

Persistence of a chimerical phenotype after hepatocyte differentiation of human bone marrow mesenchymal stem cells

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Abstract. *Objectives:* Recent studies have suggested the potential of mesenchymal stem cells (MSCs) to differentiate into a hepatocyte-like lineage. Here, we evaluate the efficacy of hepatocyte differentiation of MSCs by studying acquisition of hepatocyte-like features together with alteration of the native mesenchymal phenotype. *Material and methods:* *In vitro*, we have investigated protein and mRNA level expression of hepatocyte and mesenchymal markers of mesenchymal-derived hepatocyte-like cells (MDHLCs) and we have evaluated their functionality using metabolic assays. *In vivo*, we investigated co-expression of hepatocyte (albumin, α -foetoprotein, cytokeratin 18) and mesenchymal (fibronectin, vimentin) markers after transplantation of MSCs or MDHLCs into severe combined immune deficiency mice. *Results:* We observed that while *in vitro* these cells acquired some phenotypic and functional features of mature hepatocytes, they partially preserved their mesenchymal phenotype. After intrasplenic transplantation, engrafted MSCs with isolated expression of fibronectin and α -foetoprotein were observed. When these cells were injected into the liver, they expressed all analysed markers, confirming the chimaeric co-expression observed *in vitro*. Conversely, liver-engrafted MDHLCs conserved their hepatocyte-lineage markers but lost their chimaeric phenotype. *Conclusions:* Hepatocyte differentiation of MSCs predominantly allows the acquisition of phenotypic hallmarks and provides chimaeric cells that maintain expression of initial lineage markers. However, advanced maturation to the hepatocyte-like phenotype could be obtained *in vivo* by conditioning MSCs prior to transplantation or by infusing cells into the liver micro-environment.

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INTRODUCTION

As organ shortage remains a major limiting step in organ-derived cell transplantation, alternative candidates for liver cell therapy have been proposed, after the discovery of stem cell pluripotency (Herzog *et al.* 2003; Martin-Rendon & Watt 2003). Mesenchymal stem cells (MSCs) appear to be an appropriate candidate (Pittenger *et al.* 1999; Barry & Murphy 2004) and studies on their hepatic differentiation potential has rapidly developed in recent years. Schwartz *et al.* were the first to demonstrate *in vitro* the capacity of bone marrow stem cells to differentiate along the hepatocyte pathway (Schwartz *et al.* 2002). These results have been corroborated both *in vitro* and *in vivo* by other groups, using MSCs isolated from bone marrow (Anjos-Afonso *et al.* 2004; Lee *et al.* 2004; Wang *et al.* 2004; Sato *et al.* 2005; Aurich *et al.* 2007), cord blood (Beerheide *et al.* 2002; Kogler *et al.* 2004; Hong *et al.* 2005), placenta (Chien *et al.* 2006), and adipose tissue (Seo *et al.* 2005; Talens-Visconti *et al.* 2006). *In vitro*, characterization of conditioned cells has been based on phenotype [morphological changes, immunocytochemistry, reverse transcription-polymerase chain reaction (RT-PCR)] and on semiquantitative functional assays (albumin or urea secretion, glycogen storage, pentoxeresorufin-0-dealkylase assay, low-density lipoprotein uptake). *In vivo*, engraftment of native or *in vitro* pre-conditioned MSCs has been described to be efficient in mouse and rat models, with the exception of one recent report (Popp *et al.* 2006), and these cells were described to have acquired some hepatocyte-specific markers inside the liver. Taken together, all these data indicate the potential of MSCs to acquire elective hepatocyte features after *in vitro* or *in vivo* differentiation.

In the present study, we aimed to investigate effective yield of *in vitro* hepatocyte differentiation of human bone marrow-MSCs and to demonstrate whether or not this differentiation was associated with modifications of the initial mesenchymal phenotype. We corroborated these results *in vivo* by analysing hepatocyte and mesenchymal marker expression after transplantation of MSCs into liver-injured severe combined immune deficiency (SCID) mice and we evaluated the influence of pre-transplant conditioning of these cells on their *in vivo* expression profile. Furthermore, we studied the human origin of engrafted cells by analysis of nuclear and cytoplasmic co-expression of human markers. The study was approved by our local institutional human and animal ethical committee.

