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석사학위 논문

The role of FoxO3a on the TGF-β-mediated hepatic fibrogenesis

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The role of FoxO3a on the TGF-β-mediated hepatic fibrogenesis

간섬유화 치료 표적으로써 FoxO3a 발현 및 기능 조절 연구

2018 년 2 월 24 일

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ABBREVIATIONS

ActD	Actinomycin-D
AMPK	AMP-activated protein kinase
α-SMA	α-smooth muscle actin
CHX	Cycloheximide
CK1	Casein kinase-1
CQ	Chloroquine
ECM	Extracellular matrix
ERK	Extracellular signal-regulated kinase
FoxO	Forkhead box O
HSCs	Hepatic stellate cells
IKK	I kappa B kinase
JNK	c-Jun N-terminal kinase
MG132	Z-Leu-Leu-Leu-al
MST1	Mammalian sterile 20-like kinase
PAI-1	Plasminogen activator inhibitor 1
PI3K	Phosphoinositiol-4,5-bisphosphate 3-kinase
SBE	Smad binding element
SGK1	serum and glucocorticoid-regulated kinase-1
TGF-β	Transforming growth factor-β

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국 문 초 록

간섬유화 치료 표적으로써
FoxO3a 발현 및 기능 조절 연구

김 승 정

지 도 교 수: 기 성 환

약학과

조선대학교 대학원

간섬유화는 바이러스, 독성물질에 만성적 노출 시 진행되는 상처 회복 (wound healing) 과정을 통해 간 내부에 콜라겐, 파이브로넥틴으로 구성 된 세포 외 기질 (ECM)의 과도한 증식으로 발병하는 대사질환으로 알려져 있다. 이러한 간섬유화 과정은 간세포 내 비실질세포 중 하나인 간성상세포에서 주요하게 진행된다. 간성상세포는 정상 간에서는 휴지 상태 (quiescent state)로 존재하지만, 만성 간손상 상황에서는 활성 상태 (activation state)로 분화하며 간섬유화 진행에 주요한 역할을 담당한다. 이러한 간성상세포 활성화에 관여하는 주요 사이토카인 (cytokine)으로써 TGF-β가 보고되어 있으며, 하위 신호전달 인자로써 Smad 신호가 알려져 있다. FoxO3a 는 세포 내 여러 스트레스에 반응하여 세포 분화, 증식,

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수명에 관여하는 전사인자로 알려져 있으나, 현재까지 간섬유화 진행과정 중 간성상세포 활성화에 관한 신호전달 기전에서 FoxO3a 의 발현 및 조절기전에 대해 연구되어 있지 않다. 우리는 간섬유화 환자의 조직에서 FoxO3a 의 발현량이 증가됨을 관찰하였다. 또한 마우스 간성상세포와 HSC-T6, LX-2 세포주에서도 TGF-β 처리 시 FoxO3a 의 발현량이 상향 조절됨을 보였다. TGF-β에 의한 FoxO3a 의 발현 증가는 전사적으로 조절되었으며 또한 단백질 안정성에 의해서도 영향을 받았다. 프로테아좀 (proteasome) 억제제인 MG132 를 처리 한 결과 FoxO3a 의 축적이 관찰되었으나 라이소좀 (lysosome) 억제제인 chloroquine 처리 시 유의한 변화가 없었음을 통하여 FoxO3a 가 프로테아좀 의존적으로 분해됨을 확인하였다. FoxO3a 가 간성상세포 활성화에 영향을 줄 수 있는지 알아보기 위해, FoxO3a 를 세포 내 과발현 한 후 Smad 인산화 및 reporter 유전자를 활용하여 전사적 활성화를 관찰한 결과 Smad 의 인산화 및 reporter 활성이 증가하였다. 더 나아가 FoxO3a 를 과발현 한 결과, TGF-β에 의해 증가 된 간섬유화 관련 유전자인 PAI-1 발현량이 더욱 증가되었으며 역으로 FoxO3a siRNA 를 이용하여 세포 내 FoxO3a 발현량을 감소시킨 결과 TGF-β에 의해 증가 된 PAI-1 발현량이 감소됨을 관찰하였다. 본 연구에서는 TGF-β에 의해 매개되는 간섬유화 발병 과정 중 FoxO3a 의 발현 변화 및 역할에 대해 고찰하였다. 상기의 결과로, TGF-β에 의해 유도되는 간성상세포 활성화를 통한 간섬유화 과정의 새로운 치료 표적으로 FoxO3a 를 제시한다.

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I. INTRODUCTION

Liver fibrosis is a major disorder and fatal disease worldwide caused by chronic viral, toxins and metabolic autoimmune hepatitis [1]. It defined as development of excess fibrous connective tissue in liver, which process during regenerating the extracellular matrix (ECM) composed of collagen fibers and fibronectin, etc [2]. Upon repeated injury, the liver goes through a wound-healing response, accumulating the excessive deposition ECM and impaired organ function such as fibrosis [3]. Transdifferentiation of hepatic stellate cells is the major cellular mediator of matrix protein-secreting myofibroblasts, the major driver of liver fibrogenesis [4].

