Establishment of liver fibrosis animal model by inducing hepatic stellate cell-specific TGFβ1 using LRAT promoter

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Directed by Professor Eunae Sandra Cho

The Master's Thesis

submitted to the Department of Applied Life Science,

the Graduate School of Yonsei University

in partial fulfillment of the requirements for the degree of

M.S in Applied Life Science

Jae Eun Lee

December 2022

This certifies that the Master's Thesis
of Jae Eun Lee is approved.

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December 2022

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2022 년 12 월 이재은

CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Establishment of liver fibrosis animal model by inducing hepatic stellate cell-specific TGFβ1 using LRAT promoter

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(Directed by Professor Eunae Sandra Cho)

Liver fibrosis is a common consequence of chronic liver damage. It is a symptom in which scar tissue formation is induced as hepatic stellate cells (HSCs), activated by various inflammatory factors, such as cytokines, lose retinol and are converted into proliferative, fibrogenic, contractile myofibroblasts. Activated HSCs perpetuate their fibrogenic phenotype and the inflammatory process by the secretion of several paracrine and autocrine

factors, such as TGFβ1, a key cytokine in the activation of HSCs. In addition, they secrete and deposit excessive amounts of extracellular matrix (ECM) which induces fibrosis. In the small intestine, retinyl esters are incorporated in chylomicrons and after transport through the circulation taken up by hepatocytes. In hepatocytes, they are converted to retinol and subsequent binding to retinol binding protein 4 (RBP4) stimulates the release of the retinol-RBP4 complex back to the circulation. Via an unknown mechanism, retinol is transferred to HSCs, re-esterified, and stored in lipid droplets. Quiescent HSCs store retinol as retinyl esters, by the action of lecithin retinol acyltransferase (LRAT), in large lipid droplets. Therefore, HSCs are key cells that induce liver fibrosis, and HSCs can be targeted with LRAT. In terms of retinol metabolism in the liver, LRAT can be used as a marker for HSCs because it is specifically present in large amounts in HSCs, and can be used to identify HSCs in a quiescent state in animal models. In this study, we selected the site with the highest expression among the LRAT promoters operating specifically in HSCs, developed a system in which TGFβ1 is expressed by LRAT promoter activation, and endeavored to establish a mouse disease model in which liver fibrosis is induced by TGFβ1 by the developed system.

First, liver fibrosis induction vectors were constructed using the LRAT promoter. In the HSC-originated Lx2 cell line, the expression efficiency of the LRAT promoter was compared by size from the eC-terminal region, and LRAT-400, which showed the highest expression, was selected as the final promoter. The Tet-on system was designed to activate the LRAT promoter when treated with doxycycline, and a vector was constructed to

overexpress TGFβ1 when the LRAT promoter was activated by fusion of the mutated TGFβ1-2CS to the promoter. In the Lx2 cell lines, which originated from HSC, TGFβ1 expression increased as the LRAT promoter was activated, but in HepG2 and Hep3B cell lines of general hepatocyte origin other than HSCs, the change in TGFβ1 expression was non-significant.

When confirmed with the supernatant obtained after inducing the LRAT promoter in each cell line, there was no change in HepG2 and Hep3B, but in Lx2, 3TP-lux increased, and PAI-1, a well-known downstream target of TGFβ1, also increased significantly. The same results were obtained when confirming the induction of fibrosis by TGFβ1, and a significant increase in fibrosis markers was also shown exclusively in Lx2 cells. As a result of inducing the operation of the LRAT promoter in mouse animal experiments, TGFβ1 expression was confirmed in the liver, and it was confirmed that fibrosis was induced by comparing the expression of alpha smooth muscle actin (α-SMA).

This suggests a new method of producing a mouse liver fibrosis model that increases the induction of fibrosis by targeting HSCs. It is considered that it can be used as an effective animal model for screening fibrosis-inhibiting drugs.

Keywords: liver fibrosis, LRAT, hepatic stellate cell, TGFβ1, mouse model

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I. INTRODUCTION

Liver fibrosis results from a wound regeneration caused by acute or chronic damage to the liver due to various causes, such as chemicals, viruses, and metabolic abnormalities. Acutely damaged hepatocytes are replaced by newly generated hepatocytes, and the accumulation of extracellular matrix (ECM) is insignificant, but in the case of chronic and repeatedly damaged hepatocytes, they are replaced with ECM, such as collagen, to form

scar tissue (Bataller and Brenner 2005). During this process, hepatic stellate cells (HSCs) are activated by various types of cytokines secreted by Kupffer cells that phagocytize damaged hepatocytes and are converted into myofibroblasts, playing a pivotal role in liver fibrosis (Lee and Friedman 2011; Wu and Zern 2000; Zhang et al. 2016).

Transforming growth factor beta (TGFβ) is the cytokine that most strongly influences liver fibrosis and contributes to all stages of liver disease progression, from early liver injury at the level of inflammation and fibrosis to cirrhosis and hepatocellular carcinoma (HCC). Among the three isoforms of TGFβ, TGFβ1 is the most extensively studied in liver fibrosis and is known to be a key factor in chronic liver disease (Dewidar et al. 2015). TGFβ1 not only induces direct damage to hepatocytes but also induces HSC activation and ECM deposition, which are critical steps in the wound-healing response of damaged liver tissue (Kanzler et al. 1999).

In general, quiescent HSCs in normal liver tissue convert vitamin A (retinol) to a retinyl esters by the action of lecithin retinol acyltransferase (LRAT) and then store the retinyl esters in large cytoplasmic lipid droplets (Lee and Jeong 2012; Zolfaghari and Ross 2000). About 95% of the vitamin A absorbed by the liver is stored in HSCs in this way (Blaner et al. 2009; Ross and Zolfaghari 2004). In terms of retinol metabolism in the liver, LRAT is specifically present in HSCs in large amounts, so it can be used as a marker to identify quiescent HSCs in animal models (Mederacke et al. 2013; Nagatsuma et al. 2009).

Numerous studies have been conducted to inhibit fibrosis and recover liver function,

and various anti-fibrotic drugs have been developed, and there is a growing demand for the development of effective and standardized liver fibrosis animal models capable of screening for antifibrotic drugs (Koyama et al. 2016; Liedtke et al. 2013). Various types of animal models exist depending on the mechanism inducing liver fibrosis. These include chemical drug-induced models, such as carbon tetrachloride (CCl_4), thioacetamide, as well as the diet metabolism-induced model, and the surgical bile duct ligation model. However, there is still no standard therapy for liver fibrosis, and models that perfectly imitate all the pathological phenomena of human liver fibrosis are still lacking (Weiler-Normann, Herkel, and Lohse 2007).

