이학석사 학위논문

TIF1γ restoring vector 를 이용한 간 섬유화 유전자 치료 전략에 관한 연구.

Gene therapy strategy for liver fibrosis using TIF1γ restoring vector.

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서울대학교 융합과학기술대학원

분자의학 및 바이오제약학과

문 도 담

Abstract

Gene therapy strategy for liver fibrosis using TIF1γ restoring vector.

Dodam, Moon
Department of Molecular Medicine and Biopharmaceutical Science and Technology
The Graduate School of Seoul National University

Liver fibrosis is a phenomenon of excessive accumulation of extracellular matrix (ECM) proteins in response to liver damage. Hepatic stellate cells (HSCs) are known to be a group of cells that are activated during liver fibrosis and differentiate into myofibroblasts and play a key role in liver fibrosis. Activated HSCs express alpha smooth muscle actin (αSMA), a myofibroblast marker, secrete ECM proteins such as collagen.

Our team identified a new mechanism of liver fibrosis in previous studies. In normal liver, an anti-fibrotic factor called transcriptional intermediary factor 1 gamma (TIF1γ)

suppresses αSMA expression to inactivate in HSCs. However, during liver fibrosis, TGF(transforming growth factor)β1 decreases TIF1γ expression and increases αSMA expression, which activate HSCs. Activated HSCs overexpresses ECM proteins such as collagen and eventually causes liver fibrosis.

My hypothesis is that restoring the lowered TIF1γ expression of activated HSCs would inhibit HSCs activation and result in liver fibrosis mitigation. The TGFβ promoter−TIF1γ vector (pTGFβ−TIF1γ) was constructed to restore TIF1γ expression in activated HSCs. In vitro experiments were conducted using LX2, human HSCs cell line. As a result, αSMA, activated HSCs marker was reduced when the pTGFβ−TIF1γ vector was transfected. Next, I tried to confirm the effect of pTGFβ−TIF1γ on liver fibrosis in vivo. Before that, I used VitA−coupled liposome as a tool to deliver vectors specifically to HSCs and confirmed that VitA−coupled liposome transferred vectors specifically to HSCs. And I confirmed the fibrosis mitigation effect of pTGFβ−TIF1γ in liver fibrosis model with thioacetamide (TAA).

These results showed the importance of TIF1γ in the HSCs as anti−fibrotic factor and the mitigation effect of pTGFβ−TIF1γ

on liver fibrosis. I think this study verified the possibility of liver fibrosis gene therapy.

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Key words: VitA-coupled liposome, Gene therapy, pTGF β-TIF1γ vector, Hepatic stellate cell; HSCs, Liver fibrosis, TIF1γ (transcriptional intermediary factor 1 gamma), αSMA (alpha smooth muscle actin), aHSCs (activated HSCs-fibrosis state), qHSCs (quiescent HSCs-normal state)

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LIST OF ABBREVIATIONS

HSCs : Hepatic stellate cells.

aHSCs : Activated hepatic stellate cells (Fibrotic state).

qHSCs : Quiescent hepatic stellate cells (Normal state).

TIF1γ : Transcriptional intermediary factor 1 gamma.

CDS : Coding sequence.

αSMA : Alpha smooth muscle actin.

TAA : Thioacetamide.

VitA-coupled liposome: Vitamin A conjugated liposome.

IP injection : Intraperitoneal injection.

IV injection : Intravenous injection.

pTGFβ-TIF1γ : TGFβ promotor + CDS of TIF1γ.

ALR : Augmenter of liver regeneration.

EPLIN : Epithelial protein lost in neoplasm.

MBNL1 : Muscleblind-like protein 1.

Nm23-H1 : Nonmetastatic protein 23, homolog 1.

PIAS1 : Protein inhibitor of activated stat1.

ALT : Alanine aminotransferase.

AST : Aspartate aminotransferase.

INTRODUCTION

Liver fibrosis is excessive accumulation of extracellular matrix protein (ECM) caused by repeated liver damage. [1, 2] Excess accumulated ECM proteins during liver fibrosis destroy the hepatic architecture by forming fibrous scars and inhibit function of liver by suppressing regeneration of hepatocytes. [1, 2] Liver fibrosis further develops into liver failure, which only effective therapy is transplantation. [1, 2] However, many researchers are looking for different therapeutic strategies because of the lack of available liver for transplantation and the side effects of immunosuppressant after transplantation. [3] Recent advanced technologies have identified the cellular mechanisms of liver fibrosis. Among them, HSCs activation is the key mechanism of ECM production during liver fibrosis process.

HSCs were activated by TGFβ, which is increased during liver fibrosis, and is transformed into myofibroblast. Activated HSCs (aHSCs) accelerates liver fibrosis by increasing its proliferation and ECM production [4]

In our previous study, we confirmed that hE-MSCs (Embrionic mesenchymal stem cells) secreted a large amount of HGF (Hepatocyte growth factor), which plays an important role in liver. [5, 6] We performed in vivo experiments to determine whether hE-MSCs is effective on liver fibrosis. Mouse liver slide was stained with MT (Masson' s trichrome) staining. As a result, the liver surface undulation and fibrosis area were significantly decreased in the hE-MSCs injected group.

HSC activation is an event of fibrosis caused by TGFβ, increased pro-fibrotic factors during liver fibrosis. We found anti-fibrotic factors to evaluate the mitigation effect of hE-MSC on liver fibrosis in vitro. First, six anti-fibrotic factors candidate (ALR, EPLIN, MBNL1, Nm23-H1, PLAS1, TIF1γ) were screened by putting the 'negative regulator of TGFβ' in pubmed. Using real-time PCR, three candidates (EPLIN, NM23-H1, TIF1γ) that showed decreased expression level in activation of LX2 cells were selected. Among those factors, TIF1γ was the only factor that decreased when activated by TGFβ and increased again by hE-MSCs. Next, knock down of TIF1γ by si-RNA results in the increased level of αSMA, HSCs activation marker. Furthermore, increased

αSMA was reduced when TIF1r was overexpressed by lentivirus. Based on these results, we derived TIF1γ as anti-fibrotic factor that prevent activation in HSCs.

We have identified the HSCs activation mechanism and found TIF1γ as anti-fibrotic factor through subsequent studies. Briefly, TIF1γ could block HSCs activation by suppressing expression of αSMA in quiescent HSCs (qHSCs) under normal condition. However, when liver injury occurs, expression of TIF1r was reduced by TGFβ in HSCs. And then, expression of αSMA was increased by reducing TIF1γ. As a result, HSCs was activated and liver fibrosis is accelerated.

Based on this mechanism, My hypothesis is that TIF1γ restoration in aHSCs would return to qHSCs and relieve liver fibrosis. In vitro experiments using human hepatic stellate cell line (LX2) and in vivo experiments using mouse liver fibrosis model were performed to verify the hypothesis.

MATERIALS AND METHODS

Animal model

All animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of Seoul National University Hospital, Korea. Male 12-13-week-old BALB/c-nude mice weighing 20-25 g were used for experiments. To induce liver fibrosis, TAA was injected into mice at a 200 mg/kg concentration (Sigma-Aldrich, St. Louis, MO, USA) by intraperitoneal injection. Vector (pTGFβ-GFP, pCMV-GFP, pTGFβ-TIF1γ, pCMV-TIF1γ) was injected into mice by IP injection 1time.

VitA-coupled liposome

-VitA, liposome conjugation

10mM Retinol (R7632, Sigma) 280nmol + 1mM Lipotrust [Cationic liposome] (CSR-LEO-10-EX, Cosmobio) 140nmol per mouse. Then leave for 5 minutes at RT.

-VitA-liposome, Vector conjugation

Vortex vitA-liposome 140nmol with 18ug vector (pTGFβ-GFP, pTGFβ-TIF1γ, pCMV-TIF1γ) per mouse. Then leave for 20 minutes at RT.

-VitA-liposome-vector complex filtering

Add the solution to Vivaspin2 (concentrator, PES column, VS0221, VIVASIENCE) and 1500g centrifugation for 5min at 25℃ 3times.

