nM or with 1-850 in the concentrations .1, 1 and 10 μM. After assessing the optimal concentration via mRNA expression analysis (as described below), the cells were treated with TGFβ, GC-1, 1-850 and the dual treatment of GC-1 and TGFβ or 1-850 and TGFβ.

2.3 | Thyroid hormone-depleted serum

To obtain FBS free from TH and yet still capable of maintaining normal cell proliferation by containing peptide hormones like insulin, the FBS was filtered. Therefore, to 250 g autoclaved resin, 500 mL fetal bovine serum was added. The total content was shaken for 24 h at 37°C in an orbital shaker incubator. Next, the content was filtered through a coffee filter (Melitta, Minden, Germany). The resin-free eluate was vacuum filtrated over a polyethersulfone membrane with a .22 μm pore size (TPP Techno Plastic Products AG, Trasadingen, Switzerland). To ensure that the FBS has only low amounts of TH but still contains testosterone, estradiol, insulin and 25-OH-vitamin D, which also play pivotal roles in the liver cells, the FBS was tested with the Atellica IM 1600 Analyser in the central laboratory of the University Hospital Essen.

2.4 | Generation of Thra-knockdown

Thra-knockdown in GRX cells was generated using a lentiviral vector carrying *Thra* shRNA (Santa Cruz Biotechnology, Inc. #sc-108080), and control knockdown cells were generated using a scrambled lentiviral vector (SHC016V, Sigma).

2.5 | qRT-PCR, Western blot analysis and serum analysis

See online supplementary Materials and Methods and Table S1.

2.6 | Transcriptomic analysis by RNA sequencing

RNA was extracted from snap-frozen cell pellets (2×10^6 cells) following a standard Trizol procedure. RNA sequencing was performed at the Institut de Génomique Fonctionnelle, Lyon, France using the Ion Proton™ (Life Technologies) next-generation sequencing system. Reads were mapped via the Htseq software package[22] count feature (reserve strand setting) on the generated UCSC mouse genome assembly mm10 and count tables.

2.7 | Bioinformatic analysis

For RNA-sequencing analysis, each count table was analysed separately in the Deseq 2 R package from Bioconductor.[23] Genes were considered to be expressed only if they were above a conservative threshold of at least 10 reads in one sample; false discovery rate (.05). Genes were considered to be differentially expressed if they met a Benjamini-Hochsberg[24] *p*-adjusted value <.05 and a Log2-fold change of either >1 or <−1. Gene ontologies (GO) and functional enrichment were analysed using Gorilla[25,26] and DAVID[27] software. To reduce redundancy and identify functional categories, multiple GO terms were parsed in ReviGO to create visual representations.[28]

2.8 | Cell migration assay

Cell migration assay was performed in triplicate as described earlier with some modification.[29] Briefly, cells were grown in 35-mm glass-bottom culture dishes (Mat-Tek Corporation) until they reached confluence. Scratches were created using a 200 µL sterile pipette tip, and images were taken at 0 h, 9 h or 12 h, depending on the cell type. ImageJ (National Institutes of Health) was used to calculate the distance (µM) of migration.

2.9 | Animals and treatment

All animal experiments were approved by the *Landesamt für Natur, Umwelt und Verbraucherschutz (LANUV)* North Rhine-Westphalia (AZ: 84-02.04.2017.A157) and performed by the German regulations for Laboratory Animal Science and the European Health Law of the Federation of Laboratory Animal Science Associations. Mice were housed in individually ventilated cages at 21 ± 1°C on an alternating 12-h light/12-h dark cycle. Standard chow (Ssniff) and tap water were provided ad libitum. Homozygous $TR\alpha^{0/0}$ mice,[30] hereinafter referred to as $TR\alpha^{-/-}$, were bred and genotyped as previously described.[31] Littermates served as wild-type (WT) (C57BL/6J) controls.

Methionine-choline deficient (MCD) diet: To determine the effect of *Thra* signalling in fibrosis, male WT mice and $TR\alpha^{-/-}$ mice were fed a control chow or MCD diet for 6 weeks to induce NASH fibrosis.[32] Carbon Tetrachloride: (CCl_4) Mice received twice-weekly intra-peritoneal injections of CCl_4 (.5 mg/kg, Sigma-Aldrich) for 6 weeks to induce liver fibrosis or vehicle (mineral oil).[32] Bile duct ligation (BDL): Mice were subjected to BDL or sham surgery and sacrificed after 1 week to obtain liver tissues (from mice).[33]

2.10 | Liver immunohistochemistry

Liver tissue was fixed in formalin and embedded in paraffin. Immunohistochemical staining for TRα, alpha-1 type I collagen (Col1α1) and alpha-smooth muscle actin (αSMA) was performed using the DAKO Envision system (DAKO Corporation) according to the manufacturer's protocol. Immunostaining was performed as described previously.[32] Antigen retrieval was performed by heating 10 mM sodium citrate buffer (pH 6.0) or incubating with pepsin (00-3009; Invitrogen). The following primary antibodies were used: anti-TRα (NovusBio NBP2-22523), anti-Col1α1 (Cell Signalling #32575) and anti-αSMA (Abcam; ab32575). Negative controls included liver sections exposed to 1% bovine serum albumin instead of the respective primary antibodies. The proportion of tissue stained with αSMA and Col1α1 was assessed by morphometric analysis using NIH ImageJ version 1.48v (Rasband, W.S., ImageJ, U.S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997–2012). For morphometric quantification, 10 randomly selected 20× fields per section were evaluated for each mouse. Five-micron sections were stained with picrosirius red (Sigma, St. Louis, MO) and counterstained with fast green (Sigma, St. Louis, MO) to quantify liver fibrosis.

2.11 | Biliary cells

603B murine immature cholangiocyte cell line. The immortalized but non-transformed murine immature cholangiocyte cell line 603B was maintained in six-well plates (Corning Incorporated) in standard culture media as described previously.[33] Further experiments were performed similarly as described above with HSC, including treatment with thyroid hormone and TGFβ as knockdown experiments.

2.12 | Statistical analysis

The statistical significance was calculated using GraphPad Prism v.7.02 software (GraphPad Software, La Jolla, California, USA). Normality was assessed with the Shapiro–Wilk test and D'Agostino & Pearson normality test. A one-way ANOVA test was used for the significance between four and six samples, followed by post hoc analysis with Tukey's multiple comparison test (for normally distributed data). Kruskal–Wallis test and Dunn's multiple comparison test were used for non-parametric multiple comparisons. A two-tailed t-test was performed when the significance between the two samples needed to be calculated. A significant difference between samples was defined using a *p*-value of <.05. For correlation analysis, we used Spearman correlation. Data are presented as median values (interquartile range/IQR) or mean ± standard error of the mean (SEM).

3 | RESULTS

3.1 | Cell-specific distribution of TR in liver cells

TRβ is the predominant isoform expressed by hepatocytes, and studies to date have focused on its role in hepatocyte metabolism. HSCs are the major liver cell type responsible for scar tissue deposition and accumulation (i.e. fibrosis), but the role of TH (signalling) in HSC function is unknown. Both primary mouse hepatocytes and HSC express *Thra* and *Thrb*. As expected, TRβ is the predominant receptor in hepatocytes (Figure 1A). In contrast, HSC express significantly higher levels of *Thra*. Interestingly, immune liver cells and liver endothelia also express TRs in a specific distribution, with Kupffer cells mirroring hepatocytes and LSECs mirroring HSC (Figure 1A). In contrast to hepatocytes and Kupffer cells, HSC and LSEC express higher levels of *Thra*. Concerning *Thrb* expression, an opposite observation is shown (Figure S1).