Transforming growth factor-β (TGF-β) is a key cytokine that transmits cytostatic signals to epithelial, neuronal, and immune cells [5]. TGF-β is the most potent profibrogenic cytokine which can activate hepatic stellate cells (HSCs), which is the main producer of ECM in the liver, and is regulated by proinflammatory mediators such as cytokines that act on HSCs and liver immune cells [5, 6]. Molecular biological mechanism of TGF-β has been reported that activates a membrane receptor serine/threonine kinase complex which phosphorylates Smad2 and Smad3 [7], which triggers phenotypical HSCs transdifferentiation by paracrine and autocrine action, and induces collagen I (Col I) expression and α-smooth muscle actin (α-SMA) stress fiber organization [8]. TGF-β/Smad pathway is the most extensively investigated molecular mechanism in hepatic fibrogenesis [9]. Synergistic cooperation between Smad3 and other transcription factors can mediate the stimulatory effect of TGF-β on transcription of collagen in activated HSCs [9]. TGF-β is a potent inhibitor of ECM degradation via plasminogen activator inhibitor-1 (PAI-1) expression, which results ECM accumulation in

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ventral undersurface region of renal epithelial cells [10].

Forkhead box O (FoxO) transcription factors are one of the important stress response factor that is in charge of many regulatory events. The first discover of forkhead region was in terminal region of *drosophila melanogaster* [11]. And FoxO subfamily is conserved from *Caenorhabditis elegans* to mammals [12]. FoxOs in mammals are comprised in four FoxO subfamilies: FoxO1 (FKHR), FoxO3 (FKHRL1), FoxO4 (AFX) and FoxO6 [13]. While FoxO6 is specifically distributed to neurons, the others are widely distributed and are present ubiquitously in liver, heart, brain, lung, or else [14]. The role of three factors including FoxO1, 3, 4 was overlap in their transcriptional targets [15]. Among FoxOs, FoxO3a has several activities for plasticity of the organ, adaptation to fasting, resistance to stress, and regulation of cell proliferation, differentiation, apoptosis and longevity after transcriptional activation by nuclear translocation [14-16]. There is a study that FoxO3a activity was increased by starvation/malnutrition in hepatitis C virus infection which caused an increased expression of SOCS3 and a suppression of the interferon signaling pathway [17]. In addition, there are previous studies of FoxO3a shown that integrates profibrogenic signaling in pulmonary fibrosis [18, 19]. The major molecular mechanism of FoxO3a has been reported in phosphorylation by Akt/Phosphoinositiol-4,5-bisphosphate 3-kinase (PI3K) pathway [20]. In addition, FoxO3a act in binding with transcription factor of Smad3 effector of the TGF-β pathway [21].

In progression of liver fibrosis, TGF-β signaling pathway in HSCs is well established for the most important process of liver fibrogenesis. However, the role of FoxO3a on TGF-β signaling in HSCs activation remains elusive. Moreover, many of studies focused phosphorylation of FoxO3a/AKT signaling, but there is no report for the expression level of FoxO3a itself in TGF-β mediated liver fibrogenesis. In this study, we performed the experiments for define the role of FoxO3a in the TGF-β-mediated HSCs activation. First, we

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examined the expression level of FoxO3a in human samples suffered with liver fibrosis or HSCs cell lines. Then we investigated the regulation of FoxO3a expression on transcriptional or post-transcriptional mechanism. In sequence, we verified the role of up-regulated FoxO3a in HSCs activation and liver fibrogenesis.

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II. MATERIALS AND METHODS

1. Materials

The antibodies against FoxO3a, Phospho-Smad3 and Smad2/3, Lamin antibodies were supplied from Cell Signaling Technology (Danvers, MA, USA). Tubulin antibody was obtained from Santa Cruz (Dallas, TX, USA) Plasminogen activator inhibitor 1 (PAI-1) antibody was purchased from BD Bioscience (San Jose, CA, USA). Secondary antibodies against anti-rabbit and anti-mouse which were conjugated horseradish peroxidase were purchased from Invitrogen (Carlsbad, CA, USA). β-actin, cycloheximide (CHX), Z-Leu-Leu-Leu-al (MG132), chloroquine (CQ), and actinomycin D (ActD) were obtained from Sigma (St. Louis, MO, USA). TGF-β was purchased from R&D Systems (Minneapolis, MN, USA).

2. Cell culture

LX-2 cells (immortalized human semi-activated HSCs) were generously provided by Dr. S.L. Friedmann (Mount Sinai School of Medicine, NY, USA). HSC-T6 Cells were purchased from Merck Millipore (Darmstadt, Germany). Cells were plated in 60 mm or 6-well (Thermo Scientific, Waltham, MA, USA) plates at $1X10^5$ cells per well, and cells were grown to 70-80% confluence. Cells were maintained in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum (FBS, Atlas Biologicals, Fort Collins, CO, USA), 50 U/mL penicillin/streptomycin at 37℃ in an atmosphere of humidified 5% CO_2. Cells were then washed cold phosphate buffered saline, before sample preparation.

3. Isolation of Hepatic Stellate Cells

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HSCs were isolated from the 8-week-old ICR mice liver (Oriental Bio, Sungnam, South Korea) as previously reported [22-24]. After intubation in the portal vein, the livers were perfused *in situ* with Ca^{2+}-free Hank's balanced saline solution at 37℃ for 15 min and then perfused with solution containing 0.05% collagenase and Ca^{2+} for 15 min, at a flow rate of 10 mL/min. The perfused livers were minced, filtered through 70 μm cell strainer (BD Bioscience), and centrifuged at 50 *g* for 3 min to separate the supernatant and pellet. The pellet was then discarded. After that, the supernatant was further centrifuged at 500 *g* for 10 min, resuspended in Ficoll plus Percoll (1:10, GE Healthcare, IL, USA), and centrifuged at 1400 *g* for 17 min. HSCs were collected from the interface.

4. Patient samples

Human fibrotic or non-fibrotic liver samples obtained from 10 cancer patients were separated by a histologic examination and ultrosonography at Chosun University Hospital in South Korea, as used in the previous study [25]. The study protocol was approved by the institutional review board of Chosun Medical Center (#2013-04-005).

