약학석사학위논문

RORα agonist ODH 2-12의 간 성상세포 섬유화 억제 효과

Inhibition of Fibrogenic Activation of Hepatic Stellate Cells by a Potential RORα Agonist ODH 2-12

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약학과 병태생리학 전공

Melody Chambugong

ABSTRACT

Inhibition of Fibrogenic Activation of Hepatic Stellate Cells by a Potential RORα Agonist ODH 2-12

Melody Chambugong
College of Pharmacy
The Graduate School
Seoul National University

Hepatic fibrosis is a dynamic process characterized by the net accumulation of extracellular matrix resulting from chronic liver injury of any etiology, including viral infection, alcoholic liver disease, and nonalcoholic steatohepatitis (NASH). Activation of hepatic stellate cells (HSCs) causes transdifferentiation of quiescent cells into proliferative, and fibrogenic myofibroblasts which are now well established as a central driver of fibrosis. Recently, retinoic acid receptor-related orphan receptor alpha (RORα) has been demonstrated to have the effects on mitochondrial function in hepatocytes and M1/M2 polarization in Kupffer cells, thereby attenuates NASH. Here, I investigated the effect of a potential RORα agonist, ODH 2-12 in the activation of HSCs and in diet-induced fibrosis mice model. First,

activation of RORα by transient overexpression had an anti-fibrogenic effect in transforming growth factor-beta 1 (TGFβ1) activated human HSCs. Second, the anti-fibrotic effect of ODH 2-12 was observed in the mRNA and protein expression of the fibrogenic markers such as alpha-smooth muscle actin and collagen type 1 alpha 1 in TGFβ1 activated human HSCs. Third, the effect of ODH 2-12 was also observed in primary mouse HSCs, in that it reduced expression of the fibrogenic markers. Fourth, a diet-induced fibrosis mice model was developed by feeding the western diet. Treatment with ODH 2-12 showed reduced collagen deposition level and expression of fibrogenic markers. Finally, dual-luciferase reporter assays indicated that ODH 2-12 reduced the TGFβ1-induced reporter gene expression in the SMAD reporter. In conclusion, RORα and its agonist ODH 2-12 showed a potential anti-fibrotic effect which would provide a potential anti-fibrotic strategy.

Key Words: RORα agonist, Hepatic stellate cells, Diet-induced fibrosis

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LIST OF ABBREVIATIONS

HSCs	Hepatic stellate cells
TGFβ1	Transforming growth factor β1
RORα	Retinoic acid receptor related orphan receptor alpha
ECM	Extracellular matrix
WD	Western diet
NR	Nuclear receptor
SMAD	Suppressor of mothers against decapentaplegic
NAFLD	Nonalcoholic fatty liver diseases
NASH	Nonalcoholic steatohepatitis
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase

Ⅰ. INTRODUCTION

Liver fibrosis is a wound-healing response that involves an array of cell types and mediators to enclose the injury (Friedman 2008). It is a vigorous process designated by the net accumulation of extracellular matrix (ECM), caused by the chronic liver injury of any kinds including chronic viral infection, alcoholic liver diseases (ALD), and NASH, a progressive form of NAFLD (Tsuchida *et al,* 2017). Nash is currently arising as the looming threat in public health worldwide; however drug development in this area of disease is explosive as it needed to address complex metabolic dysfunction including fibrosis and the clinical endpoint of cirrhosis (Angulo *et al* 2015). Moreover, that unmitigated fibrosis leads to cirrhosis, with consequences of increased liver-related mortality and development of cancer or in need for liver transplantation (Wattacheril *et al* 2018).

There are diverse cell genres which are considered as the sources of ECM in liver fibrosis. However, among these cells, activated hepatic stellate cells (HSCs) are now well established as the main sources of ECM in liver fibrosis (Schuppan *et al* 2013). In normal liver, HSCs maintain a non-proliferative, quiescent, vitamin A storing phenotype but when activated, they transdifferentiate to myofibroblasts which are proliferative, contractile, inflammatory and chemotactic followed by enhanced ECM accumulation (Puche *et al* 2013). The proliferation of activated HSCs mainly follows four fibrogenic pathways such as TGFβ (transforming growth factor β), PDGF (platelet-derived growth factor), VEGF (vascular endothelial growth factor) and CTGF (connective tissue growth factor). Among

them, TGFβ1 is generally considered the most influential fibrogenic cytokine (Hellerbrand *et al* 1999). TGFβ binding and phosphorylation of the TGFR1 (type I receptor) induces phosphorylation of downstream SMAD proteins, primarily SMAD3. Upregulation of SMAD3 during HSC activation promotes transcription of type I and type III collagen (Friedman 2008).

Retinoic acid receptor-related orphan receptor alpha (RORα) is a nuclear receptor which is a member of the steroid/thyroid hormone receptor superfamily. It binds to a specific DNA sequence named ROR response element (RORE) either as monomer or homodimer in the regulatory region of target genes (Jetten, 2009). RORα ligands such as Cholesterol Sulfate, SR1078, and JC1-40, reversibly bind and increase the transcriptional activity of target genes (Solt *et al* 2012, Kim *et al* 2012). Moreover, RORα is known to have a pivotal role in liver diseases. In previous study, we demonstrated that RORα attenuates NASH by inhibiting lipid accumulation, oxidative stress and by inducing M1/M2 polarity in liver macrophages (Kim *et al* 2012, Han *et al* 2014, Han *et al* 2017). It also attenuates NASH by inducing mitochondrial functions (Kim *et al* 2017). Recently we found that in HFD fed RORα - LKO (hepatocyte knockout) mice model, the liver sections showed increased collagen deposition in Sirius red staining along with increased expression of fibrogenic marker such as αSMA, TGFβ1, MMP2, Timp1 (Kim *et al* 2017). These results indicate that RORα may have an inhibitory effect on liver fibrosis. However, the role of RORα in liver fibrosis as well as in HSCs has not been investigated to date.

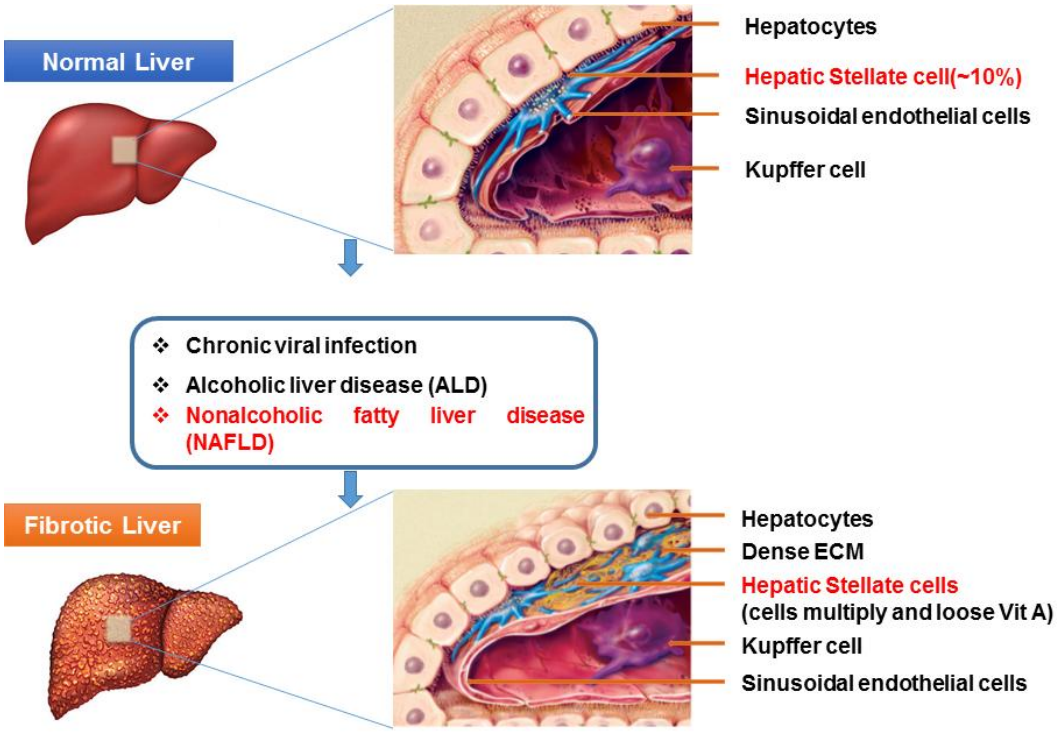

Figure 1. Cellular architecture in liver fibrosis

In liver fibrosis the cellular architecture of liver changes. Especially HSCs get activated and undergo drastic phenotypical changes. In normal liver, HSCs are non-proliferative vitamin A-storing cells. Upon liver injury, the cells start to multiply and lose their vitamin A-storing capacity. (Adopted from Puche *et al* 2013)

