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Article

Anti-fibrotic effect of Smad decoy oligodeoxynucleotide in CCl_4-induced hepatic fibrosis animal model

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Abstract: Hepatic fibrosis is the wound-healing process of chronic hepatic disease that leads to end-stage of hepatocellular carcinoma and demolition of hepatic structures. EMT has been identified to phenotypic conversion of the epithelium to mesenchymal phenotype that occurred during fibrosis. Smad decoy oligodeoxynucleotide (ODN) is a synthetic DNA fragment containing complementary sequence of Smad transcription factor. Thus, this study evaluated the anti-fibrotic effects of Smad decoy ODN on carbon tetrachloride (CCl_4)-induced hepatic fibrosis in mice. As shown in histological results, CCl_4 treatment triggered hepatic fibrosis and increased Smad expression. On the contrary, Smad decoy ODN administration suppressed fibrogenesis and EMT process. The expression of Smad signaling and EMT-associated protein was markedly decreased in Smad decoy ODN treatment mice compared with CCl_4-injuried mice. In conclusion, these data indicate the practicability of Smad decoy ODN administration for preventing hepatic fibrosis and EMT processes.

Key word: liver fibrosis; Smad; decoy; oligodeoxynucleotide; CCl_4

1. Introduction

The liver is the main organ of immense complexity responsible for the metabolism of drugs and toxic chemicals [1]. Chronic liver diseases affecting hundreds of millions of people globally are associated with a developed progress of hepatocellular carcinoma and hepatic fibrosis [2]. Hepatic fibrosis is a consequence of wound-healing responses of the liver that is caused by chronic liver injuries such as alcoholic, viral and autoimmune hepatitis [3]. Regardless of causes, direct and sustained hepatic injury induces a persistent inflammatory response and excessive deposition of extracellular matrix (ECM) in the perisinusoidal space of Disse, leading to the progression of hepatic fibrosis [4]. Hepatic fibrogenesis affects both hepatocytes and non-parenchymal cells such as hepatic stellate cells (HSCs), which are prerequisite for preserving an entire liver structure and function [5].

Hepatocyte and HSCs as the major source of myofibroblasts in injured liver play an important role in the progress of liver inflammation and development of hepatic fibrosis. Any chronic form of hepatic injury can result in transformation of hepatocytes into mesenchymal cells by an epithelial-mesenchymal transition (EMT) process. EMT is dynamic process in which fully differentiated epithelial cells undergo a phenotypic change, resulting in loss of marker proteins such as E-cadherin and zonula occludens-1(ZO-1), and acquisition of mesenchymal characteristics such as α-SMA, vimentin, matrix metalloproteinase (MMP)-2, MMP-9 and collagens [6, 7]. In addition, following damage to the epithelial cells, bile duct epithelium and hepatocytes released the profibrogenic cytokine that further activates HSCs [8, 9]. The activated HSCs lead to hepatic fibrogenesis by excessively production of ECM components such as type I collagen and fibronectin [8,9].

Carbon tetrachloride (CCl_4), a classic hepatotoxic agent, is commonly used to induce liver injury in experimental animal model to examine the pathogenesis of fibrosis and hepatic cirrhosis [1]. This liver injury is ascribed to inflammation originating from CCl_4-derived trichloromethylperoxy free radical formation in the liver [10]. CCl_4 treatment induces centrilobular necrosis which causes a wound-healing response that starts with recruitment of inflammatory and phagocytic cells recruitment in liver necrotic zones, then accumulation of ECM, release of fibrotic cytokines. Finally, continued hepatic inflammatory responses provoked by prolonged CCl_4 administration is believed to induce hepatic fibrosis, cirrhosis, and hepatocellular carcinoma [11-13].

Many studies have identified that a variety of cytokines and growth factors, including transforming growth factor-β1 (TGF-β1), epidermal growth factor (EGF) and hepatocyte growth factor (HGF), participate in EMT process [6, 14, 15]. TGF-β1/Smad signaling has been reported as a mechanism leading to hepatic fibrosis. TGF-β1 activates Smad-dependent and Smad-independent pathways to display its biological activities. For Smad-dependent pathways, TGF-β1 exerts diverse biological activities via its intracellular mediators Smad2 and Smad3, and is negatively regulated by an inhibitory mediator Smad7 [16]. Smads have been also identified to interact with other pathways such as the MAPK and NF-κB signaling pathways [17]. However, these cellular signaling processes still remain unclear in the hepatocytes. Therefore, a recent review concentrates on the regulatory mechanisms and functional role of TGF-β1/Smad pathway during the progression of hepatic fibrosis [18].

Decoy oligodeoxynucleotide (ODN) is a synthetic short DNA segment containing a consensus binding sequence that competitively combines with target transcription factor [19]. As a result, the decoy ODN binds to the specific transcription factor and inhibits gene expression by preventing the upregulation of involved genes. We previously demonstrated that inhibition of Smad and Sp1 by the decoy ODN strategy prevented renal fibrosis in mice via inhibition of the production of cytokines related to fibrosis and EMT [20]. Additionally, we examined that anti-fibrotic effect NF-κB decoy ODN in hepatic fibrosis [21]. However, the effect of Smad ODN on hepatic fibrosis in hepatocyte has not been reported. Therefore, we investigated the anti-fibrotic effect of Smad decoy ODN on hepatic fibrosis by regulating a Smad signaling pathway and EMT process, using CCl_4-induced hepatic fibrosis.

