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Identification of cromolyn sodium as an anti-fibrotic agent targeting both hepatocytes and hepatic stellate cells

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ABSTRACT

Liver fibrosis and cirrhosis, the late stage of fibrosis, are threatening diseases that lead to liver failure and patient death. Although aberrantly activated hepatic stellate cells (HSCs) are the main cause of disease initiation, the symptoms are primarily related to damaged hepatocytes. Thus, damaged hepatocytes, as well as HSCs, need to be simultaneously considered as therapeutic targets to develop more efficient treatments.

Here, we suggest cromolyn sodium as an anti-fibrotic agent to commonly modulate hepatocytes and hepatic stellate cells. The differentially expressed genes from 6 normal and 40 cirrhotic liver tissues which were collected from GEO data were assessed by pharmacokinetic analysis using a connectivity map to identify agents that commonly revert abnormal hepatocytes and HSCs to normal conditions. Based on a series of analyses, a few candidates were selected. Candidates were tested *in vitro* to determine their anti-fibrotic efficacy on HSCs and hepatocytes. Cromolyn, which was originally developed as a mast cell stabilizer, showed the potential to ameliorate activated HSCs *in vitro*. The activation and collagen accumulation for HSC cell lines LX2 and HSC-T6 were reduced by 50% after cromolyn treatment at a low concentration without apoptosis. Furthermore, cromolyn treatment compromised the TGF-β-induced epithelial mesenchyme transition and replicative senescence rate of hepatocytes, which are generally associated with fibrogenesis. Taken together, cromolyn may be the basis for an effective cure for fibrosis and cirrhosis because it targets both HSCs and hepatocytes.

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1. Introduction

Liver fibrosis results from acute damage, and the reversible wound-healing response to injury reflects the critical balance between liver repair and scar formation [1]. Because a chronic damage response leads to progressive substitution of liver parenchyma by scar tissue [1], it eventually causes liver cirrhosis. Fibrosis is also associated with an excessive wound healing response that results from the deposition of scar tissue such as excessive extracellular matrix. The spread of fibrosis throughout the liver causes liver cirrhosis, which, in turn, induces several complications like liver failure and hepatocellular carcinoma, and ultimately leads to patient death.

Hepatic stellate cells (HSCs) are an initiating cell type of the disease and a major mechanistic contributor to the development of liver cirrhosis for various disease causes such as hepatitis virus infection and alcohol consumption [2]. When HSCs are activated, extracellular matrix components, such as collagen, accumulate excessively, and this distorts liver vasculature and architecture and raises portal pressure, causing relevant symptoms. These events activate HSCs again and continuously, resulting in a harmful cycle

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with respect to the liver cirrhosis process [3]. As HSC proliferation and activation is mediated by various signaling processes [4], HSC signaling inhibitors have been studied for anti-fibrosis applications. Anti-fibrotic effects of a few synthetic chemicals and biologics are related to HSC proliferation-related growth factors and their respective signaling pathways, reducing the proliferative response of HSCs [5,6]. Some plant extracts including curcumin [7], silymarin [8], *Ginkgo biloba* extract [9], and *Salvia* extract [10] also seem to suppress connective tissue growth factor, which activates myofibroblasts and deposits them in the liver [11], and TGF-β signaling, which contributes to profibrogenic pathways by activating the release of fibrogenic components [12].

On the other hand, as fibrosis progresses by HSCs, hepatocyte damage worsens. During fibrosis, hepatocytes undergo epithelial mesenchymal transition (EMT) [13,14] and senescence owing to telomere shortening [15]. As a result, hepatocytes lose their function and liver failure occurs. Although most therapeutic studies on fibrosis or cirrhosis have focused on HSCs, hepatocytes account for up to 70% of liver tissues as parenchymal cells of the liver, whereas HSCs consists of less than 15% of the total liver [16,17]. Thus, the prevention or restoration of hepatocytes from chronic damage may be critical for the development of a radical therapy for cirrhosis.

A pharmacogenomics strategy has been developed as an efficient tool to discover modulator chemical candidates based on gene expression [18]. The approach can be used to redesign drugs for new diseases and to develop new drugs. A connectivity map is a remarkable algorithm used to rank chemical candidates that alter gene expression [19,20]. This pharmacogenomics strategy to identify candidate molecules that have a high probability of functioning *in vivo* will make drug development more efficient. Thus, we applied the connectivity map method to identify a chemical candidate mediating cirrhosis.

In this study, our aim was to identify common factors that suppress cirrhosis in clinical samples using pharmacogenomic analysis. We found that cromolyn effectively ameliorates cirrhosis by targeting both HSCs and hepatocytes.

