

# Three-dimensional Differentiated Human Mesenchymal Stem Cells Exhibit Robust Antifibrotic Potential and Ameliorates Mouse Liver Fibrosis

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## Abstract

Recently, three-dimensional (3D)-cultured adipose mesenchymal stem cells (ASCs) have provided an effective therapy for liver fibrosis. This study aimed to enhance the potential of human ASCs for antifibrosis or hepatocyte regeneration using a 3D culture system and investigate their therapeutic mechanism in experimental liver fibrosis. ASC-3Dc were generated in a 3D culture system and stimulated with four growth factors, namely epidermal growth factor, insulin-like growth factor (IGF)-I, fibroblast growth factor-2, and vascular endothelial growth factor-A. The expression levels of antifibrotic or hepatic regeneration factors were then measured using quantitative real-time polymerase chain reaction and enzyme-linked immunosorbent assay. The therapeutic effects of ASC-3Dc were determined using a liver fibrosis model induced by thioacetamide. Histological analysis was performed to elucidate the therapeutic mechanism. ASC-3Dc exhibited high levels of hepatocyte growth factor (HGF), IGF-I, stromal cell-derived factor (SDF)-I genes, and protein expression. In addition, injecting ASC-3Dc significantly prevented hepatic fibrosis and improved liver function *in vivo*. Moreover, high numbers of ki-67-expressing hepatocytes were detected in the ASC-3Dc-injected livers. Albumin-expressing ASC-3Dc engrafted in fibrotic livers augmented HGF expression. Thus, short-term 3D-cultured ASCs may be a novel alternative to the conventional treatment for liver damage in clinical settings.

## Keywords

3D culture, cell therapy, antifibrosis, mesenchymal stromal cell, liver fibrosis

## Introduction

Liver fibrosis is caused by increased extracellular matrix synthesis in the liver parenchyma. Currently, liver transplantation is the ultimate treatment option for end-stage liver fibrosis<sup>1</sup>. In practice, however, organ transplantation is not easy due to limited donors, organ rejection, surgical complications, and high costs.

Mesenchymal stem cell (MSC) therapy has been suggested as an effective alternative therapeutic approach for end-stage liver disease<sup>2,3</sup>. However, the precise therapeutic mechanisms of stem cell therapies have not yet been elucidated. Additionally, the precise therapeutic effect and lasting efficacy of these cells in clinical application are still in question.

Recently, various methods have been developed to increase the therapeutic effect of stem cell therapy. Of these approaches, three-dimensional (3D) culture systems have been reported to enhance the therapeutic potential of mesenchymal stem cells<sup>4–6</sup>. A recent study indicated that

a 3D spheroid culture improves their therapeutic effects on liver fibrosis<sup>7</sup>. In this system, the paracrine actions of mesenchymal stem cells enhance their therapeutic effects under 3D spheroid culture compared to those under regular culture conditions.

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Name	Product size (bp)	5' Primer sequence	3' Primer sequence
AFP	247	AGCTTGGTGGTGGATGAAAC	CCTCTTCAGCAAAGCAGACT
ALB	197	CATGTTTCGAGAGCTACATCAAC	ATGGAAGGTGAATGTTTCAGCA
CK8	403	CATGTTTCGAGAGCTACATCAAC	GTTGGCAATATCCTCGTACTG
HNF4A	193	TCAGCACTCGAAGGT CAAGC	CACTCAACGAGAACCAGCAG
Alu	290	AATATGGCCC AACTGCAGAA	CATCGCATTTTCACATCCAA
GAPDH	224	TCTTCACCACCATGGAGAAG	CATG AGTCCTTCCACGATAC

All the primer/probe sets were purchased from Applied Biosystems.

Previously, we reported that a novel stem cell culture condition comprising a mixture of potent growth factors and mesenchymal stem cells enhanced the potential for antifibrosis and liver regeneration<sup>8</sup>. The cytokine combination stimulated the mesenchymal stem cells, resulting in robust hepatogenic and angiogenic capacities that ameliorated fibrosis. In the present study, we developed a novel and simple 3D culture system for stem cell culture using cytokines, and investigated the therapeutic effects and mechanism of the mesenchymal stem cells in a fibrotic liver model.

## Materials and Methods

### Cell Culture

Three different cell lines of human adipose tissue-derived mesenchymal stromal cells (ASCs) were purchased from ATCC (Manassas, VA, USA). All the MSC-specific markers were evaluated by flow cytometry. ASCs were cultured at 37°C under 5% CO<sub>2</sub> in culture solution [alpha-MEM with 10% fetal bovine serum (FBS), 100 mg/ml of streptomycin, and 100 U/ml of penicillin]. To induce hepatic differentiation, cells were cultured in hepatocyte growth medium (HGM) (Promcell, Heidelberg, Germany) for 10 days.

### Culture with Cytokine Cocktail

To enhance hepatogenic potential, ASCs were cultured in culture medium [alpha-MEM with 2% FBS, 100 mg/ml of streptomycin, and 100 U/ml of penicillin] containing several cytokines including 20 ng/ml human epidermal growth factor (EGF), 100 and 20 ng/ml human vascular endothelial growth factor (VEGF)-A and human fibroblast growth factor (FGF)-2, and 20 ng/ml human insulin-like growth factor (IGF)-1 at 37°C under 5% CO<sub>2</sub> for 5 days.

### 3D Culture

3D spheroids were generated according to the previous report<sup>9</sup>. In brief, ASCs ( $1 \times 10^5$ ) were suspended in 27  $\mu$ l of conventional culture medium or cytokine cocktails culture medium and placed onto the cover of culture plates and cultured inversely for 5 days. To dissociate cells, spheroids were incubated with 0.05% trypsin/ethylenediaminetetraacetic acid for 5 min and terminated the dissociation using fresh medium (a-MEM with 10% FBS, 100 U/ml of penicillin, and

100 mg/ml of streptomycin) and these dissociated cells were used in this study.