3.2 | Thyroid hormone receptor expression is repressed during liver injury

To determine whether chronic liver injury is associated with changes in TR expression, liver tissue obtained from patients with NASH-cirrhosis (n = 5), primary biliary cholangitis (PBC) (n = 5), and normal liver (n = 5) was used to compare TR mRNA expression profiles. We found that *THRA* and *THRB* expression is decreased in chronically

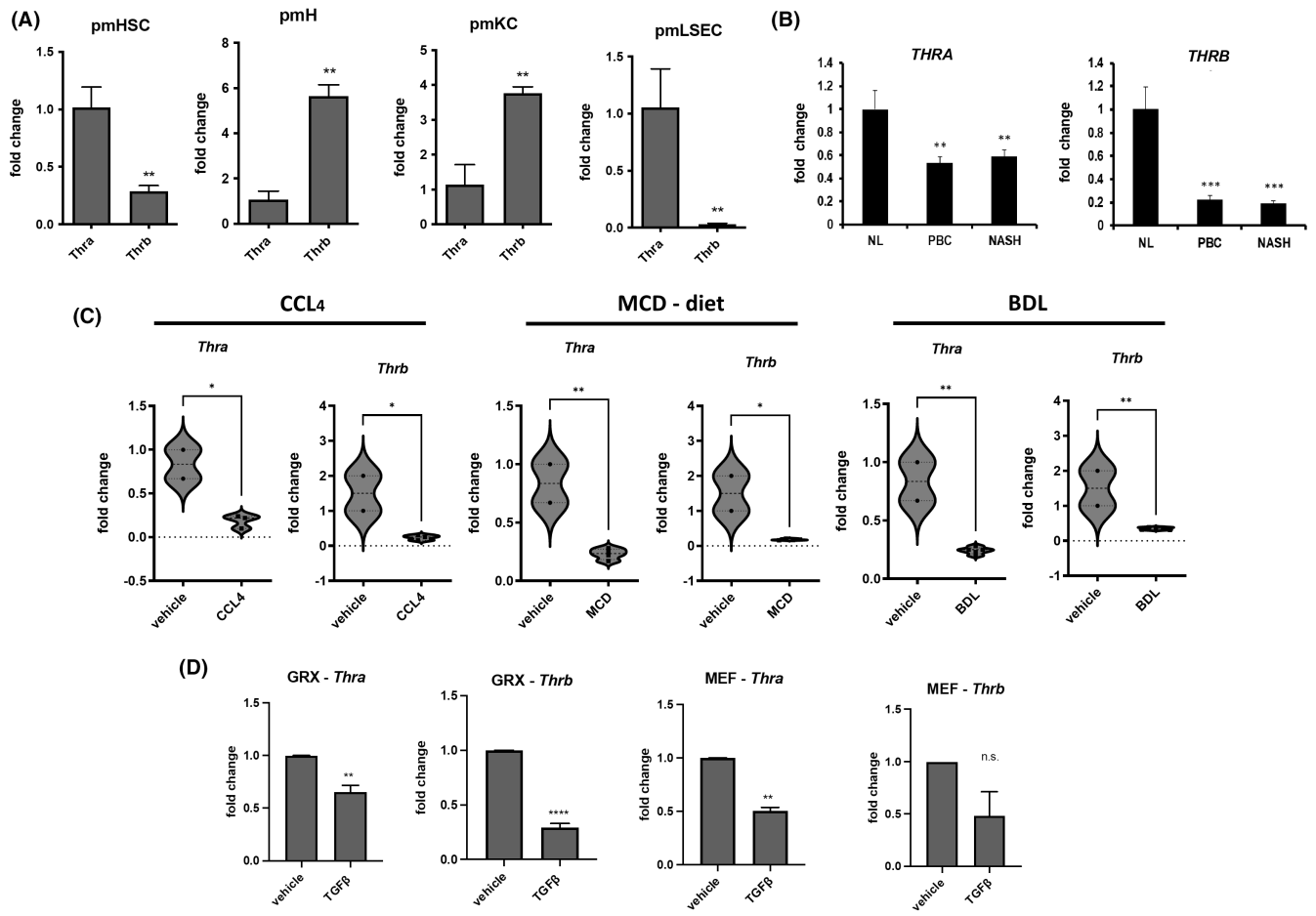

FIGURE 1 Cell-specific distribution of TRs in liver cells. (A) Expression of *Thra* and *Thrb* in liver cells. Primary murine liver cells express *Thra* and *Thrb*. Hepatocytes (pmH) and Kupffer cells (pmKC) show a significantly higher expression of *Thrb* than *Thra*. Primary hepatic stellate cells (pmHSC) and liver sinusoidal endothelial cells (pmLSEC) express significantly more *Thra* in comparison. (B) Liver tissues from explanted livers of transplant patients for end-stage NASH (cirrhosis) and primary biliary cholangitis (PBC). Normal livers (NL) were obtained from excess donor livers during transplantation. RNA was extracted and analysed by qRT-PCR for *THRA* and *THRB*. (C) Mice were exposed to three established chronic liver injury models. From obtained liver tissue, mouse primary HSC were isolated, and expression of *Thra* and *Thrb* was determined by qRT-PCR. CCl_4 liver fibrosis model: mice were treated twice weekly with an intraperitoneal injection of CCl_4 or a control substance (mineral oil) for 6 weeks. MCD NASH fibrosis model: a methionine choline-deficient (MCD) diet was given for 6 weeks. The control group received a standard diet. BDL-biliary cirrhosis model: A 14-day obstruction of the extrahepatic bile ducts by surgical bile duct ligation (BDL) was performed. (D) mouse HSCs (GRX) and mouse embryonic fibroblasts (MEF) were treated with recombinant TGFβ for 48 h. Cells were analysed for *Thra* and *Thrb* mRNA expression. Results are expressed as fold change; mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$, n.s. stands for not significant.

injured livers (Figure 1B). In addition, three murine injury-fibrosis models were used, for example, CCl_4-, MCD-diet and BDL-induced liver fibrosis. In all models, the expression of both receptors in isolated primary HSC, *Thra* and *Thrb*, decreased after induction of chronic liver injury (Figure 1C). These similarities suggest that TH signalling is disrupted in injured livers irrespective of the aetiology. We, therefore, hypothesized that changes described for NAFLD could be relevant for fibrosis or regeneration in general.

TGFβ is the prototypical profibrogenic cytokine that is highly upregulated in diseased livers. Having noted that total liver TR mRNA expression is downregulated in liver fibrosis, we evaluated whether TGFβ treatment would also downregulate TR expression in HSC. Treatment of the murine HSC line GRX and MEF resulted in repression of *Thra* and *Thrb* expression (Figure 1D).

3.3 | Triiodothyronine (T3) treatment induces TH-responsive genes and attenuates TGFβ induced activation in HSC

To assess whether HSC responds to T3, phHSCs were treated with T3 and analysed for changes in thyroid-responsive gene expression. Compared with controls, T3 treatment induced a higher TH-responsive gene (Adrenoceptor Beta 2; *ADRB2*, B-cell lymphoma 3-encoded protein; *BCL3*) expression. Treatment of phHSCs with TGFβ led to a downregulation of thyroid hormone-responsive genes. However, this downregulation was partially reversed by co-treatment with T3 (Figure 2A). To investigate whether T3 affects the fibrogenic gene response, phHSC were again treated with either TGFβ or T3 alone or the combination of TGFβ and T3. TGFβ promotes HSC activation.[34,35] As expected, treatment with TGFβ increased pro-fibrogenic gene expression (*COL1A1*, *ACTA2*, *TGFB1*). By contrast, the addition of T3 (10 nM) to TGFβ (10 nM + 5 ng/mL, respectively) repressed the expression of HSC activation markers by up to two-fold compared with TGFβ-treated phHSC (Figure 2B). Moreover, exogenous T3 reduced levels of phospho-Smad2 in TGFβ-treated phHSC (Figure 2C). Similar results were observed when studies were repeated using the human HSC cell line LX-2 (Figures S2 and S3A,B).

