Title page

Hepatic stellate cell-specific knockout of transcriptional intermediary factor 1γ aggravates liver fibrosis

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Summary: Loss of TIF1γ in HSC aggravates liver fibrosis

Non-standard abbreviations

HSC: Hepatic stellate cells

TAA: Thioacetamide

hE-MSCs: Mesenchymal stem cells derived from human embryonic stem cells

ERT2: Estrogen receptor

shRNA: Small hairpin RNA

PLA: Proximity ligation assay

SBE: SMAD-binding element

TMX: Tamoxifen

TEM: transmission electron microscopy

SEM: scanning electron microscopy

TG: Transgenic

LSEC: Sinusoidal endothelial cells

ChIP: Chromatin immunoprecipitation

Short, ~40-world summary

This study demonstrated that loss-of-TIF1γ in HSC aggravates fibrosis and TIF1γ, identified as a novel target, has potential for the development of new therapeutic approaches to inhibiting or treating liver fibrosis.

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Abstract

Transforming growth factor β (TGFβ) is a crucial factor in fibrosis, and transcriptional intermediary factor 1γ (TIF1γ) is a negative regulator of the TGFβ pathway; however, its role in liver fibrosis is unknown. In this study, mesenchymal stem cells derived from human embryonic stem cells (hE-MSCs) that secretes hepatocyte growth factor (HGF), were used to observe the repair of thioacetamide (TAA)-induced liver fibrosis. Our results showed that TIF1γ was significantly decreased in LX2 cells when exposed to TGFβ1. Such decrease of TIF1γ was significantly prevented by co-culture with hE-MSCs. Interaction of TIF1γ with SMAD2/3 and binding to the promoter of the alpha-smooth muscle gene (*αSMA*) suppressed αSMA expression. Phosphorylation of cAMP response element-binding protein (CREB) and binding on the *TIF1γ* promoter region induced TIF1γ expression. Furthermore, hepatic stellate cells-specific TIF1γ-knockout mice showed aggravation of liver fibrosis. In conclusion, loss-of-TIF1γ aggravates fibrosis, suggesting that a strategy to maintain TIF1γ during liver injury would be a promising therapeutic approach to prevent or reverse liver fibrosis.

Keywords: Transcriptional intermediary factor 1γ (TIF1γ)/site- and time-specific *TIF1γ* gene targeting/ Hepatic stellate cells (HSCs)/Liver fibrosis /

Introduction

Fibrosis is the pivotal stage of liver scarring and can progress to cirrhosis and eventually cause liver failure, for which the only effective therapy is liver transplantation (Bataller and Brenner, 2005). However, the shortage of the available donated organs and long-term post-surgical immunosuppression have forced researchers to look for alternative therapeutic strategies (Burra et al., 2012). Recent technological developments have enabled the elucidation of the cellular mechanisms of liver fibrosis as well as therapeutic approaches to liver-oriented cell therapy. Regarding the underlying mechanism, activation of hepatic stellate cells (HSCs) plays a pivotal role in extracellular matrix production during liver fibrosis. Although the activation and transdifferentiation of HSCs to myofibroblasts are regarded key pathogenic mechanisms of fibrogenesis, the key factors that play a role in the activation of HSCs remain to be fully elucidated.

The stimulation of hepatocyte regeneration by human mesenchymal stem cells (hMSCs) has been shown to be a therapeutic strategy to alleviate end-stage liver disease. However, its clinical potential is still a matter of debate (Baertschiger et al., 2009; Shi et al., 2011; Terai et al., 2005; Wang et al., 2012). In our previous study, we successfully derived hMSCs from human embryonic stem cells (hE-MSCs), and we demonstrated that hE-MSCs could be consistently produced, maintained, and expanded (Lee et al., 2010; Lee et al., 2012). In this study, hE-MSCs, which secrete hepatocyte growth factor (HGF) as the most abundant growth factor, were used as a strategic tool to screen for a target for repairing liver injury *in vivo* and *in vitro*. From these experiments, we identified transcriptional intermediary factor 1 gamma (TIF1γ) among six candidate proteins as a negative regulator of TGFβ signaling in HSCs. TIF1γ, also known as tripartite motif-containing 33, has been revealed to act as a ubiquitin E3 ligase (Xue et al., 2015). Notably, it serves as a transcriptional co-repressor by interacting with SMAD family member 2/3 (Hesling et al., 2011).

In this study, our findings on the potential suppressive role of TIF1γ in liver fibrosis were corroborated by experiments *in vitro* using LX2 HSC cells and *in vivo* using site- and time-specific *TIF1γ* gene targeting in transgenic mice. To know the role of TIF1 γ in liver fibrosis, a transgene constructs consisting of modified Cas9 conjugated to an estrogen receptor (ERT2) that is activated by tamoxifen but not estradiol (Metzger and Chambon, 2001) and under the control of the Lrat promoter to specifically knock out TIF1γ in HSCs was developed and used (Mederacke et al., 2013).

Results

Transplantation of hE-MSCs prevents TAA-induced liver fibrosis in nude mice

We previously found that hE-MSCs abundantly secrete hepatocyte growth factor (HGF) (Lee et al., 2018). Because HGF is an important growth factor in the liver (Bohm et al.), we hypothesized that hE-MSCs might be efficacious in liver therapy.

Histological analysis of collagen fibers using Masson's trichrome staining revealed that transplantation of hE-MSCs reduced liver surface undulations and the fibrotic area at day 14 of TAA-induced liver injury (0.99 ± 0.18% in control (no treatment) vs. 16.0 ± 4.4% in TAA treatment vs. 6.1 ± 3.1% in TAA/hE-MSC treatment, n = 5) (Fig. 1A). Collagen deposits in 14-day tissues were visualized and quantified using Picro-Sirius red staining, which detects collagen types I and III (Fig. 1B). The area of collagen deposit by TAA injury was significantly reduced by transplantation of hE-MSCs (2.3 ± 1.1% in control vs. 11.1 ± 1.2% in TAA treatment vs. 3.7 ± 1.0% in TAA/hE-MSC treatment, n = 5) (Fig. 1B). Fibrous septa were evaluated in liver tissues of 14-day-TAA-treated mice with or without hE-MSC transplantation. Fibrosis stage was assessed based on staining with Picro-Sirius red, according to two histological classifications, METAVIR (stages I–IV) and Ishak (stages I–V) (Bataller, 2005; Standish et al., 2006). Fibrotic expansio2n with occasional bridging/septa (Ishak stage

3/METAVIR stage F2) was detected in TAA-treated mice (Fig. 1C). However, hE-MSC transplantation prevented the formation of bridging/septa (Ishak stage 2/METAVIR stage F2) (23.7 ± 5.5 in TAA treatment vs. 10 ± 2.6 in TAA/hE-MSC treatment, n = 5) (Fig. 1C), which correlated well with a decrease in fibrous area. Injection of TAA into the peritoneum three times a week significantly elevated the levels of liver enzymes aspartate aminotransferase (AST) and alanine aminotransferase (ALT), leading to liver fibrosis. However, one systemic administration of hE-MSCs during the period of TAA injections prevented this elevation of liver enzyme (ALT; 26 ± 2.8 IU/L in control vs. 413 ± 24.0 IU/L in TAA treatment vs. 101 ± 15.6 IU/L in TAA/hE-MSC treatment, AST; 80.5 ± 16.3 IU/L in control vs. 253 ± 36.8 IU/L in TAA treatment vs. 124 ± 11.3 IU/L in TAA/hE-MSC treatment) (Fig. 1D).

TIF1γ suppresses the activation of human LX2 HSCs

To evaluate how hE-MSC transplantation suppressed liver fibrosis *in vitro*, we searched for candidate anti-fibrotic factors that were declined in TGFβ1-activated LX2 as human HSCs. It has been reported that fibrotic events are associated with a TGFβ1 signaling pathway in activated HSCs; thus, we selected and tested six proteins (Dayoub et al., 2011; Hesling et al., 2011; Netherton and Bonni, 2010; Vajda et al., 2009; Zhang et al., 2011; Zhao et al., 2013) (Table S1). Among the six candidates, we identified three proteins whose mRNA expression decreased after exposure to TGFβ1 in LX2 cells: EPLIN, a cytoskeleton-associated protein that inhibits actin filament depolymerization; Nm23-H1, nucleoside diphosphate kinase A, a metastasis suppressor; and TIF1γ (Fig. 2A). To validate the involvement of each of these three proteins in the effect of hE-MSCs on fibrosis, we cocultured hE-MSCs with TGFβ1-activated LX2 cells (Fig. S1A). When LX2 cells were exposed to TGFβ1, they were induced to myofibroblast-like cells through increased expression of alpha-smooth muscle actin (αSMA) (Fig. S1, B and C). This change was significantly prevented by co-culture with hE-MSCs (Fig.

S1, B and C). In these experiments, only TIF1γ was downregulated by TGFβ1 and upregulated in response to hE-MSCs in LX2 cells, whereas EPLIN and Nm23-H1 showed no changes at the protein level (Fig. 2B). Finally, TIF1γ was functionally validated in loss- and gain-of-function experiments. When TIF1γ expression in LX2 cells was knocked down by specific small interfering RNA (siRNA), we observed αSMA upregulation, whereas knockdown of EPLIN or Nm23-H1 did not affect αSMA expression (Fig. 2C; and S1D). TIF1γ overexpression reduced the αSMA level in TGFβ1-treated LX2 cells, suggesting that TIF1γ is a novel candidate for anti-fibrosis treatment (Fig. 2, D and E; and Fig. S1E).