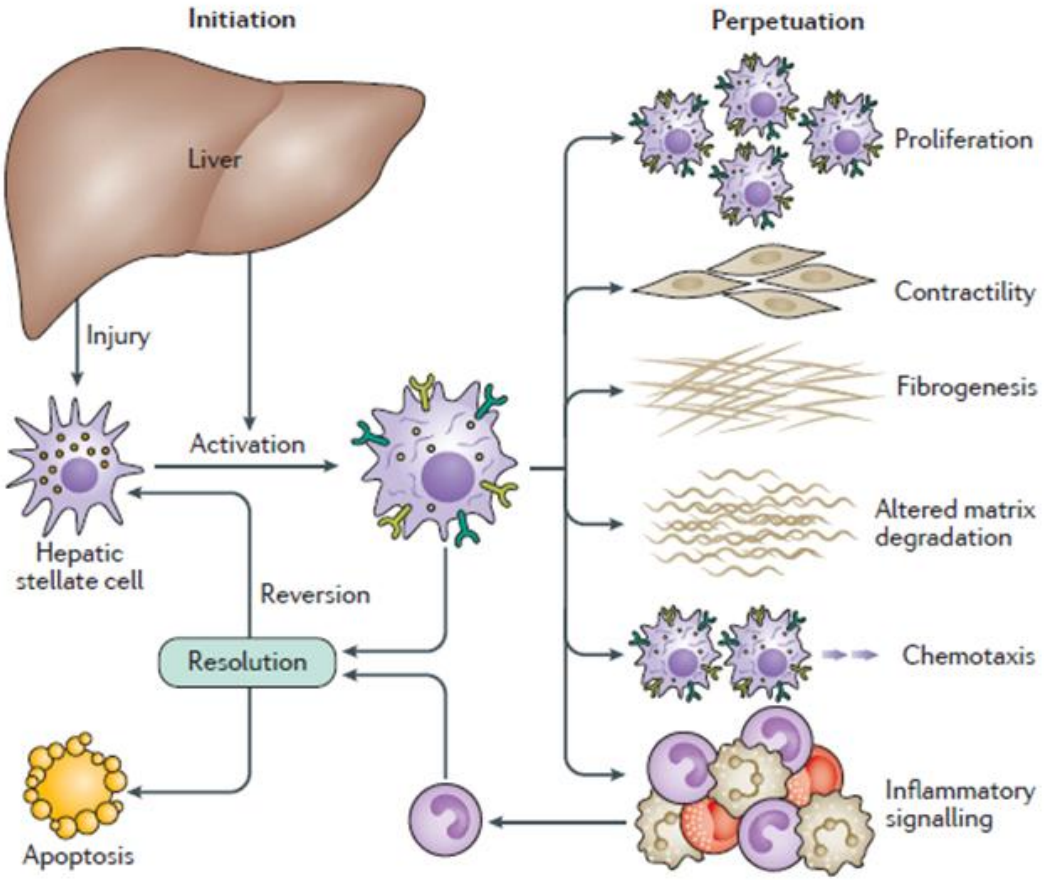

Figure 2. Phenotypical changes in HSCs activation

Liver injury initiates the activation of HSCs and cell perpetuation begins which are characterized by specific phenotypical changes including proliferation, contractility, fibrogenesis, and chemotaxis. The resolution of this phenomenon would be either the reversion or apoptosis of the cells. (Adopted from Tsuchida *et al* 2017).

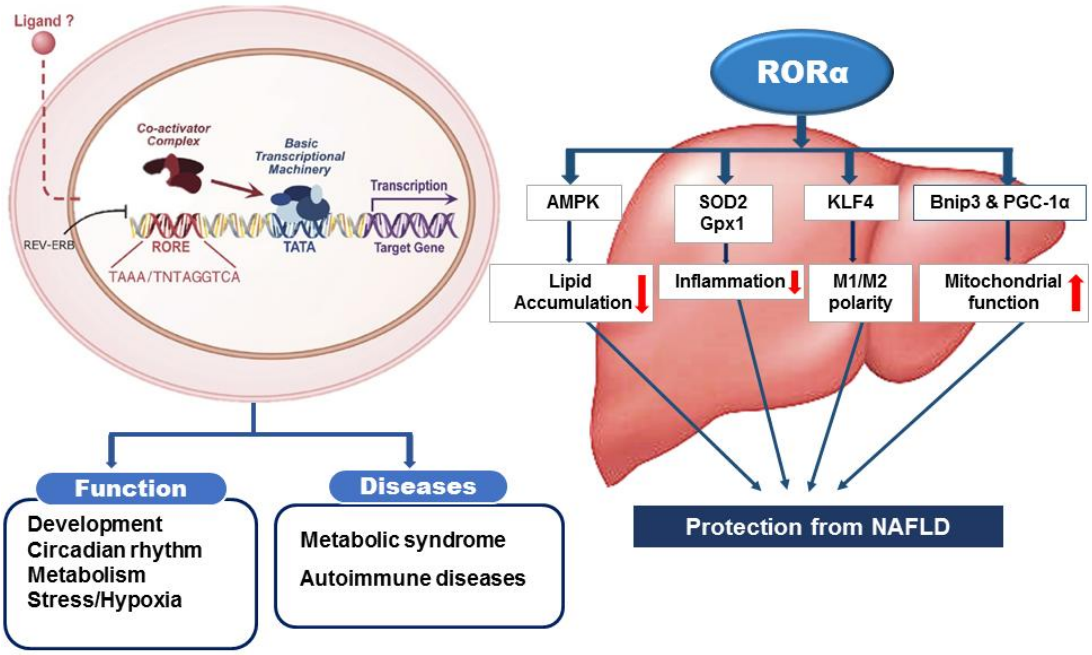

Figure 3. Physiological function and role of RORα in liver diseases

RORα is a member of steroid/thyroid superfamily of RORs. It binds to a specific DNA sequences called RORE and activate through ligand binding to regulate gene expression. RORs plays critical roles in many physiological processes such as development, circadian rhythm, metabolism, stress, and hypoxia. RORα is well known to play a major role in liver diseases including protection from NAFLD and NASH. (Adopted from Jetten *et al* 2009, Kim *et al* 2012, Han *et al* 2014, Han *et al* 2017, Kim *et al* 2017))

Ⅱ. PURPOSE OF THE STUDY

Liver fibrosis and liver cirrhosis are the major causes of morbidity and mortality in chronic liver diseases but the prevention and reversal of this condition have become a major endpoint in clinical trials in liver-specific drugs. Especially in fibrosis which is induced by advanced alcoholic or nonalcoholic steatohepatitis (NASH). As it is now evident that untreated NASH may develop into fibrosis and further to cirrhosis and hepatocellular carcinoma (HCC). Although liver fibrosis is reversible but proper drug to treat this condition is not developed yet. In our previous study, we found that RORα has a protective effect in NAFLD and NASH. We have found the effect of RORα in hepatocyte and Kupffer cells which gave us a clear idea of the role of RORα in NASH conditions. However, we also found that the pro-fibrotic factors were elevated in the liver of the RORα-LKO mice. Which took our interest to find out the effect of RORα in liver fibrosis as well as in the HSCs. There were no significant studies on the effect of RORα or its agonist in the activation of HSCs. Therefore, in this study, I aimed to identify the effect of RORα and its potential agonist ODH 2-12 in the activated HSCs as well as in the diet-induced liver fibrosis mice model. To investigate this I used RORα overexpression and SMAD reporter assay approach in hHSC cell line. In addition, I also evaluated the effect of ODH 2-12 in hHSC and primary HSCs. To further determine the effect of ODH 2-12, I developed a diet-induced liver fibrosis mouse model by feeding western diet. Thus I assessed the effect of ODH 2-12.

Ⅲ. MATERIALS AND METHODS

1. Cell culture and cell treatment

The Lx-2 cell line (an immortalized human HSCs line) was kindly provided by Professor Kim Sang Geon (College of Pharmacy, Seoul National University). The cells were cultured Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin/streptomycin. The cells were activated with recombinant human TGF-β1 from Peprotech (Rocky Hill, NJ 08553 USA) which was added to the supernatant at 5.0 ng/ml for 18 h. The Primary HSCs were isolated from 8-9 week-old, male C57/BL6J mouse liver. Under anesthesia with Zoletil & Rampoon, livers were perfused with Hank's buffered salt solution followed by continuous perfusion with a 0.1% (wt/vol) collagenase (Sigma, Type IV). The separation of stellate cells from non-parenchymal supernatant was followed by forming concentration gradient of 52/50/30% percoll (GE Healthcare, Waukesha, WI) and centrifuging at 2200 rpm for 15 minutes (Vrochides D *et al* 1996). The layer containing stellate cells was plated with Dulbecco's modified Eagle's medium with 10% FBS, 1% penicillin/streptomycin. After 24 h the cells were rinsed in the PBS and maintained in the similar medium at 37℃ in a humidified atmosphere with 5% CO_2.