### Cell Viability Assay

Viability of 3D spheroids was evaluated using propidium iodide (stains dead cells; Sigma-Aldrich, Gibco, St Louis, MO, USA). After staining, the cells were diluted in assay buffer and were then analyzed by fluorescence-activated cell sorting (BD Biosciences, San Jose, CA, USA).

### Quantitative Real-time Polymerase Chain Reaction

Quantitative real-time polymerase chain reaction (qRT-PCR) assays were conducted as previously described<sup>10,11</sup>. Briefly, total RNA was isolated from cells using RNA-stat (Iso-Tex Diagnostics, Friendswood, TX, USA) according to the previous report<sup>12</sup>. We eliminated genomic DNA contamination from RNA using DNase and its reagent (Thermo Fisher Scientific, San Jose, CA, USA). Extracted RNA was reverse transcribed using TaqMan reagents (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's protocols. The synthesized cDNA was subjected to qRT-PCR using primers and probes. The RNA levels were quantitatively measured using an ABI PRISM 7000 (Applied Biosystems). The relative mRNA expression normalized to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression. qRT-PCR primers were as follows: albumin (Hs00609411\_m1) and GAPDH (Hs99999905\_m1) for human, and albumin (Mm00802090\_m1) and GAPDH (Mm99999915\_g1) for mouse. The following paired RT-PCR primers were used:

### Enzyme-linked Immunosorbent Assay

Protein secretion levels of cultured cells were examined by using hepatocyte growth factor (HGF), IGF-1, and stromal cell-derived factor (SDF)-1 enzyme-linked immunosorbent assay (ELISA) kit (Abcam, Cambridge, MA, USA). Briefly, cells were cultured in 100 U/ml penicillin and 100 mg/ml streptomycin (Gibco) and low-glucose DMEM (Gibco, Grans Island, NY, USA) containing 10% FBS for 10 days. Culture media from each group was then centrifuged at  $1,000 \times g$  for 10 min. The supernatants were collected and used as culture medium for the study.

## Induction of Hepatic Fibrosis and Cell Injection

All animal procedures were approved by the Animal Ethics Committee of Catholic Kwandong University and performed in accordance with the Guidelines for Care and Use of Laboratory Animals of Catholic Kwandong University. We also confirmed that all experiments were performed in accordance with national guidelines and regulations. Seven-week-old male BALB/c nu mice were obtained from Koatech (Pyeongtaek, Korea). To induce liver fibrosis, mice were intraperitoneally injected with 150 mg/kg thioacetamide (TAA; Sigma, St Louis, MO, USA) two times a week for 4 weeks. After the induction of fibrotic liver (i.e., 1 week after TAA injection), cell transplantation was conducted. Cells ( $1 \times 10^6$ ) were resuspended in saline (50  $\mu$ l) and injected via the portal vein. The mice were randomly divided into three groups: PBS ( $n = 10$ ), ASC-3D ( $n = 10$ ), and ASC-3D cocktail (ASC-3Dc) ( $n = 10$ ). The sham group ( $n = 5$ ) only received 50  $\mu$ l of normal saline. Liver tissues were harvested 3 weeks after cell injection.

## Histological Analyses

The liver tissues from mice were fixed with 4% paraformaldehyde for 1 day and embedded in Tissue-Tek OCT compound (Sakura Fine Technical Co. Ltd, Tokyo, Japan). Sectioned (10  $\mu$ m thick) samples were stained with Harris hematoxylin solution (Sigma) for 3 min followed by eosin Y (Sigma) for 25 s. Collagen and hepatic fibrosis were assessed using a collagen staining kit (Chondrex, Inc., Redmond, WA, USA). Tissue sections (10  $\mu$ m thick) were fixed with Kahle's fixative for 30 min and then stained in the dye solution for 30 min. The sections were rinsed with water and incubated with a dye extraction buffer and collected eluted dye solution and then read the O.D. value at 540 and 620 nm using spectrophotometer. Masson's trichrome staining was conducted, and the areas of fibrosis were examined by MetaMorph software (Downingtown, PA, USA). Anti-Ki-67 (1:200, Dako, Carpinteria, CA, USA) was used for cell proliferation assay. To detect albumin, tissues were stained with antihuman or anti-mouse albumin (1:250, Abcam) using a secondary antibody conjugated with green fluorescent protein. Nuclei (blue) were stained with 4',6-diamidino-2-phenylindole.

## Western Blotting

Immunoblotting was performed using a previously described protocol<sup>13,14</sup>. In brief, protein extracts (100 mg each) were separated on 8% sodium dodecyl sulfate polyacrylamide gel electrophoresis gels (Bio-Rad Laboratories, Redmond, WA, USA) and electrotransferred into PVDF membranes (GE Healthcare, Menlo Park, CA, USA). The samples were probed with antibodies against the following: HGF (1:500, Abcam), p-p38 (1:250, Abcam), p38 (1:200, Abcam), and GAPDH (1:200, Abcam). The membrane was washed and incubated

with secondary antibody (horseradish peroxidase-conjugated), and the signals were detected using LAS-3000 chemi-doc system (Fujifilm, Tokyo, Japan).

## Liver Function Analysis

Blood samples were obtained from mice and centrifuged, and serum samples were collected 3 weeks after cell transplantation. The serum samples were used for liver injury markers such as alanine aminotransferase (GPT/ALT), aspartate aminotransferase (GOT/AST), ammonia, and bilirubin by a FUJIFILM DRI-CHEM 3500.

## Statistical Analysis

Statistical analyses were performed by Student's *t*-test or analysis of variance with Bonferroni's multiple comparison test using SPSS v12.0.  $P < 0.05$  was considered statistically significant. All data are shown as the mean  $\pm$  standard deviation.