2. Methods

2.1. Pharmacogenomic analysis

To define liver fibrosis- or cirrhosis-associated gene expression signatures, microarray data for 40 cirrhosis and 9 normal liver samples from GSE 25097 were utilized. Probe IDs that changed over 2.5-fold in intensity were extracted. Probe IDs were then merged into gene symbols. The 2226 gene symbols were separated into those that were 'logFC > |1.5|'-fold upregulated (710 genes) or downregulated (225 genes) in cirrhotic livers to generate a connectivity map [19]. The gene symbols for each of the two groups were converted into probe IDs corresponding to the Affymetrix HG U133A array for analysis.

2.2. Cell and chemical preparation

LX-2 and HCT-T6 cells were kindly provided by Drs. Sang-Hyun Sung (Seoul National University, Seoul, Korea) and S.L. Friedman (Mount Sinai School of Medicine, New York, NY, USA), respectively, and grown at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum and 1% penicillin and streptomycin. Murine primary hepatic stellate cell were purchased from Invitrogen (CA, US) and cultured on collagen coated dishes with William's E Medium (Invitrogen, CA, US). Primary hepatocytes were isolated from the livers of 6-week-old male C57BL6 mice and cultured in DMEM medium.

Ionomycin and cromoglicic acid were purchased from Cayman Chemical Company (MI, USA) and Santa Cruz Biotechnology (Santa Cruz, CA, US), respectively. For *in vitro* experiments, they were dissolved in dimethylsulfoxide (DMSO, Sigma–Aldrich, MO, US)

2.3. MTT assay

Cell suspensions of different cells were seeded on 96-well plates at a concentration of 5×10^4 cells per well. The 3-(4,5-dimethylthiazol-2-yl)-2,5-dipheny ltetrazoliumbromide (MTT) was first prepared as a stock solution (5 mg/mL) in phosphate-buffered saline (PBS, pH 7.2) and filtered. Then, 15 μL of MTT solution was added to each well. After incubation for 4 h at 37 °C and 5% CO_2, 185 μL of solubilization solution/stop was added to each well. The 96-well plates were read by an enzyme-linked immunosorbent assay (ELISA) reader at 620 nm for absorbance density values to determine cell viability, and the percentage of surviving cells was calculated from the ratio of the absorbance of treated to untreated cells.

2.4. LDH assay

LDH assay was performed with the culture medium at the end of experiments using the LDH Cytotoxicity Assay Kit (Cayman Chemical Company) as described by the manufacturer. Briefly, the cells were grown at 37 °C and 5% CO_2 in Dulbecco's Modified Eagle Medium supplemented with 10% heat inactivated fetal bovine serum 1% penicillin and streptomycin. The cells were seeded on 96-well plates at a concentration of 2×10^4 cells per well. After 48 h, 100 μL of each supernatant from each well of the cultured cells was transferred to a new plate and 100 μL of reaction solution was added to each well and incubated with gentle shaking on an orbital shaker for 30 min at room temperature. The absorbance was read at 490 nm with a plate reader.

2.5. Collagen detection

The Sirius Red Total Collagen Detection Kit (Chondrex, Redmond, WA, US) was used. Suspended cells were seeded in 24-well plates at a concentration of 1×10^4 cells per well and were treated with cromoglicic acid at various doses for 48 h. Diluted standard solutions or samples were added in duplicate to 1.5-mL centrifuge tubes. Each tube was incubated with 500 μL of Sirius Red Solution for 20 min at room temperature. After the supernatant was removed, the tube was washed 2 times and 200 μL of the final supernatant was transferred to a 96-well plate. The optical density value was read at 510–550 nm.

2.6. Senescence β-galactosidase (SA β-gal) staining

SA-β-gal staining was performed with the Senescence β-Galactosidase staining kit according to the manufacturer's instructions (Biovision, CA, USA). Briefly, after washing with PBS, cells were fixed with 2% formaldehyde and 0.2% glutaraldehyde in PBS for 15 min at room temperature. The fixed cells were incubated with X-gal staining solution at 37 °C for 24 h after washing with PBS. Cells were visualized and pictures were taken with a Zeiss PALM laser capture microdissection microscope (Zeiss, Germany).

2.7. Western blot

TGF-β (Calbiochem, CA, USA) treatments were used to check the level of E-cadherin in primary hepatocytes. The antibodies against E-Cadherin (H-180) were purchased from Santa Cruz and diluted 1:1000 for incubation. Western blots were performed as previously described.