HGF from hE-MSCs increases TIF1γ that interacts with SMAD2/3 leading to suppression of αSMA

To further investigate the importance of hMSC-derived HGF, we conducted *in vivo* experiments using hE-MSC-transducted with shRNA against HGF (Fig 3A). We also evaluated hHGF ELISA results (Fig. S2A). hE-MSCs were labeled with a fluorescent dye, DiI, for *in vivo* tracking as well as cell count in liver tissues. There was no significant difference in the DiI labeled cells between hE-MSCs and shHGF transducted hE-MSCs group (Fig. S2B). However, shHGF transducted hE-MSCs group showed a significant increase the fibrous area (2.38 ± 1.34 in TAA/hE-MSC vs. 4.86 ± 1.10 in TAA/shHGF hE-MSC treatment). In pCMV-hHGF vector treatment which used to show the effect of HGF *in vivo*, fibrous area was reduced compare to control group (1.65 ± 0.62 in pCMV-HGF vs. 9.39 ± 2.52 in TAA treatment)(Fig 3A). ALT and AST were also correlated with fibrous area (ALT; 11.87 ± 1.47 mU/mL in control vs. 28.19 ± 0.56 mU/mL in TAA treatment vs. 4.28 ± 1.45 mU/mL in TAA/hE-MSC treatment vs. 19.13 ± 4.55 mU/mL in TAA/shHGF hE-MSC treatment vs. 11.89 ± 4.84 mU/mL in TAA/pCMV-hHGF treatment, AST; 18.62 ± 12.27 mU/mL in control vs. 74.60 ± 1.07 mU/mL in TAA treatment vs. 25.66 ± 9.47 mU/mL in TAA/hE-MSC treatment vs. 90.86 ±

2.01 mU/mL in TAA/shHGF hE-MSC treatment vs. 33.96 ± 2.77 mU/mL in TAA/pCMV-hHGF treatment) (Fig 3B). We performed RT-PCR using liver tissue from each group to detect whether hHGF was produced as a result of hE-MSCs *in vivo*. Expression of hHGF mRNA was detected in liver tissues of mice treated with hE-MSCs or pCMV-hHGF. Its level was low in the liver of mice treated with shHGF-transduced hE-MSCs (Fig. S2C and Table S2). In order to verify the observed expression of hHGF in liver of mice treated with hE-MSCs or pCMV-hHGF *in vivo*, we measured serum hHGF level from each group (Fig 3C). ELISA results verified that hHGF was indeed detected in blood of mice treated with hE-MSCs and pCMV-hHGF. Its serum level was low in mice treated with shHGF-transduced hE-MSCs, which was correlated with mRNA expression pattern of hHGF in mice liver. Western blotting analysis of liver tissues showed that TIF1γ expression was decreased by TAA and restorated by hE-MSCs or pCMV-hHGF in mouse liver tissue (Fig. 3D). However, in the liver of mice treated with shHGF transducted hE-MSCs, TIF1γ expression was not restorated (Fig3D). The expression of αSMA was opposit to TIF1γ expression (Fig3D).

To validate the effect of HGF from hE-MSCs on TIF1γ expression in human HSCs, LX2 cells were incubated with recombinant human (rh)HGF. TGFβ1 significantly suppressed TIF1γ and increased αSMA in LX2 cells, which was significantly reversed or prevented by rhHGF (Fig. 3E; and Fig. S2D). Next, to confirm that HGF secreted from hE-MSCs is responsible for the maintenance of TIF1γ expression in HSCs, we knocked down HGF in hE-MSCs using HGF-specific small hairpin RNA (shRNA), and we cocultured the cells with TGFβ1-treated LX2 cells. While coculture of LX2 cells with hE-MSCs decreased the induction of αSMA by TGFβ1 in LX2 cells, this effect was suppressed when HGF was knocked down, suggesting that HGF from hE-MSCs is strictly required for their anti-fibrosis effect on LX2 cells or HSCs (Fig. 3F).

Although it is known that the SMAD2/3 complex is involved in the regulation of *SMA* expression by TGFβ (Pardali et al., 2017), it is not known how *SMA* is transcriptionally

regulated by TGFβ in hepatic stellate cells. We assumed that TIF1γ and SMAD2/3 interactions would be altered by TGFβ treatment and HGF treatment in LX2 cells. First, through proximity ligation assay (PLA), we attempted to determine the cellular localization of TIF1γ and SMAD2/3 and their interaction. The interacting TIF1γ and SMAD2/3 were sporadically observed and scattered throughout the nucleus and cytoplasm under basal conditions, and were drastically reduced by TGFβ treatment (total number of dots: 1329.5±143 vs. 52.7±33/100 μm^2 respectively) (Fig. 3G). When HGF was added, the interacting TIF1γ and SMAD2/3 were significantly increased and found to be concentrated in nucleus (total number of dots: 603±62.8/0.01mm^2 , and 330.7±102.2 in nucleus) (Fig. 3G). Next, we performed chromatin immunoprecipitation (ChIP) using TIF1γ antibody to identify which part of the αSMA promoter interacts with TIF1γ. The sequence of the SMAD-binding element (SBE) was used for the identification of a TIF1γ-binding site in the αSMA promoter (Fig. 4A). GPminer (http://gpminer.mbc.nctu.edu.tw/index.php) did not predict a TIF1γ-binding site in the αSMA promoter, but it did predict SBEs. PLA results suggested that SMAD2/3 forms a complex with TIF1γ; therefore, the SBE could be the TIF1γ- binding site. In ChIP data, the PCR product of –84SBE and –602SBE was increased in the HGF compared with the TGF treatment group (Fig. 4B, and quantified in C). The PLA and ChIP data indicated that binding of TIF1γ with SMAD2/3 on the αSMA promoter was augmented by HGF resulting in a decreased expression of αSMA, whereas it was reversed by TGF,.

Mechanism of upregulation of TIF1γ by HGF: pCREB binds on the TIF1γ promoter

Next, to identify the promoter region involved in transcriptional activation of TIF1γ by HGF, we screened the ~1.5-kb putative promoter region for transcription factor-binding sites. We identified binding sites for several transcription factors, including CREB, USF1, SOX5, and GATA1 (Fig. S3A). Among them, the site for CREB was regulated by HGF, which was

confirmed by deletion and point mutation (tgacg→t--cg) in a promoter study (Fig. 4D; and Fig. S3A and B). Western blot and pCREB^{s133} ChIP assays revealed that serine 133-phosphorylated CREB (pCREB^{s133}) induce transcriptions of TFI1γ by HGF (Fig. 4E and F). Ser133 phosphorylation is known as a marker for CREB activation, and pCREB^{s133} was reduced by TGFβ1 treatment and increased by HGF (Fig. 4E). In a ChIP assay, binding of pCREB^{s133} to the CREB site of the TIF1γ promoter was increased by HGF, even in the presence of TGFβ1 (Fig. 4F; and Fig. S3C).

TIF1γ is downregulated in fibrosis liver

We detected TIF1γ-positive cells in mouse livers by immunohistochemistry and observed TIF1γ-positive cells in the space of Disse (peri-sinusoidal space) (Fig. S4A). Next, we isolated primary HSCs from normal and TAA-treated mouse liver tissues. Primary HSCs from TAA-treated liver tissue were shown to have decreased TIF1γ mRNA expression levels compared to HSCs from normal liver (Fig. S4B). Interestingly, the magnitude of the decrease was much greater in BALB/c-nude than in C57BL/6N mice. As per a previously published report, liver injury in BALB/c mice or results in severe fibrosis, whereas C57BL/6 mice develop comparatively minimal fibrosis(Shi et al., 1997).

Additionally, in normal livers, most of the TIF1γ-positive cells were stained with the HSC marker CRBP1(Van Rossen et al., 2009) (Fig. 5A; and Fig. S4A). In the damaged livers of 14-day-TAA-treated nude mice, TIF1γ-positive cells were significantly reduced in number (Fig. 5B). However, transplantation of hE-MSCs significantly prevented this reduction in TIF1γ-positive cells, resulting in the maintenance of normal live architecture and cells double positive for TIF1γ and CRBP1 (15.4 ± 1.7 cells in control (without treatment) vs. 8.6 ± 1.9 cells in TAA treatment vs. 13 ± 1.6 cells in TAA/hE-MSC treatment, $n \geq 4$) (Fig. 5A and B). These data indicated that TIF1γ is a potential anti-fibrosis factor expressed in HSCs that is

downregulated by profibrotic signals such as TAA or TGFβ1 and upregulated by anti-fibrosis therapy such as hE-MSC transplantation.

To test whether our findings in the mouse model can be extrapolated to humans, we conducted TIF1γ immunohistochemistry in human normal and cirrhotic livers having liver fibrosis grade as ISHAK6/METAVIR-F4 (Bataller, 2005; Standish et al., 2006). Similar to the mouse model, we observed that TIF1γ was expressed in the space of Disse of normal liver (Fig. 5C and D) and decreased in human cirrhotic livers in parallel to an increase in α-SMA (Fig. 5D).

