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Original Article

# Hepatic cell sheets engineered from human mesenchymal stem cells with a single small molecule compound IC-2 ameliorate acute liver injury in mice

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ABSTRACT

*Introduction:* We previously reported that transplantation of hepatic cell sheets from human bone marrow-derived mesenchymal stem cells (BM-MSCs) with hexachlorophene, a Wnt/β-catenin signaling inhibitor, ameliorated acute liver injury. In a further previous report, we identified IC-2, a newly synthesized derivative of the Wnt/β-catenin signaling inhibitor ICG-001, as a potent inducer of hepatic differentiation of BM-MSCs.
*Methods:* We manufactured hepatic cell sheets by engineering from human BM-MSCs using the single small molecule IC-2. The therapeutic potential of IC-2-induced hepatic cell sheets was assessed by transplantation of IC-2- and hexachlorophene-treated hepatic cell sheets using a mouse model of acute liver injury.
*Results:* Significant improvement of liver injury was elicited by the IC-2-treated hepatic cell sheets. The expression of complement C3 was enhanced by IC-2, followed by prominent hepatocyte proliferation stimulated through the activation of NF-κB and its downstream molecule STAT-3. Indeed, IC-2 also enhanced the expression of amphiregulin, resulting in the activation of the EGFR pathway and further stimulation of hepatocyte proliferation. As another important therapeutic mechanism, we revealed prominent reduction of oxidative stress mediated through upregulation of the thioredoxin (TRX) system by IC-2-treated hepatic cell sheets. The effects mediated by IC-2-treated sheets were superior compared with those mediated by hexachlorophene-treated sheets.
*Conclusion:* The single compound IC-2 induced hepatic cell sheets that possess potent regeneration capacity and ameliorate acute liver injury.


*Abbreviations:* BM-MSCs, bone marrow-derived mesenchymal stem cells; NF-κB, nuclear factor-kappa B; IL-6, interleukin-6; STAT-3, Signal Tranducer and Activator of Transcription 3; EGFR, epidermal growth factor receptor; *AREG*, amphiregulin; CBP, CREB-binding protein; DMSO, dimethyl sulfoxide; ALT, alanine aminotransferase; AST, aspartate aminotransferase; $CCl_4$, carbon tetrachloride; PCNA, proliferating cell nuclear antigen; *A1AT*, α1-antitrypsin; *CP*, ceruloplasmin; *TF*, transferrin; *APOE*, apolipoprotein E; *C4A*, complement C4A; *RBP4*, retinol binding protein 4; *C3*, complement C3; *hGAPDH*, human glyceraldehyde 3-phosphate dehydrogenase; *mActb*, mouse actin, beta; C5aR, complement C5a receptor; HB-EGF, heparin binding-epidermal growth factor-like growth factor; TGFα, transforming growth factor alpha; ERK, extracellular signal-regulated kinase; 8-OHdG, 8-hydroxydeoxyguanosine; SOD, superoxide dismutase; GPX, glutathione peroxidase; GSH, glutathione; TRX, thioredoxin; TRXR, thioredoxin reductase; PRX, peroxiredoxin; GRX, glutaredoxin; GR, Glutathione reductase; MDA, malondialdehyde; TNFα, tumor necrosis factor alpha; LXR, liver X receptor; HGFR, hepatocyte growth factor receptor; ChoREs, carbohydrate response elements; ChREBP, Carbohydrate-responsive element-binding protein; IL-1ra, interleukin-1 receptor antagonist.

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## 1. Introduction

Liver transplantation is the best therapeutic treatment for acute as well as chronic liver failure. However, liver transplantation cannot be successfully applied to all patients with liver failure because of donor scarcity [1]. Although hepatocyte transplantation has been performed as an alternative or a bridge approach to liver transplantation, the results of hepatocyte transplantation are not satisfactory [1]. Stem cell therapy is regarded as an attractive therapy for liver disease [2,3]. In particular, mesenchymal stem cells (MSCs) are emerging as a promising cell source for treatment of acute liver failure and cirrhosis [3,4].

We previously reported that orthotopic transplantation of hepatic cell sheets derived from human MSCs ameliorated carbon tetrachloride ($CCl_4$)-induced acute liver injury [5]. These hepatic cell sheets were originally developed by fusion of two independent technologies: the first is the chemical agent that suppresses the Wnt/β-catenin signal enhancing hepatic differentiation of MSCs, and the second is cell sheets manufactured on thermoresponsive, polymer-coated culture dishes. We previously identified hexachlorophene, a Wnt/β-catenin signal inhibitor, as an inducer of hepatic differentiation in human MSCs [5]. However, in antiseptic use except for anti-bacterial ingredient of medical soap, this compound was forbidden by Food and Drug Administration (FDA), more recently [6] because of its toxicity [7]. To accomplish hepatic cell sheets in clinical use, we need to identify more effective small molecule compounds to permit clinical application. To resolve this issue, we previously screened a library of synthesized, novel, small molecules. And of many small molecule variants of Wnt/β-catenin signal inhibitors, we identified IC-2, a derivative of ICG-001, as a more effective inducer of human MSCs toward hepatic differentiation [8]. IC-2 induces the expression of hepatocyte differentiation markers in bone marrow-derived MSCs (BM-MSCs) (e.g., Albumin and tryptophan 2,3-dioxygenase) most strongly among the screened 23 compounds and induces not only gene expression but also hepatocyte function (e.g., urea production and glycogen storage) [8]. ICG-001 has been reported to specifically downregulate the Wnt/β-catenin signal pathway through inhibiting the interaction of β-catenin with CBP [9,10]. Moreover, ICG-001-derived compound PRI-724 has proven to have acceptable toxicity in a phase I clinical study [11,12]. We reported that hexachlorophene-treated BM-MSC sheets were superior in ameliorating acute liver injury compared to non-treated BM-MSC sheets [5]. In the present study, we assessed the potential of IC-2-treated cell sheets derived from BM-MSCs in comparison with hexachlorophene-treated sheets in an acute model of liver injury. Consequently, we found that engineered cell sheets using the single small molecule IC-2 have a strong protective effect on acute liver injury.

## 2. Materials and methods

### 2.1. Wnt/β-catenin signal inhibitors and cells

Hexachlorophene was purchased from Sigma–Aldrich (St. Louis, MO, USA). IC-2 was synthesized in-house. Both inhibitors were dissolved in dimethyl sulfoxide (DMSO). The final concentration of DMSO in media was 0.1%. UE7T-13 human bone marrow-derived mesenchymal stem cells (BM-MSCs) were used [5,8]. In other experiments, primary MSCs were prepared using human bone marrow mononuclear cells purchased from Lonza Walkersville, Inc. (Walkersville, MD, USA) as previously described [8].

### 2.2. Production of Wnt/β-catenin signal inhibitor-treated hepatic cell sheets derived from mesenchymal stem cell sheets

Hexachlorophene-treated cell sheets were manufactured as previously described [5]. IC-2-treated cell sheets were prepared under the same conditions except for a starting cell density of $3.6 \times 10^4$ cells/cm$^2$. The final number of cells per sheet in following the IC-2-treatment was similar to that after hexachlorophene treatment (Supplemental Fig. 1). The concentration of inhibitor was 0.8 and 15 μM for hexachlorophene and IC-2, respectively. Both cell sheets were harvested from φ60-mm-temperature-responsive culture dishes (CellSeed Inc., Tokyo, Japan) at a temperature of 20 °C for 15–30 min. Primary BM-MSC sheets with or without IC-2 treatment were prepared as follows: $CD90^+/CD271^+$ MSCs were fractioned from human bone marrow mononuclear cells (Lonza Inc.) and expanded as previously described [8]. At passage number five, cells were plated onto φ60-mm-temperature-responsive culture dishes (CellSeed Inc.) at a density of $1.8 \times 10^4$ cells/cm$^2$. One day and 5 days after seeding, medium was replaced with DMEM (Life Technologies Corp., Carlsbad, CA) containing 10% fetal bovine serum (GE Healthcare UK Ltd, Little Chalfont, UK), 100 U/ml penicillin, and 100 mg/ml streptomycin with 30 μM IC-2. Primary BM-MSC sheets without IC-2 were prepared 4 days before harvest at the same cell density as IC-2-treated primary BM-MSC sheets.