2. Results

2.1. Transfection efficiency and DNA-binding activity of Smad decoy ODN in the CCl_4-treated mouse liver

We designed ring type Smad decoy ODN and synthesized double stranded ODN that contains the sequence Smad binding element (Fig 1A). To identify the successful transfer of Smad decoy ODN, we analyzed the transfection efficiency of the FITC-labeled ODN using fluorescence microscopy. FITC-labeled Smad ODNs were administered intravenously and detected by fluorescence were shown in cytoplasm and nucleus of liver cells (Fig. 1B). These results indicate that Smad decoy ODN was successfully transfected into mouse liver.

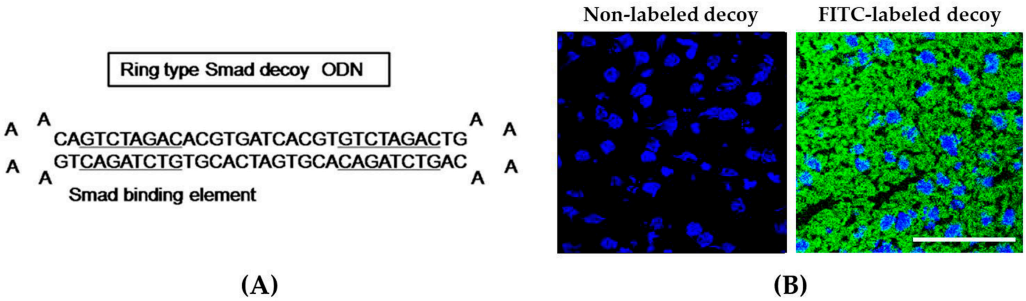

Fig 1. Synthesis of Smad decoy oligodeoxynucleotide (ODN) and transfection of Smad decoy ODN into mouse liver. (A) Design of ring-type Smad decoy ODN including GTCTAGAC which is the consensus sequence for the Smad binding element; (B) Immunofluorescence image of the transfer effect of Smad decoy ODN in liver of mice. The image on the left is the result of administration of non-labeled ODN and the image on the right green fluorescence represented successful transfection into mouse liver. Scale bar, 50 μm.

2.2. Smad decoy ODN attenuated morphological changes in CCl_4-induced hepatic fibrosis

CCl_4 administration induced centrilobular necrosis, proliferation of parenchymal cells and non-parenchymal cells, fibrosis and accumulation of ECM [18]. To identify anti-fibrotic effect of Smad decoy ODN in hepatic fibrogenesis, we used CCl_4-induced hepatotoxic model. To show histological change, we performed both H&E staining (Fig 2A) and Masson's trichrome staining (Fig 2B). The basic lobular architecture was well preserved with portal vein in the normal control group. Cellular inflammation, ballooning changes of hepatocytes and lobular necrosis had developed around the sinusoids in CCl_4-treated mouse. These changes were significantly attenuated by Smad decoy ODN treatment. Additionally, Smad decoy ODN was able to prevent the accumulation of collagen caused by CCl_4-induced liver damge. Taken together, these data indicate that Smad decoy ODN suppressed morphological changes and collagen accumulation in the CCl_4-injected mice.