2. Western diet-induced mice model

The diet-induced liver fibrosis mice were developed by feeding western diet (Research diet #D12079B) to 7 week-old, male C57/BL6J mice for 21 weeks. For control mice, low-fat diet (D12450J) with similar time-span was also fed. ODH 2-12 treatment was started

from 16 weeks of western diet feeding by oral gavage with the dose of 10 mg/kg body weight for 5 weeks once a day. After 21 weeks the mice were sacrificed. The blood serum and liver samples were collected for further assay. For histological examination, sections of liver tissue were embedded in parraffin and stained with hematoxin and eosin (H&E) and Sirius red (Hsservice, 145, Geumnanghwa-ro, Ganseo-gu, Seoul, Korea).

3. Reporter gene assay

Chang liver cells and Lx-2 cells were seeded in a 24 well plate with a density of 2.5×10^4 per well. After 24 h the cells were washed with 1X PBS and changed to a new cell media. In Chang Cells, the cells were transfected using DNA mixture of expression vectors (RORα, RORβ, RORɣ, PPARα, PPARδ, PPARɣ, LXRα) luciferase reporter promoter (Gal4-tk-Luc), and β-galactosidase vector using polyfect transfection reagent (QIAGEN). Compound treatment was done for another 18 h. In Lx-2 cells, expression vectors (myc-EV and myc-RORα) were transfected with SMAD reporter and β-galactosidase vector using polyfect transfection reagent. ODH 2-12, OCA, and TGFβ1 were treated together for another 18 h. To harvest cell lysate, 200 μl of luciferase cell culture lysis 5X reagent (E1531; Promega) was used. The luciferase reporter promoter activity was normalized by β-galactosidase.

4. Western blot assay

Cells were washed with cold 1x PBS and harvested with a RIPA lysis buffer (25 mM Tris-HCl, 150 mM NaCl, 0.1% sodium dodecyl sulfate, 1% Triton X-100, 1% deoxycholate, 5 mM EDTA) supplement

with a protease inhibitor cocktail (11.836.153.001, Roche, Switzerland) and a phosphatase inhibitor (4906845001, Roche, Switzerland) by using cell scraper. After 30 minutes of incubation on ice, lysates were centrifuged at 13,000 rpm for 15 minutes, 4℃. supernatant was separated and quantified through the BCA Protein Assay Kit (23225, Pierce, USA). Protein samples were loaded in 7% gel. After SDS-polyacrylamide gel electrophoresis, proteins were transferred onto a 0.45 μm polyvinylidene difluoride membrane by semi-dry transfer method. 1 w/w% non-fat dry milk in PBS with 0.1% Tween-20 (PBS-T) was used for blocking membrane for 1 hour under room temperature. After blocking, membranes were incubated in primary antibodies in 1 w/w% non-fat dry milk in PBS-T overnight in 4℃. Membranes were washed 3-times with PBS-T and incubated secondary antibodies for 1 h under room temperature. Amersham Prime ECL solution (RPN2232, GE healthcare, USA) was used for detection after washing 3-times with PBS-T to remove antibodies. Antibodies used in western blottings are anti-αSMA (ab7817, 1:200 dilution), Anti-COL1A1 (sc-293182, 1:2000 dilution), anti-COL1A2 (sc-8787, 1:2000 dilution), anti-RORα (sc-6062, 1:2000 dilution), anti-HSP60 (ab45134, 1:20,000 dilution).

5. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA isolation was done by EASY-BLUE™ Total RNA Extraction Kit (Intron Biotechnology, Korea) according to the manufacturer's protocol (Lx-2 cells). For primary HSCs, RNA was extracted using the RNeasy Micro Kit (QIAGEN 74004). Extracted total RNA was reverse-transcripted to synthesize cDNA using M-MLV reverse transcriptase (28025-013, Invitrogen). qRT-PCR was

performed using SYBR Green PCR master mix (4367659, Applied Biosystems). The resulting ΔCt values were normalized with 18s rRNA.

6. Statistical analyses

All data were statistically analyzed by using GraphPad Prism 5 (GraphPad Software, USA). Statistical analyses were performed using unpaired 't' test, non-parametric Mann-Whitney 'U' test and one-way Anova test for comparisons of data. $P < 0.05$ denotes statistical significance.

Table 1. Primer sequences used for quantitative RT-PCR

Gene		RT-PCR Primer sequences
hα-SMA	Forward	5′-CTT CAG GGG CAA CAC GAA-3′
	Reverse	5′-CTT CAG GGG CAA CAC GAA-3′
hCOL1A1	Forward	5′-AAC ATG ACC AAA AAC CAA AAG TG-3′
	Reverse	5′-CAT TGT TTC CTG TGT CTT CTG G-3′
hTGFβ1	Forward	5′-GGC AGT GGT TGA GCC GTG GA-3′
	Reverse	5′-TGT TGG ACA GCT GCT CCA CCT-3′
h18s rRNA	Forward	5′-GTT CCG ACC ATA AAC GA-3′
	Reverse	5′-CTC GTT CGT TAT CGG AA-3′
mα-SMA	Forward	5′-TCG TTA CCT CCA AAG GCT GCT C-3′
	Reverse	5′-ATG GCG GTG TCT GGC TAT TCA-3′
mCOL1A1	Forward	5′-GAA ACC CGA GGT ATG CTT GA-3′
	Reverse	5′-GAC CAG GAG GAC CAG GAA GT-3′
mCOL1A2	Forward	5′-AGC CAA CCG TGC TTC TCA G-3′
	Reverse	5′-TCT CCT CAT CCA GGT ACG CA-3′
mCOL3A1	Forward	5′-AAG GCT GCA AGA TGG ATG CT-3′
	Reverse	5′-GTG CTT ACG TGG GAC AGT CA-3
m18s rRNA	Forward	5′-GTA ACC CGT TGA ACC CCA TT-3′
	Reverse	5′-CCA TCC AAT CGG TAG TAG GG-3′

Ⅳ. RESULTS

1. ODH 2-12 is a potential RORα agonist

To find out a proper agonist of RORα to enhance its possible anti-fibrotic effect, I used ODH 2-12 which is structurally modified derivative of JC 1-40. In our previous study, we mentioned that JC 1-40 is a synthetic ligand of RORα and it has an inhibitory effect in lipid accumulation, oxidative stress thereby attenuates NASH (Kim *et al* 2012). To identify ODH 2-12 as a potential ligand of RORα, I performed the reporter gene assay in Chang cells with nuclear receptors which play important roles in the liver. The transcriptional activity of ODH 2-12 was higher towards RORα nuclear receptor in comparison with other nuclear receptors such as RORβ, RORɣ, PPAR α, PPARδ, PPARɣ, LXRα. From this result, I concluded that ODH 2-12 is a potential agonist of RORα.

2. RORα prevents the TGFβ1-induced fibrogenic activation of HSCs

To investigate whether RORα regulate liver fibrosis, RORα overexpression study was done in Lx-2 cells. RORα was transiently transfected for 24 h and treated with TGFβ1 (5 ng/ml) for another 18 h. After that, expression levels of pro-fibrotic markers were detected by western blotting and qRT-PCR. Overexpression of RORα reduced the protein expression of pro-fibrotic marker α-SMA, COL1A1, and COL1A2. Similarly, the overexpression of RORα reduced the mRNA expression of pro-fibrotic marker α-SMA and COL1A1. Hence, these results indicate that RORα prevents the TGFβ1 induced fibrogenic activation of HSCs.

3. ODH 2-12 inhibits the TGFβ1-induced fibrogenic activation of HSCs.

To asses the effect of ODH 2-12 in HSCs, I treated this compound in Lx-2 cells. ODH 2-12 with 5, 10, 20, 30 μM doses were treated for 18 h along with TGFβ1 (5 ng/ml). Protein assay and mRNA assay were conducted to find the effect of ODH 2-12. It reduced the protein expression of fibrogenic marker α-SMA and COL 1A1 in comparison with TGFβ1. It also reduced the mRNA expression of similar fibrogenic markers along with the mRNA expression of TGFβ 1. Hence, this observation indicates that ODH 2-12 inhibits the activation of HSCs.

4. RORα and ODH 2-12 suppresses the transcriptional activity of the SMAD reporter

To determine how RORα and ODH2-12 inhibiting the TGFβ1-induced activation of HSCs I conducted reporter gene assay on the SMAD reporter in Lx-2 cells. First, I examined the effect of RORα in the SMAD reporter to find out its effect on the SMAD reporter which was stimulated by TGFβ1 (5 ng/ml). RORα dose-dependently downregulated the transcriptional activity of the SMAD reporter Second, I carried out a similar assay with ODH 2-12 and Obeticholic acid (OCA) without RORα. Both compounds downregulated the transcriptional activity of the SMAD reporter. Finally, to explore the effect of ODH 2-12 with RORα, I performed the reporter assay in the SMAD reporter and found out that in the presence of ODH 2-12, RORα also suppresses the transcriptional activity.