### 2.3. Animal experiments

All experiments were conducted in accordance with the ethical approval of the Tottori University Subcommittee on Laboratory Animal Care. Eight-week-old NOD-SCID mice (CLEA Japan, Inc., Tokyo, Japan) were transplanted with a total of six cell sheets at two sites on the liver surface as previously described [5]. Control mice received sham laparotomy. Mice receiving carbon tetrachloride were administrated with 0.24 ml/kg (Wako Pure Chemical Industries Ltd., Osaka, Japan) dissolved in olive oil at 5% concentration by oral gavage at 1 day after transplantation. Two days after transplantation, blood samples were collected from the retro-orbital venous plexus under anesthesia with pentobarbital sodium (Kyoritsu Seiyaku Corp., Tokyo, Japan). On 2, 3, 4, and 8 days after transplantation, mice were sacrificed by exsanguination under anesthesia with pentobarbital sodium, and blood samples were collected from the inferior vena cava followed by liver resection. All mice were housed under pathogen-free conditions in a temperature-controlled room and illuminated (12 h daily) with *ad libitum* access to water and chow.

### 2.4. Biochemical tests

Blood samples were kept overnight on ice, and the serum was isolated by centrifugation at 2,000 g for 20 min. Serum aminotransferases and total bilirubin were measured as previously reported [5].

### 2.5. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA from the liver was extracted with TRIzol reagent (Life Technologies Corp.) and subjected to reverse transcription using Superscript II (Life Technologies Corp.) with oligo$(dT)_{18}$ primers. RT-PCR was performed using gene specific primers and rTaq DNA polymerase (TOYOBO CO., Ltd. Osaka, Japan). Primers used in the present study were the same as described in our previous report [5].

### 2.6. Quantitative RT-PCR analysis

UE7T-13 cells were seeded at a density of $9 \times 10^3$ cells/cm$^2$ and treated with 0.8 μM hexachlorohene, 15 μM IC-2, and 0.1% DMSO on days 1 and 4 after plating. Cells were harvested, and total RNA was extracted on days 1 and 8 after seeding. cDNA was synthesized as described earlier. Quantitative RT-PCR was performed using LightCycler® FastStart DNA Master SYBR Green I (Roche Diagnostics GmbH., Mannheim, Germany) using the LightCycler system (Roche Diagnostics GmbH.). Primers for qRT-PCR analysis were as follows: C3-Forward: 5′-CAGCACCATGGGACCCACCTCAG-3′, C3-Reverse: 5′-CTCTCCAGCCGCAAGATGTTGGG-3′; HB-EGF-Forward: 5′-GGACCGGAAAGTCCGT-3′, HB-EGF-Reverse: 5′-GCTCCTCCTTGTTTGGTGT-3′; AREG-Forward: 5′-AACGAAAGAAACTTCGACAAGAGA-3′, AREG-Reverse: 5′-ATGATCCACTGGAAAGAGGACC-3′; LXRα-Forward: 5′-GGTACAACCCTGGGAGTGAG-3′, LXRα-Reverse: 5′-TGGGGTTGATGAATTCCACT-3′, LXRβ-Forward: 5′-TCGTGGACTTCGCTAAGCAA-3′, LXRβ-Reverse: 5′-GCAGCATGATCTCGATAGTGGA-3′; IL-1ra-Forward: 5′-CAGCTGGAGGCAGTTAACAT-3′, IL-1ra-Reverse: 5′-CGCCTTCGTCAGGCATATTG-3′; GAPDH-Forward: 5′-AGCCACATCGCTCAGACAC-3′, GAPDH-Reverse: 5′-GCCCAATACGACCAAATCC-3′.

### 2.7. Western blot analysis

Ten to thirty micrograms of naive liver lysate not containing grafted cell sheets were analyzed using western blot. Primary antibodies were as follows: anti-C5aR, Glutatione peroxidase 1, Glutathione reductase, catalase (Abcam Ltd., Cambridge, UK), anti-C5a, SOD1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-TRXR1, anti-EGFR, phospho-EGFR, STAT3, phospho-Stat3 (Cell Signaling Technology Inc., Danvers, MA), anti-peroxiredoxin 2 (Sigma–Aldrich Corp., St. Louis, MO), anti-PCNA (DakoCytomation, Glostrup, Denmark), and goat polyclonal anti-Actin (Santa Cruz Biotechnology, Inc.). Anti-phospho-Stat3 (product number: #9145) recognized Tyr705 phosphorylation of STAT-3. Actin was used as an internal control. The bands were detected by ImageQuant LAS4000 (GE Healthcare UK Ltd).

### 2.8. Immunohistochemistry

Liver tissues containing the cell sheets were fixed in 4% paraformaldehyde and paraffin-embedded. Sections of 3 μm thick were used for immunohistochemistry as previously described [5]. Briefly, the sections were deparaffinized and antigens were retrieved by autoclave in citrate buffer. Except for 8-OHdG immunostaining, endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide for 15 min. Primary and secondary antibodies were identical to our previous report [5]. Anti-NF kappa B antibody (product number: sc-8008) purchased from Santa Cruz Biotechnology,Inc. recognized p65 subunit of NF-κB. Cells staining positively for NF-κB, 8-OHdG and Ki-67 were counted automatically by using inForm advanced image analysis software (PerkinElmer Inc., Waltham, MA).

### 2.9. Oxidative stress analysis

MDA adduct content was measured by OxiSelect™ MDA Adduct ELISA Kit (Cell Biolabs, Inc., San Diego, CA) according to the manufacturer's instructions. The absorbance was measured using a plate reader (Tecan Japan Co., Ltd., Kanagawa, Japan).

### 2.10. Statistical analysis

All the values in the present study were expressed as mean ± SE. Significant differences between groups were analyzed by the one-way analysis of variance post hoc test by Games–Howell using a predictive analytics software (SPSS Inc., Chicago, IL, USA) unless otherwise noted below. A P-value <0.05 was considered to be significant.

## 3. Results

### 3.1. Strong effect of orthotopic transplantation of IC-2-treated hepatic cell sheets on acute liver injury

First, we prepared IC-2-treated cell sheets using the same conditions as previous report [5], where the plating cell density was $9 \times 10^3$ cells/cm$^2$. However, the final cell numbers of IC-2-treated BM-MSCs were about a quarter of the harvests treated with hexachlorophene (Supplemental Fig. 1). To make the final cell numbers of IC-2-treated cells and hexachlorophene-treated cells roughly the same, the plating density of both conditions were changed.

To examine the effects of transplantation of IC-2-treated cell sheets, the mice underwent IC-2-treated cell sheets transplantation, hexachlorophene-treated cell sheets transplantation, and sham-operation were subject to acute liver injury by oral administration of $CCl_4$ one day after transplantation. Serum ALT and AST in mice transplanted with hexachlorophene-treated sheets were significantly reduced compared with sham-operated mice on day 2 (Fig. 1a and b). Serum ALT and AST in mice transplanted with IC-2-treated cell sheets were dramatically decreased compared with those in mice transplanted with hexachlorophene-treated cell sheets. Reduction in ALT and AST in the hexachlorophene-treated group was about 30% and 40% of the level in the sham-operated group, respectively. On the other hand, reduction in both ALT and AST in the IC-2-treated group was about 60% of the level in the sham-operated group. On day 2 after transplantation, total bilirubin was significantly reduced in mice transplanted with hexachlorophene-treated cell sheets compared with the mice in the sham-operated group. Total bilirubin was remarkably lower in mice transplanted with IC-2-treated sheets compared with the mice transplanted with hexachlorophene-treated sheets (Fig. 1c). Although the reduction of total bilirubin in mice transplanted with hexachlorophene-treated sheets was about 40% of the level of the mice in the sham-operated group, the reduction was about 85% in mice transplanted with IC-2-treated sheets. The mice transplanted with IC-2-treated cell sheets showed accelerated recovery of body weight from the reduction caused by $CCl_4$ (Fig. 1d). Indeed, the liver-to-body weight ratio on day 8 was also improved in mice transplanted with IC-2-treated cell sheets with the value being close to that of $CCl_4(-)$ mice (mean ± SE, 4.66 ± 0.33; n = 3) (Fig. 1e). These results suggest that transplantation of IC-2- treated cell sheets is more protective than that of hexachlorophene-treated cell sheets in acute liver injury.

