filtered through a 70-μm nylon filter (BD Biosciences, USA), and then cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS; Gibco, USA), 10 ng/ml bFGF (Wako, Tokyo, Japan), and antibiotic-antimycotic (Gibco). Passage three to five cells were used.

For the cell proliferation assay, ADSCs were seeded in 24-well plates in the presence or absence of bFGF, and cell numbers were counted at 8 and 72 h of incubation using a cell counter (Bio-Rad, USA). For the DNA synthesis assay, ADSCs were seeded in six-well u-slide (Ibidi, Germany) and analyzed using a Click-iT EdU Image Kit (Invitrogen). The cells were immunostained with anti-HGF antibody (Santa Cruz, Germany) and then FITC-conjugated secondary antibody (Sigma-Aldrich). EdU-positive cells were counted and compared with total cell counts. Proliferation rates were calculated from two independent experiments in duplicate.

Adipogenic differentiation was induced with an adipogenesis differentiation kit (Invitrogen) for 10 days, and assessed by Oil-Red-O (Sigma-Aldrich) staining. Chondrogenic differentiation was induced with a chondrogenesis differentiation kit (Invitrogen) for 2 weeks, and then the cells were stained with 1% Alcian Blue (Sigma-Aldrich). Cell immunophenotypes were analyzed by flow cytometer (BD Biosciences). Briefly, cells were incubated with fluorescein-coupled antibodies against

CD29, CD44, CD90, or CD45 (BD Biosciences). Appropriate antibody isotypes were used as negative controls.

Experimental protocol

Rats were randomly divided into four groups with 10 rats in each group: A (three times ADSC infusion + CCl_4); B (one time ADSC infusion + CCl_4); C [ADSC (bFGF-) infusion + CCl_4]; D (PBS + CCl_4). Hepatic cirrhosis was induced by intraperitoneal injection of 1 mg/kg body weight CCl_4 (Wako) twice a week for 8 weeks [18]. Then, 5×10^6 PKH26 (Sigma-Aldrich)- or Qtracker705 nanoparticle (Invitrogen)-labeled ADSCs in 0.5 mL PBS were infused via the caudal vein. Rats infused with 0.5 mL PBS were used as controls. Three days after infusion, the rats were sacrificed and exsanguinated to test serum levels of alanine transaminase (ALT), aspartate aminotransferase (AST), and albumin (ALB). Tissues were collected for *ex vivo* imaging analysis and histological examination.

Liver histology

Masson trichrome staining was performed to examine collagen matrix deposition in livers. To calculate the percentage of the blue-stained fibrotic area, five random fields

per section were assessed by two observers with Adobe Photoshop (Adobe Inc., USA) and Image J (NIH, USA) in a blinded manner. Next, we performed double immunofluorescence staining of HGF and PKH26 in liver sections. Briefly, tissues were incubated sequentially with the anti-HGF antibody and secondary antibody, and then mounted in Vectashield medium (Vector, USA). Unlabeled ADSC-infused liver sections served as negative controls.

Western blot analysis

Total protein (40 μg) from liver samples or cell lysates was loaded and separated in a sodium dodecyl sulfate polyacrylamide gel electrophoresis gel (Invitrogen) and then transferred to a membrane (Millipore, USA). Membranes were blocked and then incubated sequentially with primary antibodies and appropriate secondary antibodies. Primary antibodies were as follows: HGF, Proliferating cell nuclear antigen (PCNA), ALB (Santa Cruz); α- smooth muscle actin (α-SMA), β-actin (Sigma-Aldrich); c-jun N-terminal kinase (JNK) 1/2, phospho-JNK1/2, extracellular signal-regulated kinase (ErK)1/2, phospho-ErK1/2, p53, and phospho-p53 (Cell Signaling, USA).

Ex vivo fluorescence imaging assay

ADSCs were labeled by a Qtracker705 cell labeling kit (Invitrogen) according to the manufacturer's instructions. To monitor *in vivo* distribution of ADSCs, Qtracker705-labeled ADSCs were infused, whereas unlabeled ADSC-infused rats served as negative controls to adjust for the influence of auto-fluorescence. Further confirmation was obtained by performing *ex vivo* bio-fluorescence imaging using an IVIS Spectrum imaging system (Xenogen, USA) at 3 days after ADSC infusion.

