Identification of novel genes associated with progression of non-alcoholic fatty liver disease (NAFLD)

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Department of Medical Science

The Graduate School, Yonsei University

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Directed by Professor Jae-woo Kim

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ABSTRACT

Identification of novel genes associated with progression of Non-alcoholic fatty liver disease (NAFLD)

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Non-alcoholic fatty liver disease (NAFLD) is one of the most common causes of chronic liver disease worldwide. NAFLD has become an issue contributed to the fact that it can progress towards a more severe form of the disease, the non-alcoholic steatohepatitis (NASH), which can further progress towards liver cirrhosis and even cancer.

However, there is currently no approved treatment for NAFLD due to the fact that NAFLD models mimicking either steatosis or NASH-Fibrosis have been

sporadically known in *in vitro* and *in vivo*, respectively. So there have limitations in studying the progression of liver disease progression from steatosis to NASH-Fibrosis. Therefore, in many countries, people are currently conducting research with significant interest in the development and validation of cell and mouse models for NAFLD.

In this study, I found the most appropriate model among the various liver disease models previously reported. Using this model, I assessed the suitability through validation with gene markers that are mainly expressed in steatosis or NASH-Fibrosis.

Based on the previous study, oleic acids (OA) and high fat diets (HFD) were used to make model to induce steatosis in cell (Hepa1-6, primary hepatocytes) and mouse, respectively. Also, the TGFβ with FFA mixture (OA and PA) and the choline-deficient, L-amino acid-defined, high-fat diets (CDAHFD) were used to induce the NASH-Fibrosis model in cell (LX-2, hepatic stellate cells) and mouse, respectively. Ultimately, a final goal was to add a human data in addition to established cell and mouse models because it is the major purpose of this study, which is to apply to human patients suffering from liver diseases including NAFLD.

Taken together, the aim of this study is to find an unknown gene which is commonly increased in fatty liver and NASH-Fibrosis stage compared with normal liver by using integrated models (cell, mouse, and human) newly developed. By

analyzing established *in vitro* and *in vivo* models with Quant-seq, it is available to find novel genes increased in NAFLD groups compared to control groups, such as ITGAX, STC1. Also, these results provide an opportunity to determine which liver disease stage belongs to the overall NAFLD spectrum with gene expression.

Key words: Non-alcoholic fatty liver diseases, simple steatosis, NASH-Fibrosis, Fatty acid, gene expression,

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Ⅰ. INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) is the most common type of liver diseases worldwide.[1] Risk for NAFLD is increased in subjects with obesity or with metabolic syndrome.[2] As obesity results from an imbalance of energy intake and expenditure, NAFLD occurs when the rate of hepatic fatty acid uptake and de novo lipogenesis (DNL) exceeds the rate of fatty acid oxidation and export. NAFLD is classified into two groups: 1) Simple steatosis with triglyceride accumulation in

hepatocytes without inflammation, 2) Nonalcoholic steatohepatitis (NASH) with hepatocellular steatosis and fibrosis reactions.[3] Steatosis is reversible via pharmaceutical intervention, exercise, or caloric restriction. However, if it persists, it may progress into fibrosis, cirrhosis, and hepatocellular carcinoma (HCC), which are irreversible. It is, therefore, critical to prevent fibrosis and hepatic cirrhosis at the state of steatosis.[4,5]

However, there is an obstacle, which is the lack of the appropriate disease models for NAFLD, which leads to the delay in development of successful pharmacological treatment for the liver disease.[6] These make the importance of developing established models to accelerate progression of medical treatment for NAFLD.[7] Also it is necessary to develop a model of NAFLD and this model using cells and mouse can help to express human liver disease.[8]

The accumulation of triglyceride (TG) induced by oleic acids (OA) in *in vitro* is contributed to the secretion of cell death-inducing DEFA-like effector b (CIDEB), which acts as a lipogenic transcription factor, and the increase of adipose differentiation-related protein (ADRP), also known as perilipin 2. The amount of TG accumulation in hepatocytes was dose-dependent on OA, as previously described.[9,10] Also, in the *in vivo* model, C57BL6/J male mice fed on diets containing 60% fat became obese with fatty liver, compared with mice that are fed on a chow diet. [11]

On the other hand, the previous studies demonstrated that transforming growth factor beta (TGFβ) signaling is associated with hepatic stellate cells (HSC) activation and formation of fibrotic tissue in the hepatocytes.[12] Also, methionine choline-deficient diet (MCD) is frequently used to generate the mouse model of NASH, since it induces elevation of aminotransferase and finally histological changes such as hepatic steatosis and fibrosis.[13] However, the MCD diets cause a significant weight loss, which does not represent pattern of NASH patients.[14] Currently, NASH model is created by using the choline-deficient, L-amino acid-defined, high-fat diets (CDAHFD), which have advantages in terms of weight loss.[15] In the NASH-Fibrosis model, expression of both inflammation markers, TNFα, MCP1, MCM2, IL-6, and IL-1β, and fibrosis markers, αSMA, Col1a1, PAI-1, and TIMP-1, are known to be increased significantly.[16]

In this study, I sought to establish cell and mouse model showing steatosis and NASH-Fibrosis. Steatosis model was made with oleic acids and 60% HFD, and NASH-Fibrosis model was made by TGFβ with FFA mixture (OA and PA) and CDAHFD. Herein, I added human data to create a final integrated model for liver disease. Through the quant-seq data analysis of established models, the genes that are commonly increased or decreased in the cell, mouse and human are selected, and these genes are confirmed in the established models to identify their function.

Ⅱ. MATERIALS AND METHODS

1. Mice and diets

Male C57BL/6 mice were purchased from the Japan SLC (Shinzuoka, Japan). The animals were maintained according to the ethical guidelines of my institution, and the Committee on Animal Investigations of the Yonsei University approved the experimental protocol. The animals were maintained in a temperature-controlled room (22℃) on a 12:12-h light-dark cycle. First, Five-week-old mice were fed a HFD (60 kcal% fat, Research Diets, New Brunswick, NJ, USA) for 15 wk, MCD (Methionine-Choline deficient diet, Research Diets, New Brunswick, NJ, USA) for 6 wk, and CDAHFD (Choline-Deficient, L-amino acid-defined, High-Fat Diet, Research Diets, New Brunswick, NJ, USA) for 3 wk or a normal diet (Dyets, Bethlehem, PA, USA) for up to 15 wks. Body weight was measured every-day.

2. Cell culture

Three cell lines with different characteristics were used:

(1) Primary Hepatocyte

Primary mouse hepatocytes were isolated by the two-step collagenase perfusion method from livers of male C57BL/6 (8 wk old) as previously described. Hepatocytes were plated on six-well dishes at 1.0 X 10^6 cells per well and incubated

for 24hr in basal medium Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) – high glucose containing heat-inactivated 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin, 1 μM dexamethasone (Sigma), and 1 μg/ml of insulin (Roche, Penzberg, Germany) in a humidified atmosphere containing 5% CO2 at 37℃ to allow cells to attach. Cell counts and viability (Adam cell counter; Digital Bio, Seoul, Korea) were confirmed before use; viability was routinely > 85%.

(2) Hepa1-6

Authenticated Hepa1-6 murine hepatoma cell line was purchased from the American Type Culture Collection (Manassas, Virginia, USA) for this project. All cultures were maintained at 37℃ and 5% CO2. Cell lines were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Invitrogen) supplemented with heat- inactivated 10% FBS. All products used to culture cells were from Invitrogen (Carlsbad, CA, USA)

(3) LX-2

LX-2 was generated by immortalization of primary human hepatic stellate cells with the SV40 large T antigen followed by selective culture of early passaged cells in low serum media conditions. Cells are thawed in in basal medium Dulbecco's

modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) – high glucose containing heat-inactivated 10% fetal bovine serum (FBS; Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin, Once thawed, cells are expanded in 2% FBS media using the same components listed above.

3. Human sample

Information about the people was provided by Professor, Kim Won of Boramae Hospital in Seoul, Korea.

4. Induction of hepatic steatosis and fibrosis

(1) Simple steatosis

Long-chain FAs, palmitic (16:0) and oleic (18:1) were provided as sodium salts (Sigma-Aldrich, Milan, Italy). OA and PA were dissolved in EtOH 99% and volatilized with nitrogen gas to leave only pure oleic and palmitic acid. Next, OA and PA were dissolved overnight in 10 % fatty acids-free bovine serum albumin (BSA) in William's E medium with supplements (without serum). 8 mM stock solutions of PA and OA (molar ratio FA and BSA 5.33:1) were further diluted with supplemented William's E medium without fetal bovine serum to final concentrations of OA, PA or their combinations as described below. Stock solutions were kept at -20℃ before the experiments. Solutions and reagents used for cell

cultures were from GIBCO Life Technologies Ltd (Grand Island, NY, USA). Hepatic steatosis model was induced by the addition of 1200 μM or 1000 μM oleic acid for 48 hr, and lipogenesis was observed.[17]

(2) NASH-Fibrosis

HSCs, LX-2 cells were plated in 60 mm diameter dishes and cultured to 70% confluence. Cells were serum starved and supplemented with 0.2% BSA for 24 hr prior to TGFβ treatment (3 ng/ml for 30 hr). Control cells were also serum starved and supplemented with 0.2% BSA for 24 hr prior to PBS treatment. NASH-Fibrosis model was also induced by lipotoxicity in DMEM medium supplemented with PA /OA (2: 1, Final concentration; 1000 μM) for 24 hr after TGFβ treatment (3 ng/ml).[18]

5. Oil red-O staining

Cells were washed once in phosphate-buffered saline (PBS) and fixed with 3.7% formalin in phosphate-buffered saline for 5 min, rinsed with distilled water. The staining solution was prepared by dissolving 0.5 g oil red-O (Sigma) in 100 ml of isopropanol; 6 ml of this solution was mixed with 4 ml of distilled water, and filtered. The fixed cells were stained with staining solution for 1 hr. The staining solution was removed and cells were rinsed twice with distilled water.