## Results

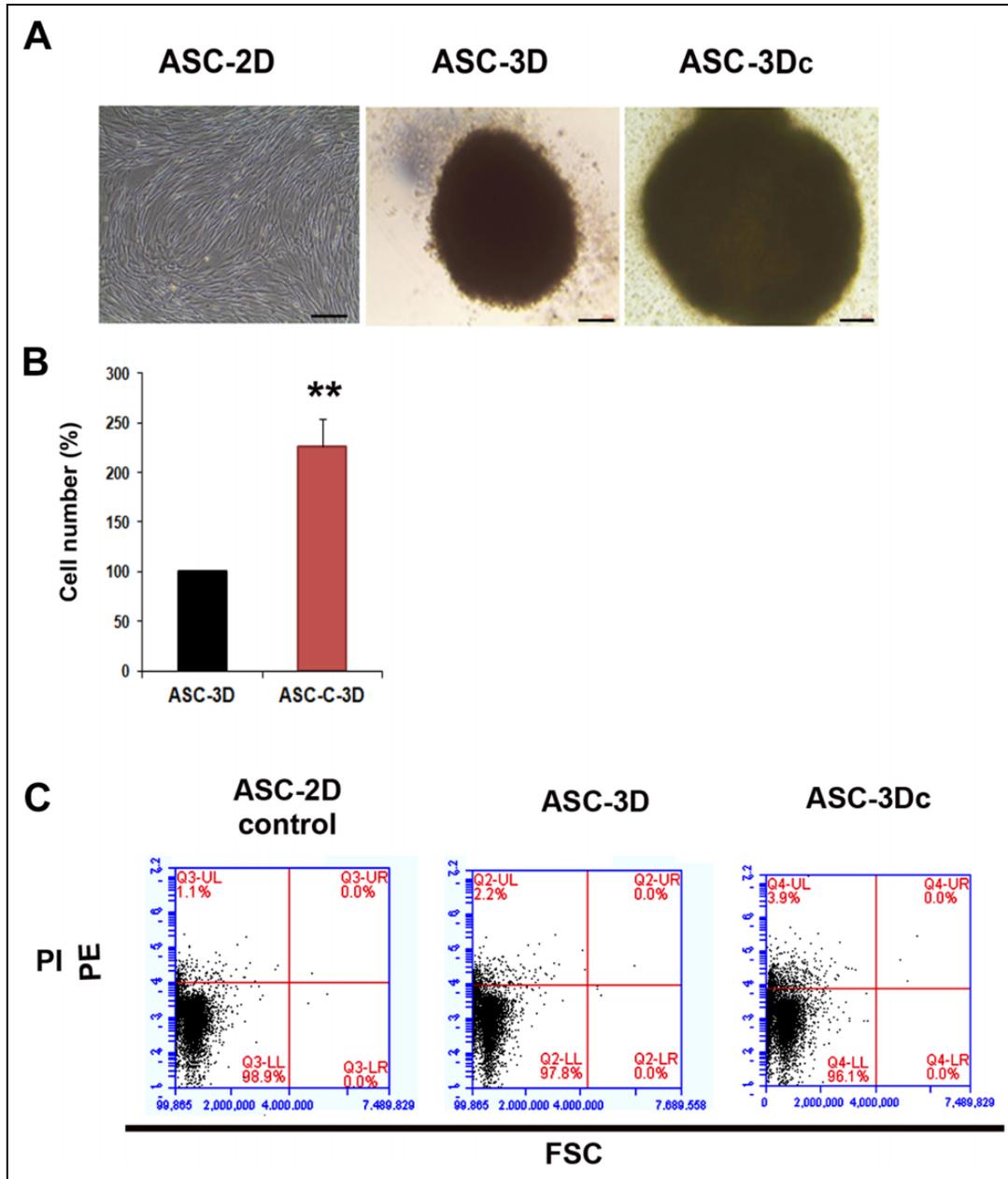
### 3D Culture Cytokine Cocktail

Previously, we found optimal combination of growth factor cocktail for hepatic protection or regeneration of stem cells<sup>8</sup>. In this study, we hypothesized whether this cytokine cocktail may stimulate the regeneration potential of stem cells. To examine the hepatic regeneration potential of ASCs in the 3D culture system, we investigated several cytokine combinations and tested one candidate cytokine cocktail containing FGF-2, IGF-2, EGF, and VEGF-A. ASCs derived from a 3D spheroid culture stimulated with this cocktail (ASC-3Dc) were compared with an ASC-3D spheroid culture without cytokines (ASC-3D). After 5 days of culture, the effect of the cytokine cocktail on cell proliferation was examined. Interestingly, spheroid proliferation was highly induced in the 3D culture containing the cytokine cocktail compared to that in the control 3D culture (Fig. 1A, B). In addition, cell viabilities of ASC-3D and ASC-3Dc were about 96% to 98% (Fig. 1C).

### ASC-3Dc Exhibit Antifibrotic or Liver Regeneration Properties

We then characterized the gene expression profile in ASC-3Dc using qRT-PCR (Fig. 2A). Interestingly, three ASC-3Dc cell lines exhibited higher levels of gene and protein expression of HGF (10.17-fold), IGF-1 (3.87-fold), and SDF-1 (4.26-fold) than control ASC-3D. To confirm the qRT-PCR results, protein levels were analyzed using ELISA. As expected, the levels of secreted HGF, IGF-1, and SDF-1 proteins were also significantly higher in ASC-3Dc supernatants than in control ASC-3D supernatants (Fig. 2B).

