이학석사 학위논문

Human embryonic stem cell-derived mesenchymal stem cells repress liver fibrosis via enhancing of TIF1γ in mice

인간배아줄기세포 유래 중배엽줄기세포의
생쥐에서 TIF1γ의 증가를 통한 간섬유화 억제

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이 지 연

Abstract

Human embryonic stem cell-derived mesenchymal stem cells repress liver fibrosis via enhancing of TIF1γ in mice

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Progressive liver fibrosis leads to cirrhosis and liver failure and requires liver transplantation, which is often indicated as the only effective therapy. However, the limitations of liver transplantation are associated with organ shortages and long-term immunosuppression. Recent technological advances have led to the disclosure of cellular and molecular mechanisms underlying

liver fibrosis and the development of prospective therapeutic approaches to reduce or reverse liver fibrosis, which would decrease the associated morbidity and demand for liver transplantation. In this study, we performed transplantation of human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) to the livers of thioacetamide (TAA)-treated mice. The result of histological analysis using Masson's trichrome staining, which detects collagen fibers, showed the recovery of TAA-induced liver fibrosis at 14 and 21 days after transplantation of hE-MSCs. To address the underlying mechanism, we screened candidate anti-fibrotic factors and selected transcriptional intermediary factor 1 (TIF1)γ. Our results indicate that TIF1γ inhibited the activation of cultured human hepatic stellate cells (HSCs), as evidenced by the decrease of smooth muscle actin (α-SMA) and collagen type I. TIF1γ was downregulated by pro-fibrotic signals such as TAA and transforming growth factor (TGF)β1 and upregulated by hE-MSCs in vivo, suggesting that TIF1γ is an anti-fibrotic factor expressed in HSCs. Using fluorescent-labeled hE-MSCs, we observed that some of the transplanted hE-MSCs survived and trans-differentiated to HSCs and could secrete paracrine hepatocyte growth factor (HGF) in TAA-treated livers. The downregulation of TIF1γ and upregulation of α-SMA shown in experimental liver fibrosis were confirmed in human cirrhotic livers. Our findings suggest that TIF1γ is a potent anti-fibrotic factor, which can be utilized in the development of novel therapeutic approaches to prevent and reverse liver fibrosis.

Keywords: liver fibrosis, human embryonic stem cell-derived mesenchymal stem cells, human hepatic stellate cells, TIF1γ, hepatocyte growth factor

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Introduction

Fibrosis is the first stage of liver scarring, which can progress into cirrhosis and eventually cause liver failure; in this case, liver transplantation is often indicated as the only effective therapy (Bataller and Brenner, 2005). However, the shortage of available organs and long-term post-surgical immunosuppression makes it necessary to look for alternative therapeutic strategies (Burra et al., 2012). Recent technological developments have provided information on the cellular and molecular mechanisms of liver fibrosis and prospective therapeutic approaches to liver-oriented cell therapy, which may reduce or reverse liver fibrosis, and thus the demand for liver transplants. Stimulation of endogenous and exogenous regeneration of hepatocytes by human mesenchymal stem cells (hMSCs) has been shown as a promising therapeutic strategy to alleviate end-stage liver disease and improve symptoms and liver function; however, its clinical application is still debated (Baertschiger et al., 2009; Shi et al., 2011; Terai et al., 2005; Wang et al., 2012). Therefore, it is important to develop an optimal expandable cell source to obtain hMSCs for the investigation of their potency and repair mechanisms for prospective therapeutic application.

Human embryonic stem cell-derived MSCs (hE-MSCs) are a self-replenishing source of cells that could potentially represent a better alternative for cell-based therapy than adult stem cells. Most adult stem cells have inherent limitations related to insufficient number of available cells and invasive procedures

required to obtain them, which limits their clinical applicability. In our previous study, we succeeded in obtaining hMSCs from human embryonic stem cells (hESCs) and demonstrated that hE-MSCs can be consistently produced, maintained, and expanded(Lee et al., 2010). Furthermore, hE-MSCs are safe in terms of tumorigenesis, and have been shown to be effective in repairing the infarcted heart and sciatic nerves in an animal model, and thus would be a useful platform in regenerative medicine(Lee et al., 2010; Lee et al., 2012).

Activation of hepatic stellate cells (HSCs) has a pivotal role in the extracellular matrix (ECM) production during the process of liver fibrosis. Although the activation and trans-differentiation of HSCs to myofibroblasts are still regarded as the key pathogenic mechanisms of fibrogenesis, the role of HSCs in hepatic fibrosis is still not fully disclosed.

In this study, we performed transplantation of hE-MSCs into the livers of thioacetamide (TAA)-treated mice and observed regression of the experimental fibrosis. To identify anti-fibrotic mechanisms in the liver, we analyzed the expression of seven genes established as potent negative regulators of epithelial-mesenchymal transition (EMT): *KLF17* (Gumireddy et al., 2009), *PIAS1* (Netherton and Bonni, 2010), *ALR* (Dayoub et al., 2011), *MBNL1* (Vajda et al., 2009), *EPLIN* (Zhang et al., 2011), *Nm23-H1* (Hesling et al., 2011; Zhao et al., 2013), and *TIF1γ* (Hesling et al., 2011). Then, we treated human HSC cultures with transforming growth factor-beta 1 (TGFβ1), which activates HSCs, and selected three genes that were downregulated in response to the TGFβ1 treatment.

Materials and methods

hE-MSC culture

This study was approved by the institutional review board of Seoul National University Hospital. hE-MSCs were obtained as previously described (Lee et al., 2010). In brief, SNUhES3 hESCs (Institute of Reproductive Medicine and Population, Medical Research Center, Seoul National University Hospital, Seoul, Korea) were cultured for 14 days in culture dishes without fibroblast growth factor-2 to establish embryonic bodies (EBs). Then, EBs were attached to gelatin-coated dishes for 16 days in low-glucose Dulbecco's modified Eagle medium (DMEM; Invitrogen) with 10% fetal bovine serum (FBS; Invitrogen) and the derived cells were expanded in EGM-2MV medium (Lonza). These cells were tested for differentiation into adipocytes, osteocytes, myocytes, and chondrocytes under the appropriate conditions to evaluate their differentiation potential as h-MSCs. We performed in vitro and in vivo experiments using hE-MSCs of passage 13 - 14.

Mouse model of liver fibrosis

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, mice were given intraperitoneal injection of 200 mg/kg TAA (Sigma Aldrich, St. Louis, MO, USA) or

phosphate-buffered saline (PBS, control) three times a week for 1 - 3 weeks. TAA-treated mice were randomly assigned into two groups receiving hE-MSCs or PBS.

Human hE-MSC transplantation

Twenty-four hours after the first TAA injection, BALB/c-nude mice were intraperitoneally anesthetized with zoletil (Virbac, France) and rompun (Bayer, Germany) and received one intracardiac injection of 5×10^4 of hE-MSCs or PBS in a total volume of 70 μl using a 31-G insulin syringe (BD, San Jose, CA, USA).

To track transplanted cells, hE-MSCs were labeled with CellTracker™ CM-DiI (Invitrogen) added to growth medium at a concentration of 4 μg/ml at 37°C for 24 h prior to transplantation. After intracardiac injection, mice were allowed to recover for 2 days and then TAA injection was continued three times a week. The livers were harvested on days 7, 14, and 21 after cell transplantation and analyzed by immunohistochemistry.

Serum assays

Blood samples were drawn from the hearts of the 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, ALT and AST activity was measured according to the manufacturer's instructions using an automatic chemistry analyzer (HITACHI 7070).

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), MT, or picrosirius red using standard protocols. MT and picrosirius red staining were used to detect collagen and visualize connective tissues. The images were obtained using a Leica light microscope (Leica, Wetzlar, Germany). To evaluate the therapeutic effect of hE-MSCs, the percentage of fibrotic liver area was estimated by quantitative image analysis of MT- and picrosirius red-stained sections using the SABIA (Metoosoft, Seoul, Korea) and ImageJ (National Institutes of Health; Bethesda, MD, USA) software. The degree of liver fibrosis was represented according to the METAVIR scale or ISHAK stage(Standish, 2006), which grade fibrosis from F0 (no fibrosis) to F4 (cirrhosis) or from 0 (no fibrosis) to 6 (cirrhosis), respectively.

Human liver tissues (purchase from SuperBioChip Lab. Seoul. Korea) and the other 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, permeablization was optionally conducted with 0.1% Triton X-100 in PBS for 10 min before blocking. Then, tissue sections were incubated with the following primary antibodies overnight at 4°C: anti-TIF1γ (1:1000, Abcam, Cambridge, UK), anti-cellular retinol-binding protein 1 (CRBP1, 1:100; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-αSMA (1:800;

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

LX2 culture

The human hepatic stellate cell line LX2 was a generous gift from Dr. Friedman (Xu et al., 2005). LX2 cells were grown in high-glucose DMEM supplemented with GlutaMax (Gibco, Grand Island, NY, USA), 5% or 10% FBS, and 1% (v/v) penicillin/streptomycin (Gibco) (LX2 complete medium) at 37°C in a humidified incubator with 5% CO_2.