6. Measurement of triglyceride amount in the hepatocytes

The liver triglyceride levels were determined by the method of Susan E. Carlson et al. with some modifications. Lipid extracts were produced by homogenizing of Hepa1-6 cells prepared by PBS. Next, it was also prepared in chloroform/methanol (2:1, v/v) with a final volume of 4ml. The homogenate was incubated with vortexing for 10 minutes, then 0.8ml of 50 mM NaCl was added, vortexed for 10 min, and then centrifuged at 4℃ for 10 min. The organic phase (25 μl of lower layer) was transferred and 25 μl of Triton X-100/chloroform (7.5:17.5, v/v) was added into samples and vortexed for 10 min. The solvents were vaporized with a vacuum evaporator. TG concentrations were determined using 25 μl of extract in a commercial colorimetric assay (Thermo Scientific, Waltham, MA, USA). Samples ad standards were vortexed and incubated at 37℃ for 30 min, and TG level were calculated from measurements of absorbance at 500nm.

7. Histological analysis

The liver tissues of each animal (chow, HFD, MCD, CDAHFD diet model) were fixed in 10% neutral-buffered formalin. Following fixation, the liver was trimmed, embedded in paraffin, sectioned, and stained with hematoxylin and ecosin (H&E) and masson's trichrome

8. RNA isolation and PCR analysis

Total RNA was isolated using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis from 5 μg of total RNA was performed using Superscript Ⅲ reverse transcriptase (Invitrogen) primed by random hexamer primer. The transcripts of ITGAX, Trem2, STC1, ADRP, CIDEB, TNFα, IL-6, IL-1β, MCM2, MCP1, TGFβ, αSMA, Col1a1, TIMP1, PAI-1, GAPDH were evaluated by PCR analysis. PCR was performed using mouse and human forward and reverse primers; 5'-CTG GAT AGC CTT TCT TCT GCT G-3', 5'-GCA CAC TGT GTC CGA ACT CA-3' (Mouse ITGAX), 5'-TTG CCA TTC GGA GGT GTT CG-3', 5'-TTG GGC CCG ATC TTC TCC AT-3' (Mouse STC1), 5'-ACT GGG ACG ACA TGG AAA AG-3', 5'–GTG CCT CTG TCA GCA GTG TC-3' (Mouse αSMA), 5'-TGT TCA GCT TTG TGG ACC TC-3', 5'-GCA GCT GAC TTC AGG GAT GT-3' (Mouse Col1a1), 5'-ACA TCA GTG CCT GCA GCT TC-3', 5'-GTG ACG GCT CTG GTA GTC CT-3' (Mouse Timp1), 5'-CAG AGG GCC CCT GGA GAA GT-3', 5'-ATT GTC TCT GTC GGG TTG TGC C-3' (Mouse PAI-1), 5'-TGA AAC GGA AGC GCA TCG AA-3', 5'-TTG CGG TCC ACC ATT AGC AC-3' (Mouse TGFβ), 5'-CAG GGG ACG AGA TAG AGC TG-3', 5'-TTG AGG AAT TGG GAC TTT GC-3' (Mouse MCM2), 5'-ACG GCA TGG ATC TCA AAG AC-3', 5'-GTG GGT GAG GAG CAC GTA GT-3' (Mouse TNFα), 5'-CCC AAT GAG TAG GCT GGA GA-3', 5'-TCT GGA CCC ATT CCT TCT TG-

3’ (Mouse MCP1), 5’-AGT TGC CTT CTT GGG ACT GA-3’, 5’-TCC ACG ATT TCC CAG AGA AC-3’ (Mouse IL-6), 5’-CTT TCC CGT GGA CCT TCC AG-3’, 5’-TAT GGG TCC GAC AGC ACG AG-3’ (Mouse IL-1β), 5’-CAG ACT GTC CTG GTC AAC GC-3’, 5’-TGC TAG ATG TGA GGA CGC CA-3’ (Mouse ADRP), 5’-GGA AAG GAC TGA CAG CTG CC-3’, 5’-ACC ATC AAG CAC GTG TCG TC-3’ (Mouse CIDEB), 5’-ACC ACA GTC CAT GCC ATC AC-3’, 5’-TCC ACC ACC CTG TTG CTG TA-3’ (Mouse GAPDH).

Real-time PCR was performed using SYBR Green Master mix (Applied Biosystems, Foster city, CA, USA) in a total volume of 20 μl. Transcripts were detected by Real-time qPCR with a Step One instrument (Applied Biosystems). A standard curve was used to calculate mRNA level relative to that of a control gene, ribosomal L32. Primer pairs for specific target genes were designed according to published data (Table 1). All reactions were performed in duplicate. Relative expression levels and S.D. values were calculated using the comparative method.

9. RNA sequencing

To induce hepatic steatosis or fibrosis, First, in the in vitro model, hepatocytes were treated with oleic acid or TGFβ with ffa mixture for 48 hr or 3 days. Second, in the in vivo model, Mice were treated by Chow, High-Fat diet or CDAHFD diet for 15 wk or 3 wk. Finally, in the case of human liver biopsies, a total of 3 groups of

patients with normal liver, fatty liver, fibrosis were used. Total RNA was isolated with Trizol reagent (Invitrogen) according to manufacturer's instructions. Total RNA was commissioned to perform RNA sequencing (E-biogen, Seoul, Korea).

10. RNA interference (siRNA)

Hepa1-6, murine hepatoma cells were plated into 60-mm-diameter dishes 24 hr prior to transfection. The following double-stranded stealth siRNA oligonucleotides (Santa Cruz Biotechnology) were used: mouse Integrin αX (ITGAX) siRNA oligonucleotides (cat# sc-35696), sense 5'-CCC CTC TGA CTC ATG CTG A-3', antisense 5'-AGT CGT ACT CAG TCT CCC C-3', mouse Stanniocalcin 1 (STC1) siRNA oligonucleotides (cat# sc-44871), sense 5'-TAG CGG AAA CTT CTC AGA GA-3', antisense 5'-TGT GAA TAA CCT CTC CCT GG-3'. Control oligonucleotides with comparable GC content were also from Invitrogen. For knockdown, cells were transfected with control or gene-specific siRNA at 50 nM in DMEM containing 20% Fetal Bovine Serum (FBS) using Lipofectamine RNAiMAX (Invitrogen), according to the manufacturer's protocol. The next day, the medium was replaced with fresh DMEM containing 100 U/ml penicillin and 100 μg/ml streptomycin. And After 48 hr of treatment with siRNA, the cells were induced Steatosis or NASH-Fibrosis for 48 hr. Total RNA extracts were prepared from the cells at the indicated time point (2 days), and RT-PCR was performed.

11. Statistical analysis

All results are expressed as mean ± s.d Statistical comparisons of groups were made were made using an unpaired Student's t test.

Table1. Sequences of oligonucleotide primers used for real time PCR (qPCR)