Generation of TG mice with inducible, HSC-specific knockout of TIF1γ: knockout of TIF1γ accelerates liver fibrosis in mice

To elucidate whether loss-of-TIF1γ acts as an accelerator in fibrosis in vivo, we generated TG mice using the Cas9-ERT2 genome editing system, which is currently widely used to target genes of interest in animals (Staahl et al., 2017). We generated a Cas9-ERT2 fusion system for induction by TMX (Fig. S4C). ERT2 is known as a nuclear receptor that is transported into the nucleus by TMX (Indra et al., 1999). To inducibly knock out TIF1γ specifically in HSCs, we selected individual sites of exons 1, 2, 3 in TIF1γ for constructing guide RNAs. Moreover, we used the ~5-kb lecithin retinal acyltransferase (LRAT) putative promoter region for exclusive expression in HSCs, because LRAT is reported to be exclusively expressed in HSCs of the mouse liver (Mederacke et al., 2013). We generated three constructs targeting each exon of the TIF1γ gene: LRAT:Cas9-ERT2: sgTIF1γ$^{\mathbf{exon1}}$, LRAT:Cas9-ERT2: sgTIF1γ$^{\mathbf{exon2}}$, and LRAT:Cas9-ERT2: sgTIF1γ$^{\mathbf{exon3}}$ (Fig. S4D). Before generating TG mice by introducing the three constructs concomitantly into embryos to knock out TIF1γ in HSCs by TMX treatment, this Cas9-ERT2 system was validated *in vitro* using 293T and LX2 cells. In CMV promoter-Cas9-ERT2-transfected 293T cells, Cas9 was detected in the nuclear fraction

in addition to the cytosol upon TMX treatment, whereas Cas9 was not detected in the nuclear fraction but only in the cytosol without TMX treatment (Fig. S4E), demonstrating that TMX treatment induces translocation of Cas9 into the nucleus.

In LRAT:Cas9-ERT2: sgTIF1γ-transfected LX2 cells, TMX treatment decreased TIF1γ and increased αSMA (Fig. S4F). Multicolor immunofluorescence microscopic imaging (Fig. S4G) showed that TMX treatment induced the trans-localization of cytosolic Cas9 protein into the nucleus, leading to a reduction in nuclear TIF1γ and induction of α-SMA in the cytosol in LRAT:Cas9-ERT2: sgTIF1γ-transfected LX2 cells as compared to non-transfected cells.

The validated DNA constructs was injected in mouse embryos to target TIF1γ by guide RNAs and to generate LRAT:Cas9-ERT2: sgTIF1γ-TG mice. We treated the mice with TAA only twice, to prevent severe fibrosis. Liver injury with TAA and TMX induction aggravated liver fibrosis in TG mice as compared with TMX/TAA-treated wild-type mice and corn oil/TAA-treated TG mice. TMX/TAA-treated wild-type mice, as well as corn oil/TAA-treated TG mice, showed a nearly normal liver phenotype, as indicated by Picro-Sirius red staining (2.9 ± 0.8% in TMX/TAA-treated wild-type mice vs. 2.1±0.4% in corn oil/TAA-treated TG mice vs. 8.1±1.6% in TMX/TAA-treated TG) (Fig. 6A and B; and Fig. S5 A-C). In the liver function test, TMX/TAA-treated TG mice showed significantly worse profiles of ALT, AST and hydroxyproline than did TMX/TAA-treated wild-type mice and corn oil/TAA-treated TG mice (ALT; 7.0 ± 2.9 in TMX/TAA-treated wild-type mice vs. 6.8 ± 0.7 in corn oil/TAA-treated TG mice vs. 17.4 ± 3.6 in TMX/TAA-treated TG) (AST; 9.1 ± 2.4 in TMX/TAA-treated wild-type mice vs. 8.6 ± 1.0 in corn oil/TAA-treated TG mice vs. 11.6.4 ± 1.8 in TMX/TAA-treated TG mice) (Hydroxyproline; 419.16 ± 27.65 in TMX/TAA-treated wild-type mice vs. 381.26 ± 34.63 in corn oil/TAA-treated TG mice vs. 570.54 ± 42.35 in TMX/TAA-treated TG) (Fig. 6C and D).

Western blotting and immunofluorescence results validated TIF1γ knockout and an increase in αSMA expression in TMX/TAA-treated TG mice (Fig. 6E and F). Limited changes in control (TMX/TAA-treated wild and corn oil/TAA-treated TG) as compared with normal wild-type mice were observed, indicating only a slight effect of the TAA treatment. However, TMX/TAA-treated TG mice showed lower TIF1γ and higher αSMA expression than control mice, which corresponded with the Picro-Sirius red, ALT, AST and Hydroxyproline results.

Structural changes within the fibrosis were analyzed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM). We observed four important pathological findings in the livers of TMX/TAA-treated TG mice compared with corn oil/TAA-treated littermates: 1) loss of fenestrae of liver sinusoidal endothelial cells (LSEC), 2) loss of microvilli on hepatocytes, 3) loss of lipid droplets in HSCs, and 4) collagen fibrils surrounding HSCs (Fig. 6G). SEM indicated loss of fenestrae of LSEC and emergence of collagen fibrils, by the TEM findings (Fig. 6G). Hepatic fibrosis showed several features in previous studies (Braet and Wisse, 2002; Elpek, 2014): 1) conversion of normal sinusoidal architecture to defenestrated capillarization of the sinusoidal endothelium, 2) transformation of fat-storing HSCs into collagen-secreting myofibroblasts, and 3) loss of hepatocyte microvilli.

Together, these findings in TG mice indicated that knockout of TIF1γ in HSCs accelerated liver fibrosis in response to injury.

Validation of anti-fibrosis action of TIF1γ using primary HSCs

We isolated HSCs from mice (Fig. 7A) using a previously reported method (Mederacke et al., 2015) to confirm whether the phenomenon observed in mice *in vivo* could be reproduced in primary HSCs. Isolated HSCs from normal mouse liver were plated on a cell-culture dish and were treated with mTGFβ and hHGF. In accordance with the LX2 *in-vitro* and the *in-vivo* results, TIF1γ was decreased by TGFβ and increased by HGF (Fig. 7B). Also we confirmed

that HSCs display a fibrotic character due to TIF1γ reduction (Fig. 7C), as α-SMA was rapidly increased by siTIF1γ, morphological changes indicating fibrosis were observed, and retinol was lost. Finally, we confirmed that HSCs prepared from Lrat:Cas9-ERT2: sgTIF1γ TG mice also showed reduced TIF1γ upon treatment with TMX *in vitro*. Similar to the results *in vivo,* knockout of TIF1γ by TMX treatment induced the activation of HSCs, including an increase in αSMA (Fig. 7D). In conclusion, these data confirmed that, in primary HSCs as well as LX2 cells, the αSMA level regulated by TIF1γ.

Discussion

Stem cell therapy is a promising treatment option for liver fibrosis, which often requires liver transplantation. However, human adult stem cells, such as bone marrow-derived MSCs, do not always show sufficient efficacy in clinical trials (Mansilla et al., 2011; Ogden and Mickliem, 1976; Rando, 2006), and the efficacy depend on donor age, underlying diseases, and individual variations.

In our previous study, we successfully established a standard method to derive a large amount of hE-MSCs from a single preparation of hESCs, which can be generated and stored according to HLA type and thus, can be used as an off-the-shelf source of allogeneic stem cells (Lee et al., 2010). Therefore, we suggest that hE-MSCs are an ideal source of stem cells for regenerative medicine avoiding some limitations of adult stem cells. In the current study, we found that transplantation of hE-MSCs in mouse fibrotic liver slowed down fibrosis and accelerated functional recovery of the injured liver. Normal serum levels of AST and ALT indicated that the effect of hE-MSCs was sustained systemically as well as at the target site, suggesting that hE-MSCs administered by intracardiac injection could reach and act in the injured organ. Moreover, we revealed that the administered hE-MSCs were homed to the damaged liver, where they released HGF, which induces TIF1γ in HSCs and prevents them

from becoming myofibroblast-like cells, which are principal producers of extracellular matrix during liver fibrosis. The mechanisms underlying stem cell-based therapies are still under investigation; nevertheless, our results suggest that the mechanism of action of hE-MSCs in the prevention of liver fibrosis may be the secretion of paracrine factors that block the molecular mechanism for progression of fibrosis.

The pathophysiologic role of TGFβ in the liver is well known; TGFβ is crucial from initial liver injury through inflammation and fibrosis, to cirrhosis and cancer (Fabregat et al., 2016). In liver fibrosis, TGFβ induces the activation and transformation of HSCs to αSMA-expressing myofibroblasts. TGFβ signaling leads to phosphorylation of the signal mediators SMAD2/3 and interaction with SMAD4 (Massague et al., 2005). The SMAD complex can bind to DNA through the SBE and activate αSMA expression (Dennler et al., 1998; Meng et al., 2016). Therefore, targeting of TGFβ signaling in HSCs might be useful in therapy to prevent liver fibrosis.

