2024 년 2 월

석사학위 논문

Role of nicotinamide N-methyltransferase on TGF-β-mediated hepatic stellate cell activation

조선대학교 대학원

약 학 과

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간 성상세포 활성화 조절 연구

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지도교수 기 성 환

이 논문을 약학 석사학위신청 논문으로 제출함

2023 년 10 월

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위 원 기 성 환 인

2023 년 12 월

조선대학교 대학원

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ABBREVIATIONS

ActD	actinomycin-D
TGF-β	transforming growth factor-β
α-SMA	alpha-smooth muscle actin
ECM	extracellular matrix
PAI-1	plasminogen activator inhibitor type 1
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HSCs	hepatic stellate cells
ALB	albumin
NNMT	Nicotinamide *N*-methyltransferase
MNAM	1-methylnicotinamide

초 록

니코틴아미드 N-메틸전이효소에 의한 TGF-β 매개 간 성상세포 활성화 조절 연구

김 나 연

지 도 교 수: 기 성 환

약학과

조선대학교 대학원

간 손상은 간염바이러스, 알코올, 비만 등 다양한 원인에 의해 발생한다. 손상 및 치유반응이 지속, 반복되면 간섬유화 단계로 진행되게 된다. 가역적 단계의 간섬유화는 손상 원인이 제거되면 다시 건강한 간으로 돌아갈 수 있지만, 간 손상이 계속되면 비가역적 단계인 간경변증, 간암까지 진행될 수 있다. 따라서 간섬유화에서의 치료는 비가역적 간질환 예방에 있어서 매우 중요한 단계로 간주된다. 간세포의 10%를 차지하는 간성상세포는 간섬유화를 유발하는데 중요한 역할을 하는 것으로 알려져 있다. 건강한 간에서의 간 성상세포는 지질방울을 함유하며 지질 대사에 관여한다. 하지만 여러 자극에 의해 활성화되면 가지고 있던 지질방울을 소실 하면서 콜라겐과 같은 세포외 기질(ECM)을 축적하는 근섬유아세포 형태로 분화한다.

TGF-β 신호 전달 경로는 간성상세포를 근섬유아세포로 분화시키는 중요한 인자로 알려져있다. 활성화된 TGF-β는 Smad2, 3 의 인산화를 유도한다. 인산화 후 smad4 와 복합체를 형성하여 핵으로 이동한후 섬유화 관련 유전자의 발현을 조절하는 것으로 알려져 있다. 최근 메티오닌 균형 조절이 간질환의 병태생리학에서 중요한 역할을 한다는 것이 보고되고 있다. 메티오닌 회로의 붕괴는 메틸 제공자 균형을 파괴하고 궁극적으로 여러 간 질환을 유발한다. 간은 메티오닌 균형을 조절하기 위해 여러 메틸전이화 효소를 발현한다. 간에서 주로 발현되는 메틸전이화 효소 중 하나인 니코틴아미드 *N*-메틸전이효소(NNMT)는 S-아데노실 메티오닌(SAM)을 메틸공여체로 활용하여 니코틴아미드 메틸화를 촉매하고 1-메틸니코틴아미드(MNAM)과 S-아데노실-L-호모시스테인 (SAH)를 생성하고, MNAM 은 *N*-methyl-2-pyridone-5-carboxamide (2PY), N-methyl-4-pyridone-5-carboxamide (4PY)로 산화되어 소변으로 배출된다. NNMT 는 간에서 세포 분화, 세포증식, 포도당합성, 지질합성을 비롯하여 다양한 생리활성을 조절하는 것으로 보고되었다. 최근 간경변 환자에서 NNMT 대사산물인 MNAM 과 2PY 의 수치가 증가했다는 결과가 발표되었으며 이는 TGF-β 신호 전달 경로와 연관성이 있는 것으로 알려지고 있다. 최근 NNMT 는 알코올성 지방간, 간암과 같은 여러 간질환 발병 및 진행에 관련이 있다는 보고가 있지만 간성상세포 활성화와 간섬유화에서의 NNMT 의 발현 및 기능에 대한 연구는 거의 없다. 본 연구에서는 간성상세포 활성화 과정에서 NNMT 의 발현 변화 유무 및 간성상세포 활성화에서 NNMT 의 효과에 대한 분자기전을 규명하고자 하였다. 우리는 간성상세포 활성화시 간의 다른 메틸전이화 효소로 알려진 글리신 *N*-메틸트랜스퍼라제(GNMT)와 NNMT 의 발현을 비교하였다. GEO 전사 데이터를 통해 NNMT 가 간성상세포 활성화시 특이적으로 증가한다는 것을 확인하였다. 또한 간성상세포주인 LX-2 세포에 TGF-

β를 처리하였을 때 NNMT 의 발현이 전사 의존적으로 증가됨을 확인하였다. 더 나아가 TGF-β에 의해 증가된 간성상세포 활성화가 NNMT 활성화에 의해 증가됨을 NNMT 억제제 또는 과발현 시스템을 통하여 증명하였다. 본 연구결과는 간성상세포 활성화 과정중 NNMT 의 조절이 간섬유화 예방 및 치료를 위한 새로운 표적이 될 수 있음을 시사한다.

I. INTRODUCTION

The liver damage is due to a diversity of causes, including hepatitis viruses, excessive alcohol consumption, and obesity. If damage continues and repeats, a healthy liver progresses to the stage of liver fibrosis [1, 2]. The reversible stage of liver fibrosis can return to a healthy liver once the cause of the damage is removed, but if the liver continues to be damaged, it progresses to irreversible stage such as liver cirrhosis. further progressing to liver cancer [3, 4]. Therefore, treatment at the stage of liver fibrosis is considered a very important step in preventing liver disease.