A major goal of current experimental research is to develop an optimal liver fibrosis model that reliably recapitulates the critical hallmarks of fibrosis (Bao et al. 2021). In mice, the pathogenesis or aspect of liver disease is different from that in humans, and since liver diseases do not occur in mice as much as in humans, creating conditions like those in humans is the key to establishing a mouse model (Jiang et al. 2020). Transgenic mouse models of liver fibrosis have been actively researched in recent years, enabling the functions and changes occurring in the fibrotic liver to be confirmed at the genetic level (Delire, Starkel, and Leclercq 2015; Faccioli et al. 2022; Popov and Schuppan 2009).

Taking advantage of the fact that HSCs are central to the development of liver fibrosis and can be targeted with LRAT, in this study, we proposed a new method for the transgenic mouse model of liver fibrosis disease that utilizes the LRAT promoter to induce HSC activation by TGFβ1.

II. MATERIALS AND METHODS

1. Plasmid DNA (pDNA) construction

LRAT promoters were obtained from genomic DNA (gDNA) isolated from NCTC 1469 cells (ATCC, CLL-9.1), a murine liver cell line. The gDNA of NCTC 1469 cells was extracted using the QIAamp® DNA Mini Kit (QIAGEN, #51304), and isolated according to the manufacturer's protocol. The LRAT region in gDNA was amplified by PCR, and four types of LRAT promoter fragments were obtained by the second round of PCR. Each DNA fragments were amplified 28 cycles under the following PCR conditions: 94°C for 45 sec, 58°C for 45 sec, 72°C for 2 min. Four LRAT promoters were constructed with different lengths of 1850, 1400, 900, and 400 bp from the N-terminal of the mouse LRAT gene (-1850, -1400, -900, -400 to +160), respectively. The 5′-primer used for the second PCR included a *Bam*HI restriction site, and the 3′-primer was designed to include the promoter 120 bp in front of the exon in the mouse LRAT gene and included an *Xba*I restriction site.

LRAT promoter vectors were constructed with the doxycycline-inducible lentiviral vector named pCW57-RFP-P2A-MCS (Addgene #78933) as a backbone. LRAT promoter fragments were inserted into the pCW57-RFP-P2A-MCS vector digested by *Xba*I and *Bam*HI (New England Biolabs, Ipswich, MA, USA), removing the hPGK promoter and puroR. Subsequently, 1.2-kb modified TGFβ1 cDNA with HA tagging was ligated to the

*Mlu*I/*Sal*I restriction site in front of the LRAT promoter region of the construct. The modified TGFβ1 cDNA has two cysteines at positions 223 and 225 mutated to serine, and these mutations primarily result in the expression of biologically active TGFβ1.

Finally, to make the LRAT promoter-dependent and doxycycline-induced vector more specific, the final vector structure was generated by reversing the direction of the entire reverse tetracycline-dependent transactivator (rtTA) of the tetracycline (Tet-on) system fused with the LRAT promoter. All constructed vectors were verified by Sanger sequencing and restriction enzyme digestion. A schematic diagram of the constructed LRAT promoter vector is shown below (Figure 1).

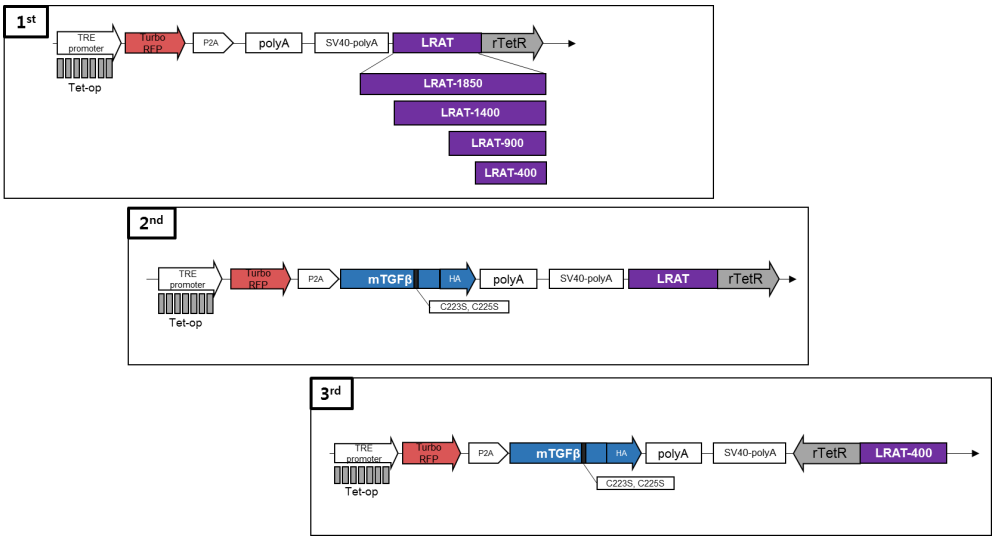

Figure 1. Schematic diagram of the LRAT promoter vector constructs.

2. Cell culture and transfection

Human HSC cell line, Lx2 was cultured in Dulbecco's modified Eagle medium (DMEM) with GlutaMax™ (Gibco,10569010) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1% (v/v) penicillin/streptomycin (P/S; BioWhittaker). Human HCC cell lines HepG2 and Hep3B were cultured in minimal essential medium (MEM) with EBSS, L-glutamine (HyClone, cytiva), 10% FBS, 1% P/S. HepG2 cell lines were maintained on plates coated with ECM (G422, Applied Biological Materials). Both the A549 cell line stabilized to express 3TP-lux as a reporter cell line and the HEK293 cell line for lentivirus production were cultured in DMEM supplemented with 10% FBS, 1% P/S. All cells were cultured in a humidified incubator with 5% CO_2, at 37°C.

To compare the expression of LRAT promoter vectors, Lx2 cells were transfected with 1μg of each LRAT promoter vector and 50 ng of Green fluorescent protein (GFP) as a transfection control in 6-well plates. Transfection was performed with Lipofectamine 2000 (Invitrogen, 11668-019) according to the manufacturer's protocol. After 16 h of transfection, 5 μg/ml of doxycycline was treated for 24 h, and the medium was replaced with fresh medium. The expression rate of the doxycycline-induced LRAT promoter was imaged with Red fluorescent protein (RFP) using a fluorescence microscope and analyzed using the ImageJ program downloaded from NIH (https://imagej.nih.gov/ij/). Each fluorescence image was quantified by averaging the fluorescence intensity of three or more

3. Lentivirus production and infection

HEK293 cells were seeded with antibiotic-free DMEM in 6-well plates 24 h before transfection. Cells were transfected with pMD.2G and psPAX2 packaging vectors and finally, the selected lentiviral vector, pCW57-RFP-P2A-reverse-rTet-mTGFβ1(2CS)-HA-LRAT-400. As the transfection reagent, Lipofectamine 2000 was used, (see section 2), and transfection was performed according to the manufacturer's protocol. The medium was replaced the next day. The lentivirus-containing supernatants were harvested 48 h after the transfection and centrifuged briefly (2000 rpm for 5 min) to remove cells and debris. Lentiviral supernatants were concentrated with the Lenti-X Concentrator (Clontech, 631231) according to the manufacturer's protocol.

