together with undifferentiated AT-MSCs. Human primary hepatocytes or HepG2 cells were used as positive controls.

RT-PCR. Total RNA (0.5μg) was reverse-transcribed using the SuperScript III Reverse Transcriptase (Invitrogen, Tokyo, Japan) according to the manufacturer's guidelines. PCR analyses were performed using the Ampli Taq Gold kit (Applied Biosystems, Tokyo, Japan). The PCR primer sequences and conditions are listed in Supplementary Table 1.

Immunofluorescence. Cells were fixed in 4% formaldehyde for 10 minutes, followed by incubation with Protein Block (DakoCytomation) for 30 minutes. Undifferentiated $CD105^+$ AT-MSCs and $CD105^+$ AT-MSC-derived hepatocytes were analyzed by immunohistochemistry with transthyretin (TTR) (1:300), albumin (ALB) (Sigma, Tokyo, Japan) (1:250), cytokeratin-18 (CK-18) (1:200) (Cosmo Bio, Tokyo, Japan), and CYP3A4 (1:2000) (Cosmo Bio) antibodies overnight at 4°C. The rhodamine (1:1000) - or FITC (1:1000)-conjugated secondary antibodies were applied for 30 minutes. Undifferentiated AT-MSCs were stained according to the same protocol used for mouse anti-human vimentin (1:50) (Sigma) and mouse anti-human CD105 (1:40) (Cosmo Bio) antibodies. Nuclei staining have been performed using DAPI (4,6-diamidino-2-phenylindole).

Western Blot Analysis. Western blot was used to detect the presence of CYP3A4, CYP1A1, CYP2C9 as well as NADPH P450 Reductase in AT-MSC-derived hepatocytes. The cells were dissolved in the Mammalian Protein Extraction Reagent (Pierce, Rockford, IL). Proteins were separated on an SDS-polyacrylamide gel and transferred to PVDF (polyvinylidine difluoride) membranes (BIORAD, Tokyo, Japan). Blots were saturated with 5% skim milk in TBS-T for 1 hour at room temperature and afterwards incubated overnight with anti-human rabbit polyclonal CYP3A4 (Cosmo Bio), goat NADPH-cytochrom P-450 reductase (CPR) (Daiichi Pure Chemicals, Tokyo, Japan), mouse CYP1A1 (Santa Cruz Biotechnology, Inc., CA, USA), or rabbit CYP2C9 (Fitzgerald, Concord, USA) antibodies, respectively. Following washing in TBS-T, the membranes were incubated for 30 minutes with sheep anti-rabbit or anti-mouse IgG-HRP-linked whole antibodies (GE Healthcare Bio-Sciences KK, Tokyo, Japan) or rabbit anti-goat IgG (H+L) HRP-linked antibodies (Southern Biotechnology Associates, Inc., Birmingham). Monoclonal antibodies against human GAPDH were used as a control of protein loading (Santa Cruz).

LDL Uptake. LDL uptake was assessed by incubating cells for 4 hours at 37°C with 10μg/ml acetylated LDL labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (Dil-Ac-LDL) purchased from Biomedical Technologies, Inc., Stoughton.

Periodic Acid-Schiff (PAS) Staining. Cells were fixed with 10% formaldehyde oxidized in 1% periodic acid for 10 minutes and rinsed twice with water. Afterwards, cells were treated with Schiff's reagent for 10 minutes, rinsed with water and stained with hematoxylin.

Ammonia Clearance. Cells were cultured in the presence of 2.5mM NH_4Cl and incubated for 30 hours. At 9th, 19th, and 30th hour of incubation, the medium was collected and tested for the concentration of NH_4Cl using Ammonia-Test Wako (Wako Pure Chemicals, Tokyo, Japan).

Albumin Production. The albumin level in the culture medium was evaluated using the Bromocresol Green (BCG) method performed by SRI Communication for the Health Company, Tokyo, Japan.

$CD105^+$ AT-MSC-Derived Hepatocyte Transplantation into Mice with CCl_4-Induced Injury. Twenty-four hours after an intraperitoneal injection of 100μL/20g body weight of olive oil containing 10μL of CCl_4, three BALB/c nude mice (SLC, Tokyo, Japan) underwent transplantation of *in vitro* generated $CD105^+$ AT-MSC (Donor#6)-derived hepatocytes at 5×10^5 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) and untreated (olive oil) mice (n = 3) were used. $CD105^+$ AT-MSC-derived hepatocytes were collagenase-dispase-treated, while AT-MSCs were trypsinized according to the standard procedure. After centrifugation, the cell pellet was rinsed in PBS (−). Histological analysis of liver tissues was conducted by serial tissue section one day after cell transplantation and stained with hematoxylin and eosin or immunohistochemically examined for human specific albumin expression. Animal studies were carried out in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute.

Statistical Analysis. Results are given as the mean ± SD. The Student *t* test was performed for statistical evaluation, with *P* less than 0.05 considered significant. All *in vitro* results were derived from at least three independent experiments.

Results

Characterization of AT-MSCs. AT-MSCs were characterized by flow cytometry, immunofluorescence and RT-PCR analysis. Flow cytometry revealed that the cells from all patients (n = 6) were: CD31 (12.9-30%),

Table 2. Expression of Stem Cell Markers in AT-MSCs, BM-MSCs, and BM CD34+ Stem Cells, by RT-PCR Analysis

	Bone Marrow-derived Mesenchymal Stem Cells	Adipose Tissue-derived Mesenchymal Stem Cells	Bone Marrow-derived CD34+ Stem Cells
CD10	+	+	−
CD13	+	+	+
CD59	+	+	+
CD105	+	+	+
CD166	+	+	−
CD49d	+	+	+
SH3	+	+	−
CD29	+	+	+
CD44	+	+	+
CD71	+	+	+
CD90	+	+	−
CD106	+	+/−	−
CD120a	+	+	+
CD124	+	+	+
CD11b	−	−	+
CD14	−	+/−	+
CD31	−	+/−	+
CD34	−	+	+
CD45	−	−	+
CD48	−	−	+
CD135	−	−	+
CD117	+	+/−	+

NOTE. +/− means that mRNA expression vary according to each patient.

CD34 (5.3%-9.9%), CD45 (0.06%-8.4%), CD29 (96%-99%) and CD105 (63.9-98.5) (Supplementary Fig. 1). The number of $CD105^+$ cells varies between donors. AT-MSCs were evaluated by the expression of stem cell markers (mesenchymal: CD10, CD13, CD59, CD105, CD166, CD49d, SH3, CD29, CD44, CD71, CD90, and CD106; and hematopoietic: CD120a, CD124, CD11b, CD14, CD31, CD34, CD45, CD48, CD135, and CD117) using RT-PCR analysis and compared with mRNA from BM-MSC and BM-HSC (Table 2). AT-MSCs share similarities in morphology (Fig. 2a,b) and marker profile with BM-MSCs (Table 2); however, AT-MSCs were found to express the mRNA of CD34, CD31 and CD14. The expression of CD106, CD14, CD31 and CD117 varies according to the donor. In addition, we compared the expression of CD105 (Fig. 2c,d) and vimentin (Fig. 2e,f) by immunostaining. The expression of both mesenchymal stem cell markers was lower in unfractionated AT-MSCs than in BM-MSCs. After MACS sorting, the $CD105^+$ fraction of AT-MSCs revealed a homogeneous morphology (Fig. 2g) and high proliferation ability (at early passages doubling time= 34-36 hours, whereas the unfractionated population = 36-40 hours). The sorting efficiency was evaluated by CD105 staining (Fig. 2h) and flow cytometry analysis before and after MACS sorting (Fig. 2j). $CD105^+$ fraction of AT-MSCs revealed expression of vimentin as well (Fig. 2i). The adipogenic differentiation potential of unfractionated and $CD105^+$ AT-MSCs was verified by performing adipogenic differentiation. This resulted in the accumulation of intracellular lipid droplets that could be stained with Oil Red O. $CD105^+$ AT-MSCs revealed a higher adipogenic induction ratio (≈60%-82%) than unfractionated AT-MSCs (≈21%-67%) (Fig. 2k). Non-induced AT-MCSs showed no presence of lipid droplets. Similar results were obtained from a different donor age. Chondrogenic induction of $CD105^+$AT-MSCs resulted in higher induction ratio than unfractionated AT-MSCs (data not shown) as well. In summary, our data suggest that $CD105^+$ fraction exhibits higher homogeneity, proliferation ability and potentiality than unfractionated AT-MSCs.

Hepatic Differentiation of AT-MSCs. We examined the hepatic-differentiation potential of $CD105^+$ AT-MSCs. During the initiation step of hepatic differentiation, the cells showed a remarkable transition from a bipolar fibroblast-like morphology (Fig. 3a-c) to a round epithelial-like shape (Fig. 3d-f). The contraction of the cytoplasm progressed further during maturation, and most of the treated cells became quite dense and round with clear or double nuclei in the late stage of differentiation. The representative morphology of differentiated $CD105^+$ AT-MSCs from 3 different donors (#1, 2, and 6 as shown in Table 1) is shown in Fig. 3d-f, and contrasted with that of primary human hepatocytes (Fig. 3g,h). We can notice clearly small round or oval-shaped cells with polyhedral structure, with tight cell to cell interactions, and visible bile canaliculi structures (Fig. 3i), similar to primary human hepatocytes (Fig. 3h). $CD105^+$ AT-MSCs derived from all three independed donors during hepatic induction underwent morphological changes. $CD105^+$ AT-MSC-derived hepatocyte-like cells from donor #2 (Age 45, male) were used for further analysis of liver functions.

RT-PCR Analysis of Hepatocyte-Specific Markers. The temporal expression pattern for a number of hepatocyte-specific genes, such as ALB, AFP, TTR, TDO2, and CYP7A1, and the hepatocyte nuclear factor (HNF)-4-alpha were analyzed by RT-PCR (Fig. 4). The expression of early endoderm differentiation marker-AFP decreased when the cells underwent maturation, whereas the expression of mature hepatocyte markers, such as ALB, TDO2, and CYP7A1 appeared at around day 6 of HIFC treatment and was maintained until day 70 (data not shown). HNF-4-alpha, essential for morphological and functional hepatocyte differentiation had been detected at day 41. Therefore, HIFC induced $CD105^+$ AT-MSCs into hepatocyte-like cells revealed a gene expression pattern of