Hepatic stellate cells (HSCs), which account for 10% of liver cells, are considered important in inducing liver fibrosis [5]. HSCs exist in a quiescent state in a healthy liver, but become activated when liver damage occurs. Activated HSCs undergo changes in myofibroblast phenotype as they lose vitamin A-containing lipid droplets [6], and excessive deposition of extracellular matrix (ECM) such as collagen and induces liver fibrosis [5]. Excessive ECM deposition disrupts the normal structure of the liver and liver function. There is still little information about the regulatory molecules and related mechanisms involved in hepatic stellate cells activation and fibrosis progression. Therefore, investigation new therapeutic target to inhibit liver fibrosis is still needed.

Transforming growth factor-β (TGF-β), considered as the master cytokine of liver fibrogenesis [7], induces fibrogenic gene expression through the Smad pathway or non-Smad signaling pathway [7, 8]. The TGF-β transmembrane receptor is composed of I and II, and upon activation, phosphorylation of Smad2 and Smad3 is promoted, and activated Smad2 and Smad3 bind to co-Smad Samd4 [8, 9]. These complex translocates into the nucleus where it binds to cofactors and regulates gene transcription such as collagen, plasminogen activator inhibitor type 1 (PAI-1) and α-smooth muscle action (α-SMA) [9, 10]

It is well known that 85% of methylation reactions occur in the liver. Regulation of

methionine balance plays an important role in the pathophysiology of the liver diseases [11-13]. Disruption of the methionine cycle impairs the methyl donor balance and ultimately causes several liver diseases such as alcoholic liver injury and nonalcoholic fatty liver disease [12, 14]. Therefore, the liver expresses several methyltransferases to regulate methyl donor balance. The liver expresses several methyltransferases, including NNMT and GNMT, to regulate methionine balance. NNMT, the major methyltransferase, uses S-adenosyl methionine (SAM) as a methyl donor and catalyzes the methylation of nicotinamide (NAM), producing 1-methylnicotinamide and S-adenosyl-L-homocysteine (SAH) [15, 16]. Additionally, the MNAM is finally oxidized to the *N*-methyl-2-pyridone-5-carboxamide (2PY) and *N*-methyl-4-pyridone-5-carboxamide (4PY) compounds, which are excreted through urine [17, 18]. NNMT is reported to play several roles in the regulation of liver physiology, including cell proliferation, cell differentiation, gluconeogenesis, and lipogenesis [11, 16, 18]. It was reported that the results showed that the levels of MNAM and 2PY, which are metabolites of NNMT, were increased in the blood and urine of patients with cirrhosis [19, 20]. Additionally, NNMT is overexpressed in alcoholic fatty liver disease and promotes the progression of fatty liver disease [21, 22]. Additionally, it is known that NNMT causes phenotypic transition to cancer-related fibroblasts in other tissues, and these results suggest that NNMT might contribute to liver fibrosis progression through the TGF-β signaling pathway [23-25]. However, to date, studies have not been conducted on the role of NNMT in HSC activation and liver fibrosis. One of the other methyltransferases, glycine *N*-methyltransferase (GNMT), like NNMT, is known to regulate methylation levels in the liver [26, 27]. Therefore, we compared the expression of NNMT and GNMT in HSCs upon activation and found that NNMT was specifically increased proven through GEO transcript data. Therefore, we examined whether NNMT expression was regulated during HSCs activation and liver fibrogenesis. Furthermore, we investigated whether NNMT affects HSC activation and the underlying concise molecular mechanism. Our study reveals a novel regulatory mechanism of NNMT in HSCs activation and elucidates its role in liver fibrosis. This suggests that NNMT might be a novel target for the

prevention and treatment of liver fibrosis.

II. MATERIALS AND METHODS

1. Materials

NNMT antibody was purchased from Proteintech (Rosemount, IL, USA). Phospho-Smad2, phospho-Smad3, Smad2/3 antibodies were provided through Cell Signaling (Danvers, Massachusetts, USA). Plasminogen activator inhibitor-1 (PAI-1) and fibronectin antibodies were supplied by BD Bioscience (Mountain View, CA, USA). alpha-smooth muscle actin (α-SMA) and β-actin, Actinomycin-D (ActD) antibody and 1-methylnicotinamide (MNAM) were obtained from Sigma-Aldrich (St. Louis, MO, USA). JBSNF was from MedChemExpress (Monmouth Junction, NJ). TGF-β was purchased from R&D Systems (Minneapolis, MI, USA). anti-rabbit or anti-mouse secondary antibodies were supplied by Invitrogen (Carlsbad, CA, USA).

2. Cell culture

Human hepatic stellate cell line (LX-2) were generously obtained by Dr. S.L. Friedmann (Mount Sinai Medical College, New York, United States). The DMEM medium was used by adding 10% fetal bovine serum (FBS, Atlas Biologics, Fort Collins, CO) and 50 U/mL penicillin/streptomycin cells. Cells were incubated at 37°C in a humidified 5% CO2 atmosphere.

3. Primary Hepatic Stellate cells isolation

ICR mice purchased from Oriental Bio (Seongnam, Korea) were used. First, healthy mice were anesthetized, and mice were fixed alive, and then a catheter was inserted into the portal vein. After perfusion of HBSS (Free Ca2+) at 37°C for 20 min, perfusion was performed with a

solution containing 0.05% collagenase and Ca2+ for 20 min. The perfused livers were removed and filtered through a 70 μM cell strainer (BD Bioscience) using DMEM medium. The hepatocytes are then separated by centrifugation (3 times for 2 min at 50 g). HSCs were isolated using picoll and percol (1:10, GE Healthcare, IL, USA). HSCs cultured for 0 days (quiescent state) and HSCs cultured for 7 days (activated state) were used.