5. Immunoblot analysis

Protein extraction, SDS polyacrylamide gel electrophoresis, and immunoblot analysis were performed as previously reported [26]. Briefly, the cell lysates were separated by electrophoresis in 7.5% and 12% gels and electrophoretically transferred to a nitrocellulose membrane (GE Healthcare, IL, USA). The nitrocellulose membrane was incubated with the indicated primary antibody and then incubated with a horseradish peroxidase-conjugated

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secondary antibody (Invitrogen, San Diego, CA, USA). Immunoreactive protein was visualized by enhanced chemiluminescence detection kit (Amersham Biosciences, Buckinghamshire, UK). Equal protein loading was verified using β-actin, lamin or tubulin.

6. RNA isolation and Quantitative RT-PCR analysis

Total RNA was extracted using TRIzol (Invitrogen) according to the manufacturer's instructions. The RNA (2 μg each) was reverse-transcribed using an oligo-d(T)18 primers to obtain cDNA. The cDNA was obtained amplified using a high-capacity cDNA synthesis kit (Bioneer, Daejeon, Korea) with a thermal cycler (Bio-Rad, Hercules, CA, USA). Amplified products were separated by using 2% agarose gel, stained with ethidium bromide (Sigma, St. Louis, MO, USA), and visualized in gel documentation system (Fujifilm, Tokyo, Japan). Quantitative PCR was performed with StepOne system (Applied Biosystems, Foster City, CA, USA) using SYBR green premix (Applied Biosystems). The relative PCR products levels were determined which were based on threshold cycle value. A melting curve analysis was done after amplification to verify the accuracy of the amplicon. Primer sequences were as follows: human FoxO3a sense 5′-GCAAGCACAGAGTTGGATGA-3′, and antisense 5′-CAGGTCGTCCATGAGGTTTT-3′; human GAPDH sense 5′-GAAGGTGAAGGTCGGAGTC-3′, and antisense 5′-GAAGATGGTGATGGGATTTC-3′ mRNA expression data was normalized to GAPDH.

7. Plasmid construction and luciferase assay

Cells were plated in 12-well plates overnight, serum starved for 4 h, and transiently transfected with SBE luciferase plasmid, pRL-TK plasmid (a plasmid that encodes Renilla luciferase and is

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used to normalize transfection efficacy) and FoxO3a or MOCK plasmid in the presence of Lipofectamine™2000 (Invitrogen) for 3 h. Transfected cells were allowed to recover in MEM with 1% FBS overnight and then exposed to TGF-β for 12 h. The firefly and Renilla luciferase activities of cell lysates were measured using the dual luciferase assay system (Promega, Madison, WI, USA) according to the manufacturer's instructions. Relative luciferase activities were measured by normalizing firefly luciferase versus that of Renilla luciferase.

8. siRNA knockdown experiment

Plasmids encoding FoxO3a was purchased from Addgene (Cambridge, MA, USA). LX-2 cells were transfected with pECE (MOCK), Flag-FoxO3a WT (FoxO3a) for 24 h using Lipofectamine™2000 (Invitrogen) according to the manufacturer's instructions. For gene silencing, cells were transfected with non-targeting control siRNA (100 pmol) or siRNA directed against FoxO3a (100 pmol) (Dharmacon, Lafatette, CO, USA) for 48 h using Lipofectamine™2000. The cells were transfected with a plasmid using Lipofectamine™2000 in Eagle's minimum essential medium (MEM) for 12 h. The medium was then changed to MEM containing 1% FBS, and the cells were further incubated for 24 h with or without TGF-β treatment for 12 h.

9. Confocal microscopy

Briefly, primary HSCs were grown on a coverslip and fixed in a 4% paraformaldehyde solution, followed by permeabilization with 0.1% Triton X-100. The cell samples were immunostained with antibodies directed against FoxO3a, overnight, followed by incubation with Alexa Fluor® 488 goat anti-rabbit IgG (Invitrogen). Tissue sections were deparaffinized and incubated with

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antibodies of FoxO3a and desmin, at 37°C, overnight and 4 h, respectively, followed by incubation with Alexa Fluor 594® goat anti-rabbit IgG (Biolegend, San Diego, CA, USA) or Alexa Fluor® 488 goat anti-rabbit IgG (Biolegend) at 37°C for 3 h. After incubation, the samples were coverslipped with mounting media. The samples were examined using a laser-scanning confocal microscope (A1, Nikon instruments Inc., NY, USA).

10. Statistical analysis

One-way ANOVA was used to determine significant differences between treatment groups. The Newman-Keuls test was used to determine the significance of the differences between the means of multiple groups. Results are expressed as mean ± standard Error (SE).

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III. RESULTS

1. Upregulation of FoxO3a on fibrotic conditions in HSCs and liver

First, we detected FoxO3a and desmin using confocal microscopy imaging. In human liver tissue was examined in the human fibrotic and adjacent normal tissue samples to determine biological significance of FoxO3a between fibrotic and normal condition. The expression of FoxO3a was observed higher in cirrhotic liver patients' sinusoidal nodule, where abundantly contains HSC (Fig 1A). Then we conducted the experiment for expression level of FoxO3a in murine primary HSC (pHSC). We isolated fresh primary HSC (pHSC) from mice liver to plate the pHSC in culture dish, then treated with TGF-β (2 ng/mL) for 12 h in quiescent state (not activated, 0 day). The FoxO3a level in pHSC was upregulated by TGF-β treatment (Fig 1B). To test the FoxO3a expression level in different types of cells, human immortalized HSC cell line, LX-2 cells and murine HSC cell line, HSC-T6 cells were used. The FoxO3a expression was increased by TGF-β treatment (2 ng/mL) in time-dependent manner, also dose-dependent manner in LX-2 or HSC-T6 cells (Fig 1C). These data suggest that expression level of FoxO3a was upregulated in hepatic fibrogenesis condition.