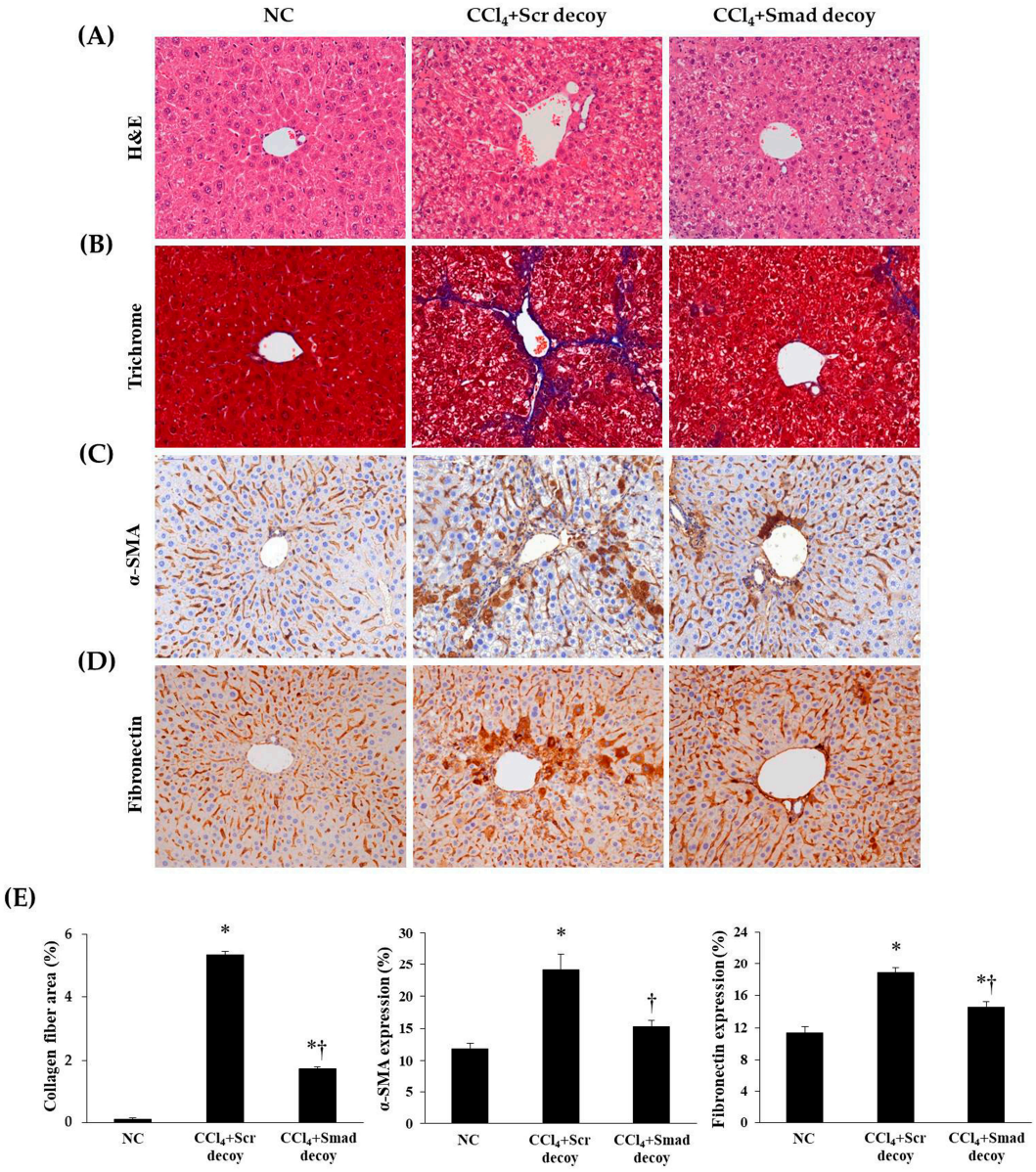

Fig 2. Smad decoy ODN attenuated morphological changes in CCl_4-induced hepatic fibrosis in mice. Paraffin-embedded liver section stained with H&E staining (A), Masson's trichrome staining (B); As a mesenchymal marker, α-SMA (C) and fibronectin (D) was detected by immunohistochemical staining; (E) Quantification of collagen, α-SMA and fibronectin expressions CCl_4 increased expression of α-SMA whereas Smad deocy ODN downregulated it. Original magnification, ×400. * $p < 0.05$ compared to normal control group; † $p < 0.05$ compared to the CCl_4+Scr group.

2.3. Smad decoy ODN suppressed ECM accumulation and EMT process in CCl_4-induced hepatic fibrosis

Following liver injury, hepatic stellate cell undergo phenotypic changes which leads to increased deposition of ECM proteins, such as α-SMA, fibronectin and type I collagen in the hepatic sinusoid [21]. Therefore, we investigated the effects of Smad decoy ODN on hepatic fibrogenesis and ECM accumulation by immunohistochemical and immunofluorescent staining in CCl_4-induced hepatic fibrosis. The expression of α-SMA as a mesenchymal marker was obviously elevated in CCl_4-injuried mice compared with normal mice. However, the administration of Smad decoy ODN resulted in downregulation of α-SMA expression (Fig 2C). The expression of fibronectin within the liver sinusoid increased in CCl_4-induced fibrosis mice, whereas administration of Smad decoy suppressed fibronectin expression (Fig 2D). The effect of Smad decoy ODN on CCl_4-induced fibrosis

in the liver was further investigated by immunofluorescent staining. The expression of α-SMA and type I collagen was increased in the liver tissue of the CCl_4-injuried mice, but this increase was inhibited by Smad decoy ODN (Fig 3).