4. Cell viability assay

To assess cytotoxicity, LX-2 cells were incubated in 12-well plates and then treated with JBSNF (10, 30, 100 μM) the next day for 12 h. The medium was then removed, and MTT solution (0.3 mg in MEM medium) was added at 1 ml per well.

When formazan crystals were formed after 10 min, the medium was removed and 300 μl of DMSO was added to each well to dissolve the formazan crystals. Then, after transferring to a 96-well plate, the absorbance of each sample was measured at 550 nm using a microplate reader (SpectraMAX, Molecular Devices, Sunnyval, CA, USA). Cell viability was defined compared to controls.

5. Immunoblot analysis

Cells were lysed for 1 h by adding RIPA buffer (inhibitor-add buffer) and then centrifugation at 12,000 x g for 10 min. Protein samples were obtained from the supernatant and quantified by Bradford assay. It was separated by electrophoresis on SDS-PAGE (7.5% and 12% gels), and transferred to a nitrocellulose membrane. Then, at 37°C, the membrane was blocked with 5% skim milk for 30 min, washed three times for 10 min, and then incubated with primary antibody overnight at 4°C. The next day, after collecting the primary antibodies, the secondary antibodies were added for 1 hour. Membranes were treated with the ECL chemiluminescence detection kit and visualized using LAS 4000 (Fujifilm, Tokyo, Japan).

6. RNA isolation and RT-PCR analysis

Total RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA, USA) following the protocol provided by the manufacturer. cDNA was obtained by reverse transcription of total RNA (2μg) using the following oligo (dT) 16 primer. The cDNA was amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA, USA). After PCR amplification, they were separated by gel electrophoresis on a 2% agarose gel, stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in a gel documentation system (Fujifilm, Tokyo, Japan). The primer sequences used are as follows: human NNMT Forward 5'-GTTTGGTTCTAGGCACTCTG-3', and Reverse 5'-GCAGGTTCTGGTCTGAGTAG-3'; human GAPDH Forward 5'-GAAGGTGAAGGTCGGAGTC-3, and Reverse 5'-GAAGATGGTGATGGGATTTC-3'. GAPDH was used as a reference gene for normalization.

7. siRNA knockdown and Transient transfection experiment

NNMT knockdown was performed using control siRNA (100pmol) or siRNA NNMT (100pmol; Bioneer, Daejeon, Korea) by transfection into LX-2 cells for 24 h. After removing the medium, it was replaced with MEM medium containing 1% FBS overnight and then treated with TGF-β for 6 hours. Plasmid pCDNA3-Flag-Smad3 was kindly provided by Professor H..S..Choi (Chonnam National University, Gwangju, Korea). LX-2 cells were transfected with Smad3 or MOCK plasmid using LipofectamineTM 2000 (Invitrogen) for 24 hours. After removing the medium, it was replaced with MEM medium containing 1% FBS overnight and then treated with TGF-β for 12 hours. For NNMT overexpression, the NNMT plasmid (Origene) was used. Transfection was performed with pCMV4 or NNMT for 24 h using LipofectamineTM 2000 (Invitrogen) according to the protocol provided by the manufacturer.

8. Statistical analysis

ANOVA and student Newman-Keuls tests were used to determined significant differences between groups and between the means of different groups. Results are expressed as mean ± standard error (SE).

III. RESULTS

1. NNMT upregulation during hepatic stellate cells activation

We found that the expression of NNMT is increased at the gene transcription level in activated mouse HSCs compare to in quiescent HSCs. On the contrary, GNMT level was reduced as evidenced by data, which was extracted from GSE34949 (Figure 1A and B). Next, NNMT levels were confirmed in primary hepatocytes and HSCs isolated from mouse liver. As a result, the expression of NNMT was higher in HSCs than in hepatocytes (Figure 1C). In addition, we found that NNMT was upregulated in accordance with α-SMA induction in primary HSCs during HSC activation (Figure 1D). We also demonstrated increased NNMT levels in primary HSCs by stimulation with TGF-β, an essential profibrotic cytokine. (Figure 1E). These results demonstrate that the expression of NNMT was increases in activated HSCs during liver fibrosis.