5. ODH 2-12 reduces the expression of fibrogenic markers in primary HSCs.

In addition, to investigate the effect of ODH 2-12 in primary HSCs I treated this compound in isolated primary HSCs. At first, I confirmed the activation of cultured primary HSCs by their morphological changes (microscopical pictures) for 6 days and extracted RNAs from 1^{st}, 3^{rd}, 5^{th}, and 6^{th} day and performed qRT-PCR assay. Notably, it is known that primary HSCs starts to activate spontaneously after 24 h of cell culture. mRNA expression studies from 1^{st}, 3^{rd}, 5^{th}, and 6^{th} day showed steady upregulation of pro-fibrogenic marker α-SMA, COL1A1 and COL3A1 expression and further confirmed the procedure. Next, I treated the cultured primary HSCs with ODH 2-12 from day 1 to day 6 and conducted qRT-PCR assay. Treatment with ODH 2-12 decreased the mRNA expression of similar fibrogenic marker α-SMA, COL1A1 and COL3A1. This observation further indicates that ODH 2-12 also inhibits the activation of primary HSCs.

6. ODH 2-12 treatment prevents diet induced liver fibrosis in vivo

To further asses the effect of ODH 2-12 in vivo I developed diet-induced liver fibrosis mice model. I fed the mice western diet for 21 weeks. Drug treatment has been done from 16 weeks to 21 weeks and after that, the mice were sacrificed and blood serum & liver tissues were collected and further studies are performed. ODH 2-12 treated WD mice shown decreased liver weight, ALT and AST level in comparison with WD fed mice. The H&E staining and Sirius red staining also showed decreased lipid, and collagen deposition in WD fed ODH 2-12 treated mice. The protein expression of fibrogenic

marker α-SMA and COL1A1 were decreased in ODH 2-12 treated mice in comparison with WD fed mice. Consistently, mRNA expression of pro-fibrogenic marker α-SMA, COL1A1, and COL1A2 also decreased in ODH 2-12 treated mice.

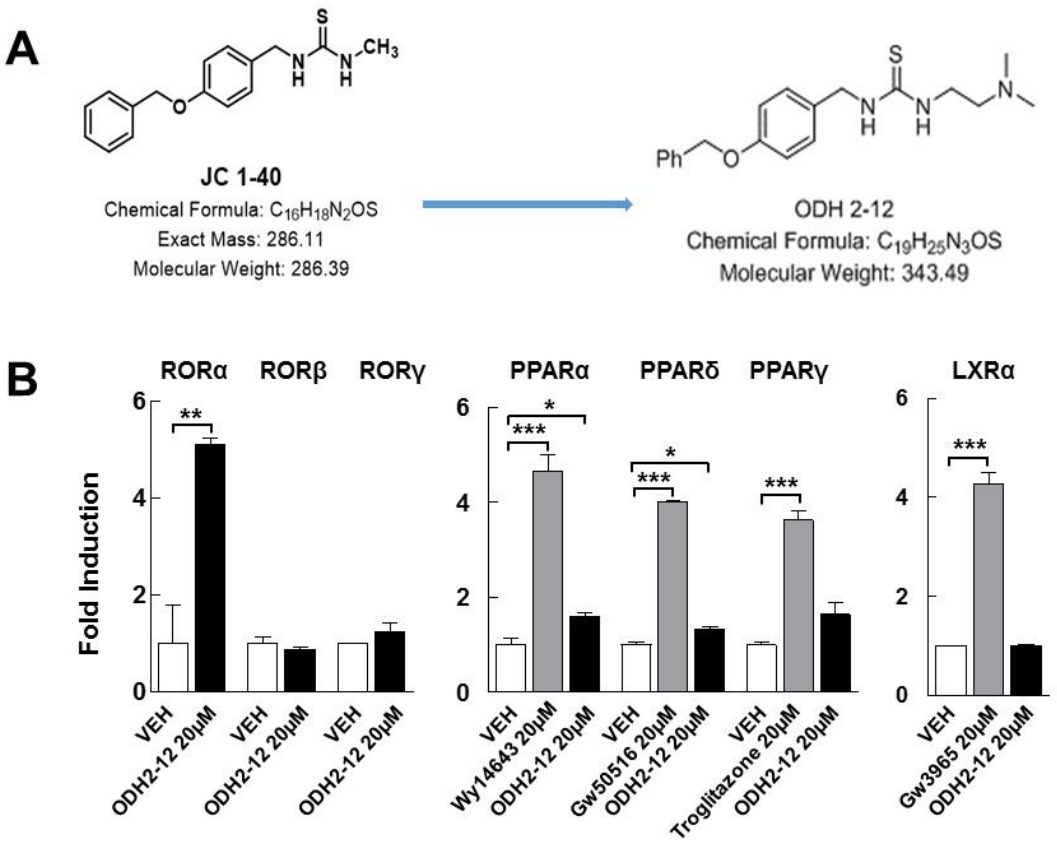

Figure 4. ODH 2-12 is a potential RORα agonist

(A) Chemical structure of JC 1-40 and its structurally modified derivative ODH 2-12.

(B) Chang cells were transfected by pM-hRORα, pM-hRORβ, pM-hRORɣ, pM-mPPARα, pM-mPPARδ, pM-mPPARɣ, and pM-hLXRα with the Gal4-tk-Luc and β-galactosidase for 24 h. After transfection, cells were treated with ODH 2-12 (20 μM), and respective agonist of each vector for 18 h. The statistical analysis was performed by unpaired 't' test, (n=3, *p<0.05, and ***, ###p<0.001).

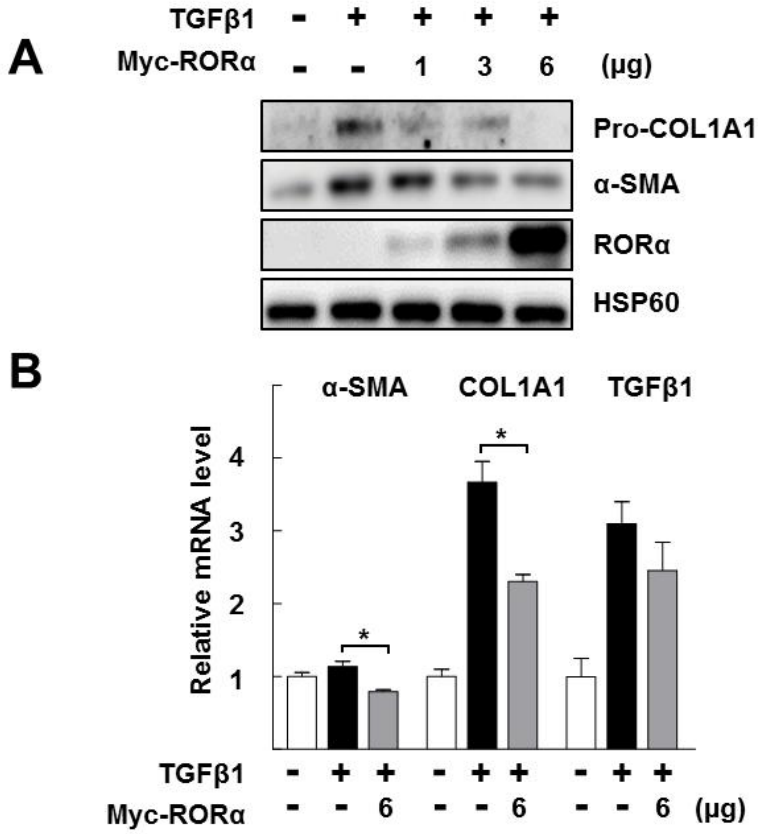

Figure 5. RORα prevents the TGFβ1-induced fibrogenic activation in hHSCs

Lx-2 cells were seeded into 60 mm plates at a density of $3x10^5$ cells per well and cells were transiently transfected with myc-EV and myc-RORα (1, 3, 6 μg for protein and 6 μg for mRNA study) for 24 h and then replaced with new culture medium which then treated with or without 5.0 ng/ml TGFβ1 for another 18 h. (A) Western blotting was performed to analyze the protein levels and (B) real-time qPCR was performed to analyze the mRNA levels of fibrogenic marker α-SMA, COL1A1 and TGFβ1. The statistical analysis was performed by following non-parametric Mann-Whitney 'U' test, (*$p<0.05$, ***, ###$p< 0.001$).

