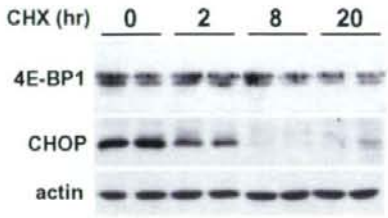

Figure S6. 4E-BP1 Protein Is Stable within Cells

MIN6 cells were incubated with thapsigargin (0.5 μM) for 24 hr, washed with PBS and treated with cycloheximide (CHX, 50 μM) for the indicated period. Cell lysates were subjected to immunoblotting with an anti-4E-BP1 antibody. Immunoblotting results for CHOP are also presented for comparison. The data are representative of three independent experiments.

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STEM CELLS

TISSUE-SPECIFIC STEM CELLS

IFATS Collection: In Vivo Therapeutic Potential of Human Adipose Tissue Mesenchymal Stem Cells After Transplantation into Mice with Liver Injury

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Key Words. Adipose • Mesenchymal stem cells • Liver regeneration • Liver function

ABSTRACT

Mesenchymal stem cells (MSCs), largely present in the adult human body, represent an attractive tool for the establishment of a stem cell-based therapy for liver diseases. Recently, the therapeutic potential and immunomodulatory activity of MSCs have been revealed. Adipose tissue-derived mesenchymal stem cells (AT-MSCs), so-called adipose-derived stem cells or adipose stromal cells, because of their high accessibility with minimal invasiveness, are especially attractive in the context of future clinical applications. The goal of the present study was to evaluate the therapeutic potential of AT-MSCs by their transplantation into nude mice with CCl_4-caused liver injury. We observed that after transplantation, AT-MSCs can improve liver functions, which we verified by changes in the levels of biochemical parameters. Ammonia, uric acid, glutamic-pyruvic transaminase, and glutamic-oxaloacetic transaminase concentrations returned to a nearly normal level after AT-MSC transplantation. These results raised the question of how AT-MSCs can achieve this. To discover the possible mechanisms involved in this therapeutic ability of AT-MSCs, in vitro production of cytokines and growth factors was analyzed and compared with MSCs from bone marrow (BM-MSCs) and normal human dermal fibroblasts (NHDFs). As a result we observed that AT-MSCs secrete interleukin 1 receptor α (IL-1Rα), IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1, nerve growth factor, and hepatocyte growth factor in a volume higher than both BM-MSCs and NHDFs. Thus, our findings suggest that AT-MSCs may account for their broad therapeutic efficacy in animal models of liver diseases and in the clinical settings for liver disease treatment. STEM CELLS *2008;26: 2705–2712*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

The establishment of stem cell therapy for the liver is of great significance. The liver is exposed to many factors such as drugs, xenobiotics, and viruses, which cause chronic hepatitis and liver cirrhosis. In most cases, these illnesses lead to hepatocellular carcinoma and, finally, to organ failure, where there is chronic inflammation, fibrosis, and no longer any ability to regenerate [1]. The only effective treatment to date is orthotropic liver transplantation, but because of the limited number of donors and organ rejection, alternative approaches are needed. One such approach for the treatment of liver failure is stem cells. After transplantation, they can support a host's liver function and thereby can open the way to further treatment and liver regeneration.

The preeminent candidate stem cells for therapy of an injured liver are mesenchymal stem cells (MSCs), which can be obtained from different sources such as bone marrow (BM) [2], umbilical cord blood [3], amniotic fluid [4], scalp tissue [5], placenta [6], and adipose tissue (AT) [7, 8] of the human body. MSCs possess both a multipotentiality and a semi-infinite proliferation ability [2–8]. Currently, attention is being given to AT as a source of MSCs for regenerative medicine [7–9]. From this tissue, a sufficient number of stem cells for stem cell-based therapy may be obtained without invasiveness or damage to a

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patient's health. The possibility for their future application in the therapy of liver diseases is very promising. MSCs can be easily obtained from a patient's own tissues, isolated ex vivo, expanded, and transplanted back into the patient as an autologous transplant. Such a possibility sidesteps the limits governing ethical issues and immunocompatibility problems. Importantly, human MSCs represent an advantageous cell type for allogenic transplantation as well, since they are immunoprivileged with low human leukocyte antigen (HLA) I and no HLA II expression, thereby reducing the risk of allogenic transplant rejection and preventing graft-versus-host disease (GVHD) [10–15]. It has been proposed that MSCs act as "trophic mediators" [16–22], which by secretion of bioactive factors act as either immunosuppressors or promoters of regeneration [17]. It is already anticipated that MSCs in animal models can engraft into the liver and ameliorate liver injury caused by CCl_4 [23–26], allylalcohol [27], or retrorsine [28] injection or by combined radiation and CCl_4 injection [29]. Mechanisms, however, are unknown and opinions remain controversial [24–31]. Among the proposed ideas, very highly convincing is the hypothesis of the paracrine effects of MSCs, by which, through secretion of active factors, amelioration of liver damage occurs [18, 20]. We have already demonstrated that human AT-MSCs have the ability to give rise to hepatocyte-like cells and that CD105 is one of the candidate mesenchymal stem cell markers [32–33].

In this study, we evaluate the therapeutic potential of AT-MSCs with respect to their future clinical usage for the treatment of liver failure. The usage of undifferentiated AT-MSCs, which are minimally manipulated ex vivo and easily obtained within a short period of time, is crucial for future clinical emergency usage. We transplanted human AT-MSCs into immunodeficient mice with acute liver failure caused by CCl_4 injection. Our results show a significant decrease of ammonia, glutamic-oxaloacetic transaminase (GOT), uric acid (UA), and glutamic-pyruvic transaminase (GPT) concentration 24 hours after transplantation of undifferentiated AT-MSCs, and also indicate that undifferentiated AT-MSCs are involved in liver regeneration in vivo. Because the mechanisms are still unclear, however, we postulate that involvement may be, in part, due to the pleiotrophic contribution through the direct and/or indirect activity of AT-MSCs. We evaluated in vitro production of cytokines/growth factors by undifferentiated AT-MSCs and compared it with BM-MSCs and normal human dermal fibroblasts (NHDFs). We detected higher secretion of interleukin 1 receptor antagonist (IL-1RA), IL-6, IL-8, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), monocyte chemotactic protein 1 (MCP-1), nerve growth factor (NGF), and hepatocyte growth factor (HGF) by AT-MSCs, as opposed to BM-MSCs and NHDFs. The vascular endothelial growth factor (VEGF) was secreted similarly by both types of MSCs, higher, however, than by NHDFs. Summarizing, our in vitro study shows a higher production of bioactive factors in AT-MSCs than in BM-MSCs. Our in vivo study shows the ability of AT-MSCs to incorporate into the liver and improve its function. The regenerative ability of AT-MSCs may be possibly due to trophic activity of AT-MSCs.

Materials and Methods

Isolation and Culturing of MSCs

AT-MSCs were derived from abdominal subcutaneous adipose tissue, which was obtained from gastric cancer patients (donor 1: female, 36 years old; donor 2: female, 55 years old; and donor 3: male, 45 years old) undergoing gastrectomy at the International Medical Center of Japan in Tokyo. The hospital's committee of ethics approved this study, and informed consent was obtained from all patients. Adipose tissue was processed as previously described [33], and banked in Dulbecco's modified Eagle's medium (DMEM) (GIBCO-BRL, Tokyo, http://www.gibcobrl.com), supplemented with 10% fetal bovine serum (FBS), 1% antibiotic-antimycotic (GIBCO-BRL), and 10% dimethyl sulfoxide (Sigma-Aldrich, Tokyo, http://www.sigmaaldrich.com). After thawing, cells were cultured in a DMEM/10% FBS, and at 70%–80% confluence, the cells were harvested with 0.05% trypsin-EDTA (GIBCO-BRL).

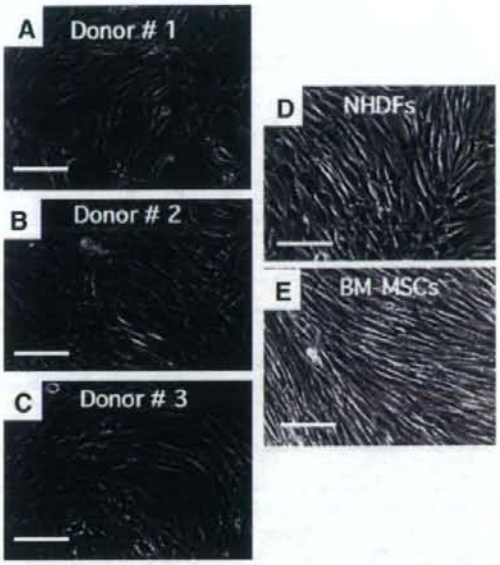

Figure 1. Morphology of adipose tissue-derived mesenchymal stem cells. Donors 1 **(A)**, 2 **(B)**, and 3 **(C)**; NHDFs **(D)**; and BM-MSCs **(E)**. Scale bars = 50 μm. Abbreviations: BM-MSCs, bone marrow mesenchymal stem cells; NHDFs, normal human dermal fibroblasts.

Human BM-MSCs (Cambrex Corp., Walkersville, MD, http://www.cambrex.com) were cultured in a mesenchymal stem cell growth medium. Normal human dermal fibroblasts (Cambrex Corp.) were cultured in DMEM supplemented with 10% FBS.

Flow Cytometry

The phenotype profile of AT-MSCs (passages 5–6) (donors 1 and 3) was evaluated by flow cytometry analysis (fluorescence-activated cell sorting, Epic XL, Software Expo 32; Beckman Coulter, Fullerton, CA, http://www.beckmancoulter.com), using CD29 (BD Bioscience Pharmingen, Tokyo, http://www.bdbiosciences.com), CD31, CD45 (eBioscience, Tokyo, http://www.ebioscience.com), CD34 (DakoCytomation, Carpinteria, CA, http://www.dakocytomation.com), and CD105 (Ancell, Bayport, MN, http://www.ancell.com) antibodies, coupled to either phycoerythrin or fluorescein isothiocyanate.

Cytokine/Growth Factor Protein Level Measurement

Cytokine/growth factor protein concentration was evaluated using Multiplex Suspension Array (Genetic Lab Corp. Ltd,. Sapporo, Japan, http://www.gene-lab.com).