Next, cells were induced with conventional hepatocyte culture medium to investigate in vitro hepatic differentiation potential of the cells. Interestingly, ASC-3Dc expressed



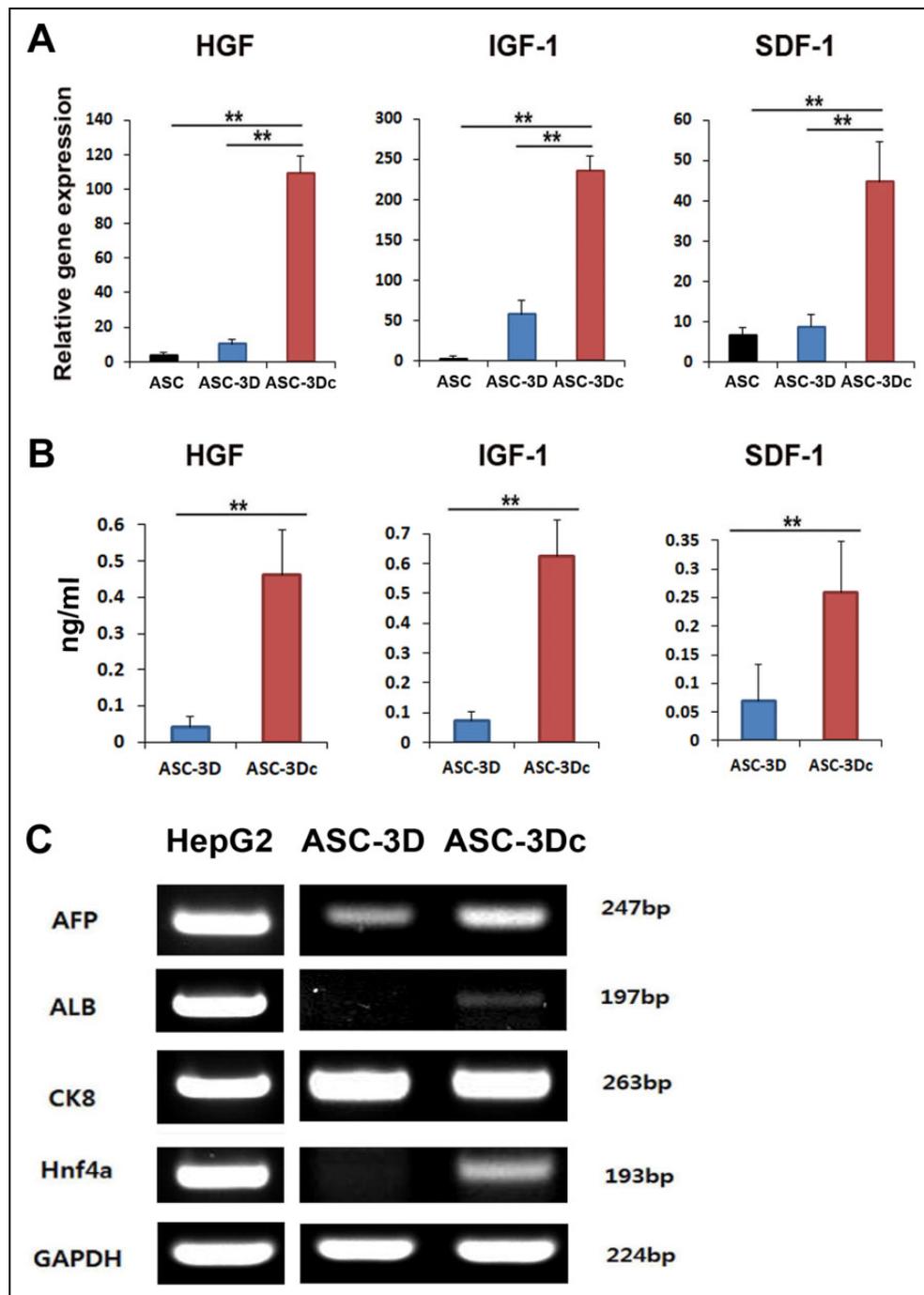
**Fig. 1.** Analysis of morphology, cell proliferation capacity, and cell viability in 3D-cultured ASCs. (A) Morphology of 2D-cultured ASCs, ASC-3D, and ASC-3Dc. (B) Analysis of cell proliferation capacity. ASCs were 3D cultured with or without cytokine cocktails for 5 days and total number of cells were then counted.  $**P < 0.01$ ;  $n = 10$  per group. Bar = 100  $\mu\text{m}$ . (C) Analysis of cell survival capacity. Representative pictures of flow cytometry. Viability of 3D spheroids was evaluated by PI staining. 3D: three-dimensional; ASC: adipose mesenchymal stem cell; FSC: forward scatter; PE: phycoerythrin; PI: propidium iodide.

hepatocyte-specific genes, AFP, ALB, CK8, and Hnf4a, 10 days after differentiation (Fig. 2C).

### Transplanted ASC-3Dc Shows Antifibrotic Property

To investigate the therapeutic potential of cytokine-induced ASC-3Dc,  $5 \times 10^5$  cells in each group were injected into mice via the portal vein 1 week after acute liver injury. As a control, mice were injected with PBS or