Reverse the column, then add 100ul of PBS and 3000g centrifugation for 2min at 25℃.

LX2 culture

The human hepatic stellate cell line LX2 was a generous gift from Dr. Friedman [7]. LX2 cells were grown in high-glucose DMEM supplemented with GlutaMax (Gibco, Grand Island, NY, USA), 2% FBS, and 1% (v/v) penicillin/streptomycin (Gibco) (LX2 complete medium) at 37° C in a humidified incubator with

5% CO2.

In order to examine the fibrosis mitigation effect of pTGFβ1-TIF1γ vector in activated HSCs, 10 ng/ml of rhTGFβ1(7754-bh-005, R&D) was pre-treated for 3days and then pTGFβ1-TIF1γ vector was transfected with Fugene HD. (Promega) and rhTGFβ1 was treated for 3days.

Antibodies

Rabbit polyclonal a-TIF1γ (ab84455, Abcam), rabbit polyclonal a-TIF1γ (ab47062, Abcam), mouse monoclonal a-αSMA (A5228; Sigma), rabbit polyclonal a-αSMA (ab5694, Abcam), rabbit polyclonal a-Collagen 1(PA5-29569, Thermo Fisher), rabbit polyclonal a-GAPDH (ab9485, Abcam) and mouse monoclonal a-GAPDH (ma5-15738, Thermo) were used for immunoblot assays.

Immunohistochemistry

Mouse livers were perfused with cold PBS and removed. The livers were fixed in a 4% para-formaldehyde solution (Wako),

embedded in paraffin, and cut into serial sections (4–5−μm thick). Paraffin sections were stained with Masson Trichrome stain (MT stain), Picro−Sirius red using standard protocols. Picro−Sirius red staining was used to detect collagen to visualize connective tissues. Images were obtained using a Leica light microscope (Leica, Wetzlar, Germany). To evaluate the therapeutic effect of pTGFβ−TIF1γ on liver fibrosis, the percentage of fibrotic liver area was estimated by quantitative image analysis of Picro−Sirius red−stained sections using the ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

qRT-PCR analysis

Total RNA was isolated from cultured cells using the Trizol (Thermo Fisher, 15596026) according to the manufacturer' s instructions. cDNA was synthesized from 1ug RNA using Reverse Transcription Master Premix (ELPIS Biotech, EBT−1512, Daejeon, Korea). qPCR was carried out using the FastStart Universal SYBR Green Master (Rox) (Roche, 04 913 914 001, Basel, Swizerland) in an ABI PRISM−7500 sequence

detection system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and protein phosphatase 1 α (*PP1A*) were used as an internal control to calculate relative changes in gene expression. Primers were designed using the Primer blast site (National Center for Biotechnology Information) and synthesized by Macrogen (Seoul, Korea).

The following primers were used:

GAPDH, forward: 5′ -AAGGTCGGAGTCAACGGATTT -3′ ,

reverse: 5′ - GTTCTCAGCCTTGACGGTGC -3′ ;

PP1A: forward 5′ - CATAATGGCACTGGTGGCAAG-3′ ,

reverse: 5′ - GCCATCCAACCACTCAGTCTT-3′ ;

αSMA, forward: 5′ -GGCAAGTGATCACCATCGGA-3′ ,

reverse: 5′ -TCTCCTTCTGCATTCGGTCG-3′ ;

TIF1γ, forward 5′ - CTCCGGGATCATCAGGTTTA -3′ ,

reverse: 5′ - TCAACATGCAAGCACTCCTC -3′ ;

Col1A: forward 5′ - CTGCCGTGACCTCAAGATGT-3′ ,

reverse: 5′ - CCGAACCAGACATGCCTCTT-3′ ;

Western Blot

Total protein was isolated from cells with RIPA cell lysis buffer

(Thermo Fischer), supplemented with complete protease inhibitor cocktail (Genedepot) and Phoshotase inhibitor cocktail (Genedepot). Lysates were incubated on ice for 15min and 13000rpm Centrifugation for 10min at 4℃, Protein concentration was determined by BCA assay (Cat. 23223, Thermo Fisher). Proteins were separated on 10%SDS-PAGE gels and transferred from gel onto polyvinyl difluoride (PVDF) membrane. The membrane was blocked with 5% normal horse serum in PBS and incubated overnight at 4℃ with primary antibodies. Detection was done using ECL HRP Chemiluminescent substrate. Western blot figures were quantified by Image J(NIH).

Serum assay

Blood samples were drawn from the hearts of anesthetized mice. Serum was separated by centrifugation at 3,000 rpm for 15 min and stored at −80°C until analysis. To test liver function after TAA treatment, ALT and AST activities were measured using an automatic chemistry analyzer (Hitachi 7070) according to the manufacturer's instructions. AST and ALT activities were

determined following the manufacturer's procedure and were expressed as mU/ml.

Statistical analysis

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Data are expressed as the mean ± SEM. Differences between groups were analyzed by the unpaired *t*-test and one-way analysis of variance. *P*-values < 0.05 were considered statistically significant.

RESULT

pTGFβ-TIF1γ vector construct strategy as a gene therapy for liver fibrosis

During liver fibrosis, increased TGFβ acts on TGFβ promotor to increase expression of TGFβ and increase αSMA expression by reducing TIF1γ expression. Increased TGFβ accelerates HSCs activation and liver fibrosis by forming the autocrine loop of TGFβ signaling [8].

pTGFβ-TIF1γ vector was constructed to rescue TIF1γ upon activation of TGFβ promoter in HSCs. I thought that TGFβ increased TIF1γ when pTGFβ-TIF1γ vector was transfected in LX2 cell. And then increased TIF1γ restores aHSCs to qHSCs, which consequently relieves liver fibrosis.

(Figure1, A)

The reason using the TGF promoter rather than commonly used CMV promoter is that the expression of the vector can be controlled depending on the state of the liver fibrosis [Normal (TGFβ↓) vs Fibrosis (TGFβ↑)].

pTGFβ-TIF1γ vector can restore quiescent state of hepatic stellate cell.

In order to confirm whether pTGFβ-TIF1γ vector can restore quiescent state of HSCs, in vitro experiments were performed using human HSCs cell line LX2. TGFβ was treated daily after transfection to confirm that the pTGFβ-TIF1γ vector works well. (Figure2, A) As a result, mRNA expression level of αSMA (aHSCs marker) and collagen1 were significantly reduced in TGFβ treated groups. (Figure2, B) And protein expression level of αSMA was also reduced in TGFβ treated groups. (Figure2, C, D) So I could confirm that pTGFβ-TIF1γ vector works well.

As the original concept is the therapy, TGFβ was pre-treated for 3days to activate HSCs and then vector

(pTGFβ-TIF1γ, pCMV-TIF1γ) was transfected. TGFβ was further treated for 2 days to make the vector work and then harvested. (Figure2, E)As a result, it was confirmed that mRNA and protein level of αSMA and collagen1 that was increased by TGFβ were significantly decreased in vector transfected group. (Figure2, F, G, H)

Based on these results, I verified that pTGFβ-TIF1γ vector can restore the aHSCs to quiescence condition.

The vitamin A-coupled liposome is a tool that transfer vectors specifically to hepatic stellate cell in vivo.