For the analyses cells, AT-MSCs (passage 5–8) (donors 1, 2, 3) (n = 3 of each donor), BM-MSCs (passage five) (n = 3), and NHDFs (passage five) (n = 3) were plated in the same concentration on 6-well plates (3×10^5cells/well). Upon confluence, they were washed with phosphate-buffered saline (PBS)(-) and incubated with serum-free DMEM/F-12 medium. After 3 days, supernatant and cells were collected for analyses of interleukins (1β, 1α, 1RA,

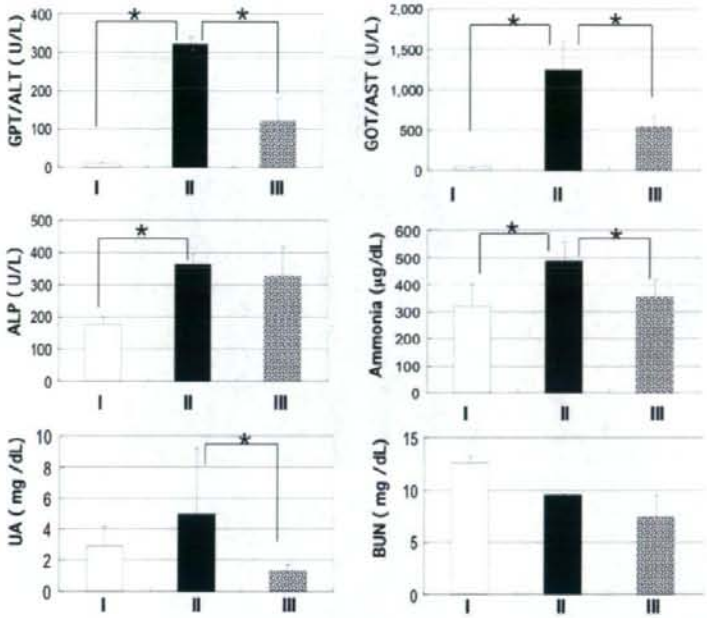

Figure 2. Biochemical analysis. Concentration of GPT/ALT, GOT/AST, ALP, ammonia, UA, and BUN in blood serum of sacrificed mice. Group (I): noninjured mice that did not undergo transplantation; group II: injured mice that underwent transplantation; group III: injured mice that received a transplant of adipose tissue-derived mesenchymal stem cells (combine data of donors 1 and 2). Data are presented as the mean ± SD and were analyzed by the Bonferroni correction; $n = 3$. (*, $p < .05$.). Abbreviations: ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BUN, blood urea nitrogen; GOT, glutamic oxaloacetic transaminase; GPT, glutamic pyruvic transaminase; UA, uric acid.

2, 4, 5, 6), hepatocyte-stimulating factors (HSFs; 7, 8, 10, 12(p40), 12(p70), 13, 15, 17), interferon (IFN) β, tumor necrosis factor (TNF) α, GM-CSF, G-CSF, eotaxin, MCP-1, macrophage inflammatory protein (MIP)-1α, MIP-1β, interferon-gamma-inducible protein (IP)-10, TNF-related activation protein (TRAP)/CD40L/CD154, transforming growth factor (TGF) β, fractaline, NGF, HGF, epidermal growth factor (EGF), and VEGF.

AT-MSC Transplantation into Mice with CCl_4-Induced Liver Injury

We used 6-week-old female nude BALB/c nude mice (SLC, Tokyo, http://www.jslc.co.jp). Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. We applied acute liver failure model, by administration of one dose of CCl_4 (supplemental online Fig. 1). At day 0, mice underwent intraperitoneal injection of 100 μL/20g body weight of olive oil containing 10 μL of CCl_4. At day 1, mice underwent transplantation of AT-MSCs (passage 10) (donor 1 [$n = 3$; group IIIA]; donor 2 [$n = 3$; group IIIB]) at a concentration of 1.5×10^6 cells per mouse (0.2 ml of the cell suspension was injected through the tail vein). As a control, CCl_4-treated mice ($n = 3$) only (group II) and nontreated (olive oil) ($n = 3$) and mice that did not undergo transplantation (group I) were used. The details of experimental groups are listed in supplemental online Figure 1. Twenty-four hours after transplantation, blood serum and liver samples were collected. Blood serum was evaluated for biochemical parameters, such as GPT, GOT, alkaline phosphatase (ALP), UA, ammonia, and blood urea nitrogen (BUN) concentration levels. The liver sections were analyzed by immunohistochemistry.

Immunohistochemical Analyses of Mice Liver Sections After Cell Transplantation

Mice livers were harvested 24 hours after cell transplantation, fixed with 10% formalin, and embedded with paraffin. Histological analyses of liver tissues were conducted by serial tissue section and stained with hematoxylin and eosin (H&E) for conventional morphological evaluation and with anti-HLA-1 antibody (Sigma-Aldrich) for detection of human AT-MSCs in immunodeficient mouse liver.

Assessment of Liver Functions

Blood samples were obtained from each mouse and centrifuged for 20 minutes at 5,000 rpm and serum was collected. Serum samples were tested for ammonia concentration level using Ammonia Test-Wako (Wako Pure Chemicals Co., Ltd., Japan, http://www.wako-chem.co.jp/english). Concentration of markers of liver injury such as GPT, GOT, ALP, UA, and BUN was analyzed using FUJIFILM DRI-CHEM 3500 machine (FujiFilm, Tokyo, http://www.fujifilm.co.jp) and FUJI DRY CHEM SLIDES (FujiFilm), respectively, for GPT/alanine transaminase (ALT)-PIII, GOT/aspartate aminotransferase (AST)-PIII, ALP-PIII, UA-PIII, and BUN-PIII.

Statistical Analysis

Results are given as the mean ± SD. Statistical analysis were conducted or by using the use of variance with the Bonferroni correction for multiple comparisons. A p value <.05 was considered significant.

RESULTS

Preparation and Characterization of AT-MSCs

AT-MSCs were cultivated in DMEM/10% FBS, and were replated when they reached subconfluence. While cultivated in expansion medium containing 10% FBS, the AT-MSCs of all three donors revealed similar morphology (Fig. 1A–C). AT-MSCs of donors 1 and 3 after expansion were trypsinized and analyzed by flow cytometry, which revealed that the cells of donor 1 expressed CD31 (30%), CD34 (5.3%), CD45 (0.06%), CD29 (99%), and CD105 (98.5%) and those of donor 3, CD31 (12.9%), CD34 (9.9%), CD45 (8.4%), CD29 (96%), and CD105 (63.9%). AT-MSCs of donors 1 and 2 were trypsinized and transplanted into nude mice with liver injury. AT-MSCs of all donors (1, 2, 3), as well as BM-MSCs, and NHDFs were plated in the same concentration (Fig. 1A–E). Upon reaching confluence, they were cultured for 3 days in serum-free medium and processed in vitro in a cytokine/growth factor protein array.

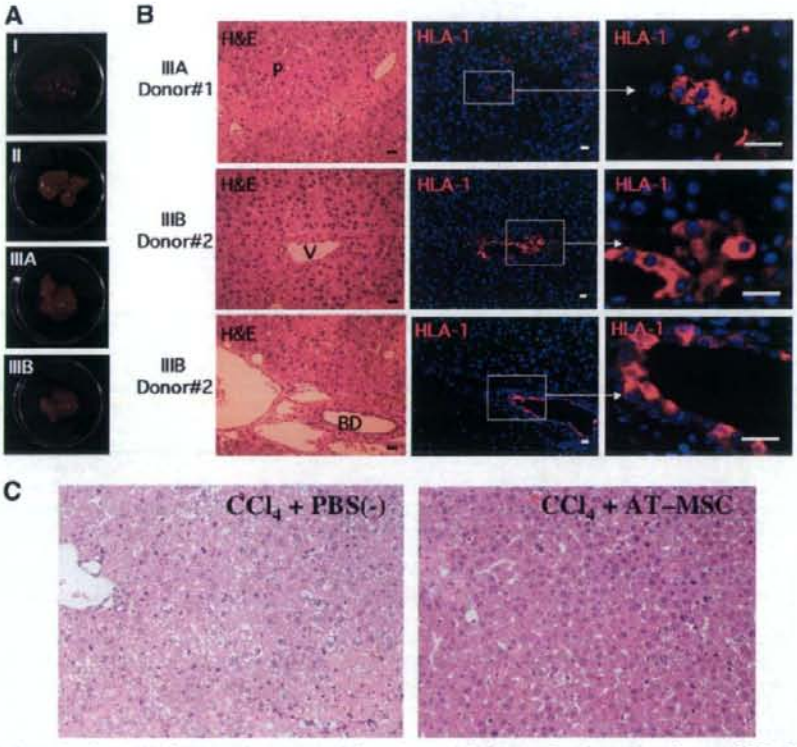

Figure 3. Morphological analysis. **(A):** Symptomatic macromorphology changes in the livers of each experimental group (I–III). **(B):** Representative immunohistological analyses for human leukocyte antigen 1 (HLA-1)-positive cells in liver sections after adipose tissue-derived mesenchymal stem cell (AT-MSC) transplantation (group IIIA and IIIB). HLA-1-positive cells were detected within P, V, and BD. On the left, H&E staining of liver sections of the same selected areas. Scale bars = 500 μm. **(C):** Hematoxylin eosin staining of CCl_4-injured liver sections from control PBS(-) (left panel) and AT-MSC transplant (right panel). Abbreviations: BD, bile duct; P, parenchyma; PBS, phosphate-buffered saline; V, vessel.

