

Secreted MMP-14 is A Predictor for Antifibrotic Effect of IC-2-Engineered Mesenchymal Stem Cell Sheets on Liver Fibrosis in Mice

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Abstract

Transplantation of IC-2-engineered bone marrow-derived mesenchymal stem cell (BM-MSC) sheets (IC-2 sheets) was previously reported to potentially reduce liver fibrosis. This study prepared IC-2-engineered cell sheets from multiple lots of BM-MSCs and examined the therapeutic effects of these cell sheets on liver fibrosis induced by carbon tetrachloride in mice. The predictive factors for antifibrotic effect on liver fibrosis were tried to identify in advance. Consequently, secreted matrix metalloproteinase (MMP)-14 was found to be a useful predictive factor to reduce liver fibrosis. Moreover, the cutoff index of MMP-14 for 30% reduction of liver fibrosis was 0.918 fg/cell, judging from univariate analysis and receiver operating curve analysis. In addition, MMP-13 activity and thioredoxin contents in IC-2 sheets were also inversely correlated with hepatic hydroxyproline contents. Finally, IC-2 was also found to promote MMP-14 secretion from BM-MSCs of elderly patients. Surprisingly, the values of secreted MMP-14 from BM-MSCs of elderly patients were much higher than those of young persons. The results of this study suggest that the IC-2 sheets would be applicable to clinical use in autologous transplantation for patients with cirrhosis regardless of the patient's age.

Introduction

Liver fibrosis is caused by an excessive accumulation of the extracellular matrix. Progression of liver fibrosis often leads to liver cirrhosis (LC) [1, 2]. Without proper treatment, LC can progress from the early to the decompensated stage, wherein liver function extremely decreases. The medical needs of decompensated LC remain unmet [3–5]. Thus, LC is a major health problem worldwide because the number of mortalities due to LC in 2010 is approximately 50,000 in the USA, 25,000 in Japan, and more than one million worldwide [6]. Most of the causes of LC in the USA include hepatitis C virus, alcoholic liver disease, and non-alcoholic fatty liver disease in adults [7]. Many congenital diseases (e.g., biliary atresia) exist in children [8]. Fontan-associated liver disease (FALD), which develops following the Fontan operation for monoventricular disease, has recently become a problem, and liver fibrosis is a universal feature of FALD [9]. The development of antifibrotic therapy not only cures liver fibrosis but also suppresses the subsequent hepatocellular carcinoma (HCC) incidence because liver fibrosis is strongly associated with HCC incidence [10].

Liver transplantation is the only treatment for decompensated cirrhosis [11]. However, liver transplantation cannot be sufficiently performed due to donor shortage. Moreover, stem cell therapy has recently attracted attention as an alternative treatment to liver transplantation [12, 13]. Stem cells include embryonic, induced pluripotent, and somatic stem cells. Mesenchymal stem cells (MSCs), which are one of the somatic stem cells, are derived from mesodermal tissues and can be obtained from bone marrow, adipose tissue, umbilical cord, and so on [14, 15]. They have distinctive features such as differentiation plasticity that differentiates ectodermal and endodermal cell lineages [16, 17], immunosuppressive ability [18], and secretion of various cytokines [19]. MSCs also have a low risk of tumorigenesis [20]. Due to these advantages, clinical trials using MSCs have been conducted in a wide range of fields, such as autoimmune diseases, cancers, and liver diseases [21–25].

Wnt/ β -catenin signaling suppression was previously reported to transdifferentiate bone marrow- and cord blood-derived MSCs into hepatocyte-like cells [26, 27]. Based on these findings, IC-2, a newly synthesized derivative of ICG-001, was identified as a potent suppressor of Wnt/ β -catenin signaling. Moreover, IC-2 efficiently induced hepatic differentiation of human MSCs [28]. A new therapy, which is a combination of IC-2 treatment and cell sheet technology, was developed to establish a novel therapeutic modality for liver diseases [29, 30]. In general, transplantation of cell sheets is superior to cell injection because transplantation of cell sheets avoids the risk of portal vein embolism, has less cell loss, maintains intercellular communications, and delivers uniform and stable cells [31]. The transplantation of IC-2-engineered bone marrow-derived MSC (BM-MSC) sheets (IC-2 sheets) was reported to exert potent regenerative ability and effectively improve acute liver injury in mice [29]. In addition, transplantations of IC-2 sheets dramatically suppressed liver fibrosis in which 47% of hydroxyproline decreased only a week after IC-2 sheet transplantation. Furthermore, matrix metalloproteinase-1 and matrix metalloproteinase-14 (MMP-1 and MMP-14) secreted from IC-2 sheets were found to play a critical role in liver fibrosis reversal [30]. Moving toward the eventual goal of clinical application, IC-2 sheets need further research and development. The efficacy of IC-2 sheets was especially examined from multiple lots of BM-MSCs derived from many donors, particularly from the elderly, and standard items were set for predicting effectiveness. This study examined the antifibrotic action of IC-2 sheets prepared from commercially purchased multiple lots of BM-MSCs on liver fibrosis. In addition, identifying factors that predict the antifibrotic effect of IC-2 sheets on liver fibrosis was attempted using the supernatants and cell lysates during the manufacturing of IC-2 sheets. Finally, cell viability, Wnt/ β -catenin signal intensity, and MMP-14 secretion were examined in response to IC-2 treatment in BM-MSCs obtained from elderly patients who underwent artificial joint replacement to find out whether IC-2 sheets manufactured from elderly donors can be used. Consequently, BM-MSC from the elderly was suggested to at least have the same or better potential as those derived from the youth.

Materials And Methods

Ethics statements

All methods were carried out in accordance with relevant guidelines and regulations. Animal experiments were conducted in accordance with ARRIVE guidelines [46] and the Tottori University Animal Care and Use Regulations. The study using human bone marrow cells collected from patients who underwent artificial joint replacement at Tottori University Hospital was approved by the Ethics Review Committee of the Faculty of Medicine Tottori University with approval number 1604A006. Informed consent was obtained from each patient.