The concentrated lentivirus pellet was re-suspended in fresh medium and infected with Lx2, HepG2, and Hep3B cell lines with polybrene, respectively. The virus infection was verified by using pLL3.7-dsRed as a positive control for lentivirus transduction. After stabilizing the cells through several passages, Lx2, HepG2 and Hep3B-LRAT-400 cell lines were treated with doxycycline (0, 5 μg/ml) in 6-well plates for 72 h to induce the LRAT promoter. The expression level of the LRAT promoter according to the origin of the cell line was imaged with RFP using a fluorescence microscope.

4. Western blotting

Doxycycline was treated for 72 h, then Lx2, HepG2 and Hep3B-LRAT-400 stable cell lines were washed twice with cold PBS and lysed with 1% Triton X-100 lysis buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100). Cell lysates were scraped and incubated on ice for 15 min. After the incubation, cell lysates were vortexed briefly and then centrifuged at 13,200 rpm for 15 min at 4°C. The cleared supernatants were transferred to a new tube. Each cell lysate was quantified by the BCA protein assay (Thermo Fisher Scientific, 23225), and 10 μg of each was sampled in 5X sample buffer according to the quantification, boiled at 100°C for 10 min, and stored on ice. All samples were loaded and separated on 15% SDS-PAGE gel and transferred to the nitrocellulose membrane (Whatman). Membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with the primary antibody for 3 h at room temperature, followed by the secondary antibody for 1 h at room temperature. Expression of each LRAT promoter was compared with TGFβ (Cell Signaling, #3711S), and actin (Santa Cruz Biotechnology, Sc-47778) was used as a loading control. For detection, WEST SAVE (AB Frontier, LF-QC0101) was used and developed with CP-BU Medical X-ray Film Blue (AGFA).

5. Conditioned medium preparation

To obtain conditioned medium, Lx2, HepG2, and Hep3B-LRAT-400 cell lines were seeded in 6-well plates and cultured overnight. After the cells had completely adhered to

the plate, the medium was replaced, doxycycline (0, 5 μg/ml) was treated, followed by incubation for 72 h, and the supernatant was harvested. The harvested conditioned medium was centrifuged at 1000 rpm for 3 min to remove cell debris, and the supernatant was transferred to a new tube. The conditioned medium was analyzed by TGFβ1 enzyme-linked immunosorbent assay (ELISA) and 3TP-lux reporter assay.

6. Quantitative real-time-PCR (qPCR) analysis and 3TP-lux reporter assay

Each RNA was extracted with TRIzol™ reagent (Invitrogen, 15596026) according to the manufacturer's protocol. cDNA was synthesized from 1 μg of RNA using CycleScript RT PreMix (dT20, Bioneer). qPCR was performed using SYBR™ Green Mix on the Applied Biosystems StepOne detection system. Each Δ*C*t value was analyzed by normalizing to GAPDH in triplicate samples. Primer specificity was confirmed by melting curve analysis after qPCR reactions. Information on qPCR primers is provided in Table 1.

For the 3TP-lux reporter assay, the A549-3TP-lux-MODC stable cell line was seeded in 12-well plates the day before, and conditioned media were harvested (see section 5) and treated with normal media at a 1:1 ratio for 16 h. As a positive control, 1 ng of rhTGFβ was treated with normal media and compared with the results in conditioned media. Luciferase activity was measured using the Luciferase Assay System (Promega, #E1500) according to the manufacturer's protocol. All results were analyzed as the average of triplicate experiments.

Table 1. Primer sequences used for quantitative real-time PCR.

Genes	Forward	Reverse
TGFβ1	CGGCAGCTGTACATTGACTT	TCCAGGCTCCAAATGTAGGG
PAI1	CAAGCAGCTATGGGATTCAAG	GCTGATCTCATCCTTGTTCCA
ACTA2	CTGCTGAGCGTGAGATTGTC	TCAAGGGAGGATGAGGATGC
Fibronectin	TGGCACTGATGAAGAACCCT	TGCCTCCACTATGACGTTGT
Col1a1	TGACCTCAAGATGTGCCACT	ACCAGTCTCCATGTTGCAGA
GAPDH	CAATGACCCCTTCATTGACC	TTGATTTTGGAGGGATCTCG

7. ELISA

To quantify and compare secreted TGFβ1, the content of TGFβ1 in conditioned medium obtained as described above (section 5) was determined by Human TGFβ1 Quantikine ELISA (R&D Systems, DB100B) according to the manufacturer's instructions. The results were analyzed as the average of triplicate experiments.

8. Plasmid DNA injection of mice

All animal experiments were conducted in accordance with the Institutional Animal Care and Use Committee of Yonsei University and approved by the Animal Care

Committee of Yonsei University School of Dental Sciences. Male BALB/c nude mice (9-week-old ;Nara Biotech) were used for plasmid tail vein injection to induce liver fibrosis. Mice were divided into three groups according to the control group without DNA injection and doxycycline administration after DNA injection. Plasmids were amplified for *in vivo* injection with Plasmid Extraction Maxi Plus Kit (Favorgen, FAPMX 020) to obtain high concentrations of DNA. The injection solution was prepared by diluting 20 μg of pDNA in 200 μl of PBS, and tail vein injection was performed on three mice per group. After the injections, in the experimental group, 100 μg of doxycycline was intraperitoneally injected twice a day for 1 week, and then 100 μg of doxycycline was injected once a day for 1 month.

All mice were sacrificed 1 month after DNA injection, and livers were harvested from each individual and stored in a deep freezer. Frozen liver tissues were prepared as frozen sections using a cryomicrotome (Lecia Biosystems, CM1860). After setting the cryomicrotome to - 20°C, frozen liver tissues were embedded with Tissue-Tek® O.C.T. Compound (Sakura, 4583) in the mold. After removing the excess of the frozen O.C.T. compound with a blade, the frozen tissue was sectioned to a thickness of 8 μm. Frozen section slides were serially sectioned with a cryomicrotome.

The prepared frozen section slides were immediately fixed in 70% ethanol, and observed with a fluorescence microscope for the expression of RFP by the LRAT promoter, and stained with TGFβ1 and alpha-smooth muscle actin by immunofluorescence staining.