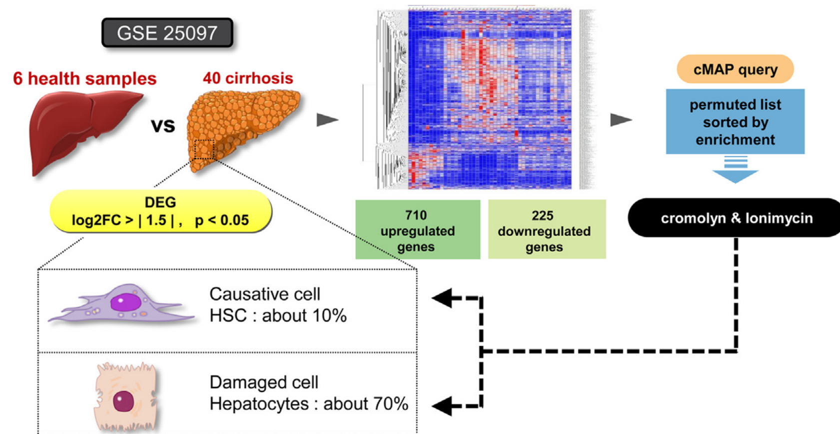

Fig. 1. Identification of candidate agents for cirrhosis treatment using a connectivity map. Differentially expressed genes edited from GSE16487 were applied to a connectivity map to obtain candidate agents with the potential to reverse cirrhosis. The top 20-ranked chemicals were included in the list.

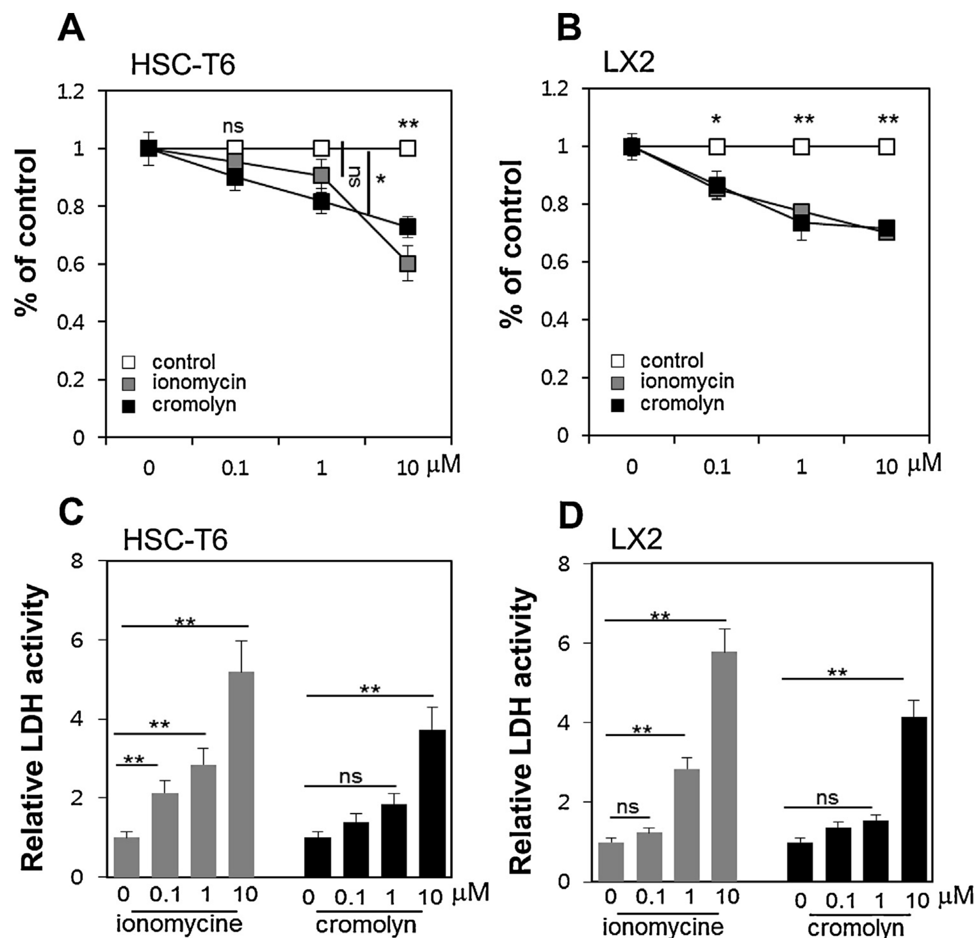

Fig. 2. Changes in proliferation and cell death induced by the candidate agents (A–B). The mitochondrial activity of HSC-T6 and LX2 was assessed using an MTT assay for various doses of the chemicals ionomycin and cromolyn. Untreated culture conditions were used for the control. (C–D). To test the cytotoxicity of increased concentrations of the drugs (up to 10 μM), cell death was measured by monitoring the lactate dehydrogenase (LDH) concentration in the culture media. ns, not significant; *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$.