TIF1γ has been suggested to inhibit TGFβ signaling through competition with SMAD4 for binding to the activated SMAD2/3 in the differentiation of embryo, stem cells, and epithelial-to-mesenchymal transition of mammary epithelial cells (He et al., 2006; Heldin and Moustakas, 2006; Hesling et al., 2011; Massague and Xi, 2012). This study revealed the interaction TIF1γ with SMAD2/3 and the binding of this complex to the *αSMA* promoter in the human HSC cell line, LX2. A PLA showed that TIF1γ interacts with SMAD2/3 in the cytoplasm as well as in the nucleus in naïve LX2. The interaction was decreased by TGFβ, whereas HGF effectively restored the interaction in the nucleus. This suggests that the interaction can occur in the cytosol and nucleus without stimulator, and in response to the positive stimulator HGF, the complex translocates to the nucleus to exert its function. Next, we performed ChIP using TIF1γ antibody to know whether TIF1γ–SMAD2/3 complex can bind the *αSMA* promoter. Structural-functional domains of TIF1γ have been reported (Heldin and

Moustakas, 2006; Venturini et al., 1999); however, a DNA-binding sequence for binding to the promoter as a transcription factor has not been reported. Therefore, the SBE sequence was used to detect the binding of TIF1γ–SMAD2/3 complex to the *αSMA* promoter after pulldown using TIF1γ antibody. The ChIP data showed that TIF1γ binds with SMAD2/3 complex to repress *αSMA* expression.

For successful repression of αSMA by TIF1γ, the mechanism regulating the TIF1γ level needs to be known. Among several transcription factor-binding sites in the *TIF1γ* promoter region, mutation of the CREB-binding site was eliminated the response to HGF, and binding of activated CREB to the *TIF1γ* promoter was enhanced by HGF.

In conclusion, we suggest the following mechanisms of TIF1γ in HSCs during fibrotic stimulation and restoration (Fig. 8): suppression of CREB phosphorylation reduces TIF1γ expression and the interaction with SMAD2/3, whereas upregulation of TIF1γ enhances the interaction with SMAD2/3 and inhibits αSMA expression. Experiments in TG mice with inducible, HSC-specific knockout of TIF1γ demonstrated that TIF1γ has potential as a novel therapeutic approach for the prevention of liver fibrosis. In addition to cell therapy using hE-MSCs, gene therapy would be feasible to directly increase or activate TIF1γ. For this purpose, we developed the construct, which induces the expression of this gene in the inflamed liver undergoing fibrosis, such as, TGFβ1-promotor-driven TIF1γ. This construct could be selectively delivered to liver stellate cells using retinol-liposome conjugate because liver stellate cells uptake and store retinol (Sato et al., 2008).

Materials and methods

Study design.

All animal study protocols were approved by the Institutional Animal Care and Use Committee (IACUC 17-0040-S1A0) of Seoul National University Hospital, Korea. Male above 12-week-

old BALB/c-nude mice (Orientbio, Korea) weighing 20–25 g were used for experiments. Mice were administered an intraperitoneal injection of 200 mg/kg thioacetamide (TAA; Sigma-Aldrich, St. Louis, MO, USA) to induce liver fibrosis or phosphate-buffered saline (PBS) as a control three times a week for 1–3 weeks. We used TAA instead of carbon tetrachloride (CCl_4), which is commonly used to establish animal models of liver fibrosis, because, in South Korea, CCl_4 has been banned from usage under the 'Montreal Protocol'. TAA-treated mice were randomly assigned into two groups receiving one time hE-MSCs or PBS via intracardiac injection. Alternatively, TAA-treated mice were injected pCMV-hHGF(18ug/head)(HG10463-UT pCMV3-HGF, Sino Biological,USA) vector via intraperitoneal at 0, 4, 12day. This study was approved by the Institutional Review Board (IRB) of Seoul National University Hospital, Korea (IRB no.1410-093-619).

LX2 culture.

The human hepatic stellate cell line LX2 was a generous gift from Dr. Friedman. 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% CO_2. Mycoplasma contamination risk of cells used in this study was assessed via MycoQsearch^{TM} Mycoplasma Real-Time PCR Detection Kit (CellSafe, Seoul, Korea).

Loss- and gain-of-function analyses.

Loss of function was analyzed in LX2 cells after siRNA-mediated knockdown of TIF1γ (sc-63127, Santa Cruz Biotechnology), EPLIN (sc-60593, Santa Cruz Biotechnology), or Nm23-H1 (sc-29414, Santa Cruz Biotechnology). LX2 cells were transfected with siRNA in DMEM GlutaMax without FBS using Metafectene-Pro (Biontex, Germany) for 7 h. Then, the medium

was changed to complete fresh medium, and cells were incubated for 1 to 4 days without a change of medium. Lentiviral vector of shTIF1γ(Cat. TL300849, OriGENE, USA) was transfected in LX2 for 24hrs. Then, the medium was changed to complete fresh medium, and cells were incubated with hTGFβ1(5ng/ml) for 3 days.

RT-qPCR analysis.

Total RNA was isolated from cultured cells using the QIAshredder and RNeasy Plus Mini kit (Qiagen, Venlo, Netherlands) according to the manufacturer's instructions. cDNA was synthesized from 1 μg RNA using the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Tokyo, Japan). qPCR was carried out using the Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA, USA) in an ABI PRISM-7500 sequence detection system (Applied Biosystems). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control to calculate relative changes in gene expression. Primers were designed using the Primer3 software (Whitehead Institute/MIT Center for Genome Research) and synthesized by Bioneer (Seoul, Korea). The following primers were used: GAPDH, forward: 5′-CAACGAATTTGGCTACAGCA-3′, reverse: 5′-TGTGAGGAGGGGAGATTCA-3′; αSMA, forward: 5′-GGCAAGTGATCACCATCGGA-3′, reverse: 5′-TCTCCTTCTGCATTCGGTCG-3′; TIF1γ, forward 5′-CTCCGGGATCATCAGGTTTA-3′, reverse: 5′ ACTGCTCAACATGCAAGCAC-3′; Nm23-H1, forward 5′-GCCTGGTGAAATACATGCAC-3′, reverse: 5′-AGTTCCTCAGGGTGAAACCA-3′; EPLIN, forward 5′-CTGCGTGGAATGTCAGAAGA3′, reverse: 5′-TTTTGCTTGCCCATAGATCC-3′; PIAS1, forward 5′-CATCGCCATTACTCCCTGTT-3′, reverse: 5′-AAGCGCTGACTGTTGTCTGA-3′; ALR, forward 5′-CCTGTGAGGAGTGTGCTGAA-3′, reverse: 5′-TCCACTTTTGAGCAGTCGAA-3′;

MBNL1, forward 5′-CAGCCGCCTTTAATCCCTAT-3′, reverse: 5′-TGTCAGCAGGATGAGCAAAC-3′.

hE-MSC culture.

hE-MSCs were obtained as previously described (7). In brief, SNUhES3 hESCs (Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University Hospital, Seoul, Korea) were cultured in culture dishes without fibroblast growth factor-2 to establish embryonic bodies (EBs) at 37°C with 5% CO_2 for 14 days. The EBs were attached to gelatin-coated dishes for 16 days in low-glucose Dulbecco's modified Eagle's medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen), and the derived cells were expanded in EGM-2MV medium (Lonza). The expanded hE-MSCs were tested for differentiation into adipocytes, osteocytes, myocytes, and chondrocytes under the appropriate conditions to evaluate their differentiation potential. For in vitro and in vivo experiments, hE-MSCs of passage (P)13–14 were used.

To knock down HGF, hE-MSCs were transducted with shHGF lentivirus (IFU $5X10^5$) specific to HGF (Cat.TL312467V, Origene, USA) and the knock downed was verified via ELISA. Mycoplasma contamination risk of cells used in this study was assessed via MycoQsearch™ Mycoplasma Real-Time PCR Detection Kit (CellSafe, Seoul, Korea).

Coculture.

LX2 cells were plated on 10-cm dishes (2×10^5 cells/mL) (Nunc, Thermo Scientific, Waltham, MA, USA)and incubated at 37°C with 5% CO_2 for 2–3 days. When cultures reached approximately 50% confluence, the medium was changed to fresh medium containing 0.5% FBS and the cells were treated with 5 ng/mL of hTGFβ1 (R&D Systems) every other day for

4 days. The medium was changed at every treatment with the cytokine. LX2 cells pretreated with hTGFβ1 were cocultured with 8×10^5 hE-MSCs per dish in a transwell insert (0.4-μm pore size) (Corning, Corning, NY, USA) in complete fresh medium containing 0.5% FBS and 5 ng/ml hTGFβ1, and samples were harvested after coculture. Alternatively, hE-MSCs treated with shRNA specific to HGF were cocultured with LX2 cells activated with hTGFβ1, after which 10 ng/mL or 20 ng/mL of rhHGF (R&D Systems) was added.

Western blot assay.

Cells or tissue samples were lysed in protein lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP-40, 0.1% SDS with protease inhibitor cocktail [Roche, Indianapolis, IN, USA]). Total protein extracts (25–30 μg) were boiled for 5 min at 95°C, separated by SDS-polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Merck, Darmstadt, Germany) using a BioRad transfer unit (BioRad, Hercules, CA, USA). Membranes were blocked with 5% skim milk diluted in Tris-buffered saline containing 0.1% Tween-20 and incubated with antibodies against TIF1γ (1:1000 Abcam, ab84455, Human TRIM33 aa 1077-1127 (C terminal)), αSMA (1:3000; SigmaAldrich, C6198, N-terminal synthetic decapeptide of α-smooth muscle actin), EPLIN (1:500; Abcam,ab50196, synthetic peptide: GVLAASMEAK ASSQQEKEDK PAETKKLRIA WPPPTELGSS GSALEEGIKM, corresponding to amino acids 502-551 of Human EPLIN), and anti-Nm23-H1 (1:1000; Santa Cruz Biotechnology, sc-465, purified nm23-H1 of human origin); anti-α-tubulin antibody (1:5000; Sigma-Aldrich, T6199, the C-terminal end of the α-tubulin isoform (amino acids 426-430)) or anti-GAPDH antibody (1:3,000; Abcam, ab9485, Full length native protein (purified) corresponding to Human GAPDH) were used to detect internal control proteins. Membranes were washed and incubated with horseradish peroxidase-conjugated

secondary antibodies(Jackson ImmunoResearch Laboratories, USA), washed, and immunoreactive bands were detected using Luminata Classicco (Merck).