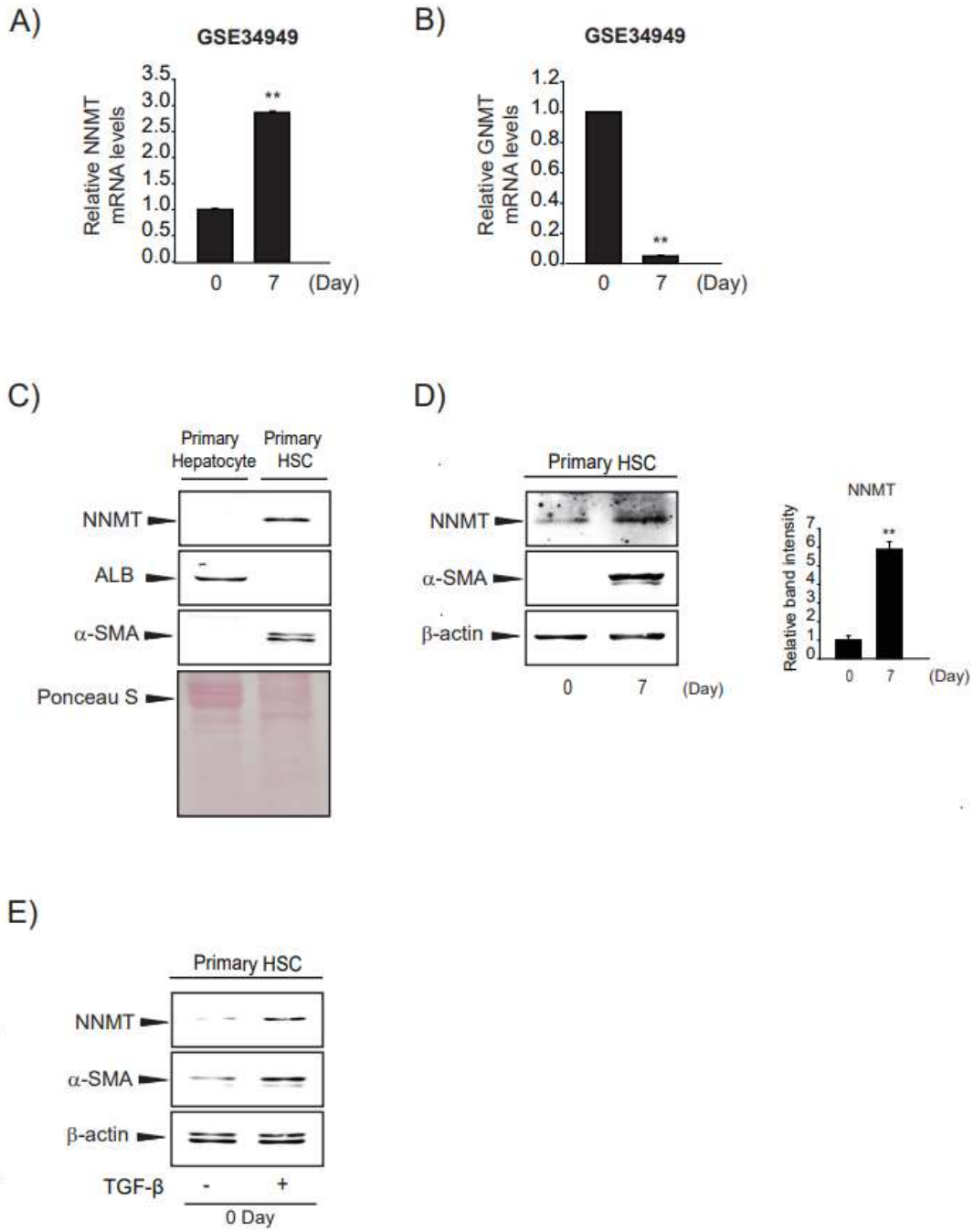


Figure 1. Upregulation of NNMT during HSC activation

(A) Transcripts level of NNMT and (B) GNMT in quiescent or activated primary HSCs from GSE34949. The data represents the mean ± SE (n = 3, significant different versus quiescent, **p<0.01). (C) We analyzed the levels of NNMT in primary hepatocytes and HSCs isolated from the livers of healthy mice. (D) NNMT was up-regulated in primary HSCs during culture activation along with α-SMA induction. (E) The effect of TGF-β on NNMT induction in primary HSCs from mice. Primary HSCs were incubated with 2 ng/mL TGF-β for 6 h, and then NNMT and α-SMA expression were assessed by immunoblotting in the cell lysates

2. TGF-β-mediated NNMT upregulation in hepatic stellate cell lines

Because, NNMT was increased in primary HSCs activation, we investigated whether NNMT was also increased by TGF-β (essential profibrogenic cytokine) in hepatic stellate cell lines LX-2 cells. First, LX-2 cells were treated with TGF-β for 3–24 h and NNMT protein levels were analyzed. NNMT was increased in a time dependent manner upon TGF-β stimulation (Figure 2A). Next, LX-2 cells were treated with TGF-β at concentrations of 0.5–2 ng/mL for 12 h and NNMT levels were analyzed. NNMT was increased in a concentration-dependent manner upon TGF-β stimulation (Figure 2B). To verify whether NNMT was regulated at the transcriptional level, LX-2 cells were treated with TGF-β and NNMT mRNA levels were analyzed. NNMT mRNA level was gradually induced by TGF-β stimulation (Figure 2C and D). Additionally, when LX-2 cells were treated with the transcriptional inhibitor actinomycin-D (ActD), TGF-β-mediated NNMT induction was inhibited (Figure 2E). Our data demonstrate that TGF-β-induced NNMT increase is transcriptionally regulated.