2.8. Animal experiments

Animal experiments were approved from the Institutional Animal Care and Use Committee at Wonkwang University (WK 2015-06-BR) and performed in compliance with the institutional guidelines. 30 male ICR mice (20 ± 2 g) were purchased from Orient Bio, Ltd. (Korea) and maintained on 12 h light/dark cycles in a temperature and humidity controlled room. Carbon tetrachlo-

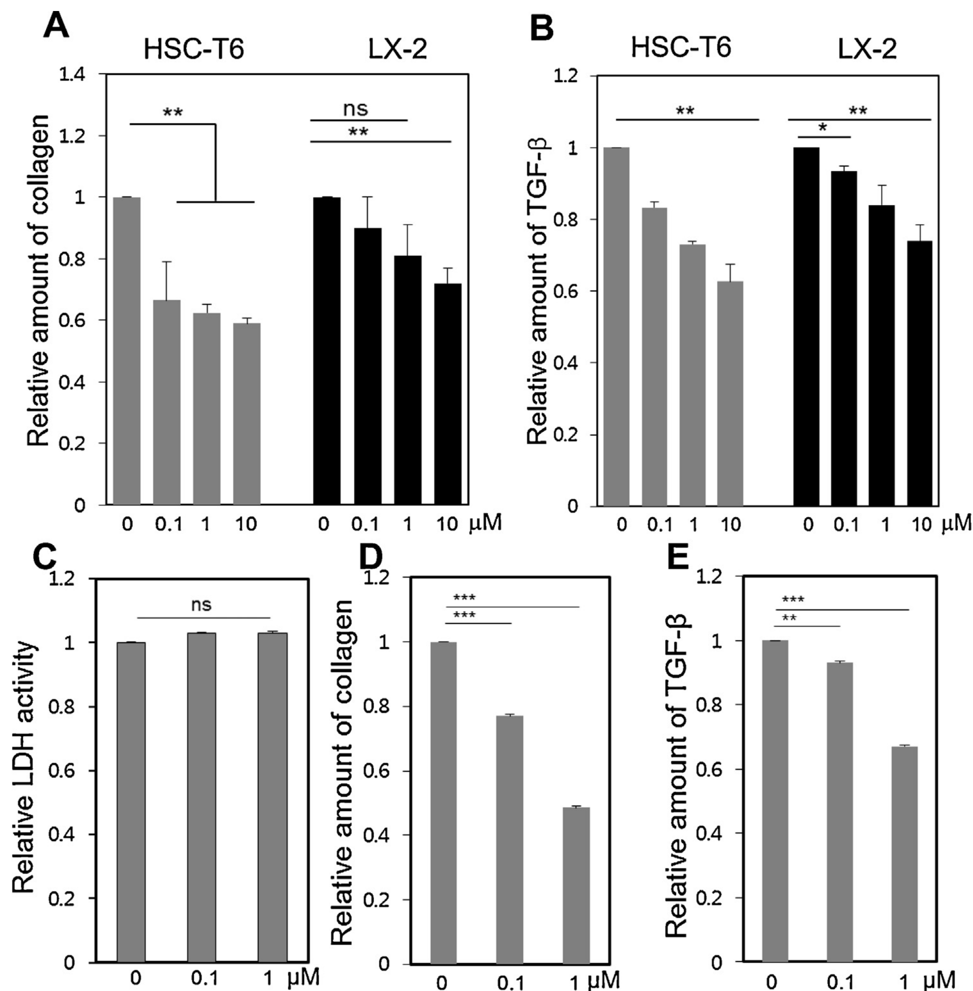

Fig. 3. Changes in the secretion level of collagen and TGF-β after cromolyn treatment. (A) Collagen deposition in culture media was analyzed by enzyme-linked immunosorbent assay (ELISA) in HSC-T6 and LX2 cell lines (B) Secretion of TGF-β was checked with ELISA in HSC-T6 and LX-2 cell lines. (C) The LDH assay was repeatedly performed on murine primary HSC. In primary HSC, secretion of (D) collagen and E.TGF-β were monitored at the different doses of cromolyn (0.1 and 1 μM). ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

ride (CCl4) were purchased from Sigma–Aldrich (US). 6 mice were used as control in healthy condition. CCl4 The other 24 mice were intraperitoneally administered with CCl4 (5 ml/kg, 20% v/v in olive oil) twice a week for 8 weeks. Then, phosphate buffered saline (PBS) were orally taken to randomly selected 8 mice and the half of the others with cromolyn of 10 mg/kg and the half with cromolyn at the higher concentration, 50 mg/kg, respectively.