Transfection of ADSCs with HGF siRNA

ADSCs were cultured in OPTI-MEM/5% FBS (Invitrogen) with or without bFGF. At 60% confluence, ADSCs were transfected for 48 h with Silencer select HGF siRNA or negative control siRNA (NC) (Applied Biosystems, USA) using Lipofectamine RNAiMAX (Invitrogen). Conditioned medium was assayed using a HGF Quantikine ELISA Kit (R&D Systems, USA). For quantitative real-time polymerase chain reaction (PCR) or reverse-transcription (RT)-PCR, total RNA was extracted using an RNeasy Plus Mini Kit (Qiagen, Germany) and analyzed using iTaq universal SYBR green one-step kit (Bio-rad) or SuperScript III one-step RT-PCR system (Invitrogen). Primers used were: HGF (NM_017017.2), HNF4a (NM_001270933), and GAPDH (NM_017008.4) (Takara, Japan).

Primary liver cell isolation and co-culture assay

Primary rat hepatocytes were obtained by collagenase perfusion and purified by iodixanol density solution (OptiPrep, Axis-shield) as described previously [19]. Hepatocytes obtained from normal liver were seeded on collagen-coated coverslips (BD Biosciences). Then, HSCs in supernatants were purified by 11.5% and 16% iodixanol density gradient centrifugation [20]. Passage one to three HSCs were used.

In co-culture experiments, 1×10^4 ADSCs were plated in the upper chamber of a cell culture insert (1 μm pore size, BD Biosciences) and co-cultured with either 1×10^5 HSCs or hepatocytes on coverslips in the bottom chamber. In parallel, cells cultured alone served as controls. Cell proliferation and apoptosis were analyzed after 3 days of culture. HSC and hepatocyte proliferation was assessed by EdU endocytosis assay as described above. Cells were also incubated sequentially with anti-α-SMA or -ALB antibodies (Santa Cruz, USA) and FITC-conjugated secondary antibodies.

The apoptosis assay was conducted by flow cytometric analysis of HSCs stained with Alexa Fluor 488-conjugated annexin V and propidium iodide (PI) (Invitrogen). Early apoptotic HSCs were annexin V+/PI−, and late apoptotic HSCs were annexin V+/PI+. Furthermore, caspase-3/7 activity in HSCs was assessed using Caspase-3/7

reagent (Essen bioscience, Japan). Cells were counterstained with an aSMA-Cy3 antibody (Sigma-Aldrich). To confirm p-JNK expression, HSCs were immunostained for p-JNK.

Statistical analyses

Results are presented as means ± standard deviation (SD). The Mann-Whitney U test was used to detect the statistical significance between two groups. Comparisons between multiple groups were made using the Kruskal-Wallis test. A value of $P < 0.05$ was considered statistically significant.

Results

bFGF facilitates the proliferation, differentiation, and HGF expression of ADSCs

In the presence of bFGF, we found a higher proliferation rate (Fig. 1A, B) and an increased fluorescence intensity of HGF (green) in ADSCs (Fig. 1B). Moreover, adipogenic and chondrogenic differentiation abilities were enhanced (Fig. 1C, D). Western blotting confirmed that HGF and PCNA expression was significantly elevated in the presence of bFGF (Fig. 1E). Furthermore, flow cytometric analysis revealed that the cells were positive for typical mesenchymal stem cell-associated markers such as

CD29, CD44 and CD90 (Fig. 1F). In other way, bFGF treatment didn't have significant influence on surface marker (Fig. 1F). These data further validated the notion that bFGF is critical for self-renewal of ADSCs [21].

bFGF-treated ADSC transplantation enhances attenuation of hepatic fibrosis and recovery of liver functions

The experimental protocol is illustrated in Fig. 2A. After 8 weeks of continuous CCl_4 administration, the liver showed typical histological changes indicating necrosis of parenchymal cells, vacuolar degeneration, and collagen deposition. Rats that received bFGF-treated ADSC infusion (groups A and B) still showed visible liver injury (Fig. 2B). However, compared with rats treated with PBS alone, histological examination revealed a significant reduction of the fibrotic area (Fig. 2B), despite a slight reduction of fibrosis in the ADSC (bFGF-) group. Furthermore, an obvious macro-morphological change was observed in ADSC-infused livers (Fig. 2C). The difference between group B and C indicated that bFGF treatment further enhanced the antifibrotic effect of ADSCs. Compared with the untreated ADSC group, western blotting showed that HGF expression was increased more significantly, and α-SMA expression was decreased in the bFGF treated ADSC group (Fig. 2D). These results indicated that elevated HGF

expression may be involved in the enhanced effects of bFGF-treated ADSCs.