Chemical compounds and cells

IC-2, a derivative of ICG-001, was synthesized in-house and dissolved in dimethyl sulfoxide (DMSO) [28]. The DMSO final concentration was 0.1%. Cell sheets were prepared using BM-MNCs that were purchased from Lonza Inc for *in vivo* study of transplantation experiments. BM-MNCs from 15 donors were passed 2

to 5 times, and 29 lots of cells were finally obtained. The cells were then cryopreserved until use for cell sheet preparation. Bone marrow cells collected from 50- to 79-year-old patients who underwent artificial joint replacement at Tottori University Hospital were used (Table S3) for *in vitro* study. Moreover, the study was approved by the Ethics Review Committee of the Faculty of Medicine Tottori University with approval number 1604A006. Informed consent was obtained from each patient. BM-MNCs were prepared by density centrifugation in Ficoll-Paque PLUS (GE Healthcare UK Ltd., Little Chalfont, UK), and cells were passed a few times and cryopreserved until use for the preparation of cell sheets.

Fluorescence-activated cell sorter analysis

Cells were washed with phosphate-buffered saline containing 0.5% bovine serum albumin and 1 mM EDTA. The cells were then incubated with the antibodies to CD34-APC, CD45-APC, HLA-DR-FITC (1:40; Thermo Fisher Scientific Inc., Waltham, MA, USA), CD90-APC (1:100; BD Biosciences, San Jose, CA, USA), and CD105-FITC (1:20; Bio-Rad Laboratories, Inc., Irvine, CA, USA) for 30 min at 4°C. IgG1-FITC (1:40; Miltenyi Biotec GmbH, Bergisch Gladbach, Germany), IgG1-APC, and IgG2a-FITC (1:40; Thermo Fisher Scientific, Inc.) were used as isotype-matched negative controls. Propidium iodide was used to remove nonviable cells. Moreover, cell surface marker expression was analyzed by Gallios (Beckman Coulter, Brea, CA, USA). Furthermore, data were analyzed using FlowJo software (Tree Star, Inc., Ashland, OR, USA).

Preparation of cell sheets and parameters

IC-2 sheets were manufactured as follows. Cells that had been frozen were passed three to five times and then plated on ϕ 60-mm temperature-responsive culture dishes (Cell Seed Inc., Tokyo, Japan) at a seeding density of 5.4×10^4 cells/cm². Furthermore, cells were treated with 30 μ M IC-2 on a ϕ 60-mm temperature-responsive culture dish the next day for a week. The medium was changed 4 days after the start of incubation. Cell sheets, called IC-2 sheets, were harvested by incubating at 20°C and storing at 20°C until transplantation. The parameters of each cell sheet included MMP-1 content, MMP-14 content, MMP-2 activity, MMP-13 activity, thioredoxin content, amphiregulin content, and C3 content in the supernatants collected from cell culture from 4 to 7 days. Active MMP-1 activity, total MMP-1 activity, active MMP-14 activity, total MMP-14 activity, MMP-2 activity, MMP-13 activity, and thioredoxin content were measured with the other cell sheets prepared in the same manner for transplantation.

Animal experiments

All animal experiments were conducted following the ethical approval of the Tottori University Subcommittee on Laboratory Animal Care (approved number: 16-Y-28). All mice were housed under pathogen-free conditions in a temperature-controlled, illuminated (12 h daily) room with ad libitum access to water and chow. To induce chronic liver injury, carbon tetrachloride (CCl₄; Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in olive oil (Wako Pure Chemical Industries Ltd.) was orally administered twice per week to 7-week-old BALB/c-nu/nu male mice (CLEA Japan, Inc., Tokyo, Japan) at a dose of 0.6 and 1.2 mL/kg for 4 and another 6 weeks, respectively.

Transplantation of cell sheets

Transplantation of cell sheets was performed 2 days after the last administration of CCl₄ in CCl₄-treated BALB/c-nu/nu mice. Three-layered cell sheets were transplanted at one site on the left lateral lobe. Control mice were operated on using sham laparotomy. CCl₄ administration was continued for another week after transplantation. Mice were sacrificed by exsanguination with sodium pentobarbital 2 days after the final administration of CCl₄, and blood samples were collected from the inferior vena cava, followed by hepatectomy. Part of the liver was fixed with 4% paraformaldehyde and embedded in paraffin for histological analysis. The remaining liver tissue was snap-frozen in liquid nitrogen.

Biochemical tests

Blood samples were maintained overnight on ice, and serum was isolated by centrifugation at 2,000×g for 20 min. Serum aminotransferase and total bilirubin levels were measured as previously reported [29, 30].

Hydroxyproline contents

Hydroxyproline contents were measured as previously described [47]. A liver tissue piece (100 mg wet weight) was homogenized in 1 mL of distilled water, and an equal volume of 12 N concentrated hydrochloric acid was added to 100 µL of this slurry. Furthermore, the mixture underwent hydrolysis in a block incubator (IKA, Staufen, Germany) at 120°C. The mixture was cooled at room temperature after hydrolysis treatment and centrifuged at room temperature at 3,000 rpm for 5 min after pipetting. After centrifugation, 10 µL of the supernatant was transferred to a 1.5-mL tube, and hydrochloric acid was removed with an evaporator (Sakuma Seisakusho, Tokyo, Japan). Subsequently, hydroxyproline was measured using a Hydroxyproline Quantification Kit (BioVision, CA, USA), according to the manufacturer's instructions. The amount of protein in the tissue homogenate was measured by the Bradford method using a protein assay concentrated dye reagent (Bio-Rad, Hercules, CA, USA). The amount of hydroxyproline per unit protein amount was determined to evaluate the fibrosis level.

Western blot analysis

Ten milligrams of proteins were subjected to western blot analysis as previously described [29, 30]. Mouse monoclonal antibodies including anti-αSMA (DakoCytomation, Agilent Technologies, Inc., Santa Clara, CA, USA) and anti-GAPDH antibody (MilliporeSigma, Burlington, MA, USA) were used. Chemiluminescent images were acquired by ImageQuant LAS-4000 (GE Healthcare UK Ltd.) with ECL Prime Western Blotting Detection Reagent (GE Healthcare UK Ltd.).

WST assay

At 24 h after seeding of BM-MSCs at a density of 5.4×10^4 cells/cm², 20–50 µM of IC-2 were added. WST assay was performed with Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan) using Sunrise Rainbow RC plate reader (Tecan Group Ltd., Mannedorf, Switzerland) 7 days after addition.