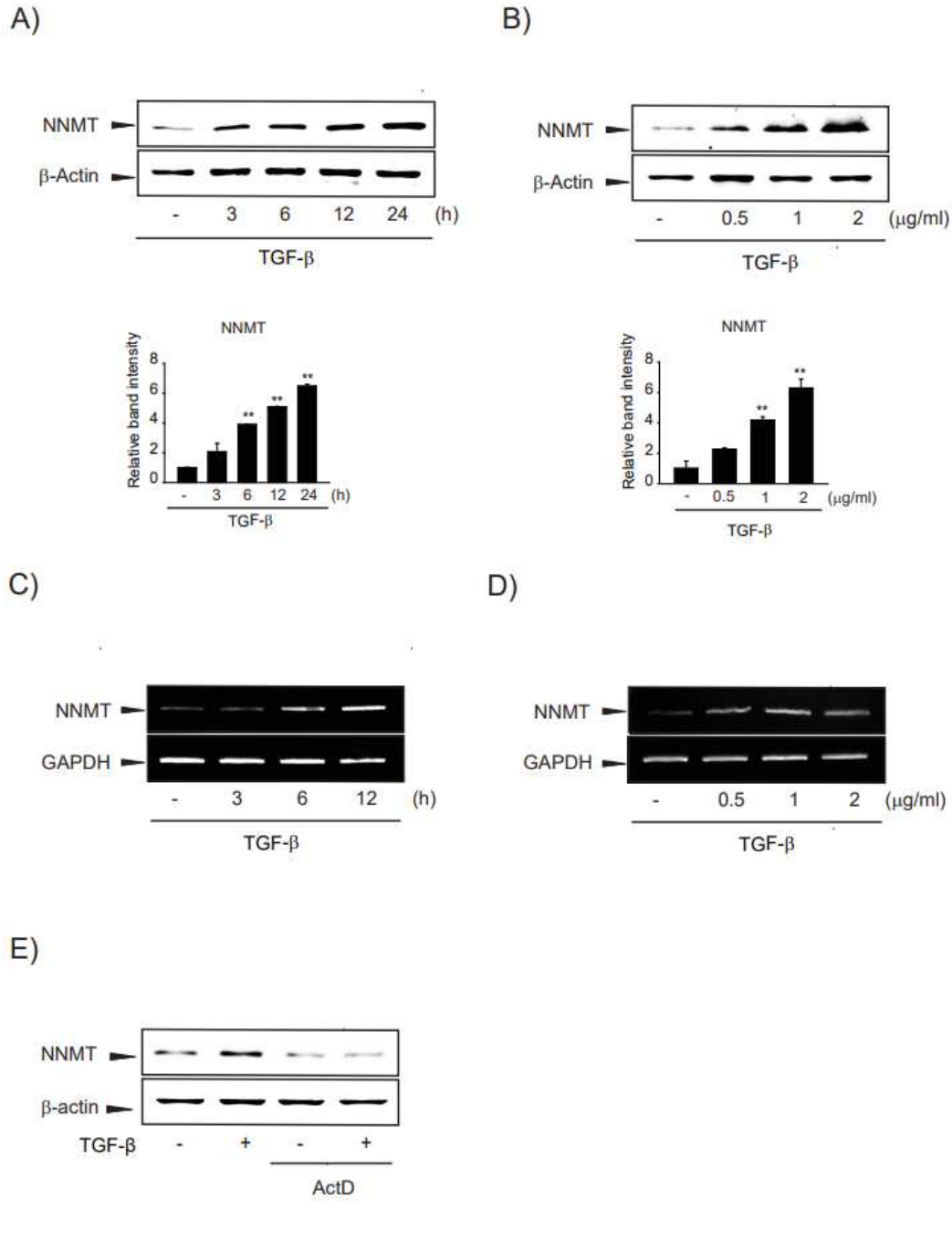


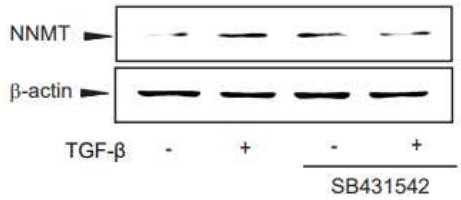
Figure 2. TGF-β mediated NNMT upregulation in LX-2 cells

(A) NNMT protein expression was assessed in LX-2 cells which were treated with 2 ng/mL TGF-β for 3–24 h. (B) NNMT protein expression was examined in LX-2 cells which were treated with 0.5–2 ng/mL TGF-β for 12 h. (C) RT-PCR analysis. Cells were treated with 2 ng/mL TGF-β for 3, 6, 12 h. (D) Cell were treated with 0.5, 1 or 2 ng/mL TGF-β for 12 h and mRNA levels of NNMT were assessed by RT-PCR using GAPDH as loading control. (E) The effect of actinomycin-D (ActD) on the NNMT induction by TGF-β in LX-2 cells. The cells were treated with 5 μg/mL of ActD in the presence or absence of 2 ng/mL TGF-β.

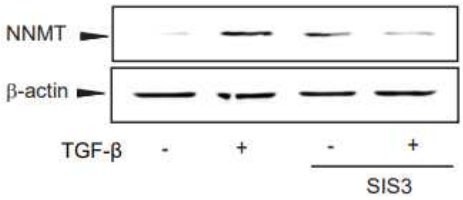
3. NNMT upregulation through TGF-β/Smad-dependent pathway

Therefore, we next, to determine the molecular mechanism of TGF-β-mediated NNMT upregulation in HSC, we investigated whether Smad, a well-known TGF-β downstream transcription factor, is involved in NNMT regulation. First, we treated LX-2 cells with SB431542, a TGF-β receptor antagonist, to investigate whether NNMT upregulation is associated with TGF-β signaling pathway. TGF-β-mediated NNMT induction was significantly attenuated by SB431542 (Figure 3A). Additionally, treatment with SIS3, an inhibitor of the TGF-β downstream transcription factor Smad3, substantially reduced NNMT levels (Figure 3B). Next, we introduced Smad3 into LX-2 cells. TGF-β-mediated NNMT increase in MOCK transfected cells were further increased by Smad3 ectopic expression (Figure 3C). Our data demonstrated that TGF-β-mediated NNMT expression is regulated through a Smad3-dependent pathway.

A)



B)



C)

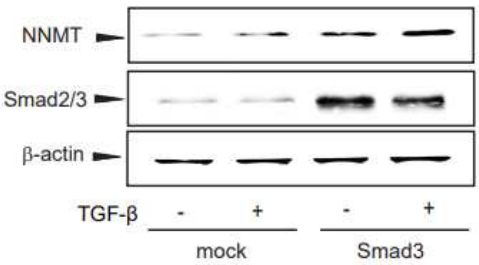


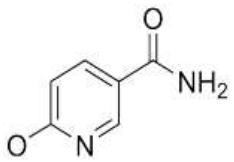
Figure 3. NNMT upregulation is mediated by TGF-β/Smad-dependent pathway

(A) The effect of TGF-β receptor antagonist (SB-431542) on NNMT expression by TGF-β. LX-2 cells were incubated with SB-431542 (10 μM) in the presence or absence of TGF-β (2 ng/mL) for 12 h, and then NNMT expression were assessed by immunoblotting in the cell lysates. (B) The effect of Smad3 inhibitor (SIS3) on TGF-β-induced NNMT expression. The cells were treated with SIS3 (5 μM) in the presence or absence of TGF-β (2 ng/mL) for 12 h. Immunoblotting was done to detect NNMT levels. (C) The effect of Smad3 overexpression on TGF-β-induced NNMT expression. LX-2 cells were transfected with Smad3 or pCDNA (MOCK), then treated with TGF-β (2 ng/mL) for 12 h.