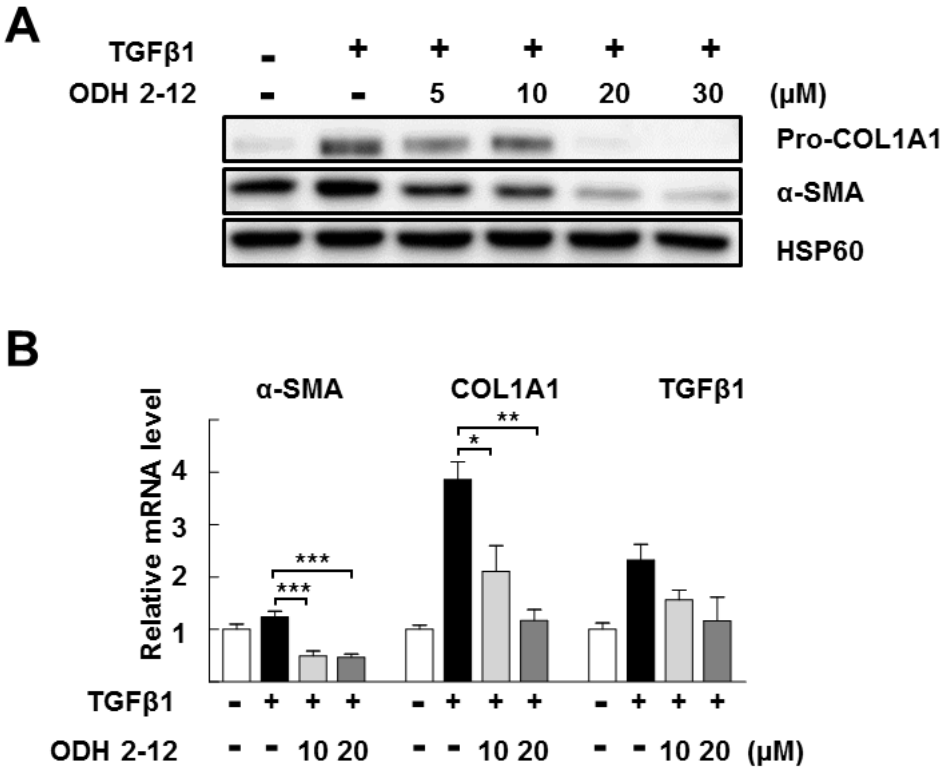

Figure 6. Treatment of ODH 2-12 inhibits the TGFβ1-induced fibrogenic activation in hHSCs

Lx-2 cells were seeded into 60 mm plates at a density of 3×10^5 cells per well, and cells were treated with ODH 2-12 (5, 10, 20, 30 μM) and with or without 5.0 ng/mL TGFβ1 for 18 h. (A) Western blotting was performed to analyze the protein levels and (B) real-time qPCR was performed to analyze the mRNA levels of α-SMA, COL1A1, and TGFβ1. The statistical analysis was performed by following one-way Anova test, (n=4, *$p<0.05$, ***$p<0.001$).

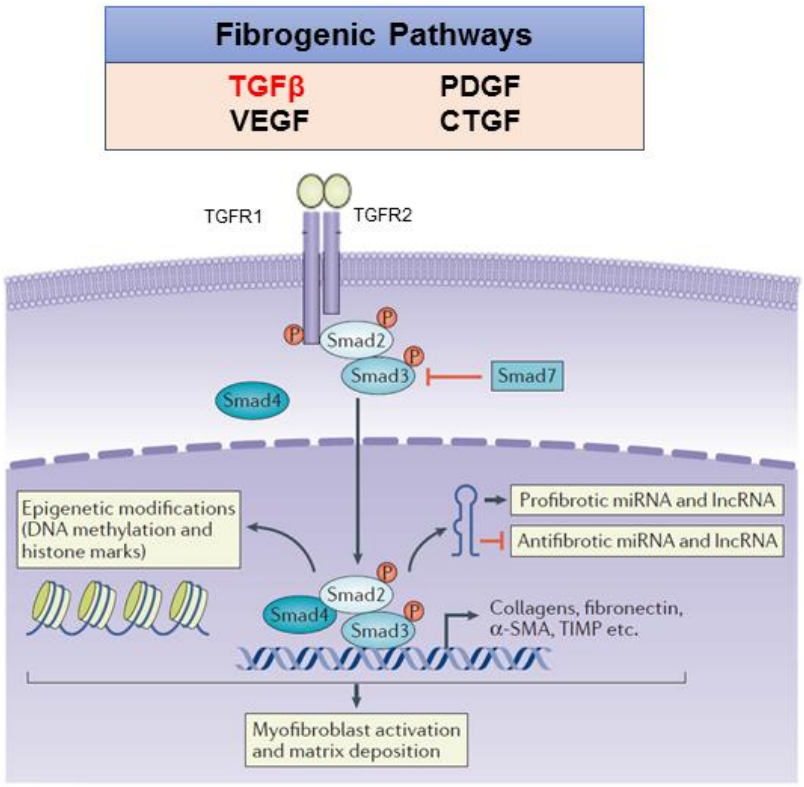

Figure 7. TGFβ1/SMAD signalling in fibrosis

The proliferation of activated HSCs mainly follows four fibrogenic pathways such as TGFβ1 (transforming growth factor β1), PDGF (platelet-derived growth factor), VEGF (vascular endothelial growth factor) and CTGF (connective tissue growth factor). Among them, TGFβ1 is considered the most potent fibrogenic cytokine. TGFβ1 binding and phosphorylation of the type I receptor induces phosphorylation of downstream SMAD proteins, predominantly SMAD3. The SMAD3 component directly binds to gene promoters to induce transcription of pro-fibrotic molecules, including α-SMA, collagen I and collagen III which induce myofibroblast activation and matrix deposition. (Adopted from Meng *et al* 2016).

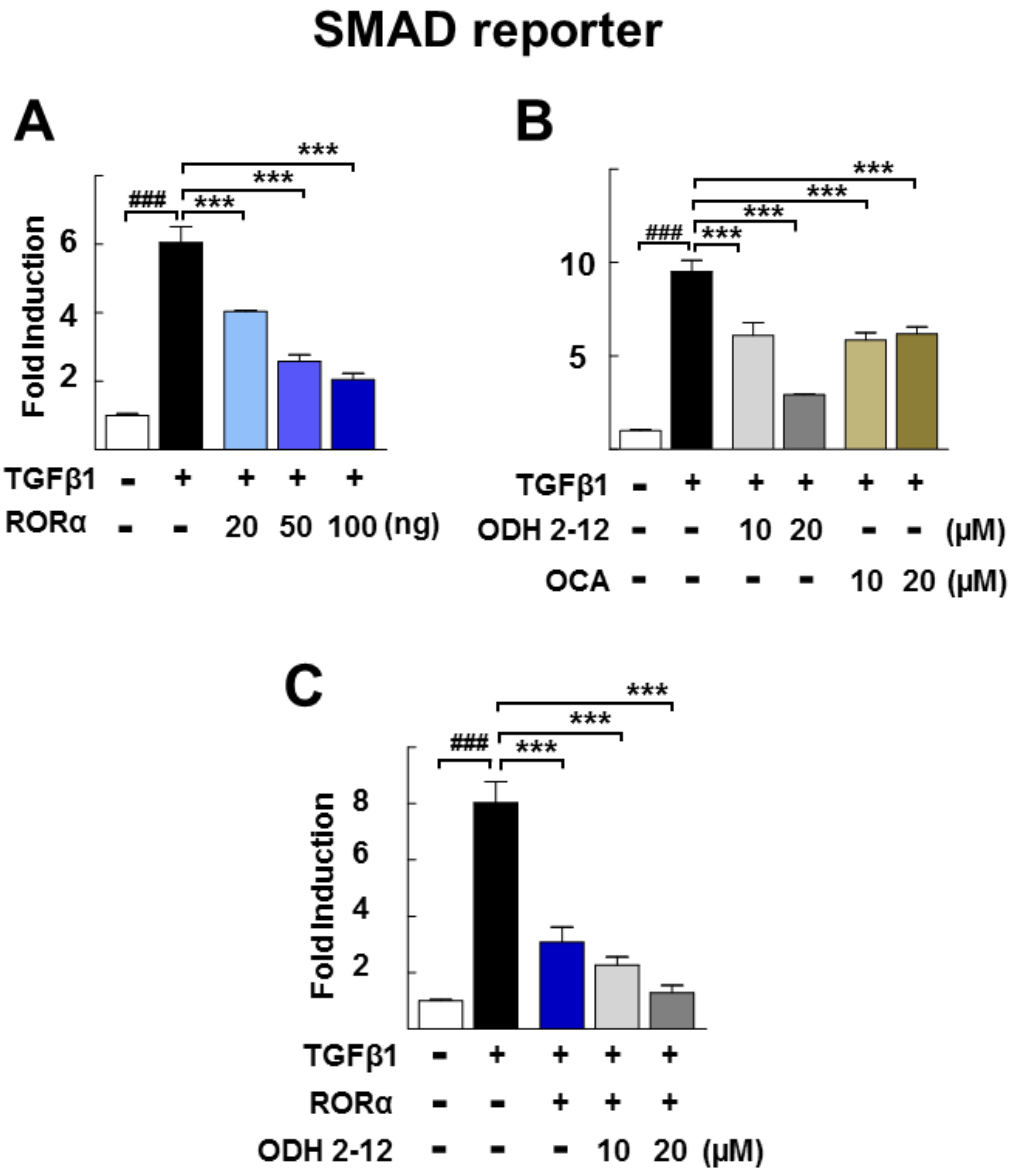

Figure 8. RORα & ODH 2-12 suppresses the transcriptional activity of SMAD reporter

(A) Lx-2 cells were transiently transfected by SMAD cignal reporter, β-galactosidase, myc-EV, and myc-RORα (20, 50, 100 ng) for 24 h and then replaced with new culture medium. After that treated with or without 5.0 ng/ml TGFβ1 for another 18 h.