Luciferase assay.

LX2 cells were seeded in 12-well plates(Nunc, Thermo Scientific, Waltham, MA, USA) and transfected with 100 ng firefly and 10 ng Renilla constructs (Promega, USA) using FuGENE HD (Promega, Madison, WI, USA) for 6 h. Luciferase activity was measured following to the manufacturer's protocols using GLOMAX 20/20 luminometer (Promega).

Enzyme-linked immunosorbent assay (ELISA).

The secretion of HGF in cell culture supernatants was analyzed by ELISA using the hHGF ELISA kit (Cusabio Biotech, China) according to the manufacturer's protocols. The absorbance at 450 nm was measured using a Multiskan GO Microplate Spectrophotometer (Thermo Scientific, Waltham, MA, USA).

hHGF in the serum was detected using hHGF ELISA kit (SHG00B/R&D) in accordance with the manufacturing instruction. The serum was prepared using serum collection tube (microtainer, 365967, BD) and diluted 1:2 with calibrator diluent RD59 buffer provided from the kit. Briefly, the samples, standard of recombinant hHGF, and positive control were added to the pre-coated 96-well, washed and treated with the detection antibody and HRP conjugated antibody. The visualization of the captured hHGF was accomplished with 3,3',5,5'-Tetramethylbenzidine (TMB) substrate solution. The yellow intensity was detected using Glomax (Promega) system at 450nm normalized at 570nm. The calculations were performed automatically in https://www.hycultbiotech.com/elisa_calculationsheet.

Subcellular protein fractionation.

293T cells (ATCC, VA, USA) were collected in a tube after trypsinization. Nuclear-cytoplasmic buffer (0.5% digitonin prepared in PBS containing a phosphatase and protease inhibitor cocktails) (Genedepot, TX, USA) was added at 4-fold the packed cell volume and pipetted carefully. After centrifugation, the supernatant was retained as the cytoplasmic fraction, and the pellet was resuspended in RIPA buffer(Thermo Scientific, USA). Lysate in RIPA was retained as the nuclear fraction. Alpha-tubulin (anti-α-tubulin antibody (1:5000; Sigma-Aldrich, T6199, the C-terminal end of the α-tubulin isoform (amino acids 426-430)) was assayed as a cytoplasmic fraction-specific marker, and lamin A/C(cat.2032S ,Cell signaling, endogenous levels of total full length lamin A) was used as a nuclear fraction-specific marker.

Proximity ligation assay.

All procedures refer to the method provided by the Duolink kit (DUO92101-1KT, Sigma-Aldrich). LX2 cell were treated with 4%PFA(163-20145-P01, Wako, USA) for 15min, permeablized with 0.5% triton X-100 (cat.T8787, SigmaAldrich) in PBS. Blocking step was performed with blocking solution provided from this kit for an hour, then primary antibody were treated anti-TIF1γ (1:100; Abcam, ab47062, Synthetic peptide corresponding to Human TRIM33 aa 600-700 conjugated to keyhole limpet haemocyanin) and anti-Smad2/3 (1:50, Santacruz, sc-6032, recombinant Smad2 of human origin) at 4℃ for 16hrs. Samples washed with wash buffer A for 3times and Duolink PLA probe plus and minus treated for a hour at 37℃. The ligation and amplification steps were performed to visualize signal. Images were acquired using a confocal microscope (LSM710; Carl Zeiss, Göttingen, Germany) and analyzed with ImageJ (National Institutes of Health, Bethesda, MD, USA) software.

Chromatin immunoprecipitation (ChIP) assay.

LX2 cells were treated with 1% formaldehyde (F8775, SigmaAldrich) for crosslinking for 10 min, and the reaction was quenched using 125 mM glycine((G8898, SigmaAldrich) for 5 min. The cells were then lysed using ChIP buffer (50 mM, NaCl 150 mM Tris-HCl pH 8.0, 0.5% Triton X-100, 1 mM EDTA, 0.1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS] including 1× Xpert protease inhibitor cocktail [GeneDEPOT, Barker, TX, USA]) and sonicated to shear the DNA to 200–500-bp fragments using a BIORUPTOR sonicator (Diagenode, Denville, NJ, USA). The target antibody was added, and the protein A/G agarose bead (Abcam) was added to pull down the target DNA. Beads were washed 3 times sequentially with different wash buffers (low-salt buffer: 20 mM Tris-HCl, 150 mM NaCl, 0.5% Triton X-100, 0.1 mM EDTA; high-salt buffer: same as low-salt buffer except for 500 mM NaCl; lithium-chloride buffer: same as low-salt buffer except for 250 mM LiCl instead of NaCl). The samples were heated to 65°C for 4 h to remove the crosslinks. The DNA fragments were recovered using a PCR purification kit (cat. 28106, Qiagen, Netherland) and analyzed using qPCR and semi-quantitative PCR.

The following primer list was used for ChIP on hSMA promoter (5’ to 3’)

-84 SBE F	GAGAGTTTTGTGCTGAGGTCCC
-84 SBE R	CCTGCTCTCCTCCCACTTGC
-602 SBE F	AAGGATGGTCCCTACTTATGCTG
-602 SBE R	GGGAGGTGAGTGGAAATAGGAA
Non SBE F	AAAGGTGGGAAATGGAAAGG
Non SBE R	TCTGCTGGTGCCGAAAAAT

Antibodies(Epitope).

Mouse monoclonal [7A9-3A3] a-CRISPR-Cas9 (ab191468; Abcam, Cambridge, UK, Recombinant fragment corresponding to Streptococcus pyogenes CRISPR-Cas9 (N terminal)), rabbit polyclonal a-HGF (ab83760; Abcam, synthetic peptide corresponding to a region within

the N-terminal sequence 108-157 (VKKEFGHEFD LYENKDYIRN CIIGKGRSYK GTVSITKSGI KCQPWSSMIP) of Human HGF, NP_001010932.), mouse monoclonal [24612.111] a-HGF (ab10678; Abcam, Recombinant human hepatocyte growth factor (rhHGF) expressed in the insect cell line Sf 21), mouse monoclonal a-CRBP1 (sc-271208; Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1-135aa of CRBP1 of human origin), rabbit polyclonal a-CRBP1 (sc-30106; Santa Cruz Biotechnology), rabbit polyclonal a-TIF1 gamma (ab47062; Abcam, Synthetic peptide corresponding to Human TRIM33 aa 600-700 conjugated to keyhole limpet haemocyanin.), and mouse monoclonal CAS9 (ab191468, Abcam, Recombinant fragment corresponding to Streptococcus pyogenes CRISPR-Cas9 (N terminal)) were used for immunofluorescence. Rat monoclonal [YOL1/34] a-alpha tubulin (sc-53030, Santa Cruz Biotechnology, raised against full length purified alpha tubulin of Saccharomyces cerevisiae origin), rabbit polyclonal a-EPLIN (ab50196, Abcam, A region within synthetic peptide: GVLAASMEAK ASSQQEKEDK PAETKKLRIA WPPPTELGSS GSALEEGIKM, corresponding to amino acids 502-551 of Human EPLIN), mouse monoclonal a-Nm23-H1 (sc-56928, Santa Cruz Biotechnology, raised against full length nm23-H1 of human origin), rabbit polyclonal a-alpha smooth muscle actin (ab5694, Abcam, raised against a synthetic peptide corresponding to N-terminus of actin from human smooth muscle), rabbit polyclonal a-TIF1gamma (ab84455, Abcam, Synthetic peptide within Human TRIM33 aa 1077-1127 (C terminal)), rabbit polyclonal a-GAPDH (ab9485, Abcam, Full length native protein (purified) corresponding to Human GAPDH.), and mouse monoclonal CAS9 (ab191468, Abcam, Recombinant fragment corresponding to Streptococcus pyogenes CRISPR-Cas9 (N terminal)) were used for immunoblot assays. Rabbit monoclonal a-CREB (9197s, Cell Signaling, recombinant protein specific to the amino terminus of human CREB-1 protein) and rabbit monoclonal a-S133 P-CREB (9198s, Cell Signaling, synthetic phosphopeptide corresponding

to residues surrounding Ser133 of human CREB) were used for ChIP assay and western blot analysis.

Immunohistochemistry.

After blood collection, mouse livers were perfused with cold PBS and removed. The livers were fixed in a 10% neutral formalin solution, embedded in paraffin, and cut into serial sections (4–5 μm thick). Paraffin sections were stained with hematoxylin and eosin (H&E) (ab24588, abcam), MT(CS-MTRI, IHC world, MD, USA), or Picro-Sirius red(365548, SigmaAldrich) using standard protocols. MT and Picro-Sirius red staining were 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 hE-MSCs, the percentage of the fibrotic liver area was estimated by quantitative image analysis of MT- and Picro-Sirius red-stained sections using the SABIA (Metoosoft, Seoul, Korea) and ImageJ (National Institutes of Health, Bethesda, MD, USA) software packages. The degree of liver fibrosis was represented according to the METAVIR scale or ISHAK stage, which grade fibrosis from F0 (no fibrosis) to F4 (cirrhosis) and from 0 (no fibrosis) to 6 (cirrhosis), respectively.