Co-culture

LX2 cells were plated on 10-cm dishes (2×10^5cells/ml) and incubated for 2 - 3days. When cultures reached approximately 50% confluence, cell medium was changed to fresh medium containing 0.5% FBS and cells were treated with 5 ng/ml of recombinant human TGFβ1 (R&D Systems, Minneapolis, MD, USA) every other day for 4days. The medium was changed at every treatment with the cytokine.

LX2 cells pretreated with hTGFβ1 were co-cultured with 8×10^5 hE-MSCs per dish in the transwell insert (0.4-μm pore size) (Corning, Corning, NY, USA) in fresh complete medium containing 0.5% FBS and 5 ng/ml hTGFβ1, and samples were

harvested after co-culturing.
Alternatively, hE-MSCs knock-downed by shRNA (sequence : ACCATTTGGAATGGAATTCCA) specific to *HGF* were cocultured with LX2 cells activated by hTGFβ1 or 10 ng/ml and 20 ng/ml of recombinant human HGF (R&D Systems) was added to LX2 cells pretreated with hTGFβ1.

Loss and gain of function analysis

Loss of function was analyzed in LX2 cells after expression knock-down performed using siRNA specific to *TIF1γ*, *EPLIN*, *Nm23-H1*, and control siRNA (Santa Cruz Biotechnology). LX2 cells were transfected with siRNA in DMEM GlutaMax without FBS using Metafectene-pro (Biontex, Planegg, Germany); 7 h later, the medium was changed to fresh complete medium, and cells were incubated for 1 to 4 days without change of medium.
Gain of function was analyzed in LX2 cells transfected with the pCMV overexpression vector carrying a *TIF1γ* cDNA clone (Origene, Rockville, MD, USA) using Matafectene-pro. After 7 h, the medium was changed to fresh complete LX2 medium, and starting the following day, 5 ng/ml hTGFβ1 was added daily to transfected cells cultured for 48 h or 96 h.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR 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). Real-time

PCR was performed 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. Real-time PCR 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′ CTGCGTGGAATGTCAGAAGA 3′,
reverse 5′ TTTTGCTTGCCCATAGATCC 3′;
KLF17,
forward 5′ GTCCCAGTCATTGCTGGTTT 3′,
reverse 5′ TGGGAGCGTTTGGTATAAGC 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′.

Western blot assay

Cells or tissue samples were lysed in protein lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 0.5% deoxycholate, 1% NP40, 0.1% sodium dodecyl sulfate [SDS] with protease inhibitor cocktail [Roche, Indianapolis, IN, USA]). Total protein extracts (25 - 30μg) were boiled for 5 min at 95°C, separated by SDS-polyacrylamide gel electrophoresis (PAGE), and transferred to polyvinylidene fluoride membranes (Millipore, Darmstadt, Germany) using a BioRad transfer unit (BioRad, Hercules, CA, USA). Membranes were blocked with 5% skim milk diluted in Tris-buffered saline (TBS) containing 0.1% Tween-20 and incubated with antibodies against TIF1γ (1:1000), αSMA (1:3000), EPLIN (1:500, Abcam), and anti-Nm23-H1 (1:1000, Santa Cruz Biotechnology); anti-α-tubulin antibody (1:5000, Sigma-Aldrich) or anti-GAPDH antibody (1:30,000, Sigma-Aldrich) were used to detect internal control proteins. Membranes were washed and incubated with horseradish peroxidase-conjugated secondary antibodies, washed, and immunoreactive bands were detected and quantified using the TINA 2.0 (RayTest) or ImageJ (National Institutes of Health) programs.

Enzyme-linked immunosorbent assay (ELISA)

The secretion of collagen1α1 to cell culture supernatant and cytokines were analyzed by ELISA using the collagen1α1 ELISA kit (Cusabio Biotech Co., 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).

Statistical analysis

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

Results

hE-MSCs repress liver fibrosis in mice

hE-MSCs were transplanted into TAA-treated immunodeficient mice to evaluate their therapeutic potential in the treatment of liver fibrosis (Figure 1A). Seven days post-engraftment, hepatotoxicity indicators such as the activity of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were downregulated in the hE-MSC transplantation group treated with TAA, and the effect was sustained for 14 and 21 days after hE-MSC transplantation (Figure 1B). Histological analysis using Masson's trichrome (MT) staining, which detects collagen fibers, showed accelerated recovery and regression of surface undulations after TAA-induced liver injury in the hE-MSC group at days 14 and 21 (Figure 1C and Supplementary Figure 1). There was also a decrease in fibrotic area at day 7, but the difference was not significant (Figure 1C). Collagen deposits in 14-day tissues were visualized and quantified using picrosirius red staining, which detects collagen type I and III (Figure 1D).

hE-MSCs repress the activation of human hepatic stellate LX2 cells

To validate the therapeutic effectiveness of hE-MSCs in liver fibrosis, we performed co-culture of hE-MSCs with TGFβ 1-activated human HSC LX2 cell line in vitro. Although smooth

muscle actin (α-SMA) is typically induced in activated HSCs, it was downregulated in LX2 cells both at the mRNA and protein levels after co-culturing with hE-MSCs compared with TGFβ 1-activated LX2 cells (Figure 2A and B). Fibrosis-related morphological changes induced by TGFβ1 treatment in HSCs were reversed by co-culturing with hE-MSCs (Figure 2C). The secretion of collagen type I, which is typically upregulated in the fibrotic liver, also decreased in LX2 cells after co-culturing with hE-MSCs (Figure 2D).

TIF1γ represses the activation of human hepatic stellate LX2 cells

The co-culture experiments indicate that hE-MSCs suppressed HSC activation. To reveal the mechanism underlying such hE-MSC activity, we analyzed the expression of candidate anti-fibrotic factors in activated HSCs. Because pro-fibrotic events in activated HSCs induce EMT, seven genes were selected based on their function as negative regulators of EMT. Among them, *EPLIN*, encoding a cytoskeleton-associated protein that inhibits actin filament depolymerization, nucleoside diphosphate kinase A (*Nm23-H1*), a metastasis suppressor, and TIF1γ were downregulated by TGF β1 treatment in LX2 cells (Figure 3A). However, only TIF1γ was downregulated by TGFβ1 and upregulated in response to hE-MSCs in LX2 cells, whereas EPLIN and Nm23-H1 showed no changes (Figure 3B). Finally, functional validation of TIF1γ was performed in loss-of-function and gain-of-function experiments. When TIF1γ expression in

LX2 cells was knocked down by specific small interfering RNA (siRNA), we observed α-SMA upregulation, whereas EPLIN or Nm23-H1 deficiency did not affect α-SMA expression (Figure 3C). TIF1γ knockdown also induced the secretion of collagen type I (Figure 3D), whereas TIF1γ overexpression reduced α-SMA levels in TGFβ1-treated LX2 cells (Figure 3E).

HGF from hE-MSCs upregulates TIF1γ

Our data indicate that hE-MSC anti-fibrotic activity was related to the upregulation of TIF1γ in HSCs, suggesting that TIF1γ is as a novel anti-fibrotic factor. Then, to reveal the mechanism between hE-MSC activity and upregulation of TIF1γ, we analyzed cytokines from hE-MSCs, which are representative cytokines of hMSCs such as HGF, VEGF, FGF-2, and detected higher expression of HGF relatively (Figure 4A) compare medium as a control. To examine the effect of HGF on TIF1γ expression in LX2 cells, the cells were incubated with recombinant hHGF. In TGFβ1-treated LX2 cells, HGF downregulated α-SMA expression, while upregulating TIF1γ levels (Figure 4B). Next, to confirm that hE-MSC-secreted hHGF regulated TIF1γ expression in HSCs, we knocked down HGF in hE-MSCs with HGF-specific small hairpin RNA (shRNA) and co-cultured them with TGFβ1-treated LX2 cells. The results indicated that the upregulation of α-SMA correlated with HGF-deficient hE-MSCs (Figure 4C).

Repression of TIF1γ in TAA-induced fibrotic mouse livers

was rescued by hE-MSC transplantation

Next, we analyzed TIF1γ levels in mouse livers by immunohistochemistry. TIF1γ-positive cells were located in the perisinusoidal space or space of Disse and were different from hepatocytes in the liver (Figure 5A). To reveal the TIF1γ expression pattern after TAA treatment and hE-MSCs transplantation, liver sections were stained with antibodies against TIF1γ and the HSC marker CRBP1 14 days after transplantation. In normal livers, most of the TIF1γ-positive cells were stained with CRBP1. In TAA-treated livers, large CRBP1-positive cells demonstrated reduced TIF1γ expression, whereas hE-MSC administration resulted in dramatic recovery of TIF1γ and reduction of CRBP1 back to normal levels (Figure 5B); this observation was substantiated by quantitative analysis (Figure 5C). Western blot analysis showed that TIF1γ expression in TAA-treated livers was upregulated by hE-MSCs transplantation. (Figure 5D).