2.9. Immunohistochemistry

After chemical induction of liver cirrhosis or fibrosis, the livers were preserved in 100 ml/L neutral buffered formalin solution and processed by embedding in paraffin. Tissue sections (4–5 μm) were immunostained by using the antibody against alpha smooth muscle actin (Abcam US, Cat.No. ab5694) and PCNA (Abcam US, Cat.No. ab18197). For detection we used a biotin-free horseradish peroxidase-labeled dextrose-based polymer complex bound to secondary antibody (DAKO EnVision Plus System-HRP, DakoCytomation, Carpinteria, CA). The slides were then developed for 5 min with 3-3′-diaminobenzidine (DAB) chromogen, counterstained with hematoxylin and coverslipped. Negative controls were stained with rabbit IgG in parallel with each batch.

3. Results

3.1. Analysis of anti-cirrhotic chemical candidates using a connectivity map

To obtain the therapeutic signature of liver fibrosis or cirrhosis based on gene expression profiles, we utilized GEO data (GSE25097). Briefly, 6 healthy liver samples and 40 cirrhotic liver samples were included. Applying a threshold fold-change of 'logFC > |1.5|', a list of differentially expressed genes was obtained for both up- and downregulation. In total, 710 genes were upregulated and 225 genes were downregulated.

A list of candidate chemical to reverse cirrhosis was generated using the connectivity map, and the 20 top-ranked chemical agents were identified and those with significant *p*-values were finally selected (Table S1). Five agents had significant *p*-values, *i.e.*, cromoglicic acid (cromolyn, CID 2882), MK-801 (Dizocilpine, CID 180081), iopanoic acid (CID 3735), ionomycin (CID 6912226), and tolazamide (CID 5503). Among them, MK-801 is an uncompetitive antagonist of a glutamate receptor [21] and tolazamide is used by type II diabetes patients to control glucose [22]. Iopanoic acid was initially developed to inhibit deiodinase enzymes, but it has been discontinued as a treatment option in the United States

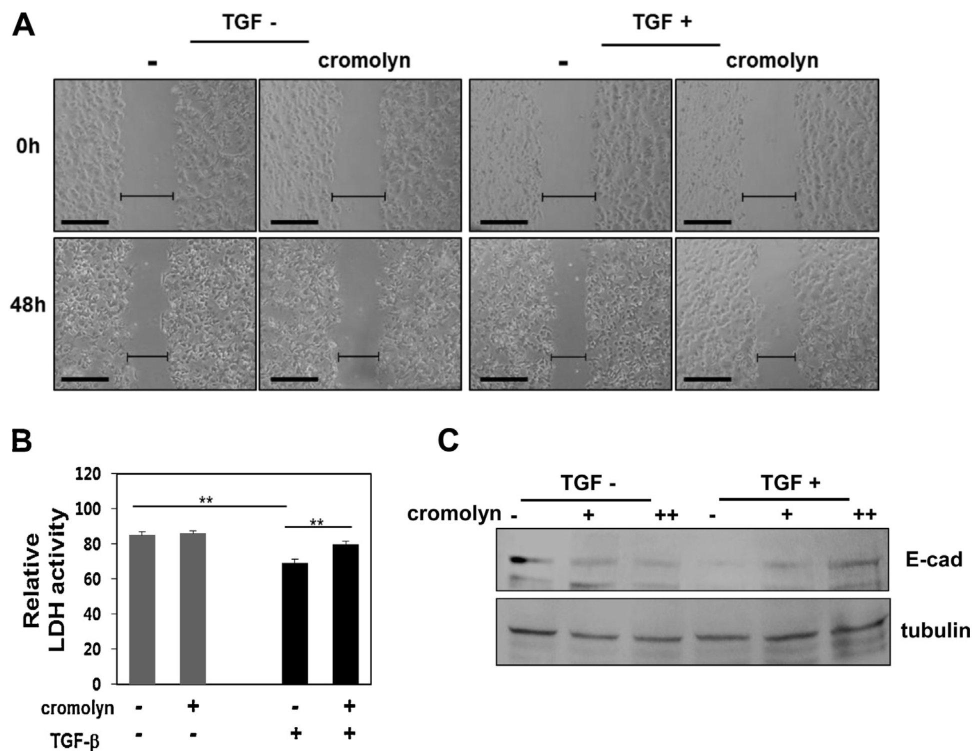

Fig. 4. Suppression of TGF-β-induced epithelial-mesenchymal transition by cromolyn. (A) In primary hepatocytes, the effect of cromolyn on cell motility was tested with wound assays between 0 and 48 h with and without TGF-β (2 ng/mL). A representative microscopy image is shown. Scale bar, 100 μm. (B) The recovery distance of wound was measured and the rate of decrease is shown as a relative percentage compared with the distance at 0 h. (C) Western blot was performed to check the change in E-cadherin expression for various combinations of cromolyn and TGF-β, as indicated. Tubulin was used as a loading control.