Reporter assay

At 24 h after seeding of BM-MSCs at a density of 5.4×10^4 cells/cm², 30 μ M IC-2 was added. The TCF/LEF reporter lentivirus (SABiosciences, Qiagen N.V., Frederick, MD, USA) and control renilla lentivirus (SABiosciences, Qiagen N.V.) were transduced, and reporter assay was performed using luciferase assay system (Promega Corp.) as previously described 4 days after addition [28].

RNA extraction and quantitative RT-PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen, Life Technologies Corp., Carlsbad, CA, USA). Reverse transcription was performed using SuperScript II First-Strand Synthesis System for reverse transcription-polymerase chain reaction (RT-PCR; Invitrogen) and oligo(dt)₁₈ primers, according to the manufacturer's instructions. Real-time PCR analysis was performed using LightCycler Fast DNA Master SYBER Green \times (Roche Diagnostics GmbH, Mannheim, Germany). Primers for qRT-PCR analysis were as follows: MMP14-forward, 5'-CCTACCGACAAGATTGATGCTG-3'; MMP14-reverse, 5'-AAACGGTAGTACTTGTTCACG-3'; TIMP1-forward, 5'-CTGTTGGCTGTGAGGAATGC-3'; TIMP1-reverse, 5'-GAGCTGGTCCGTCCACAA-3'; GAPDH-forward, 5'-AGCCACATCGCTCAGACAC-3'; and GAPDH-reverse, 5'-GCCCAATACGACCAAATCC-3'. Furthermore, PCR was performed for each assay using a 7900 HT Fast Real-Time PCR System (Applied Biosystems, Life Technologies Corp., Carlsbad, CA, USA).

Enzyme-linked immunosorbent assay

The culture supernatants of each cell culture were collected and stored at -80°C until analysis. According to the manufacturer's instructions, MMP-1, MMP-14, complement component C3, amphiregulin, and thioredoxin contents in the culture supernatants were quantified using Human MMP1 Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Abcam Ltd., Cambridge, UK), Human MMP14 ELISA Kit (Abcam Ltd., Cambridge, UK), Human Complement C3 ELISA Kit (Abcam Ltd., Cambridge, UK), Quantikine[®] Human Amphiregulin ELISA Kit (R&D Systems, Inc., Minneapolis, MN, USA), and Human TXN/Thioredoxin/TRX ELISA Kit (LifeSpan BioSciences, Inc., Seattle, WA, USA), respectively.

Matrix metalloproteinase activity assay

Culture supernatants were collected 7 days after the IC-2 treatment, and cells were homogenized in the lysis buffer provided with the following assay kits: SensoLyte[®] 520 MMP-1 Assay Kit *Fluorimetric*, SensoLyte[®] 520 MMP-14 Assay Kit, SensoLyte[®] Plus 520 MMP-2 Assay Kit, and SensoLyte[®] Plus 520 MMP-13 Assay Kit (AnaSpec, Inc., CA). Samples were stored at -80°C until analysis, and each MMP activity was measured according to the manufacturer's instructions.

Statistical analyses

All the values were expressed as means \pm SD. Student's *t*-test was performed to compare the means of response variable between the two groups using Microsoft Excel. Moreover, Steel tests (<http://www.gen-info.osaka-u.ac.jp/MEPHAS/s-d.html>) were conducted to compare the difference among the three

groups. *P* values <0.05 were considered to be statistically significant. Univariate and multivariate statistical analyses were performed using SPSS Statistics (IBM SPSS Statistics; IBM Corp., Armonk, NY, USA).

Results

Characteristics of MSCs prepared from commercial bone marrow-derived mononuclear cells (BM-MNCs)

Previous reports revealed that transplantation of IC-2 sheets consisted of hepatic differentiated BM-MSCs ameliorated CCl₄-induced acute and chronic liver injury. These observations were obtained from the data using an immortalized BM-MSC cell line or primary MSCs generated from a single donor-derived BM-MNCs [29, 30]. This study prepared BM-MNCs passed two to five times purchased from Lonza Inc. (Walkersville, MD, USA). The presence of mesenchymal lineage-associated cell surface markers was confirmed with fluorescence-activated cell sorting (FACS) analysis. The cells from Lonza 22, Lonza 26, and Lonza 30 were negative for CD34 and CD45 (Fig. 1a) but positive for CD90 and CD105. Little expression of HLA-DR was observed in the cells of three donors. The growth curve of the cells demonstrated that active cell proliferation was observed in all ten cells at passage 3 (Fig. 1c). These data suggest that the cells used in the experiments share the common features of BM-MSCs [32].

IC-2 sheets ameliorated liver fibrosis in mice

This study generated 29 lots of BM-MSCs prepared from BM-MNCs derived from 15 donors by altering the passage numbers of cells from passages 2 to 5 to evaluate whether IC-2 sheets generated from BM-MSCs from different persons have antifibrotic action. IC-2 sheets fabricated from each lot of BM-MSCs were transplanted onto the liver surface of each mouse. Consequently, mice received three-layer IC-2 sheets at one site (IC-2 group) after 10 weeks of CCl₄ intoxication. Sham-operated mice (sham group) served as the control. Liver fibrosis was markedly reduced in the IC-2 group than in the sham group, accompanied by improvement of pseudolobule-like structure observed in the sham group, regardless of the difference of cell sheets (Fig. 2a, 2b). Three outliers were excluded due to the interquartile range when 29 samples of hydroxyproline contents were statistically examined. Hepatic hydroxyproline contents were compared among 22, 26, and 3 samples for the sham, IC-2, and CCl₄(-) groups, respectively. Furthermore, hepatic hydroxyproline content was significantly reduced in the IC-2 group than in the sham group (Fig. 2c). Moreover, hepatic stellate cells (HSCs) play a critical role in liver fibrogenesis [1, 2, 33]. Alpha-smooth muscle actin (α -SMA) is a major indicator of HSC activation [1, 2, 33]. The α -SMA expression was remarkably reduced in the IC-2 group, suggesting that IC-2 sheets suppressed fibrogenesis in mice livers (Fig. 2d). In addition, serum alanine transaminase and total bilirubin were significantly decreased in the IC-2 group, whereas serum aspartate transaminase was not changed (Fig. 2e). Thus, IC-2 sheets also improved liver functions besides reducing liver fibrosis. These data suggest that IC-2 sheets generated from miscellaneous patients will be expected to exert a potent effect when applied in clinical settings.