Next, I adopted the VitA-coupled liposome as a delivery tool to specifically transfer the pTGFβ-TIF1γ vector to HSCs before conducting in vivo experiments. I briefly explain the mechanism of VitA-coupled liposome that specifically transfers vectors to HSCs in vivo. The vectors are wrapped around the liposome and VitA (Retinol) is attached to the liposome. When VitA-coupled liposomes enter the bloodstream, retinol binding proteins (RBP) in the blood

attach to VitA. HSCs is the cell that stores most retinol in the body so RBP-VitA-coupled liposomes go to HSCs in liver. Next, the RBP attached to the liposome bind to the RBP receptor in HSCs. Consequently, the vectors enter the HSCs by receptor.[9] (Figure3, A)

The pCMV-GFP vector was injected with VitA-coupled liposome through intravenous (IV) and intraperitoneal (IP) injection. Both injection pathways showed that the vector was well transferred to cells that looks like HSCs in liver. (Figure3, B, C) Retinol (HSCs marker in liver) was also seen to determine whether the GFP expressing cells are HSCs. Consequently, GFP expressing cells were co-localized with retinol, so I confirmed that VitA-coupled liposome can deliver vector to HSCs. (Figure3, D)

Moreover, I also checked through the confocal image that vector did not go to other organs. (Figure3, E) These results demonstrate that VitA-coupled liposome is a delivery tool that specifically transfer the vector to HSCs in vivo.

pTGFβ-TIF1γ vector can alleviate liver fibrosis in vivo

Previously, pTGFβ-TIF1γ vector was confirmed in vitro to restore aHSCs, and VitA-coupled liposome was confirmed in vivo to deliver the vector to HSCs specifically. Based on these results, I conducted in vivo expreiments using mouse liver fibrosis model (TAA model) to confirm whether the pTGFβ-TIF1γ vector actually alleviated liver fibrosis. In pilot study, TAA was injected three times to induce liver fibrosis. And then the vector was injected with VitA-coupled liposome. After that, TAA was injected three times to accelerate the fibrosis and sampling was performed to confirm the degree of fibrosis. (Figure4, A) Liver slides were stained through Picro-sirus red staining (red) and MT staining (blue) to see collagen deposit. As a result, liver surface undulation and fibrosis area were significantly reduced in pTGFβ-TIF1γ vector injected group. (Figure4, B) Therefore, I verified the possibility of mitigating liver fibrosis of the pTGFβ-TIF1γ vector.

Then the additional experiment was performed to increase the number of experiments. TAA was injected three times to

induce liver fibrosis. And then the vector was injected with VitA-coupled liposome. (Figure4, C) I confirmed that fibrosis was well induced at the time of vector injection (10D). (Figure4, D) After that, TAA was injected five times to accelerate the fibrosis and sampling was performed to confirm the degree of fibrosis. As a result, the liver fibrosis area was significantly reduced in pTGFβ-TIF1γ vector injected group compare to pTGFβ-GFP injected group. (Figure4, E, F) In addition, protein level of αSMA was significantly reduced in pTGFβ-TIF1γ vector treated group. (Figure4, G, H) Also ALT and AST, which are indicators of liver damage in mouse serum, were significantly reduced compared to TAA group. (Figure4, I)

Based on these results, I verified that liver fibrosis was alleviated when pTGFβ-TIF1γ was transferred to HSCs using VitA-coupled liposome.

(A) Graphical abstract

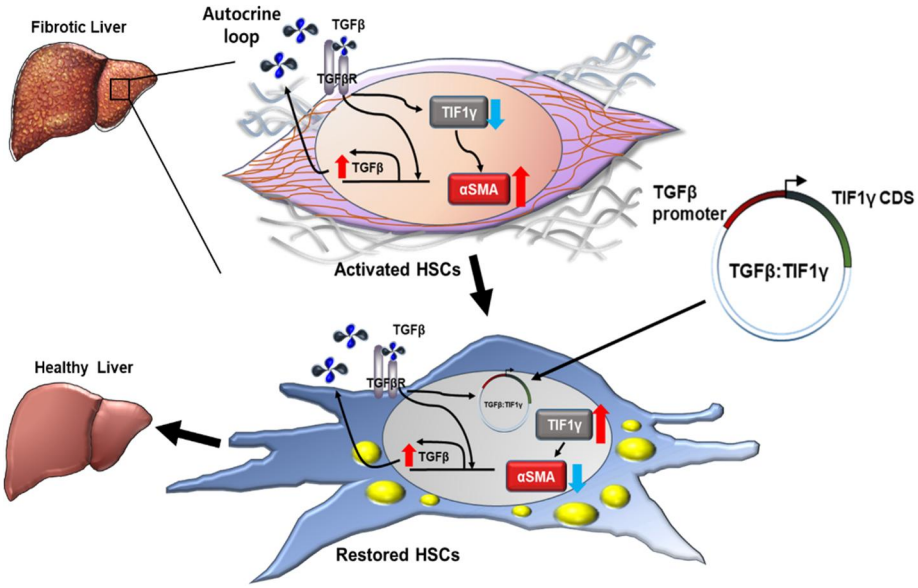
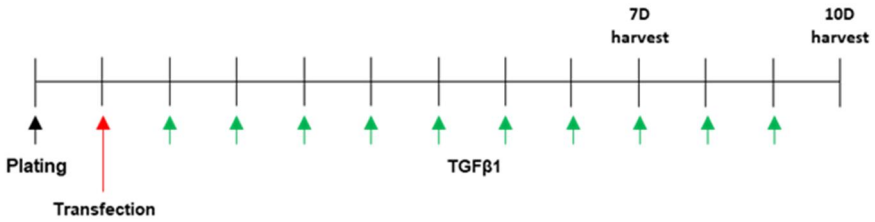


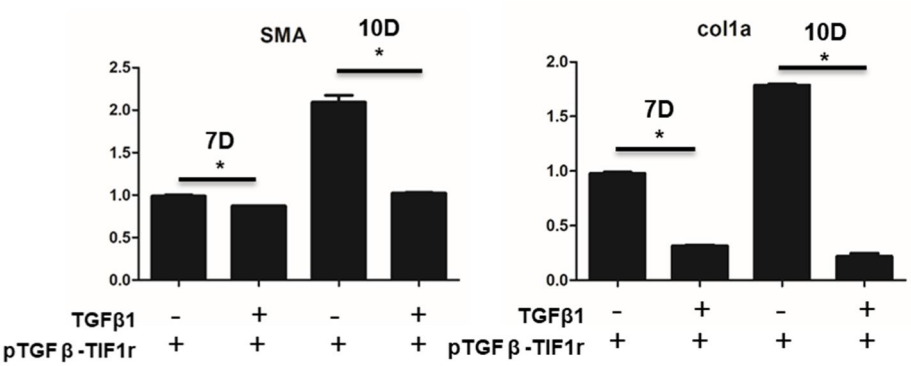
Figure1. pTGFβ−TIF1γ vector construct strategy as a gene therapy for liver fibrosis.

(A) The diagram for the strategy. TGFβ transfers the signal through the receptor into the cell, and the transferred signal activates TGFβ promoter to form an autocrine loop and decrease the TIF1γ. After Reduced TIF1γ increases αSMA and ECM proteins. Consequently, HSCs is activated and fibrosis is accelerated. When pTGFβ−TIF1γ vector is transfected to activated HSCs, TIF1r is restored by TGFβ. Restoring TIF1γ can reduce αSMA and ECM proteins. As a result, HSCs is restored quiescent state and then fibrosis is alleviated.

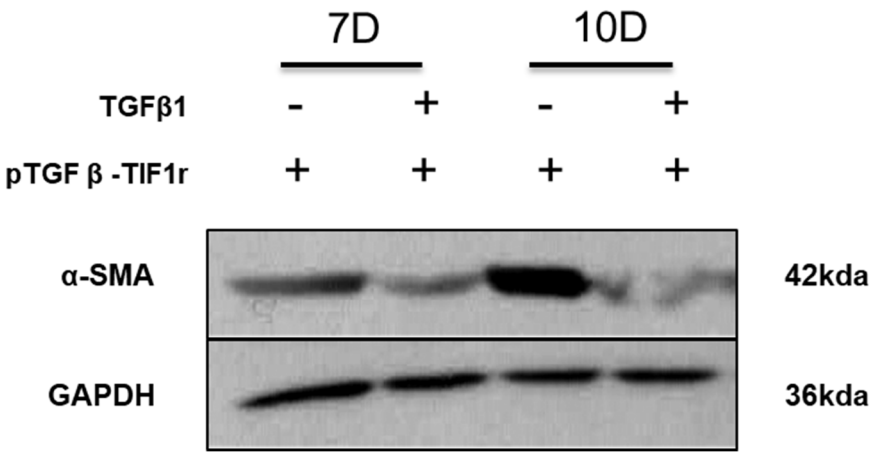
(A) Time table (LX2)