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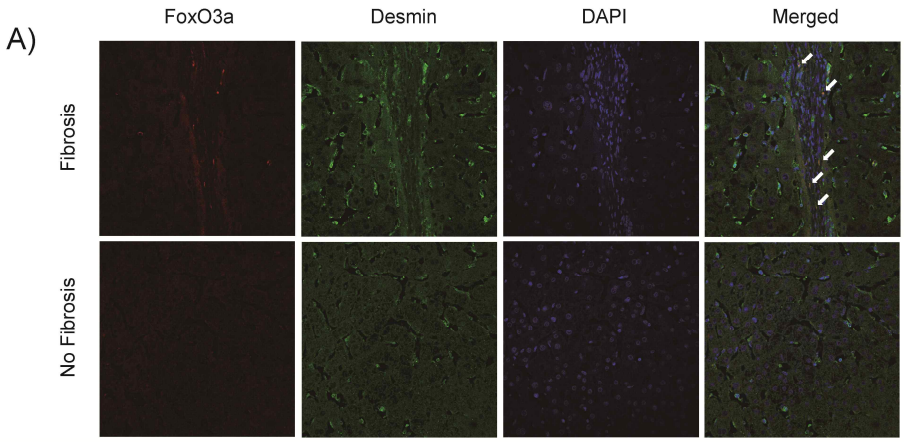

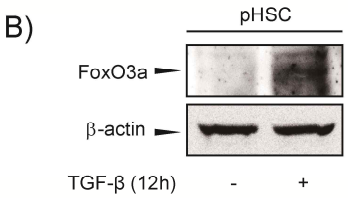

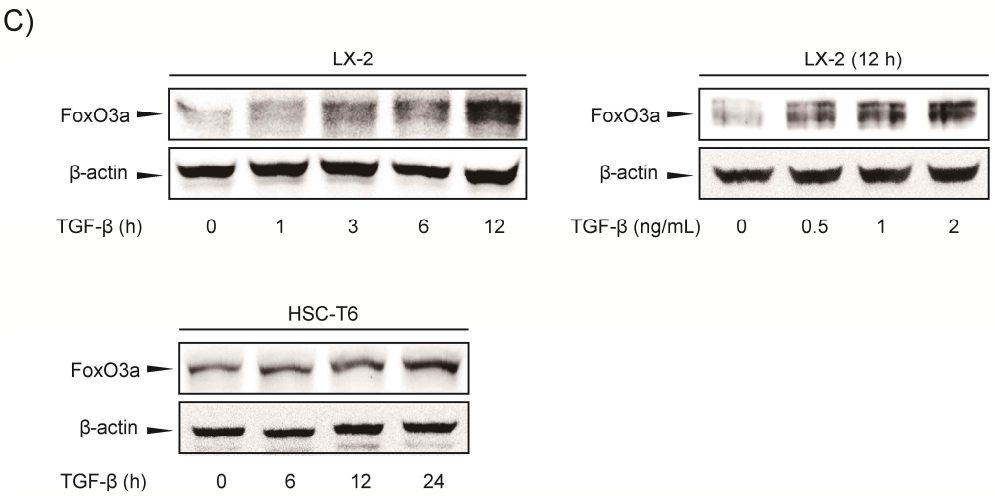

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Figure 1. Expression levels of FoxO3a in liver fibrosis patient's tissue and HSCs cell lines by treatment with TGF-β.

(A) Immunostaining in fibrotic liver patients with FoxO3a and desmin (magnification: 400X). White arrows indicate colocalization of FoxO3a and desmin. (B) Expression level of FoxO3a in primary hepatic stellate cells (pHSCs) by treatment of 2 ng/mL TGF-β. Prior to TGF-β treatment, serum starvation was conducted for 3 h. β-actin was used as an internal control. (C) Expression levels of FoxO3a by 2 ng/mL TGF-β treatment with LX-2 cells in time- or dose-dependent manner. Serum starvation was conducted for 12 h before TGF-β treatment. β-actin was used as a loading control.

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2. Localization of FoxO3a in TGF-β treated LX-2 cells

To examine the cellular localization of FoxO3a, we obtained subcellular fraction to determine the localization of FoxO3a by TGF-β treatment. Treatment with TGF-β, cytosolic FoxO3a levels in LX-2 cells were gradually increased in a time-dependent manner. At the same way, nuclear FoxO3a levels were also increased in a time-dependent manner under long exposure (Fig 2A). We carried out confocal microscopy imaging analysis to detect FoxO3a in TGF-β treated LX-2 cells. FoxO3a positive cells were increased by TGF-β treatment in both nuclear and cytoplasm of cells. These results indicate that the expression of FoxO3a was induced by TGF-β treatment, and it was abundantly localized in cytoplasm.