Transplantation of AT-MSCs into Mice with Liver Injury

To address our goal, whether undifferentiated AT-MSCs reveal therapeutic abilities to regenerate an injured liver, we transplanted AT-MSCs into mice with acute liver failure. CCl_4 injury generated oxidative stress and hepatocyte necrosis. Twenty-four hours after CCl_4 injection, mice revealed serious liver injury. Biochemical parameters such as GPT, GOT, ALP, UA, and ammonia were increased in mice with CCl_4 injury (group II) compared with noninjured mice (group I) (Fig. 2). We transplanted 1.5×10^6 cells of AT-MSCs into CCl_4-injured mice. After AT-MSC transplantation, GOT and GPT were significantly decreased to a value more than 50% lower than in group II (Fig. 2). Likewise, ammonia concentration was significantly decreased after AT-MSC transplantation (group III). ALP was also decreased (but not significantly). UA, a marker of oxidative stress, was significantly decreased up to a normal level. Markers of liver injury GOT and GPT were decreased after AT-MSC transplantation (group III), yet the liver sections were not morphologically distinguishable (H&E) from sections of the injured group II (data not shown). We also checked the concentration of BUN and detected no improvement in concentration after AT-MSC transplantation (Fig. 2). Supplemental online Figure 2 demonstrates, separately, the donor 1- and donor 2-derived AT-MSC in vivo effect on the above parameters. We could notice variations between donor 1 and donor 2 regarding their effect on ALP and ammonia concentrations. Twenty-four hours after transplantation of AT-MSCs, livers were collected and sections were examined by H&E and against HLA-1 staining. In Figure 3A, we can notice macromorphological changes in the livers of each experimental group, as claret (healthy) or beige (injured) colors. Photographs of H&E staining of the liver sections of injured mice that received a transplant of AT-MSCs (group IIIA, IIIB) show visible liver injury (Fig. 3B). Immunostaining revealed that some human AT-MSCs were detected within the host livers (Fig. 3B). Low-magnification photographs reveal HLA-1-positive cells in different areas of host livers, such as parenchyma, vessel, and bile duct. The above presented data indicate that transplanted AT-MSCs migrate into the injured liver, as well as improve markers of liver injury. Furthermore, H&E staining revealed significant morphological changes in the hepatocytes of the non-necrotic area. As shown Figure 3C, the injured livers of mice that received a transplant of AT-MSCs revealed less vacuolar degeneration caused by dilatation of mitochondria and rough endoplasmic reticulum. These observations reflect the data of the decrease of ALT and AST levels in injured mice that underwent transplantation.

In addition, to evaluate how long, and whether, the transplantation effect of AT-MSCs is sustained in a longer experimental period, kinetic analysis was performed. As shown in supplemental online Figure 4, ALT and ammonia levels were monitored for 120 hours after the cell transplantation. As a result, ALT and ammonia levels in animals that underwent AT-MSC transplantation were significantly decreased compared with levels in PBS(-) control

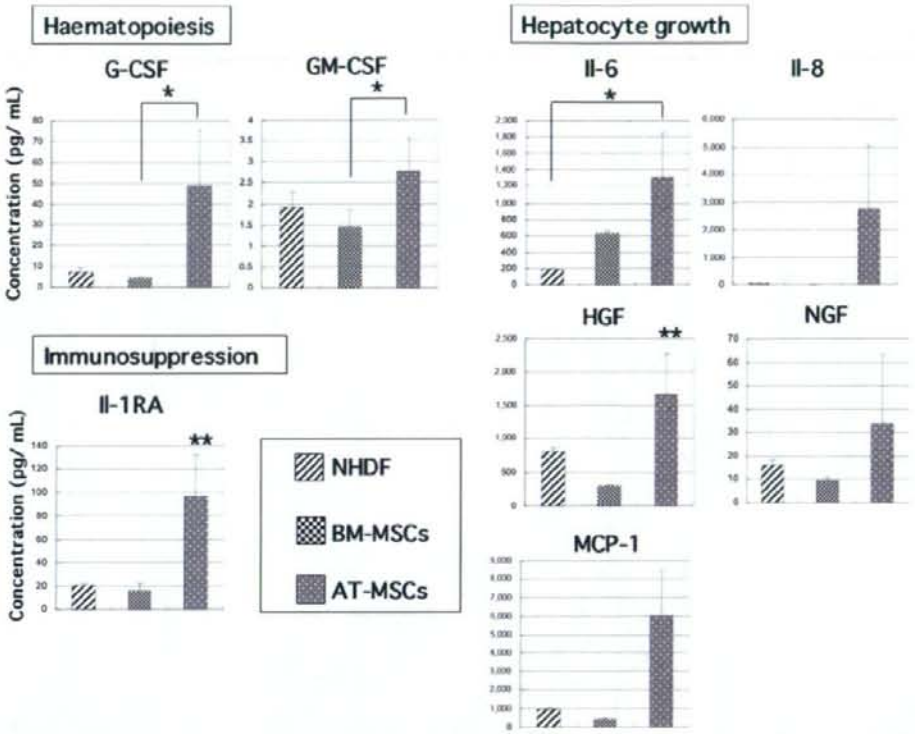

Figure 4. Proteome profile of cytokine/growth factors interleukin 1 (IL-1) receptor antagonist (RA), IL-6, IL-8, MCP-1, G-CSF, GM-CSF, HGF, and NGF concentration (pg/mL) in conditioned medium of normal human dermal fibroblasts (NHDFs), bone marrow mesenchymal stem cells (BM-MSCs), and AT-MSCs (combined data of 3 donors: 1, 2, and 3). Cells were incubated ($n = 3$) in serum-free medium for 3 days, and supernatant was collected and analyzed for the presence of selected cytokines and growth factors. Data are presented as the mean ± SD and were analyzed by the Bonferroni correction; $n = 3$. (*, $p < .05$; **, $p < .01$, vs. both NHDFs and BM-MSCs). Abbreviations: AT-MSCs, adipose tissue-derived mesenchymal stem cells; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; MCP-1, monocyte chemotactic protein 1; NGF, nerve growth factor.

animals at 24 hours, and then levels returned to normal by 48 hours. There is no change at 72, 96, and 120 hours after the transplantation. These results suggest that the recovery effect of AT-MSC transplantation is not transient.

Proteome Profile of Cytokines/Growth Factors Secreted by AT-MSCs

To determine the cytokine and growth factor profile of AT-MSCs, conditioned media of AT-MSCs (donors 1, 2, and 3) were evaluated for cytokine/growth factor protein concentration (Fig. 4, supplemental online Fig. 3, Table 1). As a control, BM-MSCs and NHDFs were analyzed. Differences in secretion between AT-MSCs and BM-MSCs were observed in the concentrations of IL-1RA, IL-6, a so-called hepatocyte stimulating factor, IL-8, G-CSF, GM-CSF, MCP-1, NGF, and HGF. These factors responsible for immunosuppression, hepatocyte growth, and hematopoiesis were secreted in a higher concentration by AT-MSCs versus BM-MSCs and NHDFs. These results suggest strong differences between AT-MSCs and BM-MSCs with respect to their stem cell properties in vitro and in vivo. High secretion of proangiogenic VEGF by AT-MSCs and BM-MSCs was similar, in contrast to NHDFs, which do not secrete VEGF. In addition, other factors such as EGF, IL-10, IP-10, MIP-1α, MIP-1β, IL-7, IL-15, eotaxin, fractaline, IL-12p40, IL-12p70, and IL-17 were also secreted, although not strongly (supplemental online Fig. 3). Importantly, the fact that undifferentiated AT-MSCs revealed regeneration ability after transplantation into mice with liver injury may be explained by their ability to secret a spectrum of bioactive factors. In addition, we observed quite a difference between analyzed AT-MSCs from different donors (supplemental online Fig. 3). For example, NGF was highly produced by donor 1, but anti-inflammatory IL-10 was secreted only by donor 2. The concentration of secreted IL-1RA, IL-6, IL-8, G-CSF, GM-CSF, HGF, NGF, VEGF, IP-10, IL-15, IL-7, and eotaxin by the three donors was significantly different (supplemental online Fig. 3). We did not detect secretion of IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-13, IFNγ, TNFα, and TRAP/CD40L/CD154 by any of the analyzed cells (Table 1). In conclusion, our results revealed a subset of cytokines/growth factors specifically excreted by AT-MSCs. Expression and secretion of those trophic proteins may likely contribute to the AT-MSC-induced therapeutic effects on animal liver injury models.

Conclusion

We have shown here that undifferentiated AT-MSCs have the ability to improve liver functions. We observed that in mice with

Table 1. Cytokine and growth factor concentration (pg/ml) in conditioned medium of NHDF BM-MSCs and AT-MSCs of three donors

					AT-MSCs					
	NHDFs (n = 3)		BM-MSCs (n = 3)		Donor 1 (n = 3)		Donor 2 (n = 3)		Donor 3 (n = 3)	
Factor	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
IL-1RA	20.14	2.46	15.61	6.57	59.37	8.00	105.28	22.93	127.74	16.01
IL-6	179.67	17.72	624.22	42.15	909.76	196.98	1110.53	316.67	1931.50	180.90
IL-7	16.67	1.19	12.61	1.66	20.27	1.17	18.19	2.11	13.29	1.64
IL-8	33.53	7.54	7.91	0.35	5222.06	2910.35	2414.26	839.00	676.27	60.64
IL-15	6.98	1.09	1.46	0.29	4.11	0.83	3.48	0.73	2.34	0.74
G-CSF	7.24	1.64	3.76	0.99	67.23	14.76	61.22	25.21	18.50	1.85
GM-CSF	1.91	0.37	1.45	0.42	3.38	0.54	3.06	0.70	1.93	0.22
Fractaline	21.85	3.34	20.42	2.60	18.64	3.65	17.54	1.28	17.60	4.18
Eotaxin	34.67	1.83	6.04	8.38	47.64	8.58	19.93	1.14	92.55	10.73
MCP-1	977.07	108.99	380.88	39.30	3414.30	3357.48	6373.97	11439.28	8289.72	10966.12
VEGF	N.D.	0	837.96	39.94	741.98	124.40	685.79	130.13	415.92	39.31
HGF	821.50	43.43	291.21	15.45	2198.45	187.50	1795.05	133.51	1026.17	51.49
NGF	16.12	2.12	9.49	1.25	68.09	7.46	14.03	1.97	19.98	1.69
IP-10	7.76	1.22	N.D.	0	1.24	1.46	5.37	0.85	6.81	2.67
IL-12p40	2.04	3.42	6.65	5.27	4.24	5.68	2.32	2.70	6.40	8.20
MIP-1α	1.15	1.24	3.76	0.28	5.04	0.85	2.61	1.53	2.69	1.62
MIP-1β	2.09	3.55	N.D.	0	2.09	3.55	6.83	4.80	5.12	4.91
EGF	N.D.	0	1.87	1.41	1.84	1.34	2.10	1.16	N.D.	0
IL-10	N.D.	0	N.D.	0	N.D.	0	43.95	1247.08	N.D.	0
IL-12p70	3.19	0.09	3.07	0.05	3.11	0.07	3.14	0.04	3.05	0.07
IL-17	0.73	0.22	N.D.	0	0.86	0.24	1.03	0.20	N.D.	0
IL-1α	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-1β	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-2	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-4	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-5	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IL-13	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
IFNγ	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
TGFα	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0
TRAP/CD154	N.D.	0	N.D.	0	N.D.	0	N.D.	0	N.D.	0