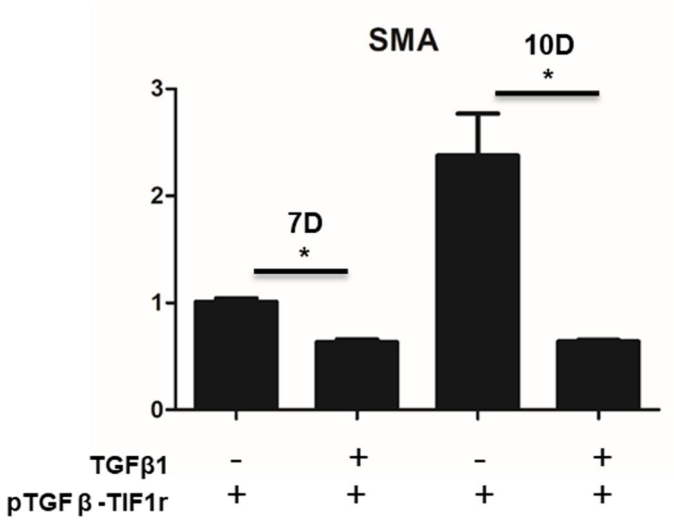
(B) Real-time PCR (LX2)



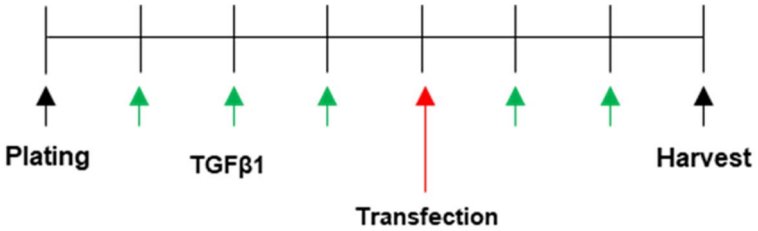
(C) Western blot (LX2)



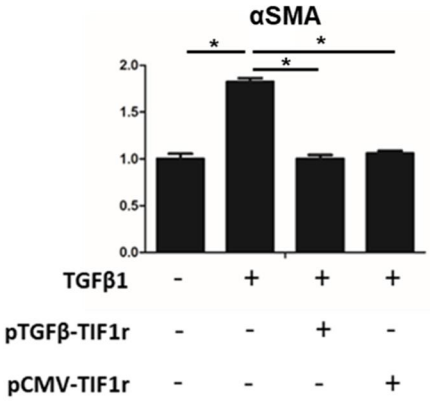
(D) Quantification-Western blot (LX2)

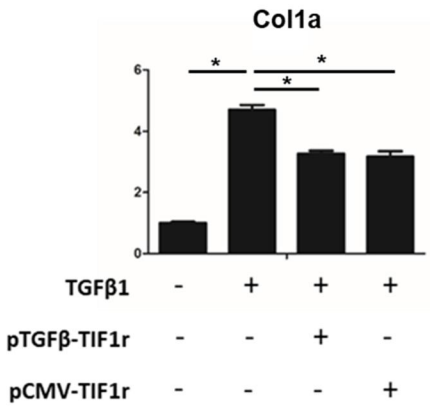


(E) Time table (LX2)

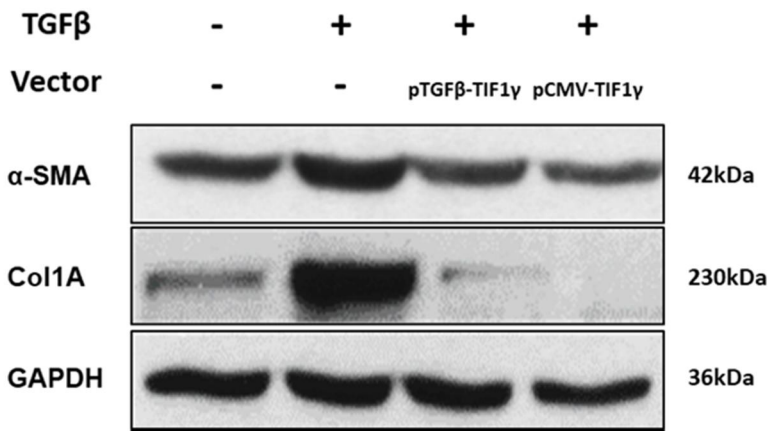


(F) Real time-PCR (LX2)





(G) Western blot (LX2)



(H) Quantification-Western blot (LX2)

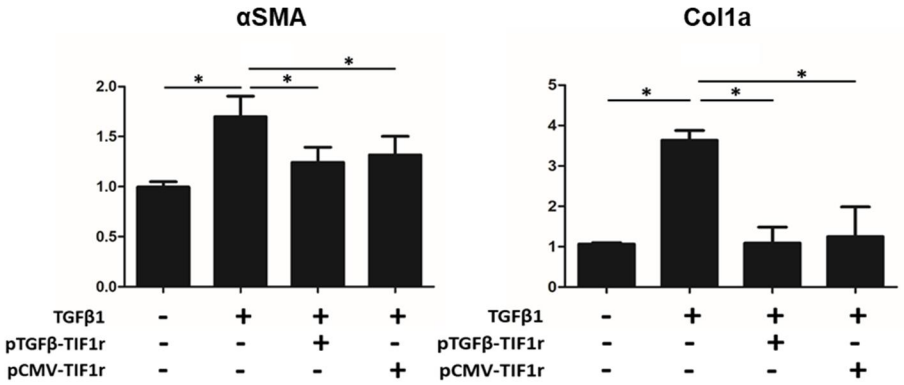


Figure2. pTGFβ−TIF1γ vector can restore quiescent state of hepatic stellate cell.

(A) TGFβ (10 ng/ml) was treated on LX2 for 7 and 10times to activate pTGFβ−TIF1γ vector after transfection.

(B) mRNA expression levels of αSMA and collagen 1 were significantly reduced in TGFβ treated group at 7 and 10days.

(C) Protein expression level of αSMA was significantly reduced in TGFβ treated group at 7 and 10days.

(D) Quantification of western blot band (αSMA) using imageJ program.

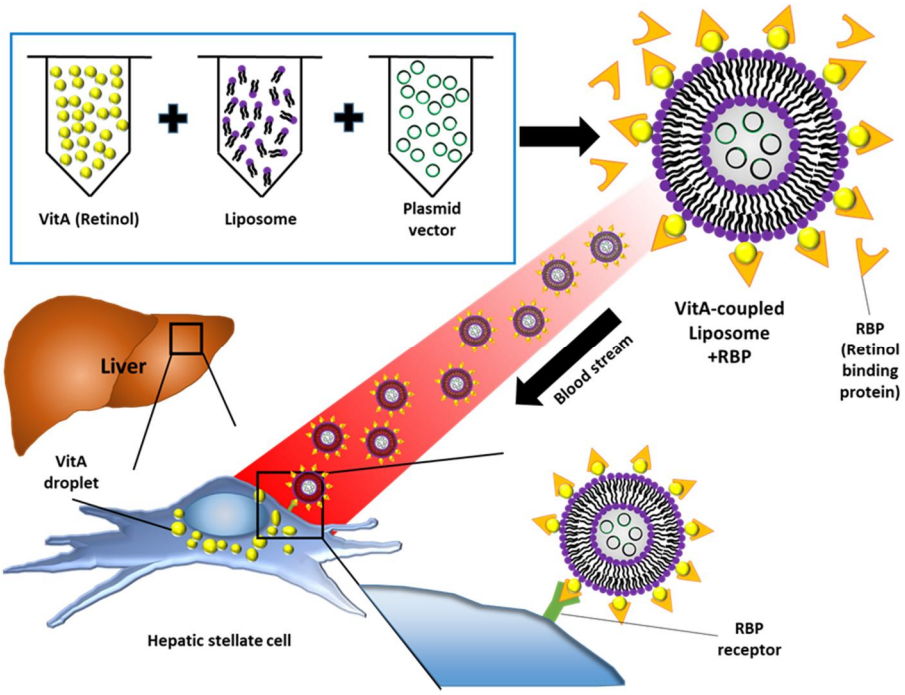
(E) TGFβ (10 ng/ml) was treated on LX2 for 3times to induce aHSCs and then pTGFβ−TIF1γ was transfected. After transfection, TGFβ was treated for 3days to activate the vector.