owing to unexpected side effects [23]. Given the strong functions and/or potential side effects of MK-801, tolazamide, and iopanoic acid for patients with cirrhosis, we focused on cromoglicic acid and ionomycin (Fig. 1).

3.2. Toxicity test for hepatic stellate cells

We used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, which measures proliferation activity, to analyze HSCs. HSC-T6 and LX2 cells were used as a representative HSCs. As the concentration of chemicals increased, the proliferation rate, as determined by the MTT assay, gradually decreased compared with the control. While the decrease was not statistically significant for a low dose (0.1 μM) of each agent in HSC-T6 cells, there was an inactivation effect for the low concentration in LX2 cell lines (Fig. 2A and B). The toxicity of cromolyn and ionomycin was then tested to determine whether the decreased MTT values (as percentages) resulted from cell death. We tested the amount of lactate dehydrogenase (LDH) secreted into the culture media for gradually increased of doses of ionomycin and cromolyn in independent cultures. While ionomycin induced cell death in a dose-dependent manner (Fig. 2C and D, left grey panel), cromolyn toxicity was mild, except for the highest dose of 10 μM in both cell lines (Fig. 2C and D right black panel). Thus, we focused on cromolyn for additional tests owing to the potential of toxicity of ionomycin.

We examined, whether cromolyn suppresses the production of collagen and TGF-β, which are the main secreted markers of activated HSCs [24]. As the chemical concentration increased, secreted collagen decreased significantly. Cromolyn treatment also gradually suppressed the production of TGF-β, as evidenced by the increased concentration detected by enzyme-linked immunosorbent assay (Fig. 3A and B). The experiments were confirmed with primary HSCs. Based on the result from LDH assay, cromolyn did not seem to seriously induce cell death under the concentration of 10 μM (Fig. 3C). The secretion of collagen and TGF-β were also significantly decreased in the dose-dependent manner (Fig. 3D and E).

3.3. Suppression of hepatocyte EMT by cromolyn

We tested the restoration effect of cromolyn on hepatocytes. Previous studies have shown that hepatocytes undergo EMT during fibrosis [14,15]; accordingly, we examined the motility of primary hepatocytes upon cromolyn treatment in the presence and absence of TGF-β.

In the absence of TGF-β, hepatocyte motility in the cromolyn-treated group was not significantly different from that in the control group. However, TGF-β induced the migration of hepatocytes, and cromolyn effectively suppressed the migratory activity of hepatocytes by 15% in terms of moving distance (Fig. 4A and B). TGF-β treatment decreased the expression level of E-cadherin, similar to the phenomenon of EMT (compare the 1st and 4th lanes of E-cadherin in Fig. 4). Incubation with cromolyn inhibited the decrease of E-cadherin in a dose-dependent manner (Fig. 4C). In the absence of TGF-β, cromolyn minimally affected the expression of E-cadherin.

3.4. Anti-senescence effect of cromolyn on hepatocytes

We also tested the anti-senescence effect of cromolyn using β-galactosidase staining because senescence is a representative phenomenon associated with fibrosis. Three days after *in vitro* culture of hepatocytes, we detected a prominent increase in the