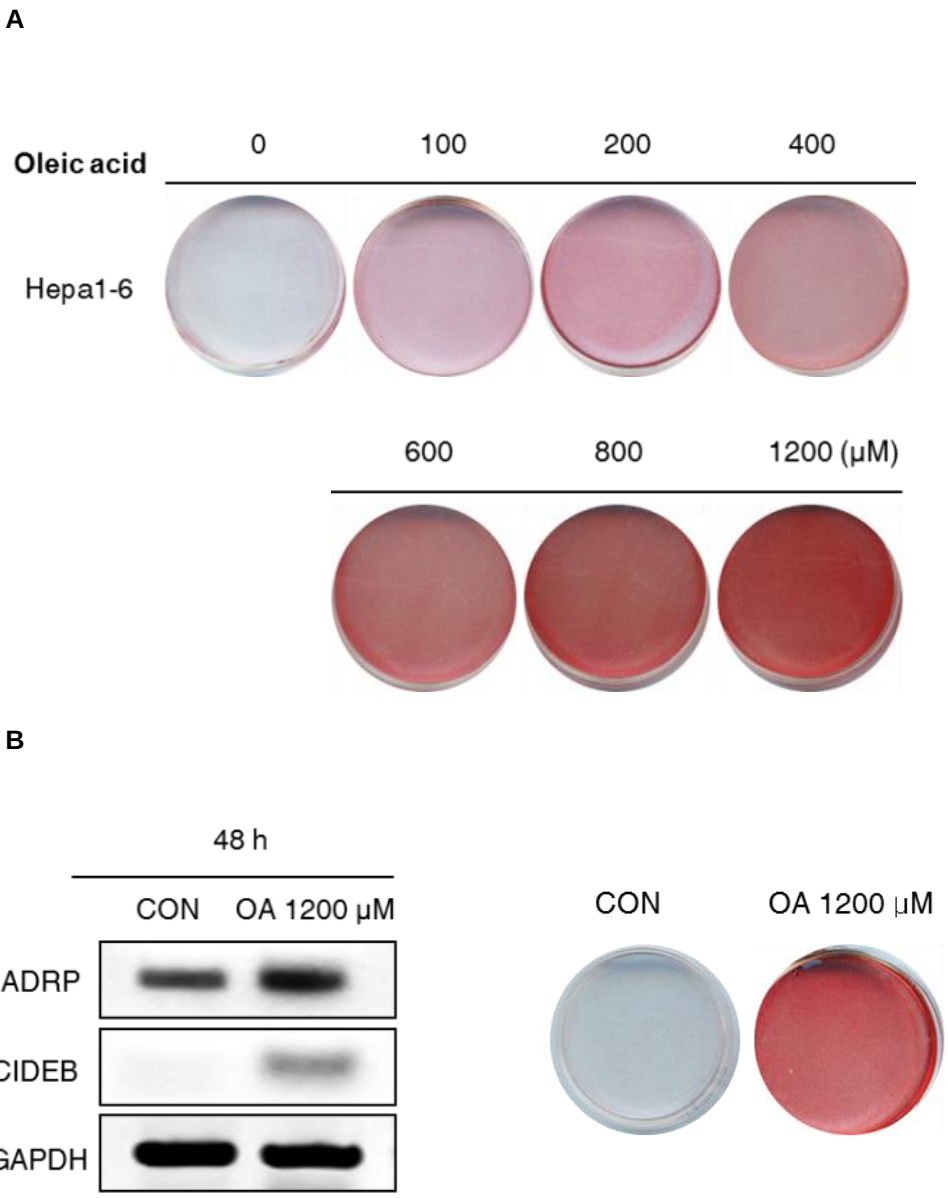
Oligonucleotide		Sequence(5′→3′)
Apoa4	Sense	CGGATGTCACTCAGCAGCTC
	Antisense	ACCAGCTTGTTGTGCACCC
CD36	Sense	TGCACCACATATCTACCAAA
	Antisense	TTGTAACCCCACAAGAGTTC
GLUT4	Sense	CATTGGCGCCTACTCAGG
	Antisense	CAAGGCACCCCGAAGATG
SCD-1	Sense	CCTTCCCCTTCGACTACTCTG
	Antisense	GCCATGCAGTCGATGAAGAA
ACCα	Sense	GCCAGACATGCTGGATCTCAT
	Antisense	CCGTCAGCTCAGATACACTTTC
MTTP	Sense	CTCCACTGAAGTGTTTCTTGATG
	Antisense	AACACAACGTCCACATCAGA
ADRP	Sense	CAGACTGTCCTGGTCAACGC
	Antisense	TGCTAGATGTGAGGACGCCA
TNFα	Sense	ATGAGCACAGAAAGCATGA
	Antisense	AGTAGACAGAAGAGCGTGGT
IL-1β	Sense	TGTGCAAGTGTCTGAAGCAGC
	Antisense	TGGAAGCAGCCCTTCATCTT
Col1a1	Sense	CGAGTCACACCGGAACTTGG
	Antisense	GGCACCAATGTCCAAGGGAG
Col1a1(Human)	Sense	ATGACTATGAGTATGGGGAAGCA
	Antisense	TGGGTCCCTCTGTTACACTTT
MCP1	Sense	AGGTCCCTGTCATGCTTCTG
	Antisense	TCTGGACCCATTCCTTCTTG
αSMA	Sense	AGGATGCAGAAGGAGATCACAG
	Antisense	CTGGAAGGTAGATAGAGAAGCC
αSMA(Human)	Sense	AAAAGACAGCTACGTGGGTGA
	Antisense	GCCATGTTCTATCGGGTACTTC
TGFβ	Sense	TGAAACGGAAGCGCATCGAA
	Antisense	TTGCGGTCCACCATTAGCAC
PAI-1	Sense	CTCTCGAAGAGGAAAGAGTCTAAC
	Antisense	CTTTGCTGAGTGAAGGCG
PAI-1(Human)	Sense	CGCCAGAGCAGGACGAA
	Antisense	CATCTGCATCCTGAAGTTCTCA
TIMP1	Sense	ACATCAGTGCCTGCAGCTTC
	Antisense	GTGACGGCTCTGGTAGTCCT
TIMP1(Human)	Sense	ACCTGCAGTTTTGTGGCTCC
	Antisense	CGGGACTGGAAGCCCTTTTC

Ⅲ. RESULTS

1. *In-vitro* model of NAFLD

(1) Establishment of hepatic steatosis model with Hepa1-6 cells and primary hepatocytes.

To analyze the development of lipid accumulation in an *in vitro* model of hepatic steatosis, Hepa1-6 cells were exposed to oleic acids in a dose-dependent manner. After 48 hr of treatment, lipid accumulation was detected by oil-red-O staining (Figure 1A-B). And it was confirmed that lipid accumulation was most effective when treated with 1200 μM oleic acids in Hepa1-6 cells. And the gene expressions related to lipogenesis (ADRP, CIDEB, Apoa4, MTTP, CD36, GLUT4, SCD1, ACCα) were constantly increased (Figure 1C). And primary hepatocytes were treated with 1000 μM oleic acids, the most effective dose of fatty acids which can be used to show lipid accumulation. (Figure 1D).



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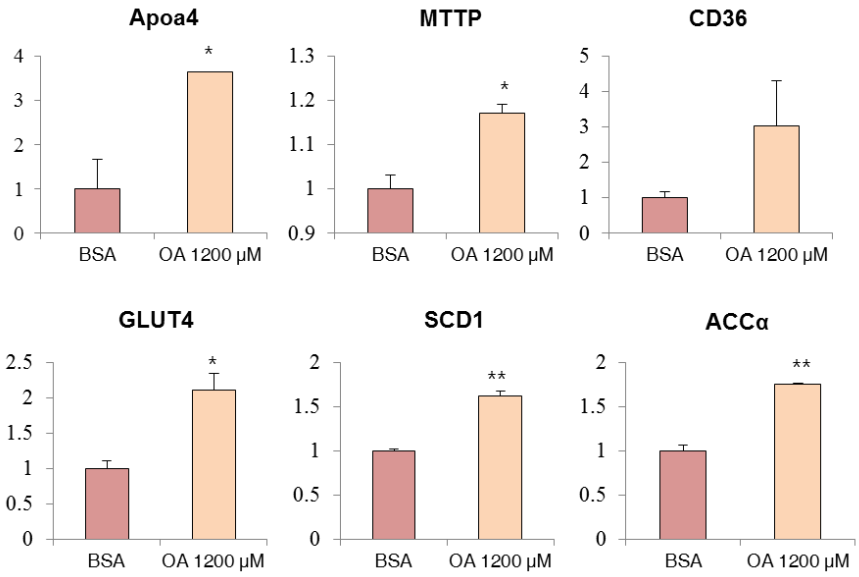

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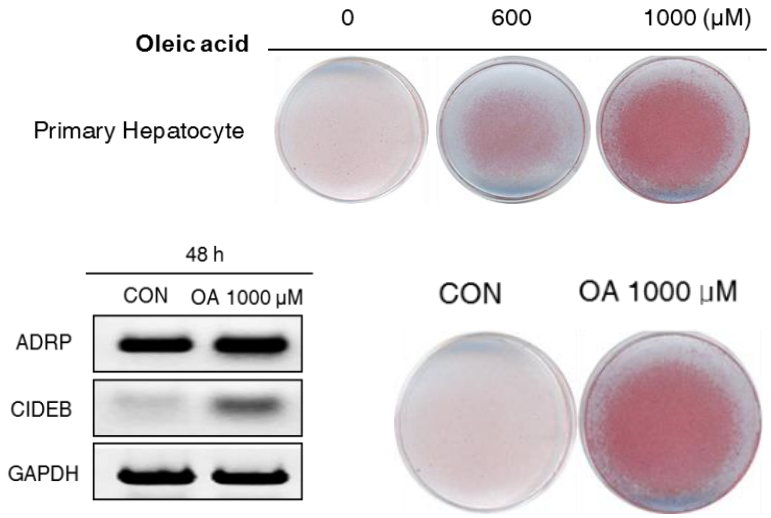


Figure 1. Effect of oleic acids on lipid accumulation in Hepa1-6 cells and primary hepatocytes. (a) Oil-red-o staining of Hepa1-6 cells treated with oleic acids. Hepa1-6 cells were incubated with an OA for 48 hr in a dose dependent manner. (b,c) Increased mRNA levels of marker genes related to steatosis in OA treated cell were analyzed by PCR. Student's test, *$p < 0.05$, **$p < 0.01$ (d) Oil-red-o staining of primary hepatocytes treated with oleic acids. Primary hepatocytes were incubated with indicated dose of OA for 48 hr. (OA: oleic acids, PA: palmitic acids)

(2) Effect of fibrogenic key factor, TGFβ on fibrogenesis in LX-2 cells.