### 3.2. Augmentation of liver regeneration by transplantation of IC-2-treated hepatic cell sheets

Since we previously reported that liver regeneration contributed to amelioration of acute liver injury [5], we examined whether IC-2-treated hepatic cell sheets promoted liver regeneration. More Ki-67-labeled hepatocytes were observed in both the mice transplanted with hexachlorophene- and IC-2-treated hepatic cell sheets on days 3 and 4 after transplantation compared with the mice in the sham-operated group (Fig. 2a). Although there was an

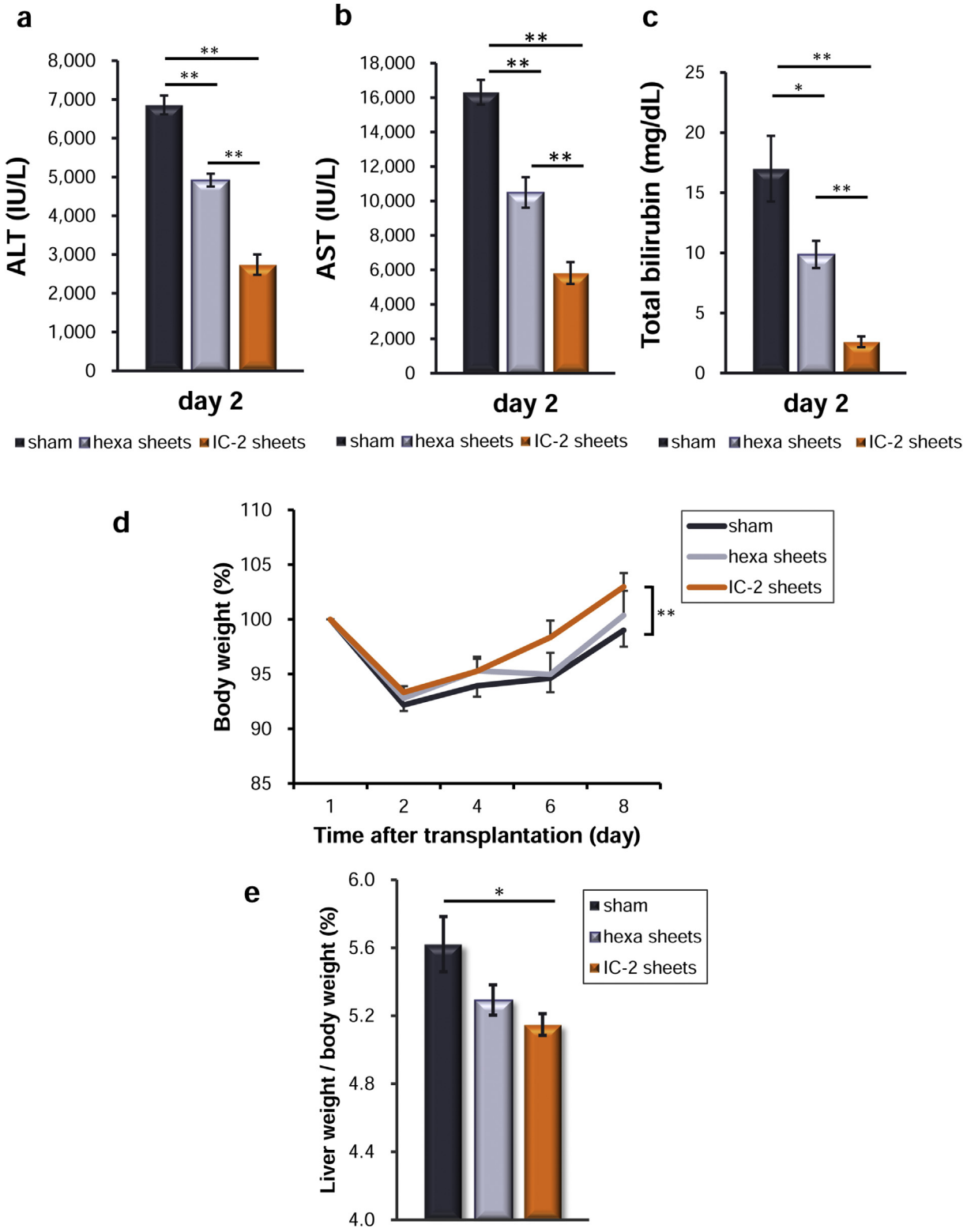


**Fig. 1.** Orthotopic transplantation of Wnt/β-catenin signal inhibitors-induced hepatic cell sheets ameliorated acute liver failure. (a, b) Serum ALT (a) and AST (b) of the recipient mice were assessed on day2 after transplantation (n = 9–10 in each group). $^{*}p < 0.05$, $^{**}p < 0.01$, examined by One-Way ANOVA. (c) Total bilirubin of the recipient mice were assessed on day2 after transplantation (n = 7–10 in each group) $^{*}p < 0.05$, $^{**}p < 0.01$, examined by One-Way ANOVA. (d) Body weight transition rate after $CCl_4$ administration (n = 9–10 on each day). The percentage was calculated with body weight of day1 as 100%. $^{**}p < 0.01$, examined by repeated measurement ANOVA. (e) Liver to body weight ratio on day8. Data are expressed as mean ± SE (n = 9–10 in each group).$^{*}p < 0.05$, $^{**}p < 0.01$, examined by One-Way ANOVA.

increasing tendency for Ki-67-positive hepatocytes in the mice transplanted with hexachlorophene-treated sheets, a significant increase was only detected in the mice transplanted with IC-2-treated sheets mice on days 3 and 4 (Fig. 2b). Furthermore, mice transplanted with IC-2-treated cell sheets showed remarkable PCNA expression with a gradual increase from day 2 to day 4, which predated the delayed peak in the mice transplanted with hexachlorophene-treated sheets (Fig. 2c). These data suggest that IC-2-treated cell sheets possess potent regenerative properties compared with hexachlorophene-treated cell sheets. In the present study, there was no significant increase in Ki-67-positive cells in the hexachlorophene-treated sheets group (Fig. 2b); however, Ki-67-positive cells were increased in our previous work [5]. Although the reason why hexachlorophene-treated sheets did not promote liver regeneration in the present study is unclear, hexachlorophene-treated cell sheets might not be able to overcome the liver injury caused by 0.24 ml/kg of $CCl_4$ administration compared with 0.2 ml/kg [13].

### 3.3. IC-2 induced complement component C3, an important factor for priming liver regeneration through NF-κB activation