These data indicate that TIF1γ is a potential anti-fibrotic factor expressed in HSCs, which is downregulated by pro-fibrotic signals such as TAA and TGFβ1 and upregulated by anti-fibrotic stimuli such as hE-MSC transplantation.

Transplanted hE-MSCs differentiate into HSCs and secrete hHGF

To track the transplanted hE-MSCs in vivo, they were labeled with a fluorescent dye (DiI), and their engraftment in

TAA-treated livers was examined 7, 14, and 21 days after transplantation (Figure 6A). Although a decrease of fluorescent cells was detected with time, they were still observed after 21 days (Figure 6B). Immunohistochemical analysis of cell lineages indicated that CRBP1 was expressed in DiI-positive cells, which did not react with the anti-hepatocyte (Hep Par-1) antibody (Figure 6C), indicating that engrafted cells differentiated into HSCs and not into hepatocytes, although the observation could not be confirmed by functional analysis in vivo. Next, we assessed the secretion of HGF by engrafted cells using hHGF-specific antibody, and detected hHGF release by DiI-positive vascular cells (Figure 6D). Interestingly, significant hHGF-positive staining was observed around the neighboring cells rather than DiI-positive cells. These results suggest that in TAA-treated mouse livers, some of the engrafted hE-MSCs survived, trans-differentiated to HSCs, and were able to secrete a paracrine HGF.

Repression of TIF1γ in human cirrhotic livers

To test whether our findings in the mouse model can be extrapolated to humans, we performed TIF1γ immunohistochemistry in human cirrhotic livers (ISHAK 6/METAVIR F4). Similar to the mouse model, we observed that TIF1γ was expressed in the human liver; moreover, TIF1γ expression was also decreased in human cirrhotic livers (Figure 7A) in parallel to the increase in α-SMA (Figure 7B). These results suggest that TIF1γ is an anti-fibrotic factor with an

important role in the maintenance of liver health, which can be utilized in the development of novel therapeutic approaches to prevent and reverse liver fibrosis.

Discussion

Therapeutic potential of hE-MSCs

Stem cell therapy is a promising treatment for liver fibrosis, which often requires liver transplantation. Several clinical trials involving human adult stem cells, including BM-MSCs, were inconclusive because the results were influenced by donor age or diseases and showed individual variations in therapeutic efficacy, leaving the recipient to depend on chance(Ogden, 1976; Rando, 2006; Mansilla, 2011). In our previous study, we have obtained a sufficient number of hE-MSCs from a single preparation of hESCs, which can be generated and stored according to the HLA type, thus serving as an off-the-shelf source of allogeneic stem cells(Lee, 2010). Therefore, we suggest that hE-MSCs are an ideal source of stem cells for regenerative medicine. Moreover, in this study, we found that transplantation of hE-MSCs in the mouse fibrotic liver resulted in fibrosis regression and acceleration of functional recovery in the injured liver. Serum levels of hepatotoxicity markers AST and ALT indicate that the effect of hE-MSCs was sustained systemically as well as in the target site, suggesting that hE-MSCs administered by intracardiac injection could reach and influence the injured organ. However, it also raises the possibility that MSCs infusion may result in ectopic tissue formation. Therefore, we monitored ectopic tissue formation in 12 mouse organs during 12 weeks after hE-MSC transplantation by PCR analysis of genomic DNA, but

did not detect any abnormal tissue formation (data not shown). Although there are many concerns about clinical application of hE-MSCs as well as other allogeneic stem cells, hE-MSCs can be obtained in sufficient numbers from a single preparation and can therefore undergo more rigorous testing than stem cells from other sources. Thus, hE-MSCs may have more clinical potential than stem cells of other origin.

TIF1γ as a novel target factor in liver fibrosis

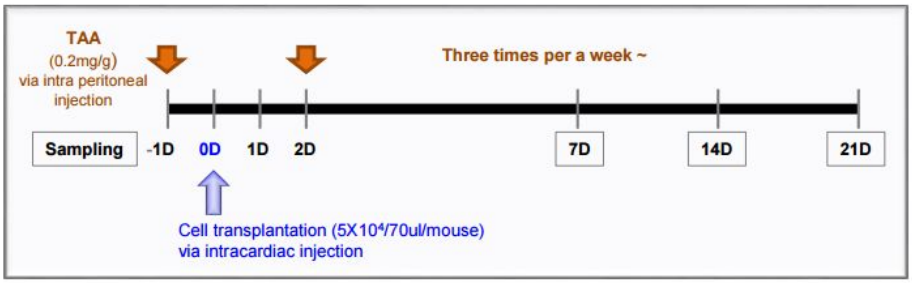
Although liver fibrogenesis is a multifactorial process, most pro-fibrotic mechanisms are associated with HSCs, which may be transformed to fibrogenic myofibroblasts via activation under various pathologic conditions; therefore, HSCs are extensively investigated to define novel drug targets (Liu, 2012; Liu, 2011; Woodhoo, 2012; Yang, 2013). Recent studies have revealed cellular and molecular mechanism of HSC activation, which may underlie the reversibility of human liver fibrosis and even cirrhosis, as evidenced by clinical research (Lee, 2015; Sohrabpour, 2012). In this study, we identified TIF1γ as a critical factor involved in HSC activation based on the loss-of-function and gain-of-function analysis and the status of liver fibrosis. In addition, TIF1γ was downregulated in TAA-treated livers and upregulated during the regression of fibrosis caused by hE-MSC transplantation. Thus, our study demonstrates that the targeted upregulation of TIF1γ identified here as a novel anti-fibrotic factor may underlie the development of new therapeutic approaches to inhibiting or treating liver fibrosis using hE-MSCs.

The role of hE-MSCs in the fibrotic response in the liver

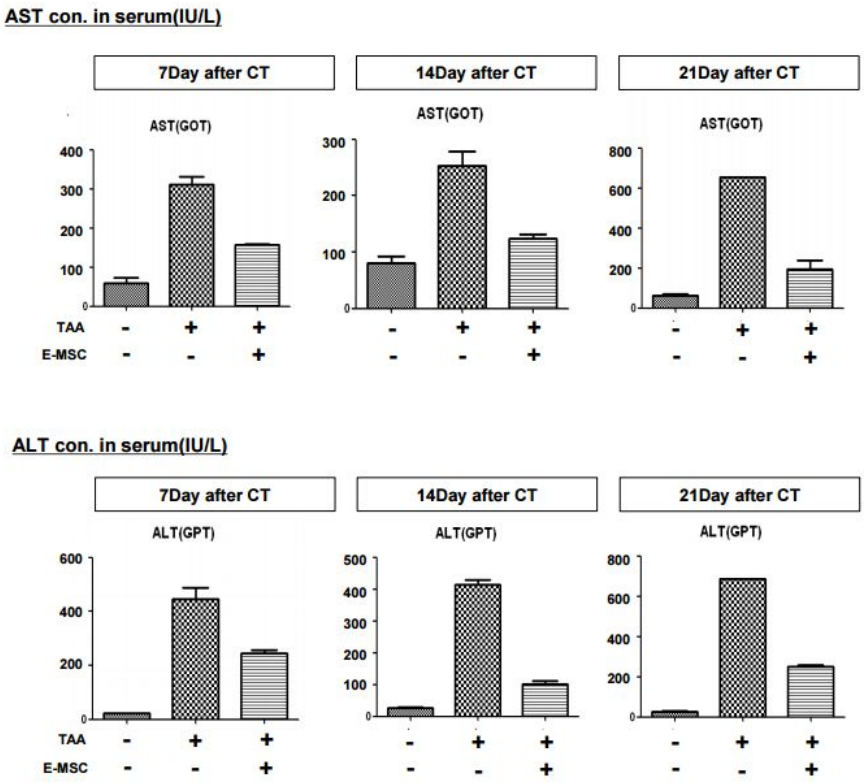
Histological analysis performed in our study revealed paracrine effects and trans-differentiation of transplanted cells. Interestingly, we observed that hE-MSCs were homogeneously engrafted among resident liver cells without forming masses and promoted the recovery of TIF1γ expression in TAA-treated mouse livers. Moreover, transplanted hE-MSCs secreted human HGF, which could upregulate TIF1γ expression in HSCs, as evidenced by in vitro experiments. The mechanisms underlying stem cell-based therapy are still under investigation; nevertheless, our results suggest that the role of hE-MSCs in the fibrotic liver may be trans-differentiation to appropriate cell types and secretion of paracrine factors that promote fibrosis regression.