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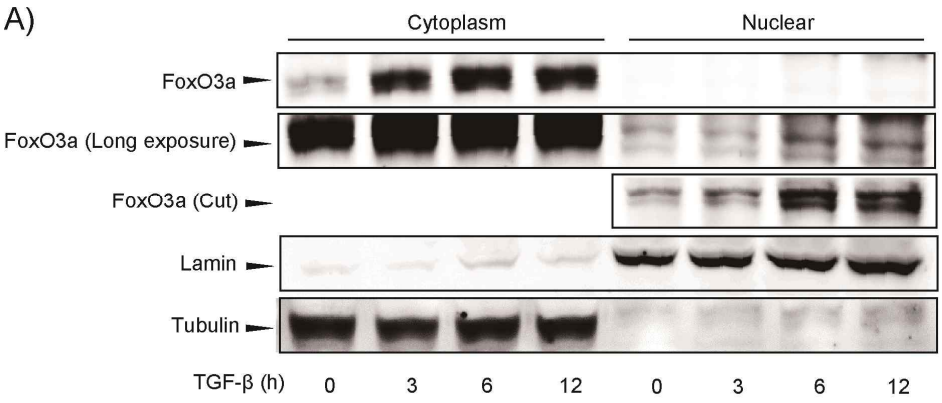

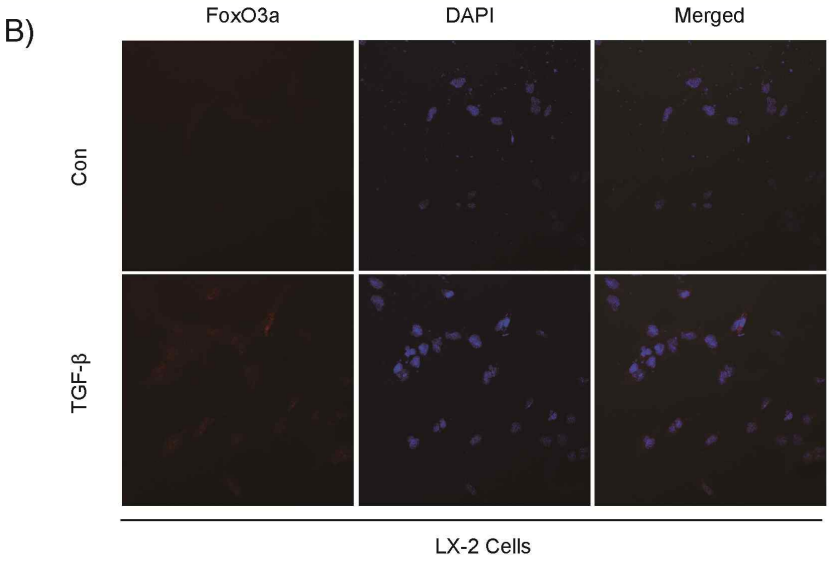

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Figure 2. Changes in FoxO3a localization in LX-2 cells by treatment of TGF-β

(A) Immunoblotting of FoxO3a in subfractionation by treatment of 2 ng/mL TGF-β in LX-2 cells. Tubulin or lamin was used as marker of cytosolic or nuclear fraction, respectively. The expression level of FoxO3a was determined. (B) Immunostaining of FoxO3a in 2 ng/mL TGF-β treated LX-2 cells were detected by confocal microscopy imaging.

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3. Transcriptional regulation of TGF-β-induced FoxO3a

To verify whether foxo3a was regulated in a transcriptional level, we examined the FoxO3a mRNA level in TGF-β-treated LX-2 cells. After 3 h treatment, the mRNA level of FoxO3a was increased to peak by TGF-β. The FoxO3a then gradually decreased up to 12 h treatment (Fig 3A). The effect of actinomycin D (ActD), a representative transcription inhibitor, on the FoxO3a expression was monitored in the presence or absence of TGF-β treatment for 12 h. TGF-β increased FoxO3a expression in absence of ActD, which was attenuated by presence of ActD (Fig 3B). Overall, these results shown that FoxO3a up-regulation by TGF-β was transcriptionally regulated.

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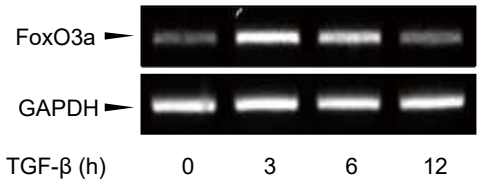

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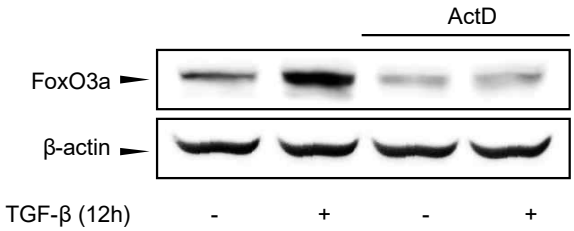

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Figure 3. Transcriptional regulation of FoxO3a expression by TGF-β treatment

(A) RT-PCR analysis. LX-2 cells were treated with 2 ng/mL TGF-β for 3-12 h. mRNA levels of FoxO3a were determined by RT-PCR using GAPDH as an internal control. (B) Effect of actinomycin-D (ActD) on FoxO3a induction by TGF-β in LX-2 cells. The cells were treated with 5 μg/mL of ActD in the presence or absence of TGF-β. The level of FoxO3a expression was determined after TGF-β (2 ng/mL) treatment for 6 h.

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4. Post-transcriptional regulation of TGF-β induced FoxO3a

Next, we investigated whether FoxO3a expression was regulated by post-transcriptional level. LX-2 cells were treated with protein synthesis inhibitor, cycloheximide (CHX) or CHX with TGF-β. Treatment with CHX rapidly decreased FoxO3a expression, however, TGF-β significantly inhibited FoxO3a decay. These result indicate that upregulation of FoxO3a by TGF-β treatment is due to decreased FoxO3a degradation (Fig 4A). Moreover, basal expression of FoxO3a was induced by proteasome inhibitor MG132, but not lysosome inhibitor chloroquine (Fig 4B). Finally, we found that MG132-mediated up-regulation of Foxo3a expression was enhanced by treatment with TGF-β (Fig 4C). These result suggest that FoxO3a degradation in proteasome is blocked by TGF-β treatment.