Liver regeneration is a sequential process comprising three main phases: initiation, proliferation/expansion, and termination [14]. These events are orchestrated by several cytokines and growth factors [14–16]. Liver is the major source of several serum proteins. Among those proteins, complements C3a and C5a are well known as the priming factors for liver regeneration [15–18]. To clarify whether hepatic cell sheets-derived serum proteins are involved in promoting liver regeneration, mRNA expression of various human-specific serum proteins from liver tissues including hepatic cell sheets was investigated. During day 2–4 after transplantation, RT-PCR using specific primer pairs for human mRNA transcripts was performed. Although gene expression of α1-antitrypsin (*A1AT*), ceruloplasmin (*CP*), transferrin (*TF*), apolipoprotein E (*APOE*), and complement C4A (*C4A*) was hardly detectable, complement C3 (*C3*) and retinol binding protein 4 (*RBP4*) were potently expressed in the liver of mice transplanted with IC-2-treated hepatic cell sheets. *RBP4* peaked transiently on day 2 after transplantation, whereas *C3* was expressed more robustly during days 2–4 when the priming and proliferation phases of liver regeneration are thought to occur (Fig. 3a). *In vitro* treatment of BM-MSCs with IC-2 caused higher expression of *C3* than the treatment with hexachlorophene (Fig. 3b). Molecules in the pathway downstream from complement C3 such as C5, C5a, and C5aR were examined [18]. On day 2 after transplantation, C5a, an active form of C5, was upregulated in the liver of mice transplanted with IC-2-treated sheets while mice transplanted with hexachlorophene-treated sheets exhibited upregulation of C5a with a 1-day delay (Fig. 3c). Expression of C5aR, a receptor of C5a, was not altered among the experimental groups. Since C5a plays a crucial role in liver regeneration through activating NF-κB and STAT3 [17], we investigated the activation of NF-κB and STAT-3 in the liver of mice transplanted with hepatic cell sheets. In accordance with the upregulation of C5a, nuclear localization of NF-κB was most abundant in hepatocytes of mice transplanted with IC-2-treated sheets (Fig. 3d). The nuclear expression of NF-κB was significantly higher in mice transplanted with IC-2-treated sheets compared with mice in the sham-operated group or mice transplanted with hexachlorophene-treated sheets (Fig. 3e). Since NF-κB is well known to activate STAT-3 pathway via transcriptional upregulation of IL-6, we examined STAT-3 activation in the liver of mice transplanted with hepatic cell sheets by western blotting. A prominent increment of phosphorylated STAT-3 was observed in the liver of mice transplanted with IC-2-treated cell sheets from day 2 after transplantation (Fig. 3f). Meanwhile, an increase in phosphorylated STAT-3 was delayed in the case of transplanted hexachlorophene-treated sheets, in accordance with the delayed expression of C5a. These data suggest that IC-2-induced complement C3 activated STAT-3 pathway via C5a-mediated nuclear translocation of NF-κB in the liver of transplanted mice. Consequently, IC-2-treated cell sheets promoted liver regeneration more robustly than hexachlorophene-treated cell sheets.

### 3.4. EGFR ligands derived from hepatic cell sheets activated EGFR pathway

As described earlier, many cytokines are important in modulating liver regeneration. We previously reported that the activation of the EGFR pathway was provoked by the transplantation of hepatic cell sheets [5]. Activation of the EGFR pathway plays a crucial role in the G1/S phase transition of hepatocyte proliferation during liver regeneration. EGFR ligands such as HB-EGF, EGF, TGFα, and amphiregulin are involved in liver regeneration [19,20]. Expression of human-specific EGFR ligands were observed in liver grafts including hepatic cell sheets. EGF and TGFα were not detected in any experimental group (TGFα data not shown); however, amphiregulin and HB-EGF were potently expressed in mice transplanted with IC-2-treated cell sheets (Fig. 4a). HB-EGF expression peaked on day 2, whereas amphiregulin was potently expressed throughout the experiment. Next, we examined whether these EGFR ligands affected the activation of the EGFR pathway in mice transplanted with hepatic cell sheets. The expression of phosphorylated EGFR was highest on day 3 in mice transplanted with IC-2-treated hepatic cell sheets. ERK was also activated in the same group on day 3 (Fig. 4b). On day 4 after transplantation, phospho-EGFR was slightly expressed in both the IC-2- and hexachlorophene-treated hepatic cell sheets transplanted groups compared with that of the sham-operated group. In addition, expression of phospho-ERK was intense in both groups of mice transplanted with treated cell sheets. These data support the mechanism that the EGFR pathway was activated by transplantation of IC-2-treated hepatic cell sheets. Next, we examined whether hepatic cell sheets-derived expression of amphiregulin and HB-EGF was induced by IC-2 treatment. Treatment of BM-MSCs with IC-2 resulted in prominent amphiregulin induction, whereas hexachlorophene treatment had no effect on the upregulation of amphiregulin expression (Fig. 4c). On the contrary, expression of HB-EGF decreased during 8 days of culture. However, the addition of IC-2 reversed expression of HB-EGF to some extent (Fig. 4d). These data suggest that direct effects of IC-2 on the induction of amphiregulin and HB-EGF play an important role in acceleration of liver regeneration.

### 3.5. Transplantation of hepatic cell sheets reduced oxidative stress

In many liver diseases, oxidative stress occurs and is a factor for exacerbation [21]. Therefore, reduction of oxidative stress may be an effective treatment in liver disease. In the present study, we used $CCl_4$, one of the hepatotoxins, to induce acute liver injury. $CCl_4$ is metabolized by cytochrome p450 enzyme to the highly reactive trichloromethyl radical ($CCl_3\bullet$), which generates oxidative stress [22]. To explore the reductive effect of hepatic cell sheets on oxidative stress, we examined the 8-OHdG-positive hepatocytes as an indicator of oxidative stress. An abundance of 8-OHdG-positive cells was observed in the liver of mice in the sham-operated group on day 2 after transplantation; however, these were reduced in both hepatic cell sheets-transplanted groups, which was particularly evident in the IC-2-treated cell sheets group (Fig. 5a). In all experimental groups, the 8-OHdG-positive cells peaked on day 2,