Abbreviations: AT-MSCs, adipose tissue-derived mesenchymal stem cells; BM-MSCs, bone marrow mesenchymal stem cells; EGF, epidermal growth factor; G-CSF, granulocyte colony-stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor; HGF, hepatocyte growth factor; IFNγ, interferon γ; IL-1RA, interleukin 1 receptor antagonist; IP-10, interferon-gamma-inducible protein 10; MCP-1, monocyte chemotactic protein 1; MIP-1α, macrophage inflammatory protein 1α; N.D., not detected; NGF, nerve growth factor; NHDFs, normal human dermal fibroblasts; TGFα, transforming growth factor α; TRAP, tumor necrosis factor-related activation protein; VEGF, vascular endothelial growth factor.

liver injury, AT-MSC transplantation promotes liver recovery. Parameters such as GOT, GPT, ALP, UA, and ammonia were decreased in injured mice after AT-MSC transplantation, suggesting that AT-MSCs have the ability to regenerate the liver. We did not, however, observe morphological improvement of the liver after AT-MSC transplantation. Although a long time course experiment with histological analyses as well as safety issues should be carefully evaluated, effect of transplantation of AT-MSCs was not transient. A lack of knowledge regarding post-transplantation modifications and risk of carcinogenesis still exists. At present, we know that AT-MSCs may undergo spontaneous transformation in vitro; however, it was observed in cells at senescent phase after a prolonged period of culturing [34]. Nevertheless, MSCs from different sources were shown to have great therapeutic potentiality [35–37], especially in stroke, myocardial infarct, and meniscus repair. However, questions such as what kind of MSCs represent the best therapeutic ability and what MSCs actually do to effect regeneration remain unclear.

By HLA-1 immunostaining, we have detected human AT-MSCs within the liver of immunodeficient mice 24 hours after transplantation. We found few positive cells in different areas of the liver such as parenchyma, vessel, and bile duct. We cannot, however, exclude the cell fusion event. Monitoring AT-MSC migration, mobilization, and engraftment for a longer time is essential and very informative. It is interesting to evaluate the effect of AT-MSCs on endogenous progenitor activation [38]. It has been proposed that there is a different response of oval cells in vivo between mice and rats as well as the injury model [39]. The possible actions of AT-MSCs in vivo are that they act directly, by causing intracellular signaling and/or indirectly by secreting or making other cells in the vicinity secrete functionally active agents. Recently, MSCs were thought to be "multidrug delivery vehicles that are injury-site sensitive and/or responsive" [17]. Liver disease is a complex, heterogeneous condition accompanied by inflammation and fibrosis, and, at present, there is no proper treatment strategy. Kupffer cells and liver macrophages are major sources of IL-1β and TNFα production. These cytokines are thought to enhance the expression of adhesion molecules in endothelial cells, which mediate the neutrophil adhesion to endothelial cells causing cell dysfunction and destruction, leading to hepatocyte necrosis [40]. It has already been demonstrated that MSCs secrete a broad spectrum of active cytokines, chemokines, and growth factors [16–22]. We observed that AT-MSCs produce significantly more bioactive factors than do BM-MSCs, and, therefore, they may have an equal or even stronger regenerative effect than BM-MSCs. This issue is very interesting and needs precise evaluation, using MSCs isolated from adipose tissue and bone marrow from the same donor. We demonstrated that in vitro AT-MSCs secrete many active factors, among them, (a) immunosuppressive IL-1RA, (b) hepatocyte-growth promoting IL-6, IL-8, HGF, NGF, and MCP-1, and (c) hematopoiesis-promoting G-CSF and GM-

CSF are secreted in a larger volume than both BM-MSCs and NHDFs (Fig. 4). IL-1RA is a strong anti-inflammatory cytokine, which binds to IL-1 receptors and inhibits the binding of IL-1α and IL-1β. Therefore, the immune activity of IL-1α and IL-1β is neutralized. Inflammatory cytokines such as IL-1 and TNFα are not produced by AT-MSCs or by BM-MSCs or NHDFs (Table 1). On the other hand, IL-6, identical to HSF, elicits an acute phase response in liver cells; IL-8 and MCP-1 are inflammatory-related factors, however, it is difficult to predict their actual in vivo activity. In fact, IL-6 together with TNFα is responsible for a hepatocyte entering to the state of replication competence [41]. It has been demonstrated that IL-6, IL-8, and MCP-1 are produced by human intrahepatic biliary epithelial cells through toll-like receptor 4, nuclear factor-kappa B, and mitogen activated protein kinase signaling pathways, and therefore are possibly mediating an innate immune system function and modulating hepatic regeneration in vivo [42, 43]. Then in the proliferation phase, HGF and TGFα stimulate proliferation of hepatocytes. HGF, considered as proapoptotic for liver stellate cells [18] as well as for myofibroblasts [44], acts as an anticirrhotic agent. Stellate cells are a major source of collagens and other extracellular matrix proteins in liver fibrosis. In addition, they regulate matrix metalloproteinases. Other highly secreted factors by AT-MSCs were C-CSF, GM-CSF, and IL-6, considered as a supporting process of hematopoiesis. The pleiotrophic G-CSF is a mobilizing factor, which promoted migration of BM-MSCs into the heart after myocardial infarction [45]. Again, in the light of much evidence that tissues contain their own multipotential stem cells [46–47], it should be emphasized that endogenous stem cells (oval cells, liver epithelial progenitors, etc.) can be the target of MSC-derived direct or indirect actions. Likewise, it is possible that bioactive factors act on oval cells and therefore provoke hepatocyte generation in vivo. Recently, G-CSF has been shown to promote rat liver repair and induce oval cell migration and proliferation [48, 49]; however, there are controversial opinions regarding the G-CSF effect [50]. Recent evidence suggests that neurotrophins, such as NGF, may have a role in hepatic regeneration [51–55], and their mRNA and protein levels become elevated in association with hepatocyte proliferation induced by, for example, administration of CCl_4 [52]. NGF, similarly to HGF, induces the apoptosis of stellate cells [52–54]. NGF is secreted by cholangiocytes and also induces their proliferation [55]. In addition, it is reported that MSCs can inhibit the proliferative and fibrogenic function of activated stellate cells in a paracrine manner [18]. This inhibition was caused by MSC-derived IL-10 and TNFα, which acted synergistically. The secretion of IL-10 by MSCs was found to be a response to IL-6 secretion by activated stellate cells. These events were not detected while stellate cells were cultured with fibroblasts [18]. The production of trophic agents by AT-MSCs exhibits donor to donor variations (supplemental online Fig. 3). In fact, we observed that the secretion of IL-10 was detected in AT-MSCs of one donor only. Recently, Akt-modified MSCs were proposed as candidate cells for paracrine-mediated actions [56]. Akt-modified MSCs are capable of up-regulating the expression of several candidate mediators such as VEGF, FGF2, HGF, and insulin-like growth factor 1 [56]. Secreted frizzled related protein 2 is the key Akt-modified MSC-released paracrine factor [56]. Of note, our data show only in vitro production of bioactive factors. In vivo MSCs may act through multiple yet unknown mechanisms to coordinate a dynamic integrated response to fibrosis. It is also likely that similar immunomodulatory mechanisms may influence the phenotype of resident hepatocytes, stellate cells, Kupffer cells, sinusoidal endothelial cells, and immune cells that infiltrate the liver during inflammation. An interesting topic for further evaluation is the effects of AT-MSCs on GVHD. It has been proposed that HGF may suppress GVHD after allogenic BM-MSC transplantation into patients with hepatocellular carcinoma [57]. In our studies we showed that AT-MSCs produce significantly more HGF than BM-MSCs and NHDFs. We propose a careful consideration of the stem cell type/source for the therapy of certain diseases, because of the composition and concentration of secreted bioactive factors by MSCs from different sources, which may promote or inhibit the state of injury.

In conclusion, our study revealed that transplantation of human AT-MSCs has functional benefits, in part because of the cells' ability to produce a large number and volume of bioactive factors. This trophic activity may result in future therapeutic usage of AT-MSCs for liver disease. We propose that trophic activity may be one of the possible actions in vivo of AT-MSCs or MSCs in general; however, this issue needs to be precisely confirmed and analyzed with a long-term course of experiments. Other mechanisms have not been excluded. In addition, further characterization, whether AT-MSCs can produce factors that inhibit fibrosis and apoptosis, promote angiogenesis, and stimulate host progenitors to divide and differentiate into functional regenerative units, is required to establish a novel therapeutic approach for liver disease treatment.

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DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicate no potential conflicts of interest.

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IFATS Collection: In Vivo Therapeutic Potential of Human Adipose Tissue Mesenchymal Stem Cells After Transplantation into Mice with Liver Injury

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HEPATOLOGY

Rapid hepatic fate specification of adipose-derived stem cells and their therapeutic potential for liver failure

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adipose, differentiation, hepatocyte, liver regeneration, mesenchymal stem cell.

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Abstract

Background and Aim: Multipotential mesenchymal stem cells (MSC), present in many organs and tissues, represent an attractive tool for the establishment of a successful stem cell-based therapy in the field of regeneration medicine. Adipose tissue mesenchymal stem cells (AT-MSC), known as adipose-derived stem cells (ASC) are especially attractive in the context of future clinical applications because of their high accessibility and minimal invasiveness during the procedure to obtain them. The goal of the present study was to induce human ASC into functional hepatocytes *in vitro* within a very short period of time and to check their therapeutic potential *in vivo*.

Methods: *In vitro* generated ASC-derived hepatocytes were checked for hepatocyte-specific markers and functions. Afterwards, they were transplanted into nude mice with liver injury. Twenty-four hours after transplantation, biochemical parameters were evaluated in blood serum.

Results: We have shown here that ASC can be differentiated into hepatocytes within 13 days and can reach the functional properties of primary human hepatocytes. After transplantation into mice with acute liver failure, ASC-derived hepatocytes can restore such liver functions as ammonia and purine metabolism. Markers of liver injury, alanine aminotransferase, aspartate aminotransferase, as well as ammonia, were decreased after ASC-derived hepatocyte transplantation.

Conclusions: Our data highlight the properties of ASC as having a special affinity for hepatocyte differentiation *in vitro* and liver regeneration *in vivo*. Thus, ASC may be a superior choice for the establishment of a therapy for injured liver.