3.4 | Chemical TR agonism ameliorates the HSC response

GC-1 is an agonist of TRs with a high affinity for TRβ. However, it also has some affinity for TRα. To assess the effect of agonism of TRs on the fibrogenic and TH-responsive gene responses in HSCs, phHSC were

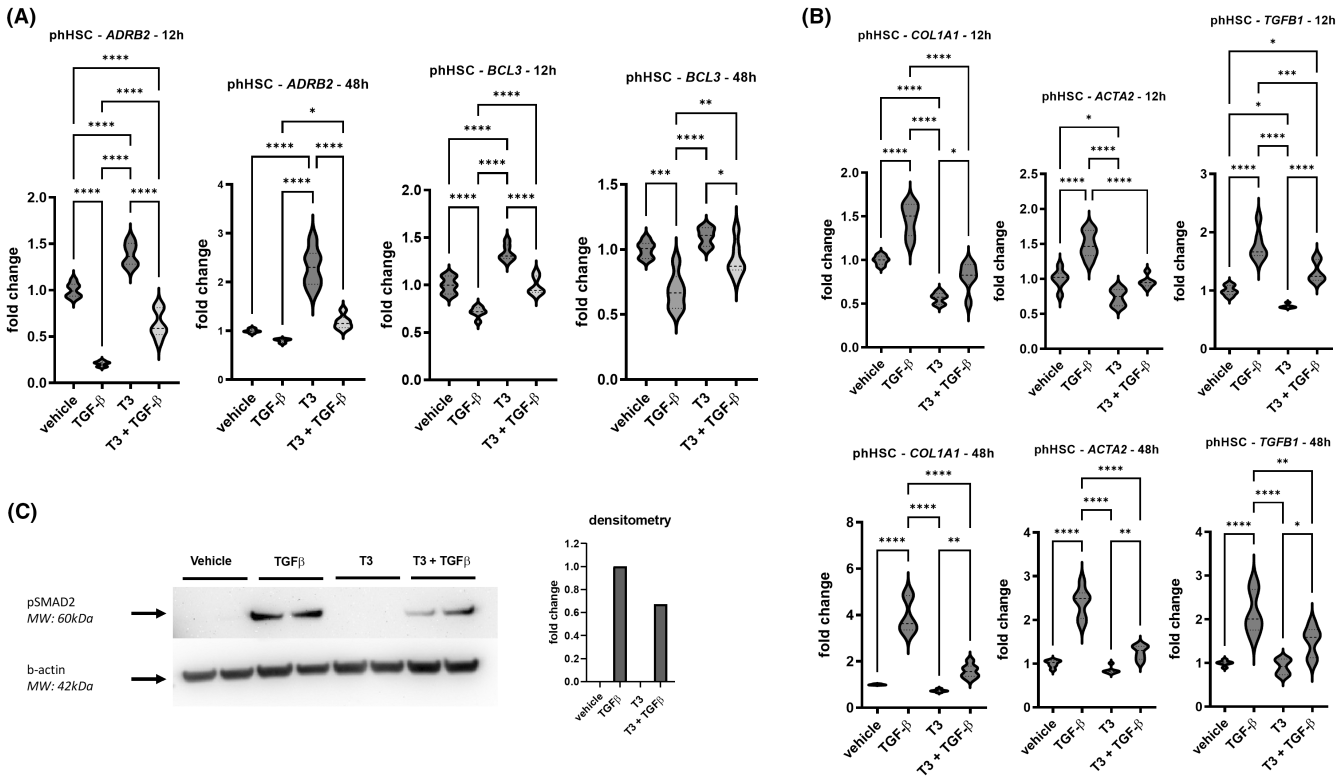

FIGURE 2 Treatment with T3 attenuates TGFβ-induced primary HSC activation and decreases levels of phospho-Smad2. phHSC were treated with T3 in the presence or absence of TGFβ for 12–48 h. Cells were analysed for thyroid-responsive and profibrogenic gene expression. (A) Thyroid responsive genes: *ADRB2*, *BCL3*. (B) pro-fibrotic genes: *COL1A1*, *ACTA2* and *TGFB1*. In addition, phHSC were treated as above, and protein expression of pSMAD2 was analysed. (C) Representative western blot images and densitometric measurement of the specific bands after 48 h treatment: phospho-Smad 2 and β-actin as the loading control. Mean ± SEM are graphed, $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

treated with GC-1. Treatment with GC-1 showed a dose-dependent effect on *COL1A1*, *ACTA2*, *DIO1* and *DIO3*. Treatment with 20 nM GC-1 resulted in the most pronounced downregulation of fibrogenic gene expression and was used in subsequent experiments (Figure 3A).

As expected, TGFβ treatment increased the fibrogenic response of phHSC by increasing the expression of *COL1A1* and *ACTA2*. However, the addition of GC-1 to TGFβ attenuated the fibrogenic responses and was comparable to the effects of T3. TGFβ decreased the expression of thyroid-responsive genes (*ADRB2*, *BCL3*), but this effect could be partially blunted by the simultaneous treatment with GC-1 (Figure 3B). These results were confirmed using the human LX-2 cells (Figure S3C).

3.5 | TR antagonists enhance HSC activation

1-850 is a TR antagonist, competitively inhibiting TRα and TRβ's activity. To examine the effect of antagonism on TRs regarding fibrogenic and TH-responsive genes, phHSC were treated with 1-850. As expected, in dose-finding experiments, the expression of TH-responsive genes *DIO1* and *DIO3* was downregulated, while pro-fibrogenic genes were upregulated in a concentration-dependent manner up to 1 μM 1-850 (Figure 4A). Higher concentrations of 1-850 (10 μM) resulted in a significant decrease of *COL1A1* and *ACTA2* expression, which is likely related to the toxicity effects of this compound. As such, we used the 1 μM dose in subsequent experiments.

We examined the effects of 1-850 alone or in combination with TGFβ. As anticipated, TGFβ treatment alone increased fibrogenic gene expression. Interestingly, the addition of 1-850 to TGFβ further enhanced fibrogenic (*COL1A1*, *ACTA2*, and *TGFB1*) responses by up to 2-fold at 48 h, respectively (Figure 4B). Similar results were observed in LX-2 cells (Figure S3D).

3.6 | Treatment with T3 attenuates TGFβ signalling in HSC in a TRα dependent manner

Since HSC activation was associated with repressed TR expression in human HSC, we evaluated whether the addition of T3 could rescue and abrogate TGFβ-induced murine HSC activation. Treatment with T3 blunted TGFβ-induced upregulation of *Col1a1*, platelet-derived growth factor receptor beta (*Pdgfrb*), and *Tgfb1* (Figure 5A) and reduced HSC migration (at 9 h) in the wound healing assay (Figure 5B). Moreover, exogenous T3 decreased levels of phospho-SMAD2 during TGFβ treatment (Figure 5C).