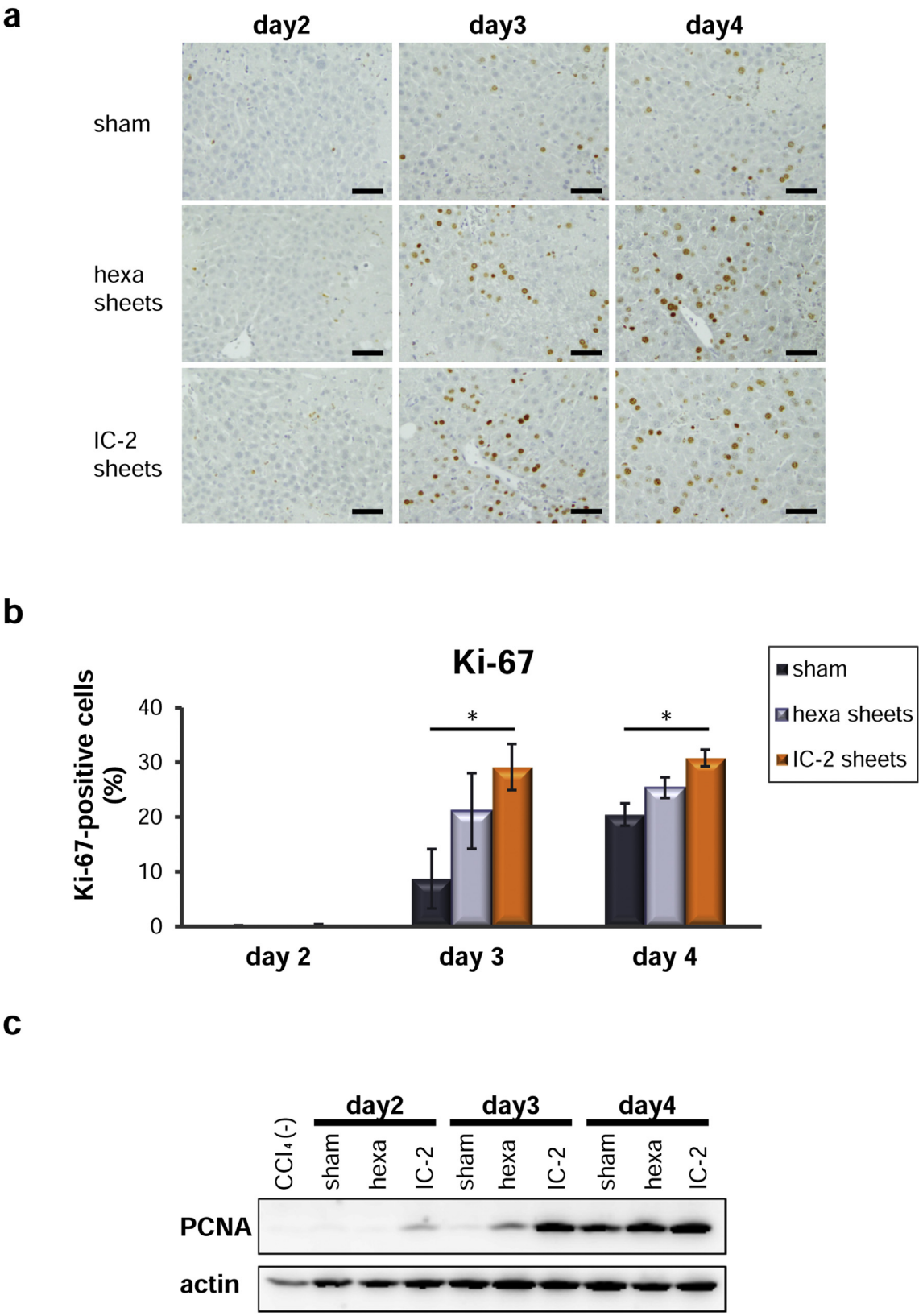


**Fig. 2.** Hepatic cell sheets promotes liver regeneration. (a) Immunohistochemistry of Ki-67. Scale bar = 50 μm. Ki-67-positive hepatocytes were gradually increased in hepatic cell sheets transplanted mice liver, especially in IC-2-treated cell sheets transplanted recipient liver after day2. (b) The ratio of Ki-67-positive nuclei during liver regeneration. Data are expressed as mean ± SE (n = 3–4 in each group, 10 fileds of individual mice were measured). *$p < 0.05$, examined by One-Way ANOVA post hoc test by Games-Howell. (c) Western blot of PCNA in recipient liver tissues.

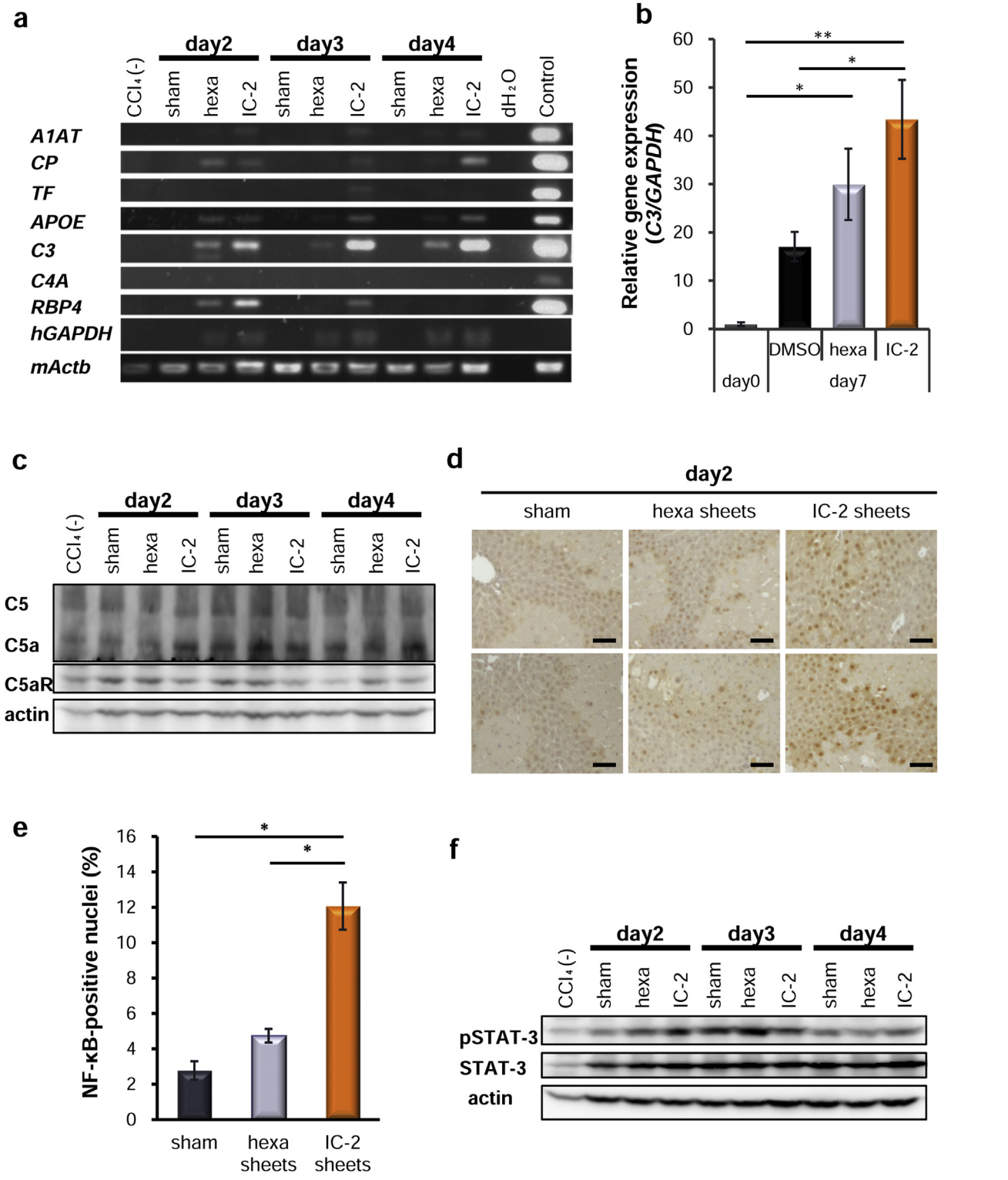


**Fig. 3.** NF-κB and STAT-3 are activated in recipient liver by complement C3 derived from hepatic cell sheets. (a) mRNA expresion of serum proteins mainly secreted from liver. Human-specific gene expression in grafted cell sheets was determined by RT-PCR analysis using primers specifically annealing human mRNA transcripts except for mouse *Actb*. Whereas Huh-7 cells were used as positive control for serum proteins, normal mice liver was used as positive control for human *GAPDH* and mouse *Actb*. (b) Quantitative RT-PCR analysis of *C3 in vitro*. UE7T-13 cells were treated with 0.8 μM hexachlorophene, 15 μM IC-2, and 0.1% DMSO respectively for 7 days. Data are expressed as the means ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, analyzed by one-way ANOVA, followed by Tukey test. (c) Western blot of recipient liver tissues. C5a was abundant in IC-2-treated cell sheets transplanted recipient liver on day2. C5aR was not altered between experimental groups. (d) Immunohistochemistry of NF-κB. Nuclear localization of NF-κB in IC-2-treated cell sheets transplanted mice hepatocytes on day2. Scale bar = 50 μm. (e) The ratio of NF-κB-positive nuclei on day2 (n = 3–4 in each group, 10 fields per each mice were measured). $^{**}p < 0.01$, examined by One-Way ANOVA. (f) STAT-3 pathway were examined by western blot. STAT3 activation was prominent in IC-2-treated cell sheets transplanted naive liver on day2.