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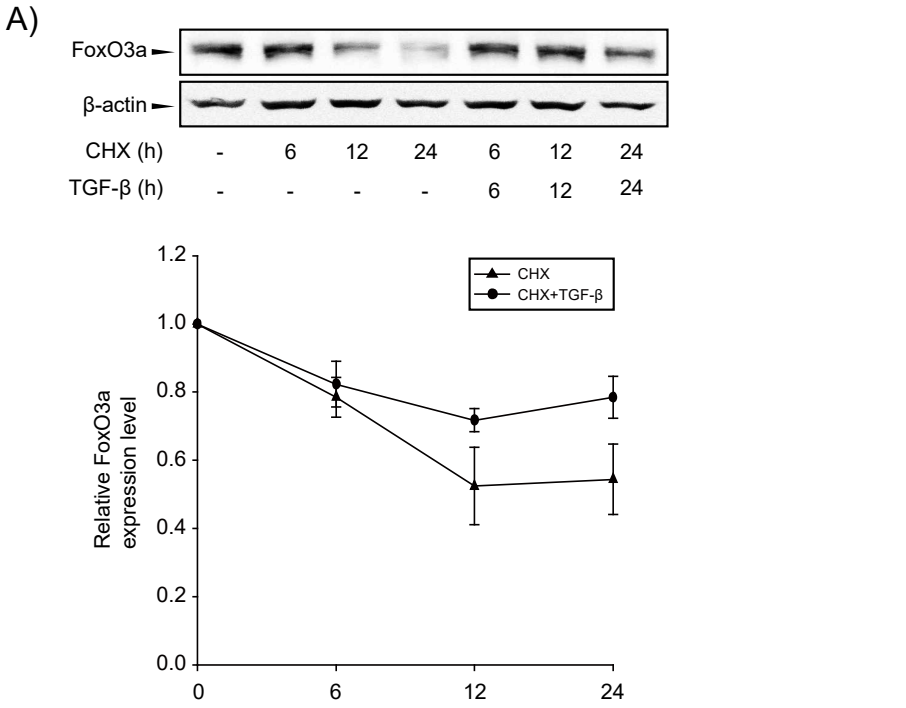

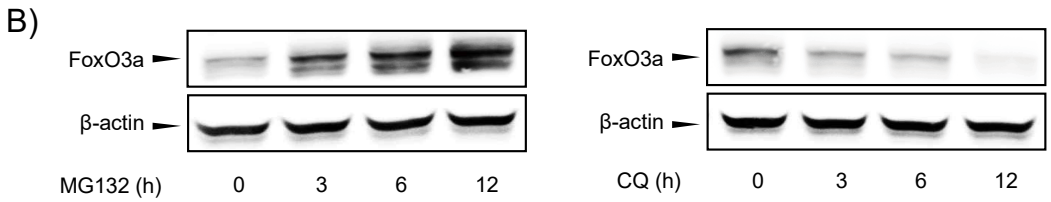

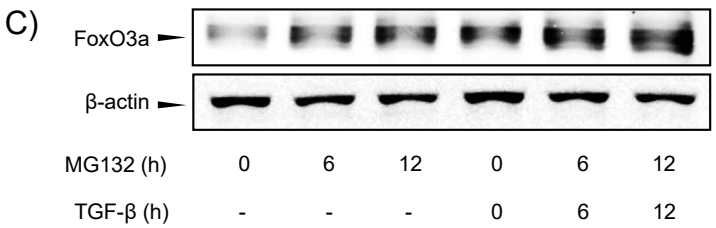

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Figure 4. Post-transcriptional regulation of FoxO3a expression by TGF-β treatment

(A) The effect of cycloheximide (CHX) on FoxO3a expression. LX-2 cells were preincubated with 0.5 μg/mL of CHX in presence or absence with TGF-β treatment. The FoxO3a expression level was measured for quantitation at the indicated time. (B) The effect of proteasome inhibition on FoxO3a upregulation. LX-2 cells were treated with 10 μM of proteasome inhibitor, MG132 or 100 μg/mL of lysosome inhibitor, Chloroquine (CQ). FoxO3a expression was assessed by immunoblotting for the indicated time. (C) Effect of MG132 in TGF-β treated LX-2 cells. LX-2 cells were treated with MG132 prior to TGF-β treatment. The level of FoxO3a was detected by immunoblotting at the indicated time.

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5. Augmentation of TGF-β/Smad signaling pathway by overexpression of FoxO3a

TGF-β/Smad signaling pathway is the most critically investigated cellular mechanism in hepatic fibrogenesis [9]. To test the role of FoxO3a in TGF-β/Smad signaling pathway, FoxO3a was introduced in LX-2 cells. pECE (MOCK)- or FoxO3a-transfected LX-2 cells were treated with or without TGF-β. Smad binding element (SBE)-driven luciferase reporter activity in MOCK- or FoxO3a-transfected cells were significantly increased by TGF-β treatment, and its activity in FoxO3a-transfected cells was significantly enhanced compare to MOCK-transfected cells. Interestingly, the SBE luciferase activity was significantly increased by FoxO3a itself without TGF-β treatment (Fig 5A). Consistently, TGF-β-induced Smad3 phosphorylation was enhanced by FoxO3a overexpression (Fig 5B). These results suggest that FoxO3a can directly upregulate the TGF-β/Smad signaling pathway.