Secreted MMP-14 was a predictor for therapeutic efficacy for liver fibrosis

Identifying parameters that predict the antifibrotic effect of IC-2 sheets was next attempted because IC-2 sheets generated from BM-MSCs originating from different persons demonstrated to have antifibrotic action. The parameters involved in fibrosis and liver regenerations measured in the supernatants of cultured cells and cell lysates were included in the analysis. Five samples were excluded from the statistical analysis because cell count was not performed in five samples (Table S1, S2). First, a univariate analysis was done between hepatic hydroxyproline contents and cell sheets-secreted factors (Table 1 and Table S1). Of the seven parameters, MMP-14 was inversely correlated with hepatic hydroxyproline contents (Fig. 3; $P = 0.012$). The MMP-14 value was measured in the conditioned media cultured from days 4 to 7 after the addition of IC-2. Consequently, hepatic hydroxyproline content was measured at 7 days after transplantation. These data suggest that secreted MMP-14 during manufacturing cell sheets is an advanced predictor for the antifibrotic effect of IC-2 sheets. Second, a univariate analysis was performed among hepatic hydroxyproline contents and factors in lysates of cell sheets (Table 1 and Table S2). The cell lysates were examined in the other cell sheets prepared in the same manner for transplantation. Of the five parameters, MMP-13 activity and thioredoxin content were inversely correlated with hepatic hydroxyproline contents (Fig. S1; $P = 0.043$ and $P = 0.017$, respectively). Consistent with the study that MMP-13 is activated by MMP-14 [34], intracellular MMP-13 activity was observed to be significantly correlated with extracellular MMP-14 contents ($P = 0.02$, data not shown). The analysis of the receiver operating curve indicated that the area under the curve for secreted MMP-14 was 0.917, suggesting that MMP-14 can predict a 30% decrease in hydroxyproline contents ($P = 0.005$; Fig. S2). The cutoff index of MMP-14 contents in the supernatants of culture media for prediction of 30% suppression of liver fibrosis was 0.918 fg/cell.

BM-MSCs derived from the elderly have similar characteristics to those derived 1 from young people.

Growth, Wnt/b-catenin signal, and MMP-14 production in response to IC-2 in BM-MSCs from the elderly were examined because the mean age of patients with cirrhosis at diagnosis is increasingly becoming older annually, which was 68.1 years old in 2014 [35]. BM-MSCs were collected from eight patients (50–79 years old) who underwent artificial joint replacement. Of the eight patients, six and two had coxarthrosis and idiopathic osteonecrosis of the femoral head, respectively (Table S3). The cell viability of BM-MSCs was decreased with IC-2 concentration, which was about 70%–80% in patients 1, 2, and 3 at 30 mM IC-2 (Fig. 4a). The Wnt/b-catenin signal intensity was suppressed at 40%–50% at 30 mM IC-2, which is similar to the data of BM-MSCs from the youth reported in reference 28 (Fig. 4b). The culture supernatants of BM-MSCs were collected at 7 and 9 days after IC-2 treatments, and MMP-14 contents were then measured in patients 1 and 8. The MMP-14 values corrected by cell number were markedly increased by the IC-2 treatment compared with the control (Fig. 5a). Surprisingly, the average values of secreted MMP-14 ranged between 3 and 6 fg/cell in 2–3 days, which were higher than the present study where BM-MSCs from young persons were used. Intracellular MMP-14 contents were also higher compared with the control in the IC-2-treated cells from patients 4 and 5 (Fig. 5b). The MMP-14 mRNA expression was also upregulated compared with the control in the IC-2-treated cells, except for patient 4 who tended upregulation without statistical significance (Fig. 5c). Taken together, these data suggest that BM-MSCs obtained from the elderly can be used as a cell source for IC-2 sheets.

Discussion

This study demonstrated the antifibrotic action of IC-2 sheets generated from commercially purchased different lots of BM-MSCs. These data suggest that [autologous transplantation](#) of IC-2 sheets will be effective for patients with LC in the future. In addition, the practical application of IC-2 sheets will be realized in elderly and young patients.

Of the seven parameters in the supernatant of the culture medium during manufacturing IC-2 sheets, MMP-14 is inversely correlated with liver fibrosis, suggesting that MMP-14 is an advanced predictor of liver fibrosis regression. In addition, the cutoff index for the prediction of the 30% suppression of liver fibrosis was 0.918 fg/cell. These data were supported by a previous report stating that MMP-14 derived from IC-2 sheets is responsible for the antifibrotic effects on liver fibrosis [30]. Moreover, MMP-14 can break down type I collagen, a major component of the extracellular matrix of the fibrotic liver [36]. Taken together, MMP-14 plays a crucial role in the fibrinolysis of the IC-2 sheets. Furthermore, MMP-13 activity in the cell lysate was found to be inversely correlated with hepatic hydroxyproline contents. Increases in MMP-13 activity may be due to the MMP-14 upregulation because MMP-13 has been reported to be activated by MMP-14 [34, 37]. Moreover, intracellular thioredoxin contents were also inversely correlated with hepatic hydroxyproline. Thioredoxin is essential for reducing oxidative stress and suppression of activated HSCs [38-40]. In a previous report, thioredoxin secretion was enhanced in mesenchymal stem cell-derived cell sheets treated with IC-2 [30]. Taken together, hepatic cells differentiated by Wnt/b-catenin signal inhibitors were converted to produce thioredoxin.

Moreover, this study found that MMP-14 was induced by IC-2 in BM-MSCs from elderly persons. Surprisingly, the average values of secreted MMP-14 ranged between 3 and 6 fg/cell, which was higher compared with the cells from young persons. These data suggest that IC-2 sheets generated from elderly persons are expected to demonstrate highly therapeutic effects. Moreover, the quality of MSCs (e.g., proliferative and differentiation abilities) declines with age [41, 42]. However, IC-2 can induce more MMP-14 production in BM-MSCs from elderly persons compared with young persons. Although the exact mechanism is unclear, this interesting phenomenon has to be clarified in the future.