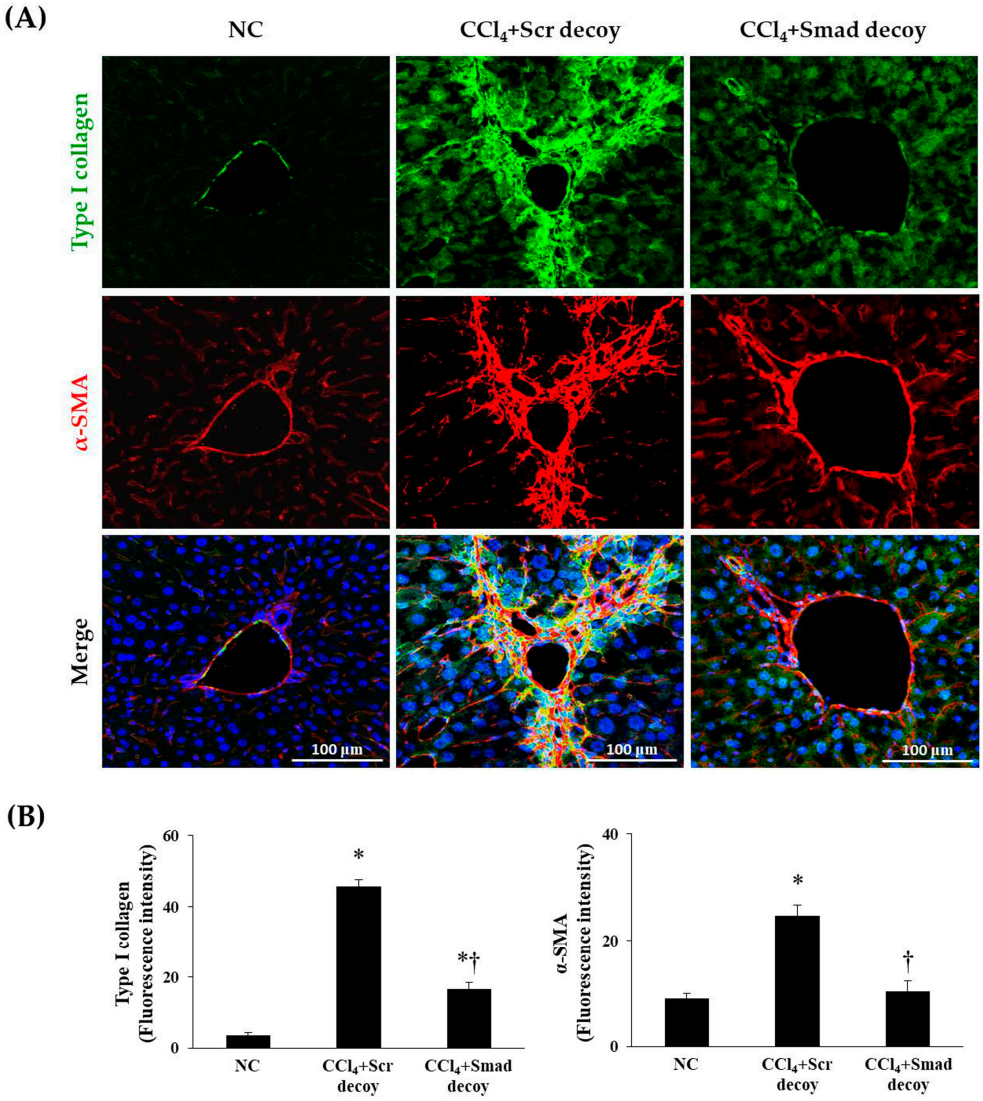

Fig 3. Expression of extracellular matrix (ECM) decreased by Smad decoy ODN in CCl_4-induced hepatic fibrosis in mice. Immunofluorescence double staining for type Ⅰ collagen (green) and α-SMA (red) showed that Smad decoy ODN administration suppressed ECM deposition around of portal vein. The nuclei were stained with Hoechst. Magnification, ×400.

During the EMT in hepatic fibrosis, intercellular junctions of epithelial cells are disrupted by down-regulation of E-cadherin which is confirmed by increase of mesenchymal phenotype, including vimentin [22]. As shown in Figure 4, the expression of E-cadherin in immunofluorescence was decreased after CCl_4 injury compared with normal control, and vimentin showed the opposite result. However, Smad decoy ODN administration suppressed this expression change in the fibrotic liver. These findings show that Smad decoy ODN has anti-fibrotic properties through downregulation of ECM expression and disruption of EMT process following liver injury.