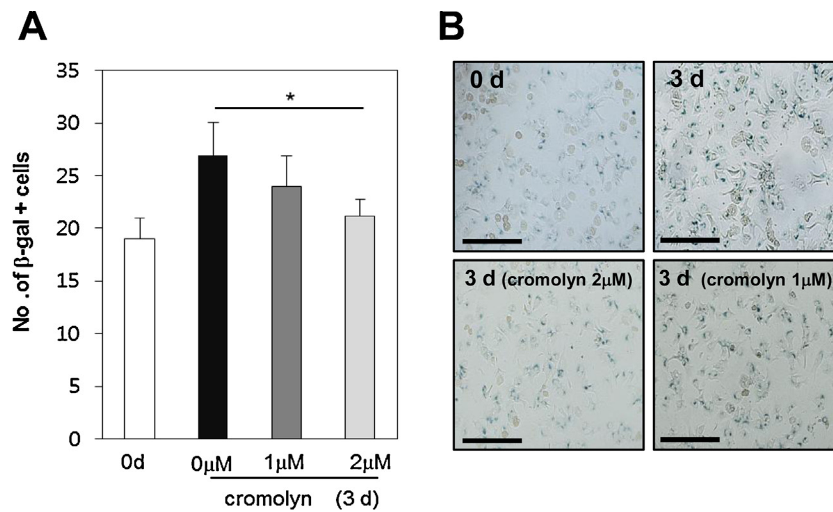

Fig. 5. Effect of cromolyn on β-galactosidase formation. (A) Senescence of primary hepatocytes was assessed using β-galactosidase staining after treatment with cromolyn in two different doses. The number of β-galactosidase-positive cells was counted. (B) Representative field of view on microscopy at 3 days after isolation of primary cell. Images showing representative β-galactosidase-positive cells. *, $p < 0.05$. Scale bar, 100 μm.

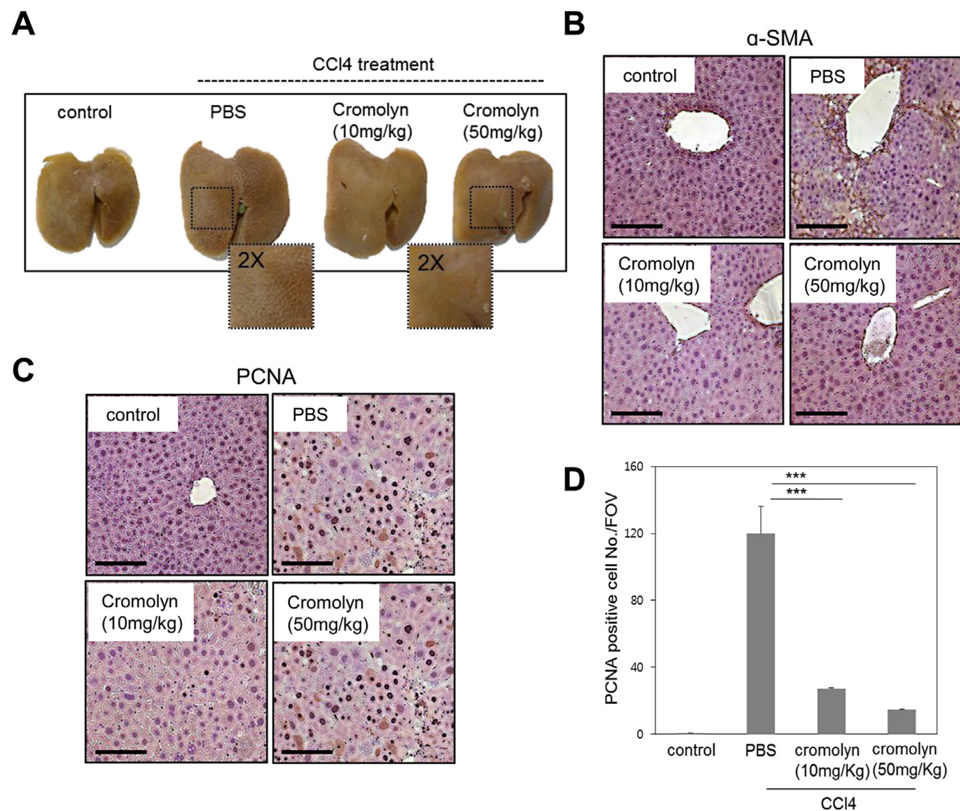

Fig. 6. *In vivo* experiments on anti-fibrotic effect of cromolyn. (A) Isolated mice liver after experiments (B) α-smooth muscle actin (C) PCNA was stained on the paraffinized tissues. (D) The number of PCNA positive cell per single field of view (FOV) of microscope was counted and expressed as a graph. ***, $p < 0.001$. Scale bar, 100 μm.

number of β-galactosidase-positive cells. The appearance of β-galactosidase-positive cells was related to cromolyn treatment in a dose-dependent manner (Fig. 5A and B).