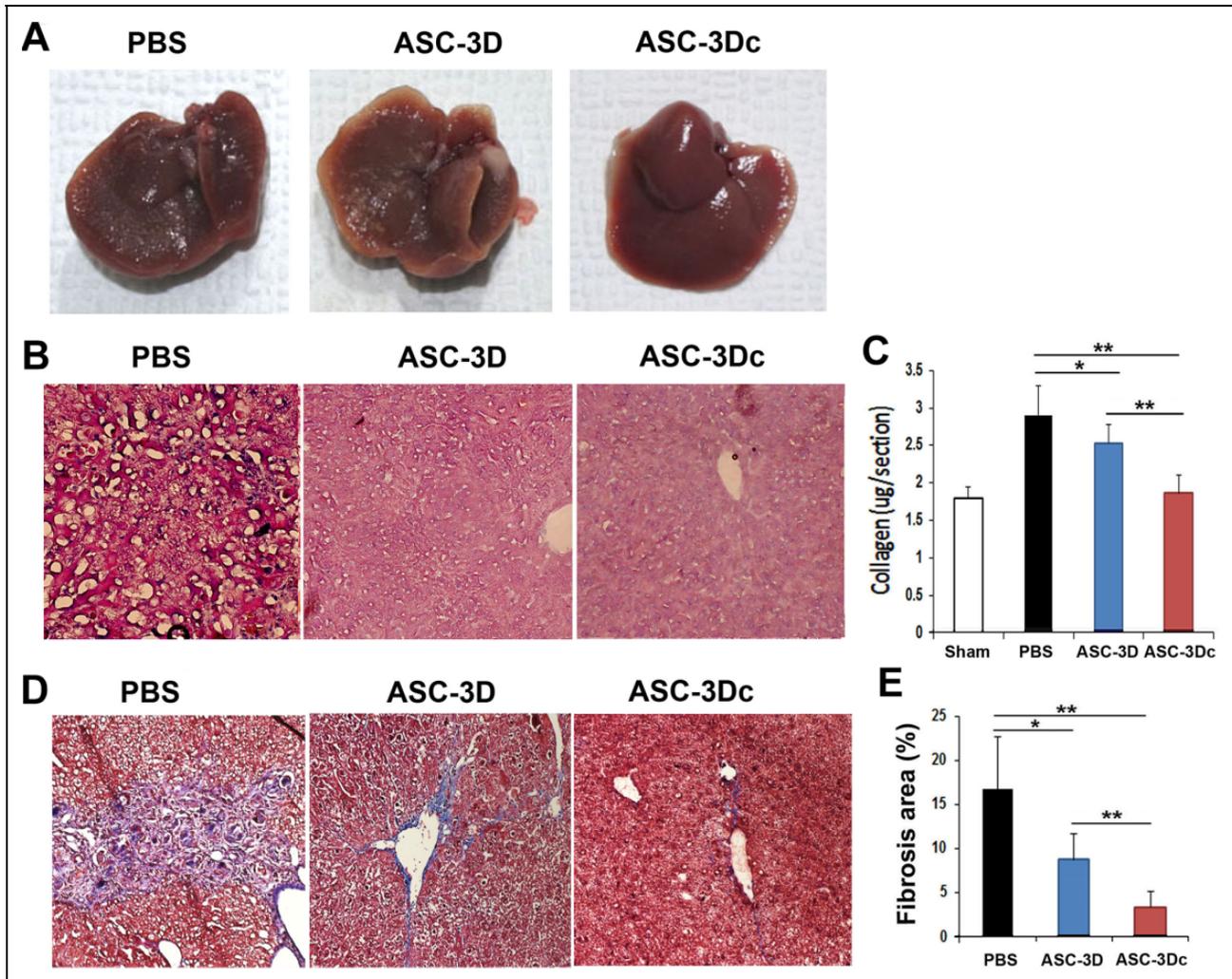
ASC-3D. Liver tissues were harvested 3 weeks after injection of stem cells and examined. Morphological changes of the liver surface were detected. Livers of PBS- and ASC-3D-treated mice showed signs of hepatocirrhosis compared to those of ASC-3Dc-treated mice (Fig. 3). In line with these morphological observations, a histological examination using hematoxylin and eosin staining also revealed damaged hepatic structures in PBS- and ASC-3D-treated livers compared to ASC-3Dc-treated



**Fig. 2.** Analysis of gene and protein expression of antifibrotic factors in ASC-3D and ASC-3Dc. (A) Gene expression patterns of multiple factors were measured by qRT-PCR. Individual values were normalized to the expression level of GAPDH.  $**P < 0.01$ ;  $n = 4$  per group. (B) Protein expression patterns of multiple factors were examined by ELISA using 10 days' culture supernatants derived from each cell type.  $**P < 0.01$ ;  $n = 4$  per group. ASC: adipose mesenchymal stem cell; ELISA: enzyme-linked immunosorbent assay; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR: quantitative real time polymerase chain reaction.

livers (Fig. 3A). The collagen content in the liver was also examined after injection of stem cells. Hepatic collagen was significantly lower in ASC-3Dc-treated livers than in PBS- and ASC-treated livers (Fig. 3B), indicating that a low-level remodeling of extracellular matrix had

occurred. To examine antifibrotic effects in the liver, Masson's trichrome staining was performed. The staining results revealed that ASC-3Dc injection significantly reduced fibrosis compared to treatment with PBS or ASC-3D (Fig. 3D).