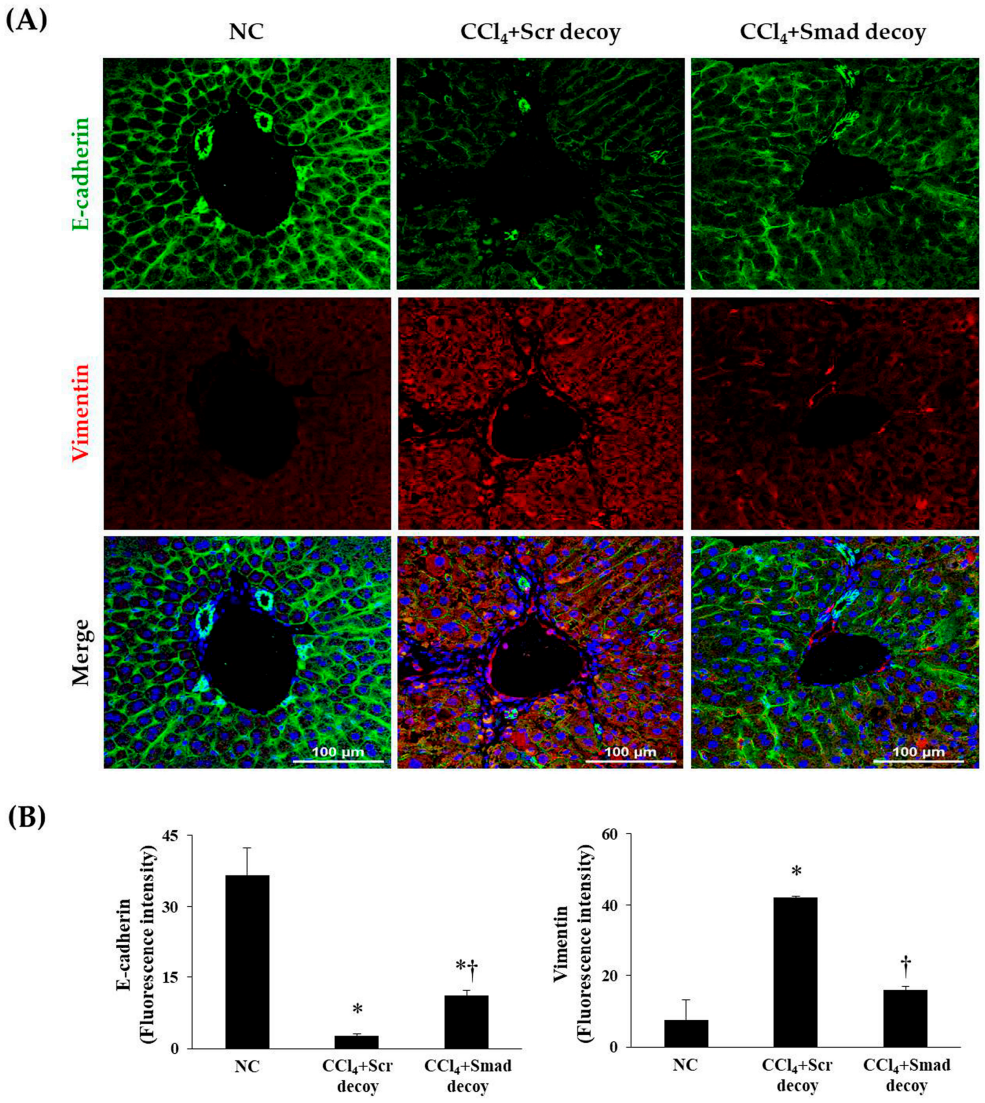

Fig 4. Effect of Smad decoy ODN on the expression of Epithelial to mesenchymal transition (EMT). Representative immunofluorescence staining showed that Smad ODN administration increased expression of E-cadherin (green) and decreased expression of vimentin (red) around of portal vein of hepatic fibrosis in mice. To counterstain, the nuclei were labeled with Hoechst 33342(blue). Image magnification, ×400.

2.4. Smad decoy ODN inhibited fibrotic genes by regulating Smad-dependent signaling pathway in CCl_4-induced hepatic fibrosis

TGF-β1/Smad signaling has been found to be a crucial mediator in EMT and subsequent fibrosis. A rapid relocation of Smad proteins by TGF-β1 signaling is followed by transcription of fibrogenesis gene [23]. To investigate the regulation of Smad decoy ODN on TGF-β1 downstream signaling pathway, we investigated the expression of p-Smad2/3 and Smad4 by Western blotting. The expressions of p-Smad2/3 and Smad4 were markedly increased in hepatic fibrosis mice compared with normal mice ($p < 0.05$, Fig 5A and B). However, injection of Smad decoy ODN attenuated CCl_4-induced fibrotic mediator production through suppression of binding Smad transcription factor in DNA binding site. In addition, quantitative real-time PCR (qRT-PCR) data shown that Smad decoy ODN inhibit Smad signaling pathway (Fig 5E). Next, fibrogenesis and EMT expression levels in the fibrotic liver were measured by Western blot (Fig 5C and D). Western blot results showed that the expression of vimentin, α-SMA, fibronectin, and type I collagen increased in fibrotic liver compared to normal control mice ($p < 0.05$). However, Smad ODN administration

decreased the tissue vimentin, α-SMA, type I collagen and fibronectin levels. On the other hand, The CCl_4 inhibited E-cadherin expression while a relative amount of E-cadherin was preserved in Smad decoy ODN treated livers. These results indicate that Smad decoy ODN inhibits hepatic EMT and fibrosis via blocking TGF-β1-stimulted Smad signaling pathway in CCl_4-induced hepatic fibrosis.