(B) The Lx-2 cells were treated with SMAD cignal reporter, β-galactosidase for 24 h. ODH 2-12 and OCA (obeticholic acid) was treated with the dose 10 and 20 μM for 18 h with TGFβ1 (5.0 ng/ml)
(C) The Lx-2 cells were transfected with SMAD cignal reporter, myc-RORα (20 ng) and β-galactosidase. Then treated with ODH 2-12 (10, 20 μM) for 18 h along with TGFβ1 (5.0 ng/ml).
The luciferase reporter promoter activity was normalized by β-galactosidase. The results are presented by following one-way Anova test, (n=3, $*p<0.05$, $***p<0.001$).

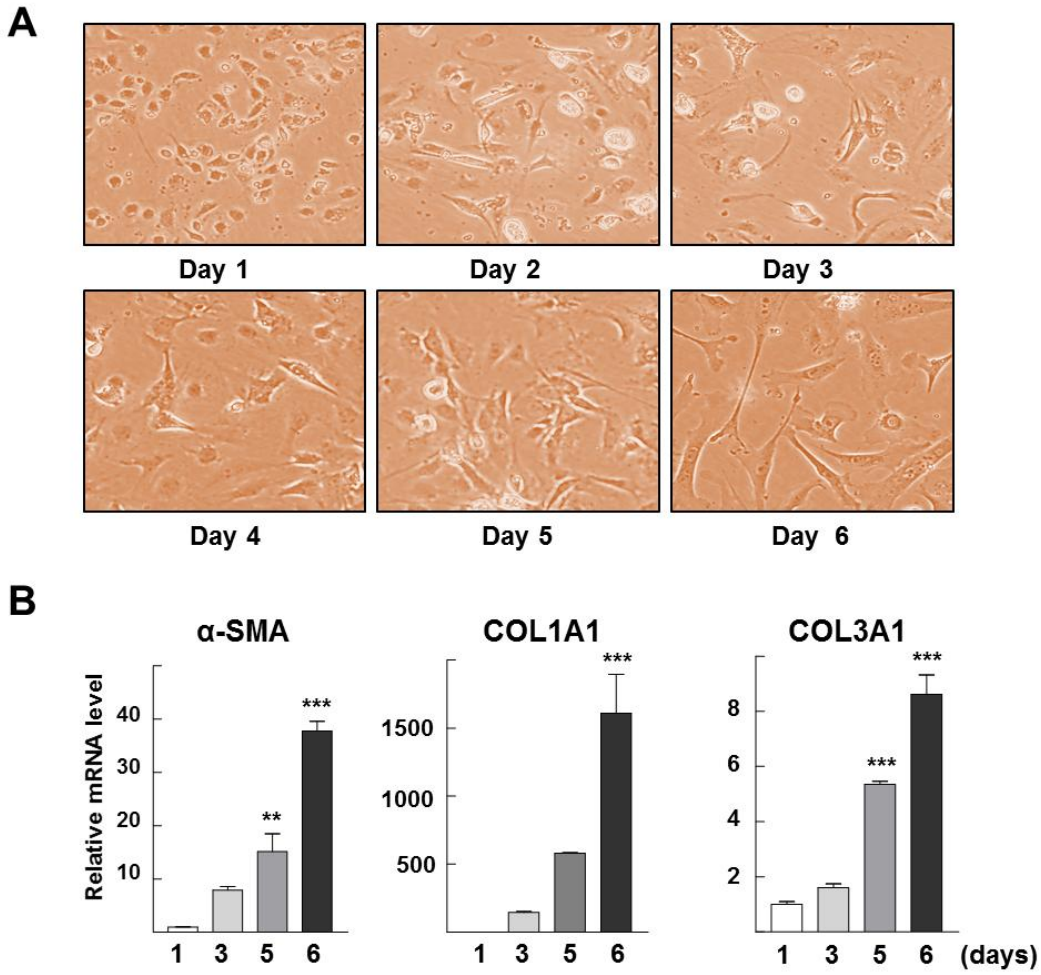

Figure 9. Isolation and activation of primary HSCs

Primary HSCs were isolated and cultured for 6 days consecutively in 12 well plate. (A) The morphological changes and activation of cells are showed through cell microscopic pictures (X400). (B) Real-time qPCR was performed to analyze the mRNA levels of α-SMA, COL1A1, and COL3A1. The statistical analysis is performed by following one-way Anova test, (n=3, *$p<0.05$, ***$p<0.001$).

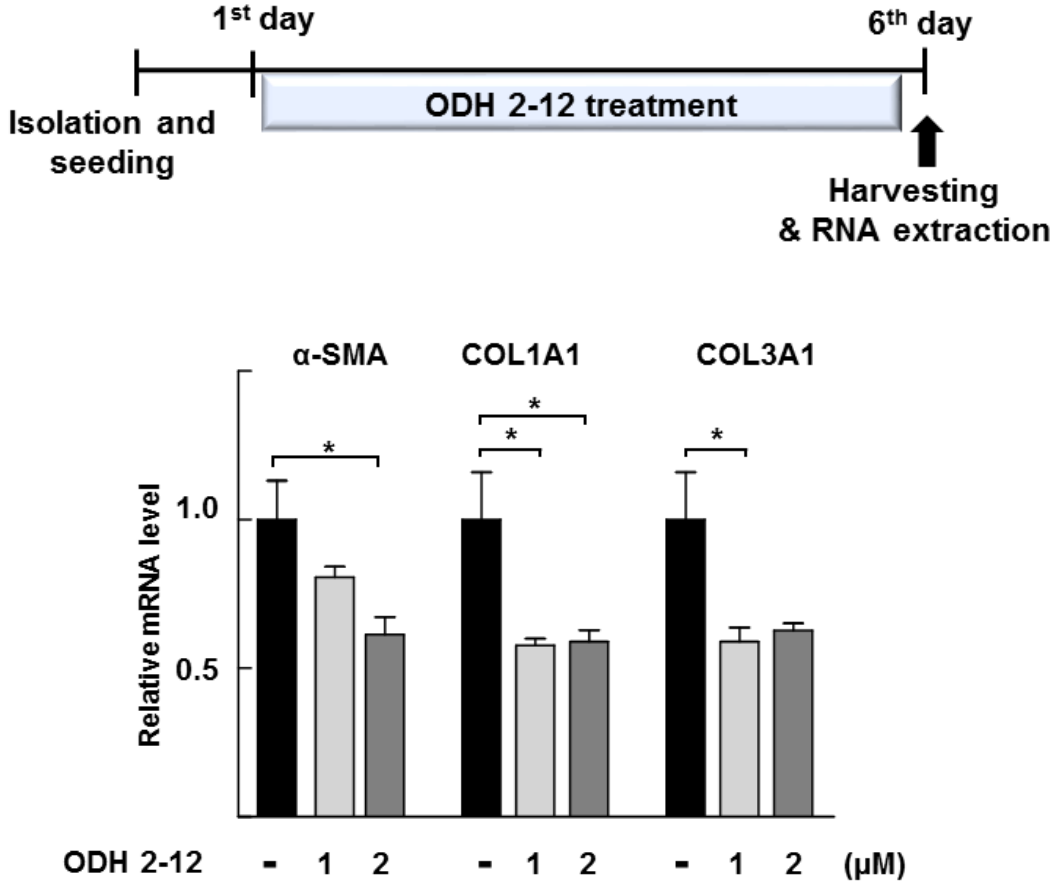

Figure 10. ODH 2-12 treatment reduces the expression of fibrogenic markers expression in primary HSCs

After isolation of primary hHSCs, ODH 2-12 (1, 2 μM) were treated for 6 days consecutively. Real-time qPCR was performed to analyze the mRNA levels of α-SMA, COL1A1, and COL3A1. The statistical analysis is performed by following one-way Anova test, (n=3, *p< 0.05).