MATERIALS AND METHODS

Cell isolation and culture

Bone marrow samples ($n = 16$) were collected by aspiration of vertebrae or iliac crests of postmortem donors aged 8–67 years. Aspirates were collected into heparinized syringes containing 10% Hanks' balanced salt solution (Invitrogen, Merelbeke, Belgium) and were processed within 48 h. Mononuclear cells (MNCs) were separated by centrifugation at 805 *g* for 20 min at 20 °C after carefully loading bone marrow on Ficoll-Paque™ PLUS (Amersham Biosciences, Uppsala, Sweden); cells were then washed twice with phosphate-buffered saline (PBS) (Invitrogen). Cell viability was always greater than 95% as assessed by trypan blue dye exclusion. MNCs were seeded in culture flasks at $1-6 \times 10^6$ cells/cm² in α -minimal essential medium (Invitrogen) containing 10% foetal calf serum (Perbio, Chester, UK) and 1% penicillin/streptomycin (P/S) (Invitrogen) (defined as MSC medium). First medium change was performed after 48–72 h and then twice weekly. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO₂.

When reaching 70% confluence, cells were detached using 0.05% trypsin–0.53 mM EDTA4Na (Invitrogen) and were re-plated at 0.5×10^4 cells/cm². Colony forming unit-fibroblast assays were performed by plating cells at 0.5–10 cells/cm² in MSC medium. Plates were examined for presence of colonies, after 10 days. Cumulative population doublings were calculated using the following equation: $[\log_{10}(N_H) - \log_{10}(N_I)]/\log_{10}(2)$, where N_I is inoculum number and N_H is cell harvest number, as described elsewhere (Bieback *et al.* 2004).

Mesodermal cell differentiation

Cells were plated at 1.5×10^4 cells/cm² in uncoated six-well dishes. To obtain osteogenic differentiation, they were incubated with MSC medium containing 0.1 μ M dexamethasone, 0.05 mM ascorbate and 10 mM β -glycerophosphate (all from Sigma, Bornem, Belgium), and changed twice a week. After 4 weeks, calcium deposition was evaluated using von Kossa and alizarin red stains. For adipogenic differentiation, cells were incubated with MSC medium containing 1 μ M dexamethasone, 0.5 mM isobutyl-methylxanthine, 0.2 mM indomethacin (all from Sigma) and 10 μ g/mL insulin (Lilly Benelux SA, Brussels, Belgium) with medium change twice a week. After 4 weeks, lipid vesicles were revealed by oil red O staining.

Hepatocyte differentiation

Hepatocyte differentiation was evaluated for MSCs at passages 1–9 (28 samples, mean passage = 3). We plated MSCs at 1.5×10^4 cells/cm² on collagen type I-coated dishes. These cells were incubated for 10 days with an induction medium containing 1% P/S Iscove's modified Dulbecco's medium (IMDM) (Invitrogen) supplemented with 20 ng/mL hepatocyte growth factor (HGF) (Biosource, Camarillo, CA, USA), 10 ng/mL fibroblastic growth factor 4 (FGF4) (Peprotech, London, UK), 1% insulin–transferrin–selenium (ITS premix, Invitrogen), and 0.61 g/L nicotinamide (Sigma); and then with 1% P/S IMDM containing 20 ng/mL oncostatin M (OSM) (Sigma), 1 μ M dexamethasone and 1% ITS premix for ≥ 20 days (maturation medium). This differentiation protocol was defined as the complete protocol. Negative controls were: serum-free IMDM and maturation medium.