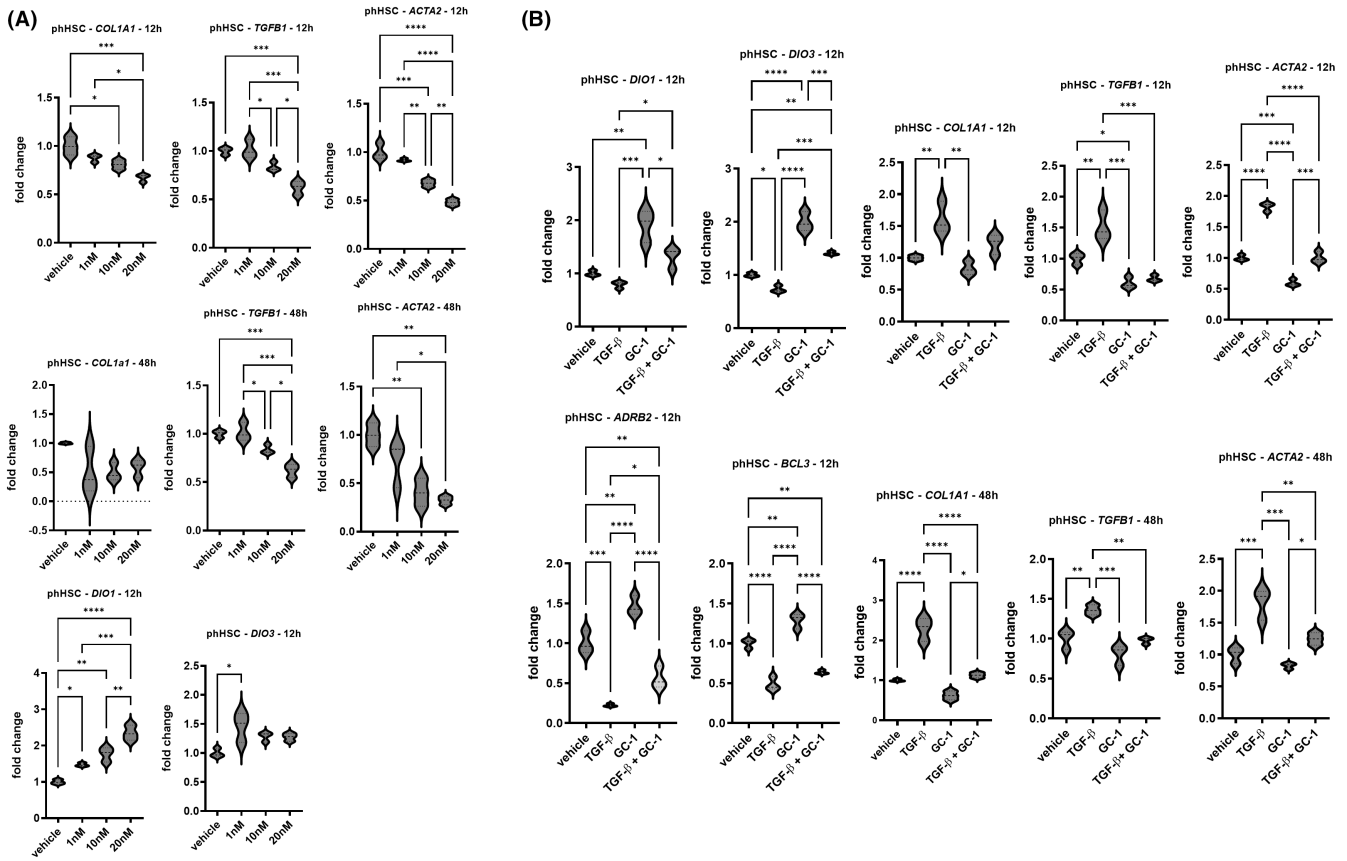

FIGURE 3 Treatment with GC-1 (thyroid hormone agonist) attenuates primary HSC activity. (A) phHSC were treated with GC-1 to assess the optimal concentration. Cells were treated with 1, 10 and 20 nM of GC-1. Cells were analysed for pro-fibrotic gene expression (*COL1A1*, *TGFB1* and *ACTA2*) and thyroid hormone receptor responsiveness (*DIO1*, *DIO3*). (B) phHSC were treated with GC-1 (20 nM) in the presence or absence of TGFβ for 12–48 h. Again, cells were analysed for thyroid-responsive and profibrogenic gene expression. Results are expressed as fold change; mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

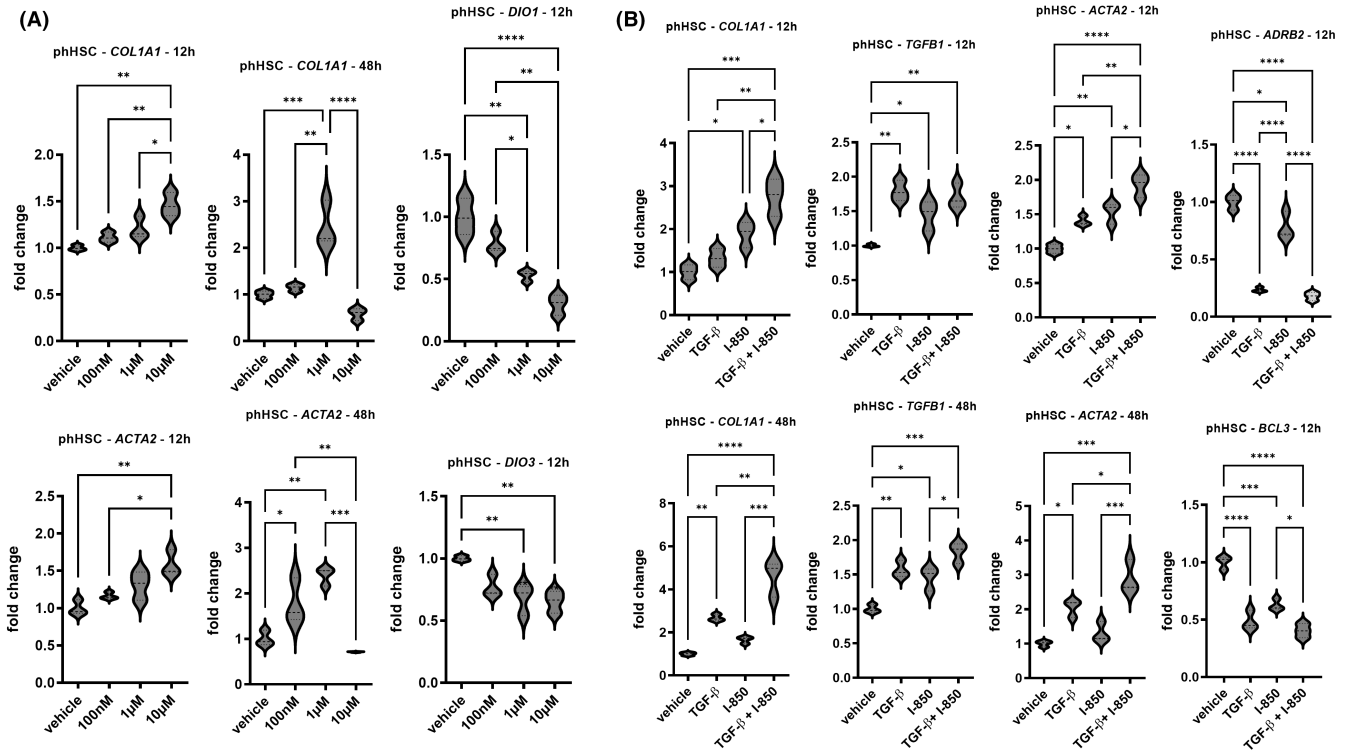

FIGURE 4 Treatment with 1-850 (thyroid hormone antagonist) activates primary HSC *activity*. (A) phHSC were treated with 1-850 to assess the optimal concentration. Cells were treated with 100 nM, 1 and 10 μM of 1–850. Cells were analysed for pro-fibrotic gene expression (*ACTA2* and *COL1A1*) and thyroid hormone receptor responsiveness (*DIO1*, *DIO3*). 10 μM concentration showed to be too toxic after 48 h of treatment (B) phHSC were treated with 1–850 (1 μM), in the presence or absence of TGFβ for 12–48 h. Cells were analysed for thyroid-responsive and profibrogenic gene expression. Results are expressed as fold change; mean ± SEM. $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$.