(F) mRNA expression levels of αSMA and collagen 1 were significantly reduced in vector transfected groups. (pTGFβ−TIF1γ, pCMV−TIF1γ)

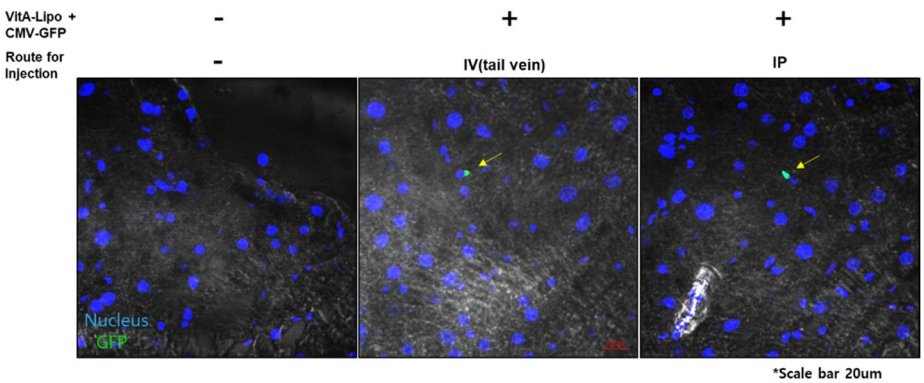
(G) Protein expression levels of αSMA and collagen 1 were significantly reduced in vector transfected groups. (pTGFβ−TIF1γ, pCMV−TIF1γ)

(H) Quantification of western blot band (αSMA, collagen1) using imageJ program.

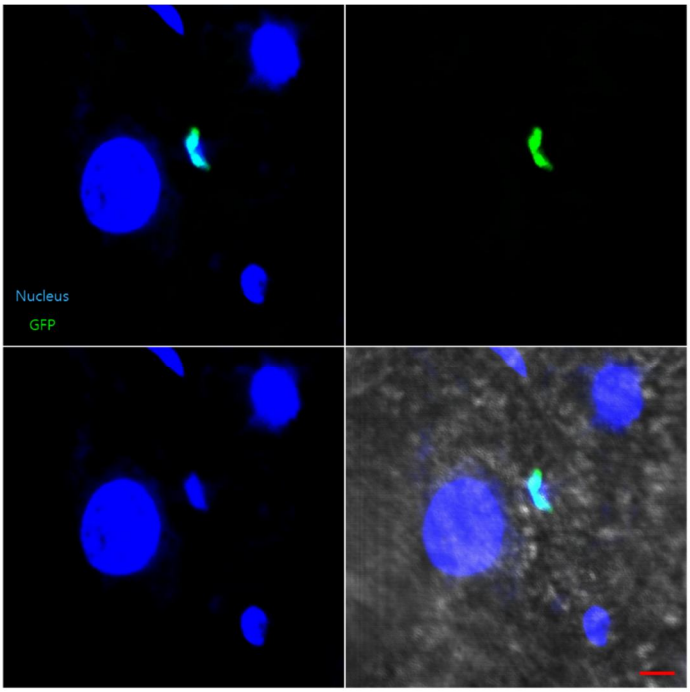
(A) Graphical abstract



(B) Confocal image (mouse liver)

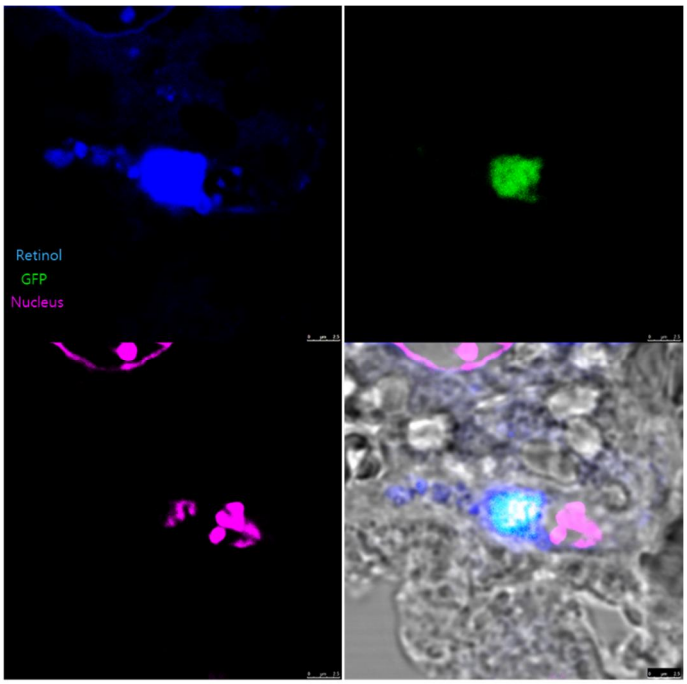


(C) Confocal image (mouse liver)



*Scale bar 2.5um

(D) Confocal image (mouse liver)



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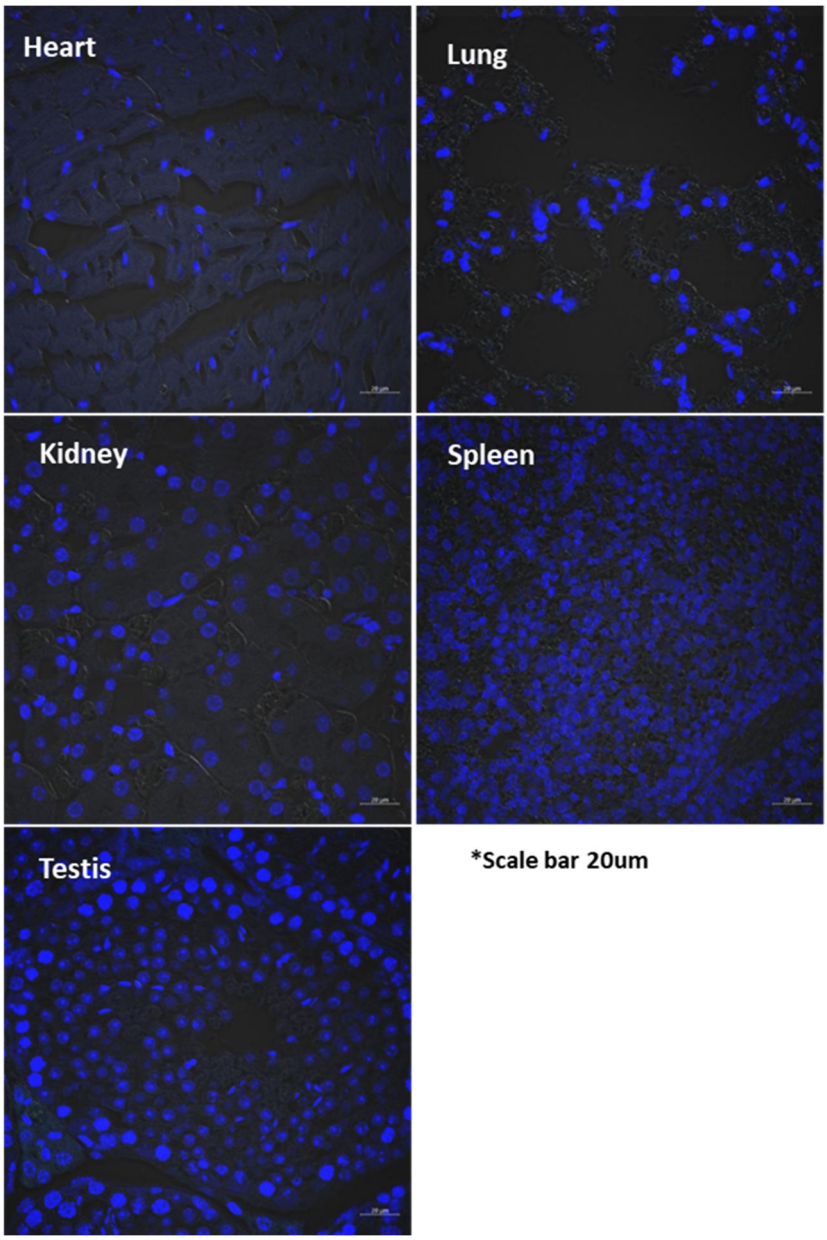
(E) Confocal image (Other organs)



*Scale bar 20um

Figure3. Vitamin A-coupled liposome is a tool that transfer vectors specifically to hepatic stellate cell.