Identifying markers to predict effects in advance is important in clinical practice, and these markers are desirably based on the mechanisms of action. In a study of autologous transplantation of skeletal muscle cell sheets for severe heart failure, the authors suggest that therapeutic effects are based on the factors that induce angiogenesis and antioxidant activity, such as stromal-derived factor 1, hepatocyte growth factor, and vascular endothelial growth factor [43–45]. Moreover, this report is believed to be the first to identify predictable markers involved in the effectiveness of therapy based on mechanisms of action in liver diseases. Taken together, the cutoff index setting of MMP-14 will greatly contribute to the improvement of IC-2 sheet therapy for LC.

Conclusions

This study found that IC-2-engineered mesenchymal stem cell sheets generated from different persons reduced liver fibrosis and secreted MMP-14 in the supernatant is a predictor for the therapeutic effect on liver fibrosis. In addition, BM-MSCs from elderly persons are expected to exert potent antifibrotic effects. Thus, the IC-2 sheets would be applicable for clinical use in autologous transplantation for patients with cirrhosis regardless of the patient's age.

Declarations

Acknowledgments

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Author Contributions

K.F, N.I., Y.K., and S.O performed the experiments. S.E, M.E. and H.N. recruited donors and collected bone marrow. K.F., N.I., N.K, J.M and G.S. wrote the manuscript. N.N. and G.S. supervised all the experiments.

Competing interest

G.S. holds more than 5% of the total shares of KanonCure Inc. and receives compensation as a member of KanonCure Inc. The other authors have no competing interests to declare.

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Tables

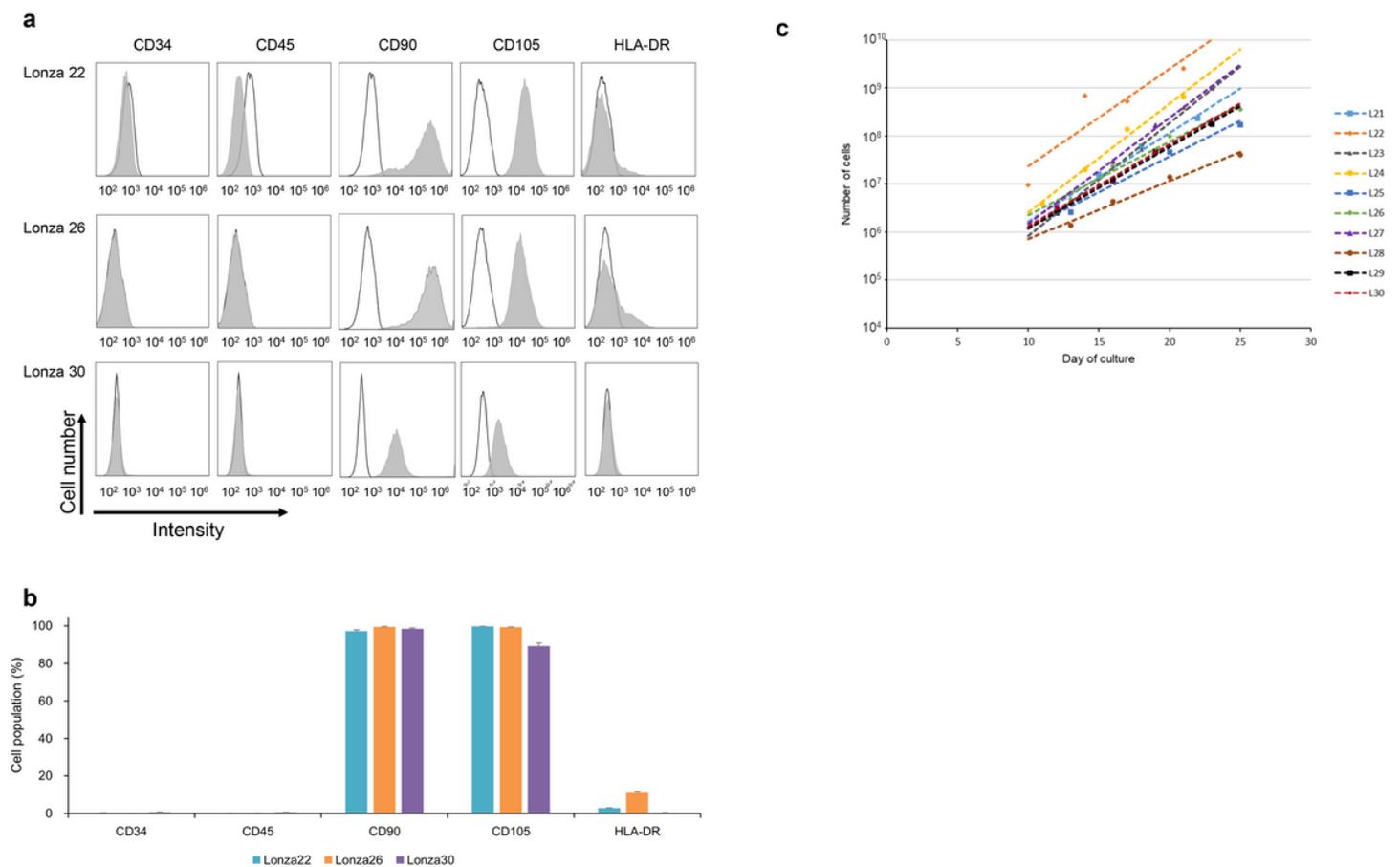
Table 1. Univariate analysis between hepatic hydroxyproline contents and cell sheets-secreted factors

Parameter	Univariate analysis		
	R-value	P-value	
MMP-1 (fg/call)	-0.214	0.351	
MMP-2 activity (fg/cell)	-0.338	0.135	
MMP-13 activity (fg/cell)	-0.331	0.143	
MMP-14 (fg/cell)	-0.578	0.012	*
Thioredoxin (fg/cell)	-0.006	0.98	
Amphiregulin (fg/cell)	-0.215	0.349	
C3 (pg/cell)	-0.226	0.325	

Table 2. Univariate analysis between hepatic hydroxyproline contents and factors in lysates of cell sheets

Parameter	Univariate analysis		
	<i>R</i> -value	<i>P</i> -value	
MMP-1 activity (fg/call)	-0.238	0.299	
MMP-2 activity (fg/cell)	-0.266	0.245	
MMP-13 activity (fg/cell)	-0.482	0.043	*
MMP-14 activity (fg/cell)	-0.380	0.089	
Thioredoxin (fg/cell)	-0.516	0.017	*

Figures



purchased from Lonza Inc. by two to five times passages. BM-MSCs from Lonza 22, Lonza 26, and Lonza 30 at passage 3 were labeled with FITC-coupled antibodies against CD34, CD45, CD90, CD106, and HLA-DR. Shaded areas indicated staining with antibodies against the indicated antigens, and solid lines indicate background fluorescence in controls stained with all antibodies except for the indicated antigen. b Grouped bar chart showing quantitation of cell surface markers on each lot of three donors-derived BM-MSCs. The data are presented as mean \pm SD from three independent FACS analyses. c The growth curve of the BM-MSCs purchased from Lonza Inc. Furthermore, ten cells of L21 (Lonza 21) to L30 (Lonza 30) at passage 3 were expanded after the start of the inoculation. Cell culture was started at a cell density of 5.0×10^3 to 1.0×10^4 cells/cm² first passed at 10–13 days and then continued every 3–4 days.