4. Antagonistic role of NNMT inhibitor on hepatic fibrogenesis

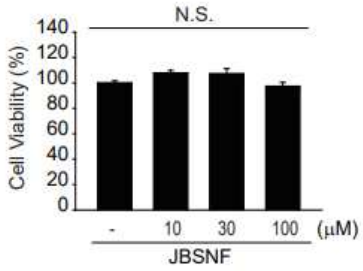
To investigate the impact of NNMT on liver fibrosis, we used an NNMT inhibitor (JBSNF). First, we conducted a toxicity evaluation of JBSNF using MTT assay in LX-2 cells. As a result, there was no cytotoxicity up to 100 μg/mL in LX-2 cells (Figure 4B). Next, we examined liver fibrognenic marker proteins to investigate whether NNMT inhibitor affects liver fibrogenesis. TGF-β-induced PAI-1, a representative fibrosis marker, expression was significantly reduced upon NNMT inhibitor JBSNF in LX-2 cells (Figure 4C). Additionally, the effect of NNMT inhibitor was confirmed with other NNMT inhibitor, MNAM. PAI-1 induced by TGF-β was also decreased by MNAM treatment (Figure 4D). Next, we confirmed the expression of fibronectin and collagen 1, which are fibrosis markers induced by TGF-β. Fibrosis markers increased by TGF-β were significantly reduced when using NNMT inhibitors. (Figure 4E). In addition, NNMT inhibitor inhibited TGF-β-induced Smad2 and Smad3 phosphorylation (Figure 4F). These results indicate that NNMT is increased by TGF-β in HSC, and NNMT inhibition can control liver fibrosis through inhibition of Smad2 and Smad3 phosphorylation.

A)

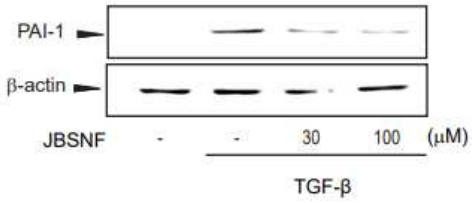


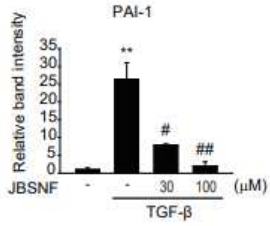
JBSNF

B)



C)





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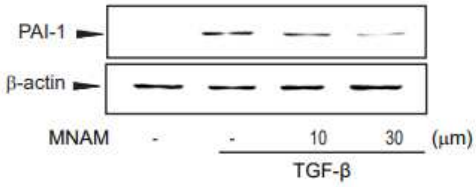

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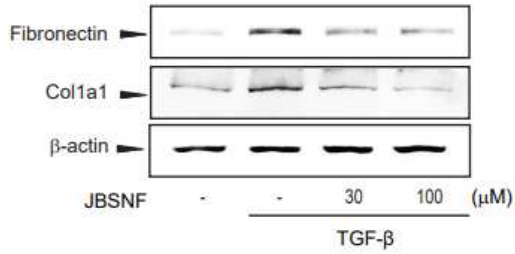

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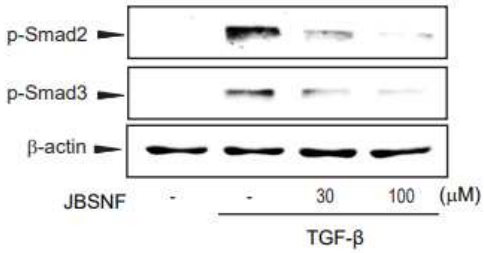


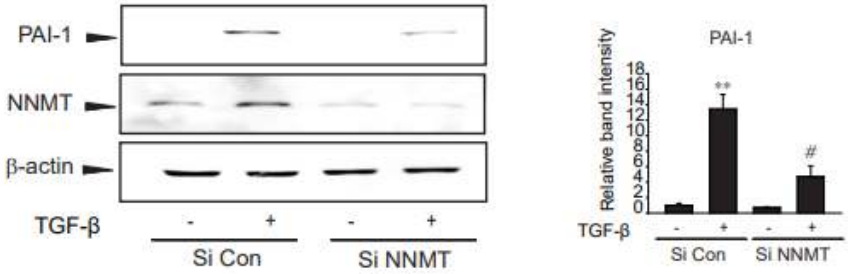
Figure 4. Effect of NNMT inhibitor on HSC activation and hepatic fibrogenesis

(A) Molecular structure of JBSNF. (B) Cytotoxicity evaluation of NNMT inhibitor (JBSNF). LX-2 cells were treated with JBSNF for 12 h, and then cell viability was assessed using with a 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) assay. Data represent means ± S.E. of three replicates. (C) The effect of JBSNF on expression of PAI-1. The cells were treated with JBSNF (30, 100 μM) in the presence or absence of TGF-β (2 ng/mL). (D) The effect of MNAM on PAI-1 expression. The cells were treated with MNAM (10, 30 μM) in the presence or absence of TGF-β (2 ng/mL). (E) Fibrogenic markers were assessed by immunoblotting. (F) The effect of JBSNF on TGF-β-induced Smad phosphorylation. The cells were treated with JBSNF (30, 100 μM) in the presence or absence of TGF-β (2 ng/mL) for 30min. p-Smad2 and p-Smad3 were detected by immunoblotting.