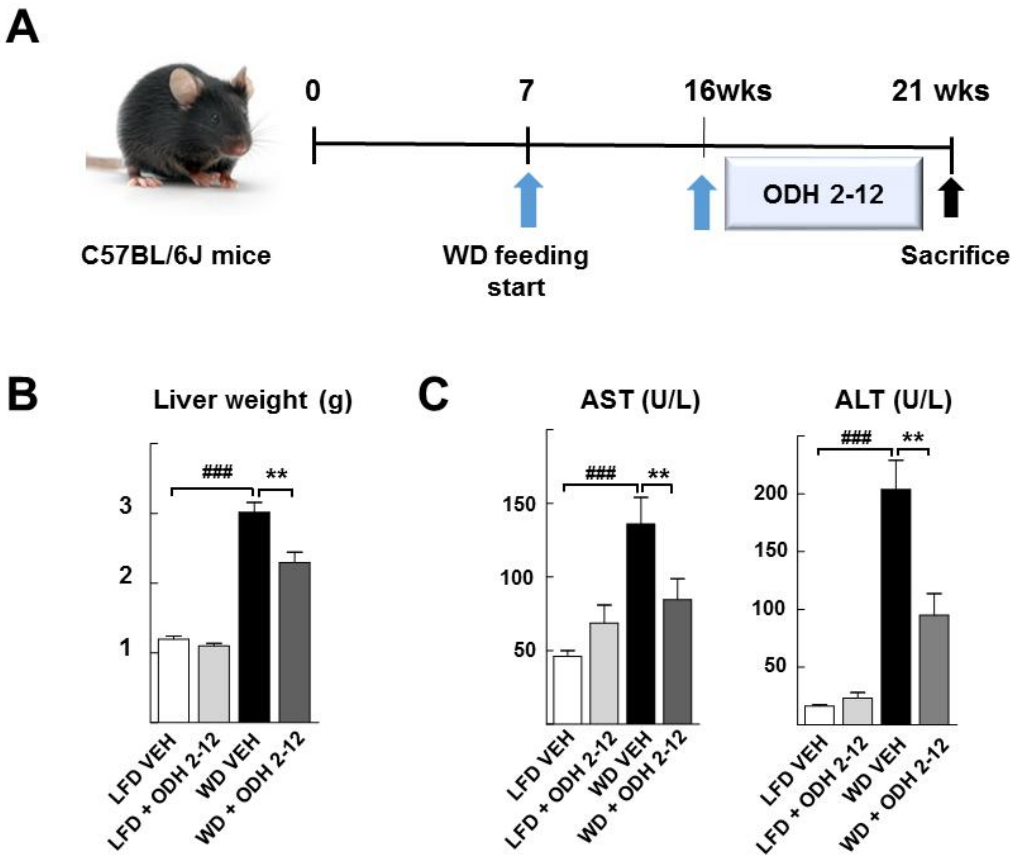

Figure 11. ODH 2-12 treatment in vivo reduces liver weight, AST, and ALT

(A) WD and LFD diet feeding started with 7 weeks old C57BL/6J mice and continued until 21 weeks. ODH 2-12 was treated from 16 weeks to 21 weeks by oral gavage (QD) 10 mg/kg body weight. After 21 weeks, animals were sacrificed. (B) Comparison of liver weight among LFD + VEH (n=11), LFD + ODH 2-12 (n=8). WD + VEH (n=14), and WD + ODH 2-12 (n=12) fed mice. (C) The AST and ALT levels were measured from blood serum with the same numbers of mice. The statistical analysis was performed following non-parametric Mann-Whitney 'U' test, (*$p<0.05$, ***, ###$p<0.001$).

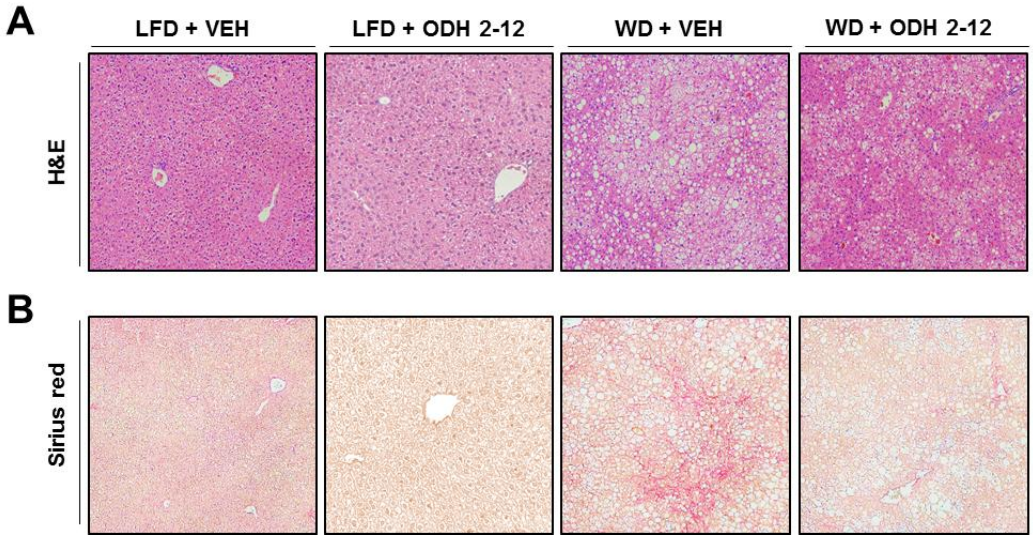

Figure 12. ODH 2-12 treatment in vivo reduces the lipid and collagen deposition

Liver tissue sections were obtained from mice described in figure 11.

(A) Representative H&E staining picture of the liver sections from LFD fed vehicle (VEH) and ODH 2-12, and WD fed vehicle (VEH) and ODH 2-12 mice.

(B) Representative Sirius red staining picture of the liver sections from LFD fed vehicle (VEH) and ODH 2-12, and WD fed vehicle (VEH) and ODH 2-12 mice.

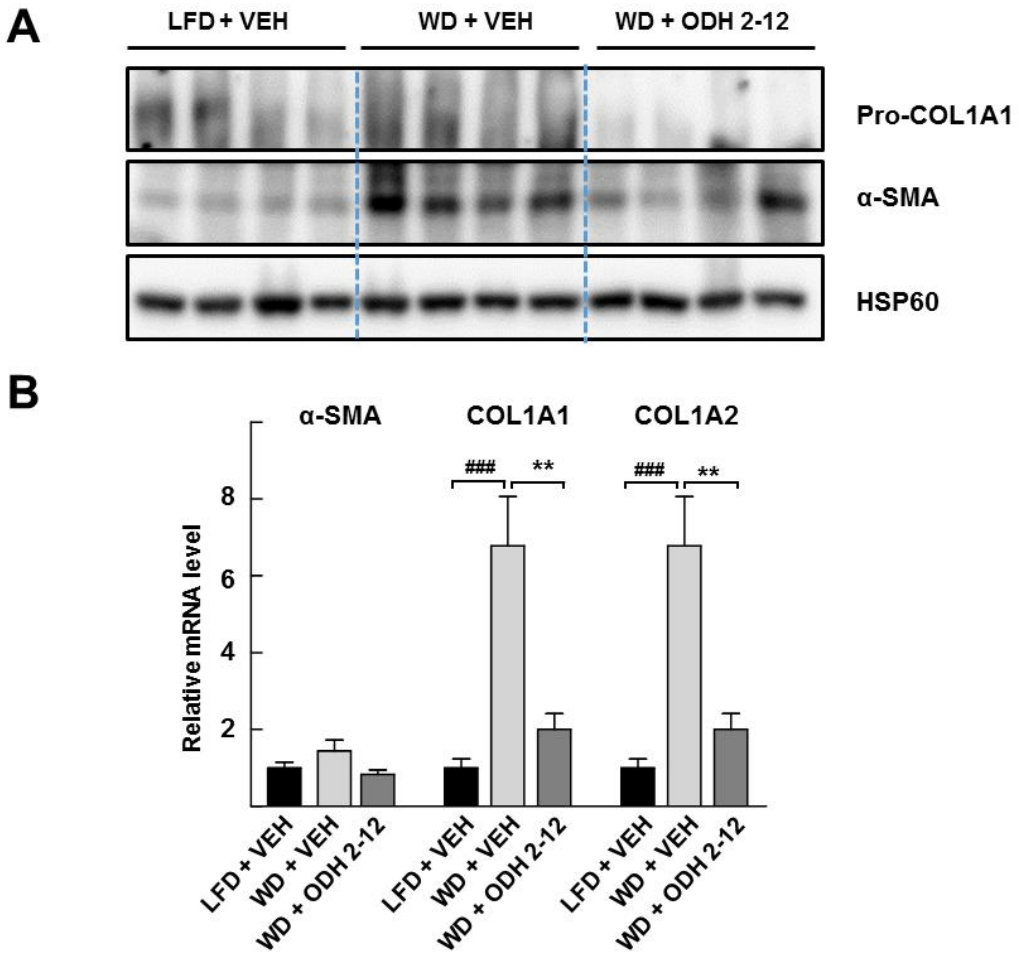

Figure 13. ODH 2-12 treatment in vivo reduces the protein and mRNA expression

WD diet-induced mice liver tissues (described in figure 11) were further analyzed to find out the protein and mRNA expression of fibrogenic markers. (A) Western blotting was performed to analyze the protien levels (n=4) and (B) real time qRT-PCR was performed to analyze the mRNA levels of fibrogenic markers α-SMA, COL1A1 and COL1A2. The statistical analysis was performed following non-parametric Mann-Whitney 'U' test, (LFD + VEH (n=9), WD + VEH (n=10) & WD + ODH 2-12 (n=11), *$p<0.05$), ***$p<0.001$).