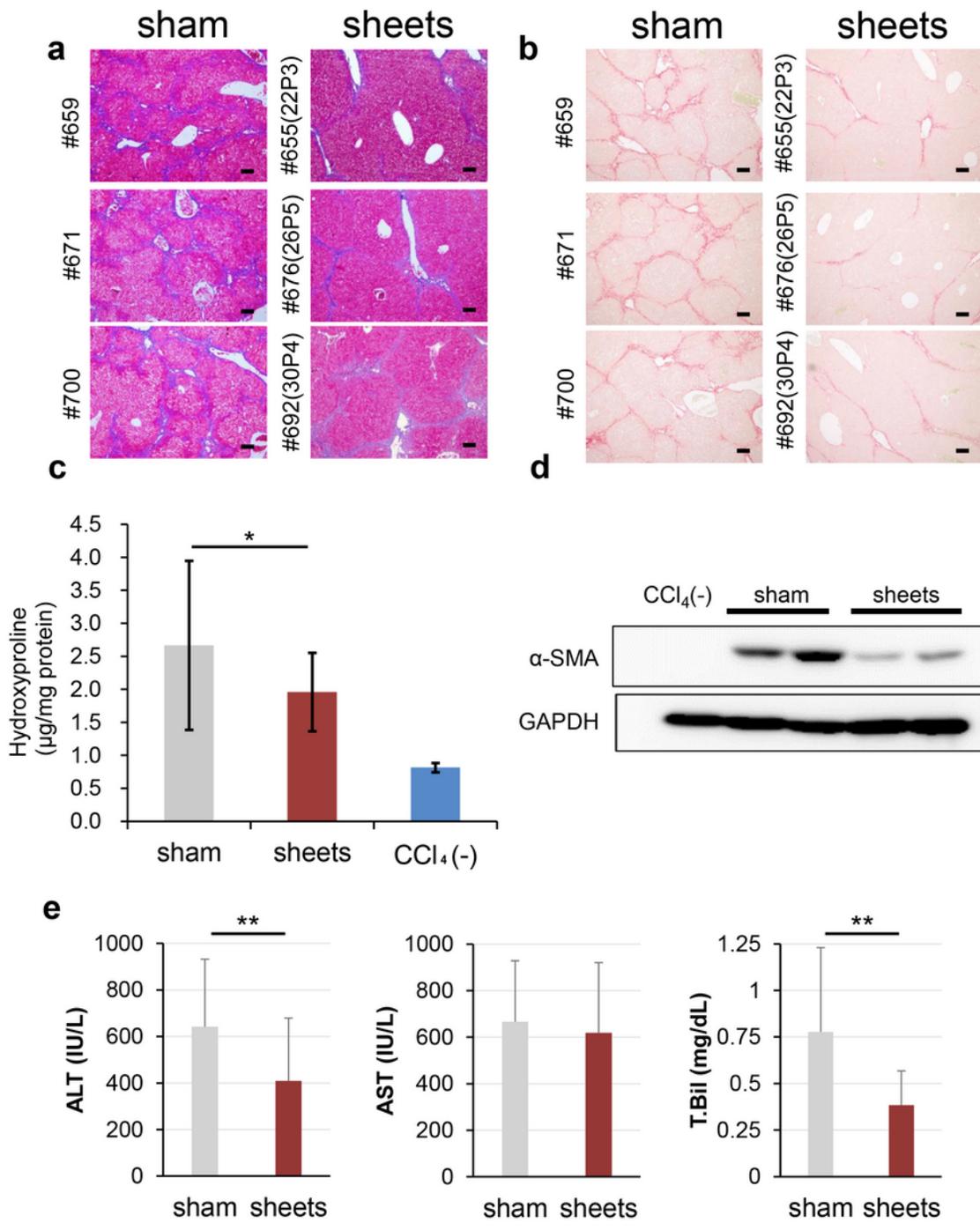


Figure 2

Regression of liver fibrosis by IC-2 sheets. a Micrographs of liver sections subjected to Azan staining. The individual number of the mouse (lot number of the cell sheet) is described on the left side of each micrograph (scale bar = 100 µm). b Sirius Red staining (scale bar = 100 µm). c Hydroxyproline contents in the liver. The number of mice examined in the sham, sheets, and CCl₄(-) groups were 22, 26, and 3, respectively. *P < 0.05 examined by Steel test. d α-SMA expression in recipient liver tissues was measured

by western blotting. Sham and sheets represent the sham and IC-2 sheets groups, respectively. e Serum aspartic aminotransferase, alanine aminotransferase, and total bilirubin were shown. The number of mice examined in the sham, sheets, and CCl₄(-) groups was 22, 26, and 3, respectively, except 24 of the total bilirubin in the sheets group. The results are expressed as mean \pm S.D. **P < 0.01 examined by Student's t-test.