Although TH signalling occurs via TRs α and β, HSC predominantly express TRα. To determine the role of TRα in murine HSCs, we used a lentiviral-mediated RNAi approach. *Thra* knockdown was >60% in HSC and was associated with an upregulation of fibrogenic genes under basal conditions, which was further enhanced by TGFβ treatment (Figure 5D).

3.7 | TRα deletion enhances MEF fibrotic response

To determine the role of TRα in alternative pro-fibrotic cells, we repeated experiments using an immortalized MEF cell line derived from $TR\alpha^{-/-}$ mice. First, we evaluated the responsiveness of MEF of control mice (WT mice) to TGFβ and T3 treatment. Intriguingly, MEF exhibited similar responses to HSC: treatment with TGFβ upregulated pro-fibrogenic gene expression while the addition of T3 blunted the fibrogenic response (Figure 6A). We next evaluated the responses of MEF isolated from $TR\alpha^{-/-}$ mice. $TR\alpha^{-/-}$ MEFs expressed enhanced levels of *Col1a1* and *Acta2* under basal conditions, and these were significantly augmented when treated with TGFβ (Figure 6C). In addition, $TR\alpha^{-/-}$ MEFs expressed increased levels of pSmad2 (Figure 6B) and exhibited a 30% greater wound healing response (Figure 6D). RNA-sequencing was used to identify gene expression changes associated with TRα-KO in MEF cells. The absence or presence of TRα affected approximately 3800 genes (Benjamini-Hochberg $p_{adj} < .05$). Among the most highly enriched pathways were several that are associated with HSC activation. Thus, the knockout of TRα in MEFs affects the expression of 187 Wnt-associated genes, 146 genes related to inflammatory processes, 115 PDGF-associated genes, and genes involved in the TGFβ signalling pathway (Figure S4). Pathway analyses also showed that KO of TRα affects the expression of 230 genes involved in metabolic processes, including glucose, lipid and protein metabolism (Figure S5).

3.8 | TRα deletion enhances MCD diet-induced liver fibrosis

To understand the physiological significance of TRα, we used whole-body $TR\alpha^{-/-}$ mice.[31] WT and $TR\alpha^{-/-}$ mice were treated with an MCD diet or standard chow, and tissue was harvested at the 6th week.[32] The schematic figure of the experimental design is presented in Figure 7A. Formalin-fixed, paraffin-embedded liver tissues from control mice were stained for TRα. Representative photomicrographs show (brown) immunoreactive cells in stroma regions. These most likely represent HSC/myofibroblasts (spindle-shaped) (Figure 7B). Determination of ALT and AST showed increased liver cell damage in the knockout mice (Figure 7D). MCD-fed $TR\alpha^{-/-}$ mice

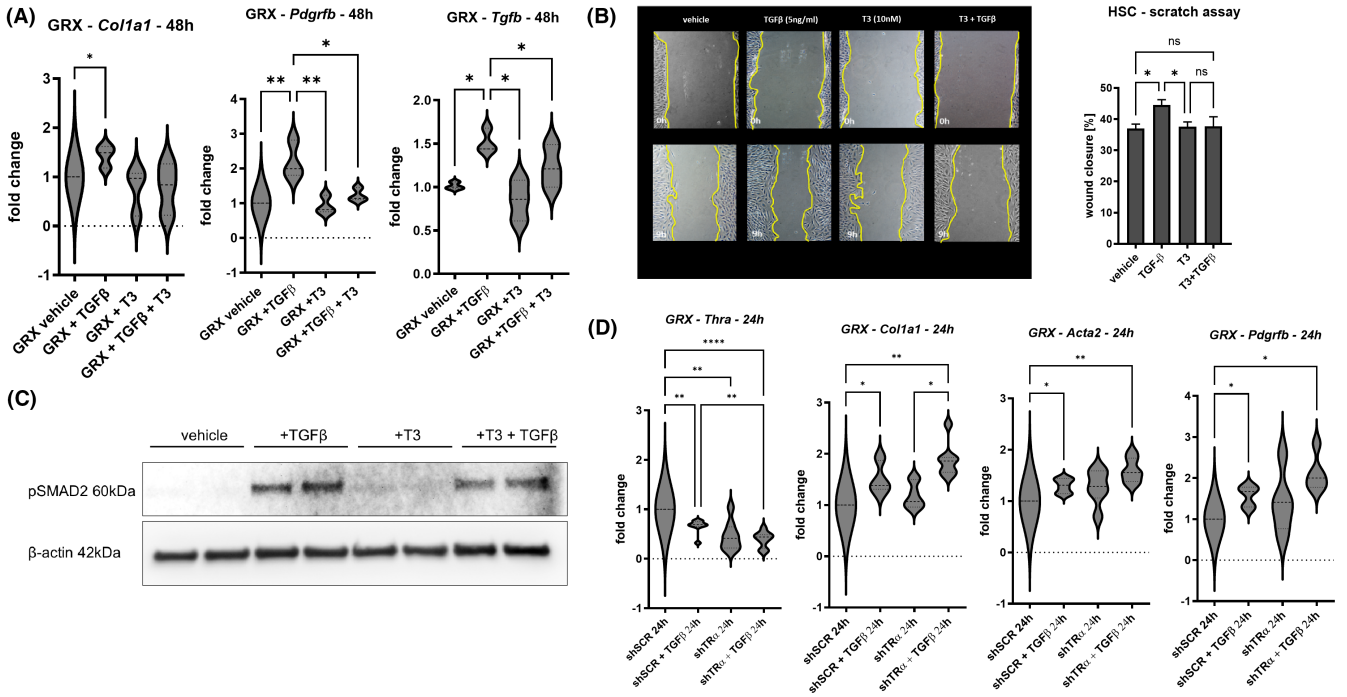

FIGURE 5 T3 attenuates TGFβ -induced fibrogenesis in an HSC cell line. (A) GRX HSC line was treated with vehicle or TGFβ and or T3 for up to 48 h. RNA was analysed by qRT-PCR for *Col1a1*, *Pdgfrb* and *Tgfb*. Results are expressed as fold change; mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$ compared with vehicle-treated GRX. (B) Wound healing under vehicle or TGFβ ± T3 conditions at time 0 (time of scratch) and 9 h later; migration quantified by measuring the distance separating two sides of the monolayer. Mean (% wound closure) ± SEM is graphed. (C) Representative western blot images showing phospho-Smad2 (pSmad2) and β-actin as a loading control. (D) GRX HSCs were first treated with lentiviral particles containing shRNA constructs specifically targeting *Thra* (GRX-*shThra*) or controls (GRX-*shScr*) (non-targeting scrambled shRNA). *Thra* knockdown (~60%) HSC (*shThra*) and control HSC (*shScr*) were then treated with recombinant TGFβ (or vehicle) for 24 h. Cells were harvested, and mRNA was analysed by qRT-PCR for *Thra*, *Col1a1*, *Pdgrfb* and *Acta2*. Results are expressed as fold change relative to *shScr* and graphed as mean ± SEM. *$p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

showed increased liver fibrosis compared to MCD-fed WT mice. This was demonstrated by induction of essential fibrogenic genes *Acta2* and *Tgfb* (Figure 7C) and by increased accumulation of αSMA (10-fold) and Col1α1 (10-fold) positive cells and increased Sirius Red staining in MCD-fed TRa−/− mice compared with MCD-fed WT mice (Figures 7E,F and 8A). In line with the results mentioned above, sections from NASH patients with fibrosis showed a reduced expression of TRα (Figure 8B).