(A) Diagram of VitA-coupled liposome vector delivery mechanism into HSCs in vivo.

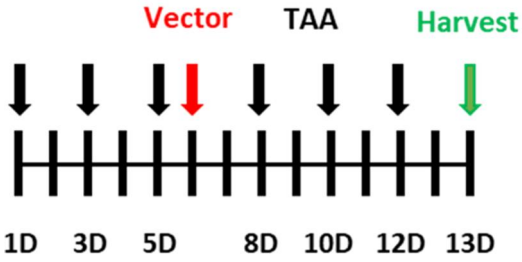
(B) Mouse liver confocal microscopy image when pCMV-GFP vector was wrapped in a VitA-coupled liposome and injected through the IP and IV routes. Nucleus was stained with DAPI (blue) and pCMV-GFP vector transfected cells expressed green fluorescence. Both injection routes well delivered the vector to liver in vivo.

(C) Cell that pCMV-GFP vector was delivered (green) in liver looks like HSCs.

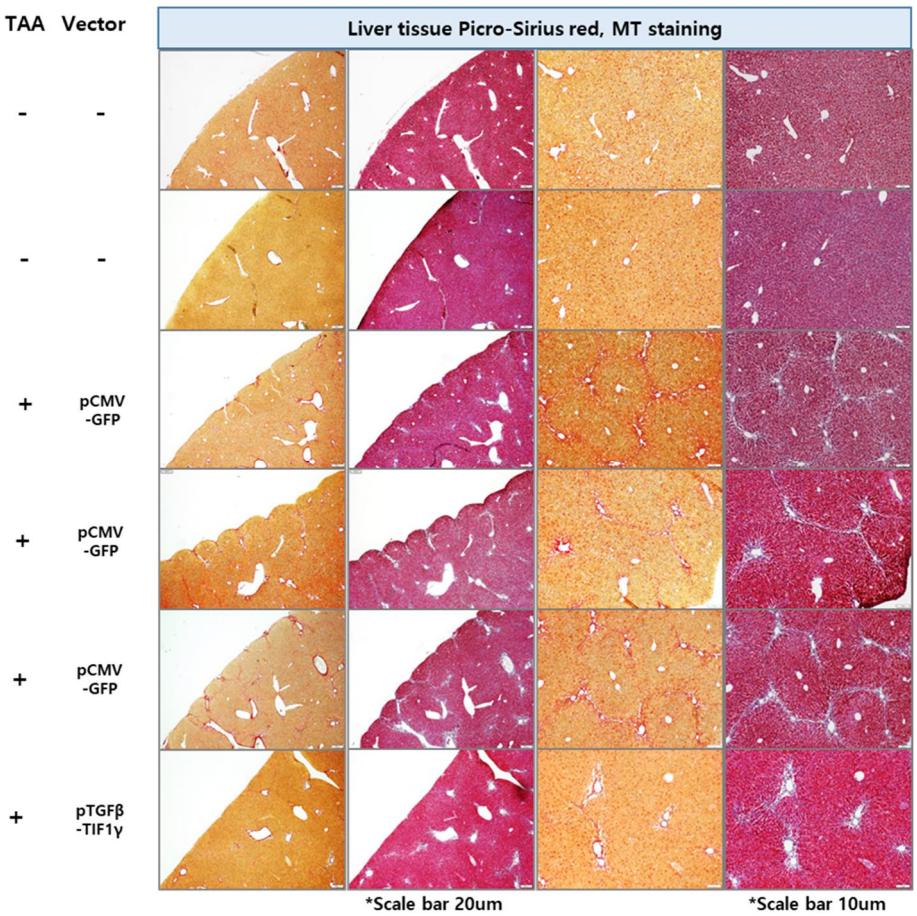
(D)GFP expressing cell was co-localized with retinol that HSCs marker in liver. Retino auto-fluorescence (excitation-335 nm, emission-458 nm)

(E) Confocal microscopy image of other organs except the liver.

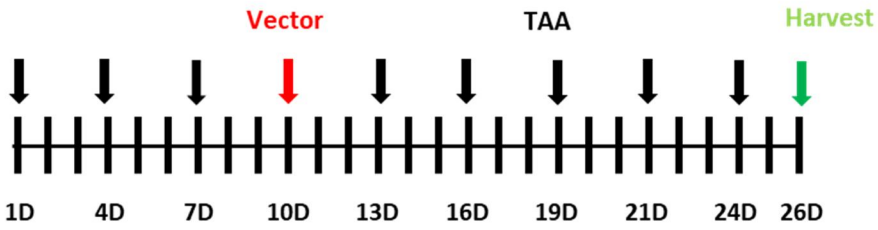
(A) Time table (mouse) - pilot



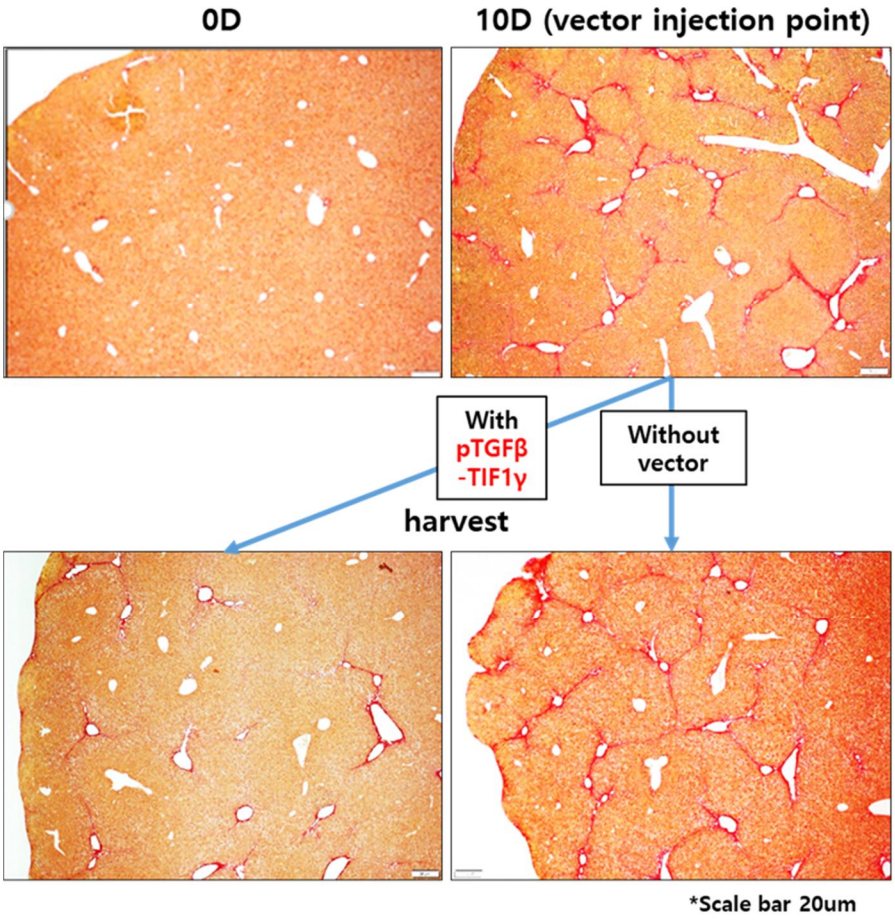
(B) Picro-Sirius red, MT staining (mouse liver)