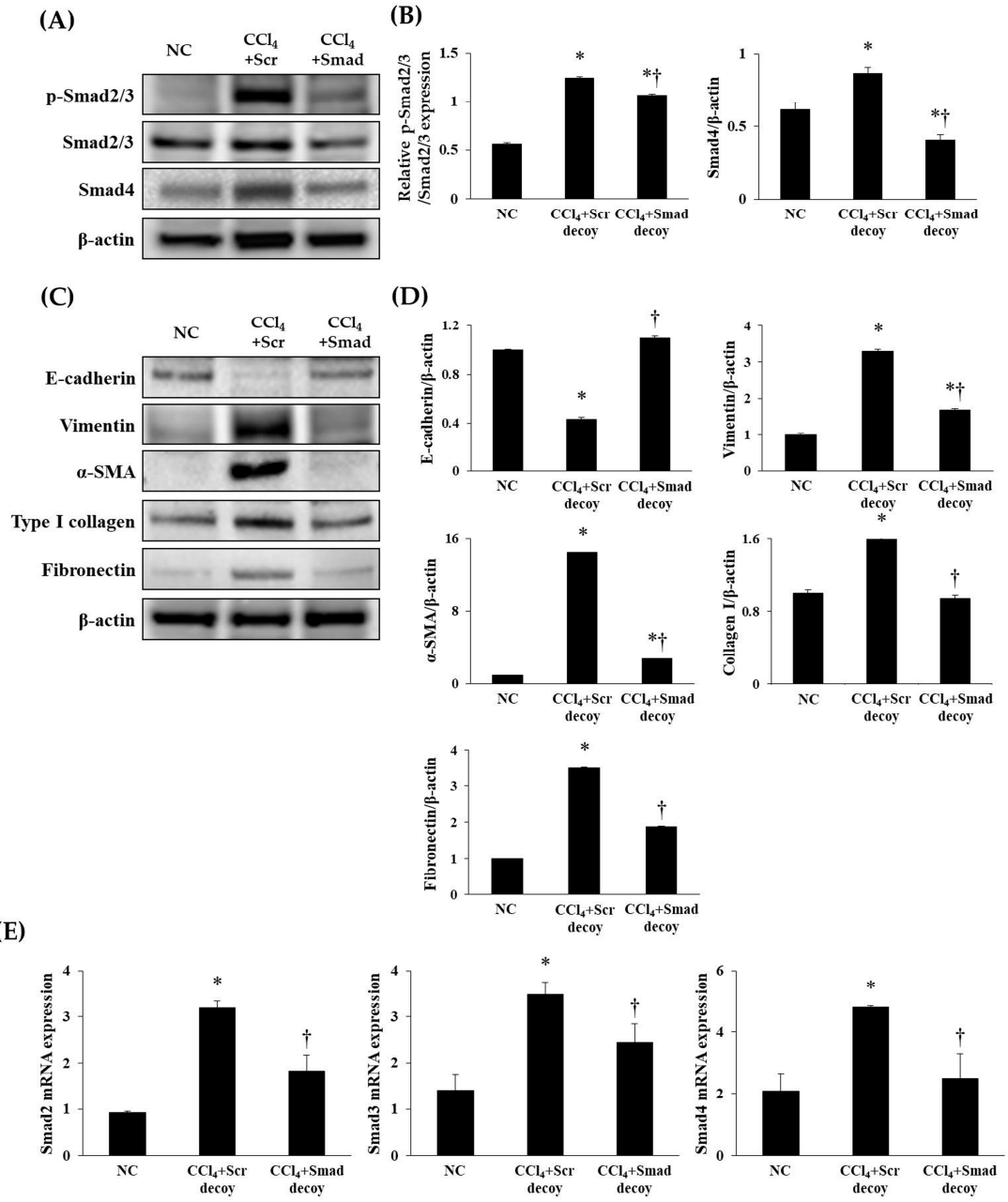

Fig 5. Synthetic Smad decoy ODN significantly suppressed fibrotic genes and ECM proteins by blocking Smad-dependent signaling pathway. (A) Western blot analysis showed that Smad decoy ODN suppressed the expression of p-Smad2/3 and Smad4. All samples were loaded the equal volumes, which was confirmed by loading GAPDH together; The results are representative of three independent experiments; (B) The expression levels of the protein from quantification of these images by Image J. The expressions of E-cadherin, vimentin, α-SMA, type I collagen and fibronectin as the representative of three independent experiments (C) and quantification of ECM proteins (D); (E) qRT-PCR analysis showed that Smad decoy ODN inhibited the mRNA expression of Smad2, Smad3 and Smad4. * $p < 0.05$ compared to normal control group; † $p < 0.05$ compared to the CCl_4+Scr group.

3. Discussion

Hepatic fibrosis is a complicated pathological process of several chronic liver diseases. Many studies have underlined the potentiality of the Smad signaling group participation in the pathogenesis of hepatic fibrosis and carcinogenesis (fibro-carcinogenesis) [24, 25]. Smad proteins a central role in the transduction of receptor signals to target genes in the nucleus [26].

Decoy ODN is a double-stranded DNA segment containing a specific transcription factor binding element [19]. Some studies reported that the induction of synthetic decoy ODN with high attraction for their target transcription factors into peculiar cells. A previous study demonstrated that NF-κB decoy ODN inhibited the EMT process in mice of CCl_4-induced hepatic fibrosis [21]. Park et al. [27] reported the effect of ring-type decoy ODNs on CCl_4-induced hepatic fibrosis. Their study showed that a ring-type Sp1 decoy ODN suppressed the level of cytokines, TGF-β1 downstream target genes and hepatic fibrosis. However, anti-fibrotic effect of Smad decoy ODN in hepatic fibrosis has not yet been demonstrated. Thus, in the current study, we synthesized Smad decoy ODN and investigated the effect of Smad decoy ODN on hepatic fibrosis. The Smad decoy ODN used in this study was a synthesized double-stranded ODN containing the consensus Smad binding element (GTCTAGAC) that binds with Smad 2/3/4 complex. In our previous study, we demonstrated that synthetic decoy ODN was effectively transfected into hepatocytes [21]. On the basis of the results of our previous study, we investigated the effect of Smad decoy ODN on CCl_4-induced hepatic fibrosis. With this aim in mind, Smad decoy ODN was transfected into liver cells.

The CCl_4-induced hepatic fibrosis model is commonly used for anti-fibrotic research with many studies showing that CCl_4, hepatotoxic agent, activated the TGF-β1/Smad signaling pathway and led to accumulation of ECM [21, 28]. Increased TGF-β1 initiated intracellular signaling by binding TGF-β receptor type Ⅱ and TGF-β1 then stimulated TGF-β receptor type Ⅰ kinase, resulting in activation of the downstream signaling pathway [29, 30]. Following binding of TGF-β1 to receptors, Smad2 and Smad3 were phosphorylated by TGF-β receptors and its complex with Smad4. This transcription factor complex was translocated to the nucleus. Complexes of p-Smad2/3 and Smad4 in the nucleus can regulate the transcription of the fibrous gene [31]. Thus, to identify anti-fibrotic effect of Smad decoy ODN, we used CCl_4 hepatotoxic animal model. Consistent with previous study, our research show that CCl_4 hepatotoxic agent accumulated ECM components and activated TGF-β1 signaling. In addition, the expression levels of p-Smad2/3 and Smad4 was increased by CCl_4, and Smad decoy ODN decreased them. In the present study, the hepatotoxic agent CCl_4 stimulated increased expression of ECM proteins and activated Smad-dependent signaling. However, Smad decoy ODN decreased the expression of type Ⅰ collagen, α-SMA and fibronectin.