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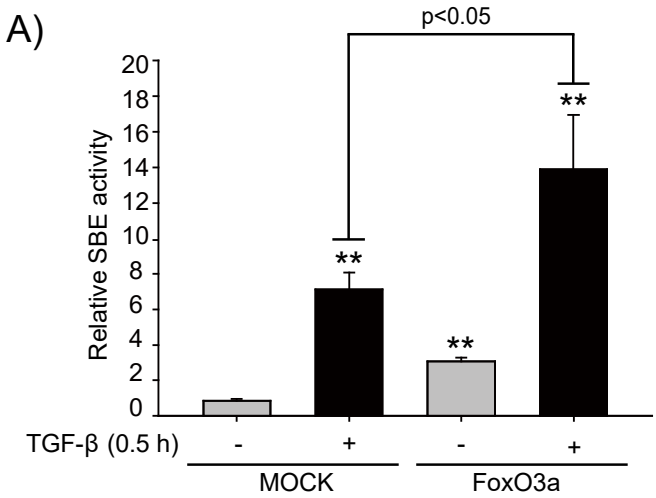

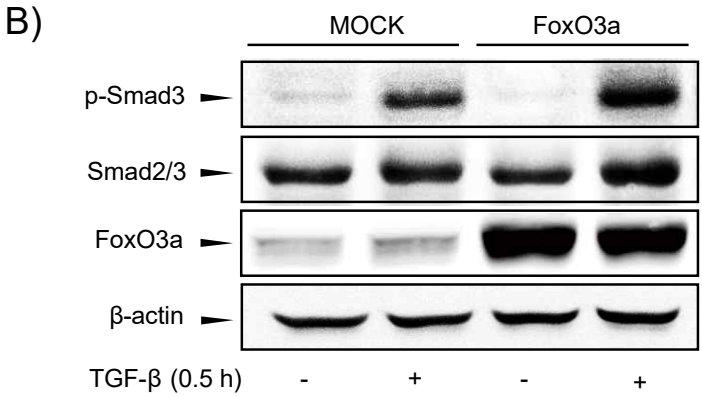

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Figure 5. Effect of FoxO3a on Smad signaling and SBE luciferase activity

(A) Effect of FoxO3a on Smad binding element (SBE) luciferase activity by TGF-β treatment. Cells were transfected with SBE luciferase construct. LX-2 cells were transfected as described in method, then treated with 2 ng/mL TGF-β for 12 h. The data represents the mean ± SE (n = 3, significant when compared with vehicle-treated controls, $^{**}p<0.01$; significant when compared with TGF-β alone). (B) Immunoblotting analysis of TGF-β induced Smad3 phosphorylation. LX-2 cells were transfected by Flag-FoxO3a WT (FoxO3a) then treated with TGF-β for 30 min. Expression level of FoxO3a was confirmed to overexpression. β-actin was used as loading control.

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6. The role of FoxO3a on hepatic fibrogenesis

To determine FoxO3a can regulate the liver fibrogenic gene expression, we detected PAI-1 expression level which is fibrotic marker in HSCs activation. Expression level of PAI-1 by treatment with TGF-β was more increased by FoxO3a overexpression. (Fig 6A). In opposite way, knock down of FoxO3a by siRNA remarkably inhibited TGF-β-induced PAI-1 expression compared to control siRNA (Fig 6B). Our data shown that FoxO3a has a regulatory role in hepatic fibrogenesis through inhibition of fibrogenic gene expression.

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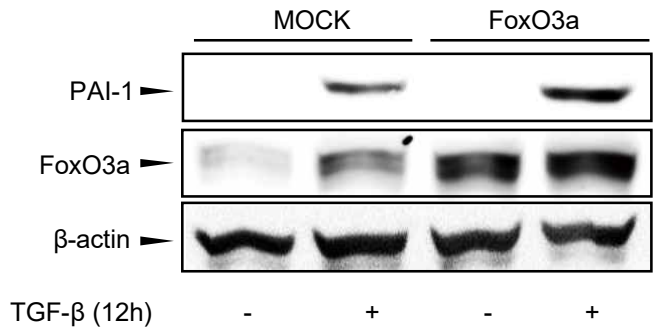

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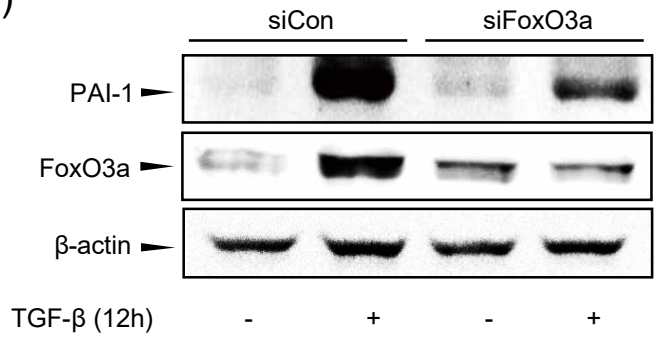

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Figure 6. Effect of FoxO3a on TGF-β induced-fibrogenic gene expression

(A) Effect of FoxO3a on TGF-β induced PAI-1 expression. LX-2 cells were transfected with MOCK or FoxO3a for 24 h and then treated with presence or absence of TGF-β for 12 h. Expression level of PAI-1 was determined by immunoblotting. FoxO3a protein was confirmed by immunoblotting with lysates from MOCK- or FoxO3a-transfected cells. (B) Effect of TGF-β-induced PAI-1 expression by FoxO3a knock down. LX-2 cells were transfected with control siRNA (siCON) or FoxO3a siRNA (siFoxO3a) for 48 h, and then treated with presence or absence of TGF-β for 12 h. Expression levels of FoxO3a was confirmed to knock down. β-actin was used as an internal control.