Flow cytometry

Cells from the first to eighth passage were trypsinized and suspended at a concentration of $0.5\text{--}1 \times 10^3/\mu\text{L}$ in PBS, and then were incubated for 30 min at 4 °C with antibodies to the following: CD45-APC, CD34-APC, CD90-PC5, CD86-PE, HLA-DR-FITC, CD73-PE, CD29-PE, CD14-PC5 (Becton Dickinson, Erembodegem, Belgium); CD105 Endoglin-FITC (Immunokontakt, Frankfurt, Germany); CD44-FITC, CD49b-FITC, CD49e-FITC, CD71-PE, CD117-PC5, CD80-PE, CD33-FITC, CD26-PE (Immunotech, Marseilles, France); HLA-ABC-PE (Dako Systems, Heverlee, Belgium); CD13-PE (Beckman Coulter, Miami, FL, USA); CD133-APC (Miltenyi, Utrecht, The Netherlands). Controls were derived by incubating the cells with corresponding isotypes: IgG1-FITC, IgG2a-PE, IgG2a-PC5, IgG2b-FITC (Serotec, Oxford, UK); IgM-FITC (Immunotech); IgG2a-FITC, IgG1-PC5 (BD Pharmingen, San Diego, CA, USA); IgG1-PE, IgG1-APC (Becton Dickinson); IgG2a-APC (Miltenyi). Cells were then washed and re-suspended in Isoton (Beckman Coulter) for reading using a FACSCalibur flow cytometer (Becton Dickinson). Intracytoplasmic human albumin staining was performed as follows: 2×10^5 cells were incubated in 200 μ L Cytotfix/Cytoperm (Becton Dickinson) for 20 min at room temperature, were washed with 1 mL $1 \times$ Perm/Wash solution (Becton Dickinson) and were centrifuged for 5 min at 805 g. Cell pellets were re-suspended in 50 μ L Perm/Wash and were incubated with 1 μ L FITC-labelled rabbit antihuman albumin antibody (Cedarlane Laboratories, Ontario, Canada) or 1 μ L FITC-labelled IgG1 control isotype (BD Pharmingen), for 25 min at 4 °C. After washing

cells with 1 mL PBS and centrifuging for 5 min at 2000 r.p.m., cells were fixed in 250 μ L Cellfix (Becton Dickinson) and were analysed using the FACSCalibur flow cytometer.

Immunocytochemistry

Cells were fixed with 3.5% formaldehyde for 15 min, permeabilized with 1% Triton X-100 (Sigma) in Tris-buffered saline (TBS) buffer (pH 7.5) for 15 min and then were rinsed with TBS. After 1 h incubation with blocking solution (3% dry milk in TBS), cells were rinsed with TBS and then incubated for 1 h at room temperature with primary mouse antihuman monoclonal antibodies as follows: 1/50 vimentin, 1/50 albumin (Sigma); 1/50 α -smooth muscle actin (ASMA), 1/50 HepPar1 (Dako Cytomation); 1/50 CK18 (Progen, Heidelberg, Germany); 1/200 connexin-32 (CX-32), 1/50 CK8 (Chemicon, Temecula, CA, USA); 1/20 dipeptidylpeptidase-IV (DPP-IV) (Ansell, Bayport, MN, USA); 1/100 E-cadherin (BD Pharmingen) and rabbit polyclonal antihuman antibodies as follows: 1/50 laminin, 1/100 fibronectin, 1/100 α -foetoprotein (α FP) (Dako Cytomation) and 1/100 albumin (Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Cells were washed five times and primary antibody locations were revealed by 1 h incubation with Cy3-coupled antimouse IgG (Jackson ImmunoResearch Laboratories) and FITC-coupled antirabbit IgG (Sigma). After rinsing, nuclei were visualized by 30 min staining with 4',6-diamidino-2-phenylindole (DAPI, Sigma). For immunoperoxidase staining, formaldehyde-fixed cells were incubated with 3% hydrogen peroxide (Sigma) for 3 min, washed with distilled water and then were permeabilized with 1% Triton X-100. After 1 h block in 1% bovine serum albumin (BSA, Sigma), cells were incubated 1 h at room temperature with primary antibodies in 0.1% BSA, were washed with PBS, and then exposed for 30 min to antirabbit or antimouse Dako Envision HRP antibody (Dako Cytomation). Staining was performed by 3 min exposure to 3,3'-diaminobenzidine (Sigma). After rinsing, nuclei were stained with Mayer's haematoxylin (10 min) and cells were mounted. Each antibody was tested on fresh (< 24 h) cultures of human hepatocytes as positive controls and MSCs as negative controls.

Reverse-transcription polymerase chain reaction

Total RNA was extracted from cells ($n = 0.9 \times 10^6$) grown in six-well plates using the TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium), and cDNA was generated using a reverse transcription kit (Thermoscript RT, Invitrogen), according to the manufacturer's instructions. Polymerase chain reaction (PCR) amplifications were performed using specific primers (Table 1) and polymerase elongase (Invitrogen) in a final volume of 25 μ L. All PCR experiments were performed at 30 cycles. Samples were electrophoresed on 1% agarose gel and nucleic acids were visualized by ethidium bromide staining.