5. The role of NNMT on liver fibrogenesis

To further verify the effect of NNMT in liver fibrogenesis, we transfected with siRNA against NNMT and NNMT overexpression plasmids. LX-2 cells were transfected with SiNNMT with or without TGF-β1. The level of PAI was increased upon TGF- β1 treatment, but the PAI expression level was significantly decreased by treatment with SiNNMT (Figure 5A). Fibronectin and collagen, markers of liver fibrogenesis increased by TGF-β, were also decreased in SiNNMT transfected cells (Figure 5B). In contrast, TGF-β1-mediated PAI-1 expression level was markedly increased by NNMT (achieved using NNMT overexpression plasmid) (Figure 5C). Additionally, the increased p-Smad3 level upon TGF-β treatment was suppressed by NNMT (Figure 5D). Collectively, these results suggest that NNMT may be involved in hepatic stellate cell activation and liver fibrogenesis.

A)



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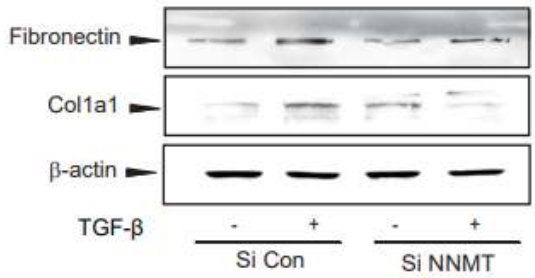

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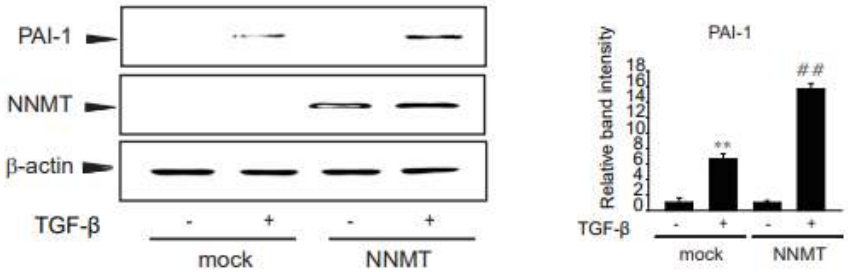

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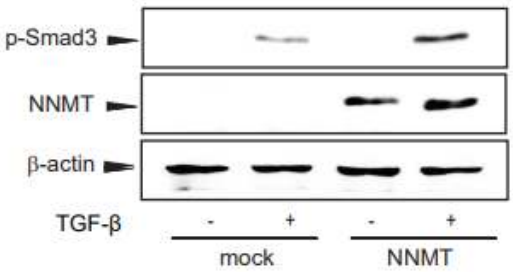

Figure 5. Effect of NNMT knockdown or overexpression on hepatic fibrogenesis

(A) The effect of NNMT knockdown on TGF-β-induced PAI-1. LX-2 cells were transfected with control siRNA (SiCon) or NNMT siRNA (SiNNMT) for 24 h and then treated with TGF-β (2 ng/mL) for 6 h. (B) The effect of NNMT knockdown on TGF-β-induced fibronectin and collagen. LX-2 cells were transfected with control siRNA (SiCon) or NNMT siRNA (Si NNMT) for 24 h and then treated with TGF-β (2 ng/mL) for 12 h. (C) The effect of NNMT overexpression on TGF-β-mediated PAI-1. LX-2 cells were transfected with NNMT or pCMV4 (MOCK) for 24 h and then cultivated with TGF-β (2 ng/mL) for 6 h. (D) The effect of NNMT on TGF-β-induced Smad phosphorylation. LX-2 cells were transfected with NNMT or pCMV4 (MOCK) for 24 h and then cultivated with TGF- β (2 ng/mL) for 30min.