The mean starting weight of the WT mice was higher than that of the knockout littermates (26.46 g vs. 21.26 g). The $TRa^{-/-}$ mice have a known growth retardation. Having the same starting weight would have resulted in a significant age difference as a possible BIAS. The weight loss was more severe in the WT mice due to the different starting weights (−43.9 vs. −36%).

3.9 | Ductular Liver Progenitor cells

In the initial experiments, we have shown that cells other than hepatocytes and stellate cells also express TR (Figure 1). The ductular reaction is responsible for pathological liver repair in cholangiopathies, leading to fibrosis. Therefore, we investigated the influence of the TH status in the immortalized but non-transformed murine immature cholangiocyte cell line 603B, which is known to have progenitor properties.[33] Interestingly, T3 treatment inhibited TGFβ-mediated activation (Figure S6A–C), and TRα knockdown promoted TGFβ-mediated activation of LPC (Figure S6D).

4 | DISCUSSION

In this work, we evaluated the role of TH and TRs in hepatic fibrogenesis. In vitro and in vivo data show that TRα and TRβ are downregulated in the liver during hepatic fibrogenesis in humans and mice. In cell culture experiments, treatment of HSC with TGFβ also downregulated the expression of both receptors. HSC express predominantly TRα, while hepatocytes mainly express TRβ. Loss of TRα promoted fibrogenesis. Interestingly, the fibrogenic response of HSC to treatment with TGFβ is attenuated by simultaneous treatment with T3. Treatment with T3 leads not only to decreased expression of mRNA of pro-fibrotic genes but also to decreased phosphorylation of SMAD2, which is an essential downstream target in the activation of the canonical TGFβ signalling pathway.

Furthermore, we could show that functional properties, such as the TGFβ induced migration, is also reduced by activating TRs. Consistent with these results, the knockdown of the *Thra* (encoding

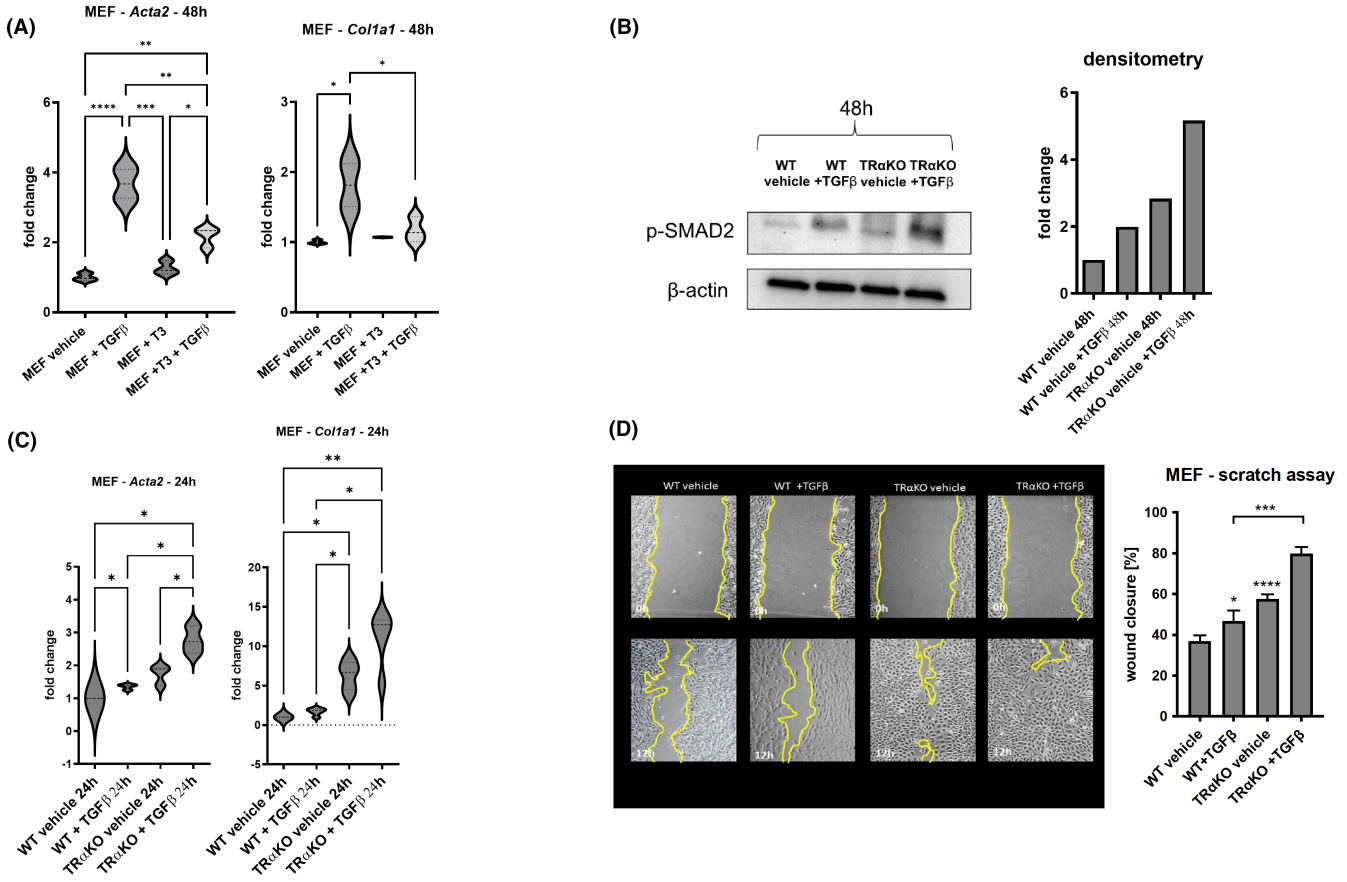

FIGURE 6 MEF is responsive to TGFβ and T3 treatment (A) WT MEF were cultured in TH-stripped serum for 24 h and then treated for 48 h with T3 (10 nM) and TGFβ (5 ng/mL). At the end of treatment, RNA was analysed by qRT-PCR. *Acta2* and *Col1a1* are shown. Results are graphed as fold change; mean ± SEM; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$ compared with vehicle treated MEFs. $TR\alpha^{-/-}$ MEF (TRαKO) exhibit enhanced fibrogenic responses: MEF from $TR\alpha^{-/-}$ mice and littermates were treated with TGFβ for 24-48 h. At the end of treatment, cells were extracted for mRNA and protein analysis. (B) Immunoblot showing representative images of phospho Smad2 (pSMAD2) and β-actin and corresponding densitometry. (C) *Col1a1* and *Acta2* expression. Results were presented as fold change; mean ± SEM; $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$, $^{****}p < .0001$ compared with vehicle-treated littermate WT MEFs. (D) Wound-healing assay under vehicle or TGFβ conditions at time 0 (time of scratch) and 12 h later.

for TRα) leads to increased fibrogenic activity of murine stellate cells. These results were confirmed in $TR\alpha^{-/-}$ MEF cells treated analogously to the previous experiments. The interaction of TRα with TH regulates the phenotype of HSC to an extent via the TGFβ signalling pathway so that the TH-TR interaction may be attractive for future therapy of liver fibrosis. RNA sequencing data from $TR\alpha^{-/-}$ MEF suggest that investigating the contribution of other potential signalling mediators in the future may be worthwhile, such as Wnt and Hedgehog signalling. For example, TR-dependent proliferation in hepatocytes is linked to the Wnt/β-Catenin pathway.[36] These observations suggest that TH regulation is not restricted to liver fibrogenesis.