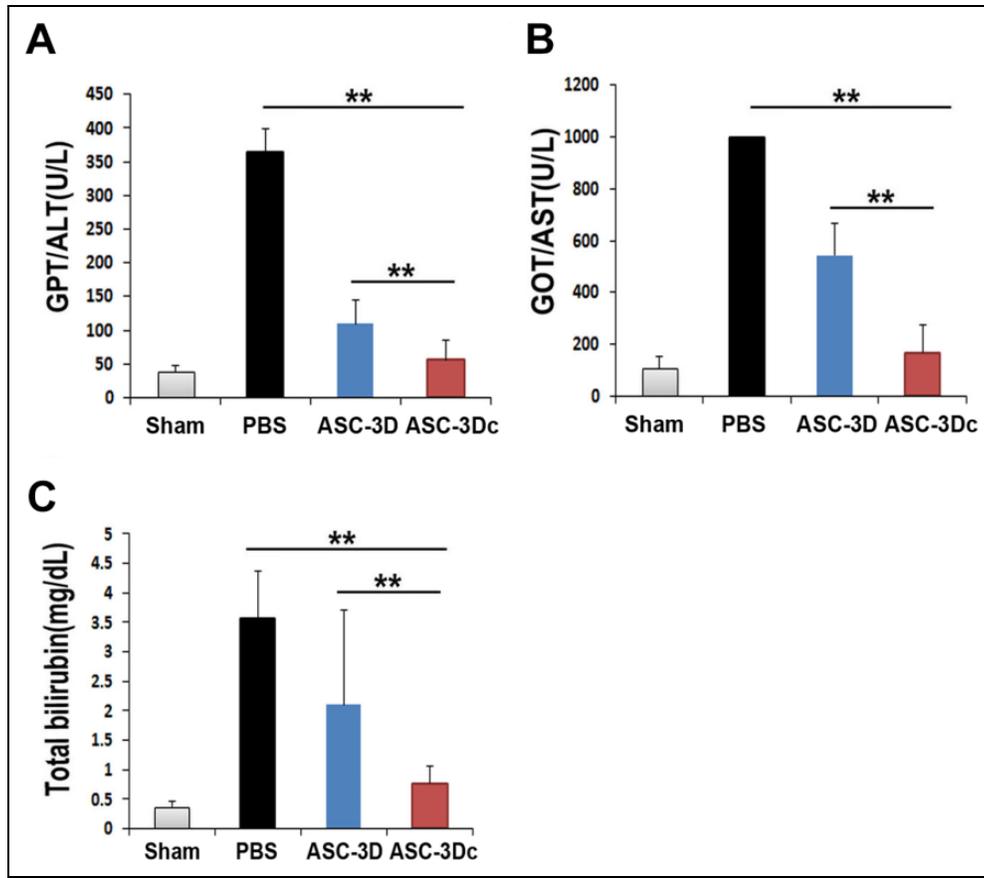
**Fig. 3.** In vivo therapeutic effects of ASC-3Dc in acute fibrotic liver. (A) Representative morphology of the liver after the induction of fibrosis and cell transplantation. (B) Representative H&E staining of liver sections after cell transplantation. An undistorted hepatic structure was observed in ASC-3Dc-treated livers compared to PBS-treated livers. Bar = 100  $\mu$ m. (C) Quantitative analysis of collagen content in liver tissues after injection of stem cells. The hepatic collagen content was measured by Sirius red/fast green collagen staining method. We analyzed five sections from six animals per each group.  $n = 6$  per group.  $**P < 0.01$ ;  $*P < 0.05$ . (D) Representative images of MT stained fibrotic liver after injection of stem cells. Bar = 100  $\mu$ m. (E) Quantitative analysis of the fibrotic area. We analyzed five sections from six animals per each group.  $n = 6$  per group.  $**P < 0.01$ ;  $*P < 0.05$ . ASC: adipose mesenchymal stem cell; H&E: hematoxylin and eosin; MT: Masson's trichrome.

### Transplantation of ASC-3Dc Maintains Normal Hepatic Function

To evaluate the therapeutic effect of ASC-3Dc on hepatic function, blood sera of mice were analyzed biochemically. Interestingly, the concentrations of GPT/ALT and GOT/AST were significantly decreased at 2 weeks after ASC-3Dc injection compared to that after ASC-3D or PBS treatment (Fig. 4A, B). Similarly, total bilirubin was also significantly decreased in ASC-3Dc-injected mice compared to that in ASC-3D- or PBS-injected mice (Fig. 4C). These data indicate that ASC-3Dc are protective against liver damage.

### Transplantation of ASC-3Dc Enhances Hepatic Regeneration

To elucidate the therapeutic mechanisms of ASC-3Dc in liver damage, hepatocyte proliferation was evaluated. Histological analysis revealed significantly higher numbers of Ki-67-positive hepatocytes in ASC-3Dc-treated livers than in ASC- or in PBS-treated livers (Fig. 5A). To further delineate the therapeutic mechanisms of ASC-3Dc in antifibrosis or hepatic regeneration, we performed western blotting on fibrotic liver tissues 3 weeks after ASC-3Dc injection. Interestingly, HGF, a representative antifibrotic or hepatic regenerating factor, was significantly increased in



**Fig. 4.** Biochemical analyses after cell transplantation. (A to C) Concentrations of GPT/ALT, GOT/AST, and total bilirubin in mice serum at 3 weeks after injection of stem cells. \*\* $P < 0.01$ ;  $n = 10$  per group.

ASC-3Dc-injected tissues compared to that in PBS-injected tissues (Fig. 5B). These data suggest that humoral factors derived from ASC-3Dc augmented HGF expression in mouse livers and enhanced antifibrosis and hepatic regeneration.

### *In Vivo Transdifferentiation of ASC-3Dc*

To determine the fate of the transplanted ASC-3Dc, histological analysis was conducted on liver tissues engrafted with ASC-3Dc expressing human albumin. Dil-labeled ASC-3Dc were successfully engrafted in the peri-portal or the peri-sinusoid area and expressed human albumin (Fig. 6A). To confirm the engraftment of ASC-3Dc in liver tissues, PCR using genomic DNA and primers targeting human-specific Alu DNA sequences was performed. The PCR detected the human-specific Alu DNA in the recipient liver tissues (Fig. 6B), indicating the hepatic transdifferentiation potential of ASC-3Dc in liver tissue.