Introduction

The liver is exposed to many factors such as drugs, xenobiotics and viruses, which cause chronic hepatitis and liver cirrhosis. In most cases these lead to hepatocellular carcinoma and finally to organ failure, where there is chronic inflammation, fibrosis and no longer any regeneration ability.[1]

At present, liver transplantation is the only effective treatment for severe liver injuries. However, because of organ rejection and lack of donors, alternative strategies are urgently needed.

Human primary hepatocytes are commercially available; however, maintaining them in *in vitro* culture is very difficult, if not nearly impossible. After a few days of *in vitro* culturing they lose their functions. Additionally, their usage does not solve the problem of rejection. These factors limit their experimental applications and exclude their clinical usage.

In the last few years, extrahepatic cell populations with the potential to impact liver diseases have been discovered. The potential candidate stem cells for therapy of an injured liver are mesenchymal stem cells (MSC), which can be obtained from different sources such as bone marrow (BM),[2] umbilical cord blood (UCB),[3] amniotic fluid (AF),[4] scalp tissue,[5] placenta,[6] or adipose tissue (AT)[7,8] of the human body. These cells reveal a multipotentiality and semi-infinite proliferation ability. The hepatogenic differentiation capacity of MSC has been confirmed in many independent studies on BM-MSC,[9–14] UCB-MSC,[15–16] and adipose-derived stem cells (ASC).[17–19] The possibility for their future application in the therapy of liver diseases is very promising. MSC can easily be obtained from a patient's own tissues, isolated *ex vivo*, expanded, differentiated toward hepatocytes, and transplanted back into the patient in the form of either undifferentiated MSC or MSC-derived hepatocytes. Such a possibility sidesteps the limits regarding ethical issues and immunocompatibility problems. Importantly, MSC represent an advantageous cell type for allogenic transplantation as well, because they are immuno-privileged with low major histocompatibility complex (MHC) I (histocompatibility

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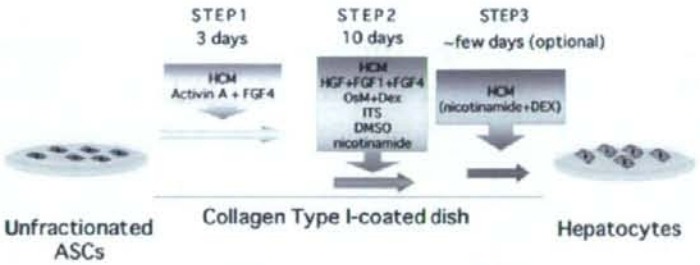

Figure 1 Improved and modified hepatogenic induction strategy. At present, approximately 2 weeks are required to induce hepatogenic characteristics in adipose-derived stem cells (ASC). Unfractionated ASC were plated on collagen type I-coated dishes and were treated with Activin A and FGF4 at step 1, followed by step 2, treatment with hepatocyte growth factor (HGF), fibroblast growth factor (FGF)1, FGF4, oncostatin M (OsM), dexamethasone, insulin-transferrin-selenium (ITS), dimethyl sulfoxide (DMSO), and nicotinamide. At this point, cells may be maintained a few days in hepatocyte culture medium (HCM) alone (or optionally supplemented with 10^{-5} mol/L dexamethasone and 0.05 mmol/L nicotinamide). MSC, mesenchymal stem cells.

lymphocyte antigen [HLA I]) and no MHC II (HLA II) expression, therefore reducing the risk of allogenic transplant rejection.[20–27]

Currently, attention is being given to adipose tissue (AT) as a source of MSC for regenerative medicine. From adipose tissue, a sufficient number of stem cells for a stem cell-based therapy may be obtained without invasiveness or damage to a patient's health. We have already demonstrated that human ASC have the ability to give rise to hepatocyte-like cells and that CD105 is a candidate mesenchymal stem cell marker.[19] However, this *in vitro* differentiation method is not applicable to a practical, clinical use, as more than 1 month is required to induce ASC into cells with hepatic functions.

In the present study, we evaluate the therapeutic potential of ASC-derived hepatocyte-like cells after transplantation into mice with liver injury. Clinical applications in the future would require a special approach, such as shortening as much as possible *ex vivo* manipulations, including cultivation and direct hepatic fate. Therefore, we improved and modified our hepatocyte differentiation strategy, based on the current knowledge on *in vivo* mouse fetal liver development. At present, a period as short as 13 days is required and that strategy is enriched by pretreatment with Activin A (PeproTech, EC, London, UK) and fibroblast growth factor (FGF)4 (PeproTech) (one of the factors secreted by septum transversum mesenchyme (STM) and cardiogenic mesoderm at the early stage of endoderm development *in vivo*). Additionally, we reorganized the content of the growth factor cocktail and enriched it with the addition of dimethyl sulfoxide (DMSO), nicotinamide and insulin-transferrin-selenium. Using the present protocol, we obtained functional hepatocyte-like cells in a much shorter period of time. Finally, we transplanted ASC-derived hepatocyte-like cells into immunodeficient mice with liver injury/non-severe acute liver injury. Our results showed a significant decrease of ammonia, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and uric acid (UA) in the blood plasma of mice after ASC-derived hepatocyte-like cell transplantation. The results show a very important step towards future establishment of an alternative and successful therapy for liver disease.

Methods

Isolation and culturing of ASC

Adipose-derived stem cells were derived from abdominal subcutaneous adipose tissue, which was obtained from two female gastric cancer patients (Donor #1 [36 years old] and Donor #2 [45 years old]), undergoing gastrectomy at the International Medical Center of Japan, Tokyo. The hospital's committee of ethics approved this study, and informed consent was obtained from both patients. Adipose tissue was processed as previously described.[19] For *in vitro* differentiation, the cells (ASC062801, ASC012202, ASC0025) obtained from DS Pharma Biomedical Co., Osaka, Japan) were also analyzed.

Hepatic differentiation

At passage five to 10, the cells were plated on collagen type I-coated dishes at a concentration of $3.0–4.0 \times 10^4$ cells/cm^2 (Fig. 1). When the cells reached confluency, hepatogenic induction was carried out over a period of 2 weeks. First, the cells were treated for 3 days with DMEM (GibcoBRL, Tokyo, Japan) (serum free) supplemented with 20 ng/mL Activin A and 20 ng/mL FGF4 (PeproTech EC, London, UK). Afterwards, the cells were cultured for 10 days in a hepatocyte culture medium (HCM), containing 5 μg/mL transferrin, 10^{-6} mol/L hydrocortisone-21-hemisuccinate, 0.5 mg/mL bovine serum albumin, 2 mmol/L ascorbic acid, 20 ng/mL epidermal growth factor, 5 μg/mL insulin, 50 μg/mL gentamicin (Cambrex Corp., Walkersville, MD, USA) and supplemented with 150 ng/mL hepatocyte growth factor (HGF), 100 ng/mL FGF1, 25 ng/mL FGF4, 30 ng/mL oncostatin M (OsM; PeproTech), (2×10^{-5} mol/L) dexamethasone (Dex; Sigma, Tokyo, Japan), 1 × insulin-transferrin-selenium (ITS; Gibco), 0.05 mmol/L nicotinamide (Sigma), and 0.1% DMSO (Sigma). For the next few days, the cells were maintained with HCM alone. For *in vivo* transplantation, hepatocyte-like cells from two donors (#1 and #2) were harvested by treatment with a 0.05% collagenase/1000 U/mL dispase solution for 3–5 min, dissolved in

PBS (–) and injected intravenously into mice with liver injury caused by CCl_4 injection.

Quantitative real-time PCR

In order to confirm the regulation of the hepatocyte-specific genes in ASC-derived hepatocytes, we performed real-time polymerase chain reaction (PCR) for albumin (ALB) and tryptophan 2,3-dioxygenase (TDO2), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference gene. After retro-transcription, cDNA was subjected to real-time PCR by using Platinum Quantitative PCR Super Mix-UDG (Invitrogen, Tokyo, Japan) and specific primers for ALB (NM_000477): F:GTCACCAAATG CTGCACAGA, R:ACGAGCTCAACAAGTGCAGT for TDO2 (NM_005651): F:GTGTGCATGGTGCACAGAAT, R:GGGTT CATCTTCGGTATCCA, for FOXA2 (NM_021784): F:GGGA GCGGTGAAGATGGAAG, R:TGCCAGCGCCCACGTA and for GAPDH (NG_007073): F:GAAGGTGAAGGTCGGAGT, and R:GAAGATGGTGATGGGATTTC, based on the human genome database. The PCR conditions were as follows: denaturation at 95°C for 30 s, annealing at 56°C or 60°C for 30 s, and extension at 72°C for 30 s for up to 45 cycles. Real-time PCR was carried out by using the Applied Biosystems (Tokyo, Japan) PRISM 7700 Sequence Detection System.

Immunofluorescence

Cells were fixed in 4% formaldehyde for 10 min, followed by incubation with Protein Block (DakoCytomation, Carpinteria, CA, USA) for 30 min. ASC-derived hepatocytes were analyzed by immunohistochemistry using monoclonal anti-human specific albumin ALB (clone HAS-11, 1:250; Sigma) antibody overnight at 4°C. The Alexa Fluor 488 (green, 1:1000)-conjugated secondary antibody (Invitrogen, Tokyo, Japan) was applied for 30 min. Nuclei staining was performed using 4,6-diamidino-2-phenylindole (DAPI; Vector Laboratories, Burlingame, CA, USA).

Albumin production

Albumin production was evaluated by enzyme linked immunosorbent assay (ELISA, E80-129; Bethyl Laboratories, Montgomery, TX, USA). The antibody is human specific and does not cross-react with mouse, rat, bovine, goat, and pig albumin. Briefly, the supernatant during hepatogenic induction was collected every 3 days at days 3, 6 and 9, and ELISA assay was performed. Data are reported as the mean ± SD and were analyzed by Student's *t*-test, $n = 3$ ($^*P < 0.05$).