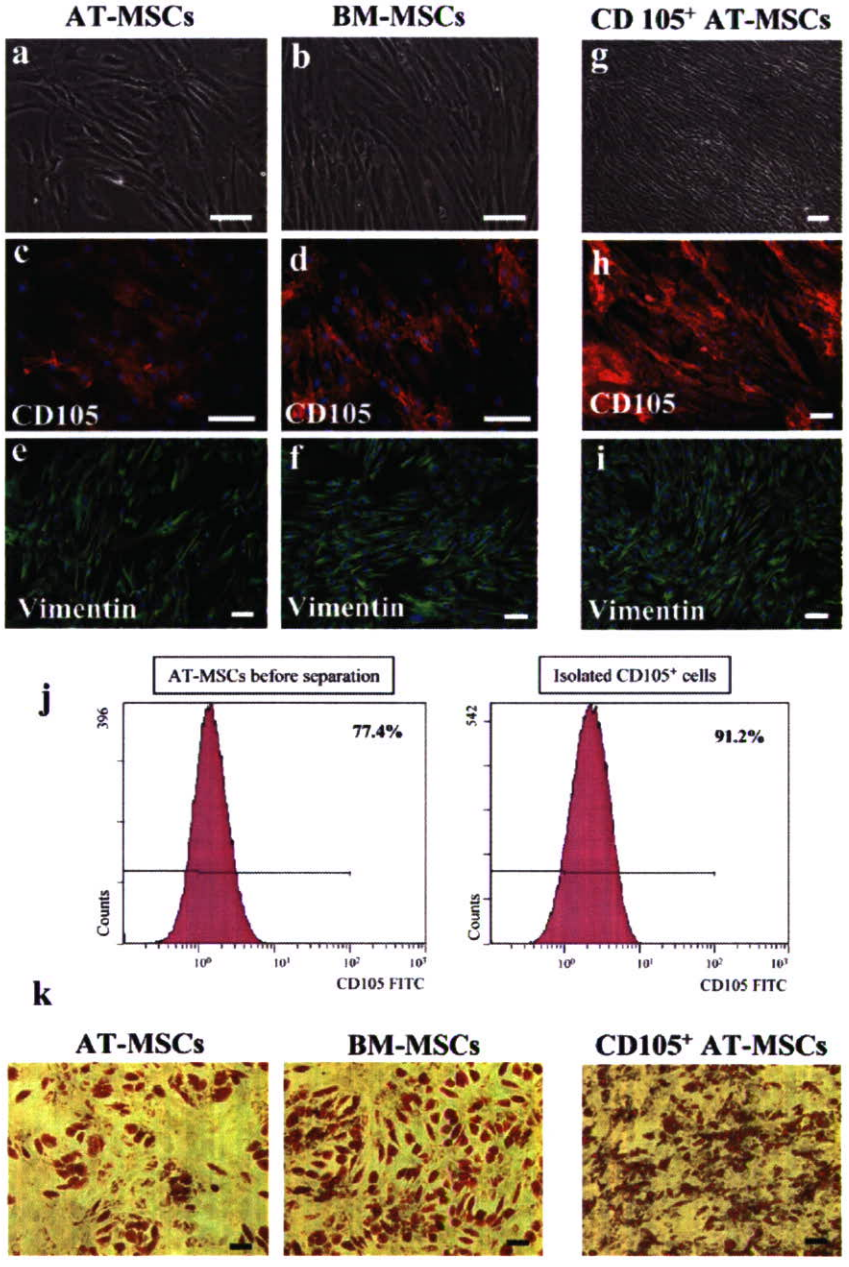

Fig. 2. Morphology of AT-MSCs (a) and BM-MSCs (b). Both types of cells were stained for CD105 (rhodamine, red) and vimentin (FITC, green); AT-MSCs (c and e) and BM-MSCs (d and f). MACS sorting resulted in achievement of a homogeneous population (g). CD105$^+$ AT-MSCs were stained for CD105 (h) and vimentin (i). Before and after MACS sorting cells were analyzed by flow cytometry for CD105 (j). BM-MSCs, AT-MSCs unfractionated, and CD105$^+$ AT-MSCs were cultured for 3 weeks in an adipogenic induction medium and analyzed by Oil red O staining (k). Scale bars represent 50μm.

hepatocyte-specific markers and transcription factor similar to human hepatocytes.

Immunostaining and Western Blot Confirmation of Hepatic Differentiation. In the next experiments, we examined whether, CD105$^+$ AT-MSCs expressed hepatocyte-specific proteins after hepatic induction. Markers such as ALB, TTR, CYP3A4, and CK-18 were positively stained using immunofluorostaining. We detected double-positive cells for ALB/CYP3A4, CK-18/TTR (Fig. 5A), and ALB/TTR (data not shown) at day 40. Undifferentiated cells were not stained on all these four markers (data not shown). To further confirm efficient hepatic induction, we checked the protein expression of the hepatocyte-specific enzymes CYP1A1, CYP3A4, CYP2C9 and NAPDH P-450 reductase by Western blot analysis (Fig. 5B). A clear band for all four proteins was detected in CD105$^+$ AT-MSC-derived hepatocyte-like cells (Fig. 5B, lane 2). Undifferentiated CD105$^+$ AT-MSCs (Fig. 5B, lane 1) express a low, detectable level of CYP1A1 as well, as had been observed in RT-PCR analysis (data not shown).

Functional Characterization of AT-MSC-Derived Hepatocytes. Afterwards, we examined whether hepatocytes derived from CD105$^+$ AT-MSCs are functionally

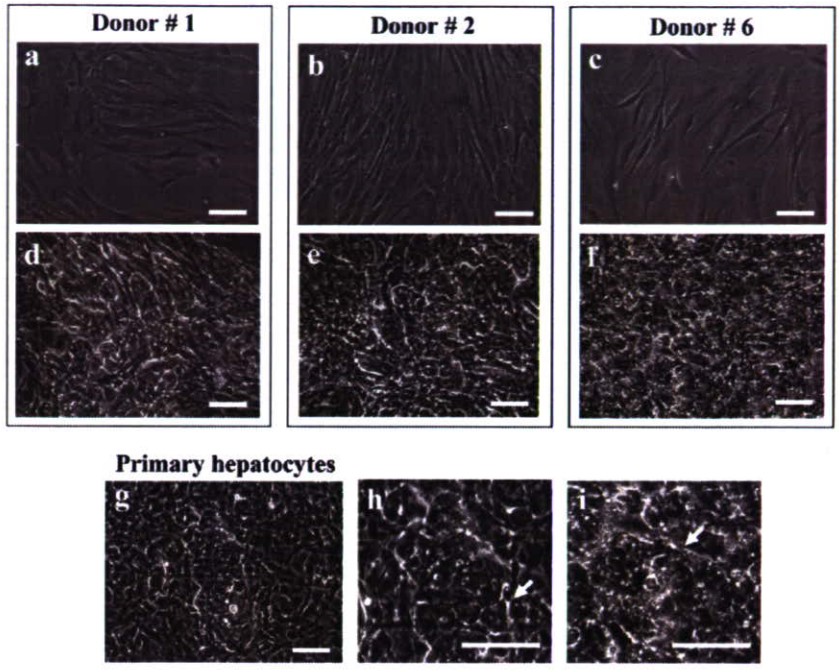

Fig. 3. Undifferentiated CD105$^+$ AT-MSCs from 3 donors (a,b,c) and the same cells after 40 days of hepatic induction (d,e,f). Photograph (i) represents higher magnification of (f). The morphology of CD105$^+$ AT-MSC-derived hepatocyte-like cells represents many similarities with the primary human hepatocytes (g). Photograph (h) represents higher magnification of (g). Arrows indicate bile canaliculi structures. Scale bars represent 50μm.

competent. After 2 weeks of hepatic induction, almost 20% of the cells could incorporate LDL, and, during maturation (40 days), almost all of the induced cells turned out to be competent for LDL uptake (Fig. 5C). At 35 days, CD105$^+$ AT-MSCs were analyzed for their glycogen-storage ability by PAS staining. As shown in Fig. 5D, almost all CD105$^+$ AT-MSC-derived hepatocytes were strongly positive for PAS staining, while undifferentiated CD105$^+$ AT-MSCs were weakly positive. Moreover, we examined their abilities to produce albumin and detoxify ammonia. Our analyses indicate that AT-MSC-de-

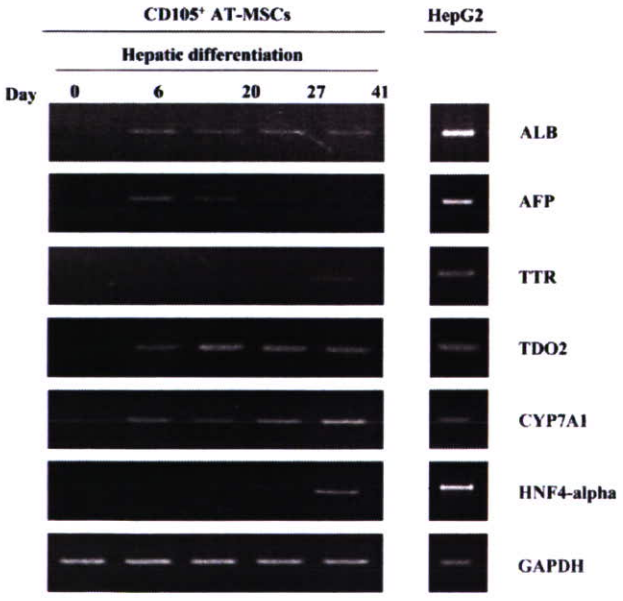

Fig. 4. RT-PCR of a temporal gene expression pattern of hepatocyte-specific markers during hepatic differentiation of CD105$^+$ AT-MSCs. RNA was isolated from undifferentiated CD105$^+$ AT-MSCs (day 0) and from CD105$^+$ AT-MSCs during hepatic induction at days 6, 20, 27, and 41.

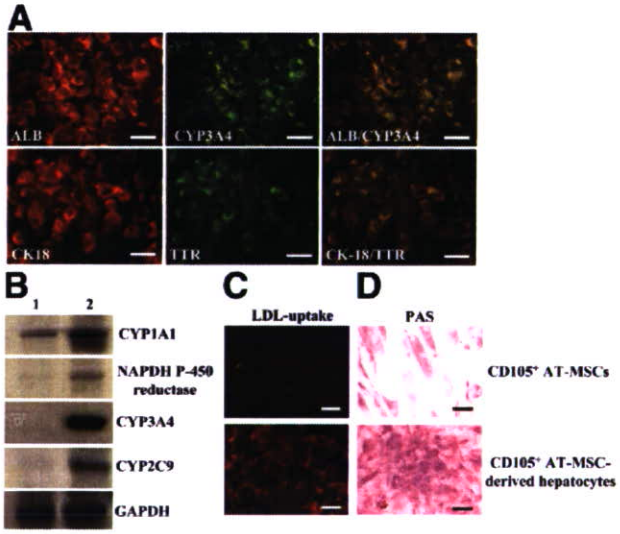

Fig. 5. Immunofluorostaining analysis of hepatocyte-specific markers in differentiated CD105$^+$ AT-MSCs (A). At day 40, cells were fixed and stained with monoclonal antibodies against sequentially ALB, CK-18 (red), CYP3A4, and TTR (green). Western blot analysis indicated that CYP1A1 (58.2kDa), NADPH P-450 reductase (76.5 kDa), CYP2C9 (56 kDa) and CYP3A4 (57 kDa), were actively synthesized in CD105$^+$ AT-MSC-derived hepatocytes (lane 2), GAPDH was used as an internal control (35 kDa) (B) (lane 1). CD105$^+$ AT-MSCs-derived hepatocyte-like cells were analyzed for ability of LDL-uptake (C) and glycogen storage ability, using PAS (periodic acid Schiff) staining (D). Scale bars represent 50μm.

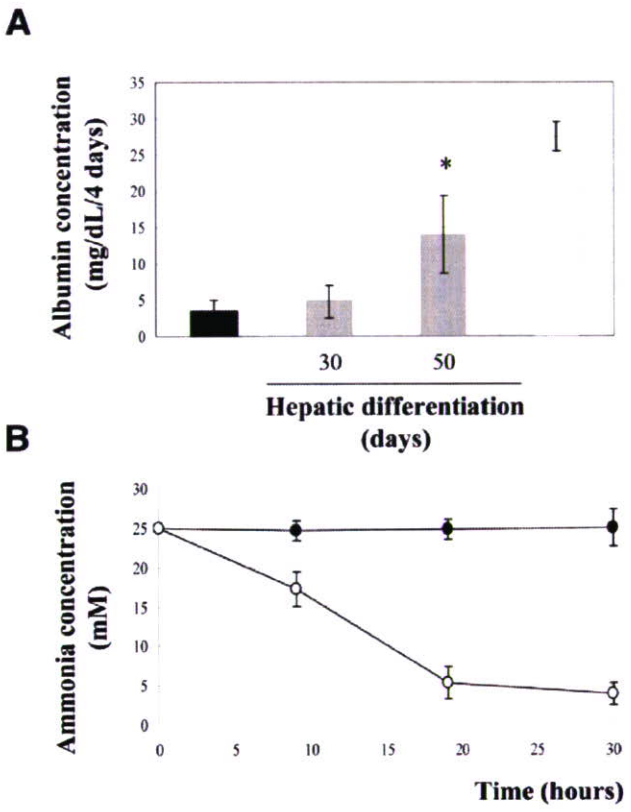

Fig. 6. (A) Albumin production of CD105$^+$ AT-MSCs (shaded square) during hepatic differentiation (at day 30 and 50), undifferentiated CD105$^+$ AT-MSCs (filled square), and primary human hepatocytes (open square). Graph (B) shows ammonia detoxification by CD105$^+$ AT-MSC-derived hepatocytes (open circle). Undifferentiated CD105$^+$ AT-MSCs (filled circle) did not reveal the ability to clear ammonia from the culture medium. Data are reported as the mean ± SD and were analyzed by the Student t test, n = 3. * $P < 0.05$).

rived hepatocytes have the capacity to secrete albumin (Fig. 6A) and clear ammonia from culture media (Fig. 6B). In addition, although data are not shown, we detected the CYP3A4 activity of CD105$^+$ AT-MSC-derived hepatocytes. These data taken together indicate that CD105$^+$ AT-MSC-derived hepatocyte-like cells exhibit liver-specific functions similar to normal matured human hepatocytes.