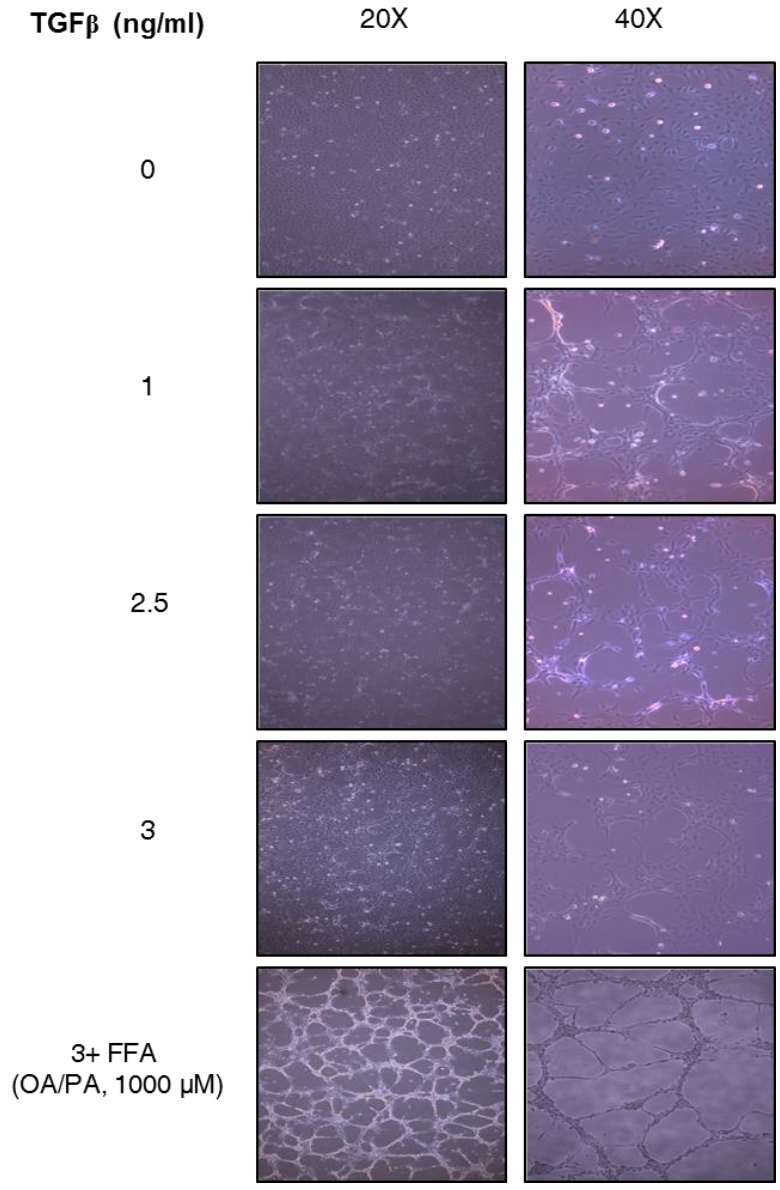
Hepatic stellate cells (HSCs) are known to play a key role in the liver fibrosis by secreting fibrogenic factor. And their activation in damaged liver leads to formation of scar tissue, leading to chronic fibrosis or cirrhosis.[19]

I first confirmed the induction of liver fibrosis by treating TGFβ which was the most fibrogenic factor in human hepatic stellate cells, LX-2 cells as reported in the previous studies.

TGFβ, a fibrosis inducer, was treated in LX-2 cells in a dose dependent manner (ranging from 0 to 3 ng/ml), and morphology of NASH-Fibrosis was shown to be severe depending on the concentration. In addition, treatment of TGFβ with a mixture of OA and PA which was lipotoxicity inducer resulted in a much more dramatic induction of NASH-Fibrosis than treated with TGFβ alone. (Figure 2A)

And activation of HSCs was correlated with enhanced mRNA gene expression of alpha-SMA, col1a1, TIMP-1, and PAI-1, known as fibrosis inducers, as well as ADRP and CD36, involved in lipogenesis in LX-2 cells (Figure 2B).[9]

A



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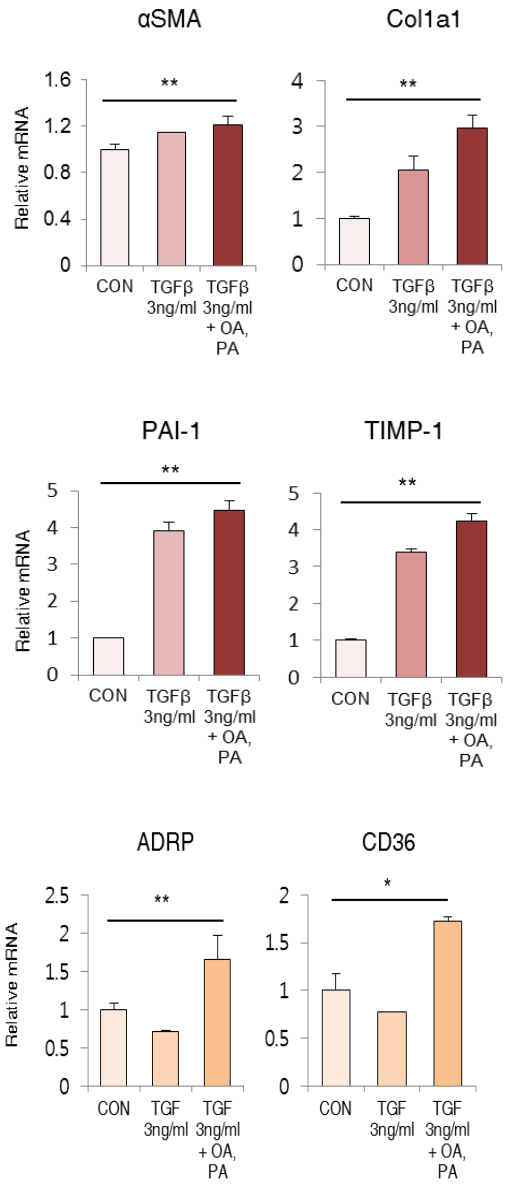


Figure 2. Effect of different concentrations of TGFβ on Fibrosis in LX-2 cells. (a) Morphology of LX-2 cells treated with TGFβ and fatty acids in a dose dependent manner. (TGFβ : Transforming Growth Factor β) (b) LX cells were examined for their responsiveness to TGFβ as measured using quantitative real time reverse transcription-polymerase chain reaction (see methods) on fibrosis genes (αSMA, Col1a1, PAI-1, TIMP-1), lipogenesis genes (ADRP, CD36). Untreated controls were used as a reference point and the fold induction after 3 ng/ml TGFβ treatment with FFA mixture for 48 hours (PA/OA=2:1, 1000 μM) is shown. Student's test, *$p < 0.05$, **$p < 0.01$

2. *In-vivo* model of NAFLD

Effect of HFD and CDAHFD on mouse liver

(1) High-Fat diet (HFD)

According to previous studies, high-fat diet is effective to induce NAFLD rodent model that it can cause obesity, insulin resistance, fasting hyperglycemia, dyslipidemia, and altered adipokine profile, which are commonly observed in NAFLD patients.[20]

When normal, young (5-wk-old) mice are fed a diet containing 60% fat for 15 wk, they form fatty liver compared with mice that are fed on a normal chow diet (Figure 3A). Also, expression of lipogenic genes was significantly up-regulated in HFD group rather than control group (Figure 3B,C). Taken together, feeding HFD to mice for 15 wk contributed to development of fatty liver.

(2) Choline-Deficient, L-amino acid-defined, High-Fat Diet (CDAHFD)

Until now, it is accepted that Methionine-Choline Deficient diet, MCD rodent model is considered the most reliable model for studying the inflammatory and fibrotic aspects of the NAFLD spectrum.[21]

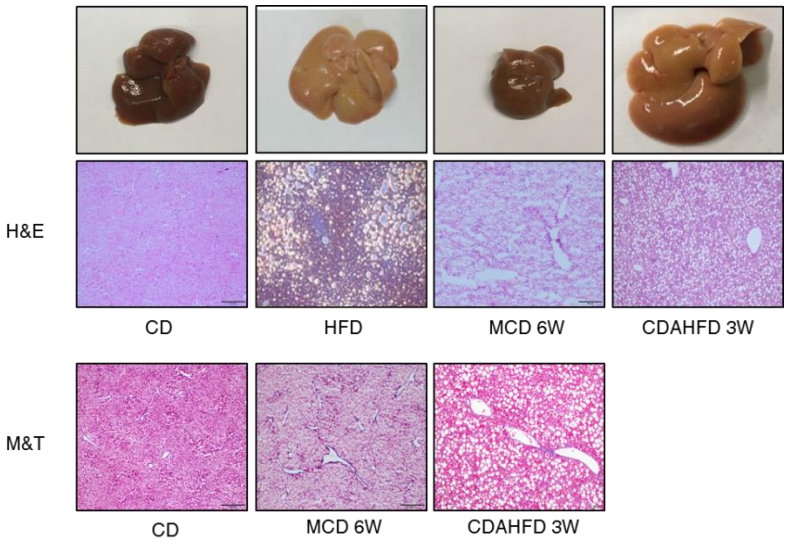
Studies suggest that MCD impairs mitochondrial β-oxidation as well as hepatic very low density lipoprotein (VLDL) secretion. Initially, I examined the expression

of genes related to inflammation and fibrosis in livers of mouse which was fed on MCD diets. The NASH-Fibrosis livers exhibited increased mRNA levels of both inflammation markers and fibrosis markers (Figure 3B,C).

Based on previous studies, I conducted CDAHFD diets for 3 wk to induce the NASH-Fibrosis model in *in vivo*.[22] As a result, mice with CDAHFD diets showed little difference in body weight compared to a mouse with a chow diet. (Data was not shown). In addition, the mRNA level of inflammation maker genes and fibrosis marker genes are significantly increased compared to the MCD diet, which is well-known to induce the NASH-Fibrosis model (Figure 3B-C).