ASC-derived hepatocyte transplantation into mice with CCl_4-induced injury

Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Six-week-old female BALB/c nude mice (CLEA Japan Inc., Tokyo, Japan) were used. An acute liver failure model was produced by giving one dose of CCl_4. At day 0, mice underwent i.p. injection of 100 µL/20 g bodyweight of olive oil containing 10 µL CCl_4. At day 1, mice underwent transplantation of ASC-derived hepatocyte-like cells (Donor #1 ($n = 4$), or Donor #2 ($n = 4$) at a concentration of 1.5×10^6 cells per mouse (0.2 mL cell suspension was injected through the tail vein). As a control, non-transplanted CCl_4-treated mice ($n = 3$) and non-transplanted CCl_4-non-treated (olive oil) ($n = 3$) mice were used. Twenty-four hours after transplantation, blood serum was collected and evaluated for biochemical parameters, such as AST, ALT, UA and ammonia concentration levels.

Assessment of liver functions

Blood samples were obtained from each mouse, centrifuged for 20 min at 400 *g* and serum was collected. Serum samples were tested for ammonia concentration level by using the Ammonia Test-Wako (Wako Pure Chemicals, Tokyo, Japan). The concentration of markers of liver injury such as ALT, AST and UA was analyzed by using a FUJIFILM DRI-CHEM 3500 machine and FUJI DRI-CHEM Slides for ALT/ALT-PIII, AST/AST-PIII, and UA-PIII, respectively (Fujifilm Co., Tokyo, Japan).

Statistical analysis

The results are given as the mean ± SD. Statistical analyses were conducted using either the variance with the Bonferroni correction for multiple comparisons or the Student's *t*-test. The statistical analysis of quantitative relative expression was evaluated by using the Pair Wise Fixed Reallocation Randomization Test©, Relative Expression Software Tool-XL = REST-XL© (http://www.gene-quantification.info/). A P value < 0.05 was considered significant.

Results

Hepatic fate specification of ASC

A direct fate hepatic specification (Fig. 1) was performed within 13 days. After that, ASC-derived hepatocyte-like cells could be maintained for a few days in HCM alone (optionally supplemented with Dex 10^{-8} mol/L and nicotinamide 0.05 mmol/L). After 3 days of pretreatment with FGF4 and Activin A, ASC expressed FOXA2 (Fig. 2a), the expression of which was decreased at day 6 of the induction system (3 days of pretreatment with FGF4 and Activin A, followed by 3 days of treatment with a cocktail containing HGF, FGF1, FGF4, OsM, Dex, ITS, DMSO and nicotinamide) (Fig. 2a). FOXA2, so-called hepatocyte nuclear factor 3β (HNF-3β) is an essential transcription factor for endoderm specification as well as hepatogenic fate. Similarly, ALB (hepatocyte-specific protein) and TDO2 (hepatocyte-specific enzyme, expressed by mature hepatocytes) were also detected by quantitative PCR at day 3 and their expression increased at day 6 of the induction system (Fig. 2a). The representative morphology of the ASC-derived hepatocyte-like cells of either a cancer patient's ASC or from the commercialized cells at the 13th day of induction is shown in Figure 2b. Importantly, 24 h of incubation with our new cocktail (Step II) alone is enough to dramatically influence the morphology of ASC (Donor #2) from fibroblast to epithelial (Fig. 3a). The pretreatment with Activin A and FGF4, however, is very important, because it induces the endodermal fate and alters further morphological changes and maturation of hepatocyte-like cells. As shown

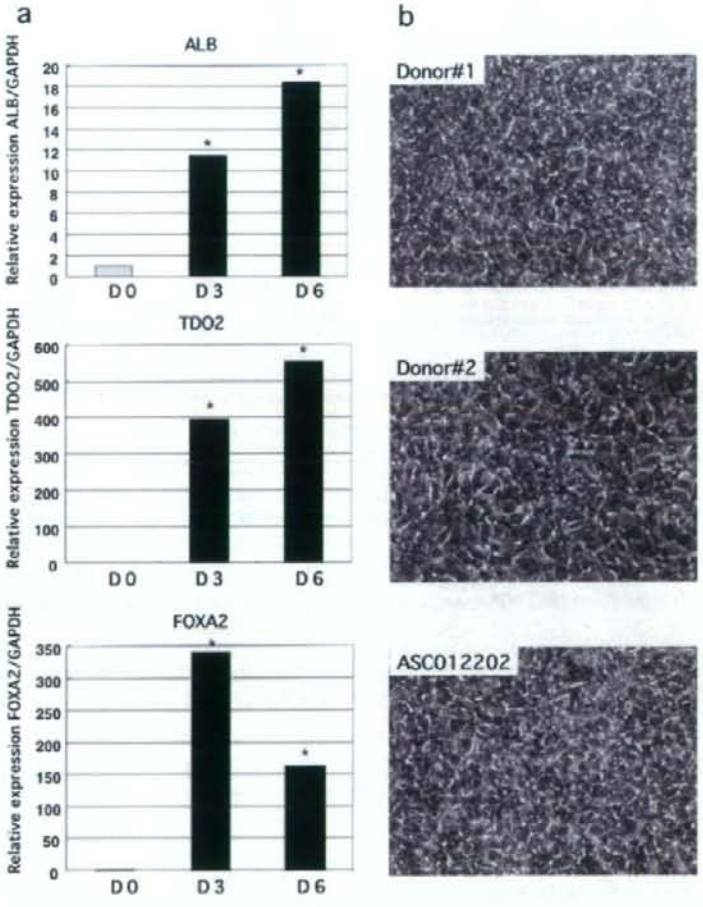

Figure 2 (a) Expression of albumin (ALB), tryptophan 2,3-dioxygenase (TDO2) and FOXA2 at day 3 (D3) (pretreatment with fibroblast growth factor [FGF]4 and Activin A) and day 6 (D6) (3 days of pretreatment with FGF4 and Activin A, and 3 days of treatment with hepatocyte growth factor [HGF], FGF1, FGF4, oncostatin M [OsM], dexamethasone [Dex], insulin-transferrin-selenium [ITS], dimethyl sulfoxide [DMSO], and nicotinamide) (■). Undifferentiated adipose-derived stem cells (ASC) (D0) (▨). Data were analyzed by the Pair Wise Fixed Reallocation Randomization Test©, $n = 3$. $^*P < 0.05$. (b) Morphological features of ASC-derived hepatocyte-like cells of ASC derived from Donor #1, Donor #2, and commercially available ASC012202.

in Figure 3(a), changes in the morphology of ASC-derived hepatocyte-like cells (Donor #2) at days 0, 4, 9 and 16 of hepatogenic induction indicate hepatocyte maturation. At day 13, ASC-derived hepatocyte-like cells expressed albumin (Fig. 3b), which was detected by immunostaining, using anti-human specific antibody. Undifferentiated ASC, however, did not express albumin (data not shown). We also checked the functionality of ASC-derived hepatocyte-like cells. Figure 3(c) represents the albumin production at days 3, 6 and 9 of the induction process. ASC-derived hepatocyte-like cells also revealed an ability to uptake low-density lipoprotein (LDL) and store glycogen (Fig. 4).

Transplantation of ASC-derived hepatocyte-like cells into mice with liver injury

To address whether ASC reveal therapeutic abilities to regenerate an injured liver, we transplanted ASC-derived hepatocyte-like cells of Donors #1 and #2 into nude mice with acute liver failure. CCl_4 injury generated oxidative stress and hepatocyte necrosis. Twenty-four hours after CCl_4 injection, mice revealed serious liver injury. Biochemical parameters such as ALT, AST, UA and ammonia were increased in mice with CCl_4 injury compared with non-injured mice (Fig. 5). We transplanted 1.5×10^6 cells of ASC-derived hepatocyte-like cells into a CCl_4-injured mouse. After transplantation, ALT and AST were significantly decreased to a value more than 50% lower than in non-transplanted and injured mice (Fig. 5). Likewise, ammonia concentration was significantly decreased after ASC-derived hepatocyte-like cell transplantation. UA, a marker of oxidative stress, was significantly decreased up to a normal level after transplantation of ASC-derived hepatocyte-like cells (Fig. 5). Hematoxylin-eosin staining revealed that the level of injury was the same in the injured, non-transplanted mice (Fig. 6b,e) as well as in the injured transplanted mice (Fig. 6c,f), in contrast to the non-injured non-transplanted mice (Fig. 6a,d). Significant morphological changes between those mice, however, were detected in the hepatocytes of the non-necrotic area. The

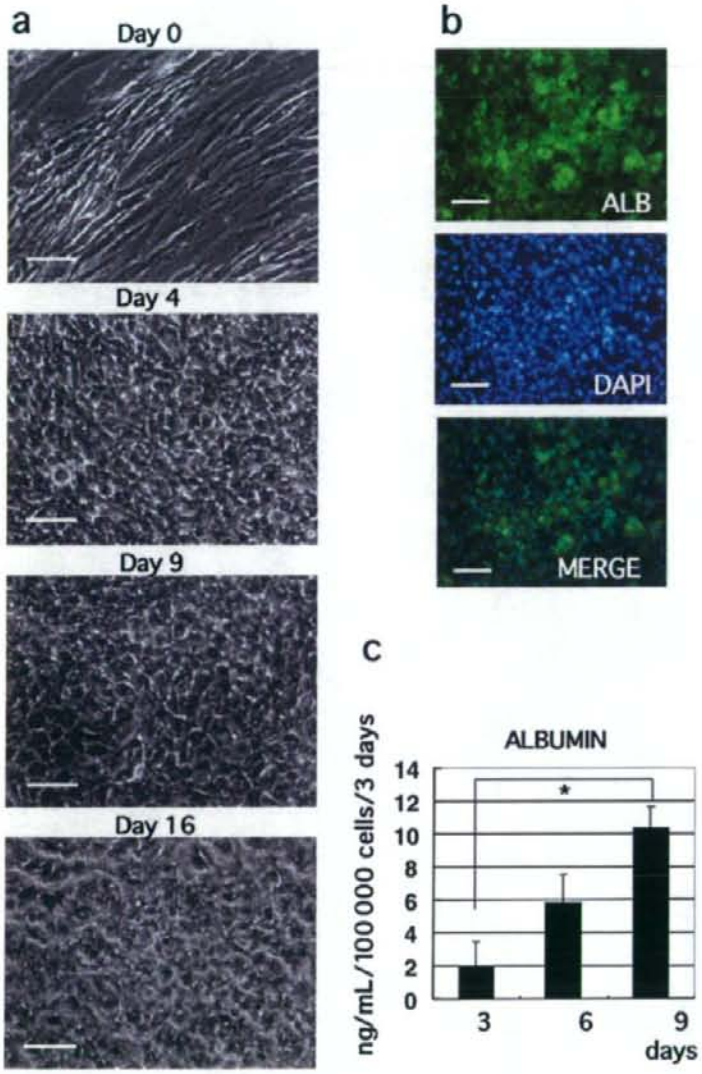

Figure 3 (a) Morphology of adipose-derived stem cells (ASC) (Donor #2) at days 0, 4, 9 and 16 during the hepatogenic induction process. (b) Albumin immunostaining analyses of ASC-derived hepatocyte-like cells at day 13 of induction. (c) Albumin production by ASC-derived hepatocyte-like cells at days 3, 6 and 9 of induction. Data are reported as the mean ± SD and were analyzed by Student's *t*-test, $n = 3$. $^*P < 0.05$). ALB, albumin; DAPI, 4,6-diamidino-2-phenylindole. Bar, 50 μm.

livers of injured, transplanted mice revealed less vacuolar degeneration caused by dilatation of mitochondria and rough endoplasmic reticulum. These observations reflect the data of the decrease of ALT and AST levels in injured transplanted mice. Therefore, transplantation of ASC-derived hepatocyte-like cells provided protection against CCl_4-induced hepatic injury. The above results indicate that ASC-derived hepatocyte-like cells generated within 13 days reveal hepatocyte-specific markers and functions *in vitro*, and improve liver function *in vivo*.