Transplantation of CD105$^+$ AT-MSC-Derived Hepatocytes into Mice with CCl_4 Injury. Finally, we addressed our ultimate goal, namely, examining whether our generated hepatocytes were therapeutically applicable. We transplanted 5 × 10^5 CD105$^+$ AT-MSC-derived hepatocytes into CCl_4-injured mouse. Hematoxylin-eosin staining showed damage to the liver after a CCl_4 injection (Fig. 7a-c). Twenty-four hours after transplantation of undifferentiated CD105$^+$ AT-MSCs and CD105$^+$ AT-MSC-derived hepatocytes liver sections were examined by human specific albumin immunostaining (Fig. 7d-f) and revealed that CD105$^+$ AT-MSC-derived hepatocytes were incorporated into host livers. In those mice, some liver functions were improved such as ammonia concentration level, as well as a marker of the damaged liver-glutamic-pyruvic aminotransferase (GPT) (data not shown) level in the peripheral blood. Some human albumin-positive cells have been found in liver sections after implantation of undifferentiated CD105$^+$ AT-MSCs; however, these cells exhibited no typical hepatocyte morphology (Fig. 7b,e). In summary, our AT-MSC-derived hepatocyte-like cells are applicable for transplantation.

Discussion

Transplantation of hepatocytes might become easier, efficient, and safer than whole organ transplantation to cure patients suffering from end-stage liver dysfunction. The

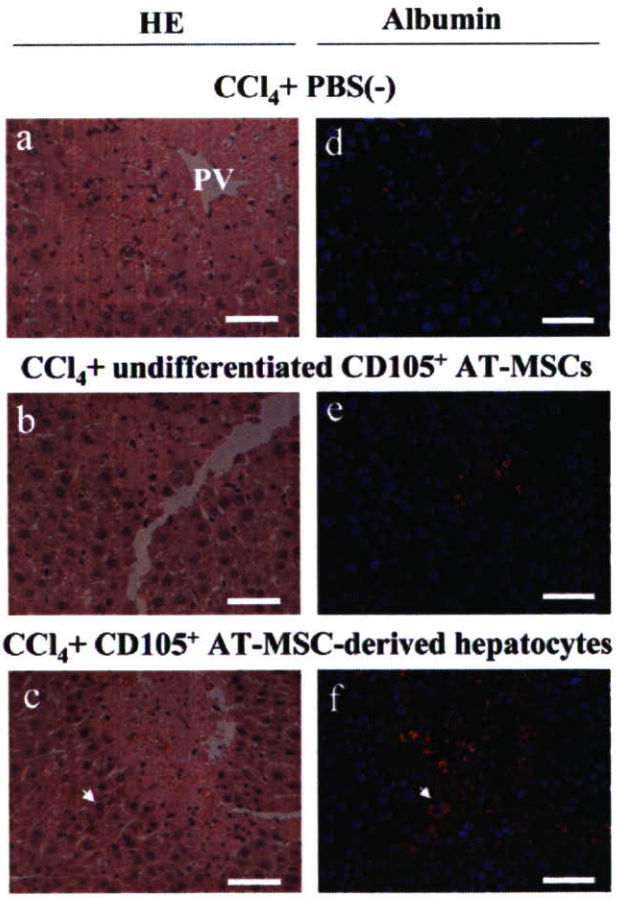

Fig. 7. Transplantation of CD105$^+$ AT-MSC-derived hepatocytes into mice with CCl_4 injury. Histological sections were stained with anti-human specific albumin antibodies. Hematoxylin Eosin (HE) staining of a liver section from CCl_4-treated mice administered with PBS (−) (n = 3) (a), HE staining of a liver section from CCl_4-treated mice 1 day after transplantation of undifferentiated CD105$^+$ AT-MSCs (n = 3) (b) and CD105$^+$ AT-MSC-derived hepatocytes (n = 3) (c). Panels (d), (e), (f) represent the subsequent human ALB immunostaining of serial sections of (a), (b), (c). The arrows point to the stained cells integrated with hepatic areas in clusters. PV, portal vein. Scale bars represent 50μm.

maintenance of hepatocyte function *in vitro* is difficult if not nearly impossible. The generation of stem cell-derived hepatocytes holds considerable promise for future clinical applications. The hepatogenic potential of MSCs from different sources has been previously described.[7-10] There have been two reports so far indicating the endoderm differentiation capacity of AT-MSCs (insulin-, somatostatin-, and glucagon-expressing cells[40] as well as ALB- and AFP-expressing hepatocyte-like cells with the ability to synthesize urea and uptake LDL[33]); however, the second reported system includes the use of DMSO, which has been shown to maintain hepatic morphology. In contrast, our system simulates *in vivo* endoderm development of the liver, as previously reported.[2,3] Concerning the donation procedure, isolation ratio, and minimal decline in donor health, we postulate that AT-MSCs represent an attractive tool for studies on stem cell therapy for the liver. MSCs from a predominant source, such as bone marrow, reveal a lower proliferation capacity and frequency than AT-MSCs,[28] and their frequency is influenced by age, gender, the presence of osteoporosis, and prior exposure to high-dose chemotherapy or radiation. Importantly, from 200ml of lipoaspirate, it is possible to obtain approximately 40-fold more stem cells than from 40 ml of marrow.[21] Even though adipose tissue provides a more heterogeneous population of stem cells, the potential number of AT-MSCs is sufficiently large to allow the selection/sorting of the multipotential fraction of AT-MSCs. The $CD105^+$ fraction from BM-MSCs has been reported to have more homogeneity and a greater ability to form CFU-Fs.[37-39] In order to select multipotent AT-MSCs, some scientists have used other isolation procedures[41]; we selected a $CD105^+$ fraction using magnetic beads. Insofar as the $CD105^+$ fraction of BM-MSCs is age-resistant,[39] sorting might be very useful because many patients with liver failure or hepatocarcinoma are elderly. Large amounts of fat are discarded during liposuction, and, in the future, this valuable resource could be used for the isolation of AT-MSCs, which might then be stored in each patient's cell bank.

We have demonstrated the ability of $CD105^+$ AT-MSCs to undergo hepatic differentiation, resulting in the achievement of functional and transplantable hepatocytes (60%-85%). Unfractionated AT-MSCs revealed hepatogenic potentiality as well; however, the ratio of differentiation was lower (20%-60%). The hepatogenic potentiality of AT-MSCs was confirmed by the detection of hepatic-specific markers and biochemical functions. The major protein produced by the hepatocytes, ALB, was synthesized and secreted into a medium at days 30 and 50 (subsequently: 5 and 14 mg/dl/4days/10^6 cells). The expression and activity of the microsome CYP enzymes involved in drug, xenobiotic metabolism, and sterol and bile acid synthesis indicates hepatocyte specificity. Generated from $CD105^+$ AT-MSCs, hepatocytes express CYP7A1, CYP1A1, CYP2C9, CYP3A4, and NADPH-cytochrome P450 reductase. Therefore, these findings suggest that there might be value in *in vitro* preclinical drug investigations.

We then implanted *in vitro* generated hepatocytes into CCl_4-injured nude mice and observed direct incorporation into the liver, which was confirmed by human albumin immunostaining. Our induction system does not require co-culturing, thus undesired cell-cell interactions are reduced; nevertheless, further studies examining the *in vivo* mechanism of homing, engraftment, and liver regeneration need to be performed in order to eliminate post-transplantation complications. We observed that some *in vivo* functions, such as the serum ammonia level and GPT, decrease after transplantation of undifferentiated AT-MSCs (data not shown) as well. These findings indicate the possibility of *in vivo* differentiation of AT-MSCs caused by a regeneration microenvironment. The subsequent interaction of transplanted undifferentiated MSCs with liver parenchyma cells also needs to be evaluated in the context of the promotion of fibrosis,[42] cancer, or liver dysfunction. Another topic of interest deserving evaluation is the correlation between factors such as donor age, cancer record, and the differentiation potentiality of stem cells. It should be emphasized that three independent donor-derived $CD105^+$ AT-MSCs showed similar hepatic differentiation ability. In the context of clinical application, the usage of AT-MSCs, which can be obtained from a patient's own tissue in contrast to highly potent ES cells, would eliminate many obstacles such as ethical issues, rejection and the risk of teratoma and tumor formation.

In summary, we have presented *in vitro* production of functional and transplantable hepatocytes from adipose tissue-derived mesenchymal stem cells. Our findings, combined with the development of tissue engineering technologies, may support stem cell-based therapy for liver injuries and for the establishment of a bioartificial liver.

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SPECIAL ISSUE REVIEWS—A PEER REVIEWED FORUM

Stem Cell Plasticity: Learning From Hepatogenic Differentiation Strategies

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Many studies on stem cell plasticity are challenging the concept that stem cells contain an intrinsically predefined, unidirectional differentiation program. This means that the developmental fate of a stem cell is dependent on the general potential of the cell (pre-determined stem cell fate) as well as on microenvironmental cues, such as stimuli from growth factors (stem cell niche). Here, we reviewed reports that examined the hepatocyte differentiation ability of stem cells from two different sources: embryonic stem cells and adult stem cells. All of those stem cells revealed the ability to give rise to hepatocyte-like cells using different induction strategies. However, it is still not clear which of those stem cells would be the best source for hepatocyte replacement or which would be the best protocol. We herein present the current knowledge regarding available protocols and factors used in order to obtain functional hepatocytes from stem cells. *Developmental Dynamics 236:3228–3241, 2007.* © 2007 Wiley-Liss, Inc.

Key words: stem cells; hepatocytes; plasticity; differentiation

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INTRODUCTION

Stem cells compose a "reservoir" of potential cells at various stages of development that can be used for the restoration and regeneration of damaged tissues and organs. Under proper conditions, stem cells may differentiate into specialized tissues and organs. They are self-sustaining and can replicate themselves for a long time. These unique features make them a promising tool for studies on therapy for diseases such as chronic liver disease, heart stroke, spinal injuries, stroke, Parkinson's disease, Alzheimer's disease, retinal degeneration, and diabetes mellitus.