V. DISCUSSION

It has been well established now that HSCs plays major roles in liver fibrosis. Activation of these cells is the main driver for the accumulation of fibroblasts and extracellular matrix which results in liver fibrosis (Friedman 2006). Upon liver injury, activated stellate cells undergo a perpetuation phase which includes proliferation, contractility of myofibroblasts which produce TGFβ1 (Tsuchida *et al* 2017). On the other hand, TGFβ1 stimulates the further uncontrolled activation of cells to multiply and to increase the amount of ECM per cells (Puche *et al* 2013). Therefore to prevent the fibrogenic activation of these cells became a critical point in the treatment of liver fibrosis. In this study, I aimed to inhibit the fibrogenic activation of HSCs in order to prevent the overall liver fibrosis.

Although, there are several studies about inhibiting the activation of stellate cells or suppression of TGFβ1/SMAD signaling by using nuclear receptors including PPARɣ (Hazra *et al* 2004), NR4A1 (Zerr *et al* 2014), NRF2 (Prestigiacomo *et al* 2018). However, in this study, for the first time, I demonstrated that RORα has an inhibitory effect in HSCs fibrogenic activation as well as in liver fibrosis. RORα is a multifunctional nuclear receptor which plays a critical role in many physiological processes. Notably, it has a crucial role in liver diseases. We previously demonstrated that it plays a protective role in NAFLD and NASH. Recently, we also showed that in HFD fed RORα-LKO (hepatocytes specific) mice model, there were considerably more collagen deposition and upregulated α-SMA expression (Kim *et al* 2017). Following these findings, we came with the hypothesis that

RORα has an inhibitory effect in the liver fibrosis. To prove this hypothesis, I sought out the effect of RORα in the fibrogenic activation of HSCs. However, there were no significant previous studies on the effect of RORα in HSCs.

In this study, I activated HSCs (Lx-2 cells) by TGFβ1 and performed the RORα overexpression study. The protein and mRNA expression study revealed that overexpression of RORα reduces the expression of key pro-fibrotic markers α-SMA and COL1A1 significantly. However, RORα is a ligand binding NR which by binding to its agonist promote the transcriptional expression of the target gene, therefore proper agonist is needed which can activate the RORα. In this study, I used the ODH 2-12 compound with the hope that it might be an appropriate agonist of RORα in order to boost the anti-fibrotic effect of RORα. To prove ODH 2-12 is an agonist of RORα I carried out reporter gene assay to find out the selective transcriptional activity of ODH 2-12 in different liver important NRs. The reporter gene assay results showed that ODH 2-12 has higher transcriptional activity towards RORα comparing other RORs (RORβ and RORɣ) and other important NRs such as PPARs and LXRα. Despite this study, further ligand binding studies are needed to confirm ODH 2-12 as a selective agonist of RORα.

In resemblance to RORα, treatment of ODH 2-12 in Lx-2 cells has shown similar inhibitory effect in both protein and mRNA expression of the fibrogenic marker which was induced by TGFβ1. Additionally, to find the mechanism of how RORα and ODH 2-12 expressing anti-fibrotic effect I conducted SMAD reporter assay which expresses

the transcriptional activity of SMAD reporter. RORα and ODH 2-12 both suppress the transcriptional activity of the SMAD reporter. However, this study does not define the exact mechanism of how RORα and ODH 2-12 are suppressing the SMAD. Nevertheless, this finding is indicative of the role of RORα in the TGFβ1 and SMAD signaling. Additionally, detailed mechanism studies are needed to find out the exact mechanism of RORα mediated suppression of SMAD pathway. Furthermore, to find out the effect of ODH 2-12 in vivo, I developed a fibrosis mice model which is induced by WD. Although there is another diet system, named as MCD (Methionine and choline-deficient) diet, which is also known to induce fibrosis in the liver. However, I choose WD because it contains high fat, cholesterol, and a combination of high-fructose corn syrup, sucrose, fructose, or glucose which eventually leads to metabolic syndrome along with NAFLD with fibrosis, making this diet most suitable for my research goal. On the other hand, the MCD diet is incapable of inducing metabolic syndrome and also does not induces NAFLD and fibrosis consistently. Therefore, the clinical significance of the MCD diet is questionable (Stephenson *et al* 2018).

Treatment of ODH 2-12 in vivo has shown significant anti-fibrotic effect in the diet-induced liver fibrosis mice model. It reduced the liver weight and AST and ALT levels which indicates that it improved the NAFLD condition in vivo. Moreover, in the histological liver tissue examination, marked decreased of collagen deposition in Sirius red staining indicates attenuation of fibrosis. Finally, the decreased expression of fibrogenic markers in both protein and mRNA assay has provided further evidence of the anti-fibrotic effect of ODH

2-12. Although, in animal model, I only evaluated the effect of ODH 2-12 in liver tissues, not specifically in the HSCs. Therefore, further studies can be conducted either on the HSCs of this diet-induced mice model or in mice, where RORα is conditionally knockout from other liver cells except HSCs.

In conclusion, I have demonstrated that ODH 2-12 could be a promising anti-fibrotic agent although further studies are needed to find out the full potential of this compound in vivo and in vitro. In addition, it has the potential to be a proper agonist of RORα which can signify the anti-fibrotic role of RORα in the liver.

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국문초록

간 섬유화는 바이러스 감염, 알콜성 간질환 및 비알콜 성 지방 간염 (NASH)을 비롯한 모든 원인의 만성 간 손상으로 인한 세포 외 기질의 순 축적을 특징으로하는 동적 과정이다. 간 성상 세포의 활성화 (HSCs)는 비활성 세포를 섬유증의 주 요인인 증식성의 섬유 myofibroblasts로 전이 분화시킨다. 최근 retinoic acid receptor related orphan receptor α (RORα)은 간세포에서의 미토콘드리아 기능 및 쿠퍼 세포에서의 M1 / M2 분극화에 영향을 미침으로써 NASH를 약화시키는 것으로 나타났다. 여기에서, 우리는 활성화 된 HSCs 및 식이 유도 된 섬유증 쥐 모델에서 잠재 RORα 작용제, ODH 2-12의 효과를 조사하였다. 첫째, 우리는 RORα 과발현이 형질전환 transforming growth factor beta 1 (TGFβ1)로 활성화 된 인간 HSCs에서 항 섬유화 효과가 있고 ODH 2-12가 RORα의 전사 활성을 유도한다는 것을 관찰했다. 둘째, 우리는 ODH 2-12의 항섬유화 효과를 관찰하고, TGFβ1-활성화 인간 HSCs에서 Alpha Smooth Muscle Actin 및 Collagen type 1 Alpha 1과 같은 섬유성 마커의 mRNA 및 단백질 발현을 감소시키는 것으로 나타났다. 셋째, 우리는 일차 마우스 HSCs에서 ODH 2-12의 효과를 조사하였으며 섬유화 마커에 대해 유사한 감소 효과를 보였다. 다음으로, 우리는 식이요법으로 유도된 섬유증 마우스 모델을 서양 식단에 먹이로 개발했습니다. ODH 2-12 처리는 간 조직학에서 감소된 콜라겐 침착을 보여주었고 fibrogenic 마커의 mRNA와 단백질

발현도 감소시켰다. 마지막으로, 이중 루시퍼 라제 리포터 분석은 ODH 2-12가 SMAD 신호 전달 경로에서 TGFβ1-유도 리포터 유전자 발현을 감소시킨다는 것을 나타내었다. 결론적으로, ODH 2-12는 잠재적인 항섬유화 효과를 나타내므로 잠재적 항섬유화 제제의 후보 물질이 될 수 있다.

주요어: RORα agonist, Hepatic stellate cells, Diet-induced fibrosis

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