Transmission electron microscopy

Samples of MSCs and MDHLCs were trypsinized and centrifuged at 13 400 *g* for 3 min at room temperature. After supernatant removal, cell pellets were fixed with 2.5% EM-grade glutaraldehyde (Agar Scientific, London, UK) buffered in 0.1 M sodium cacodylate for 48 h, were post-fixed in 1% osmium tetroxide (Agar Scientific), and then embedded in Epoxy embedding medium (Fluka Chemie, Buchs, Switzerland). Ultrathin sections were contrasted with uranyl acetate and lead citrate before examination by transmission electron microscopy using a Zeiss EM109 microscope (Carl Zeiss Inc., Oberkochen, Germany).

Glycogen staining

After 3.5% formaldehyde fixation, cells were incubated for 10 min in 1% periodic acid (Sigma), were washed with distilled water and incubated with Schiff's reagent (Sigma) for 15 min. After

Table 1. Primers used for RT-PCR characterization of mesenchymal-derived hepatocyte-like cells

Primer	Sequence	Size (bp)	Cycle
Albumin	S: 5'-CCTGGTGTGATTGCCTTTGCTC-3' AS: 5'-CATCACATCAACCTCTGGTCTCACC-3'	308	94 °C/1 min; 55 °C/1 min; 72 °C/2 min
G6Pase	S: 5'-TCAGCTCAGGTGGTCTCTT-3' AS: 5'-CCTCCTTAGGCAGCCTTCTT-3'	291	94 °C/1 min; 55 °C/1 min; 72 °C/2 min
CK-8	S: 5'-AAGGGCTGACCGACGAGATC-3' AS: 5'-GATCGCCACCTACAGGAAGC-3'	537	94 °C/30 s; 50 °C/30 s; 72 °C/60 s
CK18	S: 5'-CCATGCGCCAGTCTGTGGAG-3' AS: 5'-AGCAGATTGAGGAGAGCACCAC-3'	322	94 °C/30 s; 65 °C/45 s; 72 °C/45 s
α1-AT	S: 5'-TCGCTACAGCCTTTGCAATG-3' AS: 5'-TTGAGGGTACGGAGGAGTTCC-3'	142	94 °C/30 s; 55 °C/1 min; 72 °C/2 min
αFP	S: 5'-TGAAATGACTCCAGTAAACCC-3' AS: 5'-AATGAGAAACTTTGCTTCATC-3'	199	94 °C/30 s; 55 °C/1 min; 72 °C/2 min
CYP 3A4	S: 5'-TTAGTGTCTCAACCTTACC-3' AS: 5'-GGTGGTGATGATTCCAAGCTA-3'	802	94 °C/120 s; 50 °C/60 s; 72 °C/60 s
CYP 2B6	S: 5'-CCTCTTCCAGTCCATTACCG-3' AS: 5'-GCCATACACAGAGGCAGTCA-3'	551	94 °C/120 s; 50 °C/60 s; 72 °C/60 s
TAT	S: 5'-TGAGCAGTCTGTCCACTGCCT-3' AS: 5'-CTCAGATCCTCCTCATTACAT-3'	358	94 °C/40 s; 56 °C/50 s; 72 °C 60 s
TDO	S: 5'-ATACAGAGCACTTCAGGGAGC-3' AS: 5'-GATACCGAAGATGAACCCAACCA-3'	316	94 °C/40 s; 56 °C/50 s; 72 °C/60 s
HNF4	S: 5'-CCAAGTACATCCAGCTTTC-3' AS: 5'-CTTTGACCCAGATGCCAA-3'	295	94 °C/40 s; 56 °C/50 s; 72 °C/60 s
c-met	S: 5'-CAATGTGAGATGTCTCCAGC-3' AS: 5'-TCTGCCTGCAATCTACAAGG-3'	560	94 °C/40 s; 55 °C/40 s; 72 °C 60 S
PEPCK	S: 5'-TTAGATGGGACAAAGCCTG-3' AS: 5'-GCAAGACGGTGATTGTAATC-3'	226	94 °C/40 s; 55 °C/40 s; 72 °C/60 s
Vimentin	S: 5'-CTACATCGACAAGGTGCGCTT-3' AS: 5'-TGCCAGAGACGCATTGTCAA -3'	301	94 °C/30 s; 58 °C/30 s; 72 °C/60 s
ASMA	S: 5'-ATGTGTGAAGAAGAGGACAG-3' AS: 5'-ACTCGTAACTTTCTCAAGG-3'	730	94 °C/30 s; 55 °C/30 s; 72 °C/30 s
Fibronectin	S: 5'-ACTGGAGCCTCGAAGAGCAA-3' AS: 5'-TGCAAGTGATGCGTCCGTG-3'	607	94 °C/30 s; 55 °C/30 s; 72 °C/30 s
GAPDH	S: 5'-CGGAGTCAACGGATTTGGTTCGTAT-3' AS: 5'-AGC CTT CTC CAT GGT GGT-3'	307	94 °C/1 min; 58 °C/1 min; 72 °C/2 min