Stem cells can be classified into two major categories according to their developmental status: embryonic and adult (postnatal). Each represents a

ABBREVIATIONS: AAT α-1-antitrypsin **AFP** α-fetoprotein **ALB** albumin **AT** adipose tissue **BM** bone marrow **BMP** bone morphogenic protein **CCl_4** carbon tetrachloride **CD** cluster differentiation **CK** cytokeratin **CYP** cytochrom p450 **DEX** dexamethasone **Dlk-1** delta-like protein 1 **DMN** dimethylnitrosoamine **DMSO** dimethylsulfoxide **EB** embryoid body **ECM** extracellular matrix **EGF** epidermal growth factor **EPCAM** epithelial cell adhesion molecule **ES** embryonic stem **FACS/MACS** fluorescence/magnetically activated cell-sorting **FBS** fetal bovine serum **FGF** fibroblast growth factor **FISH** fluorescence in situ hybridization **GATA** GATA binding protein **GFP** green fluorescent protein **GVHD** graft-versus-host disease **G6P** glucose-6-phosphatase **HAT** histone acetyltransferase **HDAC** histone deacetylase **HGF** hepatocyte growth factor **HIFC** hepatic induction factor cocktail **HNF/FOXA** hepatocyte nuclear factor/forkhead box **HSC** hematopoietic stem cells **ICG** indocyanin-green **IGF** insulin-like growth factor **ITS** insulin/transferrin/selenium **LDL** low density lipoprotein **LIF** leukemia inhibitory factor **MSC** mesenchymal stem cell **NCAM** neural cell adhesion molecule **NGF** nerve growth factor **OC** oval cell **OsM** oncostatin M **RA** retinoic acid **STM** septum transversum mesenchyme **TAT** tyrosine aminotransferase **TDO2** tryptophan-2,3-dioxygenase **TGF** transforming growth factor **TNF** tumor necrosis factor **TTR** transthyretin **UCB** umbilical cord blood.

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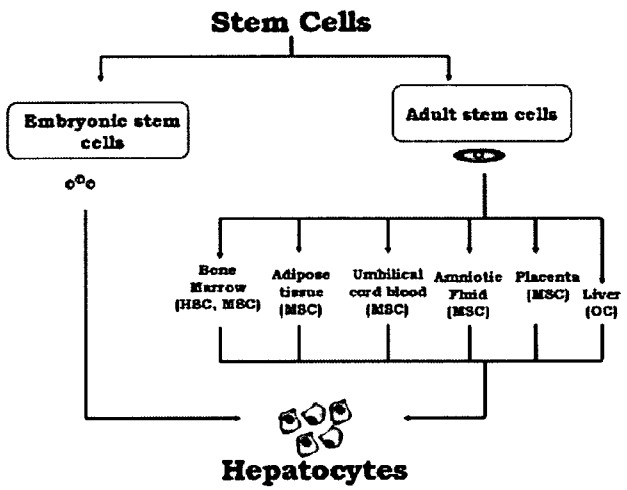

Fig. 1. Hepatic stem cells. The stem cells have already been differentiated in vivo and/or in vitro into hepatocyte-like cells. Embryonic stem (ES) cells, stem cells, and adult stem cells, such as mesenchymal stem cells (MSCs), or hematopoietic stem cells (HSCs) from bone marrow, adipose tissue, placenta, amniotic fluid, and umbilical cord blood are shown. In addition, the detection of hepatocyte progenitors/oval cells (OCs) within the adult liver has been shown in various reports.

diverse differentiation potential status and a different potential application. The liver is one target for which the development of stem cell-based therapy is of great significance. Even though an injured liver is highly regenerative, many debilitating diseases lead to hepatocyte dysfunction and organ failure. Treatments such as resection are usually arrested because of too little remaining liver function. Liver transplantation is the only effective treatment for severe liver injuries. However, because of organ rejection and the limited number of donors, alternative therapeutic approaches are needed. Stem cells could offer a potentially unlimited and minimally invasive source of cells for hepatocyte replacement and liver regeneration.

Many types of stem cells have been differentiated in vivo and/or in vitro into hepatocyte-like cells (Fig. 1) using different induction strategies. However, there is no defined strategy to produce hepatocytes from stem cells, and more study is needed.

In this review, we present the current status of information regarding hepatocyte differentiation protocols using stem cells from different sources. We also highlight the superior strategies regarding the functions of generated hepatocytes and the best types of stem cells for liver regeneration.

EMBRYONIC DEVELOPMENT OF THE LIVER

Based on an actual understanding of embryonic development of the liver (Lemaigre and Zaret, 2004; Zhao and Duncan, 2005), many studies in vivo and in vitro have indicated the therapeutic potential of stem cells for liver regeneration. Knowledge of embryonic liver development basically includes studies on rodent embryos, but, recently, many studies have been done on chicks, zebrafish, and frogs. During mouse development, the induction of hepatic genes occurs in a segment of the definitive endoderm at about 8.5 days of gestation (Lemaigre and Zaret, 2004; Zhao and Duncan, 2005) (Fig. 2). The induction requires signaling cues from cardiogenic mesodermal cells in the form of fibroblast growth factors (FGFs) and from septum transversum mesenchyme (STM) in the form of bone morphogenic proteins 2 and 4 (BMP2, BMP4). Afterwards, the endodermal cells start to proliferate and bud into the STM, where there is essential interaction with endothelial cells. However, the role of endothelial cells is yet unknown. Hematopoietic stem cells (HSCs) also have a significant role; these cells migrate into the liver bud and proliferate there, apparently emitting the signal for further liver development. When the hepatic endoderm is specified and the liver bud is growing, the cells within the so-called hepatoblasts are bipotential and capable of differentiating into hepatocytes and bile duct cells (cholangiocytes).

There are numerous signals required for the growth of the fetal liver and the prevention of apoptosis (Lemaigre and Zaret, 2004; Zhao and Duncan, 2005). Each of those cues generated by STM, cardiac mesoderm, endothelial cells, HSCs, and a microenvironment rich in extracellular matrix (ECM) is crucial for liver development.

DEVELOPMENT OF DIFFERENTIATION CONDITION MEDIA FOR HEPATOCYTE INDUCTION IN VITRO

Numerous cytokines and growth factors have been shown to have a potent effect on hepatic growth and differentiation in vitro. They include the hepatocyte growth factor (HGF), epidermal growth factor (EGF), transforming growth factor (TGF)-β, acidic FGF/FGF1, insulin, insulin-like growth factor (IGF), and oncostatin M (OsM). It is noteworthy that for each of the cytokines/growth factors, the timing, dosage, and combinations must be carefully chosen according to the stem cell type and desired effect. Among the non-proteinaceous chemical compounds that are known to promote hepatic differentiation and/or maintenance in vitro are dexamethasone (DEX), retinoic acid (RA), sodium butyrate, nicotinamide, norepinephrine, and dimetylsulfoxide (DMSO). Sequential treatment with growth factors that mimic in in vivo development of the liver has been demonstrated. Lastly, the important factor during liver development, growth, and regeneration is ECM, which also undergoes extensive remodeling under pathological conditions. Hence, the introduction of appropriate ECM (collagen, fibronectin) during in vitro differentiation may enhance the differentiation process.

Specific transcription factors of early endoderm, bipotential cells, and, finally, hepatocytes are expressed at specific stages of development. Early differentiation markers include the

hepatocyte nuclear factor (HNF)-3beta/forkhead box A2 (FOXA2), GATA binding protein 4/6 (GATA4/6), α-fetoprotein (AFP), albumin (ALB), and cytokeratin 8/18 (CK8/18), and there are other factors, such as α-1-antitrypsin (AAT), tyrosine aminotransferase (TAT), transthyretin (TTR), tryptophan-2,3-dioxygenase (TDO2), glucose-6-phosphatase (G6P), and a sort of cytochrom P450 (CYP)-metabolizing enzyme. When specific markers are expressed, the biochemical and metabolic functions are of importance. These include albumin and urea synthesis, glucose production, glycogen storage ability, and CYP activities.

HEPATIC STEM CELLS: EXTRINSIC AND INTRINSIC SOURCES

The liver involves three distinct sources of cells that participate in regeneration. The first source is mature hepatocytes, which respond rapidly to liver injury. They represent unipotent stem cell–like properties, although their division potential is limited. It is important to mention here that, for stem cell–based therapy, the primary culture of hepatocytes does not replicate sufficiently in vitro to produce the number of cells necessary for transplantation and does not maintain its properties in vitro.

The cell source, which is activated when extensive and chronic damage occurs, is located within the biliary tree and gives rise to bipotential so-called oval cells (OCs), which can differentiate into hepatocytes and biliary epithelial cells (cholangiocytes) (Beltrami et al., 2007; Evarts et al., 1987; Farber, 1956; Fausto, 2004; Fougere-Deschatrette et al., 2006; Herrera et al., 2006; Kojima et al., 2005; Shafritz et al., 2006; Wang et al., 2003; Yovchev et al., 2007; Zhou et al., 2007). The term "oval cell" was introduced by Farber (1956) who found non-parenchymal cells with the characteristic morphological appearance after treating rats with carcinogenic agents. Farber believed that OCs are not the hepatocyte progenitors. However, the group of Thorgeirsson showed evidence of the bipotentiality of OCs (Evarts et al., 1987), and other authors confirmed their observation (Fougere-Deschatrette et al., 2006). The existence and profile of OCs in adult liver is still debated. The isolation of such progenitors is difficult due to the lack of surface markers. OCs express the markers of biliary epithelial cells (CK 7, CK 19, and OV-6), immature fetal hepatoblasts (AFP, ALB, gamma-glutamyltranspeptidase [GGT]), hematopoietic stem cells (CD34, CD90, Sca-1, CD117, and the flt-3 receptor), and markers not expressed on other types of cells, for example, chromogranin A, the neural cell adhesion molecule (NCAM), and delta-like protein 1 (Dlk-1). Information relative to surface markers on progenitors within the adult liver is constantly increasing (Herrera et al., 2006; Yovchev et al., 2007). Herrera et al. isolated hepatic progenitors from adult human liver expressing mesenchymal stem cell markers ($CD29^+$, $CD73^+$, $CD44^+$, $CD90^+$, $CD34^-$, $CD45^-$, $CD11^-$, and $CD133^-$) together with hepatocyte-specific markers (ALB^+, AFP^+, $CK8^{low+}$, and $CK18^{low+}$) (Herrera et al., 2006). Beltrami et al. isolated multipotent stem cells from adult human liver which had the characteristics of pluripotent stem cells (Beltrami et al., 2007). Rogler's research group identified transit bipotential cells ($NCAM^+$, $CK\ 19^-$, and $HepPar\ 1^-$); when these cells became committed to cholangiocytes, they expressed: $NCAM^+$, $CK\ 19^+$, and $HepPar\ 1^-$, and while committed to hepatocytes, they expressed: $NCAM^-$, $CK\ 19^-$, and $HepPar\ 1^-$ (Zhou et al., 2007). Miyajima's research group has documented several important molecules responsible for the differentiation and maturation of fetal hepatocytes (Kojima et al., 2005; Watanabe et al., 2007). These findings make the isolation of liver progenitors easier. However, the number of OCs in the normal liver is extremely low; therefore, many researchers have characterized the hepatoblasts from fetal liver (Dan et al., 2006; Nierhoff et al., 2007). Fausto's research group determined the immunophenotype of fetal liver stem cells as $CD34^+$, $CD90^+$, $CD117^+$, the epithelial cell-adhesion molecule $(EPCAM)^+$, $c\text{-}met^+$, $SSEA\text{-}4^+$, $CK18^+$, $CK19^+$, ALB^-, AFP^-, $CD44h^+$, and $vimentin^+$ (Dan et al., 2006).

A third source is exogenous stem cells, which may be derived from bone marrow (BM) or other organs or tissues. How these cells from different sources integrate to achieve a homeostatic balance remains unexplained. Thus, the question of whether those stem cells transdifferentiate into hepatocytes under patho-physiological conditions or only in experimental strategies remains to be answered (Fausto, 2004; Fausto et al., 2006; Shafritz et al., 2006). In addition, the question of how homeostasis is disrupted by pathologic conditions also needs to be answered. Seeking a proper inner balance is also important. Answering these questions is essential for understanding how the body functions. At present, the answers to the above questions remain elusive; however, below are discussions of the various stem cells and their hepatogenic potentials.