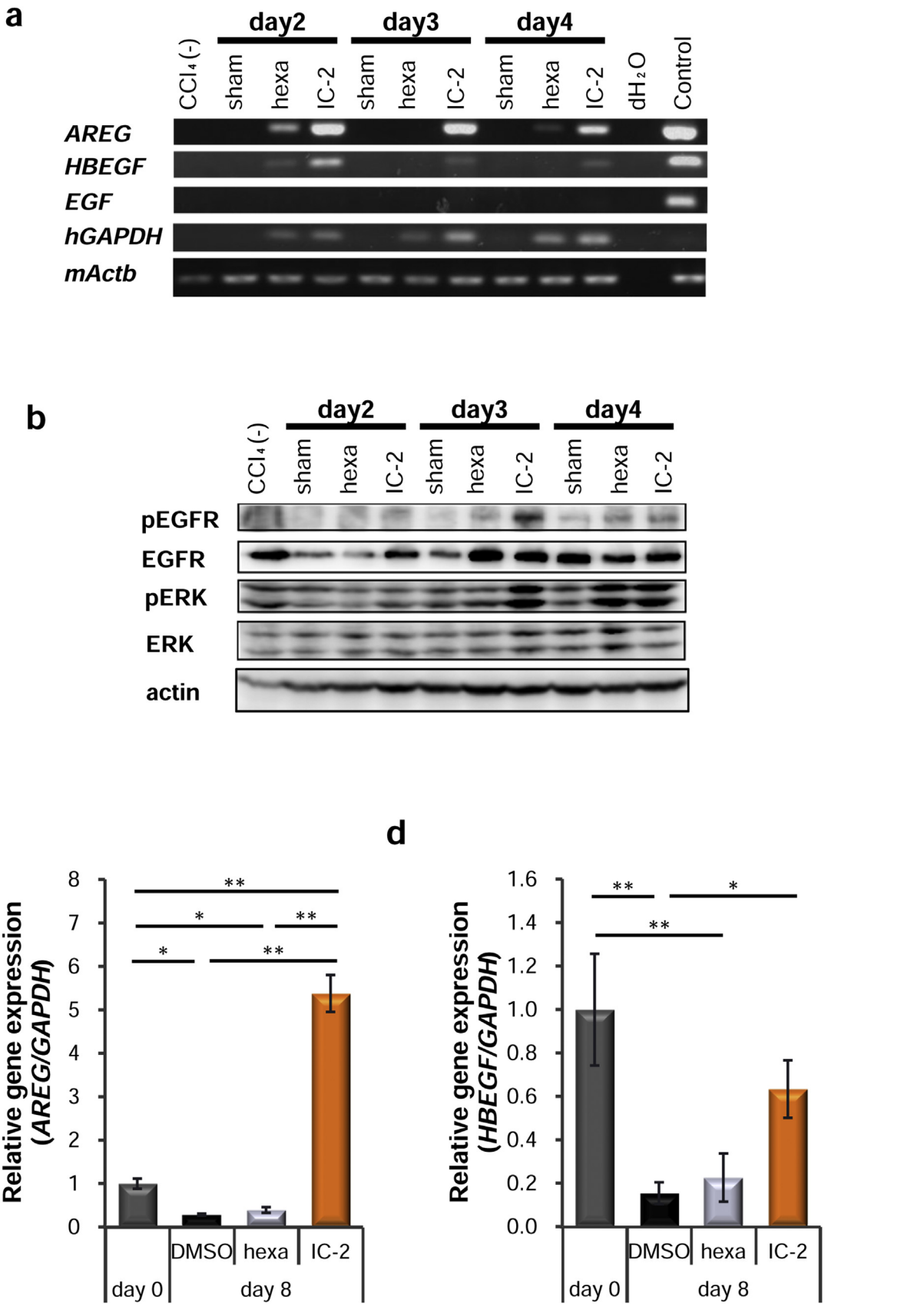


**Fig. 4.** Hepatotrophic factors activated EGFR pathway through EGFR ligands. (a) RT-PCR analysis of human-specific EGFR ligands expression. RT-PCR analysis was conducted using primers specifically annealing human mRNA transcripts except for mouse *Actb*. Huh-7 cells were used as positive control for EGFR ligands. Normal mice liver was used as positive control for human *GAPDH* and mouse *Actb*. (b) Western blot of EGFR pathway. EGFR pathway was activated in IC-2-treated cell sheets transplanted naive liver on day3. (c, d). Quantitative RT-PCR analysis of Amphiregulin (c) and HB-EGF (d) *in vitro*. UE7T-13 cells were treated with 0.8 μM hexachlorophene, 15 μM IC-2, and 0.1% DMSO respectively for 7 days. Data are expressed as the means ± SD (n = 3). $^{*}p < 0.05$, $^{**}p < 0.01$, analyzed by one-way ANOVA, followed by Tukey test.

and then gradually decreased. Judging from the 8-OHdG-positive cell rate, IC-2-treated hepatic cell sheets exhibited a potent antioxidative effect compared with the other groups on day 2 and 3 (Fig. 5b). Among antioxidative proteins, major enzymatic antioxidants such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), are involved in redox regulation [23]. Among them, SOD and catalase have been reported to contribute to the reduction of oxidative stress by transplantation of MSCs in a mouse model of acute liver injury [13,24]. In addition to these major enzymes, mammalian cells have two important thiol-dependent antioxidant systems, glutathione (GSH) and TRX [25]. Thioredoxin reductase (TRXR) and peroxiredoxin (PRX) are essential for the TRX system. Glutaredoxin and glutathione reductase (GR) are also involved in glutathione system. Since reduction in oxidative stress was observed in mice transplanted with hepatic cell sheets, we examined the involvement of these antioxidative proteins. Focusing on day 2, prominent expression of TRX and TRXR1 was detected in the liver of mice transplanted with IC-2 treated cell sheets, whereas SOD and catalase were not altered among the experimental groups (Fig. 5c). GPX1 was expressed in both hexachlorophene- and IC-2-treated cell sheets transplanted groups at a similar level. These results suggest that the TRX system is the main contributor to the reduction of oxidative stress. The 8-OHdG-positive cells on day 2 were also decreased in hexachlorophene-treated hepatic cell sheets. This may be caused by upregulation of the glutathione system accompanying higher GR expression because the TRX and GSH systems have been reported to be functionally related [25]. Taken together, these data suggest that the antioxidant property of IC-2-treated hepatic cell sheets is involved in their remarkable suppressive effect on acute liver injury.

### *3.6. Primary human mesenchymal stem cell-engineered hepatic cell sheets with IC-2 also ameliorated acute liver injury*

Since above results were obtained using cell sheets manufactured from UE7T-13 cells which were immortalized BM-MSCs with hTERT and E7 gene, we assessed whether the manufacturing procedure of hepatic cell sheets could be applied to clinical use. $CD90^+/CD271^+$ bone marrow-derived mononuclear cells have been reported to include MSCs with growth and differentiation capacity toward osteoblasts, chondrocytes, and adipocytes [26]. Furthermore, we previously reported that IC-2 could induce hepatic differentiation of primary $CD90^+/CD271^+$ BM-MSCs [8]. To examine whether IC-2 is applicable for primary human BM-MSCs, the effects of IC-2-treated hepatic cell sheets and non-treated cell sheets were assessed, in which IC-2-engineered cell sheets from $CD90^+/CD271^+$ BM-MSCs and non-treated cell sheets from $CD90^+/CD271^+$ BM-MSCs were employed. In mice transplanted with non-treated cells, ALT were decreased; however, neither AST nor total bilirubin were significantly reduced (Fig. 6a–c). On the contrary, transplantation of IC-2-engineered hepatic cell sheets strongly reduced serum transaminases and total bilirubin. In addition, liver-to-body weight ratio on day 8 was also significantly improved in mice transplanted with IC-2-treated cell sheets with almost the same value as that without $CCl_4$ treatment, suggesting that body weight reduction caused by severe liver injury was rapidly recovered by transplantation of IC-2-engineered cell sheets. MDA adduct, the final products of lipid peroxidation showed significant reduction by mice transplanted with IC-2-treated hepatic cell sheets. These results suggest that IC-2-engineered hepatic cell sheets from primary $CD90^+/CD271^+$ BM-MSCs are also effective in limiting acute liver injury.

## 4. Discussion

In the present study, we developed IC-2-engineered hepatic cell sheets derived from human BM-MSCs. IC-2-treated cell sheets are significantly superior to hexachlorophene-treated cell sheets. In our previous study, we reported that hexachlorophene-treated cell sheets prevented and ameliorated acute liver injury by acceleration of liver regeneration [5]. Acceleration of liver regeneration by hexachlorophene-treated cell sheets is caused by the following three mechanisms. First, high expression of complement C3 and its downstream signals such as C5a, NF-kB, and STAT3 were activated. Second, phosphorylation of EGFR is enhanced although the relevant ligands of EGFR were not defined. Third, the TRX oxidation and reduction cycle is activated, leading to the contribution of redox state [5]. In the present study, we found that IC-2-treated cell sheets exhibited remarkable activity in the reduction of acute liver injury. Surprisingly, all three factors such as C5a, phospho-EGFR and TRX were amplified by IC-2-treated cell sheets. These data reveal the underlying mechanisms whereby IC-2-treated cell sheets ameliorate liver damage, and may open a new avenue toward novel cell therapy for liver disease.