Human liver tissues purchased from SuperBioChip Laboratories (Seoul, Korea) and the paraffin-embedded tissue sections were deparaffinized in xylene and rehydrated in graded alcohol. After sections were subjected to heat-mediated antigen retrieval with citrate buffer (DAKO, Glostrup, Denmark), non-specific binding sites were blocked with 1% bovine serum albumin in PBS containing 0.01% Triton X-100. Depending on the antibody used, permeabilization was optionally conducted with 0.1% Triton X-100 in PBS for 10 min before blocking. Then, the tissue sections were incubated with the following primary antibodies overnight at 4°C: anti-TIF1γ (1:1000; Abcam), anti-cellular retinol-binding protein 1 (CRBP1, 1:100; Santa Cruz Biotechnology), anti-αSMA (1:800; Sigma-Aldrich), anti-hepatocyte

(Hepatocyte Paraffin-1; Hep Par-1) (1:300; DAKO), anti-CAS9 (1:50; Abcam), or anti-HGF (1:100; Abcam). After washing, the sections were incubated with secondary Alexa Fluor-conjugated antibodies (Invitrogen) for 2 h at room temperature, washed with PBS, and mounted in fluorescence mounting medium with 4′,6-diamidino-2-phenylindole (IHC World, Woodstock, MD, USA). Images were acquired using a confocal microscope (LSM710; Carl Zeiss, Göttingen, Germany).

Construction of TMX-inducible TIF1γ- knockout vector.

pCAG-ERT2CreERT2 (cat. No. 13777) and pX330-U6-Chimeric_BB-CBh-hSpCas9 (cat. No. 42230) plasmids were purchased from Addgene, and murine TIF1γ CRISPR/Cas9 KO plasmid (sc-430111) was purchased from Santa Cruz Biotechnology. The murine LRAT promoter sequence was predicted (http://gpminer.mbc.nctu.edu.tw/index.php), and a region (–5,500 to +72 bp) with predicted high activity was selected. The LRAT promoter was obtained by PCR (F: 5′-GACATTGATTATTGACTAGTCCTTAAAGAGAGGCATCCGGGGTC-3′, R: 5′-GTTCTTCTCCTTTGCTAGCCATGACGCTCACGCTAAAGAGCTTGAAG-3′) using normal murine DNA (C57BL/6N). To analyze LRAT promoter activity, the CMV promoter of pcDNA DEST47 was replaced with the LRAT promoter. To generate the LRAT promoter-dependent and TMX-induced TIF1γ-knockout construct, the CAG promoter and Cre of pCAG-ERT2CreERT2 were replaced with the LRAT promoter and SpCas9. Next, three gRNAs for TIF1γ with the U6 promoter (gRNA 1: 5′-GGTGCGGCTGGGCCCGACGA-3′, gRNA 2: 5′-CTACATTCTTGACGACATAC-3′, gRNA 3: 5′-GAAGATAATGCAAGTGCAGT-3′) were inserted into the plasmids. pLRAT:Cas9-ERT2: sgTIF1γ constructs were confirmed by Sanger sequencing.

Generation of TMX-inducible TIF1γ- knockout mice.

Transgenic (TG) mice expressing pLRAT:Cas9-ERT2: sgTIF1γ were generated and interbred in pathogen-free conditions at Macrogen (Seoul, Korea). All manipulations were conducted with the approval of Macrogen Institutional Animal Care and Use Committee. To prepare embryos, C57BL/6N female mice of 5–8 weeks of age were intraperitoneally injected at 48h intervals with pregnant mare serum gonadotropin (7.5 IU) and human chorionic gonadotropin (5 IU) for superovulation. After the injections, the female mice were mated with C57BL/6N stud male mice. Female mice with vaginal plugs were sacrificed, and fertilized embryos were harvested. The three pLRAT:Cas9-ERT2: sgTIF1γ DNAs were linearized, and the same concentrations of these constructs were microinjected into one cell of each embryo using standard microinjection procedures (Macrogen, Seoul, Korea). Briefly, 4 ng/μL of a mixture of the three constructs was injected directly into the pronucleus of the zygote using a micromanipulator, and microinjected embryos were incubated at 37°C for 1–2 h. Fourteen to sixteen injected one-cell-stage embryos were transplanted by surgical methods into the oviducts of pseudopregnant recipient mice (ICR). Founders were identified by PCR using tail genomic DNA and primers specific to Cas9-ERT2 (F: 5′-TGCTACAGAACAGTTGCAGCC-3′, R: 5′-ACCTTGTACTCGTCGGTGATC-3′) and TIF1γ gRNAs (U6-F: 5′-GTCGACGAGGGCCTATTTCCCATGATT-3′, gRNA1-R: 5′-TCGTCGGGCCCAGCCGCACC-3′, gRNA2-R: 5′-GTATGTCGTCAAGAATGTAG-3′, and gRNA 3-R: 5′-ACTGCACTTGCATTATCTTC-3′). After generation to F2, male 12-week-old mice were used for experiments.

Serum assays.

Blood samples were drawn from the hearts of anesthetized mice at 14 days after transplantation. 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. Alternatively, AST (ab105135, Abcam) and ALT (ab105134, Abcam) assays were performed using the colorimetric method in TG mice. The substrates, glutamate, and pyruvate, were used for generating standard curves to measure enzyme amounts. AST and ALT activities were determined following the manufacturer's procedure and were expressed as mU/ml.

Hydroxyproline Assay

The hydroxyproline from collagen was detected using hydroxyproline assay kit (ab222941/abcam) according to the manufacturing instructions. Briefly, 10 mg of liver tissue was homogenized with distilled water and boiled including the same volume of 10N NaOH for 1hr and neutralized using 10N HCl. The precipitant was centrifuged and collected. An amount of 33ug/10uL samples then were dried on 65 ℃ hot plate. Measurement at 560nm absorbance was obtained using GloMax (Promega) system and the following formula was used for calculation. Hydrolyzed hydroxyproline concentration = B (Amount of hydroxyproline) / V (sample volume) × D (Dilution factor).

Electron microscopy.

Liver samples for electron microscopy were fixed with 2.5% glutaraldehyde and cut into ~1-mm^3 pieces for TEM. Briefly, after dehydration, thin sections were stained with saturated uranyl acetate and lead citrate and observed under a JEM-1400 Plus transmission electron microscope. For SEM, each sample was ion-sputter-coated and observed with a Hitachi S-4700 scanning electron microscope.

Primary HSC isolation and culture.

Primary HSCs were cultured following the protocol reported by Mederacke et al(Mederacke et al., 2015). For *in situ* digestion, mice were sequentially perfused with EGTA, pronase, and collagenase solutions. Liver tissues were separated and digested *in situ* with 1% DNase for 25 min. After passage through a strainer, the cells were washed and aspirated with GBSS two times. HSCs were separated by centrifugation at 1,380 × *g* on Nycodenz density gradient for 17 min without a brake. HSCs were cultured in Dulbecco's modified Eagle's medium containing 10% FBS.

Statistical analysis.

Statistical analysis was performed using the GraphPad Prism 6 software (GraphPad Software, La Jolla, CA, USA). Data expressed as the mean ± standard deviation. Differences between groups were analyzed by the unpaired *t*-test or one-way analysis of variance (1-way ANOVA). *P*-values <0.05 were considered statistically significant.

A short summary paragraph of supplemental material

Fig. S1 shows the process for selection of *TIF1γ* using in vitro cell system. Fig. S2 shows the expression of hHGF by hE-MSC or shHGF or pCMV-hHGF in vivo and in vitro. Fig. S3 shows the screening and analysis of transcription factor on TIF1γ promoter. Fig. S4 shows the expression of TIF1γ in normal mouse liver or HSC, and validation for development of TG mice with tamoxifen-inducible and HSC-specific knockout of *TIF1γ*. Fig. S5 shows the observation of TG mice with tamoxifen-inducible and HSC-specific knockout of *TIF1γ*. Table S1 shows six candidate of anti-fibrosis factors. Table S2 shows primers for mRNA of hHGF or mHGF in mouse liver.

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Figure Legends

Figure 1. hE-MSC transplantation represses liver fibrosis after injury in mice. (**A**) Experimental scheme of hE-MSC transplantation into mice having a liver injury by TAA administration. Quantification of liver fibrosis by MT staining in 3 groups (normal, TAA and TAA/hE-MSCs. Mice, n = 5 in each group, complied from two independent experiments). Quantification of the fibrotic area is presented as the blue portion (%) in total area, including red+blue portions. *P < 0.05. Scale bar 200 ㎛. (**B**) Picro-Sirius red staining of fibrotic areas in the livers at 14 days after hE-MSC transplantation. Quantification of liver fibrosis by Picro-Sirius red staining in 3 groups (normal, TAA and TAA/hE-MSCs. Mice, n = 5 in each group, complied from two independent experiments). *P < 0.05. Scale bar 200 ㎛. (**C**) Measurement of fibrous septa using Picro-Sirius red staining. Portal-to-portal fibrous bridging/septa were counted per 2.7 × 3.4 mm^2 (TAA vs. TAA/hE-MSCs. n=5). Scale bar 200 ㎛. Data were expressed as the mean ± standard deviation. *P < 0.05. (**D**) Serum levels of hepatotoxicity indicators AST and ALT at 14 days (normal, TAA and TAA/hE-MSCs. Mice, n = 5 in each group, complied from two independent experiments). (A,B,D) Statistically analyzed by 1-way ANOVA. C Statistically analyzed by unpaired Student's *t* test.