Taken together, 3 wk of CDAHFD-diet feeding induced significant hepatic steatosis, inflammatory cell infiltration, and fibrosis in WT livers. Also, hepatocyte inflammation and hepatocellular fibrogenesis were observed in a mouse with MCD diet and CDAHFD diet through H & E staining of liver tissue. In addition, it is confirmed that the mice with CDAHFD diets were more predominantly macro-vesicular steatosis than those from the MCD diets (Figure 3B)

A



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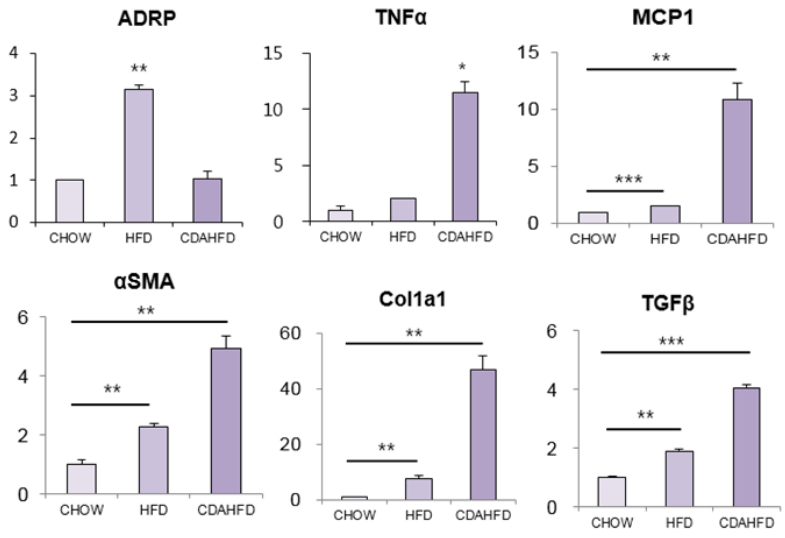

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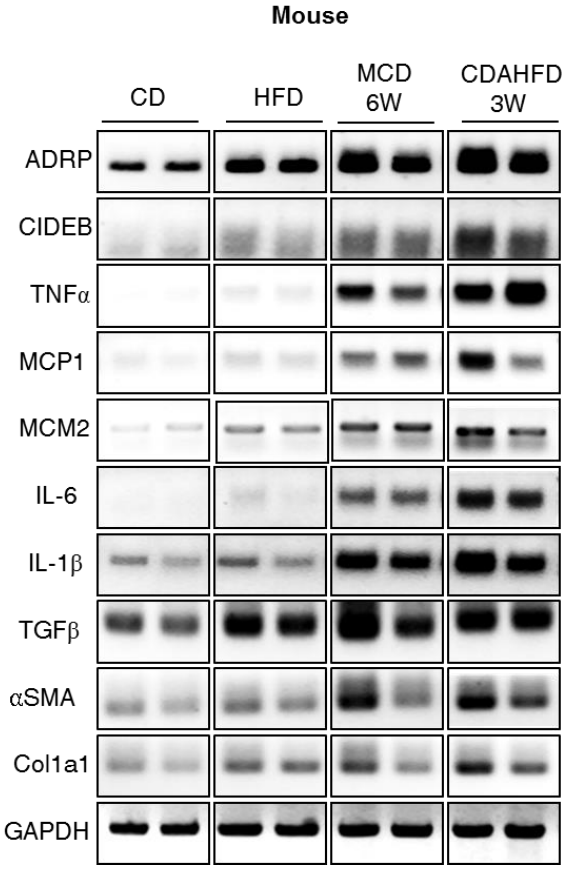


Figure 3. Effect of HFD and CDAHFD on mouse liver. (a) Representative liver histology stained with both hematoxylin and eosin stains (H&E staining) and masson's trichrome stains (M&T staining) in a mouse model of NAFLD. (b,c) The mRNA expression of the genes in mouse liver fed with chow, 60% HFD for 15 wk, MCD for 6 wk, and CDAHFD for 3 wk. The relative mRNA level of ADRP, CIDEB showing the FA biosynthetic activity, TNFα, MCP1, MCM2, IL-6, IL-1β which are important components of inflammation induction and TGFβ, αSMA, Col1a1 participating in the fibrogenesis. Student's test, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$

3. Dataset of hepatic gene expression in integrated model of cell and mouse.

OA was treated to Hepa1-6 cells and mice were fed on HFD. Also TGFβ with FFA mixture (OA and PA) was treated to LX-2 cells and mice were fed on CDAHFD. In these samples, fold changes of relative gene expression was measured by RNA sequencing. After 48 hr, in *in vitro* and 3 or 15 wk in *in vivo* from induction of either steatosis or NASH-Fibrosis, if the fold change value is greater than 1, gene expression is defined as up-regulated. Also If the value is less than or equal to 1, it is defined as down-regulated. The union of *in vitro*, *in vivo*, and human groups was selected as candidate genes.

(1) Simple steatosis

First, I compared up-and down-regulated genes between *in vitro* and *in vivo* groups of hepatic steatosis from Quant-seq data (Figure 4A). Many genes were up- or down-regulated in both *in vitro* and *in vivo* model. Furthermore, the established both *in vitro* and *in vivo* models were finally filtered with human data to find overlapping genes in all three models, *in vitro*, *in vivo* and in humans.

As a result, 898 genes were up-regulated and 627 genes were down-regulated simultaneously in all three models of steatosis (Figure 4B).

(2) NASH-Fibrosis

First, I compared up-and down-regulated genes between *in vitro* and *in vivo*

groups of NASH-Fibrosis from Quant-seq data (Figure 4C). Many genes were up- or down-regulated in *in vivo* model but not in *in vitro* model. In addition, the established both *in vitro* and *in vivo* models were finally filtered with human data to find overlapping genes in all three models of NASH-Fibrosis, *in vitro*, *in vivo* and in humans

Together, 392 genes were up-regulated and 831 genes were down-regulated simultaneously in all three models of NASH-Fibrosis (Figure 4D).

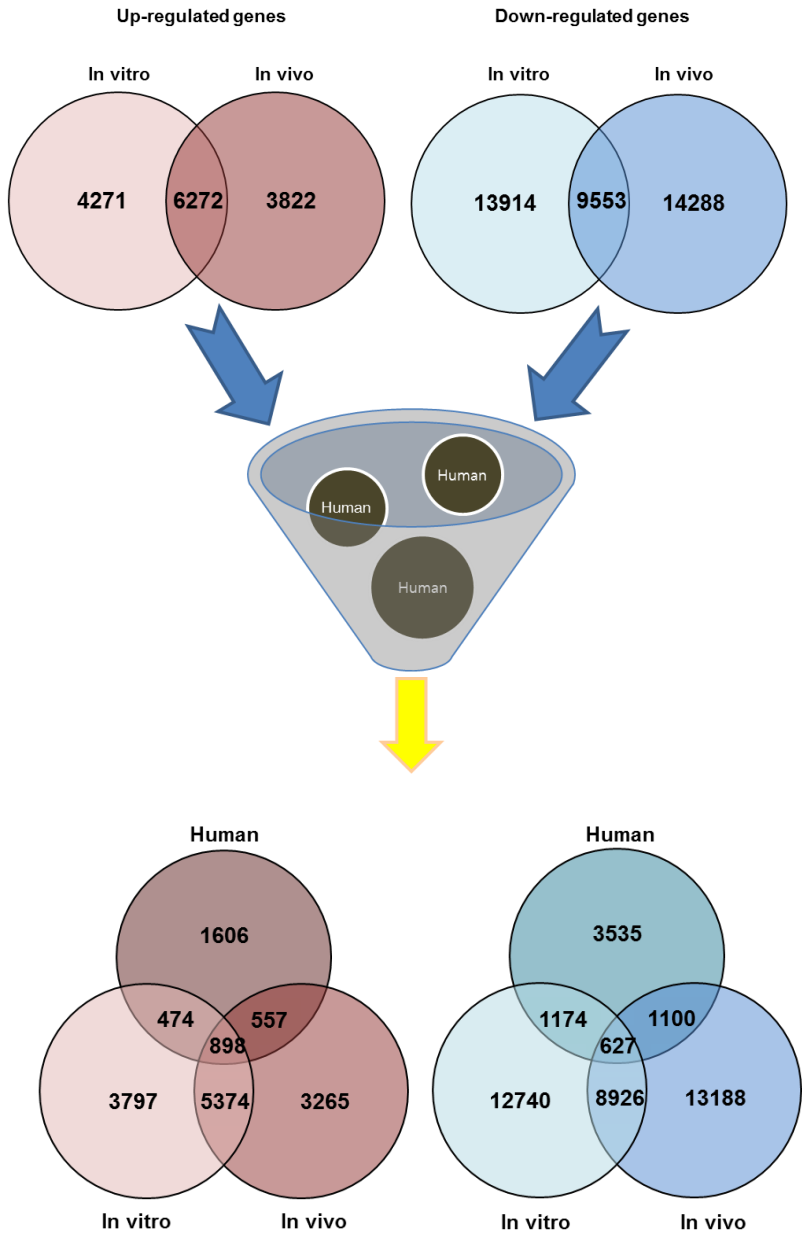
A

Gene symbol	Fold change OA-1200plusIns /BSA (*In vitro*)
Srms	13.743
Serpini1	10.578
Grk4	8.462
Caly	8.283
Acta1	6.317
Cpne5	5.260
Snora26	4.202
Col2a1	4.150
Ccl4	3.140
Scara3	2.075
Serpina3n	2.072
Pate4	2.052
Mcm8	1.886
Kiss1	1.876
Vegfb	1.783
Cers6	1.662
Lpin2	1.492
Lage3	1.404
Nrg1	1.251
Serp2	1.200
Acat1	1.191
Itgax	1.008
Smarce1	0.799
Kras	0.792
Stc1	0.732
Wnt10a	0.664
Urb1	0.651
Mcm9	0.511
Ripk4	0.495
Kcnq1	0.494
Alox12b	0.328
Clec3b	0.327
Ugt2b1	0.196
Dmtn	0.163
Notch4	0.132
Fut7	0.122
Serpina1b	0.089
Cav1	0.051

Gene Symbol	Fold change HFD/Chow (*In vivo*)
Cyp2b9	926.427
Cidea	133.115
Spink1	21.445
Mmp17	10.612
Trem2	9.913
Foxm1	8.684
Fasn	7.803
Nuf2	7.443
Ccl4	7.383
Mrc2	7.282
Stc1	4.973
Pparg	4.713
Mcm2	4.250
Itgax	3.324
Mctp1	2.282
Sept10	2.194
Sod3	2.106
Psd4	1.931
Ptms	1.751
Grk5	1.715
Rem1	1.708
Btg1	1.695
Gsn	1.463
Msra	1.299
Srgn	1.232
Wnt2	0.851
Mocs2	0.790
Psmg2	0.778
Zbtb32	0.642
Pinx1	0.624
Sowaha	0.560
Ston1	0.530
Rhbdd2	0.470
Pacsin1	0.361
Lncbate1	0.280
Fmn2	0.233
Adra2a	0.121
Txk	0.092
Acot1	0.034