In the present study, we performed transplantation of cell sheets before $CCl_4$ treatment, since transplantation of cell sheets followed by administration of $CCl_4$ is technically easier than the reverse order. Strictly speaking, transplantation of cell sheets after liver injury may be preventive but not therapeutic. Initial $CCl_4$ administration followed by transplantation of cell sheets may fit “therapy of clinical scenes.” However, transplantation of cell sheets followed by $CCl_4$ treatment may cause more severe liver injury than the reverse order, since cytochrome P450 produces cytotoxic radicals in the cells which have liver functions [22]. In our previous report, we demonstrated that transplantation of cell sheets in both before and after liver injury really ameliorated liver injury [5]. Briefly, in transplantation of cell sheets followed by $CCl_4$ administration, ALT and AST reduced at 1 day after $CCl_4$ administration by 49% and 38.5%, respectively. On the other hand, in $CCl_4$ administration followed by transplantation of cell sheets, ALT and AST reduced at 1 day after $CCl_4$ administration by 28.1% and 29.8%, respectively. In the present study, ALT and AST drastically reduced about 60% in cell sheets transplantation followed by liver injury. Taken together, these data suggest that cell sheets transplantation even after liver injury seems to potently reduce liver injury, and could be enough curative treatment for liver injury.

To initiate liver regeneration, several priming factors, such as TNFα, IL-6, C3a, and C5a participate [15–17]. Among these factors, C3 and C5 are predominantly supplied by hepatocytes [27,28]; therefore, *C3* expression might be induced as a result of hepatic differentiation. In the promoter region of the human *C3*, there is a liver X receptor (LXR)-responsive element, and LXR-dependent regulation of *C3* expression has been reported [29]. LXR expression is upregulated in accordance with hepatic differentiation of iPSCs [30,31]. Moreover, LXR regulates differentiation of hepatic cells [32]. In case of our hepatic differentiation procedure, expression of LXRα and LXRβ were induced in UE7T-13 human BM-MSCs by treatment with IC-2 treatment in an *in vitro* study (Supplemental Fig. 2a and b). These data suggest that complement C3 induced by IC-2 triggers liver regeneration through upregulation of LXR. Complement C3 followed by activation of C5, NF-κB, IL-6 and STAT3 potently promotes liver regeneration in IC-2-mediated hepatic cell sheets system.

To promote liver regeneration, growth factors following priming factors are required for entering the S-phase of cell cycle. During this phase, EGFR and HGFR (c-met) are two major pathways for promoting liver regeneration [20]. In the present study, the EGFR

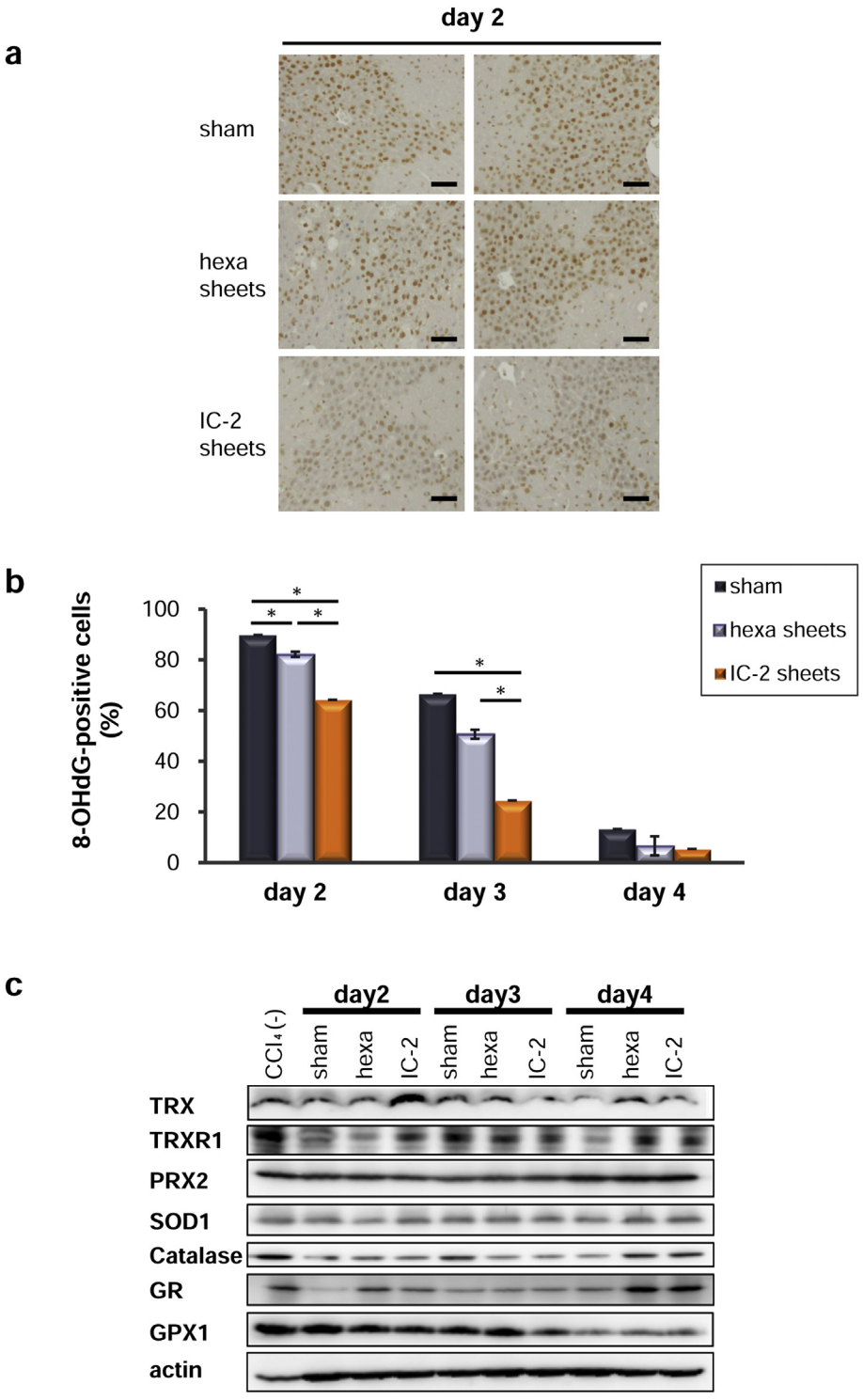


**Fig. 5.** Oxidative stress was reduced in cell sheets transplanted mice liver through thioredoxin oxidation and reduction cycle. (a) Immunohistochemistry of 8-OHdG. Scale bar = 50 μm 8-OHdG-positive nuclei were diminished in cell sheets transplanted mice, especially in IC-2-treated cell sheets transplanted recipient liver on day2. (b) The ratio of 8-OHdG-positive nuclei on day2. Data are expressed as the means ± SE (n = 3, 10 fields per each mice were measured). *$p < 0.05$, examined by One-Way ANOVA. (c) Western blot of antioxidant proteins in recipient liver tissues. TRX and TRXR were abundant in IC-2-treated cell sheets transplanted recipient liver on day2.

pathway was potently activated in the liver of mice transplanted with IC-2-treated hepatic cell sheets. What ligands for EGFR pathway operate in our system? Two molecules are raised as candidates for ligands for EGFR in our system. One is amphiregulin, and the other is HB-EGF. The *in vitro* study in Fig. 4c and d suggests that amphiregulin is a plausible ligand for EGFR. However, it was reported that amphiregulin is a target gene of Wnt/β-catenin signaling pathway [33,34]. Although the reason why expression of amphiregulin was induced by IC-2, a Wnt/β-catenin signaling inhibitor, remains to be solved, other mechanisms might be involved in transcriptional activation of amphiregulin. The transcriptional regulatory elements of amphiregulin include carbohydrate