STEM CELLS FOR HEPATOCYTE DIFFERENTIATION

Embryonic Stem Cells

Embryonic stem (ES) cells were first isolated from mouse embryos (Evans and Kaufman, 1981). Pluripotent ES cells isolated from the inner cell mass of blastocysts are capable of giving rise to cells found in all three germ layers of the embryo. They are considered to have the greatest range of differentiation potential. The isolation of human ES cells several years ago expanded the potential of ES cells as a source of cells for not only developmental studies but also stem cell–based therapy (Thomson et al., 1998).

The pluripotency of mouse ES cells has been proven in vivo and in vitro. In vivo, an injection of ES cells generates teratomas harboring derivatives of all three embryonic germ layers. In vitro, after removal from the feeder layer or from the leukemia inhibitory factor (LIF), mouse ES cells aggregate in a suspension to form spheroid clumps of cells called embryoid bodies (EBs). The cells within the EBs differentiate spontaneously and express molecular markers specific for the three embryonic germ layers.

Here, we present examples of hepatic induction (in vitro and in vivo) using mouse and human ES cells utilized by different groups (Fig. 3) (Lavon and Benvenisty, 2005; Ter-

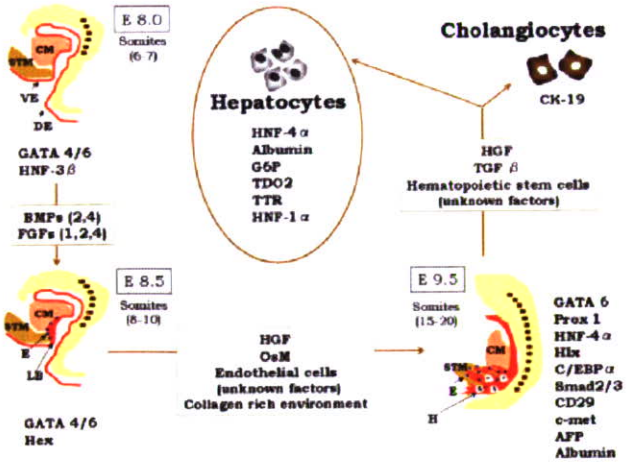

Fig. 2. Early stages of liver development in mouse embryo. By day E8.0, in response to inductive cues derived from cardiogenic mesoderm (CM, pink) and septum transversum mesenchyme (STM, brown), the ventral definitive endoderm (VE) undergoes direct hepatic fate specification. By day E8.5, the specified hepatic endoderm forms a liver bud (LB, red), which expresses several liver markers, including albumin. Endothelial cells (E, blue) surround the bud and are necessary for its expansion. By day E9.5, hepatoblasts, which form cords, invade the septum transversum mesenchyme, the source of mesodermal stellate cells, and endothelial cells (E, white), which form vessels. Transcription factors responsible for specific liver development are listed. The factors directing the developmental stages are listed on the arrows. CM, cardiogenic mesoderm; STM, septum transversum mesenchyme; VE, ventral endoderm; DE, dorsal endoderm; LB, liver buds; E, endothelial cells/angioblasts; H, hepatoblasts; HSCs, hematopoietic stem cells; BMP, bone morphogenic protein; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; OsM, oncostatin M; TGF, transforming growth factor; CD29, integrin 1β; Hex, hematopoietically expressed homeobox; Prox-1, prospero related homeobox 1; Hlx, H2.0-like homeobox gene; C/EBPα, CCAAT enhancer binding protein α G6P, glucose-6-phosphatase; CK, cytokeratin; TDO2, tryptophan-2,3-dioxygenase; TTR, transthyretin; AFP, αfetoprotein.

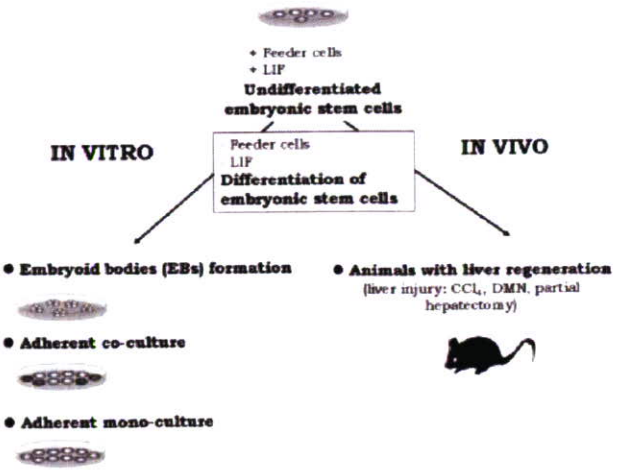

Fig. 3. Strategies of hepatic induction from embryonic stem (ES) cells. In vivo strategies include the use of factory farm animals with liver injury or liver regeneration. In vitro hepatic differentiation strategies include the formation and co-culture of embryoid bodies (EBs) and adherent monoculture models.

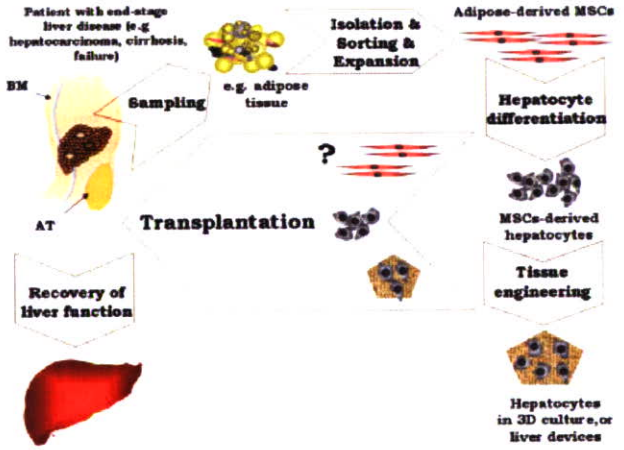

Fig. 4. Schematic representation of mesenchymal stem cell–based therapy. MSCs can be obtained from a patient's own tissues (bone marrow [BM], adipose tissue [AT]), isolated, purified (MACS, FACS system), and induced into a hepatic lineage. The generated hepatocytes may be directly implanted or, after using tissue-engineering technologies, transplanted as liver devices back into the patient. The contribution of undifferentiated MSCs in liver regeneration is still unknown; however, the usage of undifferentiated MSCs is not excluded.

amoto et al., 2005). In vitro strategies include differentiation through EB formation, co-culture, and mono-culture systems, while in vivo strategies utilize animals with liver injury or liver regeneration. All progress regarding ES cell differentiation is summarized in Table 1.

Mouse ES cells.

In vivo differentiation. ES cells have a propensity to develop teratomas when implanted into animals. Teratomas form tumors and finally cause the death of the host animal, which severely limits their clinical use. Chinzei et al. (2002) demonstrated that cells isolated from EBs nine or more days after LIF removal expressed a panel of hepatic markers and were capable of producing albumin and urea. After transplantation into partial hepatectomy of female mice pretreated with 2-acetylaminofluorene, ES cell–derived cells survived and expressed ALB, whereas teratomas were found in mice transplanted with ES cells or EBs up to day six. These authors demonstrated that, while ES cells always developed teratomas in recipient mice, the incidence was decreased with implantation of EBs; but this improvement depended on the culture period of the EBs. The in vivo differentiation of ES cells carrying green fluorescent protein (GFP) in the AFP locus was achieved by Yin et al. (2002). They selected a subpopulation of GFP-positive and AFP-expressing cells from differentiating in vitro ES cells. After transplantation into partially hepatectomized lacZ-positive ROSA26 mice, GFP-positive cells engrafted and differentiated into lacZ-negative and ALB-positive cells. In this case, no teratomas were observed. Furthermore, using an animal with an injured liver (regenerative condition), Yamamoto et al. (2003) reported efficient differentiation of ES

TABLE 1. Differentiation Potential of ES Cells Towards Hepatocyte-Like Cells[a]

Differentiation protocol	Hepayocyte specific markers/functions	Reference
Mouse ES cells *in vivo*		
EBs into hepatectomized mice, FGF1+FGF2, HGF, OsM+DEX	ALB, AFP, TAT, Urea synthesis	Chinzei R et al., [2002]
ES cells into hepatectomized mice, FACS sorting of AFP-GFP$^+$ fraction	ALB, AFP	Yin Y et al., [2002]
Transplantation of ES cells into CCl_4-treated mouse	ALB, TDO2, AAT, TTR, glucose production, ammonia detoxification, bilirubin metabolism	Yamamoto H et al., [2003]
ES cells into CCl_4-intoxicated mice	ALB, AAT, CK18, HNF4α, Dlk	Moriya K et al., [2007]
Mouse ES cells *in vitro*		
EBs / FGF1 / HGF / OsM+DEX	ALB, G6P, TAT, TTR, AAT	Hamazaki T et al., [2001]
EBs formation	AFP, ALB, HNF-4α, Transfferin	Jones EA et al., [2002]
EBs, RA, HGF, NGFβ	ALB, G6P, TTR, HNF-4α, ATT	Kuai XL et al., [2003]
EBs formation	AFP, ALB, TTR, AAT, G6P, GST, FOXA1/2/3	Miyashita H et al., [2002]
EBs formation	AFP, ALB, TTR, AAT, TDO2, CPS-1, PEPCK, LST1, FOXA2, ICG uptake	Yamada T et al., [2002]
HNF-3β transfected ES cells / EBs, FGF2+DEX + nicotinamide	ALB, C3, P450, PEPCK, PXMP1-L, CK18, Urea & lipid synthesis, PAS staining	Ishizaka S et al., [2002]
HNF-3β transfected ES cells / EBs, FGF2	AFP, ALB, TTR, AAT, TDO2, PEPCK, FOXA2, HNF-4α, ICG uptake	Kanda S et al., [2003]
Co-cultured with mesenchymal cell, nicotinamide + DEX + dHGF + OsM	AFP, FOXA2, TAT, TDO2, G6P, ALB, PAS staining, ammonia detoxification	Ishii T et al., [2005]
Adherent monoculture / HGF + FGF1 + FGF4 / OsM + DEX	ALB, TTR, TAT, G6P, TDO2, CK8, LST-1, CPS1, PEPCK, CYP1A1, FOXA2, HNF-4α, CK-18, ALB & fibrinogen synthesis	Teratani T et al., [2005]
EBs + Activin A. FACS sorting. High density culture: BMP-4 + FGF2 + Activin A	FOXA2, ALB, AFP, TAT, CPS1, CYP7A1, CYP3A11, ALB production, PAS staining	Gouon-Evans et al., [2006]
EBs, nicotinamide + DEX, FACS sorting of GFP + cells	ALB, AFP, G6P, TDO2, HNF-4α, AAT, CYP2E, ABCG2, Cx32	Heo J et al., [2006]
EBs, Activin A + FGF2. dHGF + non-parenchymal liver cell-derived growth factors + DEX	ALB, CK18, TAT G6P, FOXA2, HNF-4α, CYP7A1, CK19, AFP, ammonia detoxification, glucose production, metabolism of lidocaine and diazepam	Sato-Gutierrez et al., [2006, 2007]
Human ES cells		
EBs / serum free medium supplemented with NGFβ or HGF	AFP, AAT (by NGFβ and HGF), ALB (by NGFβ)	Schuldiner M et al., [2000]
EBs / HCM with: sodium butyrate + DMSO	ALB, AAT, AGRP, HNF-4α, TTR, C/EBPα, C/EBP β, CK18, CK8, ALB synthesis, CYPIA2 activity, PAS staining	Rambhatla L et al., [2003]
EBs / Matrigel + biodegradable scaffolds, Activin A or IGF	ALB, AFP	Levenberg S et al., [2003]
Activin A + ITS / FGF4 + BMP-2 / HGF/OsM + DEX	AFP, ALB, CK8 CK18, G6P, AAT, HNF-4α, PEPCK, TDO2, TAT, CYP7A1, CYP3A4, CYP2B6, LDL uptake, ICG uptake, PAS staining, PROD	Cai J et al., [2007]
DMSO / HCM + HGF + EGF / HCM + HGF + OsM	AFP, ALB, AAT, TDO2, HNF-4α, C/EBPα, TTR, Hepar 1, CYP3A4 activity, PAS staining, ICG uptake & excretion	Hay DC et al., [2007]

[a]GST, glutathione -S-transferase; CPS-1, carbamyl phosphate synthetase 1; PEPCK, phosphoenolpyruvate carboxykinase; LST1, liver specific organic anion transporter 1; C3, complement 3; PXMP1-L, peroxisomal membrane protein 1-like protein; dHGF, HGF with deletion of 5 amino acids; ABCG2, ATP-binding cassette subfamily G member 2; Dlk-1, delta-like protein 1; Cx32, Gap junction protein; c-Met, Met-protooncogene/HGF receptor; ApoE, apolipoprotein E.

cells into hepatocytes with therapeutic properties. They harvested a GFP$^+$ fraction of in vivo differentiated ES cells and characterized them. Those cells revealed hepatocyte-specific markers and therapeutic potential (Table 1). Similar studies with in vivo transplantation of GFP$^+$ ES cells were conducted by Moriya et al. (2007). These authors reported the presence of ES cells within the liver on days 10 and 20, after liver injury; however, on day 30 they could not detect any. In addition, until day 30, no tumors were detected, and fibrosis decreased.