Fold	Color index Color
>10	
>7.5	
>5	
>3	
>2	
>1.75	
>1.5	
>1.25	
1	
<0.8	
<0.6666	
<0.5714	
<0.5	
<0.3333	
<0.2	
<0.1333	
<0.1	

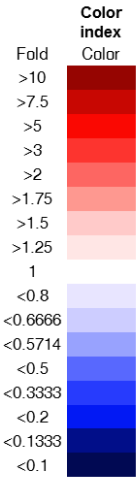
B



C

GENE Symbol	Fold change NASH-Fibrosis/Control (*In vitro*)
PMEPA1	126.294
FOXS1	115.038
SH2D2A	77.793
ANGPTL4	63.226
ABCA4	42.388
MYCT1	9.733
UNC13A	9.676
AMZ1	8.853
MYH16	7.221
LCE3D	7.182
BDKRB1	4.938
PRDM1	4.922
STAC3	4.841
MATN3	2.993
BMPR2	2.976
APBB2	1.994
FSTL3	1.993
CPEB3	1.749
SUV39H2	1.748
DNTTIP2	1.499
STC1	1.465
SMCR8	0.828
APOA1BP	0.800
STARD7-AS1	0.670
NHS	0.669
SOAT1	0.610
CCDC97	0.609
EMP1	0.500
PIM1	0.335
IKZF2	0.334
SDPR	0.205
MATN2	0.204
GPRC5A	0.134
ANKRD29	0.133
TSACC	0.105
MT1F	0.105
DKK1	0.069
C6orf201	0.069
FAM65B	0.043

GENE Symbol	Fold change NASH-Fibrosis/Control (*In vivo*)
Gpnmb	730.684
Cenpe	195.455
Cxcl5	140.503
Trem2	82.103
Itgax	38.792
Ccl4	22.728
Shisa4	9.591
Olfml3	8.711
Hepacam2	7.508
Panx1	7.447
Stc1	7.262
Wnk2	5.932
Chrnb1	3.008
Timp2	2.562
Nxpe4	2.004
Panct2	1.995
Col19a1	1.936
Adam28	1.759
Tmem258	1.673
Serpina9	1.501
Trim8	1.498
Nup85	1.251
Srrm1	0.805
Ubr1	0.800
Dusp19	0.667
Narfl	0.570
Psmd1	0.522
Lrrc3	0.499
Mpc2	0.419
Cend1	0.338
Dio3	0.256
Frem3	0.179
Tbx18	0.134
Serpine3	0.123
Mup10	0.100
Cxcl13	0.097
Krt79	0.055
Kiss1	0.029
Scd1	0.016



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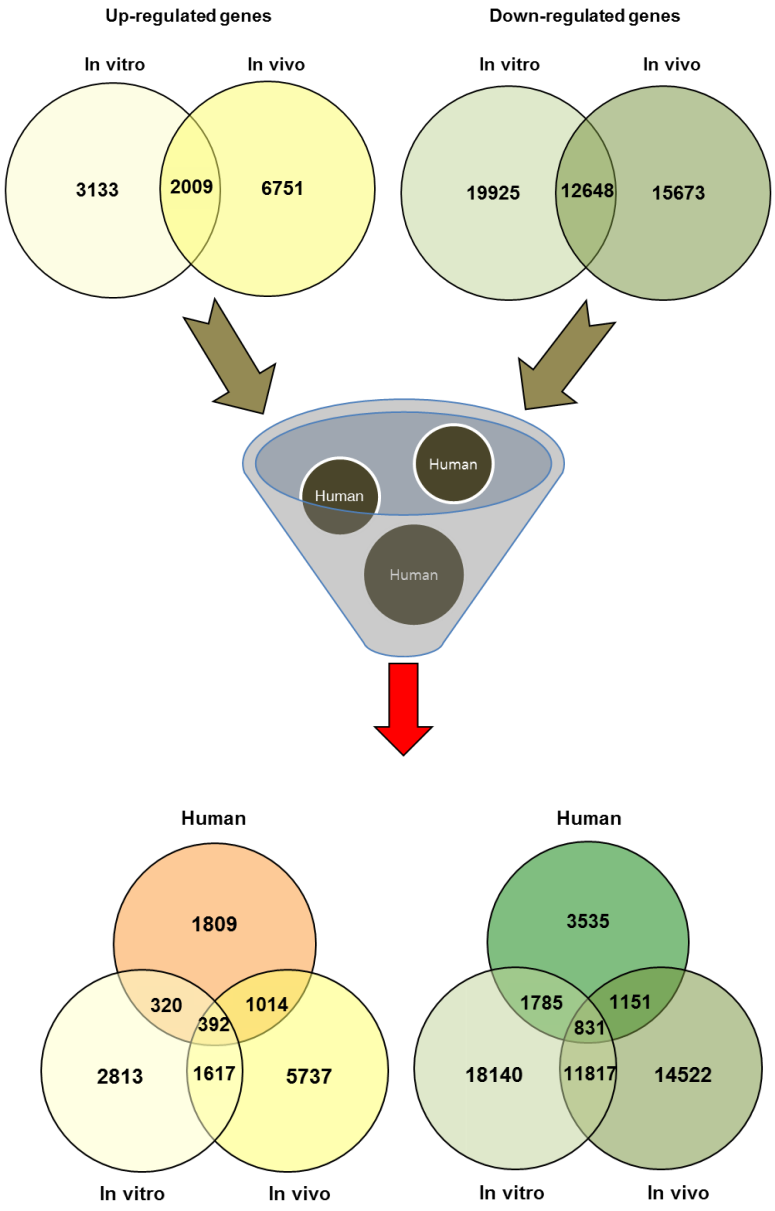


Figure 4. Relative gene expression in established cell and mouse model. (a,c) Visualization of the gene expression level. Red means high and blue means low expression. All number means fold changes. (b) In the case of steatosis, the Venn diagrams show overlapping patterns of probe sets that were equally up-regulated or down-regulated in *in vitro, in vivo* and human. (d) In the case of NASH-Fibrosis, the Venn diagrams show overlapping patterns of probe sets that were equally up-regulated or down-regulated in *in vitro, in vivo* and human.

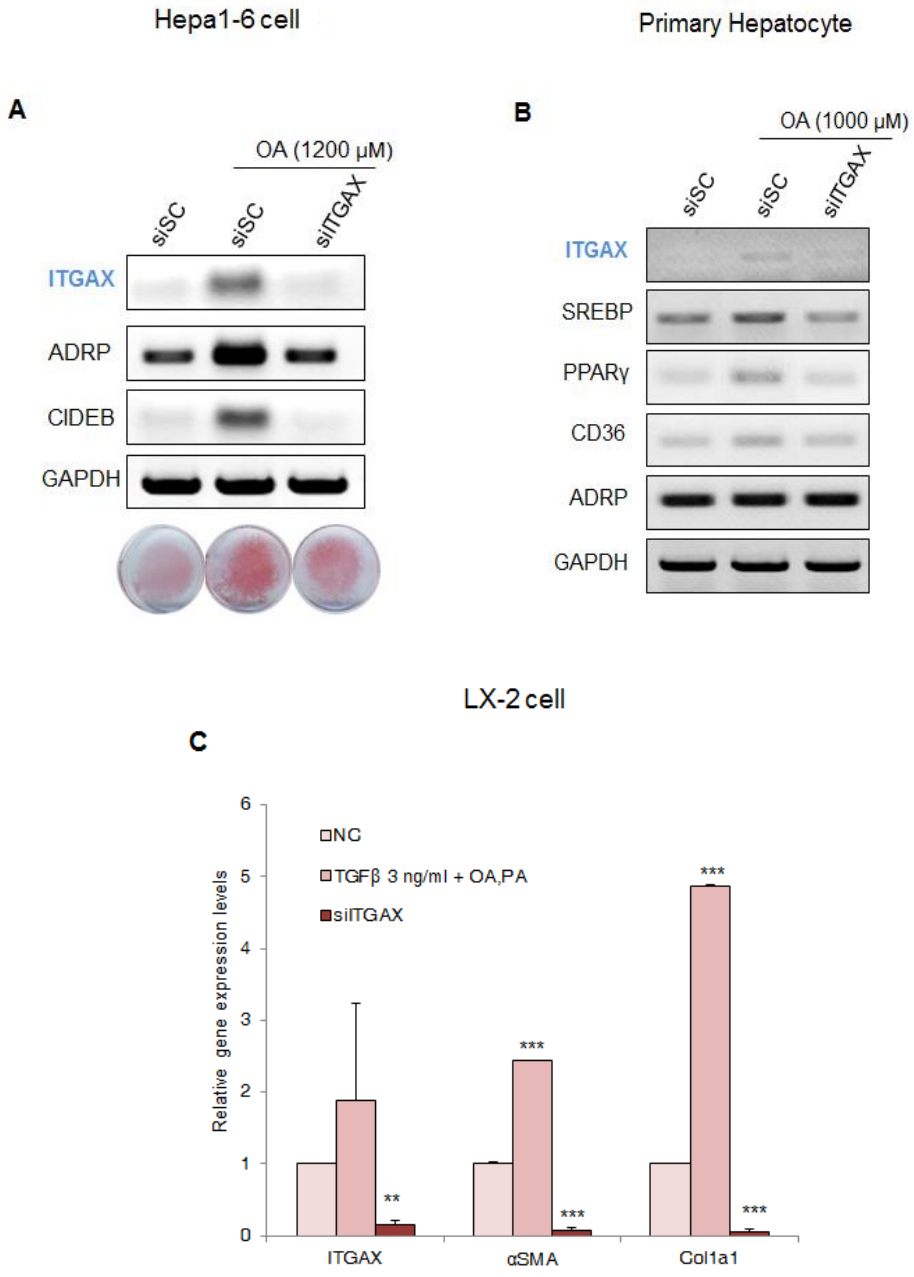
4. Expression patterns of selected genes, ITGAX and STC1 in hepatocytes

Of the genes obtained from the quant-seq data (Figure 3), two genes, ITGAX (Integrin, alpha X, CD11c) and STC1 (Stanniocalcin 1) significantly increased compared to the control group were selected.