Liver injury parameters such as serum ALT and AST levels were markedly increased after CCl_4 administration (~16-fold increase in ALT levels and ~15.7-fold increase in AST levels compared with those in normal rats; data not shown). In the ADSC (bFGF-) group, ALT and AST levels were significantly lower than those in the PBS group, and further reduced in the bFGF treated ADSC group. In addition, rats that underwent bFGF treated ADSC infusion showed a gradual increase in ALB levels (Fig. 3B). More importantly, the ALB level was elevated further by three time infusions of bFGF treated ADSCs. AST and ALT levels in rats infused three times with bFGF treated ADSCs were notably decreased even more. However, there were no significant difference in ALB levels between ADSC alone and PBS groups. Taken together with the potent promotion of the proliferation and HGF expression ability of ADSCs by bFGF, these findings strengthened that bFGF treatment is beneficial for the biological and therapeutic effects of ADSCs.

Infused ADSCs engraft in the injured liver

Fluorescence images showed that most of Qtracker705 nanoparticles were incorporated into ADSCs, indicating a high incorporation efficiency (Fig. 3A). To track the *in vivo*

distribution of ADSCs, fluorescent signals from livers were examined by *ex vivo* imaging analysis. Compared with normal liver, a significantly higher fluorescence signal from cirrhotic liver indicated promotion of infused ADSC migration into the injured site, despite a small proportion of ADSCs were trapped in the lung (Fig. 3B, C). Our data suggest that intravenously infused ADSCs have the potential to migrate into normal and injured livers, particularly under the conditions of liver damage.

The auto-fluorescence of the integrated cells were too faint to be detected, and no significant difference was found between the auto-fluorescence signal from the integrated ADSCs and parenchymal cells (Fig. 4A). In contrast, significant PKH26-HGF fluorescence signals were detected in PKH26-labeled ADSC-infused livers. As shown in Figure 4B–D, numerous ADSCs were localized along collagen fibers, parenchyma, and portal tracts, further confirmed that the infused ADSCs migrated to the injury site. Moreover, double-positive yellow cells with a stretching morphology were observed in ADSC-infused livers, indicating that the ADSCs were still viable and express HGF efficiency *in vivo*. Quantitative analysis revealed more ADSCs in livers when infused three times (Fig. 4D). These results suggested that HGF may be involved in ADSC recruitment to the liver. These data are consistent with previous reports that HGF is an important regulator of cell migration [22].

Quantitative analysis of HGF expression and knockdown efficiency

Next, we determined the amount of HGF in ADSC-conditioned medium. A total of 2×10^5 ADSCs were cultured for 72 h, resulting in secretion of 2137.2±261.5 pg/mL HGF (Fig. 5B). In addition, bFGF stimulation significantly enhanced the secretion of HGF to 4776.2±532.4 pg/mL, indicating that ADSCs are sensitive to bFGF stimulation and up-regulate HGF expression efficiently. Transfection of ADSCs with HGF siRNA partially abrogated the effects of bFGF on both mRNA (Fig. 5A) and protein (Fig. 5B) levels.

ADSCs enhance hepatocyte proliferation but suppress HSC survival via HGF

When co-cultured with ADSCs, hepatocytes showed a higher efficiency for DNA synthesis even without significant engraftment (Fig. 6A). For co-cultured HSCs, we found a decrease in the DNA synthesis rate (~7%, Fig. 6B) but a significant increase in the apoptosis rate as indicated by caspase-3/7 staining (~9%, Fig. 6C) and flow cytometry analysis (~8%, Fig. 6D). We transfected ADSCs with HGF siRNA to identify whether HGF mediated the effects of ADSCs. Importantly, the observed effects were abrogated by downregulation of HGF expression (Fig. 6A–D). Collectively, these

results further confirmed that paracrine HGF is critical for the effect of ADSC on HSC and hepatocyte.