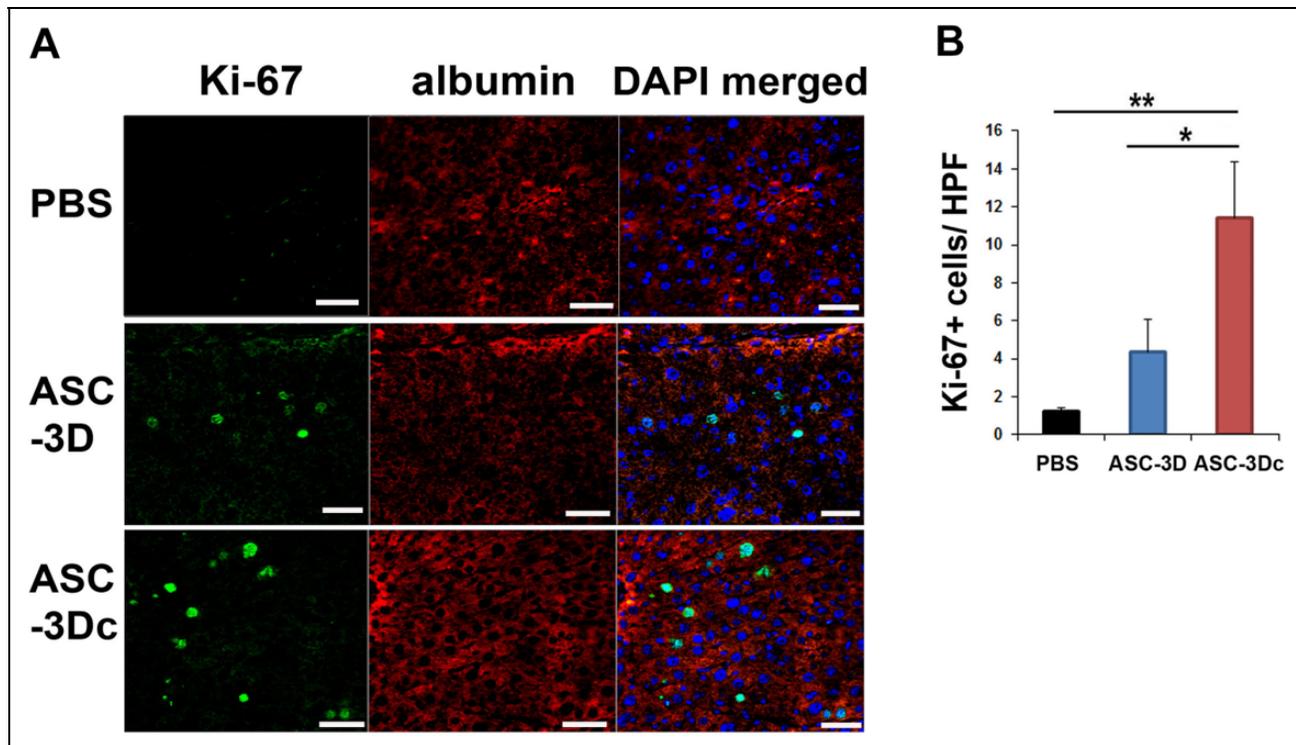
Next, to investigate the mechanism responsible for the therapeutic activity, we analyzed the mRNA expression levels of human and mouse albumin from the cell injected liver tissues. The expression levels of human and mouse albumin

were significantly higher in ASC-3Dc-injected liver tissue compared to the ASC-3D- or PBS-injected liver tissues (Fig. 6C). These data indicate that ASC-3Dc might have both transdifferentiation and paracrine properties.

### **Discussion**

In this study, we report for the first time that ASCs derived from a cytokine-stimulated 3D culture system exhibit enhanced antifibrosis in a fibrotic liver. The important findings of the present study are: (1) a cocktail containing four cytokines increased the proliferation of ASCs in spheroid culture, (2) genes encoding antifibrotic factors were enriched in ASCs-3Dc, (3) the local injection of ASCs-3Dc into fibrotic liver resulted in better antifibrosis and liver function, and (4) ASCs-3Dc showed hepatic transdifferentiation potential *in vivo*.

Numerous studies have demonstrated that stem cell-based therapy is effective for treating liver cirrhosis<sup>15,16</sup>. However, it has been also reported that stem cells show little therapeutic effects in fibrotic liver<sup>17</sup>. In fact, a recent clinical trial revealed that the transplantation of MSCs had no or limited benefit in liver disease<sup>18</sup>. These contradictory outcomes prompted us to study



**Fig. 5.** Transplantation of ASC-3Dc promotes liver regeneration. (A) Representative images of Ki-67-positive cells in fibrotic liver by immunohistochemistry 3 weeks after injection of stem cells. Bar = 400  $\mu$ m. (B) Quantitative analysis of Ki-67-expressing hepatocytes. \*\* $P < 0.01$ ;  $n = 6$  per group. ASC: adipose mesenchymal stem cell; HPF: high power field.

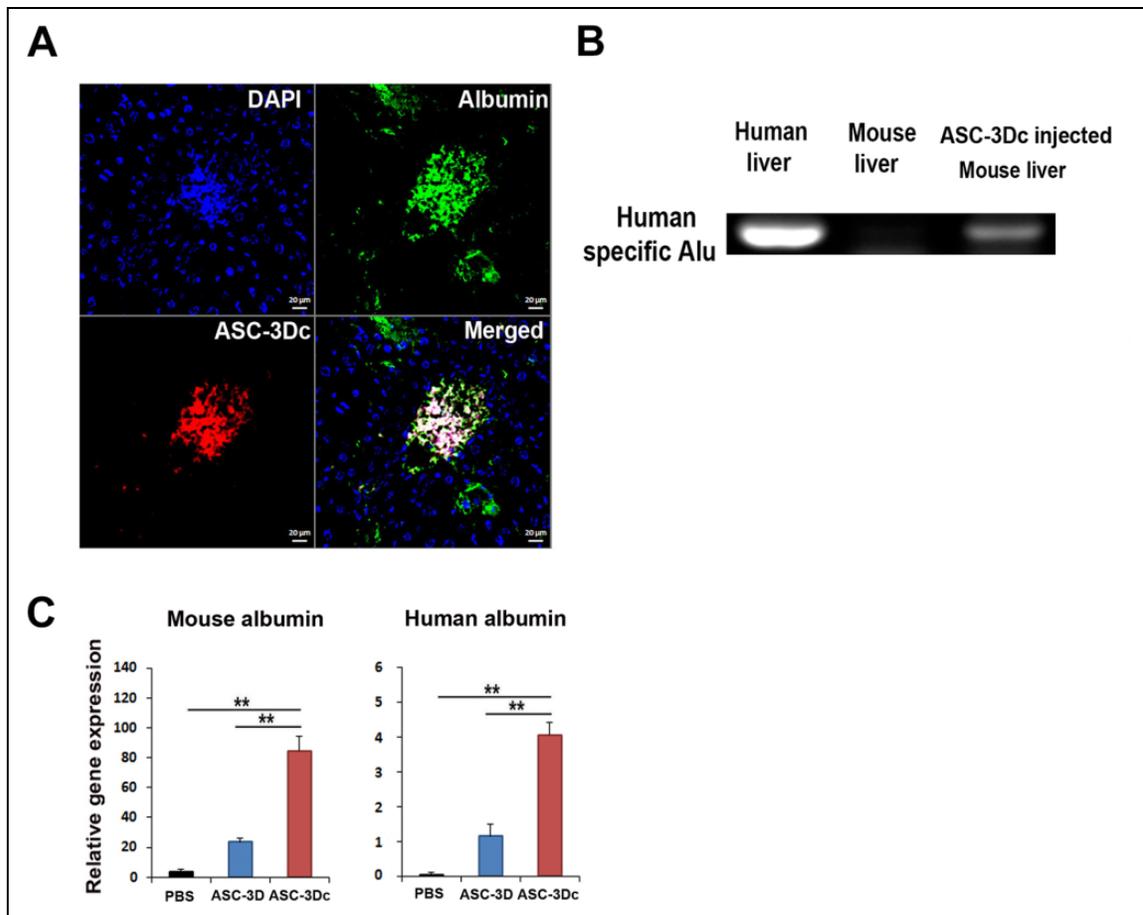
the therapeutic effects of ASCs and their mechanism in cell therapy. To this end, we developed a novel and simple culture technique using a cytokine cocktail and a 3D culture system.