Figure 1

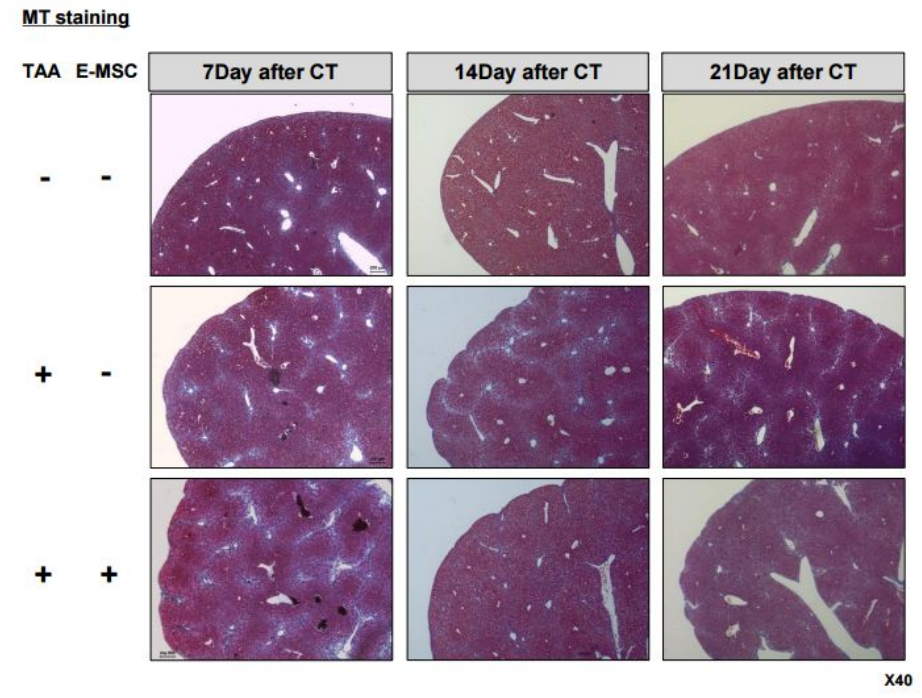
A. Scheme

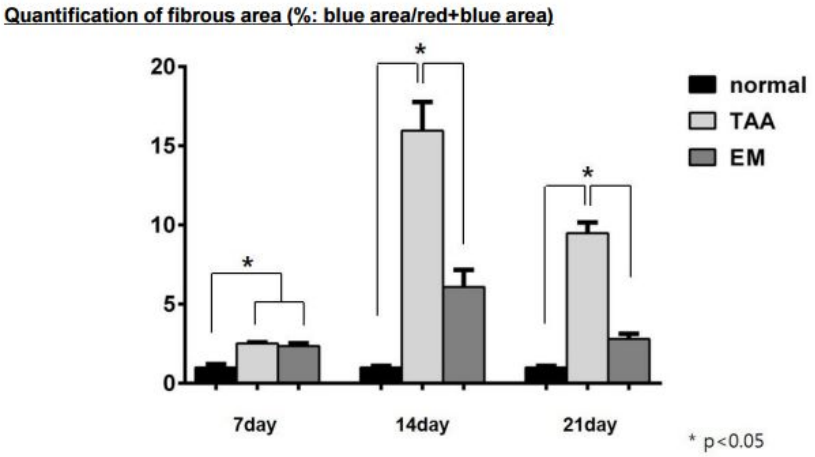


B. Hepatotoxicity parameters

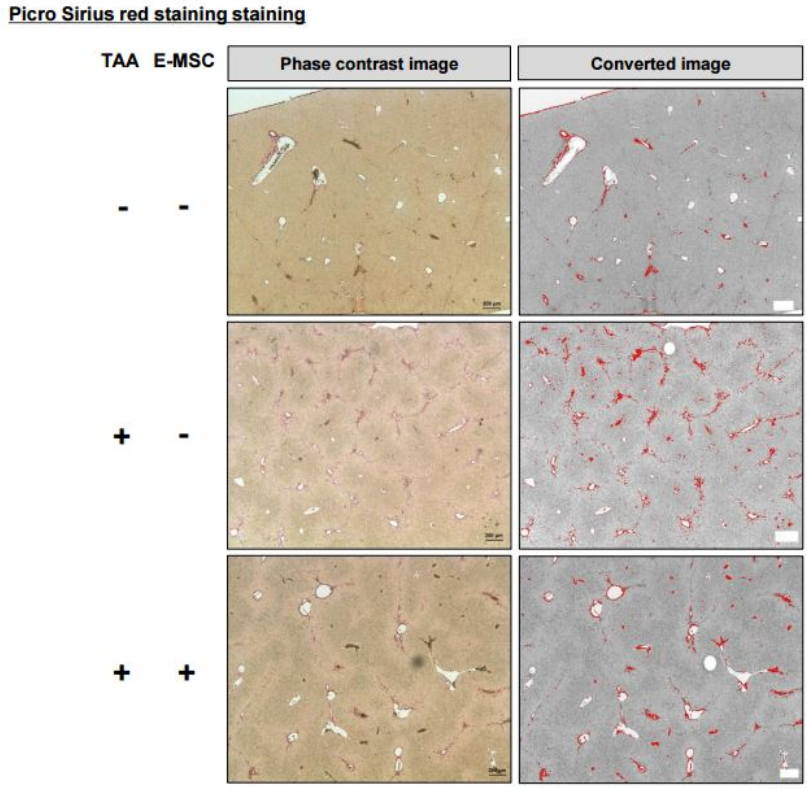


C. Quantification of Fibrosis - MT staining





D. Quantification of fibrosis - Picro Sirius red staining



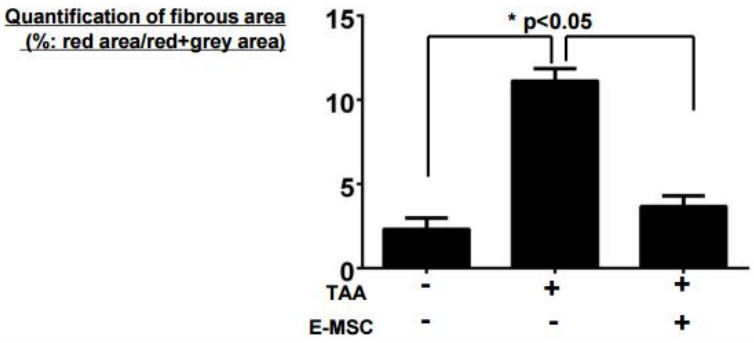
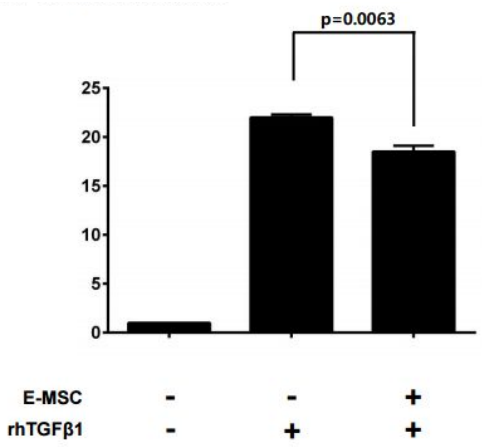


Figure 1. Human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) repress liver fibrosis in mice.

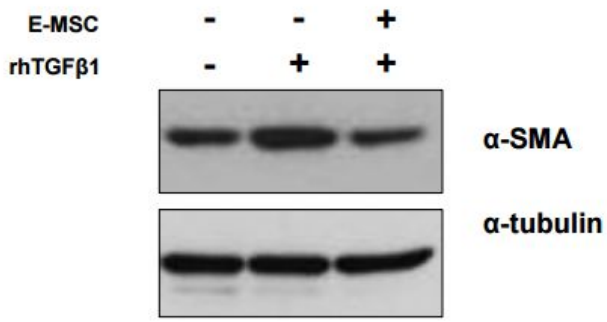
(A) Experimental scheme of hE-MSC transplantation into the livers of thioacetamide (TAA)-treated mice via intracardiac injection. (B) Hepatotoxicity indicators aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were increased after TAA treatment but decreased after hE-MSCs transplantation. (C) Quantification of fibrosis using Masson-trichrome (MT) staining. At days 14 and 21 post-transplantation, the livers of the hE-MSC group demonstrated recovery from TAA-induced injury with the regression of surface undulations. There was no significant difference between the TAA and TAA-hE-MSC groups at day 7 post-transplantation. (D) Picrosirius red staining of fibrotic areas in the livers 14 days after hE-MSC transplantation.