TABLE 2. Differentiation Potential of Mesenchymal Stem Cells Towards Hepatocyte-Like Cells[a]

Source/species	Differentiation protocol	Hepatocyte specific markers/functions	Reference
BM-MSCs			
Human, Mouse, Rat	linoleic acid + DEX + ascorbic acid 2-phosphate + EGF, Matrigel, HGF + FGF4	HNF-1α, ALB, AFP, CK18, TTR, CK8, urea synthesis, PAS staining, LDL-uptake, CYP activity	Schwartz RE et al., [2002]
Human	Transplantation of MSCs into Allylalcohol intoxicated rats	ALB, AFP, CK18	Sato Y et al., [2005]
Human	EGF + FGF2 / HGF + FGF2 + nicotinamide / OsM + DEX	AFP, ALB, CK18, TAT, TDO2, G6P, HNF-4α, urea synthesis, CYP activity. LDL-uptake, PAS staining	Lee KD et al., [2004]
Human	FGF4 + HGF + DEX	AFP, ALB, CK18, HNF-1α, CYP1A1, CYP2B1, urea synthesis, PAS staining, CYP activity	Snykers S et al., [2006]
Human	Co-culture with rat liver, HGF/OsM + DEX + nicotinamide	AFP, CK18, ALB, TDO2, TAT, AAT, HNF-4α, urea synthesis, PAS staining, CYP activity	Ong SY et al., [2006]
Rat	HGF + DEX	AFP, ALB	Oyagi S et al., [2006]
Rat	Transplantation of MSCs into CCl_4 and DMN-intoxicated rats	Liver fibrosis was improved by MSC treatment	Zhao DC et al., [2005]
UCB-MSCs			
Human	HGF + DEX + ITS / IMDM + OsM + DEX	AFP, TAT, ALB, CK18, LDL-uptake	Lee OK et al., [2004]
Human	HGF + DEX + ITS / OsM + DEX	ALB, CK18, AFP, TAT, HGF, PEPCK. CPS-1, LDL-uptake	Hong SH et al., [2005]
Human	EGF + FGF2 / HGF + FGF2 + nicotinamide/OsM + DEX	AFP, ALB, CK18, TAT, TDO2, G6P, HNF-4α, urea synthesis, CYP activity, LDL-uptake. PAS staining	Lee KD et al., [2004]
Human	FGF4 + HGF + FBS	CK18, ALB, AFP, urea synthesis, PAS staining	Kang XQ et al., [2005]
Human	FACS sorted β2m-c-Met$^+$ cells, Co-culture with liver non-parenchymal cells + HGF + DEX	AFP, ALB, CK18, CYP1B1, CK8, urea synthesis, ICG-uptake	Wang Y et al., [2005]
AT-MSCs			
Human	DEX + EGF + ascorbic acid + HGF, OsM	AFP, ALB, urea synthesis, LDL-uptake	Seo MJ et al., [2005]
Human	EGF + FGF2 / HGF + FGF2 + nicotinamide/ OsM + DEX	ALB, AFP, CK18, CYP3A4, CYP2E1, c/EBPβ, HNF-4α	Talens-Visconti R et al., [2006]
Human	MACS sorted CD105$^+$ AT-MSCs / HGF + FGF1 + FGF4 / OsM + DEX	ALB, AFP, TTR, HNF-4α, TDO2, CK18, TTR, ammonia detoxification, PAS staining. LDL-uptake	Banas A et al., [2007]
Amniotic fluid MSCs			
Human, Mouse	HGF + OsM + DEX + FGF4	ALB, AFP, HNF-4α, MDR1, urea synthesis	De Coppi P et al., [2007]

[a]K2m, K2 -microglobulin; MDR, multidrug resistance.

In vitro differentiation.

EB formation. EB cells mature by the processes of spontaneous differentiation and cavitation and acquire markers for a variety of differentiated cell types. Dissociating EBs and plating the differentiated cells as a monolayer yields many cell lineages. Several growth factors and transcription factors have been shown to be capable of directing the differentiation of mouse ES cells. Different matrix proteins may dramatically influence the generation and survival of these cells (Flaim et al., 2005). Usually, collagen is used as the matrix for culturing the cells towards a hepatic lineage because the liver bud proliferates and migrates into STM, which is composed of loose connective tissue containing collagen. Hamazaki et al. (2001) demonstrated that mouse EBs can be differentiated into hepatocyte-like cells, when cultured on collagen-coated plates, with early (FGFs), middle (HGF), and late (OsM, DEX, and insulin+transferring+selenium [ITS]) differentiation stage factors. Jones et al. (2002) confirmed these observations by culturing ES cells carrying a gene trap vector insertion into an ankyrin-repeat-containing gene. This modification induces beta-galactosidase expression when hepatocyte differentiation begins. Kuai et al. (2003) reported that the nerve growth factor (NGF)-β also promotes hepatic differentiation, which

is increased in the presence of HGF and RA.

Miyashita et al. (2002) also demonstrated in vitro hepatic differentiation through the formation of EBs without using hepatocyte-specific cytokines. Yamada et al. (2002), using an ES cell line carrying the enhanced GFP gene, identified indocyanin-green (ICG) uptake by cells differentiated from mouse EBs and reported the presence of liver-specific markers using RT-PCR and immunocytochemistry. Ishizaka et al. (2002) demonstrated that when transfected with HNF-3β, mouse ES cells were able to differentiate into hepatocytes with liver-specific metabolic functions after stimulation with FGF2, DEX, L-ascorbic-2-phosphate, and nicotinamide. The same genetically modified ES cells were differentiated through EB formation into hepatic-like cells by Kanda et al. (2003) using an attached culture system. Importantly, later on, they discovered that HNF-3β transfected ES cell–derived hepatic-like cells have infinite proliferating potential, resulting in tumor formation and, finally, the death of the animals after transplantation.

EBs offer the advantage of providing a three-dimensional (3D) structure, which enhances cell–cell interactions that may be important for hepatocyte development. However, the complexity of EBs is a problem because of the cytokines and inducing factors generated within these structures that induce differentiation of other cell lineages as well. Until now, none of the hepatic differentiation systems based on EB formation has revealed sufficiently efficient induction of functional hepatocytes for an experimental therapeutic study.

A direct co-culture. A co-culture strategy was used by Ishii et al. (2005). They produced in vitro mature hepatocytes from ES cells (carrying GFP in the AFP locus) entirely via isolation of AFP-producing cells and subsequent maturation of these cells by co-culture with Thy1-positive (CD90) mouse fetal liver cells. These hepatic-like cells produced and stored glycogen and neutralized ammonia.

In a co-culture system, differentiation takes place in contact with assisting cells (fetal liver cells or stromal cells) because of the presence of differentiation factors; however, undefined factors produced by these supportive cells may influence the differentiation of ES cells into other than hepatocyte cell types. An additional disadvantage is the difficulty entailed in separating the ES cell–derived hepatocytes from assisting cells.

An adherent mono-culture. Teratani et al. (2005) showed that ES cells can differentiate into functional hepatocytes without requiring EB formation, in vivo transplantation, or a co-culture system. By comparing the genes in CCl_4-treated and untreated normal mouse liver, their group identified a hepatic induction factor cocktail (HIFC). ES cell–derived hepatocytes, after HIFC induction, expressed multiple liver-specific markers as well as hepatocyte nuclear factors. In addition, AFP expression appeared in the early and TDO2 at the late stage of differentiation, which means that ES cell–derived hepatocytes mimic normal liver development. The functionality shown by their ability to produce glucose and clear ammonia and to synthesize urea displays the characteristics of mature hepatocytes. In addition, in this case, no teratomas were observed, and karotype analysis showed a normal chromosome number. Most importantly, the transplantation of ES cell–derived hepatocytes in mice with cirrhosis generated by dimethylnitrosoamine (DMN) showed a significant therapeutic effect. On the basis of the HIFC differentiation system, Yamamoto et al. (2005) compared the gene expression profile of ES cell–derived hepatocytes with adult mice liver and found significant similarities in a gene expression profile. Using small interfering RNA (siRNA) technology, they found that HNF-3β/FOXA2 is essential for in vitro hepatic differentiation, which also indicates that this system progresses via endoderm differentiation, imitating hepatic development in vivo: step (0) pluripotent ES cells, step (1) endoderm specification (HNF-3β), step (2) immature hepatocytes (AFP, ALB), and step (3) mature hepatocytes (ALB, TDO2).

Selection of ES cell-derived hepatic progenitors. BMP-4 has been found to be crucial in the hepatic specification of the mouse ES–derived definitive endoderm (Gouon-Evans et al., 2006). When treated with Activin A in serum-free condition, mouse ES cells generate an endoderm progenitor population that co-expresses T Brachyury, HNF-3β, c-kit/CD117, or c-kit/CD117 and CXCR4. Those progenitors, when treated with BMP-4, FGF2, and Activin A, developed into a highly enriched population (45–75%) of functional hepatocyte-like cells, positive for AFP and ALB, producing albumin and storing glycogen. The authors demonstrated that BMP-4 is required to induce a hepatic fate in the GFP-Brachyury$^+$/CD4-FOXA2^{high}/c-kit^{high} embryoid body population. Those progenitors, when treated with FGF2 and Activin A, developed into a highly enriched population of functional hepatocyte-like cells, producing albumin. After transplantation into injured livers, they engrafted and proliferated within the parenchyma. Another study concerning hepatic progenitors derived from ES cells was presented by Heo et al. (2006). The authors used mouse ES cells transfected with the GFP reporter gene regulated by the ALB promoter. After culturing these cells in a serum-free chemically defined medium without growth factors or in feeder layers, they observed the formation of EBs and hepatic differentiation. The FACS-sorted GFP$^+$ fraction developed into functional hepatocytes without evidence of cell fusion and participated in the repair of liver injury. Moreover, the GFP$^+$ fraction also differentiated into biliary epithelial cells. This event confirms the success of their selection of progenitor cells. Impressive studies have been published by Sato-Gutierrez et al. (2006). Their differentiation protocol (specifically described by Sato-Gutierrez et al., 2007) utilized EB formation and then treatment with Activin A and FGF2. After that, it required indirect co-culturing with human liver non-parenchymal cell lines (cholangiocytes, stellate cells, and liver endothelial cells) and treatment with HGF, DMSO, and DEX (Sato-Gutierrez et al., 2006). The final step involved sorting of the most hepatocyte-reminiscent cell population (GFP$^+$ cells under the control of the ALB promoter). Those cells displayed metabolic capacity (albumin production and ammonia detoxification) and rescued animals with 90%-hepatectomized livers.

Human ES Cells.