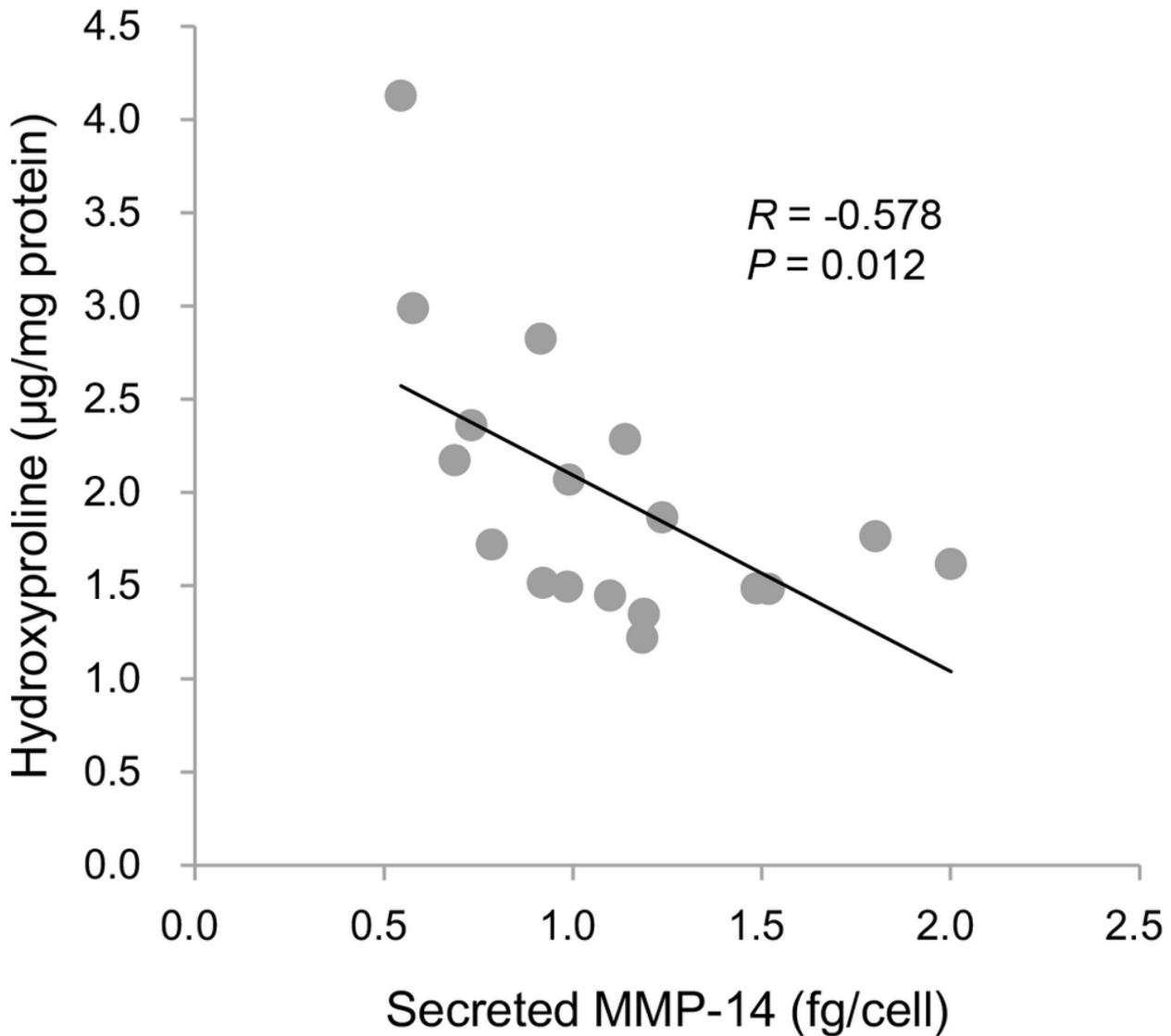


Figure 3

Secreted MMP-14 from IC-2 sheets is inversely correlated with hepatic hydroxyproline contents. Secreted MMP-14 contents in the conditioned media during manufacturing IC-2 sheets from days 4 to 7 were measured and expressed as per cell. Hepatic hydroxyproline contents at 7 days after transplantation were analyzed.