Figure 2

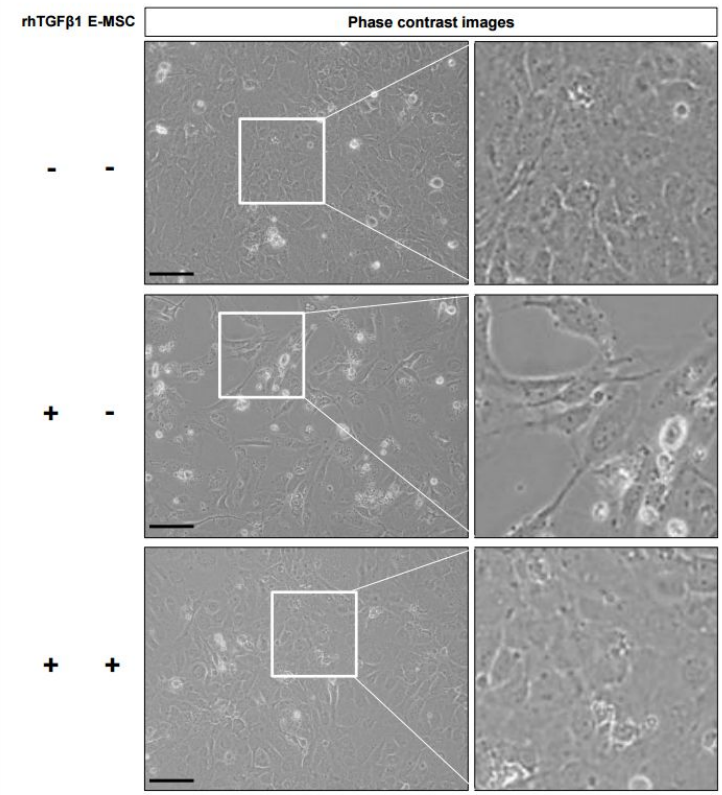
A.


p=0.0063
25
20
15
10
5
0
E-MSC - - +
rhTGFβ1 - + +

B.


E-MSC - - +
rhTGFβ1 - + +
α-SMA
α-tubulin

C.


rhTGFβ1 E-MSC
Phase contrast images
- -
+ -
+ +

D.

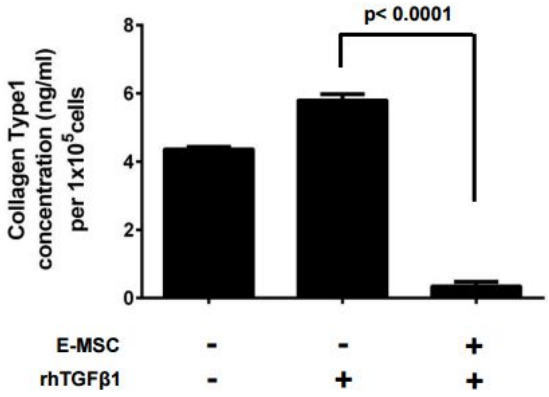
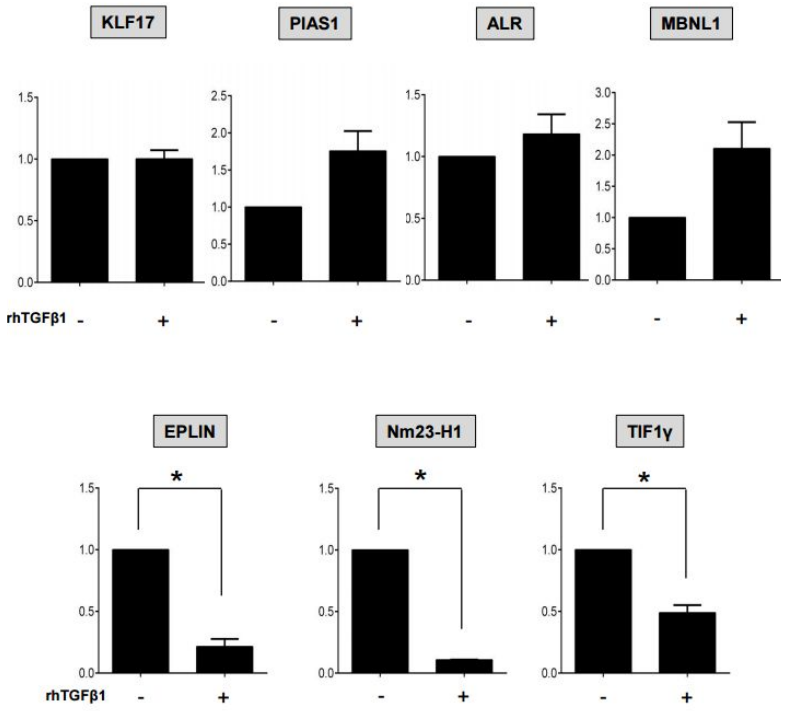

p< 0.0001
Collagen Type1 concentration (ng/ml) per 1x10^5cells
8
6
4
2
0
E-MSC - - +
rhTGFβ1 - + +

Figure 2. Human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) inhibit activation of cultured human hepatic stellate LX2 cells.

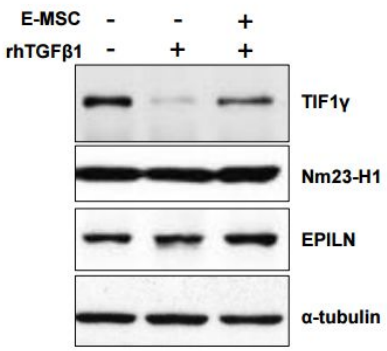
(A) Real-time PCR analysis of mRNA expression in LX2 cells co-cultured with hE-MSCs; *α-SMA* mRNA was down-regulated in TGFβ1-treated LX2 cells. (B) α-SMA protein expression analyzed by western blotting. α-SMA protein increase in activated LX2 cells was reversed by co-culturing with hE-MSCs. (C) Morphological changes of LX2 cells treated with TGFβ1 and co-cultured with hE-MSCs. (D) Collagen type I secretion analyzed by enzyme-linked immunosorbent assay. The secretion of collagen type I was decreased in LX2 cells co-cultured with hE-MSCs.

Figure 3

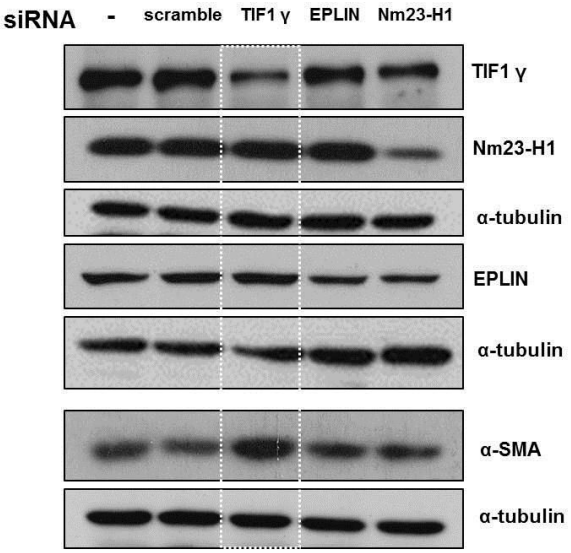
A.

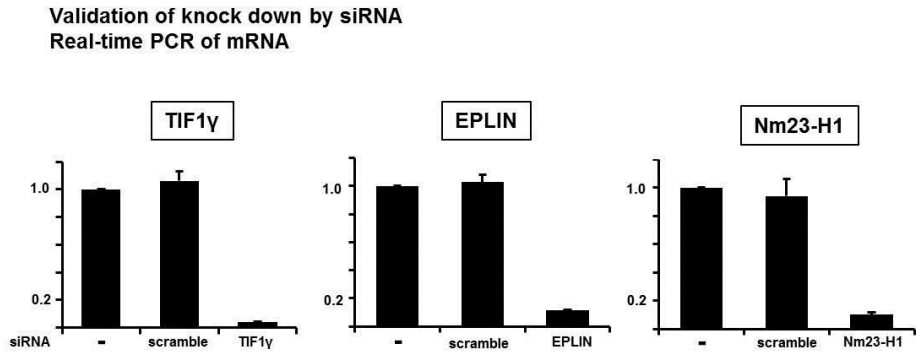

KLF17
PIAS1
ALR
MBNL1
rhTGFβ1 - + - + - + - +
EPLIN
Nm23-H1
TIF1γ
*
rhTGFβ1 - + - + - +

B.

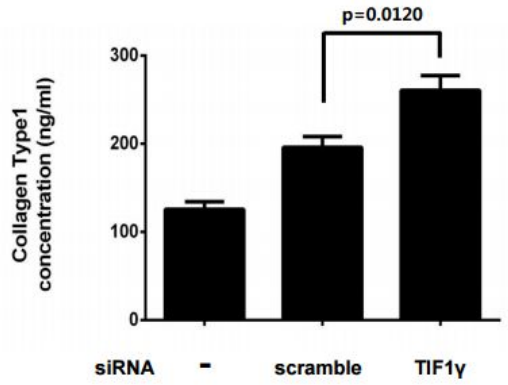

E-MSC - - +
rhTGFβ1 - + +
TIF1γ
Nm23-H1
EPILN
α-tubulin

C.

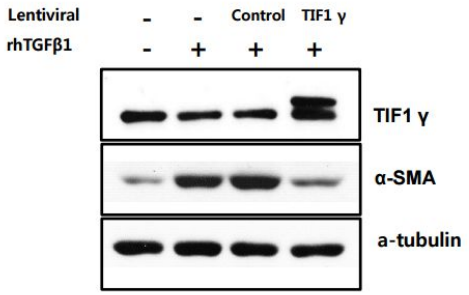

siRNA
-
scramble
TIF1 γ
EPLIN
Nm23-H1
TIF1 γ
Nm23-H1
α-tubulin
EPLIN
α-tubulin
α-SMA
α-tubulin


Validation of knock down by siRNA
Real-time PCR of mRNA
TIF1γ
EPLIN
Nm23-H1
1.0
0.2
siRNA
-
scramble
TIF1γ
EPLIN
Nm23-H1

D.


p=0.0120
Collagen Type1 concentration (ng/ml)
300
200
100
0
siRNA
-
scramble
TIF1γ

E.