We next investigated whether the JNK pathway was involved in ADSC-induced HSC apoptosis. Western Blot analysis showed that co-culture with ADSC didn't have significant effect on activation of ErK which was involved in the prevention of apoptosis. However, expression of p-JNK in HSCs was increased significantly (Fig. 6E, F), and its downstream molecule p53 was phosphorylated as well, followed by activation of the effector caspase-3 (Figure 6C), which induced HSC apoptosis and inhibited αSMA expression (Figure 6E). These results suggested that ADSC-induced HSC apoptosis was involved in activation of JNK and p53 signaling but did not significantly affect ErK activity. Taken together with the results of HGF RNA-interference experiments, HGF from ADSCs may contribute to the inhibitory effects on HSCs. These findings are consistent with a previous study demonstrating that the JNK pathway mediates growth inhibition and apoptosis of HSC [23].

Finally, we determined whether ADSCs treated with bFGF could differentiate into hepatocytes. Western blot and RT-PCR analyses revealed that ADSCs treated with bFGF for 6 days didn't show significant hepatocyte-specific marker expression compared with hepatocytes as the positive control (Supplementary Figure). These

results suggest that addition of bFGF alone may be insufficient to induce hepatocyte differentiation.

Discussion

Activated HSCs play a significant role in collagen and extracellular matrix (ECM) deposition that controls the development of liver fibrosis, and such excessive deposition of collagen and ECM was generally considered irreversible [24,25]. However, recent clinical studies found that liver cirrhotic patients who have been treated effectively demonstrate recovery with remodeling of fibrosis [26]. This finding indicates that it is feasible to augment the capacity for fibrotic tissue remodeling towards recovery. As a therapeutic approach, HGF is considered to be essential for tissue regeneration [27,28], and HGF can be up-regulated by the microenvironment upon tissue damage [29]. In liver disease, HGF promotes HSC apoptosis, which is associated with its antifibrotic effect [30]. However, in neoplastic tissues, HGF functions as an antiapoptotic cytokine [31,32]. In the current study, ADSCs treated with bFGF showed significant up-regulation of HGF expression to physiologically relevant levels that enhanced the therapeutic effect on hepatic cirrhosis.

It appears reasonable that systemic ADSC infusion ameliorated liver injury,

resulting in the histological and serological changes. To date, the mechanisms by which ADSCs exert their beneficial effects remain controversial. A previous study suggested that ADSCs exert their therapeutic effects on liver disease by either differentiating into functional hepatocyte-like cells or in a paracrine manner by secreting cytokines to promote repair [33]. However, the present study didn't find obvious hepatic differentiation characteristics when ADSCs were treated with bFGF. In contrast, we found that bFGF promoted HGF expression in ADSCs, indicating that the therapeutic effects of bFGF-treated ADSCs on liver disease may be mediated in a paracrine manner. Our study also demonstrated that the infused ADSCs migrated into the liver and localized to the fibrosis area. Furthermore, double immunofluorescence revealed that the ADSCs expressed HGF efficiency *in vivo*. Moreover, co-culture experiments showed enhancement of hepatocyte proliferation but suppression of HSC survival by ADSCs, which could be responsible for the liver histological changes in ADSC-infused rats. In addition, the absence of HGF resulted in abrogation of the biological effects induced by ADSCs, which supports the notion that HGF secreted by ADSCs is important for the anti-fibrosis effect. Similarly, ADSCs accelerate tissue reperfusions in a limb ischemia model by secreting HGF, which also indicates HGF as an important factor for tissue repair [34]. In another study, activated HSCs showed increased nerve growth factor

expression and induced apoptosis [35]. We found that HGF from ADSCs was involved in HSC apoptosis. These two distinct cytokines may cooperatively regulate apoptosis of HSCs. However, the contribution of other cytokines to HSC apoptosis remains unclear, and the potential involvement of other cells should be considered in future studies.

The JNK pathway is critical in mediation of HSC apoptosis [36,37]. Our data indicated the induction of JNK-p53-dependent apoptosis of HSC by ADSC. Together with the increased caspase-3/7 activity and the results from HGF RNA-interference experiments, this study demonstrated that overexpress HGF by bFGF-treated ADSCs induced HSC apoptosis via activation of JNK-p53 signaling, leading to the resolution of liver fibrosis.