α1-AT, α1-antitrypsin; αFP, α-fetoprotein; ASMA, α-smooth muscle actin; CK, cytokeratin; CYP, cytochrome P450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; HNF4, hepatic nuclear factor 4; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosin aminotransferase; TDO, tryptophan-2,3-dioxygenase.

rinsing for 10 min in tap water, nuclei were stained for 10 min with Karazi's haematoxylin. Cells were then washed with tap water and mounted. Freshly cultured (< 24 h) human hepatocytes and MSCs served, respectively, as positive and negative controls.

Metabolic assays

Urea concentrations of culture supernatants were screened by a colorimetric assay (Gentaur, Brussels, Belgium), handled according to the manufacturer's instructions, and were analysed using a HTS7000PLUS absorbance reader (Perkin Elmer, Wellesley, MA, USA). Serum-free IMDM, incubated with freshly cultured (< 24 h) mouse hepatocytes, and serum-free IMDM alone, respectively, served as positive and negative controls.

Human albumin concentration was screened using ELISA assay (Cygnus Technologies, Plainville, MA, USA, or Bethyl Laboratories, Montgomery, TX, USA) as per manufacturers' instructions and was analysed by Multiskan EX absorbance reader (Thermo Fisher Scientific, Zellik, Belgium). Controls were designed as for urea assay.

For revelation of glucose-6-phosphatase (G6Pase) activity, cells were incubated for 5 min in solution containing 0.1 g glucose-6-phosphate (G6Pate, Sigma) and 0.15 g lead nitrate (Acros, Geel, Belgium) in a Tris-maleate buffer at pH 6.7, at 37 °C. Brownish precipitates of lead sulphide were obtained by incubating the cells in a solution of 1% ammonium sulphide (Sigma) (Sokal *et al.* 1989). They were then mounted for viewing by light microscopy. Controls were performed as for periodic acid-Schiff staining.

Functional gluconeogenesis assay was performed on freshly isolated mouse hepatocytes from fasted mice, MDHLCs and MSCs. Cells were first incubated with their corresponding medium (see above) containing pentachlorophenol (Sigma) at the following concentrations (0, 0.15 and 0.30 mM) for 4 h and then were washed twice in PBS. For gluconeogenesis testing, cells were incubated for 2 h in a Krebs glucose-free solution (pH 7.4) containing lactate (10 mM) and pyruvate (1 mM). Gluconeogenesis was evaluated *via* glucose formation, measured using the glucose oxidase method, as reported by Lefebvre *et al.* (1994).

Cell transplantation

Cell transplantations were performed on 8–12-week-old female SCID mice as summarized in Table 2. Five transplantation groups were designed: group A, MSCs injected into spleens of hepatectomized mice; group B, MSCs injected into spleens of retrorsine-allyl alcohol-treated mice; group C, MSCs injected into livers of hepatectomized mice and groups D and E, MDHLCs injected, respectively, into spleens or livers of hepatectomized mice. Hepatectomies were performed on left or anterior liver lobes, when associated, respectively, to spleen or liver infusion. Retrorsine-allyl alcohol treatment consisted of two 14-day separated intraperitoneal injections of 30 mg/kg retrorsine, followed 4 weeks later by a single intraperitoneal dose of 0.62 mmol/kg allyl alcohol the day before cell injection (Yavorkovsky *et al.* 1995; Laconi *et al.* 2001; Menthen *et al.* 2004; Piscaglia *et al.* 2005). Each group of mice consisted of three control mice receiving 100 µL PBS and six sample mice receiving injections of 1×10^6 cells suspended in PBS. After 1 month, serum was collected and tissues were removed, fixed and prepared routinely before paraffin wax embedding, for further analyses.