Lentiviral
- - Control TIF1 γ
rhTGFβ1
- + + +
TIF1 γ
α-SMA
a-tubulin

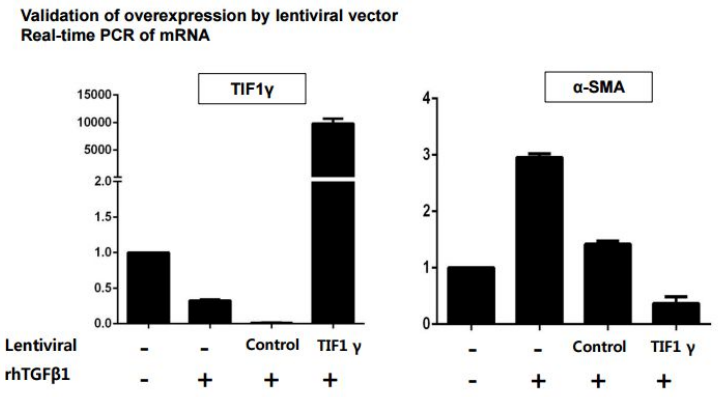
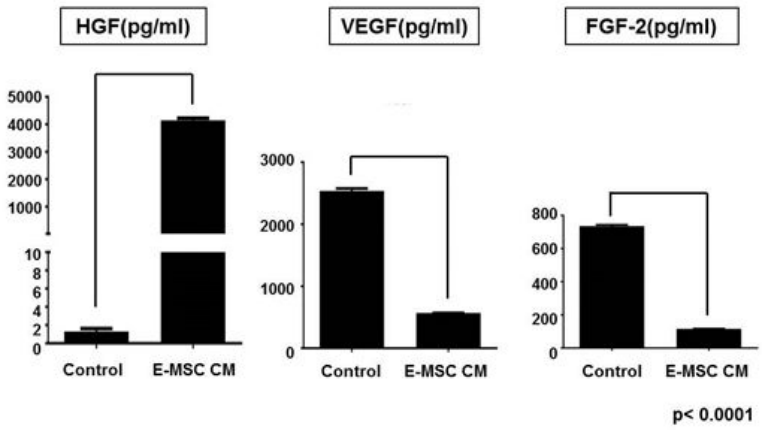

Validation of overexpression by lentiviral vector
Real-time PCR of mRNA
TIF1γ
α-SMA
15000
10000
5000
2.0
1.5
1.0
0.5
0.0
4
3
2
1
0
Lentiviral
- - Control TIF1 γ
rhTGFβ1
- + + +

Figure 3. TIF1γ represses activation of human hepatic stellate LX2 cells.

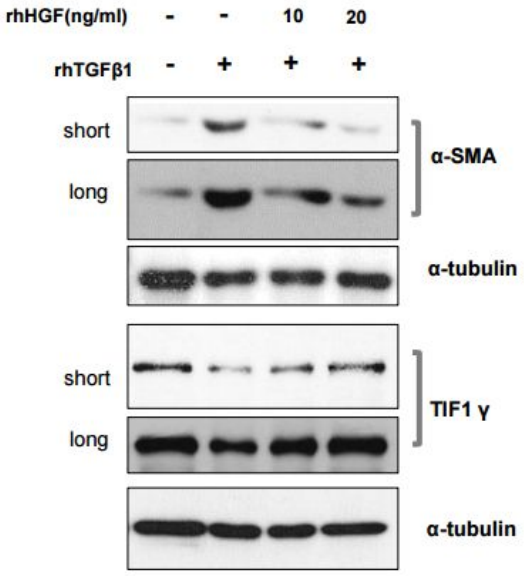
(A) Real-time PCR analysis of mRNA expression in TAA-treated LX2 cells. *EPLIN, Nm23-H1*, and *TIF1γ* were downregulated, whereas *KLF17, PIAS1, ALR*, and *MBNL1* were upregulated by TGFβ1 treatment. (B) Western blotting analysis of TIF1γ, EPLIN, and Nm23-H1 protein expression. TIF1γ was downregulated in TGFβ1-activated LX2 cells and upregulated after co-culturing with hE-MSCs. (C) siRNA-mediated knock-down of EPLIN, siNm23-H1, and siTIF1γ expression in LX2 cells. Expression knock-down was confirmed by real-time PCR and western blotting. α-SMA protein was upregulated in TIF1γ-deficient but not in EPLIN- or Nm23-H1-deficient LX2 cells. (D) Enzyme-linked immunosorbent assay analysis of collagen type I secretion revealed an increase in TIF1γ-deficient LX2 cells. (E) TIF1γ overexpression in LX2 cells (confirmed by real-time PCR and western blotting) downregulated α-SMA mRNA and protein expression in TGFβ1-treated LX2 cells.

Figure 4

A.


HGF(pg/ml)
VEGF(pg/ml)
FGF-2(pg/ml)
5000
4000
3000
2000
1000
10
8
6
4
2
0
3000
2000
1000
0
800
600
400
200
0
Control
E-MSC CM
Control
E-MSC CM
Control
E-MSC CM
p< 0.0001

B.


rhHGF(ng/ml) - - 10 20
rhTGFβ1 - + + +
short
long
α-SMA
α-tubulin
short
long
TIF1 γ
α-tubulin

C.

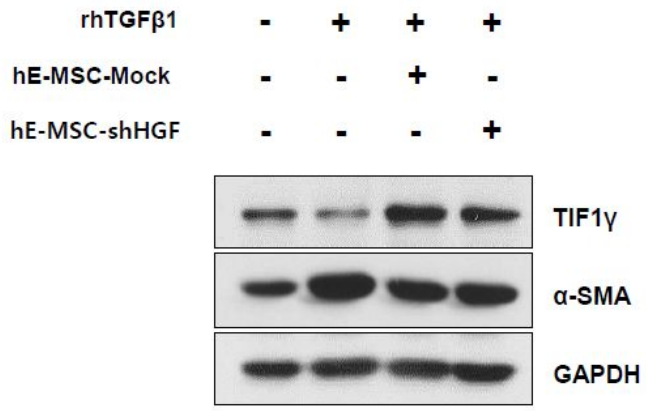


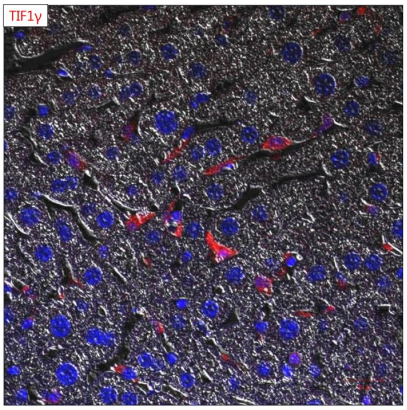
Figure 4. Human embryonic stem cell-derived mesenchymal stem cell (hE-MSC)-secreted hepatocyte growth factor (HGF) upregulates TIF1γ.

(A) ELISA result of hE-MSC culture supernatant. HGF is a unique growth factor that is abundantly secreted from hE-MSCs while other factors are not.

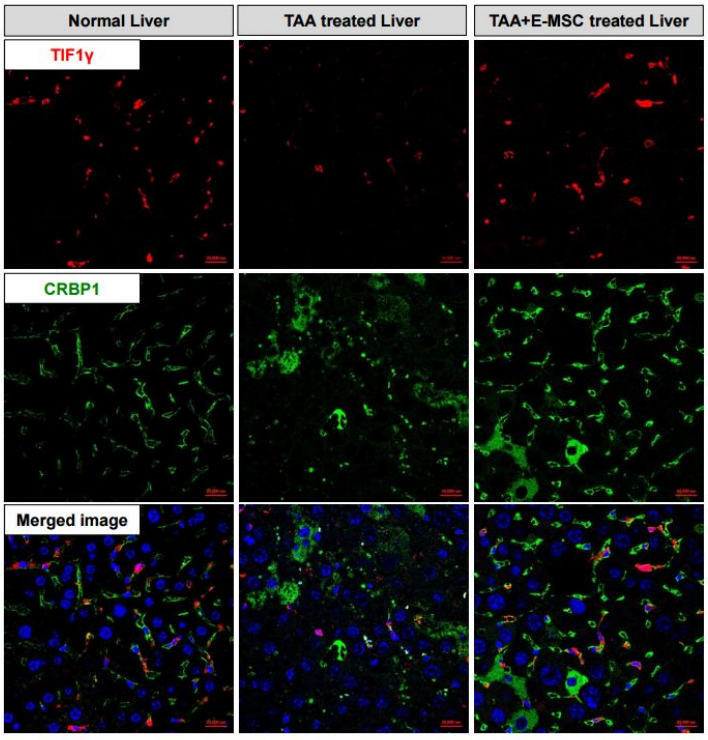
(B) TGFβ1-treated LX2 cells were incubated with recombinant human HGF (10 and 20 ng/ml) and analyzed for α-SMA and TIF1γ expression. (C) HGF was knocked down by specific shRNA in hE-MSCs, which were then co-cultured with LX2 cells. HGF-deficient hE-MSCs upregulated α-SMA and downregulated TIF1γ expression in LX2 cells.