Discussion

Transplantation of hepatocytes generated from stem cells might become an easier, more efficient, and safer way than whole organ transplantation to cure patients suffering from liver disease. ASC can be very easily obtained with minimal invasiveness from a patient's own adipose tissue. Such a possibility sidesteps the obstacles regarding the risk of rejection, ethical issues, and availability of stem cells. We have already demonstrated mouse

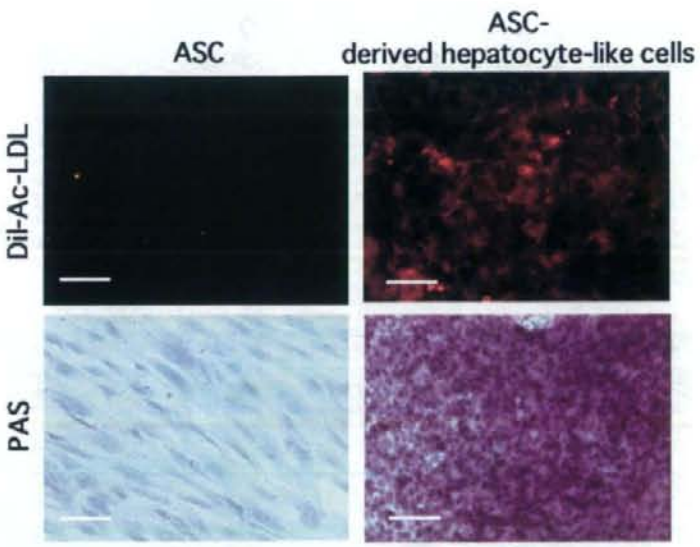

Figure 4 Low-density lipoprotein (LDL) uptake ability and glycogen storage ability (PAS) of adipose-derived stem cells (ASC)-derived hepatocyte-like cells at day 13 of induction. Dil-Ac-LDL, 1,1′-dioctade-cyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Dil)-labeled acetylated LDL. Bar, 50 μm.

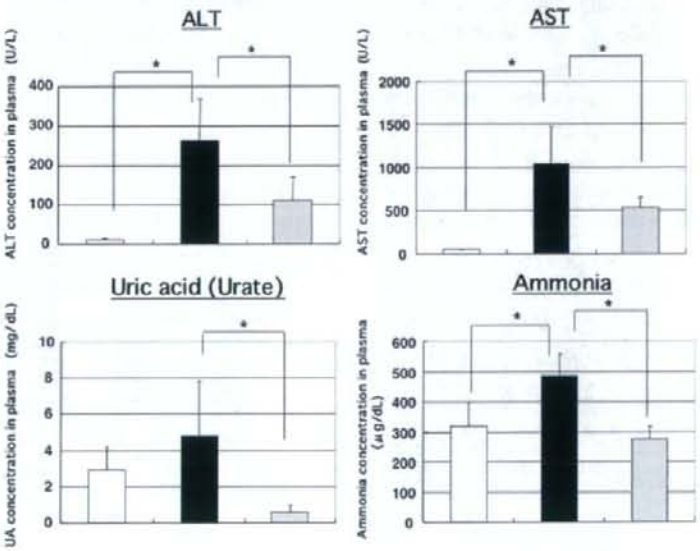

Figure 5 Biochemical analysis. Concentration of ammonia, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and UA (uric acid/urate) in blood serum of killed mice. □, non-injured, non-transplanted mice; ■, injured and non-transplanted mice; ▨, injured and transplanted with adipose-derived stem cells (ASC)-derived hepatocyte-like cells (combined data of Donors #1 and #2). Data are presented as the mean ± SD and were analyzed by the Bonferroni correction $n = 3$. (* $P < 0.05$).

embryonic stem cell[28–30] and human adult ASC[19] hepatogenic differentiation.

In the present study, we presented induction within a very short time of human ASC into hepatocyte-like cells. Thirteen days is sufficient to generate *in vitro* cells, which reveal hepatocyte-specific morphology, marker profile, and functionality. This is first time for such a short hepatogenic differentiation protocol to be presented. At the beginning we treated the cells with Activin A together with FGF4, which are important factors at early stages of endoderm formation in mouse liver development. Afterwards we used a number of factors essential for hepatogenic specification and hepatocyte morphology maintenance. We compared the hepatocyte-like cells obtained by a new rapid protocol with the hepatocyte-like cells of an original protocol,[19] and have found that they reveal all the analyzed functions, albeit much earlier. We observed that 24 h of *in vitro* cocktail treatment (HGF, FGF1,

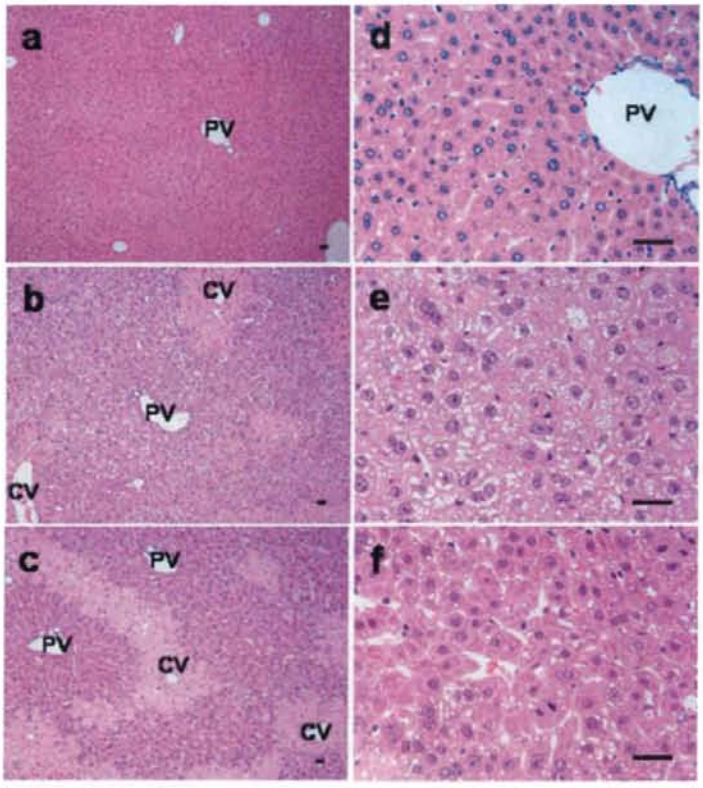

Figure 6 Hematoxylin-eosin staining of liver sections from (a,d) non-injured non-transplanted, (administered with olive oil and phosphate-buffered saline [PBS] [–]) mice ($n = 3$); (b,e) injured non-transplanted (administered with CCl_4 and PBS [–]) mice ($n = 3$); (c,f) injured transplanted (administered with CCl_4, 1 day after 1.5×10^6 ASC-derived hepatocyte-like cells transplantation) mice ($n = 4$). Panels a–c lower magnification 100 ×, panels d–f higher magnification 400 ×. CV, central vein; PV, portal vein. Scale bars represent 50 μm.

FGF4, OsM, Dex, ITS, nicotinamide, and DMSO) induces a dramatic change in morphology followed by little production of albumin at day 6 and a significant increase in the albumin level at day 9. However, using a previous protocol, albumin production was detected at days 30–50.

Prior to *in vivo* transplantation, it is important to induce hepatic fate within a short period of time and transplant the cells as fast as possible back into the patient with liver disease. Such a short period of time does not require large quantities of growth factors and may save much on expenses. Additionally, it will serve as hope and a great chance for a patient's total recovery. Significant morphological changes and albumin production as early as within 9 days suggest that it may be possible to even shorten the hepatic fate prior to transplantation. In the context of future clinical usage, a short period of stimulation to induce hepatic fate may be sufficient, because cells after transplantation may undergo further maturation in a regeneration environment.

Transplantation of *in vitro*-generated hepatocyte-like cells into CCl_4-injured nude mice resulted in the improvement of liver function *in vivo*. Interestingly, *in vivo* liver functions illustrated by the concentrations of ALT, AST, UA and ammonia were significantly decreased after ASC-derived hepatocyte-like cell transplantation (Fig. 5). The functional benefits of ASC-derived hepatocyte-like cell transplantation may be because of the functional support of the transplanted cells. It is still not clear by which mechanisms the transplanted cells improve the functioning of the liver. Fusion with host hepatocytes is not excluded. Likewise, the support and activation of endogenous progenitors are possible. Further studies examining the *in vivo* mechanism of homing, engraftment, and liver regeneration need to be conducted. It has been reported that in recipient liver, partial portal embolization, not partial portal ligation, improves engraftment of transplanted hepatocytes in a monkey primate preclinical model.[31] This provides new possibilities and strategies for future cell transplantation. It is essential to exclude any post-transplantation complications prior to any clinical trials. A long-term course experiment as well as safety issues should be carefully evaluated. Interestingly, in another study,[32] we observed that parameters such as ALT, AST, UA and ammonia were also decreased after undifferentiated ASC transplantation and we postulate that undifferentiated ASC per se compose a very attractive tool for the establishment of successful therapy for the liver.[32] We also speculate that the therapeutic potential of ASC may be due to the trophic activity of ASC.[32] These findings require additional studies with respect to safety issues post-

transplantation; however, they give great promise for future clinical applications.