9. Immunofluorescence staining and fluorescence imaging

Frozen section slides were incubated for 1 h at room temperature in PBS containing 5% goat serum and 0.1% Triton X-100 buffer for blocking. Afterward, primary antibodies (anti-HA, anti-α-SMA) diluted in PBS containing 1% goat serum and 0.1% Triton X-100 were bound overnight at 4°C. After washing thrice with PBS, the secondary antibody (anti-rabbit-Alexa Fluor-594) diluted in the same condition was bound for 30 min at room temperature in the dark. After washing the secondary antibody with PBS, tissues were mounted with a mounting solution containing DAPI (ProLong™ Diamond Antifade Mountant with DAPI, Invitrogen, P36962). Fluorescence was monitored using confocal microscopy (Zeiss LSM700). The antibodies used in the experiment were as follows. HA (901501, BioLegend), α-SMA (ab124964, abcam)

10. Statistical analysis

Data were analyzed with Prism 9.4.1 software (GraphPad). All statistical analyzes of qPCR, reporter assay, and TGFβ1 ELISA assay were performed by one-way ANOVA; data are presented as mean ± standard deviation (SD). A *P*-value less than 0.05 was considered statistically significant (****, $P < 0.0001$, ***, $P < 0.001$, **, $P < 0.01$).

III. RESULTS

1. LRAT promoter vector construction and screening

Expression of vectors constructed with 1850, 1400, 900, and 400 bp from the C-terminal region of the LRAT promoter was compared by RFP fluorescence in the Lx2 cell line of human HSC origin. All groups were co-transfected with GFP as a control for the transfection of each vector, and RFP was expressed as the LRAT promoter. Expressions were compared by the number of particles of RFP per GFP. Comparing the expression rate of RFP itself, LRAT-400 showed the highest expression rate, and the expression of RFP per GFP was also highest in LRAT-400. Comparing the shape and growth of each cell in the brightfield image, the cell growth was the lowest in LRAT-400, which had the highest RFP intensity due to the influence of TGFβ1 induced by the LRAT promoter (Figure 2a). Based on the above results, LRAT-400 was selected as the final promoter.

To increase the expression efficiency of the LRAT promoter, reverse TetR and LRAT promoter were cloned in the opposite direction. By comparing this with the conventional type of vector, an experiment was conducted to compare the LRAT promoter expression rate with RFP fluorescence intensity. As a result, RFP expression was measured to be higher in the vector that changed the direction of the entire promoter than in the conventional vector (Figure 2b). Through this, the reverse-rTetR-LRAT-400 vector was determined as the final form.

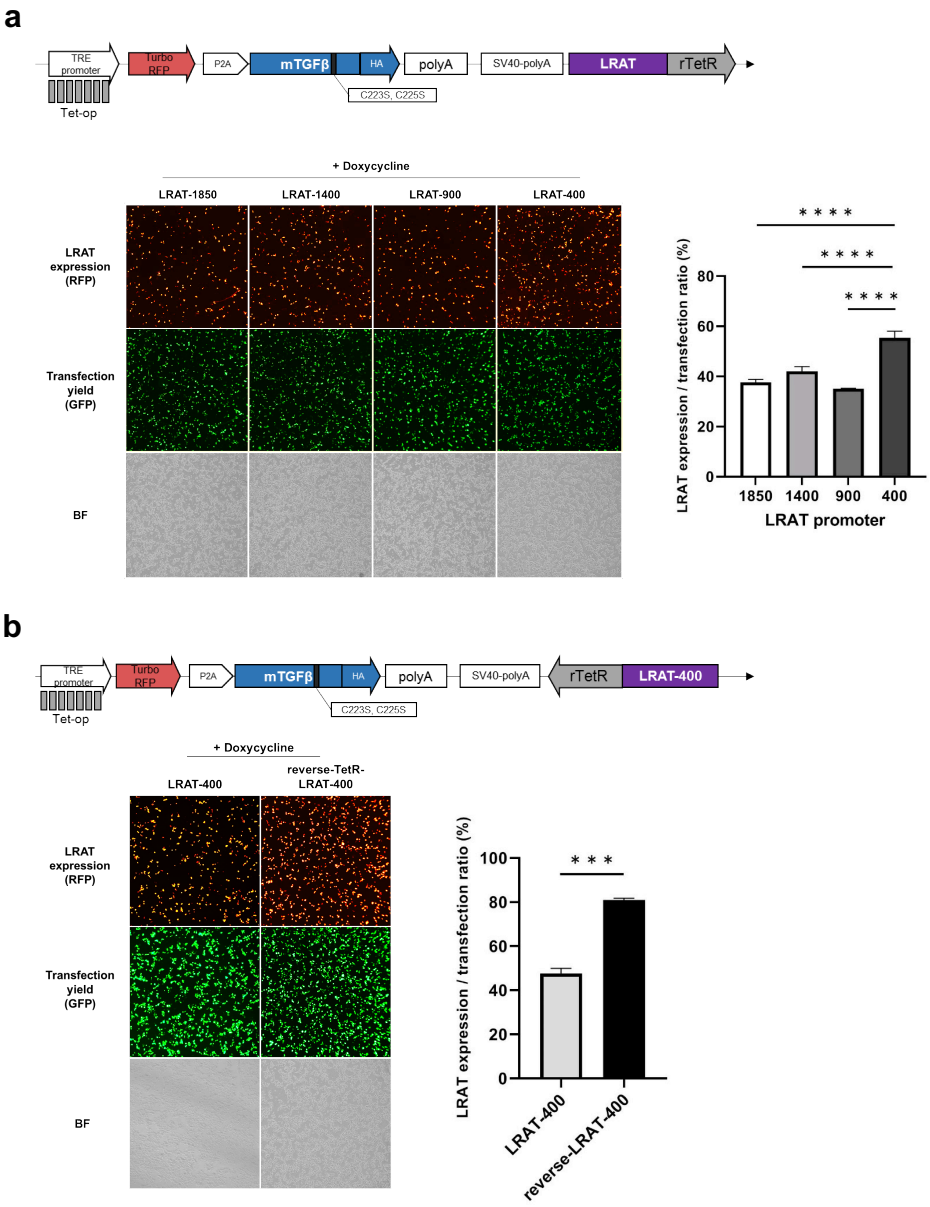

Figure 2. Comparison of RFP fluorescence expression between LRAT promoters.

Schematic diagrams of the LRAT promoter inducing TGFβ1-2CS consisting of a Tet-on

system are placed on top of each. (a) Using a fluorescence microscope, expression rates according to the LRAT promoter site (-1850, -1400, -900, -400) were compared with the RFP intensity and normalized to GFP as the transfection control (left). LRAT promoter expression ratios were quantified using ImageJ (right). Statistical significance compared to each promoter was indicated by ****, $P < 0.0001$ by one-way ANOVA. (b) Expression rates between LRAT-400 and reverse-LRAT-400 were compared as described in (a). Statistical significance compared to each promoter was indicated by ***, $P < 0.001$ by paired *t*-test.