3.5. In vivo *validation on anti-fibrotic effect of cromolyn*

Carbon tetrachloride (CCl4) was used to induce liver fibrosis for 8 weeks and the isolated livers showed coarse surface. Oral administration of cromolyn for 4 weeks obviously compromised the surface coarseness (Fig. 6A). And the tissues were immunostained upon α-smooth muscle actin (SMA) and PCNA, the representative markers of liver fibrosis. While α-SMA was definitely increased compared with control, the cromolyn-treated livers presented moderate staining signal (Fig. 6B). PCNA also showed similar pattern with the results of α-SMA. Although CCl4 successfully developed PCNA positive cells, treatment of cromolyn mitigated the number of PCNA

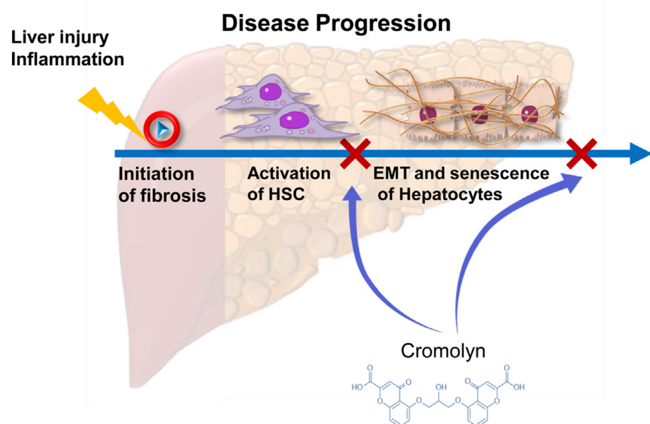

Fig. 7. Concept of cromolyn action Several sources of stress, such as viral infections, activate hepatic stellate cells (HSCs) and initiate fibrosis. Fibrosis continuously activates HSCs. This results in a deleterious cycle. The aberrantly activated HSCs deposit collagen around hepatocytes. Hepatocytes senesce and undergo EMT. Cromolyn may have two targets, HSCs and hepatocytes.

positive cells (Fig. 6C and D). Although the tests were performed at different doses, the 10 mg/kg and 50 mg/kg, the anti-fibrotic effect of cromolyn was observed similarly at both doses.

4. Discussion

Liver cirrhosis results from the progression of chronic hepatitis and is currently regarded as an irreversible disease [25]. For successful treatment that targets the causes of liver cirrhosis, it is necessary to systemically determine the component cells associated with the disease. In this study, we used a bioinformatics approach to identify a candidate agent to discontinue the progression of fibrosis and ultimately recover liver function by targeting both HSCs—the initial disease-causing cells—and hepatocytes—the primary damaged cells. Cromolyn was selected as a possible therapeutic candidate in this study, and we validated its potential as a therapeutic agent for fibrosis or cirrhosis *in vitro*. The studies on cromolyn, the stabilizer of mast cell, have reported its potential as a remedy for pulmonary fibrosis and cardiac fibrosis [26,27].

The mast cell is a known effector of allergic reactions, but recent evidence indicates that mast cells are involved in wound healing and fibrosis [28].

Cytokines/mediators in the cytoplasmic granule of the mast cell, such as histamines, chymases, tryptases, heparin, TNF-α, and TGF-β, are inflammatory and profibrogenic mediators, and cause histological changes by inducing various immunological reactions. The association between mast cells and liver fibrosis has been speculated based on the findings of a few recent studies [29–32]. Mast cells are integral players in the onset of liver fibrosis via MMP2 expression [32] and a large number of mast cells are deposited in fibrotic areas of tissue in hepatitis C patients, suggesting mast cells contribute to the onset of liver fibrosis [29,33]. Although mast cell is a critical mediator of liver inflammation, mast cells-independent anti-inflammatory effect of cromolyn has been also reported [34]. Thus, based on the fact that inflammation is a main cause of liver fibrosis, treatment of cromolyn may compromise the progress of fibrosis through anti-inflammatory effect.

In our study, although the role of mast cells in the pathogenesis of fibrosis was not clarified, treatment with cromolyn suppressed HSC activation and restored their function *in vitro*. Taken together, these results indicate that cromolyn could be have clinical applications for the development of an anti-cirrhotic agent in the future.

We utilized a connectivity map, which has been recently developed to predict the tripartite linkage between disease, gene expression, and small chemicals [19], to identify candidate agents that have anti-fibrotic or cirrhotic effects on the liver. In previously studies, gene expression signatures in healthy and cirrhotic liver tissues were used and the threshold *p*-value was modulated to make it more probable. The differentially expressed gene profiles obtained from tissue samples was limited because different cell types were mixed in the small biopsy fragments [35,36]. This can cause low data reproducibility among experiments *in vitro* or *in vivo*. However, we paid specific attention to microarray data for those error-prone biopsy tissues. Based on the presence of various cell types, with additional *in vitro* experiments, we independently validated the effect on different cells, *i.e.*, hepatocytes and HSC. Cromolyn sodium was effective for both HSCs and hepatocytes and did not exhibit noticeable toxicity (Fig. 7). Although many therapeutic agents are used to treat liver cirrhosis, we suggest that cromolyn is a potential agent for radical therapy of liver cirrhosis.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phrs.2015.10.002.

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