According to UCSC data, expression of gene, ITGAX is originally high in lung, lymph node, spleen, bone marrow but not in the liver. However, 1200 μM oleic acids treated in *in vitro* model result in the high expression of ITGAX in hepatocytes. On the other hand, STC1 was reported to show the highest expression in ovary but not in liver. However, high expression of STC1 in hepatocytes can be confirmed by treating TGFβ and FFA mixture (OA+PA) in *in vitro*. This suggests that ITGAX, STC1 may contribute to progress NAFLD including both steatosis and NASH-Fibrosis in *in vitro* and *in vivo*.

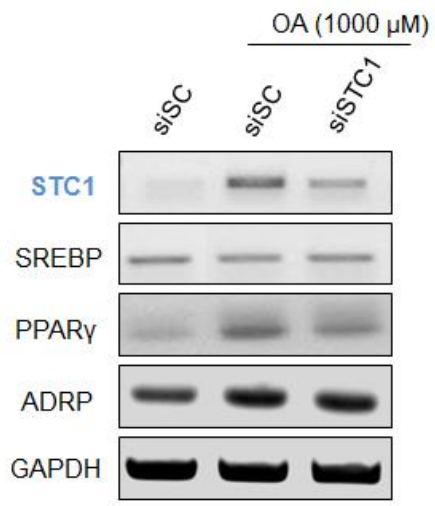
To examine whether ITGAX, STC1 affect NAFLD, these genes were knocked down in primary hepatocytes and LX-2 cells using siRNA. As a result, ITGAX-knock down cells including both hepa1-6 cells and primary hepatocytes were induced decrement in steatosis progression evidenced by mRNA levels (ADRP, CIDEB, SREBP, PPARγ, CD36) and oil-red-O staining (Figure 5A,B). Furthermore, we performed experiments on hepatic stellate cells, LX-2 cells, to determine whether progression to NASH-Fibrosis was inhibited as well as steatosis when ITGAX gene was knocked down. After induction of fibrosis in LX-2 and

knockdown of ITGAX, we confirmed that αSMA and col1a1, fibrosis markers, decreased (Figure 5C). This suggests that inhibition of ITGAX from steatosis inhibits progression to NASH-Fibrosis. On the other hand, in the case of STC1, the steatosis model was induced by using oleic acid in primary hepatocytes after then, STC1 gene was knocked down. However, it was confirmed that there was no change in the lipogenesis maker, ADRP, SREBP, PPARγ (Figure 5D). After induction of fibrosis with TGFβ and FFA mixture (OA+PA) in LX-2 cells and knockdown of STC1, the RNA levels of fibrosis markers, αSMA, Col1a1 and TIMP1 were decreased (Figure 5E). These results indicate that STC1 is a gene affecting NASH-Fibrosis progression significantly. Taken together, these results indicated that ITGAX and STC1 is the most powerful candidate gene expected to facilitate NAFLD progression among the RNA sequencing data.



Primary Hepatocyte

D



LX-2 cell

E

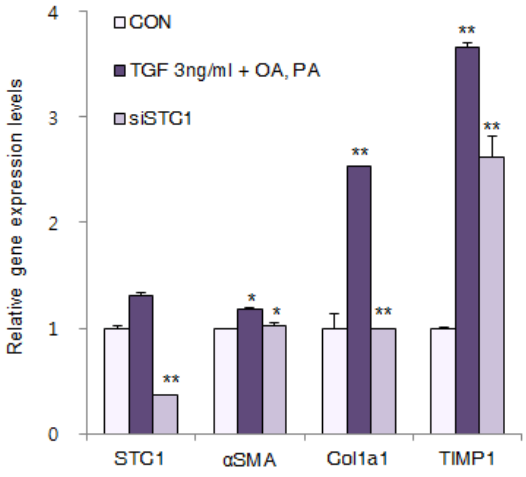


Figure 5. Knockdown of ITGAX and STC1 in hepatocytes. ITGAX and STC1 was the most powerful candidate genes expected to facilitate NAFLD progression. Knockdown of ITGAX in hepa1-6 cell (a), primary hepatocyte (b) with steatosis, and LX-2 cell with NASH-Fibrosis (c) was assessed by RT-PCR, qPCR and Oil-red-O staining. Knockdown of STC1 in primary hepatocyte with steatosis (d), LX-2 cell with NASH-Fibrosis (e) was assessed by RT-PCR and qPCR. Student's test, *p < 0.05, **p < 0.01, ***p < 0.001

Ⅳ. DISCUSSION

Nowadays, Nonalcoholic fatty liver disease (NAFLD) is now a well-known metabolic risk factor with obesity worldwide. The landmark of steatosis is the accumulation TG in the liver and it promotes liver fibrogenesis. The signs of NASH-Fibrosis, which are more advanced in simple steatosis, are the accumulation a large amount of fat, inflammatory infiltration, and fibrosis.[23] Therefore, it is necessary to develop experimental cell and mouse model in which liver damage occurs and there is still no accurate model to date.[24]

In this study, there were established *in vitro* and *in vivo* models to study the effects of NAFLD, especially simple steatosis and NASH-Fibrosis.

Although simple steatosis is a benign non-inflammatory liver disease, it has been reported that lipid accumulation causes nonalcoholic steatohepatitis and fibrosis, and that the severity of fatty liver in humans is related to the stage of liver fibrosis. However, it remains unclear that the precise mechanism of NAFLD and the process of alleviating patients with nonalcoholic fatty liver disease in search of unknown and important genes that cause NAFLD are necessary. For this reason, it is necessary to establish an *in vitro* and *in vivo* model for NAFLD studies to resolve metabolic problems

In this study, in *in vitro*, three types of hepatocytes, primary hepatocytes, Hepa1-

6 cells, and LX-2 cells were used to produce steatosis, NASH and hepatic fibrosis models and to prove and verify the characteristics of NAFLD in this model.

I used gene markers known from previous studies to demonstrate that these models represent steatosis and NASH-Fibrosis. These typically include ADRP, CIDEB, which cause lipogenesis, TNFα, MCP1, which are involved in inflammation, and αSMA, Col1a1, which are related to fibrogenesis.

According to the results, steatosis was induced in the oleic acid-treated group in *in vitro*. On the other hand, NASH-Fibrosis model was induced in the group treated with TGFβ which is key factor of fibrosis with FFA mixture. So I confirm that it is possible to influence the plasma TG concentration or induce the lipotoxicity effect by triggering the early activation of stress-related kinases and apoptosis depending on which type of fatty acid is used.

Next, in *in vivo,* the marker genes related to lipogenesis, inflammation, and fibrogenesis in the liver tissues of HFD, MCD, and CDAHFD diets were increased compared to the control, and the CDAHFD group used to derive the NASH model was showed more dramatic gene expression than MCD group. High-carbohydrate diet, high cholesterol diet, and high fat diet (60%) were used to induce the simple steatosis model in *in vivo*. In the case of high cholesterol diet, it is basically a diet made to study atherosclerosis. Also it shows necrosis and fibrosis as well as liver steatosis. Also, high carbohydrate diets have a similar pattern to high fat diets, but

they have more caloric consumption than high fat diets. For this reason, mice were given 60% high fat diets for 15 wks, resulting in an effective diet mimicking simple steatosis in an *in vivo* model. In addition, the MCD diet is known to be the most effective diet to induce the NASH-fibrosis model. As a result, it showed significant inflammation and fibrogenesis, but does not reflect the metabolic profile of NAFLD in humans. In conclusion, the MCD model mimics only histopathological features but lacks the main pathogenic factors of human disease. To overcome these problems, CDAHFD diet was used and by using the CDAHFD diet, mouse liver tissue showed morphological character of NASH-Fibrosis more rapidly.

The reason for establishing the *in vitro* and *in vivo* models for mimicking steatosis and NASH-Fibrosis is to select the novel genes that increase or decrease at the same time in three models by adding the human model and ultimately to improve NAFLD patients. So I performed RNA sequencing with three liver models *in vitro*, *in vivo*, and human. Herein, overall gene-expression profile was compared between NAFLD groups including steatosis and NASH-Fibrosis and control group. To filter out RNA sequencing data, I set up criteria. I selected meaningful genes which are expressed more than two-fold higher in NAFLD groups. As a result, 1525 candidate genes in the case of steatosis as well as 1223 candidate genes in the case of NASH-Fibrosis were screened from RNA sequencing data applying the criteria. Although it was focused on ITGAX and STC1 in this study, the other 2746

candidate genes are worthy enough to be researched further. Also, in this study, we identified candidate gene expressions and conducted gene silencing experiments with established *in vitro* and *in vivo* model, furthermore we will reveal the gene function deeply.