Table 2. Description of cell transplantation procedures

Groups	Transplantation procedures		
	Cells	Injection site	Hepatic stimulus
A	MSCs	spleen	HTM
B	MSCs	spleen	R-AA
C	MSCs	liver	aHTM
D	MDHLCs	spleen	HTM
E	MDHLCs	liver	HTM

(a) HTM, (anterior lobes) hepatectomy; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells; R-AA, retrorsine-allyl alcohol.

Immunohistochemistry

Liver sections (5 μm) were deparaffinized with Histosafe, isopropanol and methanol solutions (VWR). Epitope retrieval was performed by incubating sections in 97 °C, in heated 0.02 M citrate buffer (pH 5.8) for 75 min. Endogenous peroxidase was inactivated by treating with 0.3% hydrogen peroxide in methanol for 15 min at room temperature. Non-specific binding was blocked by incubating sections for 30 min at room temperature in PBS with 1% BSA. Tissues were stained with mouse monoclonal antihuman albumin (1/250) or antihuman vimentin (1/50) antibodies using the avidin-biotin technique with Dako Cytomation Animal Research Kit according to the manufacturer's instructions. Polyclonal antihuman antibodies were incubated overnight at room temperature at the following concentrations: 1/3000 rabbit antihuman fibronectin, 1/1000 rabbit antihuman αFP and 1/200 guinea pig antihuman CK18 (Progen). Sections were rinsed with PBS and then were incubated for 30 min at room temperature with antirabbit Dako Envision HRP antibody or for 45 min with HRP-conjugated goat antiguinea pig antibody (Progen). Sections were washed and immunoreactivity was revealed by adding diaminobenzidine (Sigma), forming a brown precipitate on labelled cells. After rinsing, nuclei were counterstained with Mayer's haematoxylin for 10 min and sections were mounted with coverslips. Control immunohistochemistry was performed on human or PBS-injected mouse liver sections using primary antibodies, the corresponding isotypes or without primary antibodies. Control isotypes used were: mouse IgG1 (BD Pharmingen) for antihuman CK18; mouse IgG2a (BD Pharmingen) for antihuman vimentin and albumin.

Detection of human Alu sequences by *in situ* hybridization

Three micron liver sections were deparaffinized and re-hydrated in graded alcohols. Antigen retrieval was performed by incubating sections in citric acid monohydrate solution (pH 6.0) twice in a microwave oven for 10 min. After washing in PBS, sections were treated with 3% hydrogen peroxide in 70% ethanol for 20 min; they were immersed in water for 10 min and then dehydrated in increasing grades of ethanol. After air drying for 15 min, sections were covered with human Alu-DNA-Probe (PR-1001-01, Innogenex, San Ramon, CA, USA) and were sealed with coverslips. Slides were warmed at 95 °C for 5 min to denature DNA and hybridization was performed at 37 °C. After overnight incubation, coverslips were removed and slides were washed in three baths of 2 \times SSC (pH 7.0) (room temperature for 10 min, 56 °C for 10 min and room temperature for 15 min). Non-specific binding was prevented by incubation in PBS 3% BSA for 1 min and by a two-step Avidin/Biotin block (SP-2001, Vector Laboratories Ltd., Peterborough, UK). Sections were incubated 1 h with biotinylated goat anti-FITC antibody (1 : 100, BA-0601, Vector Laboratories Ltd.), washed in PBS and exposed for 15 min to streptavidin-HRP (Animal Research Kit, Dako Cytomation). Visualization was performed using a short incubation in 3,3'-diaminobenzidine. After rinsing, nuclei were counterstained with Mayer's haematoxylin for 10 min and sections were subsequently mounted.

Cell counting

Cell numbers were obtained by counting positive cells (for hepatocyte-like morphology and albumin or glycogen staining) in three different random fields (magnification $\times 200$) of each three different culture samples. Total cell counts are provided for each experiment.