2. HSC-specific operation of the newly constructed LRAT promoter

LRAT is known to be involved in retinol metabolism in mouse and human liver tissues and is exclusively expressed in HSCs. Therefore, as a control, HepG2 and Hep3B, which are non-HSC-derived hepatocytes, were compared with the HSC-originated Lx2 cell line. To confirm the effect of the vector (section 1), three cell lines (Lx2, HepG2, Hep3B) were infected with the lentivirus produced with reverse-rTetR-LRAT-400 vector with high expression efficiency to construct a stable cell line. Then, the LRAT promoter was induced with doxycycline, and the difference in TGFβ1 expression among the three cell lines was confirmed by fluorescence imaging, western blot analysis, TGFβ1 transcript level, and TGFβ1 ELISA (Figure 3). The activation level of the LRAT promoter for each cell line was confirmed by RFP using a fluorescence microscope, and western blotting was performed with TGFβ1 antibody in the cell lysates. TGFβ1 ELISA was performed with the supernatant to quantitatively compare the amount of secreted TGFβ1 mature form.

As a result, as the LRAT promoter operated in the HSC-derived Lx2 cell line, the expression of RFP was significantly increased compared to the other two cell lines (Figure 3.a). Additionally, the expression of TGFβ1 in cell lysates was specifically increased only in the Lx2 cell line (Figure 3b). As expected, in the quantitative comparison of TGFβ1 transcript level and secreted TGFβ1, there was no significant difference in expression in HepG2 and Hep3B, which are derived from non-HSC hepatocytes, but there was a dramatic and significant increase in Lx2 cell line compared to controls (Figure 3c, d). Thus, it was confirmed that the final form of LRAT-400 promoter acts specifically in HSCs.

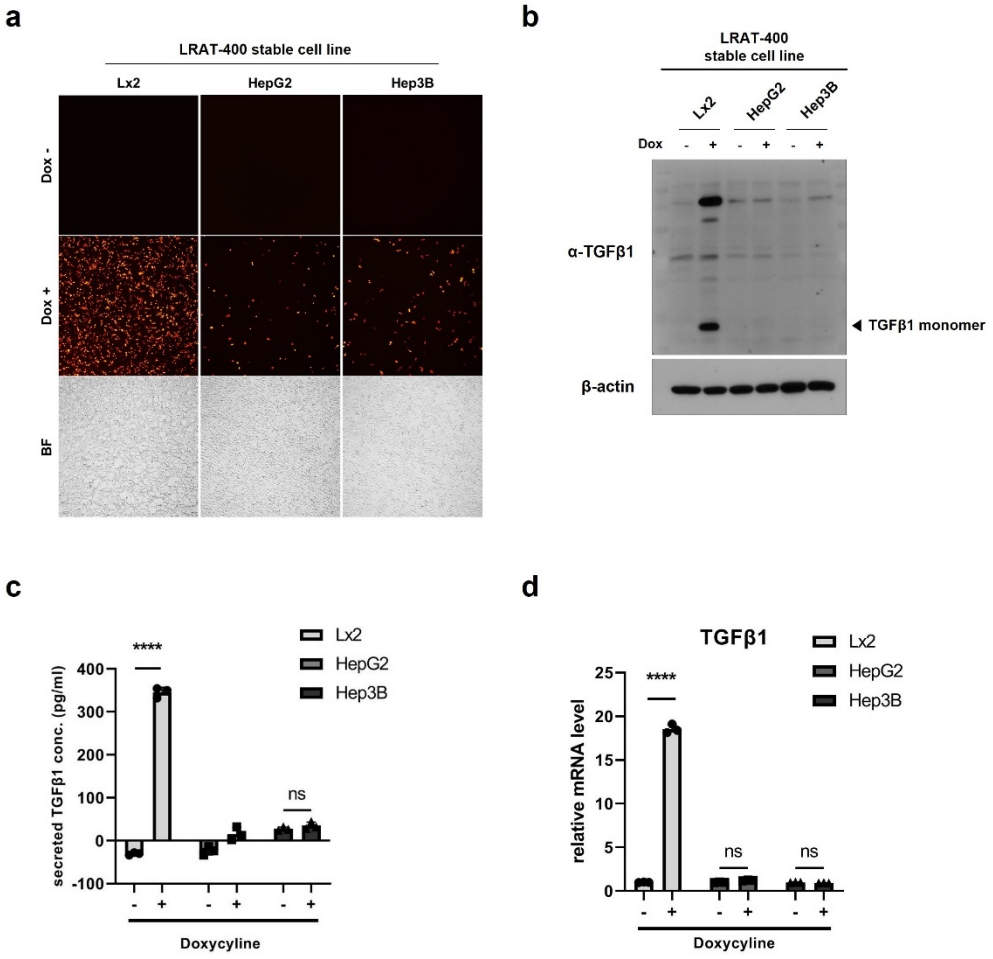

Figure 3. Comparison of LRAT promoter activation level between HSC-originated cell line and hepatocyte-originated cell lines.

(a-d) LRAT promoter induced by doxycycline activity was compared in three cell lines in which the LRAT-400 promoter was transduced with lentivirus. (a) Comparison of RFP fluorescence intensity. (b) Western blot shows TGFβ1 expressed in each cell line. The

TGFβ1 monomer represents the size of the secreted TGFβ1 monomer. (c) Concentration of extracellular secreted TGFβ1 measured by TGFβ1 ELISA. Statistical significance for the effect of LRAT promoter induction in each cell line was indicated by ****, $P < 0.0001$ by one-way ANOVA. (d) Relative TGFβ1 transcript level. Statistical significance for the effect of LRAT promoter induction in each cell line was indicated by ****, $P < 0.0001$ by one-way ANOVA.

3. Lx2 cell-specific functional activity of TGFβ1 induced by the LRAT promoter

In three cell lines (Lx2, HepG2, Hep3B) stabilized after transduction of the LRAT promoter vector with lentivirus, the degree of functional expression of TGFβ1 in the supernatant obtained after inducing the LRAT promoter was evaluated by 3TP-lux reporter assay and comparison of PAI-1 transcript level. Experiments were conducted using the A549-3TP-lux stable cell line, and analysis was performed by treating A549-3TP-lux cells with conditioned media obtained from Lx2, HepG2, and Hep3B-LRAT-400 stable cell lines for 16 h, respectively (Figure 4a).