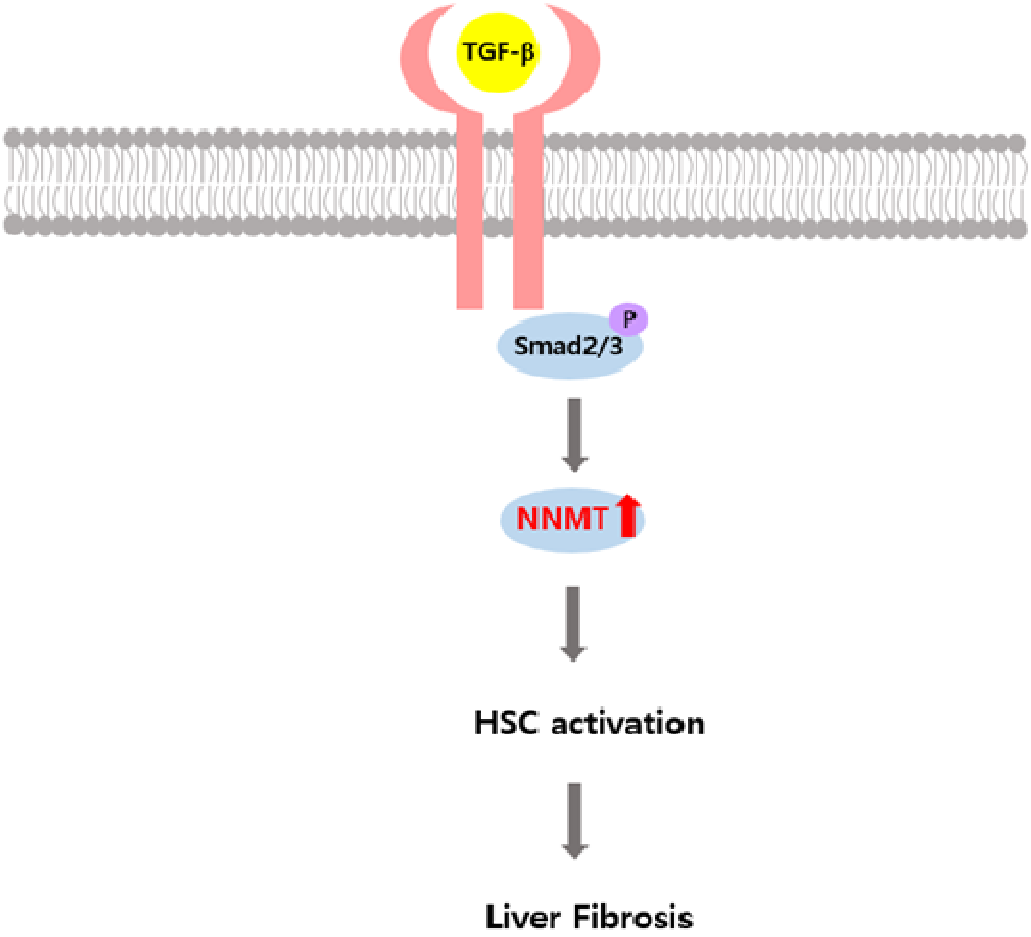

Figure 6.

Schematic diagram showing the role of NNMT for hepatic stellate cell activation and liver fibrosis.

IV. DISCUSSION

Hepatic stellate cells (HSCs) activation is important in the progression of liver fibrosis [3, 5]. TGF-β cytokine, which is considered important for ECM production, activates HSCs and regulates the expression of ECM-related genes, contributing to the progression of liver fibrosis [8, 28]. Recently, the expression of nicotinamide methyl metabolites has increased in patients with cirrhosis [19, 29] and the role of NNMT in various liver diseases has been revealed [21, 30, 31], but there is no study on the expression and molecular mechanism of NNMT in liver fibrosis. Therefore, we revealed the role of TGF-β-mediated NNMT in HSCs activation and liver fibrosis. When HSCs were isolated and activated from the mouse, the expression of NNMT was increased (Figure 1D). In addition, when LX-2 cells, a hepatic stellate cell line, were treated with TGF-β, the expression of NNMT protein was increased in a time- and concentration-dependent manner (Figure 2A and B), and NNMT mRNA expression also increased (Figure 2C and D). And it was proven to be regulated at the transcriptional level through ActD treatment (Figure 2E). Next, we investigated the effect of TGF-β-mediated NNMT on HSCs activation. HSC activation marker, and fibrosis marker, which were increased by TGF-β, decreased upon treatment with NNMT inhibitor (Figure 4C and E). It was speculated that this effect of NNMT inhibitors occurs through Smad inhibition (Figure 4F).

NNMT is specifically expressed in large quantities in the liver [15, 32, 33], and is also expressed to a small extent in various tissues such as adipose tissue[34], breast[35], and gallbladder [36]. We demonstrated that it is expressed more in HSCs, which are non-parenchymal cells, than in hepatocytes, which are parenchymal cells among liver cells (Figure 1 C). Our results indicate that NNMT, which is specifically abundant in the liver, plays a more important role in HSCs than in hepatocytes. Moreover, we found that NNMT expression level in HSCs is much higher than parenchymal hepatocytes. These results implicated that NNMT might play an essential role in hepatic fibrogenesis.

Next, we found that the TGF-β-mediated NNMT expression was regulated by the Smad signaling pathway using Smad overexpression or Smad inhibitor (Figure 3B and C). However, the direct relationship between Smad and NNMT needs to be elucidated in further studies. Additional studies should be conducted to investigate the presence of a Smad binding element (SBE) in the NNMT promoter region and direct binding of Samd in the SBE from NNMT through chromatin immunoprecipitation (ChIP) analysis. Furthermore, we plan to examine whether non-canonical pathway including MAPK [37], which is a Smad-independent pathway activated by TGF-β, is related to the regulation of NNMT expression.

It was well established that NNMT metabolites were changed by NNMT hyperactivity [38]. We thus investigated the effect NNMT substrate and its metabolites (SAM, SAH, NAM, and MNAM) on HSC activation (data not shown). Treatment of LX-2 cells with NAM and SAH had no effect on the expression of PAI-1 by TGF-β. SAM serves as a methyl donor for several methyltransferases, and the ratio of SAM plays an important role in methionine cycle balance [39, 40]. We confirmed the effect exogenous SAM on HSC activation and TGF-β-mediated PAI-1 expression was decreased by SAM treatment. These results support that the decreased levels of SAM and methionine imbalance due to NNMT hyperactivity are the result of restoration of methionine balance upon provision of exogenous SAM [41, 42].

MNAM, a metabolite of NNMT, acts as a negative feedback inhibitor and inhibits NNMT when MNAM increases excessively due to excessive activity of NNMT [43, 44]. MNAM is commonly used as an NNMT inhibitor. JBSNF, a nicotinamide analogue, acts on the same mechanism as MNAM to inhibit NNMT. JBSNF inhibits NNMT specifically and with high sensitivity[44]. Our results clearly showed that both NNMT inhibitors have ability to abolish PAI-1 increases by TGF-β (Figure 4C). In addition, we can obtain the similar results which were shown when knockdown and overexpression of NNMT (Figure 5). However, these results should be confirmed *in vivo*. Currently, *in vivo* efficacy studies are being conducted in a mouse model of liver fibrosis using NNMT inhibitor JBSNF. Additionally, we recently generated NNMT knockout mice to reveal the molecular mechanism of the NNMT using *in vivo*.

In conclusion, our results demonstrated that TGF-β-induced NNMT increase is transcriptionally regulated through the Smad signaling pathway. NNMT induction by TGF-β causes HSC activation and hepatic fibrogenesis. However, NNMT inhibition has the antagonistic effect of TGF-β-mediated HSC activation. These findings suggest that NNMT acts as a novel promising target for the prevention and treatment of liver fibrosis.

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