Short-term hepatogenic induction methods may also have great usage in drug metabolism studies and toxicological analyses. In fact, we have already observed that ASC-derived hepatocyte-like cells reveal cytochrome activities (data not shown).

In conclusion, our study revealed that ASC have a special affinity towards hepatocyte differentiation *in vitro* and hepatocyte regeneration *in vivo*. Thus, ASC may be a superior choice for the establishment of therapy for an injured liver.

Acknowledgments

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SPECIAL ISSUE REVIEWS–A PEER REVIEWED FORUM

Pleiotropic Function of FGF-4: Its Role in Development and Stem Cells

Nobuyoshi Kosaka,[1] Hiromi Sakamoto,[2] Masaaki Terada,[2] and Takahiro Ochiya[1]

Fibroblast growth factors (FGFs) were initially recognized as fibroblast-specific growth factor, and it is now apparent that these growth factors regulate multiple biological functions. The diversity of FGFs function is paralleled by the emerging diversity of interactions between FGF ligands and their receptors. FGF-4 is a member of the FGF superfamily and is a mitogen exhibiting strong action on numerous different cell types. It plays a role in various stages of development and morphogenesis, as well as in a variety of biological processes. Recent studies reveal the molecular mechanisms of FGF-4 gene regulation in mammalian cells, which is involved in the developmental process. Furthermore, FGF-4 also acts on the regulation of proliferation and differentiation in embryonic stem cells and tissue stem cells. In this review, we focus on the diverse biological functions of FGF-4 in the developmental process and also discuss its putative roles in stem cell biology. *Developmental Dynamics 238:265–276, 2009.* © 2008 Wiley-Liss, Inc.

Key words: FGF-4; embryogenesis; stem cell

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INTRODUCTION

The fibroblast growth factor-4 (FGF-4) gene was identified as *HST-1* gene from human stomach cancers and Kaposi's sarcoma by a NIH3T3 transforming assay (Sakamoto et al., 1986; Delli Bovi et al., 1987). FGF-4 is also known as kFGF and HBGF-4. The deduced amino acid sequence of the FGF-4 is 43%, 38%, and 40%, identical, respectively, to human fibroblast growth factor-2 (FGF-2), human fibroblast growth factor-1 (FGF-1), and mouse fibroblast growth factor-3 (FGF-3) protein. The *FGF-4*, *FGF-3*, and *FGF-19* genes are located on human chromosome region 11q13 as clustered FGFs (Fig. 1) (Wada et al., 1988; Huebner et al., 1988; Katoh, 2002; Itoh and Ornitz, 2008). FGF-4 has a classic signal peptide sequence, whereas FGF-1 and FGF-2 have neither this sequence nor internal hydrophobic domains. This indicates that, unlike FGF-1 and FGF-2, the FGF-4 protein is cleaved after a signal peptide, glycosylated, and efficiently secreted as a mature protein of 176 amino acids excluding the 30 amino acid residue of a signal peptide (Fig. 1) (Yoshida et al., 1987; Taira et al., 1987). Inhibition of glycosylation impaired secretion, and the stability of the secreted FGF-4 were greatly enhanced by the presence of heparin in a cultured medium (Delli-Bovi et al., 1988; Miyagawa et al., 1988). However, unglycosylated FGF-4 was cleaved into two NH2-terminally truncated peptides of 13 and 15 kDa, which appeared to be more biologically active than the wild-type protein (Fuller-Pace et al., 1991; Bellosta et al., 1993). These two proteins also showed higher heparin binding affinity than that of wild-type FGF-4. Although it is not obvious whether cleavage of FGF-4 to generate these two proteins occurs in vivo, this could represent a novel mechanism of modulation of FGF-4 mediated biological activity.

FGFs constitute a large family of multifunctional, heparin-binding proteins that show diverse patterns of interaction with a family of receptors (FGFR-1 to -4) that are subject to alternative splicing. FGFR binding specificity is a vital mechanism in the regulation of FGF signaling and is achieved through usage of two alternative exons, IIIc and IIIb, for the second half of immunoglobulin-like domain 3 (D3) in FGFRs. While FGF-4 binds and activates the IIIc splice forms of FGFR1 to -3 at comparable

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Developmental Dynamics

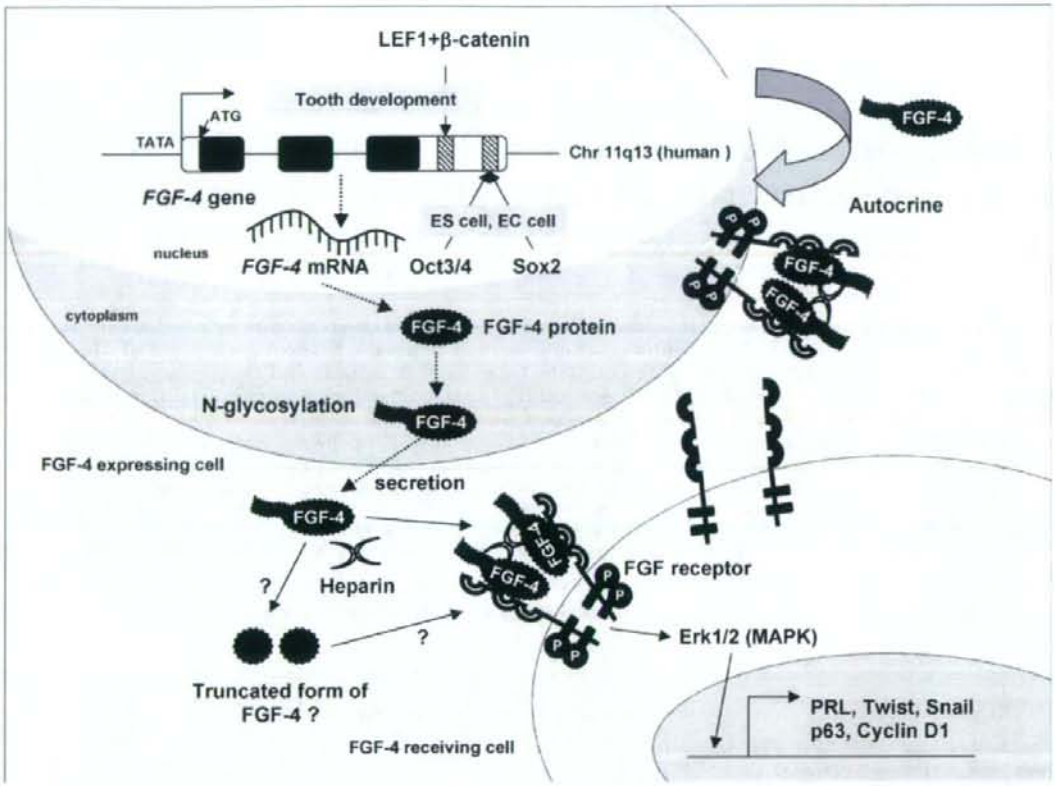

Fig. 1. Regulation and action of fibroblast growth factor-4 (FGF-4). The *FGF-4* gene is encoded by three exons (solid box). The distal enhancer is located in the untranslated region of the third exon (diagonal stripe). Initial binding of SOX-2 and Oct-3/4 to their cognate sequences in the FGF-4 distal enhancer enhances the transcription of *FGF-4* gene in embryonal carcinoma cells (EC cells) or embryonic stem cells (ES cells; Lamb and Rizzino, 1998). Intraepithelial Wnt10a and/or Wnt10b signals mediate, through LEF1 and β-catenin, the transcriptional activation of the *Fgf-4* gene in the epithelium (Kratochwil et al., 2002). FGF-4 protein is glycosylated, cleaved after a signal peptide (Yoshida et al., 1987; Taira et al., 1987). Stability of the secreted FGF-4 was enhanced by the presence of heparin (Delli-Bovi et al., 1988; Miyagawa et al., 1988). However, the unglycosylated FGF-4 was immediately cleaved into two NH2-terminally truncated peptides of 13 and 15 kDa, which appeared to be more biologically active than the wild-type protein (Fuller-Pace et al., 1991; Bellosta et al., 1993). These two proteins also showed higher heparin binding affinity than that of wild-type FGF-4. FGF-4 binds the IIIc splice forms of FGFR-1 to -3 (Ornitz et al., 1996). FGF-4 induced the phosphorylation of Erk1/2 not only in ES cell (Kunath et al., 2007), TS cell (Yang et al., 2006) and mesenchymal cells (Bobick et al., 2007) during embryogenesis, but also in Sertoli cells and germ cells (Hirai et al., 2004). FGF-4 induces the expression of prolactin (PRL) in primary rat pituitary cell (Shimon et al., 1996), and of Twist and Snail in the mesenchyme (Rice et al., 2005).

levels, it shows little activity toward the IIIb splice forms of FGFR-1 to -3 (Fig. 1; Ornitz et al., 1996; Itoh and Ornitz, 2004).

The *FGF-4* gene was originally discovered by assaying human tumor DNA for dominantly transforming oncogenes. Several groups reported the expression of FGF-4 in testicular germ cell tumors. Yoshida et al. reported the expression of FGF-4 in a human teratoma cell line and in five of nine surgically resected human testicular germ cell tumors including seminomas and embryonal carcinomas (Yoshida et al., 1988a). Strohmeyer et al. showed 70 testicular germ cell tumors analyzed at the DNA and RNA levels for the *c-kit*, *FGF-4*, and *FGF-3* oncogenes. There are significant differences in oncogene expression between seminomas and nonseminomas, with *c-kit* being expressed in 24 of 30 (80%) seminomas but in only 3 of 40 (7%) nonseminomatous tumors, and *FGF-4* being expressed in 24 of 38 (63%) nonseminomas but only 1 of 24 (4%) of seminomas, demonstrating an inverse relationship in the expression pattern of these 2 oncogenes in human testicular germ cell tumors. A significant association between tumor stage and *FGF-4* expression in the nonseminoma group was found. No gross alterations in the *c-kit*, *FGF-4*, and *FGF-3* loci were found in the DNA (Strohmeyer et al., 1991). Another group reported that the human teratocarcinoma cell line Tera 2 could be induced to differentiate in vitro after exposure to retinoic acid (Schofield et al., 1991). While the *FGF-4* oncogene is expressed in undifferentiated cells, the addition of retinoic acid rapidly downregulates the expression of this gene.

Developmental Dynamics