As a result, 3TP-lux reporter activity and PAI-1 transcript level increased as the LRAT promoter operated exclusively in the Lx2 cell line, identical to the results of the previous experiment. Similarly, in HepG2 and Hep3B, there were no significant changes in 3TP-lux reporter or PAI-1 transcript levels by doxycycline (Figure 4b, c). This indicates that as the LRAT promoter operates, TGFβ1, which was fused with the LRAT-400 promoter vector, is not only expressed but functional.

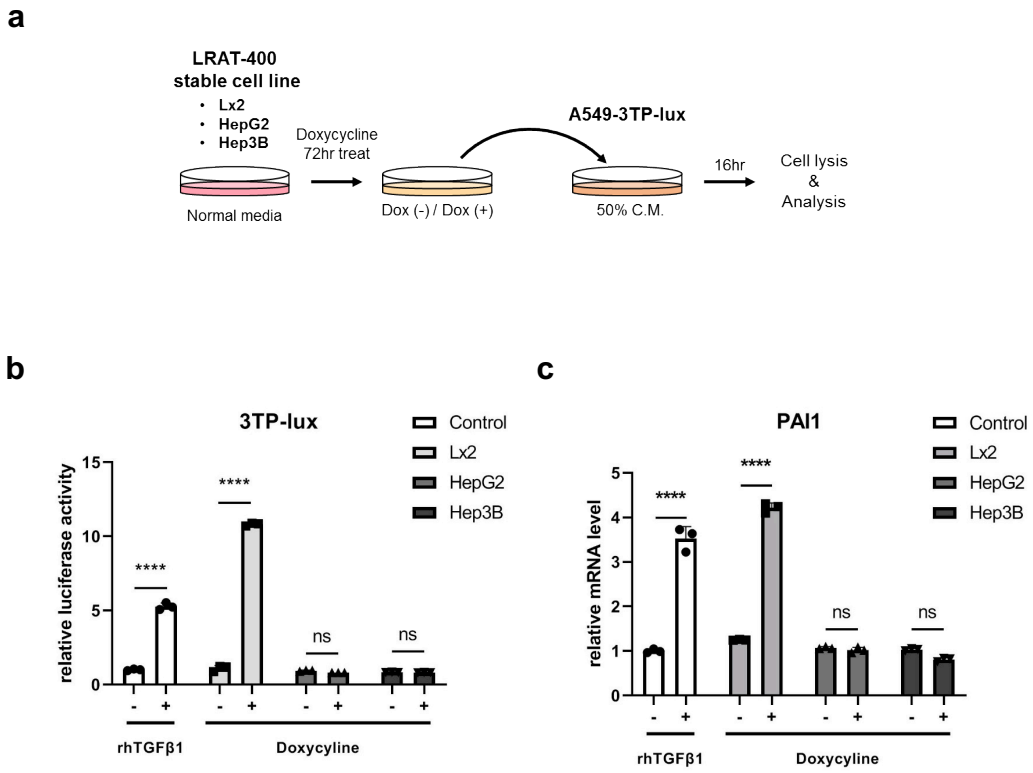

Figure 4. Comparison of functional activity of TGFβ1 by LRAT promoter activation between HSC-originated cell line and hepatocyte-originated cell lines.

(a) Schematic diagram showing the conditioned medium production and transfer process. (b) 3TP-lux reporter activity by the operation of the LRAT promoter induced by doxycycline in each stable cell line. rhTGFβ1 was used as a positive control. Statistical significance for the effect of LRAT promoter induction in each cell line was indicated by ****, $P < 0.0001$ by one-way ANOVA. (c) Relative PAI-1 transcript level. Statistical significance for the effect of LRAT promoter induction in each cell line was indicated by ****, $P < 0.0001$ by one-way ANOVA.

4. Upregulation of fibrosis marker expression by TGFβ1 stimulation induced by LRAT promoter

TGFβ is a cytokine activated in fibrotic diseases and is well-known as a key mediator of the fibrotic process (Desmouliere 1995; Meng, Nikolic-Paterson, and Lan 2016). Therefore, tissue fibrosis progressed due to TGFβ induction, and activation was observed and studied in several fibrosis animal models and clinical data. TGFβ plays a critical role in the conversion of fibroblasts to myofibroblasts in fibrotic tissue, and it is known that the expression of fibrosis markers is up-regulated in response. (Walton, Johnson, and Harrison 2017).

Based on this, the induction of fibrosis by TGFβ1 overexpressed from the LRAT promoter constructed in this study was confirmed by qPCR. Three cell lines (Lx2, HepG2, Hep3B) stabilized to express the LRAT promoter vector were treated with 5 μg/μl of doxycycline for 72 h to sufficiently induce the operation of the LRAT promoter. Afterward, transcript levels for α-SMA, fibronectin, and collagen type 1, which are the most well-known fibrosis markers, were compared for each cell line. The LRAT promoter was also activated specifically for the Lx2 cell line, and as a result of TGFβ1 expression, fibrosis markers were significantly increased in the Lx2 cell line. Consistent with the results of the previous experiment, there were no significant changes in HepG2 and Hep3B (Figure 5).