The Smad-dependent signaling response was correlated with the EMT process during hepatic fibrogenesis. In addition, Sung et al. [20] demonstrated that inhibition of Smad signaling attenuated the EMT process and accumulation of ECM in renal fibrosis. Some research also suggested that chronic hepatic injury resulted in transformation of hepatocytes into myofibroblasts by the EMT process [32]. During EMT, symptoms such as increased migratory capacity, invasiveness, enhanced resistance to apoptosis and greatly increased ECM production occur. EMT has been classified into three dissimilar biological subtypes built on biological circumstances. The type 2 EMT, classified as organ fibrosis, is associated with organ repair and is involved in secondary morphologic change of epithelial or endothelial cells to resident mesenchymal or fibroblast cells in response to persistent inflammation. This processes lead to loss of epithelial marker proteins and acquisition of mesenchymal characteristics [7]. In present study, the epithelial markers decreased and myofibroblast markers increased by CCl_4 administration. It suggested that hepatocytes changed into myofibroblasts via the EMT process. However, this result can be interpreted differently. For example, loss of E-cadherin may have been the result of CCl_4-induced hepatocyte injury, and proliferation of myofibroblasts may have occurred in response to hepatocyte-secreted cytokines. In addition, several studies investigated that there is a contradiction in the EMT process [33]. The controversy surrounding the EMT process means that it has become one of the most debated topics

in hepatic fibrosis study today [34]. In current study, we just investigated the anti-fibrotic effect of Smad decoy ODN and observed the EMT processes in an animal model of hepatic fibrosis. Therefore, to support inhibition effect of Smad decoy ODN on EMT process more experiment needs through *in vivo* and *in vitro*.

In summary, this study confirmed that Smad decoy ODN inhibited hepatic fibrosis by blocking the TGF-β1/Smad signaling pathway which was activated by CCl_4 administration. CCl_4-treatmed mice induced inflammation response and hepatic failure such as accumulation of ECM, centrilobular necrosis, activation of fibrotic genes and EMT processes. However, effectively transfection of Smad decoy ODN attenuated immune responses and pathophysiological changes in the liver. But, in our study, only the histological examination was carried out, and thus it is considered that further study on the expression level of cytokine is needed. In addition, Smad decoy ODN suppressed EMT processes and the production of ECM proteins in CCl_4-induced hepatic fibrosis. Therefore, these results indicate that Smad decoy ODN is able to protect liver against hepatic injury.

Taken together, our results demonstrate that Smad decoy ODN suppressed Smad-mediated hepatic fibrosis by blocking Smad signaling pathway and reducing EMT processes. Given this fact, Smad decoy ODN gene therapy might provide a new therapeutic strategy to prevent hepatic fibrosis. However, more studies are needed to further determine the relationship between the therapeutic use of Smad signaling and hepatic fibrosis to chronic hepatic diseases.

4. Materials and Methods

4.1. Synthesis of ring-type Smad decoy ODNs

Decoy ODNs were synthesized by Macrogen (Seoul, Korea). Smad and scrambled (Scr) decoy ODN sequences used were listed below in Table 1 (consensus sequence is underlined). This section is not mandatory, but can be added to the manuscript if the discussion is unusually long or complex.

Table 1. Sequences of decoy used in this study

Decoy	Sequence
Scr	5'-GAATTCAATTCAGGGTACGGCAAAAAATTGCCGTACCCTGAATT-3'
Smad	5'- GAATTCGTGTCTAGACTGAAAACAGTCTAGACAC-3'

These structures were annealed for 6 h, while temperature was decreased from 80 ˚C to 25˚C. These decoy ODNs were predicted to form a stem-loop structure (Figure 1). To obtain a covalent ligation for ring-type decoy ODN molecules, each decoy ODN was mixed with T4 ligase (Takara Bio, Otsu, Japan) and incubated for 18 h at 16˚C.

4.2. Animal models and Smad decoy ODN transfer

Animal protocols were approved by the Institutional Animal Care and Use Committee of the Catholic University of Daegu (EXP-IRB number: 2014-0001-CU-AEC-04-A). Male C57BL/6 mice (6 weeks old, 20-22g; Samtako, Daejeon, South Korea) were housed in a room with controlled humidity and temperature, and a 12h light-dark cycle. To examine the in vivo transfection efficiency of synthetic Scr ODN, Smad decoy ODN and FITC-labeled Smad decoy ODN were injected into mice intravenously (using the tail vein). The mice were sacrificed 24 h after injection. Optimum cutting temperature compound (Sakura Finetek Japan, Tokyo, Japan) was used to embed liver tissue samples prior to frozen sectioning. Cryosections of liver, which were transferred with FITC-labeled Smad decoy ODN, were examined by fluorescence microscopy.

Mice were randomly divided into three groups as follows: (1) normal control (NC) group, (2) group treated with CCl_4 and Scr decoy ODN (CCl_4+Scr), and (3) group treated with CCl_4 and Smad decoy ODN (CCl_4+Smad). Chronic liver injuries were induced by intraperitoneal injection of CCl_4 (2 ml/kg, dissolved in corn oil [at a ratio of 1:3] three times a week.

One week after the first CCl_4 injection, Scr or Smad decoy ODN (10μg) was transferred biweekly via the mouse tail vein, using an in vivo gene delivery system (Mirus Bio, Madison, WI, USA). Mice were sacrificed 8 weeks after the first CCl_4 injection. All experiments were anesthetized with Isoflurane (O2 0.5 L/min, Isoflurane 2%) inhalation anesthesia and Avertin (2, 2, 2-Tribromoethanol + 2-methyl-2-butanol + Saline Mix → After Filter 250 mg/kg intraperitoneal administration) to reduce animal pain. When the animal suffers an unbearable pain, it was euthanized using CO_2.

4.3. Histology analysis

All liver tissues were fixed in 10% formalin at room temperature. After fixation, sections perpendicular to the anterior-posterior axis of the liver were dehydrated in graded ethanol, cleared in xylene, and embedded in paraffin. Paraffin-embedded tissues were cut into 4μm sections and deparaffinized. Liver tissue sections were stained with hematoxylin and eosin (H&E) and Masson's trichrome according to standard protocol.