Figure 2. TIF1γ is a repressor for myofibroblastic activation of human hepatic stellate LX2 cells. (**A**) RT-qPCR analysis of 6 genes after TGFβ1 treatment. *P < 0.05. Statistically analyzed by unpaired Student's *t* test. Mean vales ± SD were calculated from three independent experiments, with biological triple in each experiment. (**B**) Representative western blot of TIF1γ, EPLIN, and Nm23-H1 expression in TGFβ1 treated LX2 cells with or without hE-MSCs. Quantified by Image J program. Reproducible result from three independent

experiments was shown. Mean vales ± SD were calculated from three independent experiments, with biological triple in each experiment. *P < 0.05. ****P < 0.0001. (**C**) Representative western blot of TIF1γ, EPLIN, and Nm23-H1, and αSMA expression in LX2 cells transfected with siEPLIN, siNm23-H1, or siTIF1γ for gene knockdown. Quantified by Image J program. Reproducible result from three independent experiments was shown. Mean vales ± SD were calculated from three independent experiments, with biological triple in each experiment. *P < 0.05. **P < 0.01. **(D)** Representative western blot of TIF1γ and αSMA expression in TGFβ1 treated LX2 cells transduced by lentiviral vector CMV- TIF1γ. Quantified by Image J program. Results represent the mean ± standard deviation of three independent biological experiments. *P < 0.05. **P < 0.01. **(E)** RT-qPCR analysis of αSMA gene in TGFβ1 treated LX2 cells transduced by lentiviral vector CMV-TIF1γ. *P < 0.05. Results represent the mean ± standard deviation of three independent experiments, with biological triple in each experiments. Statistically analyzed by 1-way ANOVA.

Figure 3. Induction of TIF1γ by HGF and complex formation with SMAD. (**A**) Experimental scheme of hE-MSC or shHGF hE-MSCs or pCMV-hHGF vector transplantation into mice having a liver injury by TAA administration. Representative histochemical staining of MT and Picro Sirius Red staining in each group (Each panel from independent individual). Quantification of liver fibrosis by Picro Sirius Red staining in 5 groups (normal, TAA and TAA/hE-MSCs, TAA/shHGF hE-MSCs, TAA/pCMV-hHGF. n=3, 5, 5, 5, 5, respectively) was presented as the red portion (%) in total area. One or two fields in each sample were measured by Image J program. Mean vales ± SD were calculated from measured fields of each sample in each group. Scale bar 10 μm. ***P < 0.001. ****P < 0.0001. (B) Serum levels of hepatotoxicity indicators AST and ALT at 14 day (normal, TAA and TAA/hE-MSCs, TAA/shHGF hE-MSCs, TAA/pCMV-hHGF. n=3, 3, 3, 4, 4, respectively). Mean vales ± SD were calculated from three

or four samples with technical duplicates in each sample. $^{**}P < 0.01$. $^{***}P < 0.001$. (C) ELISA of hHGF in mouse serum (normal, TAA and TAA/hE-MSCs, TAA/shHGF hE-MSCs, TAA/pCMV-hHGF. n=3, 3, 3, 4, 4, respectively). Recombinant mHGF used as a negative control (625, 2500, 10000 pg/ml) which did not show reactivity with hHGF antibody. hHGF antibody(AF-294-NA, R&D system) used in ELISA reactive with hHGF not mHGF. Mean vales ± SD were calculated from three or four samples with technical duplicates in each sample. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. (D) Representative western blot of TIF1γ and αSMA expression in 5 groups (normal, TAA and TAA/hE-MSCs, TAA/shHGF hE-MSCs, TAA/pCMV-hHGF). Each lane presented independent individual. Reproducible result from two independent western blot was shown. Quantified by Image J program. Mean vales ± SD were calculated from two or six bands in two independent western blot. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. **(E)** Representative western blot for TIF1γ and αSMA in LX2 cells treated with rhTGF β1(5 ng/mL) or/and rhHGF (10 or 20 ng/mL). Quantified by Image J program. Results represent the mean ± standard deviation of two independent biological experiments. $^{**}P < 0.01$. $^{***}P < 0.001$. $^{****}P < 0.0001$. **(F)** Representative western blot of TIF1γ and αSMA in LX2 co-cultured with shHGF- or mock-transfected hE-MSCs. Quantified by Image J program. Results represent the mean ± standard deviation of two independent biological experiments. $^{*}P < 0.05$. $^{**}P < 0.01$. $^{***}P < 0.001$. **(G)** Representative proximity ligation assay to confirm interaction of TIF1γ and SMAD2/3 in LX2 cells. Rabbit TIF1γ antibody and mouse SMAD2/3 antibody were used. Anti-rabbit-plus and anti-mouse-minus probes bound to primary antibodies. Ligase used to ligate the probes and polymerase used to amplify the specific sequence that could hybridize to the fluorescent probe to visualize interactions as dots. Red dots represent endogenous interactions between TIF1γ and SMAD2/3. The graph shows the total number of dots in the nucleus (grey) and the cytoplasm (black). The dots were counted using Image J program in 4 random filed per a group. Mean vales ± SD were

calculated from 4 fields in two independent experiments, with biological duplicate in each experiments. *$P < 0.05$. Number of total dots compared by 1-way ANOVA. Scale bar 10μm for upper panel and 2μm for bottom panel.

Figure 4. Interaction of TIF1γ with SMAD and binding to the promoter of the αSMA suppressed αSMA expression and binding of pCREB on the TIF1γ promoter region induced TIF1γ expression. **(A)** Diagram showing the location of primers in αSMA promoter that can amplify each SBE. **(B)** Representative ChIP analysis of αSMA promoter region with TIF1γ antibody and specific primers. The SBE sequence was analyzed using GPminer v. 2.0. TIF1γ antibody used to pull down the protein-DNA complex and the precipitated DNA fragments were analyzed with specific primers (indicated in the diagram) to confirm the binding sites. **(C)** Quantification of ChIP data. Results represent the mean ± standard deviation of three independent biological experiments. *$P < 0.05$. Statistically analyzed by 1-way ANOVA. **(D)** Identification of CREB-binding sites in the putative promoter region (~1.5 kb) of TIF1γ. Luciferase assay of a CREB-site point-mutated fragment (deletion of ga in tgacgcca), evaluated using TGFβ1 or/and HGF. Results represent the mean ± standard deviation of three independent biological experiments. *$P < 0.05$. Statistically analyzed by 1-way ANOVA. **(E)** Representative western blot for total CREB and pCREB^{s133} in LX2 cells treated with TGFβ1 or/and HGF. Results represent the mean ± standard deviation of three independent experiments. **$P < 0.01$. **(F)** ChIP using primers for the CREB-binding site in the TIF1γ promoter, with the pCREB^{s133} antibody. Results represent the mean ± standard deviation of three independent experiments. *$P < 0.05$. Statistically analyzed by 1-way ANOVA.

Figure 5. TIF1γ expression in liver analyzed by immunofluorescence. (A) TIF1γ and CRBP1 staining in normal, TAA-treated, and hE-MSC-transplanted mouse livers after TAA treatment.

Scale bar 20 ㎛. (B) Quantification of TIF1γ-positive cells in the livers at 14 days (normal, TAA and TAA/hE-MSCs. n = 3 in each group). TIF1γ-positive cells per 0.125 mm^2 were counted using immunofluorescence images (n ≥ 4 for each slide). Mean vales ± SD were calculated from counted fields of each sample in each group. (C) Immunofluorescence of TIF1γ in human normal liver. TIF1γ-positive cells (yellow arrow) were located in the space of Disse. Scale bar 10 ㎛ (D) TIF1γ and αSMA double staining in human normal (n = 4) and cirrhotic (n = 6) liver tissues. Human liver tissues purchased from SuperBioChip Laboratories (Seoul, Korea). Black scale bar 20 ㎛. White scale bar 10 ㎛.