Experiments involving human ES cells are limited because of obvious ethical concerns. Nonetheless, the number of published reports using human ES cells is rising. Schuldiner et al. (2000) showed the potential of human ES cells to differentiate into three embryonic germ layers after stimulation with different growth factors. EBs were dissociated and plated onto fibronectin-coated dishes and treated with growth factors, none of which induced differentiation into any specific cell type (Schuldiner et al., 2000). Rambhatla et al. (2003) used sodium butyrate to induce hepatocyte differentiation in human ES cells through EB formation. The characteristics of hepatocyte morphology and glycogen accumulation have been observed; however, sodium butyrate induced significant cell death. Levenberg et al. (2003) used biodegradable scaffolds of PLGA-poly(lactic-co-glycolic acid) and PLLA-poly(L-lactic acid) to induce tissue-like structures after seeding ES cells or EBs, and they found hepatocyte differentiation after stimulation with Activin A and IGF. Fourteen days after the implantation of 2-week-old constructs into SCID mice, immunostaining analysis of CK and AFP indicated that the implanted constructs continued to express these human proteins. Cai et al. (2007) used a strategy of sequential treatment (Activin A+ ALB/Activin A + ITS/ FGF4 + BMP-2/ HGF/OsM + DEX) under a serum-free condition. The generated hepatocytes revealed hepatocyte-specific functions. In addition, they were readily infected by the human immunodeficiency hepatitis C virus pseudotype. Another report also showed effective sequential treatment of human ES cells using a conditioned medium supplemented with FGF2, an unconditioned medium supplemented with DMSO, then HCM supplemented with HGF and EGF, and finally HCM supplemented with HGF and OsM (Hay et al., 2007).

Despite the great potential of ES cells, their use has been limited because of difficulty regarding their availability, problems related to histocompatibility, and ethical issues.

In addition, there is a problem with post-transplantation uncontrolled differentiation followed by tumorigenesis. Such handicaps might be sidestepped in the future by somatic cell nuclear transfer of a patient's own skin cells into donated oocytes (Fu, 2007; Gurdon et al., 2003). Notably, genetic manipulations on mouse fibroblasts in order to achieve pluripotency have succeeded (Takahashi and Yamanaka, 2006). The authors transfected mouse embryonic fibroblasts with Oct3/4, Sox2, c-Myc, and Klf4. Afterwards, they generated ES cells and cloned animals from fertilized mouse eggs (Okita et al., 2007). The use of ES cell–like cells (induced pluripotent stem iPS cells) instead of human ES cells might provide wide applications in the field of regenerative medicine. Additionally, donor oocyte mitochondria may present another technical hurdle since they will put non-self antigens in the nuclear transfer cells (Bowles et al., 2007; Harvey et al., 2007).

Further investigations concerning genomic stability, differentiation fidelity, and cellular "reprogramming" need to be performed in order to verify the safety of using ES cell.

Adult Stem Cells

Nearly all postnatal organs and tissues contain populations of stem cells, which have the capacity for renewal after trauma, disease, or ageing. In adults, there is a spectrum of stem cells with a different scale of quantity and potentiality (uni-, di-, tri-, and multi-potent). They are ready to receive signals from circulating blood to control homeostasis. Some of the adult stem cells have already shown their ability for hepatocyte differentiation (Fig. 1). Initially, research mainly focused on bone marrow (BM) as a source of stem cells for liver regeneration when its contribution to liver regeneration in vivo was described. Petersen et al. (1999) showed that the transplantation of unfractionated male BM into the livers of lethally irradiated female rats, whose livers had been injured by 2-acetylaminofluorene and CCl_4, rescued the animals from radiation-induced BM ablation and simultaneously produced small numbers of BM-derived hepatic stem cells. Later on, many studies were performed to confirm the contribution of these cells to liver regeneration on unfractionated and fractionated BM-derived stem cells (Alison et al., 2000; Lagasse et al., 2000; Petersen et al., 1999; Theise et al., 2000a,b). However, some researchers postulate that this event is not transdifferentiation but, rather, a nuclear transfer via cell fusion between BM-derived stem cells and recipient hepatocytes (Vassilopoulos et al., 2003; Wang et al., 2003). At the present time, some researchers have difficulty differentiating BM stem cells into liver cells (Rountree et al., 2007; Vig et al., 2006) and others claim that HSCs transdifferentiate towards hepatocytes without cell fusion (Jang et al., 2004). A relevant summary describing the contribution of BM-HSCs in liver regeneration can be found in a review by Thorgeirsson and Grisham (2006).

An adherent fraction of cells, first found in the stroma of BM, so-called mesenchymal stem cells (MSCs), colony-forming units-fibroblasts (CFU-F), and stromal cells, emerged as a remarkable tool for regenerative medicine (Pittenger et al., 1999). Such cells have also been found in other than BM sources, such as amniotic fluid (De Coppi et al., 2007), placenta (In't Anker et al., 2004), adipose tissue (AT) (Zuk et al., 2001, 2002), umbilical cord blood (UCB) (Bieback et al., 2004), and many fetal tissues and organs (Campagnoli et al., 2001; In't Anker et al., 2004). Even though isolated from different tissues, these cells share similar surface markers with small variability ($SH3^+$, $CD29^+$, $CD44^+$, $CD71^+$, $CD90^+$, $CD105^+$, $CD106^+$, $CD120a^+$, $CD124^+$, $CD14^-$, $CD31^-$, $CD34^-$, and $CD45^-$) (Kern et al., 2006; Wagner et al., 2005). Such small variations are related to the tissues from which they have been obtained, the donors, and the age of the donors. The ability of MSCs to differentiate toward hepatocytes has been confirmed in many independent studies (Table 2). The possibility for their future application in the therapy of liver diseases is very likely (Fig. 4). MSCs can be easily obtained from a patient's own tissues, isolated ex vivo, expanded, differentiated toward hepatocytes, and transplanted back into the patient. In the future, after the development of tissue-engineering technologies, they might be implanted

back into the patient as 3D cultures (Huang et al., 2006) or liver devices (Sato-Gutierrez et al., 2006). Such a possibility sidesteps the limits regarding ethical and immunocompatible problems. Currently, attention is being given to adipose tissue (AT) as a source of MSCs for regenerative medicine (Banas et al., 2007; Seo et al., 2005; Talens-Visconti et al., 2006; Zuk et al., 2001, 2002). From AT, a sufficient number of stem cells for stem cell–based therapy may be obtained without invasiveness or damage to a patient's health. Below, we present the current strategies of hepatocyte differentiation from MSCs from different sources, emphasizing those strategies that utilize MSCs from sources of high availability, as well as pointing out the functions of generated hepatocytes.

Bone Marrow Mesenchymal Stem Cells (BM-MSCs).

Schwartz et al. (2002) showed that rat, mouse, and human BM–derived multipotent adult progenitor cells (MAPCs), cultured with FGF4 and HGF on Matrigel, can differentiate into cells expressing several liver-specific markers. These cells had functional characteristics of hepatocytes, e.g., albumin and urea secretion and CYP activity. Sato et al. (2005) showed that human BM-MSCs xenografted into liver of a rat treated with allylalcohol differentiated into human hepatocytes, which express liver-specific markers, without cell fusion. These studies excluded spontaneous fusion between human MSCs and rat hepatocytes, by identification of both human and rat chromosomes using fluorescence in situ hybridization (FISH). Lee KD et al. (2004) demonstrated the evidence of hepatogenic induction of BM-MSCs using sequential treatment with factors. They showed the characteristics of hepatocyte-like cells, including albumin and urea secretion, glycogen storage, low-density lipoprotein (LDL) uptake, and CYP activity. Using the same strategy, they obtained similar results by differentiating umbilical cord blood (UCB) MSCs (Lee KD et al., 2004). Other studies using sequential treatment have been performed by Snykers et al. (2006). They have used HGF, FGF4, DEX, and ITS and achieved functional hepatocytes. Other interesting studies have been conducted by Ong et al. (2006). Prior to treatment with HGF and OsM, these researchers co-cultured human BM-MSCs with rat liver slices derived from gadolinium chloride ($GdCl_3$)-treated rats. They detected a significant alteration in hepatocyte functions, such as albumin and urea production, after coculture with liver slices derived from intoxicated animals. This fact may suggest the use of some pro-inflammatory, such as tumor necrosis factor (TNF) α, in order to upgrade the differentiation ratio. The next two reports show the therapeutic effect of BM-MSCs on liver injury (Oyagi et al., 2006; Zhao et al., 2005). Oyagi et al. (2006) showed that, after treatment with HGF, transplanting BM-MSCs into a rat with CCl_4 intoxication resulted in improved liver function (albumin and glutamic-oxaloacetic transaminase [GOT] levels) and decreased fibrosis. Similarly, Zhao et al. (2005) have demonstrated a protective effect of MSCs isolated from rat BM on fibrosis caused by CCl_4 and DMN.

Umbilical Cord Blood Mesenchymal Stem Cells (UCB-MSCs).

Human UCB has already been shown to be applicable to the clinical treatment of various hematopoietic diseases. As well as HSCs, UCB contains stem cells with the properties of MSCs (Kern et al., 2006; Wagner et al., 2005). It has been reported that, after stimulation with HGF, DEX, and OsM, UCB-MSCs differentiate into hepatocyte-like cells (Hong et al., 2005; Lee OK et al., 2004). Functional hepatocytes, together with BM-MSCs, were generated by another group (Lee KD et al., 2004). Kang et al. (2005) used FGF4 and HGF in a 1% (fetal bovine serum) FBS supplemented medium. They generated functional hepatocytes as well. Wang et al. (2005) sorted the β_2m^-, c-Myc^+ fraction of UCB-MSCs, which, after coculture with non-parenchymal liver cells in the presence of HGF, differentiated into functional hepatocytes secreting ALB and urea. Since UCB is normally discarded at birth, the highly proliferative UCB-MSCs can be obtained easily from newborns and used for therapy.

Adipose Tissue Mesenchymal Stem Cells (AT-MSCs).

AT represents an attractive source of MSCs for future stem cell–based therapy (Fig. 4) (Zuk et al., 2001, 2002). Large numbers of human AT-MSCs can be obtained with minimal invasiveness. The ability of AT-MSCs to differentiate into hepatocytes was first observed by Seo et al. (2005). They used HGF, OsM, and DMSO and demonstrated that generated hepatocytes had the ability to uptake LDLs and synthesize urea. Next, the protocol of differentiation of BM-MSCs and UCB-MSCs toward hepatogenic lineage by Lee KD et al. (2004) was also confirmed on AT-MSCs (Talens-Visconti et al., 2006). Our group recently reported hepatogenic differentiation of AT-MSCs from cancer patients' own $CD105^+$ AT-MSCs (Banas et al., 2007). Although AT-MSCs are a more heterogeneous fraction than BM-MSCs, after selection/sorting, we obtained a homogeneous, highly proliferative, and potent population of stem cells. The hepatic differentiation protocol was based on previously established ES cell differentiation by Teratani et al. (2005). The generated cells revealed hepatocyte-specific morphology (Fig. 5B), markers detected by RT-PCR, immunostaining (Fig. 6B), and functions such as ammonia detoxification and albumin production (Banas et al., 2007). Xenobiotic metabolic enzymes, such as CYP1A1, CYP3A4, CYP2C9, and NADPH P-450 reductase, were detected by Western blot analysis. Adipose tissue is an attractive source of MSCs for liver therapy. Important to note is the fact that undifferentiated AT-MSCs express some hepatocyte-specific markers, which raises the possibility that AT-MSCs might contain a large number of hepatic progenitors or that they are predisposed toward a hepatic lineage because they are in close vicinity to the liver. These speculations need evaluation; however, they seem very intriguing and promising.

Mesenchymal Stem Cells From Other Sources.