4.4. Immunohistochemical staining

Paraffin-embedded sections were deparaffinized with xylene and dehydrated in gradually decreasing concentrations of ethanol. For immunohistochemical analysis, the dehydrated tissue sections were immersed in a 10mM sodium citrate buffer (pH 6.0) for 5 min at 95°C. The last step was repeated using a fresh 10 mM sodium citrate solution (pH 6.0). The sections were allowed to remain in the same solution while cooling for 20 min, and they were then rinsed in phosphate-buffered saline (PBS). Next, sections were incubated with a primary antibody (1:100 dilution) for 1h at 37 °C. Primary antibodies were follows: anti-fibronection (BD Biosciences, Burlington, Canada) and anti-α-SMA (A2547, Sigma-Aldrich, St.Louis, MO, USA). After three serial washes with PBS, the sections were processed by an indirect immunoperoxidase technique using a commercial Envision System kit (DAKO, Carpinteria, CA, USA). Immunohistochemical images were viewed with an Eclipse 80i microscope (Nikon, Tokyo, Japan).

4.5. Immunofluorescent staining

Paraffin-embedded liver tissue sections were deparaffinized with xylene and dehydrated in gradually decreasing concentrations of ethanol. Tissue sections were then placed in blocking serum (10% donkey serum) at room temperature for 1 h. The sections were immunostained with primary antibodies (1:500 dilution) against type Ⅰ collagen (Novous Biologicals, Littleton, CO, USA), E-cadherin (Cell Signaling Technology, Danvers, MA, USA), Vimentin (BD Biosciences) and α-SMA (Abcam) at room temperature for 2h. After washing, sections were incubated with the secondary antibodies (1:200 dilution) conjugated with Alexa Fluor 488 or Alexa Fluor 555 (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min at 37 °C. Slides were then mounted using Dako fluorescence mounting medium. Specimens were examined and photographed with a confocal microscope (Nikon, Tokyo, Japan).

4.6. Western blot analysis

Frozen liver tissues were homogenized in cell lyticTM M lysis reagent (Sigma-Aldrich, St.Louis, MO, USA) and samples were centrifuged at 12,000 rpm for 20 min at 4°C after incubation for 30 min on ice. The supernatant was collected, and the residual protein concentration was determined by using Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA). Total protein was separated on 8% to 12% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membrane (GE Healthcare, Little Chalfont, Buckinghamshire, UK) using the standard SDS-PAGE procedure. The membranes were blocked in 5% bovine serum albumin in TBS-T (10 mM Tris, 150 mM NaCl, and

0.1% Tween-20) for 1 h at room temperature. Then, the membrane was probed with a primary antibody (1:1000 dilution) overnight at 4 ˚C. The membrane was further probed with horseradish peroxidase conjugated secondary antibody (1:2000 dilution) for 2 h at room temperature. The signals were detected using an enhanced chemiluminescence detection system (Amersham, Piscataway, NJ, USA). The primary antibodies used were anti-fibronectin, anti-vimentin (BD Biosciences), anti-p-Smad2/3, anti-Smad2/3, anti-Smad4 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-type I collagen (Novous Biologicals), anti-α-SMA (Abcam), anti-E-cadherin (Cell Signaling Technology), and anti-glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) (Cell Signaling Technology).

4.7. Qiamtotatove real-time PCR (qRT—PCR)

Total RNA was isolated from liver tissues using TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Reverse transcription reaction was performed by using AccuPower RT Premix and Oligo dT18 primer (Bioneer, Daejeon, Korea) according to the manufacturer's recommendations. The PCR mixtures contained 100ng of cDNA and 100pM each of forward and reverse primers. The samples were denatured at 95C for 10 min, followed by 45 cycles of annealing and extension at 95℃ for 20s, 60℃ for 20s, and 72℃ for 20s. Real-time PCR was performed in a LightCycler nano System (Roche Applied Science, Mannheim, Germany) by using LightCycler DNA Master SYBR GREEN I (Roche Applied Science). Expression values were normalized to β-actin. The real-time PCR was performed in a LightCycler nano System (Roche Applied Science, Mannheim, Germany) by using LightCycler DNA Master SYBR GREEN I (Roche Applied Science). The primer sequences were as follows:

Table 2. Sequences of PCR primer used in this study

Gene	Sequence
Smad2	forward, 5′-TGCATTCTGGTGTTCAATCG -3′ reverse 5′-CGAGTTTGATGGGTCTGTGA -3′
Smad3	forward 5′-GTCAACAAGTGGTGGCGTGTG-3′ reverse 5′-GCAGCAAAGGCTTCTGGGATAA-3′
Smad4	forward 5′-TGACGCCCTAACCATTTCCAG-3′ reverse 5′-CTCCTAAGAGCAAGGCAGCAAA-3′

4.8. Statistical analysis

All data are presented as means ± SE. A Student's t-test was used to assess the significance of independent experiments. Differences with $p < 0.05$ were considered significant.

Author Contributions: Mi-Gyeong Gwon, Jung-Yeon Kim and Kwan-Kyu Park conceived and designed the experiments; Mi-Gyeong Gwon and Jung-Yeon Kim is co-first author. Mi-Gyeong Gwon, Jung-Yeon Kim, Hyun-Jin An, Woon-Hae Kim, Hyemin Gu pergormed the experiments; Min-Kyung Kim, Sok-choen Park discussed the study and analyzed the data. All authors have read and approved the final version of this manuscript.

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Conflicts of Interest: The authors declare no conflict of interest.

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