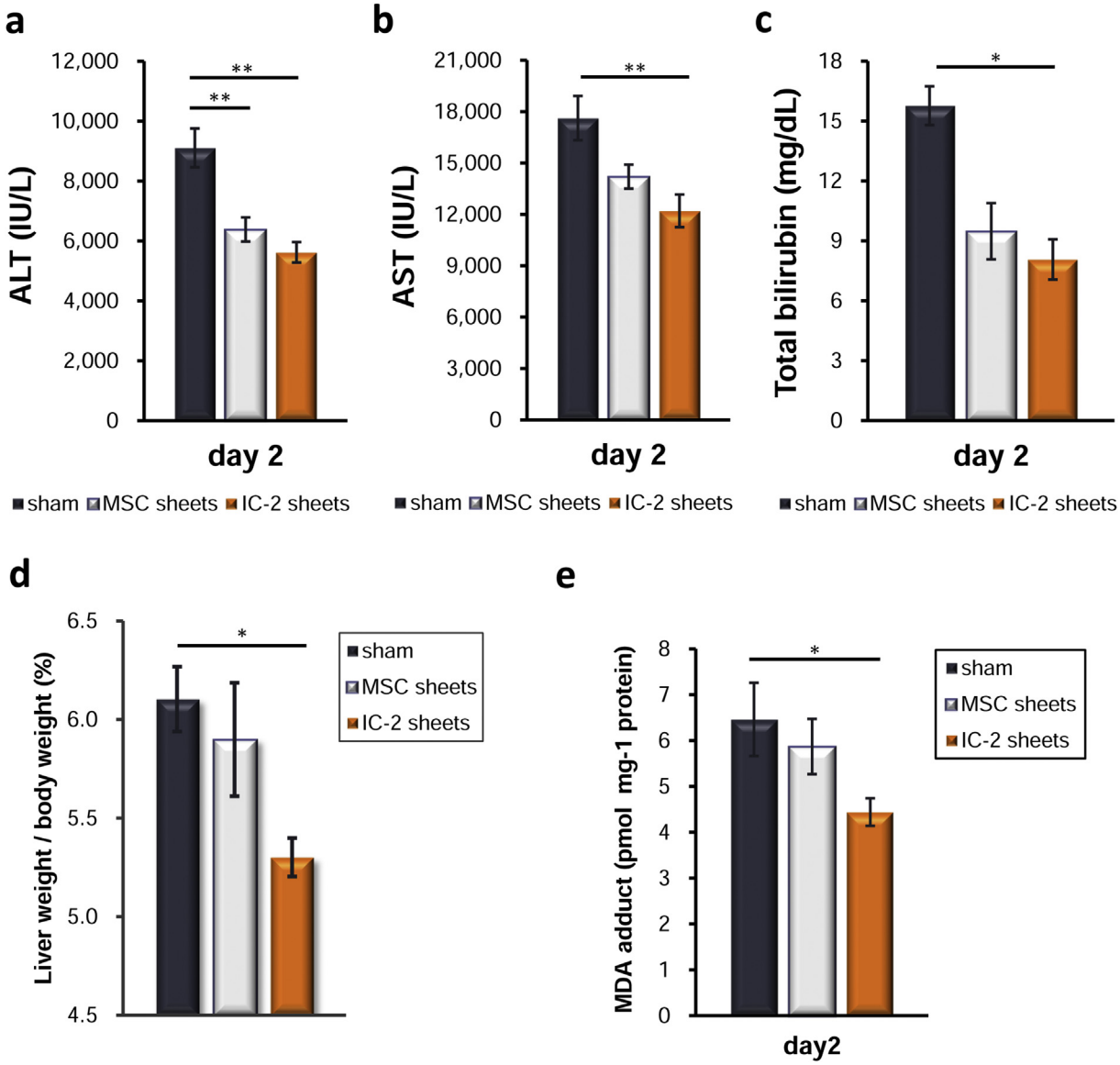


**Fig. 6.** IC-2-treated primary BM-MSC sheets ameliorated acute liver failure. (a, b) Serum ALT (a) and AST (b) of the recipient mice were assessed on day2 after transplantation (n = 7–8 in each group). *$p < 0.05$, **$p < 0.01$, examined by One-Way ANOVA, followed by Tukey test. (c) Total bilirubin of the recipient mice were assessed on day2 after transplantation (n = 4–7 in each group). *$p < 0.05$ examined by One-Way ANOVA, followed by Tukey test. (d) Liver to body weight ratio on day8. Data are expressed as mean ± SE (n = 6–8 in each group).*$p < 0.05$, **$p < 0.01$, examined by One-Way ANOVA. (e) MDA adducts of recipient liver tissues on day2. MDA adducts were measured by ELISA. The columns were expressed as mean ± SE (n = 3). *$p < 0.05$, examined by Steel analysis.

response elements (ChoREs) where ChREBP binds [35]. Since the LXR response elements exist in the promoter regions of ChREBP, ChREBP is transcriptionally regulated by LXRα [36]. Therefore, increased expression of LXRα in accordance with hepatic differentiation by IC-2 treatment may induce amphiregulin expression via ChREBP/ChoRE interaction. Increased expression of LXRα may support this hypothesis; however, further studies are required. Otherwise, amphiregulin may directly protect liver injury through inhibition of apoptosis [37]. Leastwise conclude that hepatic cell sheets-derived amphiregulin might participate in promoting liver regeneration through activating the EGFR pathway or inhibition of apoptosis.

In the present study, enhanced expression of TRX and TRXR was observed in hepatic cell sheets transplanted liver. In our study, the TRX system mainly contributes to the antioxidative action. Expression of TRX and TRXR may be enhanced via the NF-κB pathway. Putative NF-κB binding sites are present on the TRX promoter and TRXR promoter regions [38]. There have been no reports that NF-κB directly regulates TRXR. However, NF-κB is involved in TRX production under oxidative stress, since the production of TRX was completely abrogated in NF-κB signal-suppressing cells, which resulted in exacerbated oxidative stress by TNFα treatment [39]. Since NF-κB-expressing hepatocytes were clearly increased in IC-2-treated hepatic cell sheets-transplanted mice, reduction of oxidative stress may occur as a result of TRX production through NF-κB activation. Hepatic cell sheets derived from BM-MSCs are supposed to produce many other factors than complement C3, amphiregulin and TRX. Interleukin-1 receptor antagonist (IL-1ra) was also enhanced by IC-2 treatment (Supplemental Fig. 3). Since administration of recombinant human IL-1ra to $CCl_4$-induced acute liver failure mice was reported to elicit a decrease of centrilobular necrotic areas and increase of

hepatocyte proliferation [40], IL-1ra might be involved in protection against acute liver injury in this system.

In the present study, hepatic cell sheets manufactured using a novel molecule potently inhibited acute liver failure. The humoral factors mentioned above were crucially important in explaining the significant effects of IC-2-engineered hepatic cell sheets on liver injury. However, grafting as a cell sheet itself is also important in transplantation of hepatic cells because hepatic functions can be retained under cell sheet condition [41]. Furthermore, tissue engineering based on cell sheet technology enabled us to transplant abundant cells compared to intra-portal or intra-venous injection of cell suspensions without the risk of embolization. It is attractive that cell sheet transplantation is capable of attachment to desired organ without suture. The sites of transplanted is also important. In the present study, we conducted orthotopic transplantation. Subcutaneous transplantation of cell sheets may be less invasive than orthotopic transplantation with laparotomy [42].

Recently, several groups have reported the efficacy of cell sheet transplantation in a mouse model of liver failure [42,43]. To our knowledge, our hepatic cell sheets-manufacturing procedure is distinct from others, in the view of functional enhancement with a single compound. Taken together, originally manufactured hepatic cell sheets comprised BM-MSCs, IC-2, and cell sheet technology are of clinical significance in regenerative therapy for liver disease.

## 5. Conclusion

We found that the single small molecule IC-2-induced hepatic cell sheets derived from BM-MSCs prominently reduced $CCl_4$-induced acute liver injury. Our data suggest that the de novo humoral factors produced by IC-2-induced hepatic cell sheets play an important role in promoting liver regeneration and reducing acute liver injury. Since manufacturing procedure of hepatic cell sheets were applied to primary BM-MSCs, protective efficacy-enhanced hepatic cell sheets with just a single compound would provide clinical application for regenerative medicine of liver disease.

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## Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.reth.2018.07.001.

## Disclosure of potential conflicts of interest

GS owns more than 5% stocks of the total shares of KanonCure Inc. YK is representitive director of KanonCure Inc. The other persons have declared that no competing interests exist.

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