Ⅴ. CONCLUSION

In summary, these findings suggest that oleic acid as well as high fat diet promote hepatic steatosis and TGFβ with FFA mixture as well as CDAHFD diet induce NASH-Fibrosis. The NAFLD research using established models in this study will play an important role in alleviating the disease degree of NAFLD patients.

In addition, using RNA Quant-seq analysis, I found out novel genes especially ITGAX, STC1 and they are involved in NAFLD progression including hepatic steatosis and NASH-Fibrosis.

REFERENCES

1. Buzzetti E, Pinzani M, Tsochatzis EA. The multiple-hit pathogenesis of non-alcoholic fatty liver disease (NAFLD). Metabolism. 2016; 65(8):1038-48.
2. Hassan K, Bhalla V, El Regal ME, A-Kader HH. Nonalcoholic fatty liver disease: a comprehensive review of a growing epidemic. World J Gastroenterol 2014; 20:12082–101.
3. Jonathan C. Cohen, Jay D. Horton, and Helen H. Hobbs. Human Fatty Liver Disease: Old Questions and New Insights. Science. 2011; 332(6037): 1519–1523.
4. Takahashi Y, Fukusato T. Histopathology of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. *World J Gastroenterol* 2014; 20(42): 15539-15548
5. Angulo P, Kleiner DE, Dam-Larsen S, Adams LA, Bjornsson ES, Charatcharoenwitthaya P, et al. Liver fibrosis, but no other histologic features, is associated with long-term outcomes of patients with nonalcoholic fatty liver disease. Gastroenterology 2015; 149:389–97
6. Prasanna K. Santhekadur, Divya P. Kumar, Arun J. Sanyal. Preclinical Models of Nonalcoholic Fatty Liver Disease. Journal of Hepatology 2018;

68(2):230-237

7. Kucera O, Cervinkova Z. Experimental models of non-alcoholic fatty liver disease in rats. *World J Gastroenterol* 2014; 20(26): 8364-8376

8. Prasanna K. Santhekadur, Divya P. Kumar, Arun J. Sanyal. Preclinical Models of Nonalcoholic Fatty Liver Disease. Journal of Hepatology 2018; 68(2):230-237

9. Motomura W1, Inoue M, Ohtake T, Takahashi N, Nagamine M, Tanno S, Kohgo Y, Okumura T. Up-regulation of ADRP in fatty liver in human and liver steatosis in mice fed with high fat diet. Biochem Biophys Res Commun. 2006 ;340(4):1111-8.

10. Li JZ, Ye J, Xue B, Qi J, Zhang J, Zhou Z, Li Q, Wen Z, Li P. Cideb regulates diet-induced obesity, liver steatosis, and insulin sensitivity by controlling lipogenesis and fatty acid oxidation. Diabetes. 2007 ;56(10):2523-32.

11. Undi Hoffler, Kristen Hobbie, Ralph Wilson, Re Bai, Akef Rahman, David Malarkey, Greg Travlos, and Burhan I Ghanayem. Diet-Induced Obesity in mice is associated with hyperinsulinemia, hepatic steatosis, and glomerulopathy in C57Bl/6J mice. Endocrine. 2009 ; 36(2): 311–325.

12. Scott L. Friedman. Mechanisms of Hepatic Fibrogenesis. GASTROENTEROLOGY 2008 ;134:1655–1669.

13. Rinella, M. E., M. S. Elias, R. R. Smolak, T. Fu, J. Borensztajn, and R. M.Green. Mechanisms of hepatic steatosis in mice fed a lipogenic methionine choline-deficient diet. J. Lipid Res. 2008 ;49: 1068–1076.

14. Lee SJ, Kang JH, Iqbal W, Kwon O-S. Proteomic Analysis of Mice Fed Methionine and Choline Deficient Diet Reveals Marker Proteins Associated with Steatohepatitis. PLoS ONE 2015 Apr 7;10

15. Ayae Ikawa-Yoshida, Saori Matsuo, Atsuhiko Kato, Yusuke Ohmori, Atsuko Higashida, Eiji Kaneko and Masahiko Matsumoto. Hepatocellular carcinoma in a mouse model fed a choline-deficient, L-amino acid-defined, high-fat diet. Int. J. Exp. Path. 2017 ;98, 221–233

16. Enomoto H, Bando Y, Nakamura H, Nishiguchi S, Koga M. Liver fibrosis markers of nonalcoholic steatohepatitis. *World J Gastroenterol* 2015 ; 21(24): 7427-7435

17. Ricchi M, Odoardi MR, Carulli L, Anzivino C, Ballestri S, Pinetti A, Fantoni LI, Marra F, Bertolotti M, Banni S, Lonardo A, Carulli N, Loria P. Differential effect of oleic and palmitic acid on lipid accumulation and apoptosis in cultured hepatocytes. J Gastroenterol Hepatol. 2009 ;24(5):830-40.

18. T L Xu, A Y Hui, E Albanis, M J Arthur, S M O'Byrne, WS Blaner, P Mukherjee, S L Friedman, F J Eng. Human hepatic stellate cell lines, LX-1 and LX-2: new tools for analysis of hepatic fibrosis. Gut 2005 ;54:142–151.

19. Steven Dooley & Peter ten Dijke. TGF-β in progression of liver disease. Cell Tissue Res 2012 ;347:245–256

20. Anna M. Diehl, M.D., and Christopher Day, M.D. Cause, Pathogenesis, and Treatment of Nonalcoholic Steatohepatitis. N Engl J Med 2017 ;377:2063-72.

21. Yoshihisa Takahashi, Yurie Soejima, and Toshio Fukusato. Animal models of nonalcoholic fatty liver disease/nonalcoholic steatohepatitis. World J Gastroenterol. 2012 ; 18(19): 2300–2308.

22. Masahiko Matsumoto, Natsuko Hada, Yoshiyuki Sakamaki, Akiko Uno, Toshihiko Shiga, Chiaki Tanaka, Tsuneo Ito, Asao Katsume and Masayuki Sudoh. An improved mouse model that rapidly develops fibrosis in non-alcoholic steatohepatitis. Int. J. Exp. Path. 2013 ;94, 93–103

23. Duvnjak M, Lerotic I, Barsic N, Tomasić V, Virović Jukić L, Velagić V. Pathogenesis and management issues for non-alcoholic fatty liver disease. World J Gastroenterol 2007;13(34):4539–4550

24. Kucera O, Cervinkova Z. Experimental models of non-alcoholic fatty liver disease in rats. World J Gastroenterol 2014 ;20(26): 8364-8376

ABSTRACT (IN KOREAN)

비 알코올성 지방간 질환에 관련된 새로운 유전자 발굴 및 기능 분석

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한 지 윤

비 알코올성 지방간 질환 (NAFLD)은 전 세계적으로 만성 간 질환의 가장 흔한 원인 중 하나이다. NAFLD는 심각한 건강 문제가 되어 더 심한 형태의 질병으로 진행될 수 있으며, 비 알코올성 지방성 간염과 간 경변 및 암으로 진행될 수 있다.

비 알코올성 지방간 질환 연구를 위해 개발된 많은 세포 및 마우스 모델이 있지만, 현재 비 알코올성 지방간 질환에 대한 승인 된 치료법은 없다.

비 알코올성 지방간 질환에 대한 치료제의 개발이 더딘 이유는 인간 간 질환 상태를 나타낼 수 있는 승인 된 전임상 모델이 없기 때문이다. 따라서 많은 국가에서 사람들은 현재 비 알코올성 지방간 질환에 대한 세포 및 마우스 모델의 개발 및 검증에 상당한 관심을 가지고 연구를 진행하고 있습니다.

이 연구에서, 나는 비 알코올성 지방간 질환의 세포 모델과 마우스 모델의 개발을 확립했다. 이전의 연구에 기초하여, 올레인산과 고지방식이는 각각 지방간을 유도하는 모델로 사용하였고, CDAHFD식이 및 TGFβ와 지방산 혼합물 사용하여 지방성 간염· 간 섬유화 모델을 유도 하였다. 궁극적으로 최종 목표는 비 알코올성 지방간 질환을 가지는 환자의 상태를 개선하는 것이 연구의 주요 목적이기 때문에 기존의 세포 및 동물 모델에 추가로 인간 모델을 추가하여 분석하였다.

종합적으로, 이 연구의 목적은 세포, 동물, 사람 3 가지 모델을 통합하여 지방간, 지방성 간염 및 간 섬유화 단계의 상태에서 정상간과 비교를 통해 공통적으로 증가하는 알려지지 않은 유전자를 찾는 것이다. 그런 다음 정상적인 간 그룹에 비해 비 알코올성 지방간 질환 그룹에서 증가하는 새로운 유전자를 찾기 위해 확립 된 세포 및 동물 모델과 사람의 간 조직에서 Quant-seq 분석을 진행한다.

또한, 지방간이 지방성 간염으로 진행되는 과정에 대한 분자생물학적 근거가 완전하지 않다. 나는 비 알코올성 지방간 질환의 진행 과정에서 중요하게 작용하는 유전자를 찾았으며, 여기에는 대표적으로 ITGAX, 및 STC1이 포함되고, 정상간에 비해 지방간 그리고 지방성 간염 및 간 섬유화 상태의 간에서 현저하게 높이 발현된다. 더 나아가, 지방간 모델 (올레인산 처리) 및 지방성 간염 및 간 섬유화 모델 (TGFβ와 지방산 혼합물을 함께 처리)로 채택 된 간세포에서 ITGAX, STC1의 결손은 비 알코올성 지방간 질환을 개선시킨다.

이러한 이유로 ITGAX, STC1은 지방 생성 및 간세포 항상성의 중요한 조절 인자임을 발견하였다.

핵심 되는 말: 비 알코올성 지방간 질환, 지방간, 지방간 염증 및 간 섬유화, 지방산, 유전자 발현