Recent studies (preclinical and clinical) suggest that there is a strong association between subclinical and overt hypothyroidism and the incidence and severity of NAFLD in adults and children.[8,37,38] Further studies found that hypothyroidism is also associated with the presence and severity of NAFLD-related fibrosis.[5–7,39] Two recent meta-analyses confirmed those results and claimed that hypothyroidism may play a vital role in the progression and development of NAFLD.[40,41] Intriguingly, another meta-analysis proved that low thyroid function was associated with an increased likelihood of liver, heart, and lung fibrosis.[42] Our data offer cellular evidence for these observations by showing that disrupted TH signalling leads to an activation of HSC and that other fibrogenic-like cells may respond similarly.

Ongoing clinical studies with TRβ selective agents suggest that in NASH, selectivity for TRβ is associated with the liver metabolic benefits of TH. These benefits range from the reduction of hepatic fat, atherogenic lipids, and lipoproteins while avoiding unwanted systemic actions of excess TH in other organ systems, which are mainly regulated by TRα. In a 36-week phase 2 NASH clinical trial, Resmetirom (MGL-3196; a TRβ-agonist) demonstrated statistically significant reductions in MRI–proton density fat fraction (PDFF, a measure of liver steatosis), liver enzymes, atherogenic lipids, Lp(a), markers of inflammation and fibrosis.[12,13] The two major TR isoforms, TRα and TRβ, show tissue- and cell-specific distributions. TRβ mediates the metabolic actions of T3 and is the major receptor isoform expressed in the liver.[43] Our data confirm TRβ's dominance in

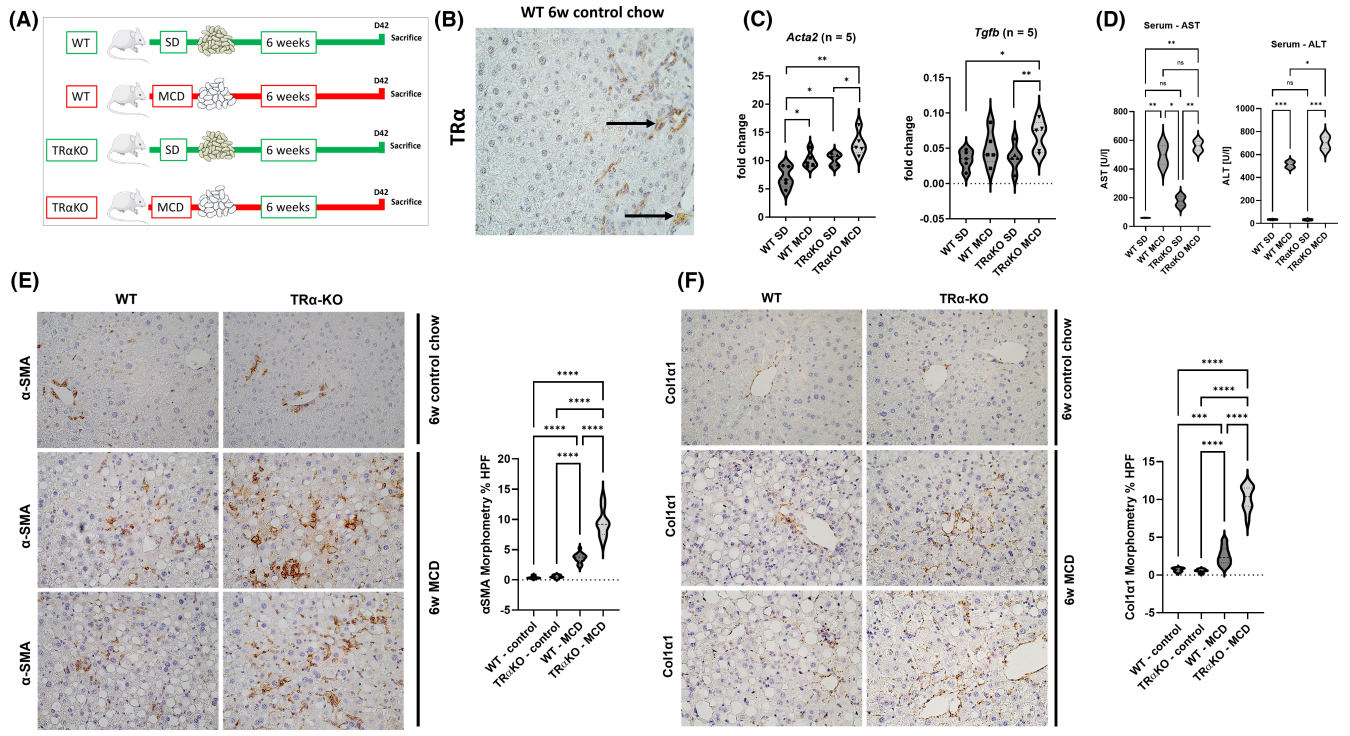

FIGURE 7 TRα-knockout alleviates liver fibrosis induced by methionine choline-deficient diet in mice: Wild type (WT) and TRα knockout ($TR\alpha^{-/-}$) mice (TRαKO) were fed control chow or the methionine-choline-deficient (MCD) diet for 6 weeks. The livers were harvested for IHC and qRT-PCR. Representative staining is shown. (A) Scheme of the experimental model (n = 5/group). (B) Formalin-fixed, paraffin-embedded liver tissues from standard chow-treated WT mice were stained for TRα. Representative micrographs show (brown) immunoreactive cells in stromal regions. These are most likely HSC/myofibroblasts (spindle-shaped) and are consistent with the findings in Figure 1. (C) Transcript levels of pro-fibrotic genes in the liver of the different groups. (D) Serum markers of liver injury. (E) α-smooth muscle actin (αSMA) and collagen (F) 1α1 (Col1α1) immunoreactivity and morphometry (20× fields for analysis). Results are expressed as fold change relative to control mice and presented as mean ± SEM. *$p < .05$ versus control mice. Mean ± SEM were plotted. $p < .05$, **$p < .01$, ***$p < .001$, ****$p < .0001$.

hepatocytes. However, a direct effect of a TRβ selective agonist on fibrogenesis has not been conclusively demonstrated, especially as HSC (the primary driver of fibrogenesis) predominantly express TRα. Considering this, these compounds could indirectly affect fibrosis by improving the metabolic situation rather than directly reducing HSC activation. However, TRβ-specific agonists may also cause activation of TRα, albeit to a lesser extent, as treatment with GC-1 in HSC resulted in a similar activity-inhibitory response, although TRβ expression is very low. GC-1 is a synthetic TH analogue that is relatively selective for both the binding and activation functions of TRβ1 over TRα1 (KD values are 67 and 440 pM, respectively). However, GC-1 is 5 and 100 times more potent than the endogenous agonist T3 at TRα1 and TRβ1 in vitro.[44] MGL-3196 is 28-fold selective for TRβ (EC50 = .21 μM) over TRα (EC50 = 3.74 μM) in a functional assay.[45] The very potent hepatic thyromimetic effect of MGL-3196 is a result of its´ TRβ selectivity and hepatocyte-specific transport mediated by OAPT1B1, although MGL-3196 is about 60–100 times less potent than the endogenous T3.[14] Therefore, it is unknown whether MGL-3196 has a similar effect in HSC as GC-1.