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7. Schematic diagram

Schematic diagram illustrating the mechanism by which FoxO3a regulates TGF-β-induced liver fibrogenesis.

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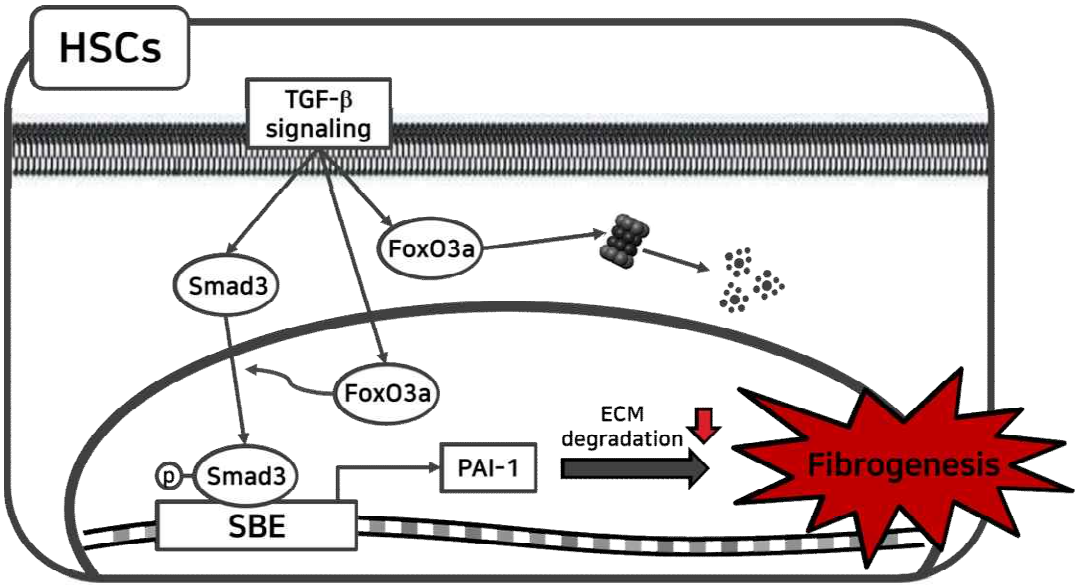

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Figure 7. Schematic diagram

Schematic diagram of shown results.

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IV. DISCUSSION

FoxO3a activity was mainly regulated in several phosphorylation modification including Akt, extracellular signal-regulated kinase (ERK), serum and glucocorticoid-regulated kinase-1 (SGK1), casein kinase-1 (CK1) and I kappa B kinase (IKK), which resulting increase in translocation of FoxO3a into cytoplasm [27-29]. In opposite way, c-Jun N-terminal kinase (JNK), AMP-activated protein kinase (AMPK) and mammalian sterile 20-like kinase (MST1) lead to FoxO3a subcellular location in nucleus for transcriptional activity [28-30]. Interestingly, we demonstrated first time that FoxO3a expression level was increased in liver fibrotic condition and activated HSCs.

Our data has shown that TGF-β-induced FoxO3a was transcriptionally regulated. And FoxO3a expression was also regulated in post-transcriptional ways that identified by treatment with Protein synthesis inhibitor, cycloheximide (CHX) or proteasome inhibitor, MG132 and lysosome inhibitor, chloroquine (CQ). The expression level of FoxO3a in LX-2 cells was quickly decreased by CHX, however, treatment with TGF-β markedly inhibited FoxO3a degradation. Furthermore, FoxO3a was accumulated by MG132 treatment, but not was CQ. These result proposed that up-regulated FoxO3a by TGF-β treatment was modulated by ubiquitin-proteasome system. Mouse double minute 2 (mdm2), which was reported as E3 ubiquitin ligase for p53 [31], promotes FoxO3a ubiquitination and degradation [32]. Thus, we tried to test the binding affinity between mdm2 and FoxO3a in HSCs, but there was no significant change in TGF-β treated HSCs (data not shown). Further study is still required to identify concise molecular mechanism for FoxO3a ubiquitination in HSCs.

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One of the important roles of FoxO3a is to act in regulation of cell proliferation during oxidative stress exposure [33]. We hypothesized the FoxO3a expression induced by TGF-β has an effect to proliferation of HSCs. We investigated cell prolifeation in TGF-β treated LX-2 cells by BrdU incorporation assay and flow cytometry technique. But there was no significant proliferation change was observed by TGF-β treatment in HSCs (data not shown).

TGF-β/Smad signaling has a key role that transmits intracellular signals in liver fibrosis. Our findings that upregulation of Smad signaling pathway by TGF-β treatment enhanced by FoxO3a overexpression, leading to SBE-driven luciferase activity and fibrogenic gene expression including PAI-1. PAI-1 plays a major role in hypofibrinolysis including liver fibrosis [34], which was activated by several factors such as alcohol [34], non-alcoholic steatohepatitis [35] and infection of HCV [36], driving to ECM accumulation which lead to liver fibrosis and progression to liver cirrhosis. Our current data showed that PAI-1 expression was regulated by overexpression or knock-down of FoxO3a.

In conclusion, FoxO3a is transcriptionally and post-transcriptionally regulated in HSCs. Increased FoxO3a enhanced TGF-β/Smad signaling pathway to increase fibrotic gene expression and may participate in HSCs activation. These findings present FoxO3a as a novel biomarker and therapeutic target for liver fibrosis.

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