Paracrine activity in stem cells is an important therapeutic mechanism of repairing or regenerating damaged tissue. However, these activities in stem cells are limited to standard 2D culture conditions. Recently, 3D culture systems promoted differentiation or paracrine activity such as the production of angiogenic and anti-inflammatory factors<sup>4,9</sup>. In addition, these systems produce favorable therapeutic effects on several diseases such as myocardial infarction, peritonitis, kidney injury, and fibrotic liver<sup>7,9,19,20</sup>. Based on these pieces of evidence, we used the method to investigate maximum paracrine activity in hepatic fibrosis and found that the 3D culture stimulated with the cytokine cocktail was highly effective in antifibrosis and for maintaining liver function.

The aim of 3D culturing of ASCs is to enhance paracrine capacities. In this study, enhanced expressions of paracrine/humoral factors (HGF, IGF-1, and SDF-1) were observed in ASC-3Dc compared to the nonstimulated ASC-3D. We reason that these factors might directly or indirectly activate resident hepatic progenitor cells to induce antifibrosis and hepatic regeneration. HGF is a paracrine growth factor known to regulate cell growth and motility by activating a tyrosine kinase via the c-Met receptor<sup>21,22</sup>. In addition, HGF plays a central role in the development and regeneration of

livers<sup>21,22</sup>. HGF also induces hepatic stellate cell apoptosis, which is associated with antifibrosis<sup>23</sup>. In fact, adenoviral vector-mediated transduction of HGF in bone marrow MSCs not only reduced liver fibrosis but also improved function in hepatocytes<sup>24</sup>. Furthermore, MSCs overexpressing HGF prevented liver failure and reduced mortality<sup>25</sup>. Therefore, a high expression of HGF in ASC-3Dc is beneficial for cell-based therapy without genetic modifications. IGF-1 is also associated with various biological functions regulating cell proliferation, migration, apoptosis, and differentiation<sup>26,27</sup>. Administration of IGF-1 mitigated cirrhosis in rats via hepatoprotective effects<sup>28</sup>. In addition, IGF-1 shows therapeutic potential in acute liver failure by inhibiting proinflammatory cytokines<sup>29</sup>. Another chemokine, SDF-1, is a known powerful chemoattractant of hematopoietic stem cells and is widely expressed in many tissues including the liver<sup>30,31</sup>. Stem cell homing and mobilization to liver are mainly involved in the interaction of SDF-1 and HGF<sup>32</sup>.

Another therapeutic mechanism is the transdifferentiation of injected stem cells. Recently, it was reported that ASCs had a special affinity for hepatocyte differentiation in vitro and liver regeneration in vivo<sup>33</sup>. Similarly, adipose-derived MSCs also showed hepatic differentiation potential<sup>34</sup>. In the present study, we traced the fate of the injected ASC-3Dc in vivo and found engrafted ASC-3Dc expressing human ALB in the mouse livers. These data



**Fig. 6.** Hepatic transdifferentiation of ASC-3Dc in vivo. (A) ASC-3Dc expressing human albumin were detected in fibrotic liver tissue 3 weeks after injection of stem cells. (B) Engraftment of ASC-3Dc in liver tissues. PCR was performed using genomic DNA and primers targeting human-specific Alu DNA sequences. The PCR detected human-specific Alu DNA in the recipient liver tissue. (C) Gene expression levels were determined by qRT-PCR using liver tissues. Individual values were normalized to the expression level of GAPDH.  $**P < 0.01$ ;  $n = 6$  per group. ASC: adipose mesenchymal stem cell; GAPDH: glyceraldehyde 3-phosphate dehydrogenase; qRT-PCR: quantitative real time polymerase chain reaction.

suggest that ASC-3Dc have transdifferentiation capacity, properties that might contribute to their antifibrotic effects in fibrotic liver.

In conclusion, we report that a novel, cytokine cocktail-stimulated ASC in a 3D culture system exhibited robust antifibrotic capacities in fibrotic liver. Accordingly, ASC-3Dc might be a new therapeutic alternative for liver cirrhosis. However, further investigation is needed to demonstrate whether ASC-3Dc can ameliorate liver cirrhosis in clinical trials.

### Ethical Approval

This study was approved by Catholic Kwandong University institutional review board.

### Statement of Human and Animal Rights

This article does not contain any studies with human subjects. All procedures regarding animal study were conducted in accordance

with the protocol approved by the Animal Ethics Committee of Catholic Kwandong University.

### Statement of Informed Consent

There are no human subjects in this article and informed consent is not applicable.

### Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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