Recently, intriguing evidence emerged for a direct relationship between T3 and TGFβ signalling in a fibrotic context. Previous results have already demonstrated a repressing function of both isoforms (TRα and TRβ) on TGFβ signalling after binding of T3.[11] Another group reported a reduced expression of TR in human NASH.[46] Alonso-Merino and co-workers detected a direct interaction of TRs with Smad2/3 and Smad4 in a way that, if bound to T3, inhibition of TGFβ related recruitment of SMADs occurs. In this context, the group further demonstrated that liver fibrosis induced by CCl_4 is attenuated by TH administration in mice.[11]

Conversely, mice lacking TRs exhibited increased collagen deposition within the liver.[11] Hypothyroidism-induced steatosis, glycogen loss, fibrosis and a minor vascularization in the liver of rabbits. In contrast, hypothyroidism increased the proliferation of hepatocytes and the infiltration of mast cells but did not modify the number of immune cells in the sinusoids. These changes were associated with a minor anti-FXRα immunoreactivity of periportal hepatocytes and pericentral immune cells.[47] We show for the first time that the fibrogenic response to chronic liver injury is more potent in a TRα-specific model. This ultimately underlines the relevance of TRα in the development of fibrosis and contrasts with the previous assumption of an exclusive relevance of TRβ.

Evidence suggests that NASH may be, in part, a condition of diminished liver TH levels or hepatic hypothyroidism, which is controlled by deiodinases. Indeed, compared to healthy controls,

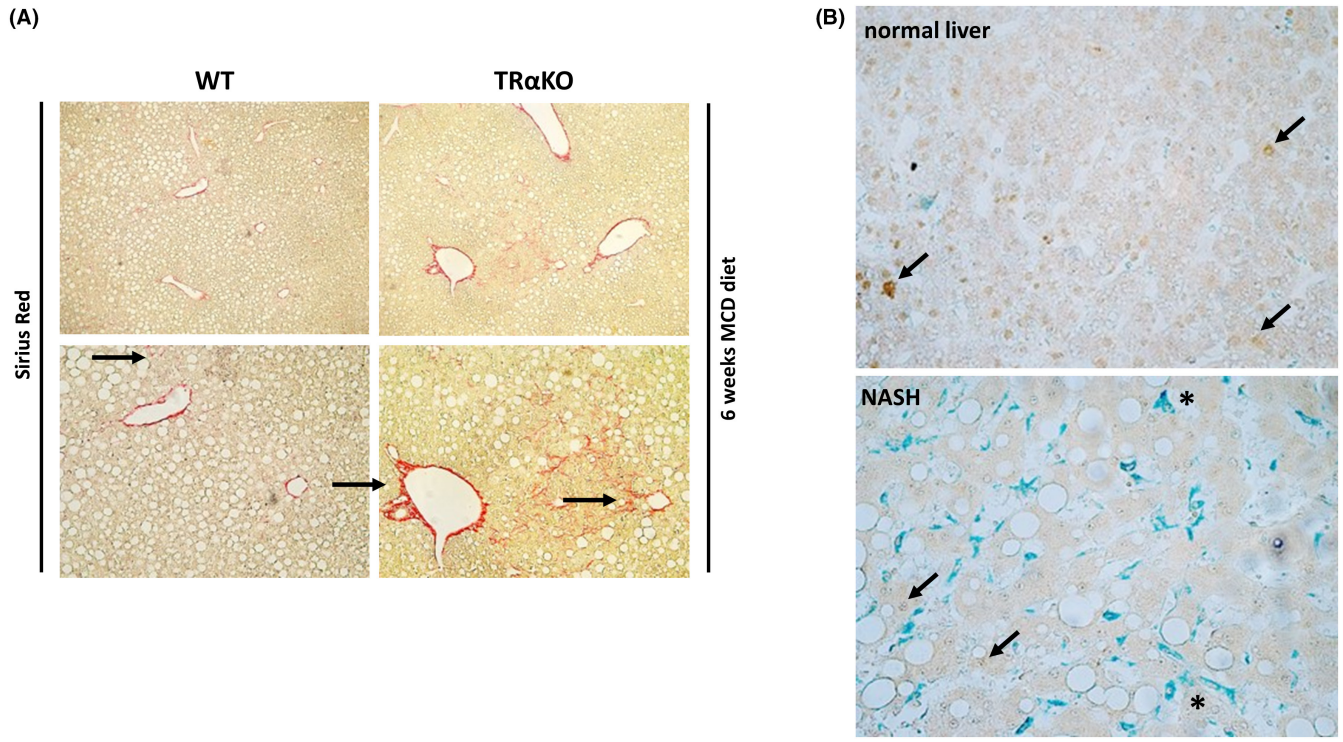

FIGURE 8 (A) Representative Sirius-red staining; black arrows indicate Sirius-red stained fibrils. (B) Representative double immunostaining (TRα and αSMA) from advanced NASH patients and normal liver as a control; black arrows indicate TRα-positive cells, * indicates αSMA-positive cells

NAFLD patients demonstrated reduced expression of *DIO1* and increased expression of *DIO3*.[10] These data imply the presence of intra-organ (i.e. intrahepatic) TH regulation. Indeed, the present data reveal that HSCs are responsive to TH and harbour the intracellular mechanisms to regulate it.

There are several limitations of our study. Although the expression of TRβ is very low in HSC, this does not preclude the possibility that it may still modulate HSC fibrogenesis. TRβ knockdown experiments are challenging to accomplish due to their low expression in HSC. As such, it would be important to study the fibrogenic actions of $TR\beta^{-/-}$ mouse-derived HSC. In addition, although the current work has focused on TGFβ-dependent mechanisms, likely TRα signalling may also be modulating fibrogenesis via other mechanisms. Also, the critical question arises whether TRα mediated effects on hepatocytes are synergistic to those of TRβ or even partly antagonistic. This is of major importance for therapeutic application in vivo and needs to be analysed in a comparative gene pathway analysis. This information is particularly crucial for treating metabolic liver disease with TR agonists. Finally, it remains unclear how T3-TR interactions suppress the phosphorylation of SMAD2. We know that TH mainly mediate their function via their TRs, which act as ligand-dependent transcription factors. However, it was recently demonstrated that ligand-bound TRs also mediate the activation of the phosphatidylinositol-3-kinase (PI3K) pathway, which represents a noncanonical TH/TR action more rapid and independent from DNA-binding.[48–50]

In summary, our analyses in cell culture, mice and humans show that TH modulates HSC function by regulating TGFβ signalling. TRα plays a specific role in HSC fibrogenesis, suggesting that the role of TRα in chronic liver injury and fibrosis should not be neglected.

AUTHOR CONTRIBUTIONS

Paul Manka, Jason D. Coombes, Svenja Sydor, Cindy Wang, Marzena K. Swiderska-Syn, Sebastian Hönes, René J. Boosman, Andrea Tannapfel, Anja Figge performed experiments and contributed intellectually to the study; Jan Best, Karine Gauthier, Leo A. van Grunsven, Ye H. Oo, Anna M. Diehl, Lars C. Moeller, Klaas N. Faber, Oliver Goetze, Patricia Aspichueta, Christian M. Lange, Ali Canbay provided samples and contributed to the study design, analysis and interpretation of data and funding of the studies. Paul Manka, Jason D. Coombes, Ali Canbay, Wing-Kin Syn co-wrote the manuscript. Wing-Kin Syn is the lead investigator, who designed and supervised the overall project and substudies, performed experiments, wrote the manuscript and is the senior author and guarantor of the manuscript. All authors reviewed the draft for important intellectual content and approved the final article for submission.

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CONFLICT OF INTEREST STATEMENT

Nothing to report.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

INSTITUTIONAL REVIEW BOARD STATEMENT

Liver samples from PBC and NASH patients were acquired from the Liver and Hepatobiliary Unit in Birmingham in accordance with local ethical approval REC 06/Q2702/61 (University of Birmingham RG-06235/Trust Ref RRK3181). The liver specimens for hepatic stellate cell isolation were obtained from fresh tumour resections at the University Hospital Essen (reference number: 12-5232-BO).

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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