Figure 5

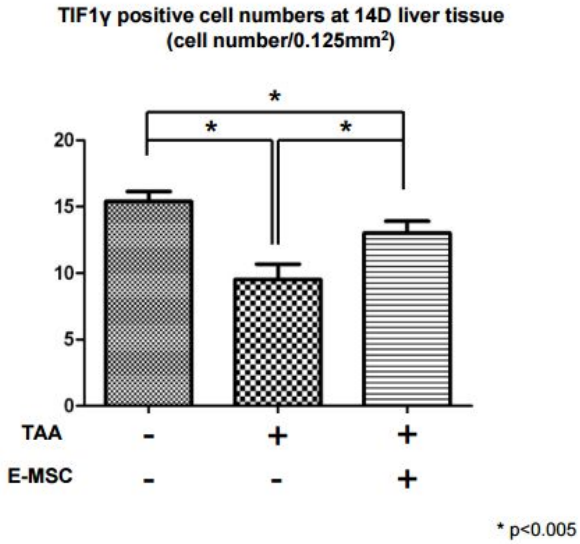
A.


TIF1γ

B.


Normal Liver
TAA treated Liver
TAA+E-MSC treated Liver
TIF1γ
CRBP1
Merged image

C.


TIF1γ positive cell numbers at 14D liver tissue
(cell number/0.125mm²)
*
*
*
20
15
10
5
0
TAA - + +
E-MSC - - +
* p<0.005

D.

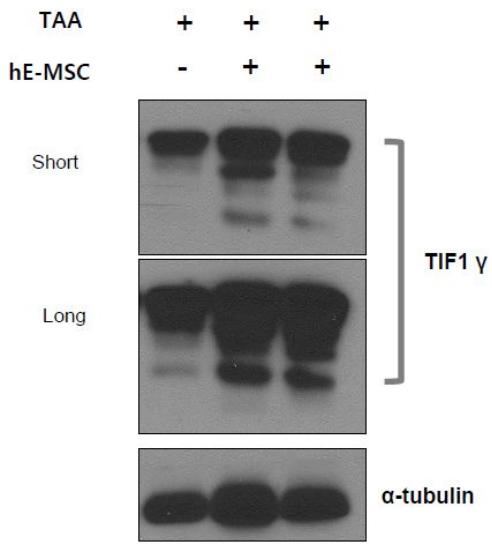
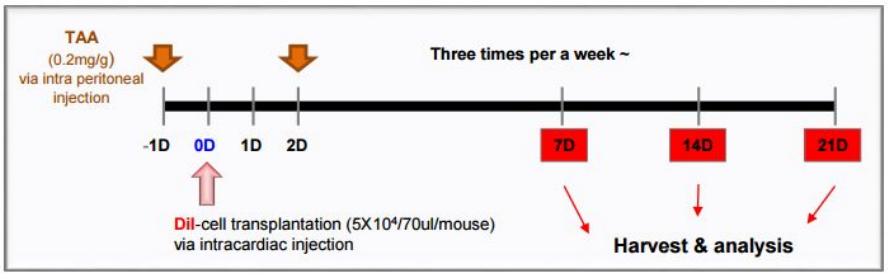

TAA + + +
hE-MSC - + +
Short
Long
TIF1 γ
α-tubulin

Figure 5. TIF1γ downregulation in the fibrotic livers of thioacetamide (TAA)-treated mice is rescued by human embryonic stem cell-derived mesenchymal stem cell (hE-MSC) transplantation.

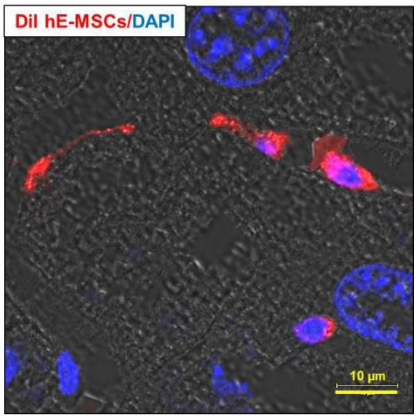
(A) TIF1γ expression in the normal mouse liver analyzed by immunohistochemistry. TIF1γ-positive cells were observed in the space of Disse. (B) TIF1γ and CRBP1 expression in the livers 14 days after hE-MSC transplantation. TIF1γ colocalized with CRBP1 in the normal liver. CRBP1 abnormal expression and TIF1γ downregulation observed in TAA-treated fibrotic livers were reversed by hE-MSC transplantation. (C) Quantification of TIF1γ-positive cells in the livers 14 days after hE-MSC transplantation. Significant upregulation of TIF1γ expression in the liver was detected in the TAA-hE-MSC-treated mice compared with TAA-treated mice and control ($p < 0.05$). (D) Western blotting analysis of mouse livers. TIF1γ expression in TAA-treated livers was increased by hE-MSCs transplantation.

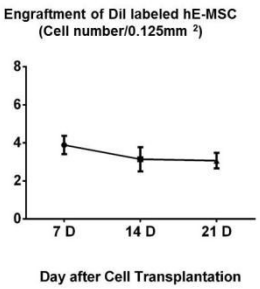
Figure 6

A. Scheme

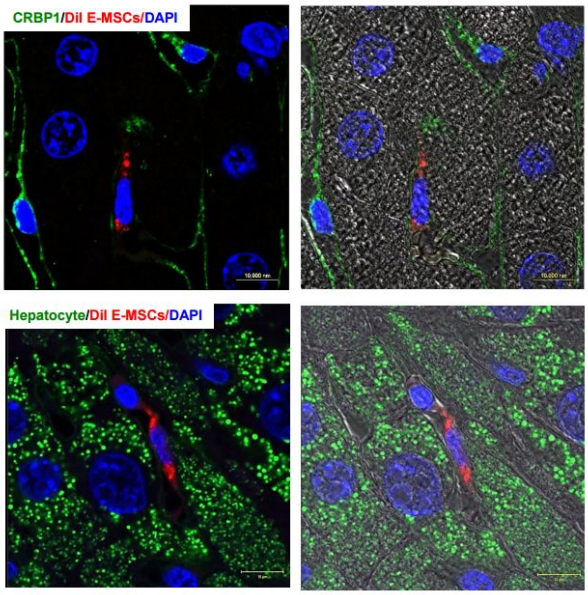


B.





C.


CRBP1/DiI E-MSCs/DAPI
Hepatocyte/DiI E-MSCs/DAPI

D.

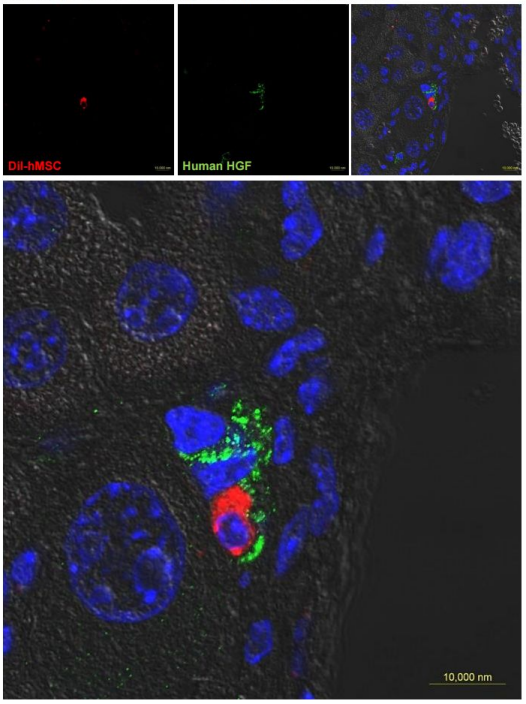
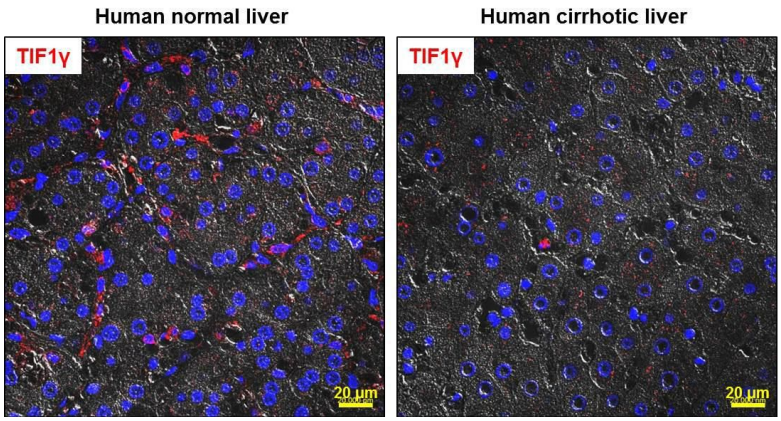

DiI-hMSC
Human HGF
10,000 nm

Figure 6. Transplanted human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) differentiate into hepatic stellate cells (HSCs) and secrete human hepatocyte growth factor (hHGF).