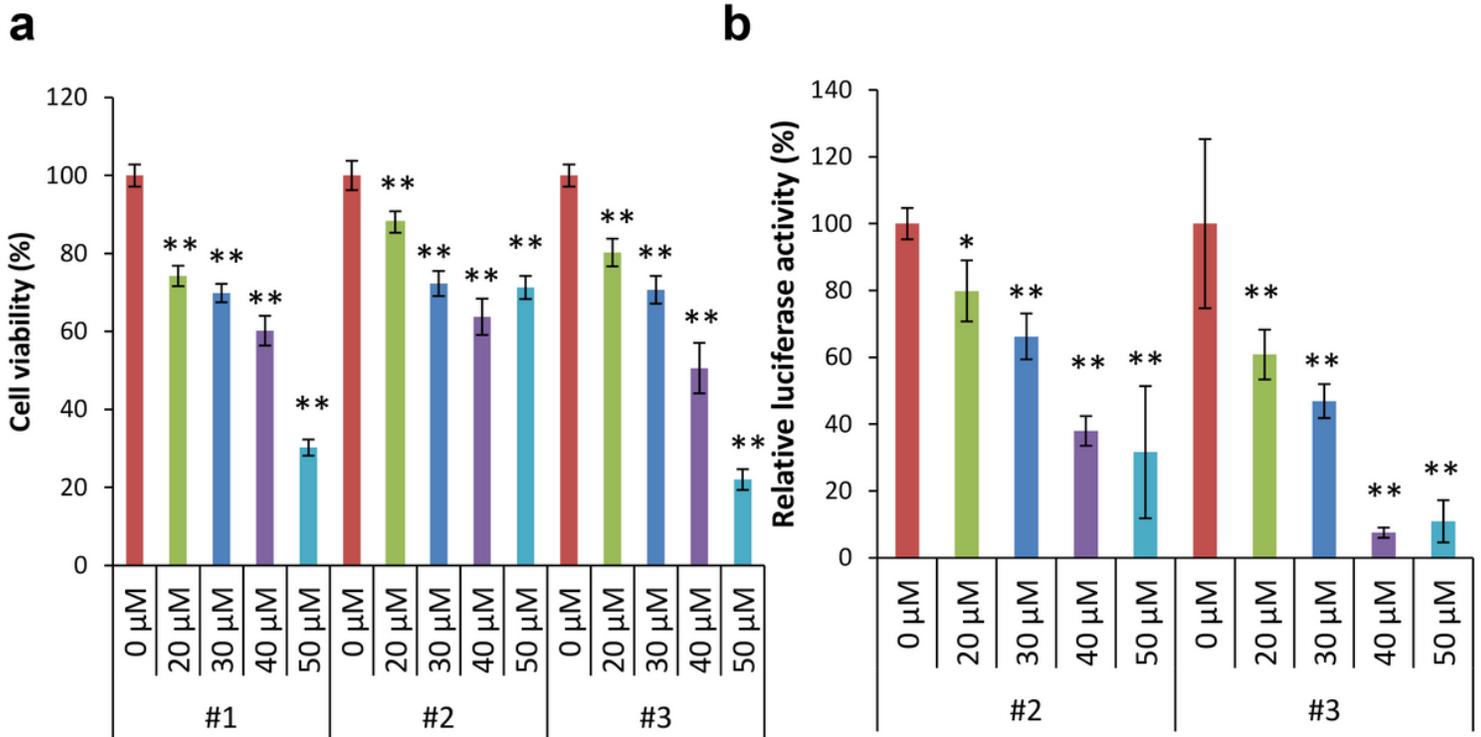


Figure 4

Cell viability and Wnt/ β -catenin signal intensity in response to IC-2. a Cell viability by WST assay in various concentrations of IC-2 (n = 3). Mean \pm SD, *P < 0.05, and ** P < 0.01 compared with 0.1% DMSO, examined by Dunnett's test. b Wnt/ β -catenin signal intensity in response to various concentrations of IC-2 by reporter assay (n = 3). Mean \pm SD, *P < 0.05, and **P < 0.01 compared with 0.1% DMSO, examined by Dunnett's test. Relative luciferase activity was expressed as corrected firefly luciferase activity with the internal control renilla luciferase activity calculated against 0.1% DMSO.

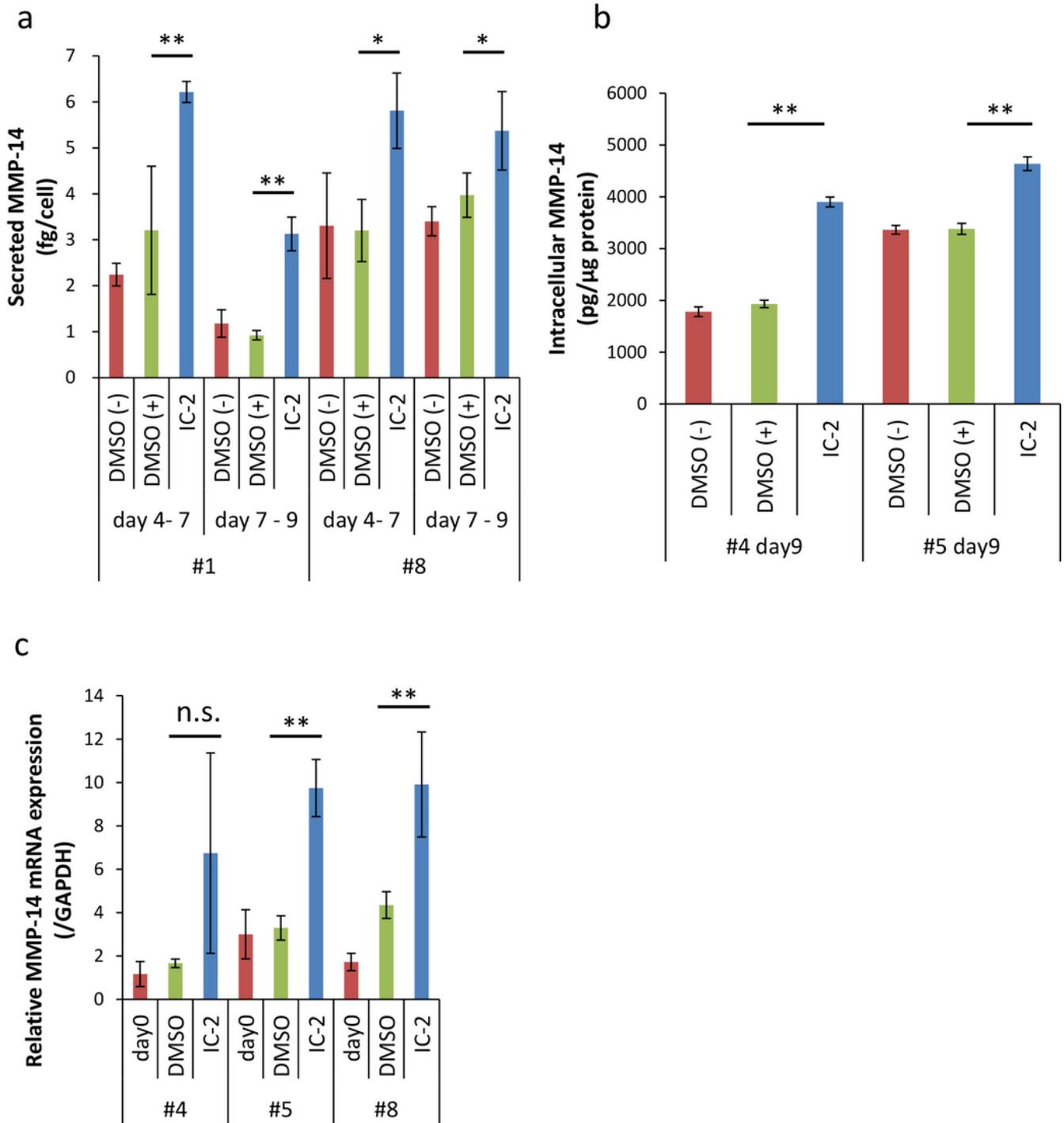


Figure 5

MMP-14 in response IC-2 in BM-MSCs from the elderly. a Secreted MMP-14 in the culture supernatant of BM-MSCs from the elderly in IC-2-contained medium (n = 3). Mean ± SD, *P < 0.05, and **P < 0.01 compared with ±DMSO medium examined by Student's t-test. b Intracellular MMP-14 contents of BM-MSCs from the elderly with IC-2 treatment (n = 3). Mean ± SD, *P < 0.05, and **P < 0.01 compared with ±DMSO medium examined by Student's t-test. c MMP-14 mRNA expression of BM-MSCs from the elderly

after IC-2 treatment (n = 3). Mean \pm SD, *P < 0.05, and ** P < 0.01 compared to day 0 or DMSO-containing medium after IC-2 treatment, examined by Student's t-test.

Supplementary Files

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