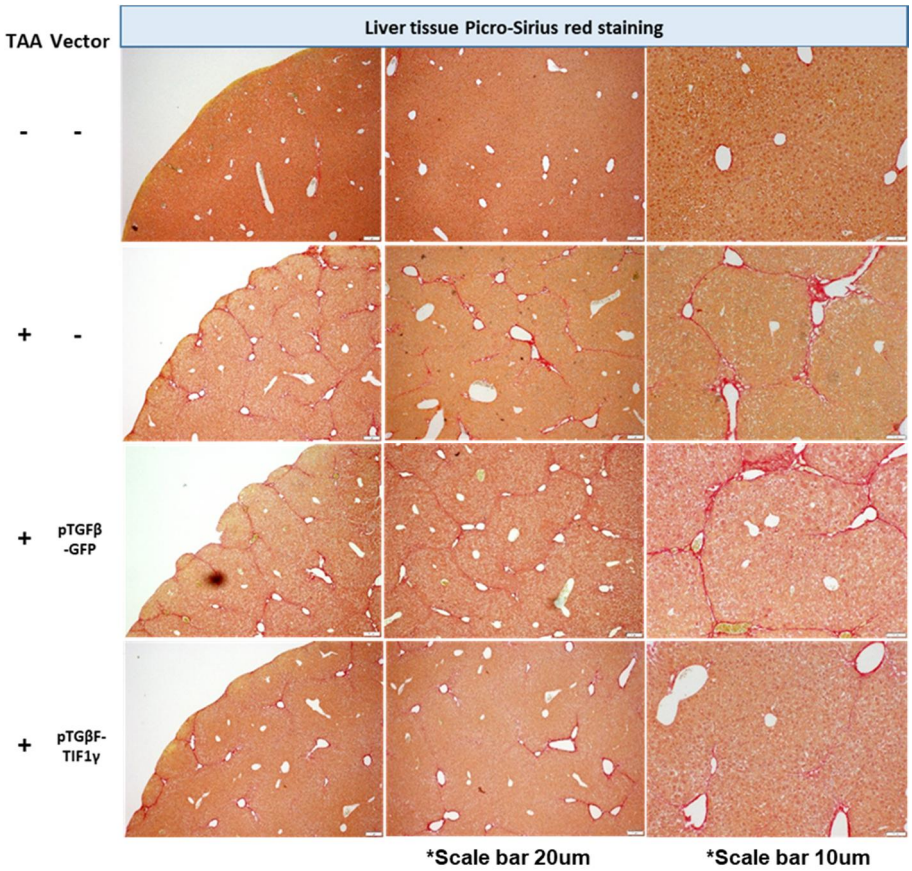
(C) Time table (mouse)



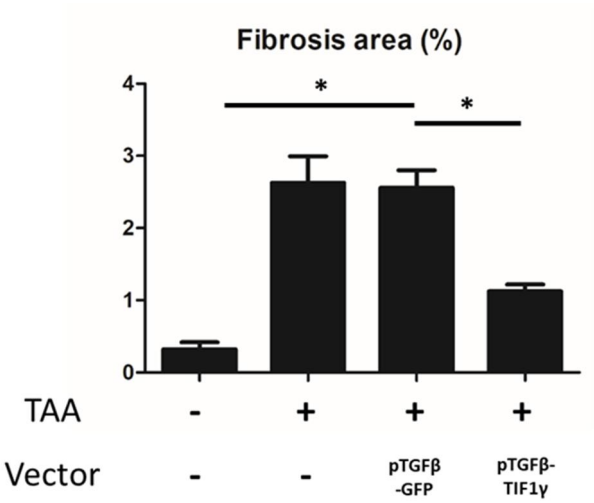
(D) Picro-Sirius red staining (mouse liver)



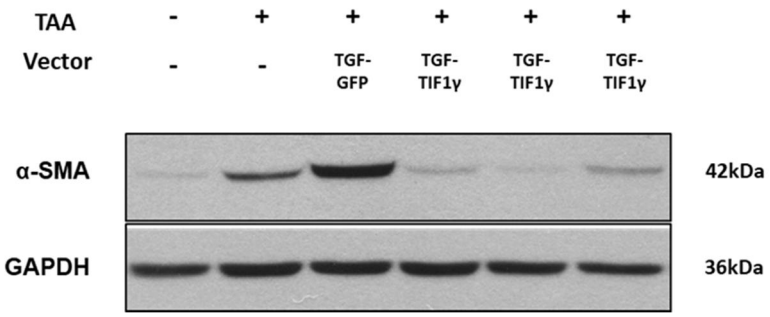
(E) Picro-Sirius red staining (mouse liver)



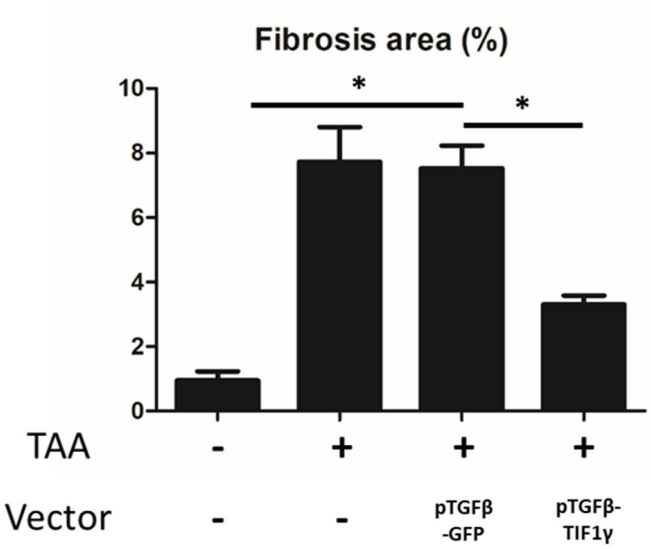
(F) Quantification of fibrosis area



(G) Western blot (mouse liver)



(H) Quantification-Western blot (mouse liver)



(I) ALT, AST levels (mouse serum)-IU/L

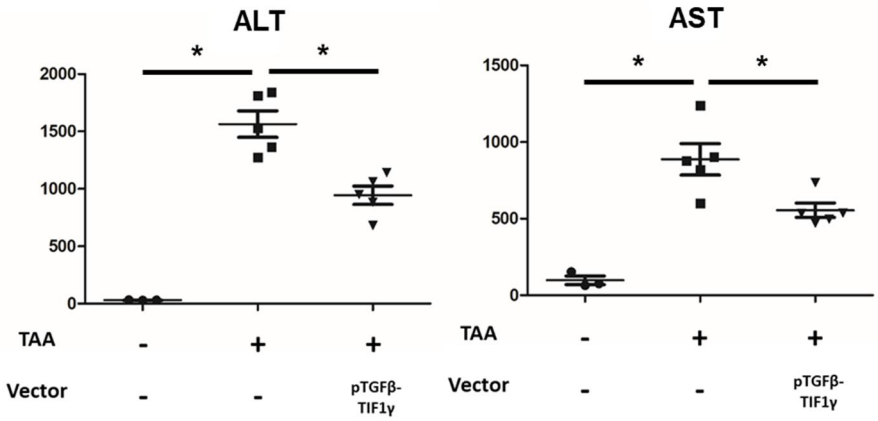


Figure4. TGFβ-TIF1γvector can alleviate liver fibrosis in vivo.

(A)Timetable showed schedule of animal experiment. TAA (200 mg/kg) was injected 3times through IP injection to induce fibrosis. And then, vector(pCMV-GFP, pTGFβ-TIF1γ) was injected 1time through IP injection. Next TAA was injected 3times to Aggravate fibrosis and harvested liver after 2days.

(B)Picro-sirius red and MT staining showed reduction of collagen accumulation by treatment of pTGFβ-TIF1γ on mouse liver fibrosis model.

(C) Timetable showed schedule of animal experiment. TAA (200 mg/kg) was injected 3times through IP injection to induce fibrosis. And then, vector(pTGFβ-GFP, pTGFβ-TIF1γ) was injected 1time through IP injection. Next TAA was injected 5times to more Aggravate fibrosis and harvested liver after 2days.