Finally, compared with single infusion therapy, our work shows the first evidence that repeated administration of bFGF-treated ADSCs reduces hepatic injury and fibrosis formation more efficiently in animal model. Moreover, a potential therapeutic mechanism of ADSCs requires HGF production rather than engraftment. Previous work shows that ADSCs can be safe and highly efficient in clinical applications [38]. Our findings provide a rationale for the potential therapeutic application of ADSCs in patients with hepatic cirrhosis, suggesting that ADSC infusion could be a novel therapeutic approach for hepatic cirrhosis.

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Disclosure

The authors report no potential conflict of interest in this work.

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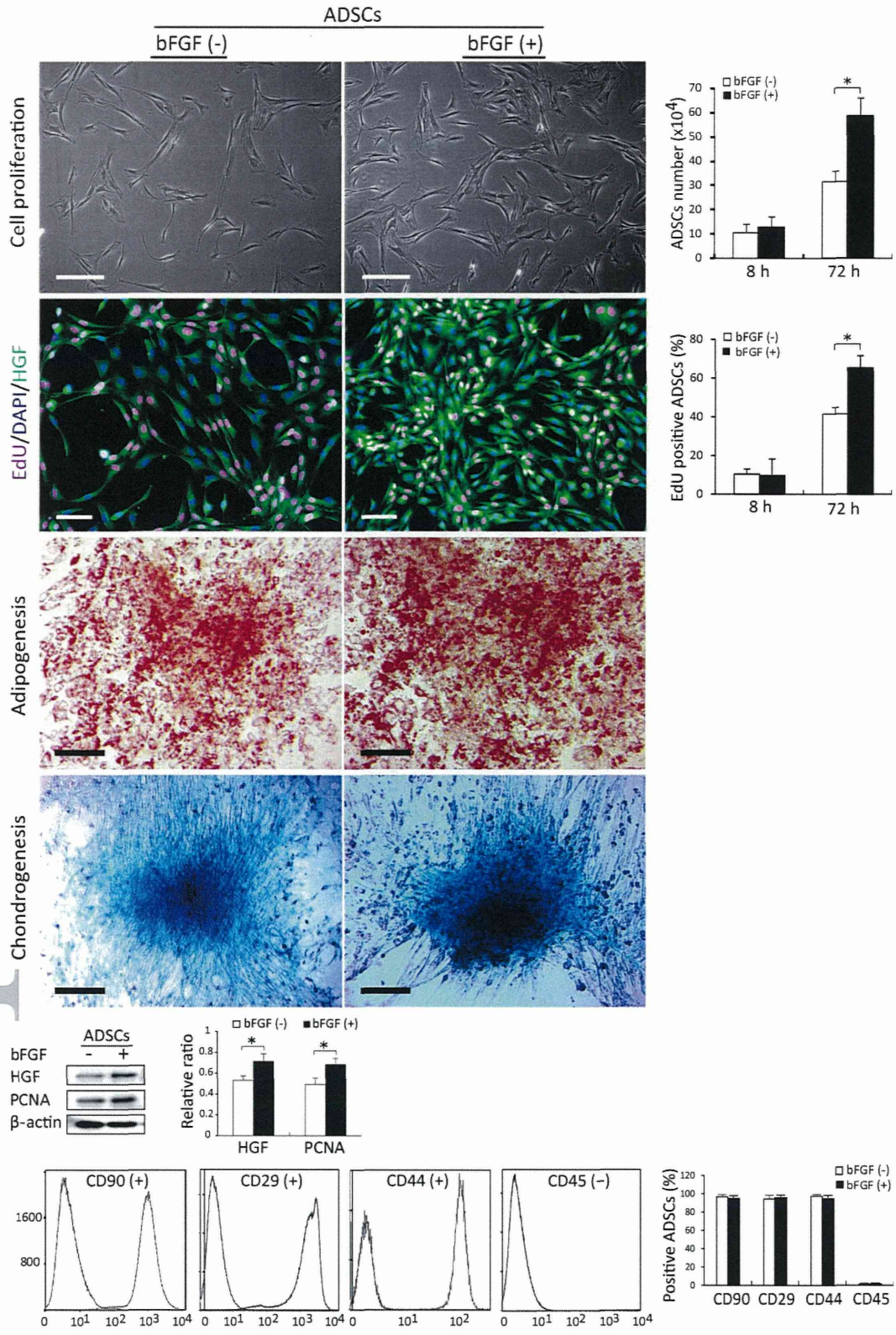

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