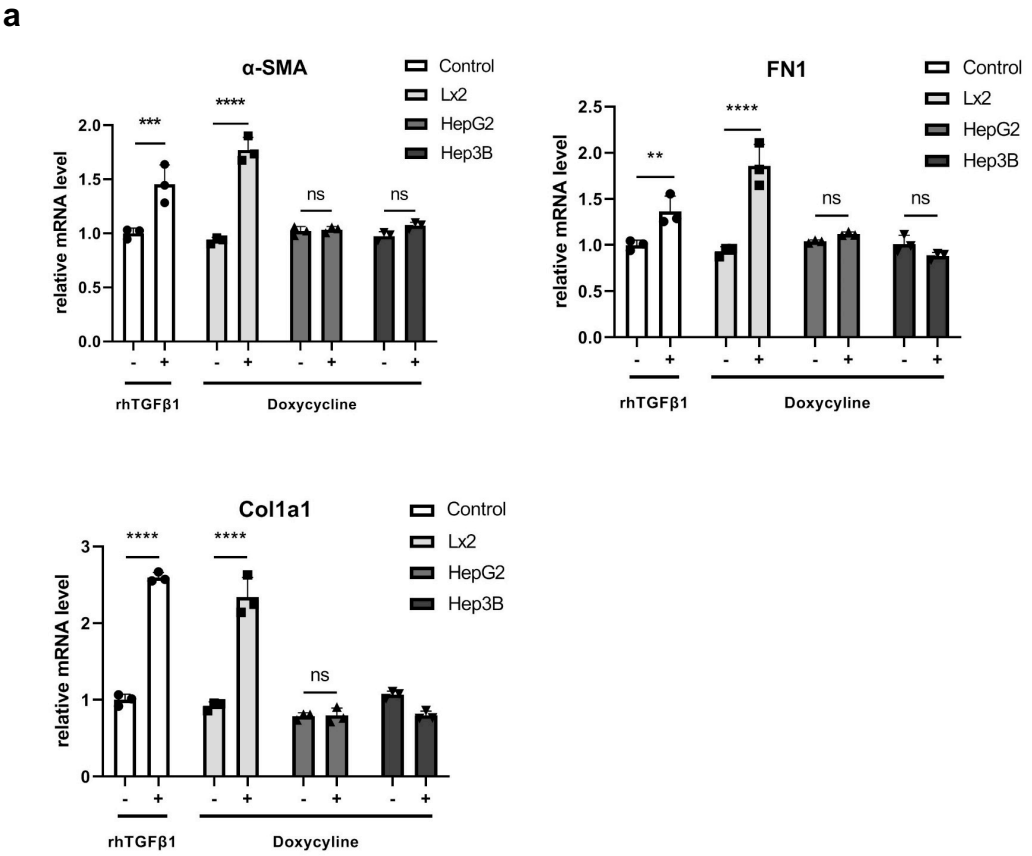

Figure 5. Expression of fibrosis-related markers by TGFβ1 stimulation induced by the LRAT promoter.

Relative α-SMA, fibronectin, and collagen type 1 transcript levels. rhTGFβ1 was used as a positive control. Statistical significance for the effect of LRAT promoter induction in each cell line was indicated by ****, $P < 0.0001$; ***, $P < 0.001$; **, $P < 0.01$ by one-way ANOVA.

5. *In vivo* mouse liver fibrosis disease model induced by the LRAT promoter

Next, we injected pDNA into BALB/c nude mice via the tail vein and observed the expression and functioning of the LRAT promoter vector *in vivo*. After injecting the LRAT promoter vector, doxycycline was injected intraperitoneally for 1 month to induce the activity of the LRAT promoter in the vector. At 5 weeks after DNA injection, mice were sacrificed, and livers were harvested. The LRAT promoter operation was confirmed by comparing the RFP intensity by fluorescence imaging of slides prepared by frozen sectioning of liver tissue. As a result of comparing RFP expression in liver tissue, it was observed that RFP was expressed only in the doxycycline-injected group, and the LRAT promoter vector was observed to work well *in vivo* after the tail vein-injected pDNA was introduced into the liver (Figure 6a).

Next, by observing the expression level and co-localization of TGFβ1 and α-SMA in mouse liver tissues, it was confirmed whether TGFβ1 secreted by the LRAT promoter directly induced fibrosis. Frozen liver tissue was serially sectioned, and immunofluorescence was performed on adjacent tissue slides with TGFβ1-tagged HA and α-SMA antibodies, respectively. As a result, it was observed that TGFβ1 was highly expressed in doxycycline-positive (Dox+) tissues, and α-SMA was expressed in the same region (Figure 6b). In Dox+ tissues, α-SMA was not confined to the perivascular area but also appeared in the TGFβ1-expressed region, which is interpreted as a result of fibrosis induction by TGFβ1 secreted by the operation of the LRAT promoter.

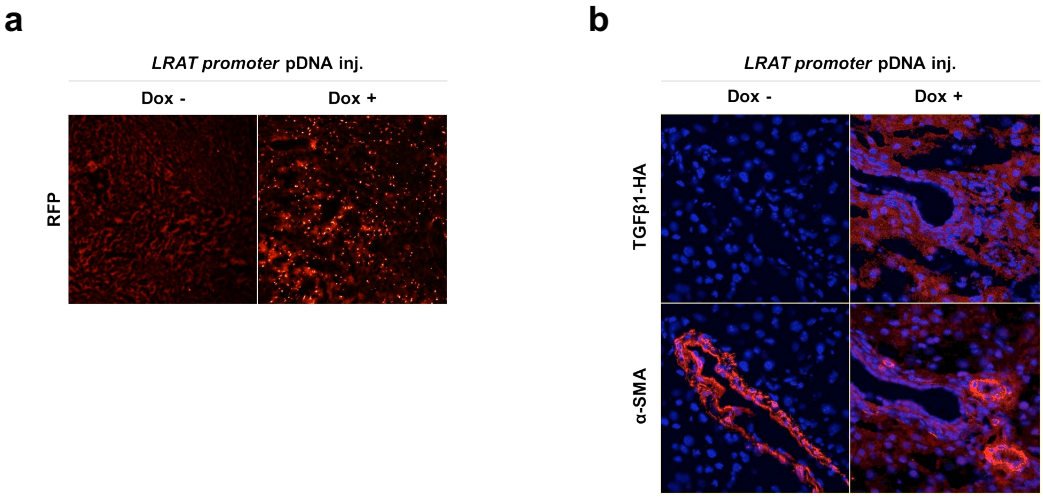

Figure 6. Effect of LRAT promoter expression and fibrosis induction *in vivo*.

(a) Comparison of the operation of LRAT promoter vectors *in vivo* by RFP intensity. (b) *In vivo* expression of α-SMA and HA-tagged TGFβ1 by immunofluorescence. Co-localization between the TGFβ1 expression site and the α-SMA-induced site was confirmed on the serial section slide.

IV. DISCUSSION

As the global prevalence of liver disease continues to increase over the decades (Williams 2006), a number of prior studies have been conducted to prevent the progression of chronic hepatitis to cirrhosis, an irreversible condition. Most of the causes of death from liver disease are known to be due to advanced complications after HCC or liver cirrhosis (Byass 2014). Therefore, suppressing the progression of the disease in the process of hepatic fibrosis at a reversible level is a key factor. From studies on the mechanism of liver fibrosis, it has been found that activated HSCs are at the center of the fibrotic process, and research on the development of antifibrotic drugs that block the fibrotic process by inhibiting the activity of HSCs is actively underway (Kisseleva and Brenner 2021; Ray 2014).

Animal models using various etiologies of liver fibrosis have been developed to support the analysis of liver fibrosis mechanisms and molecular biological understanding. Animal models that perfectly match the morphology of liver fibrosis in humans have been limited in their implementation so far. Even so, the gap is closing with the development of fibrosis models in various pathways, such as hepatotoxicity, autoimmunity, and metabolic problems (Weiler-Normann, Herkel, and Lohse 2007). In particular, studies using transgenic mouse models that induce genetic defects or overexpression of specific genes are being actively conducted.

The transgenic mouse model is a model for precisely analyzing the function of the gene to be transformed *in vivo* and more accurately realizing the disease state in humans. However, its disadvantages are that it takes considerable time to build an experimental model and requires a lot of financial investment (Doyle et al. 2012; Lampreht Tratar, Horvat, and Cemazar 2018).