Figure **6.** Confirmation of TG mice with TMX-inducible and HSC-specific knockout of *TIF1γ*. (**A)** Observation of fibrosis and bridging structures by Picro-Sirius red staining upon knockdown of TIF1γ. Representative histochemical staining of Picro Sirius Red and H&E in each group. There were 3 groups; 1) TMX/TAA in wild-type, n = 3; 2) corn oil/TAA in TG, n = 3; 3) TMX/TAA in TG, n = 6. Two experiments. Scale bar 20 ㎛ (**B)** Quantification of fibrosis using Picro Sirius red staining images. Fibrosis region was measured by Image J program (TMX/TAA in wild-type, n = 3; corn oil/TAA in TG, n = 3; TMX/TAA in TG, n = 6). Data were expressed as the mean ± standard deviation. Differences between groups were analyzed by 1-way ANOVA (Lane1,2 n=3, lane3 n=6). ***P* < 0.01. **(C)** Serum levels of AST and ALT at 35 day in the experimental scheme. AST was measured through absorbance of 450nm and ALT was detected through 570nm (TMX/TAA in wild-type, n = 3; corn oil/TAA in TG, n = 3; TMX/TAA in TG, n = 6, Two experiments). Data were expressed as the mean ± standard deviation. Differences between groups were analyzed by 1-way ANOVA (Lane1,2 n=3, lane3

n=6). *P < 0.05. ***P < 0.001. (D) Hydroxyprolne assay from liver tissues at 35 day in the experimental scheme. The hydrolyzed hydroxyproline from homogenized liver tissue was detected at 560nm absorbance using GloMax (Promega) system (TMX/TAA in wild-type, n = 3; corn oil/TAA in TG, n = 3; TMX/TAA in TG, n = 6, each sample measured one or two times, one experiment). Differences between groups were analyzed by 1-way ANOVA (Lane1,2 n=5, lane3 n=10). ****P < 0.0001. **(E)** Representative western blot analysis and **(F)** immunofluorescence of TIF1γ and α-SMA protein in livers at 35 day. CRBP1 used as a HSC marker in immunofluorescence. Quantified by Image J program. Results represent the mean ± standard deviation of two independent biological experiments. Scale bar 25μm for 400X image (left lower magnification), 2 μm for 2000X image (right higher magnification) **P < 0.01. (**G**) Representative TEM and SEM images of TG mice liver after corn oil/TAA treatment at 35 day. The yellow circle indicates fenestrae of LSEC. Red asterisks indicate collagen deposits in the periphery of HSCs. Scale bar 2 μm for 12000X magnification, 1um for 20000X and 30000X magnification, 500nm for 40000X magnification in TEM images. Scale bar 10 μm for 550X, 800X and 900X magnification, 1μm for 4500X and 5000X and 12000X magnification. Reproducible result from two independent experiments was shown.

Figure **7.** Validation of TIF1γ effect in primary HSC. **(A)** Schematic drawing illustrating the procedure of preparation of primary HSCs. (B) Representative immunofluorescence staining of primary HSCs prepared from a C57BL6/N wild-type mouse. Reproducible result from two independent experiments was shown. TIF1γ or αSMA-positive area % per 0.068mm^2 were quantified using Image J program. Scale bar 25 ㎛. **P < 0.01. ***P < 0.001. **(C)** Representative immunofluorescence staining of wild-type HSCs transfected with TIF1γ siRNA for 3 days. Reproducible result from two independent experiments was shown. TIF1γ or αSMA-positive area % per 0.076mm^2 were quantified using Image J program. Scale bar 25 μm

for low magnification, and 10 μm for high magnification. **P < 0.01. ***P < 0.001. **(D)** Representative immunofluorescence staining of HSCs from TG mouse. HSCs treated with 10 nM TMX for 4 days after HSC isolation from TG liver. Reproducible result from two independent experiments was shown. TIF1γ or αSMA-positive area % per 68 μm^2 were quantified using Image J program. Scale bar 25 μm. *P < 0.05. **P < 0.01.

Figure 8. Role of TIF1γ during HSC transformation and restoration. TIF1γ expression in HSCs is decreased by TGFβ and increased in the presence of HGF. Phosphorylation of cAMP response element-binding protein (CREB) and binding of pCREB on the TIF1γ promoter region induced TIF1γ expression. Interaction of TIF1γ with SMAD2/3 and binding of the complex to the promoter of the αSMA suppressed αSMA expression. Therefore, TIF1γ has potential for the development of new therapeutic approaches to inhibiting or treating liver fibrosis.

Supplemental figure legends

Figure S1. (A) Experimental scheme for validation of anti-fibrosis potential of hE-MSCs in vitro. (B) RT-qPCR analysis of αSMA expression. Results represent the mean ± standard deviation of three independent biological experiments. (C) Western blot analysis of αSMA protein expression. Quantified by Image J program. Results represent the mean ± standard deviation of three independent biological experiments. (D) RT-qPCR analysis for the validation of knockdown by siRNA. Results represent the mean ± standard deviation of three independent experiments. *P < 0.05 (E) RT-qPCR analysis for validation of TIF1γ overexpression by lentiviral vector. Results represent the mean ± standard deviation of three independent experiments. *P < 0.05

Figure S2. (A) Validation of shHGF hE-MSCs using ELISA of hHGF. Results represent the mean ± standard deviation of two independent experiments. (B) Counting of DiI-labeled he-MSCs in liver tissue. Randomized three or four fields ($6.2mm^2$) were counted using immunofluorescence (TAA/hE-MSCs, TAA/shHGF hE-MSCs. n=5, 5, respectively. Three TAA individual tissues used negative control). (C) RT-PCR of hHGF in mouse liver tissues. Using specific hHGF primers or mHGF primers, mRNA of hHGF or mHGF in mouse liver was detected. Each lanes were presented each individual mouse liver tissue. (D) RT-qPCR of TIF1γ in human HSCs, LX2 cells which treated hTGFβ1 (5ng/ml) or/and hHGF (10 ng/ml or 20 ng/ml). Results represent the mean ± standard deviation of two independent experiments. $*P < 0.05$

Figure S3. (A) Scheme of the promoter study of TIF1γ. (B) Promoter study by measuring luciferase reporter activity. Reproducible result from two independent experiments was shown. (C) Experimental setup for CREB (phospho) ChIP. To detect CREB binding site at residue 1497, PCR was performed with CREB 1548 F-GCGAGGAGCACGGCTTGAG and CREB 1392 R-AGTGTTCCCAAGAAAGGTGCTGTAA primers, after chromatin pull-down with antibody against phosphorylated (S133) CREB.

Figure S4. (A) TIF1γ expression in mouse liver analyzed by immunofluorescence. TIF1γ-positive cells were located in the space of Disse. Co-staining of TIF1γ and CRBP1 (HSC marker) in mouse normal liver. Scale bar 20 ㎛. (B) RT-qPCR of TIF1γ of primary HSC from normal and TAA-treated mouse liver tissues (BABL/c nude n=2, C57BL/6N n=4, technically triplicate). **P < 0.01. ***P < 0.001. ****P < 0.0001. Dot shows replication. Statistically analyzed by 1-way ANOVA. (C) Comparison between CRISPR/CAS9 and inducible CAS9. (D) Vectors and experimental setup used for the establishment of TMX-inducible TIF1γ-knockout TG mice. (E) Validation of working Cas9-ERT2 system using 293T cells transfected with the CMV-promoter-Cas9-ERT2 construct. Lamin A/C was used as a nuclear faction

control and α-tubulin as cytosolic faction control. Results represent the mean ± standard deviation of three independent experiments. (F) Representative western blot validation of effective silencing by the Cas9-ERT2:sgTIF1γRNA system in LX2 cells transfected with Lrat:Cas9-ERT2: sgTIF1γRNA construct. Results represent the mean ± standard deviation of three independent experiments. (G) Immunofluorescence imaging analysis of the Cas9-ERT2/sgTIF1γ system in LX2 cells. Scale bar 7.5 μm. Comparison of a transfected (ROI 1) and a non-transfected (ROI 2) cell after TMX. Reproducible result from two independent experiments was shown.

Figure S5. (A) Wild-type mouse livers after TAA injury induced with TMX were stained with HE, MT, and Picro-Sirius red, demonstrating mild fibrosis. Scale bar 20 μm. (B) Lrat:Cas9-ERT2:sgTIF1γ-TG mouse livers after TAA injury induced with vehicle corn oil were stained with HE, MT, and Picro-Sirius red, demonstrating mild fibrosis, as observed in wild-type mice. Scale bar 20 μm. (C) Lrat:Cas9-ERT2:sgTIF1γ TG mouse livers after TAA injury induced with TMX were stained with HE, MT, and Picro-Sirius red. Scale bar 20 μm. Supplementary data of Fig 6A. There is a duplication for better clarity.

Supplemental tables

Table S1. Six candidate anti-fibrosis factors.

Candidates	Full name	Used sample or cell line	Ref.
ALR	AUGMENTER OF LIVER REGENERATION	Human Hepatocellular Carcinoma	[Mol Med. 2011]
EPLIN	EPITHELIAL PROTEIN LOST IN NEOPLASM	human prostate cancer (PCa)	[Oncogene. 2011]
MBNL1	MUSCLEBLIND-LIKE PROTEIN 1	atrioventricular canal endocardial cells (chick embryo)	[Dev Dyn. 2009]
Nm23-H1	NONMETASTATIC PROTEIN 23, HOMOLOG 1	human lung adenocarcinoma cell line	[Exp Cell Res. 2012]
PIAS1	PROTEIN INHIBITOR OF ACTIVATED STAT1	mouse mammary epithelial NMuMG cells	[PLoS One. 2010]
TIF1γ	TRANSCRIPTIONAL INTERMEDIARY FACTOR 1-GAMMA	human mammary epithelial cell lines	[EMBO Rep. 2011]

Table S2. Primers for mRNA of hHGF or mHGF in mouse liver.

Primer	Sequence	Size(bp)	TM(°C)
mHGF Forward	CAGAAGGACAGAAGAAAAGAAGAA	150	60
mHGF Reverse	CCCCTGTTCCTGATACACCTGT		
hHGF Forward	TGGGATCATCAGACACCACAC	177	60
hHGF Reverse	CATAGTATTGTCAGCGCATGTTTT		