(A) Experimental scheme for the transplantation of DiI-labeled hE-MSCs. (B) Transplanted DiI-labeled hE-MSCs in liver tissue were observed using confocal microscopy. DiI-positive cells decreased with time, but could be detected even 21 days post-transplantation. (C) Immunofluorescence staining of liver tissue 14 days post-transplantation using CRBP1 and hepatocyte antibodies. DiI-positive cells were stained with CRBP1 but not with hepatocyte antibody. (D) Human HGF-specific staining in the liver. hHGF from DiI-positive cells was detected around neighboring cells.

Figure 7

A.


Human normal liver
Human cirrhotic liver
TIF1γ
TIF1γ
20 μm
20 μm

B.

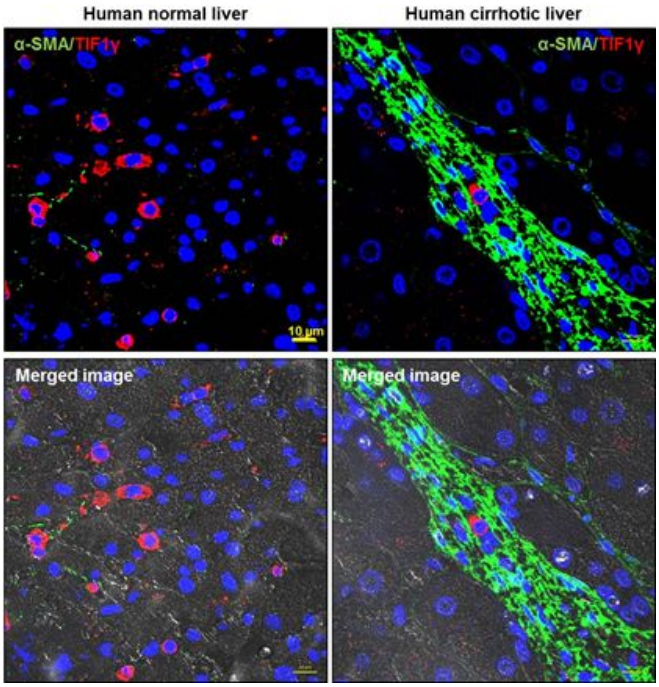
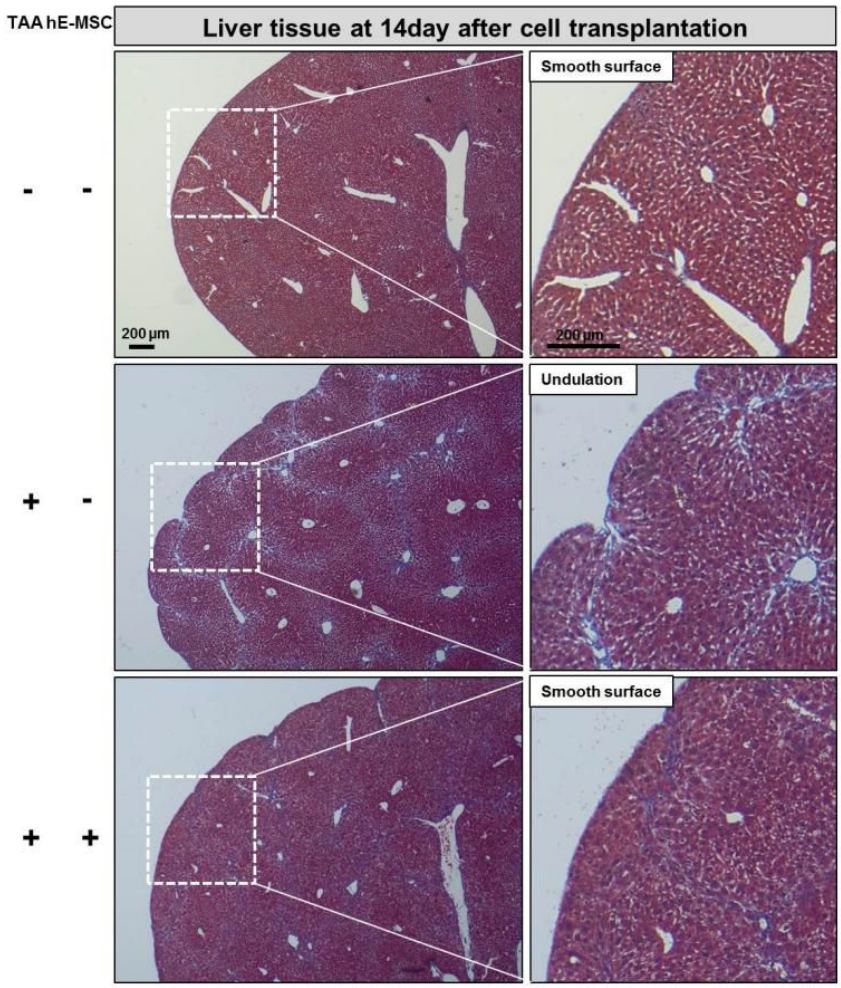

Human normal liver
Human cirrhotic liver
α-SMA/TIF1γ
α-SMA/TIF1γ
10 μm
Merged image
Merged image

Figure 7. Inhibition of TIF1γ expression in the human cirrhotic liver.

(A) TIF1γ staining in human liver tissues. TIF1γ expression decreased in the human cirrhotic livers characterized by morphological destruction. (B) TIF1γ and α-SMA double staining in human liver tissues demonstrates TIF1γ downregulation and α-SMA upregulation in the cirrhotic livers compared to the normal livers.

Supplementary Figure

sFig1.



Supplementary figure 1. Human embryonic stem cell-derived mesenchymal stem cells (hE-MSCs) reduced surface undulation induced by TAA in mice.

High magnification view of mice liver by stained Masson-trichrome (MT). Transplantation of hE-MSCs reduced surface undulation of liver induced by TAA at days 14 after cell transplantation.

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국문초록

진행성 간 섬유화는 간 경변증 및 간부전에 이르게 되며, 효과적 치료법으로 간 이식을 필요로 한다. 그러나 간이식은 장기부족과 장기간의 면역억제라는 한계점이 있다. 최근 기술적 발전은 간 섬유화의 세포·분자적 기전을 밝혔으며, 간 섬유화를 줄이거나 회복시키는 전망적인 치료적 접근법 개발을 이끌었고, 이는 사망률과 간 이식에 대한 수요를 감소시킬 것이다. 본 연구진은 티오아세트아마이드(thioacetamide, TAA)가 처리 된 쥐의 간에 인간 배아줄기세포 유래 중배엽 줄기세포(hE-MSC)를 이식하였다. 콜라겐 섬유를 검출하는 Masson's trichrome 염색을 이용한 조직분석은 이식 후 14일과 21일째 hE-MSCs가 처리된 쥐에서 TAA의해 유도된 간 섬유화가 회복된다는 것을 보여주었다. 기전을 설명하기 위해, 본 연구진은 항 섬유화 인자 후보군을 선별하였고, transcriptional intermediary factor 1 gamma(TIF1ɣ)를 선택하였다. 실험 결과들은 TIF1ɣ 가 alpha smooth muscle actin(α-SMA)와 collagen type I 을 감소시켜 배양된 인간 간 성상세포(Hepatic stellate cell)의 활성화를 저해시킨다는 것을 보여준다. TIF1ɣ는 in vivo에서 TAA 혹은 transforming growth factor (TGFβ1)와 같은 섬유화 신호에 의해 감소되었고, hE-MSCs에 의해 증가되었다. 이는 TIF1ɣ가 간 성상세포에서 발현되는 항 섬유화 인자 임을 나타낸다. 형광 표지된 hE-MSC를 이용해, 이식된 hE-MSC가 생존하여 일부가 간 성상 세포로 분화되었음을 관찰하였고, TAA가 처리된 간에서 이식된 세포가 hepatocyte growth factor (HGF)를 분비하고 있음을 보여주었다. 실험적인 간 섬유화에서 보여졌던 TIF1ɣ의 감소와 αSMA의 증가는 인간 간경화 조직에서도 나타났다.

본 연구진의 발견은 TIF1ɣ가 간 섬유화를 예방하거나 회복시키는

새로운 치료적 접근법 개발에 이용될 수 있는 강력한 항 섬유화 인자임을 보여준다.

주요어 : 간 섬유화, 인간 배아 줄기세포 유래 중배엽 줄기세포, 간 성상 세포, TIF1γ, 간 성장 인자

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