In this study, we developed a transgenic mouse model production system that causes liver fibrosis *in vivo* by inducing HSC-activation by TGFβ1 using the LRAT promoter. Based on the lentiviral vector with the Tet-on system, a model system was established to selectively induce fibrosis. In addition, a site showing the highest expression among HSC-specific LRAT promoters was selected, and vectors were cloned to express an active TGFβ1 mutant by the LRAT promoter. Ultimately, this system targeted HSCs, key cells in inducing liver fibrosis, with the LRAT promoter to maximize the effect of inducing liver fibrosis.

In addition, TGFβ1-2CS mutations, which generate bioactive TGFβ1 without an acid activation process and make it constitutively active, are fused to the vector so that HSCs are continuously stimulated by TGFβ1. TGFβ1-2CS(C223S/C225S) mutants containing two-point mutations from cysteine to serine prevent latency-associated peptide (LAP) from binding to the mature TGF β1 dimer , allowing it to initiate downstream signaling immediately after expression (Brunner et al. 1989; Hall et al. 2010; Samuel et al. 1992).

Summarizing the above results, the LRAT promoter was activated *in vitro*, specifically for the HSC-derived Lx2 cell line, and fibrosis was induced by TGFβ1 expressed from the

LRAT promoter vector. In an *in vivo* model, it was also shown that fibrosis was induced in the liver by the LRAT promoter system. In this study, the LRAT promoter system was induced *in vivo* by direct injection of plasmid DNA. In case of injecting lentivirus with this system, it is expected that the expression efficiency *in vivo* can be further increased. The transgenic modeling system using the LRAT promoter vector could be simple and more efficient than the general transgenic mouse model system, suggesting new methods and applications in liver fibrosis research.

V. CONCLUSION

The LRAT promoter vector that works specifically in HSCs was constructed, and its function was confirmed *in vitro*. TGFβ1-2CS mutated to secrete the mature form of TGFβ1 was fused to a vector to construct a system that induces fibrosis by directly stimulating HSCs with TGFβ1. This study proposes a new approach to the liver fibrosis mouse model, and it is thought that the LRAT-TGFβ1 promoter method can be used as an effective animal model for screening fibrosis inhibitory drugs.

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ABSTRACT (IN KOREAN)

LRAT promoter 를 이용한 간성상세포 특이적인 TGFβ1 의 liver fibrosis 동물 모델 구축

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이 재 은

간섬유화는 만성 간 손상에 의해 발생하는 일반적인 결과로, 사이토카인과 같은 다양한 염증 인자에 의해 활성화된 간성상세포(HSC, Hepatic stellate cell)가 근섬유아세포로 전환되면서 흉터 조직 형성이 유도되는 증상이다. 간섬유화 발생기전에서 간성상세포는 TGFβ1 과 같은 사이토카인에 의해 활성화되며, 간성상세포의 활성으로 다량의 세포 외 기질(ECM, Extracellular matrix)이 분비되어 섬유화가 유도된다.

레티닐 에스테르는 소장에서 흡수되어 간세포로 이동하여 레티놀로 전환되며, 레티놀 결합 단백질 4 (RBP4)에 결합해 레티놀-RBP4 복합체를 형성한다. 이 후, 레티놀은 휴지기의 간성상세포에서 레시틴-레티놀 아실 트랜스퍼 레이스 (LRAT)의 작용에 의해 다시 에스테르화되어 지질방울 (LD)에 장기적으로 저장된다. 이러한 간에서의 레티놀 대사측면에서 볼 때, LRAT 가 간 세포 중 특히 간성상세포에 특이적으로 다량 존재하기에 간성상세포의 마커로 적용할 수 있으며, 동물 모델에서 휴지기의 간성상세포를 식별하는 역할을 수행할 수 있음을 의미한다.

따라서 본 연구자는 간성상세포가 간섬유화를 유발하는 핵심 세포이며 LRAT 로 간성상세포를 표적 할 수 있다는 것에 주목하였다. 본 연구에서는 간성상세포에서 특이적으로 작동하는 LRAT promoter 중 가장 활성이 높은 부위를 선정하고, LRAT promoter 활성화에 의해 TGFβ1 이 발현되는 시스템을 개발하여 생체 외 실험에서 간성상세포 특이적으로 TGFβ1 이 발현됨을 확인하고, 개발한 시스템에 의해 TGFβ1 에 의한 간섬유화가 유도되는 마우스 질병 모델을 수립하고자 하였다.

먼저 LRAT promoter 를 이용한 간섬유화 유도 벡터를 제작하였다. 간성상세포 기원인 Lx2 세포주에서, LRAT promoter 를 c-terminal 영역에서부터 크기 별로 발현 효율을 비교하여 가장 높은 발현을 보인 LRAT-400 을 최종 promoter 로 선정하였다. Tet-on system 에 의해 doxycycline 을

처리하였을 때 LRAT promoter 가 작동되도록 설계하였고, TGFβ1 의 2CS 돌연변이 형태를 융합시켜 LRAT promoter 가 작동되었을 때 TGFβ1 이 과발현되도록 벡터를 구성하였다.

간성상세포기원인 Lx2 세포주의 경우 LRAT promoter 가 작동함에 따라 TGFβ1 발현이 증가하였으나, 간성상세포가 아닌 일반 간세포기원의 HepG2 와 Hep3B 세포주에서는 LRAT promoter 가 작동하지 않아 TGFβ1 발현에 변화가 유의미하지 않은 수준으로 나타났다. 각 세포주에 LRAT promoter 를 유도한 후 수득한 상등액으로 분석하였을 때, HepG2 와 Hep3B 에서는 변화가 없고, Lx2 특이적으로 3TP-lux 가 증가하며, TGFβ1 의 다운스트림으로 잘 알려진 PAI-1 또한 증가하였다. TGFβ1 에 의한 섬유화 유도 여부를 분석하였을 때, 역시 Lx2 세포에서만 특이적으로 섬유화 마커들의 유의미한 증가를 보였다.

마우스 생체 내 실험으로 LRAT promoter 의 작동을 유도하였을 때 간 내부에서 TGFβ1 의 발현이 관찰되었고, 그로 인한 섬유화 유도를 α-SMA 로 분석하였다.

이는 마우스 간섬유화 모델 제작의 새로운 방식을 제안하며, 간성상세포를 표적함으로써 섬유화 유도를 증가하여 섬유화 억제 약물 스크리닝에 효과적인 동물 모델로 사용할 수 있을 것으로 사료된다.

핵심어: 간섬유화, LRAT, 간성상세포, TGFβ1, 마우스 모델.