(D) Picro-sirius red staining that stain collagen in red was performed. Fibrosis was induced at vector injection point(10D). Fibrosis area (red) of pTGFβ-TIF1γ vector injected group was reduced compare to TAA group.

(E) Picro-sirius red staining that stain collagen in red was performed. Fibrosis area and liver surface undulation were reduced in pTGFβ-TIF1γ group compare to TAA group and pTGFβ-GFP group.

(F) Fibrosis area was quantified by measuring the ratio of fibrosis area (red area / total area). The fibrosis area of pTGFβ-TIF1γ group was significantly reduced compared to the pTGFβ-GFP group.

(G) Protein expression levels of αSMA was significantly reduced in pTGFβ-TIF1γ vector group.

(H) Quantification of western blot band (αSMA) using imageJ program.

(I) ALT and AST serum levels were measured using automatic chemistry analyzer (Hitachi 7070). ALT and AST serum levels were significantly reduced in pTGFβ-TIF1γ group compare to TAA group.

*** Group of in vivo study (pilot)**

- Non treat (N=2)
- TAA+pCMV-GFP (N=3),
- TAA+pTGFβ-TIF1γ (N=1)

***Group of in vivo study**

-Non treat (N=3)
-TAA 10D (vector injected point, N=3)
-TAA 26D (N=5)
-TAA+pTGFβ-GFP (N=2)
-TAA+pTGFβ-TIF1γ (N=5)

DISCUSSION

HSCs activation plays a key role in pathological progression of liver fibrosis. HSCs activation is triggered by increased in TGFβ level during liver fibrosis. The pathophysiologic role of TGFβ in the liver is well-known. TGFβ initiates inflammation and fibrosis, and ultimately plays an important role in the progression to cirrhosis and cancer.[10] In liver fibrosis, TGFβ induces activation and transformation of HSCs to turn into myofibroblast expressing αSMA. TGFβ phosphorylates SMAD 2/3 and interacts with SMAD4.[11] The SMAD2 / 3-SMAD4 complex is attached to the (SMAD binding element)SBE site of DNA to activate αSMA expression.[12, 13] Therefore, targeting HSCs activation mechanisms that interfere with the signaling pathway may be useful in liver fibrosis therapy.

TIF1γ is known to be a factor that inhibits the TGFβ signaling pathway by binding to phosphorylated SMAD2 / 3 through competition with SMAD4 [14-16] In previous studies, we confirmed that TIF1γ interacts with SMAD2 / 3 and bind to the αSMA promotor in human HSCs cell line (LX2). And TIF1γ-

SMAD2 / 3 complex inhibits αSMA expression by binding to the αSMA promoter. Thus, TIF1γ inhibited αSMA expression in HSCs, indicating that it is an anti-fibrotic factor that prevents HSCs activation. (In previous study)

Based on the results of previous studies, I established a therapeutic strategy for liver fibrosis using TIF1γ. I want to restore TIF1γ in activated HSCs during the fibrosis process. I focused TGFβ, which increases during liver fibrosis progression and plays a key role in HSCs activation. Once TGFβ signal is turned on, TGFβ secretion is amplified through positive autocrine feedback. [8, 17] Based on the TGFβ signaling mechanism, a vector was constructed by conjugating TIF1γ CDS to the TGFβ promoter, in which TGFβ promoter is activated and TIF1γ is restored when exogenous TGFβ acts on HSCs.

The anti-fibrotic effect of the pTGFβ-TIF1γ vector was verified by the reduction of αSMA and collagen 1 when the vector was transfected into LX2. To validate the anti-fibrotic effect of the vector in vivo, we needed a delivery tool to specifically delivery vector to HSCs. Vitamin A (retinol) complexes with retinol binding protein (RBP) in the body and is

delivered to the HSCs, retinol reservoir. Based on this mechanism, we used the Vitamin A-coupled liposome as a tool to specifically delivery vector to HSCs. [18, 19] pTGFβ-TIF1γ vector was tested in vivo using a vitamin A-coupled liposome, and liver fibrosis was alleviated in the vector treated group.

In conclusion : In previous studies, we identified TIF1r, which plays a key role in the HSCs activation mechanism. And I established a liver fibrosis therapy strategy at the cellular level that restores TIF1r in activated HSCs during liver fibrosis.

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국문초록

TIF1γ restoring vector 를 이용한 간 섬유화 유전자 치료 전략에 관한 연구

서울대학교 융합과학기술 대학원
분자의학 및 바이오제약학과
분자의학 및 바이오제약전공
문 도 담

간 섬유화는 간 손상에 대한 반응으로 extracellular matrix (ECM) 단백질이 초과로 쌓이는 현상입니다. 간 성상 세포는 간 섬유화 과정 중 활성화 되어 myofibroblast 로 분화되고, 간을 경화 시키는데 핵심 역할을 하는 세포 군으로 잘 알려져 있습니다. 활성화된 간 성상 세포는 myofibroblast marker 인 alpha smooth muscle actin (αSMA) 을 발현하며 collagen 과 같은 ECM 단백질을 분비하여 간을 경화 시키는데 기여합니다.

저희 팀은 이전 연구에서 새로운 간 섬유화 기전을 밝혔습니다. 간이 정상인 상태에서는 간 성상 세포 내 transcriptional intermediary factor 1 gamma (TIF1γ) 라는 항 섬유화 인자가 αSMA 발현을 막아 비활성 상태를 유지하고 있습니다. 하지만 간

섬유화 과정에서는 TGFβ1 에 의해 TIF1γ 발현양이 감소하게 되고 그로 인해 억제되고 있던 αSMA 발현양이 증가하게 됨으로써 간 성상 세포가 활성화 됩니다. 활성화 된 간 성상 세포는 collagen 과 같은 ECM 단백질을 초과 생성하게 되고, 결국에 간 섬유화가 진행됩니다.

저는 이전 연구에서 밝힌 간 섬유화 기전을 바탕으로 활성화 된 간 성상 세포 내에 낮아진 TIF1γ 발현을 다시 회복시켜준다면 간 성상 세포의 활성화가 억제되고, 그 결과로 간 섬유화가 완화될 것이라고 생각하였습니다. 간 성상세포에서 TIF1γ 발현을 회복시키기 위하여 TGFβ promotor-TIF1γvector (pTGFβ-TIF1γ)를 제작하였습니다. 그리고 in vitro 에서 pTGFβ-TIF1γ가 TGFβ에 의해 증가한 αSMA (활성화된 간 성상세포 표지자) 를 감소 시키는 것을 사람의 간 성상 세포주인 LX2 에서 확인하였습니다. 이 후 pTGFβ-TIF1γ의 간 섬유화 완화 효과를 in vivo 에서 확인하고자 하였습니다. 그 전에 vector 를 간 성상 세포에 특이적으로 전달하는 도구로 Vit A-coupled liposome 을 채택하였고, 간 성상 세포에 특이적으로 vector 를 전달하는 것을 in vivo 실험에서 확인하였습니다. 그 후 TAA(Thioacetamide) 간 섬유화 동물 모델에서 pTGFβ-TIF1γ의 섬유화 완화 효과를 확인하였습니다.

이러한 결과를 통해 저는 간 섬유화 과정 중 간 성상 세포 내 항

섬유화 유전자 TIF1γ의 중요성 및 pTGFβ-TIF1γ의 간 섬유화 완화 효과를 확인 할 수 있었습니다. 저는 이 연구가 간 섬유화 유전자 치료의 가능성을 확인 시켜주었다고 생각합니다.

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주요어 : VitA-coupled liposome, 유전자 치료, pTGFβ-TIF1γ vector, 간 성상 세포; HSCs, 간 섬유화, TIF1γ (transcriptional intermediary factor 1 gamma), αSMA (alpha smooth muscle actin), aHSCs (활성화 된 상태의 간 성상 세포), qHSCs (비활성화 상태의 간 성상 세포)

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