Of interest are recent studies demonstrating the evidence of hepatogenic differentiation of MSCs from amniotic fluid (De Coppi et al., 2007). Those mul-

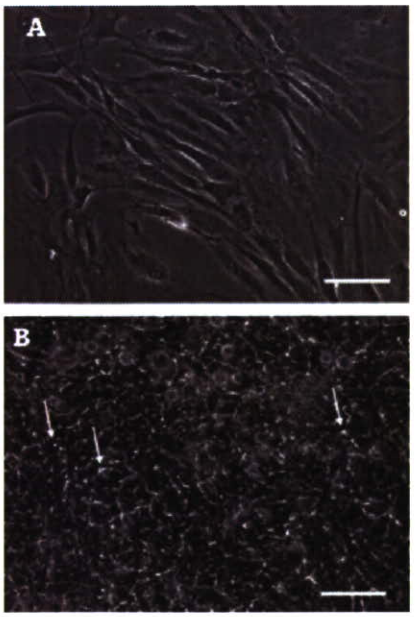

Fig. 5. Photograph of undifferentiated AT-MSCs (**A**) and those after hepatocyte induction (**B**). The morphology of AT-MSCs during hepatocyte induction diametrically changed in shape from spindles to rounds. The bile canaliculi structures are visible. The arrows indicate the bile canaliculi structures. Scale bars = 50 μm.

tipotential stem cells were identified by immunoselection of c-kit/CD117, and they expressed both mesenchymal (CD29, CD105) and embryonic (SSEA-4, Oct3/4) stem cell markers, while hematopoietic (CD45, CD34) stem cell markers were not expressed. The studies showed evidence of hepatogenic differentiation, which expressed the hepatic marker ABC transporter MDR1 and HNF-4α and revealed the ability to synthesize urea. Until now, amniotic fluid similar to UCB has been a medical waste; instead, it might be used as a stem cell source to cure liver dysfunction.

In addition, MSCs from placenta, by culturing in a hepatocyte culture medium, have been differentiated into hepatocyte-like cells (Chien et al., 2006).

Mesenchymal Stem Cells Are an Ideal Source for Stem Cell Therapy.

Because it is easy to obtain from patients, the autologous MSCs are a particularly promising source of cells for many clinical applications in the evolving field of regenerative medicine. Furthermore, MSCs represent an advantageous cell type for allogenic transplantation since MSCs are immune-privileged, with low MHC I and no MHC II expression, therefore reducing the risks of transplant rejection. MSCs have been found to be immunosuppressive, through a mechanism thought to involve paracrine inhibition of T- and B-cell proliferation and as such have been used in trials investigating their effects on autoimmune diseases and graft-versus-host disease (GVHD) (Ucelli et al., 2007). Co-infusion of donor-derived MSCs together with HSCs has been shown to reduce the incidence and severity of GVHD in sibling allografts (Le Blanc et al., 2007). It was reported that a patient suffering from severe therapy-resistant GVHD was treated with human AT- MSC and revealed a complete recovery (Fang et al., 2007). The hypo-immunogenic properties of MSCs are considered to be sufficient to allow transplantation even between individuals who are not HLA-compatible. Therefore, MSCs represent a population of cells with the potential to contribute to future treatments for a wide range of acute or degenerative diseases including liver.

STEM CELL DIFFERENTIATION TOWARDS CHOLANGIOCYTES

The liver consists of two types of endodermal epithelial cells, hepatocytes and cholangiocytes. Both cells originate from bipotential hepatoblasts (OCs) (Beltrami et al., 2007; Evarts et al., 1987; Farber, 1956; Fausto, 2004; Fougere-Deschatrette et al., 2006; Herrera et al., 2006; Kojima et al., 2005; Shafritz et al., 2006; Wang et al., 2003; Yovchev et al., 2007; Zhou et al., 2007). Although cells derived from bone marrow cells (rat), MSCs (mouse), or HSCs (mouse) can differentiate into hepatocytes, whether these non-hepatic cells can directly differentiate into cholangiocytes remains controversial (Krause et al., 2001; Moritoki et al., 2006; Theise et al., 2000a,b). Little is known regarding the molecular pathways required for biliary epithelial cell development. However, it has been reported that the Notch signaling pathway is involved in the development of intrahepatic bile ducts (Kodama et al., 2004). In fact, a primary culture of hepatoblastic cells, which overexpressed a constitutively active form of Notch, resulted in preferential differentiation into cholangiocytes (Tanimizu et al., 2004). Recently, the same group reported that hepatoblastic cells can be converted into cholangiocyte-type epithelial polarity in a matrigel 3D culture (Tanimizu et al., 2007). Despite confidence that hepatoblastic cells and extra-hepatic stem cells may differentiate into cholangiocytes, the mechanistic details of this process remain poorly understood. Moreover, the physiological importance and therapeutic utility of this phenomenon need to be investigated.

THE ROLE OF "EPIGENETIC MODIFIERS" AND "ENVIRONMENTAL NICHES" IN HEPATOGENIC DIFFERENTIATION

The pool of stem cells in the human body has a highly diverse developmental status (Fig. 7). The change from pluripotent to multipotent and to more developmentally restricted states is accompanied by global changes in gene expression. Genes active in earlier progenitors are gradually silenced at developmentally later stages, and a subset of cell type-specific genes is turned off (Dennis and Charbord, 2002) (Fig. 7).

This progression is the result of the selective expression of transcription factors together with chromatin remodeling and modification (involvement of histone acetyltransferases [HATs] and histone deacetylases [HDAC]) and DNA methylation of CpG dinucleotides, localization of chromatin to specialized nuclear domains, and the recently discovered miRNA involvement. These epigenetic mechanisms allow cells to maintain their identity even when exposed to signals that would induce alternative cell fate in less determined cell lineages.

None of the studies we have discussed address epigenetic mechanisms in liver stem cell biology. Some progress on the mechanisms involved is beginning to be published. For example, the role of epigenetic modifiers

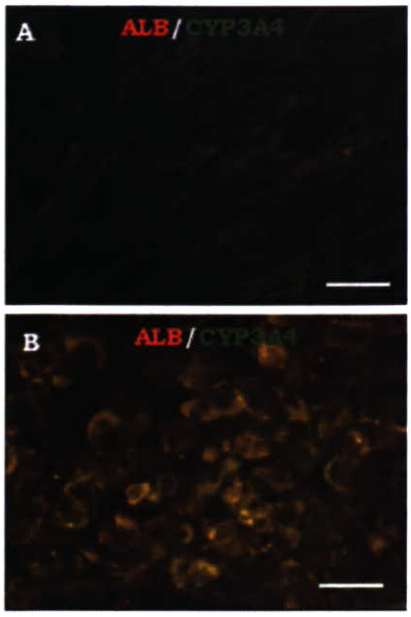

Fig. 6. Photographs of ALB immunostaining; undifferentiated (**A**) and after hepatocyte induction (**B**). At 40 days of induction, cells were fixed and co-stained with monoclonal antibodies against ALB (red) and CYP3A4 (green). Scale bars = 50 μm.

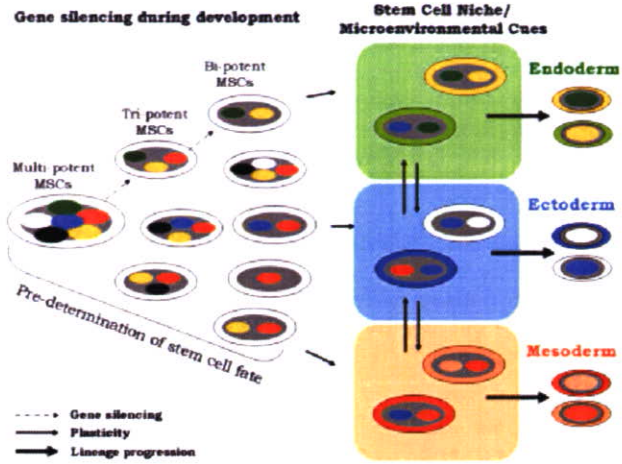

Fig. 7. Epigenetic mechanisms involved in stem cell fate determination and plasticity. Model showing the hierarchical tree of stem cells (MSCs) in the adult human body, where there are sub-populations of MSCs with developmentally different gene expression. During development, by series of epigenetic mechanisms (gene silencing and chromatine modifications), cells partially reach their destination (Dennis and Charbord, 2002). However, this fate is not totally determined and can be influenced by a different microenvironment (stem cell niche). This ability of stem cells, called plasticity, is a great property, which allows for the future development of stem cell–based therapy. Colors indicate different lineage gene sets. Red: mesoderm; blue: ectoderm; green: endoderm. The gene set (represented by different colors within the nucleus) determines the potentiality of MSCs. While MSCs are in contact with specific factors and environments (stem cell niche), which direct stem cell differentiation towards a specific lineage (endoderm, green; ectoderm, blue), they undergo further gene expression changes and phenotypic changes.

(hypomethylating agent 5-aza-2-deoxycytydine [5azadC] and deacetylating agent-histone deacetylase inhibitor Trichostatin A [TSA]) has been shown in neurogenic differentiation (Alexanian, 2007). In hepatogenic differentiation, factors such as TSA (Snykers et al., 2007), DMSO (Sato-Gutierrez et al., 2006, 2007; Seo et al., 2005) and sodium butyrate (Rambhatla et al., 2003), which are the inhibitors of HDAC, are often used to manipulate cell fate. Importantly, a report has been published regarding the alteration of hepatic differentiation of MSCs using TSA (Snykers et al., 2007). Nevertheless, generalizations about the developmental mechanisms of epigenetic change associated with potency restriction have not emerged; at present this important area of study is enigmatic.

A parallel important area of research is the interaction of stem cells with their physical environment as they travel to and/or arrive at their final destination. Specifically, the connective tissue/matrix environs of niche compartments must be intensively examined. The cells that make up and create the niche are as important as the stem cells that occupy and respond to the niche environment. Here again, generalizations regarding developmental and molecular mechanisms do not exist.

The pool of mesenchymal stem cells (MSCs) within the human body may be a highly effective tool to realize the potential of regenerative medicine using autologous cells. To do this, an understanding of the molecular basis of the stability of the differentiated state and of niche compartments, along with elucidation of stem cell mobilization and homing, must be in place. The interesting unknown is whether what works to reconstitute the hepatic system with stem cells will yield insight into the reconstitution of any other organ, or that, much like the hoped-for cure for cancer, regenerative medicine with stem cells will be done one organ system at a time.

CONCLUSIONS AND FUTURE PERSPECTIVES

The field of hepatic stem cell study has undergone tremendous growth during the past decade. Despite many reports on the differentiation potential of stem cells, there is little understanding of the molecular basis of stem cell plasticity. Array-based gene expression analyses of "stemness" have been reported (Boquest et al., 2005; Ramalho-Santos et al., 2002; Urs et al., 2004). Under normal conditions, the expression of "stemness genes" is tightly regulated by a dynamic array of mediators, including the spatial and temporal expression of inhibitors and the epigenetic modulation of the genome. Once stem cells are exposed to microenvironmental cues of tissue or organ injury and regeneration, the balance of regulatory mediators is restored, with the plasticity of stem cells being induced towards differentiation into a specific cell lineage. In the natural milieu, the hepatic differentiation of stem cells involves multiple pathways. This may be mimicked in vitro by using a combination of various factors and techniques. It is anticipated that, over the next few years, we will see profound investigations of hepatic stem cells and, particularly, of their mechanisms, transduction pathways, and epigenetic modulations. Hepatic differentiation may certainly be enhanced by further studies and a combination of various techniques, including tissue-engineering technologies. However, while stem cell plastic-

ity is certainly a hot issue these days, its potency for clinical application is even hotter. In considering stem cell–based therapy, MSCs have emerged with great potential. An assortment of studies has documented their contribution in hepatogenic generation in vivo and in vitro. Preliminary results of only a few clinical studies on the administration of bone marrow stem cells to liver cirrhotic patients seem to be very promising, but additional well-designed and controlled studies are needed.

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