Statistics

Results are expressed as mean \pm SD and statistically ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$) significant differences were assessed using the unpaired Student's *t*-test.

RESULTS

Characterization of human bone marrow mesenchymal stem cells

We obtained growing cultures of MSCs from all bone marrow samples (Fig. 1a). These cells grew up to 34 cumulative population doublings at the 8th passage. After passage 10, growth and morphology of the cells altered markedly with loss of differentiation capacities (Fig. 1b). This alteration was not impeded by growth factor treatment (bFGF, EGF). Immunophenotype was analysed at each passage and appeared to be stable with high expression of CD44, CD29, CD73, CD90, CD13, HLA-ABC, moderate expression of CD105, CD49e, CD71, CD26, CD49b, and no expression of CD45, CD34, CD14, CD33, CD117, CD133, CD80, CD86 and HLA-DR (Fig. 1o). Immunocytochemistry confirmed mesodermal origin of MSCs as they consistently expressed vimentin, fibronectin, ASMA and laminin (Fig. 1c–f). Mesenchymal phenotype was further confirmed in all MSC samples (up to passage 8) by efficiently differentiating these cells into osteocytes and adipocytes (Fig. 1g–n).

In vitro hepatocyte differentiation of human bone marrow mesenchymal stem cells

We determined our complete protocol after having tested serial combinations ($n = 30$, each performed twice or more) of various factors that have been described to play a role in hepatocyte differentiation (HGF, EGF, aFGF, bFGF, FGF4, IL-6, OSM, DMSO, nicotinamide, ascorbic acid 2-phosphate, dexamethasone, ITS) (Zaret 2001; Heng *et al.* 2005; Lavon & Benvenisty 2005). We elicited the protocol which achieved best results of hepatocyte-like morphology, urea secretion, glycogen storage and albumin expression (Fig. 2). Highest levels of albumin immunopositive and glycogen-producing cells, and of urea secretion were observed in MDHLCs differentiated using the complete protocol, as compared to cells differentiated with the other tested protocols (Fig. 2).

We estimated that $61.1 \pm 1.1\%$ of MDHLCs ($n = 910$) displayed hepatocyte-like morphology, defined as round or polygonal shaped cells reduced in size, containing cytoplasmic granulations and central nucleus with prominent nucleolus (Fig. 3a). This phenotype was observed consistently in each sample induced to differentiate and could be maintained in culture up to 10 weeks, but reverted when cells were incubated in growth factor-free medium. These morphological changes were also noted with maturation medium but here the cells obtained did not display other hepatocyte-like features (Fig. 2c). Ultrastructural analysis demonstrated that MDHLCs acquired morphological features of high metabolic activity (prominent nucleolus, highly developed rough endoplasmic reticulum, enhanced content of mitochondria) and of mature hepatocytes (deposition of glycogen) (Fig. 3a).

Immunocytochemical analysis demonstrated that MDHLCs expressed albumin (9/14 samples tested), α FP (5/10), DPPiV (7/7), E-cadherin (3/3) and connexin-32 (4/4) (Fig. 3b) but did not express other epithelial markers such as CK8, CK18 and HepPar-1 (Fig. 3b). Albumin positivity rate was calculated to be $71.1 \pm 14.5\%$ by flow cytometry ($n = 5$) in MDHLC populations while no staining was noted in MSCs ($2.6 \pm 2.0\%$, $n = 3$) (Fig. 3c).

Analysis of hepatocyte-specific marker expression by RT-PCR revealed that MDHLCs expressed albumin (3/5), α FP (5/6), α 1-antitrypsin (3/5), G6Pase (2/5), PEPCK (2/5), TAT (3/5), TDO (2/4) and c-met (2/5) (Fig. 4). Although absent at the protein level, CK8 expression was noted in 2/5 samples. MDHLCs did not express CYP3A4, CYP2B6, HNF4 nor CK18 ($n = 5$ each).

Differences in the expression pattern of hepatocyte markers between MDHLC samples were not dependent on culture age, as we compared MDHLC populations of similar passages (passage 1–3). Moreover, these variations were observed up to passage 8 and could not be correlated to bone marrow sample characteristics (donor's age, cell proliferation rate) (Table 3).

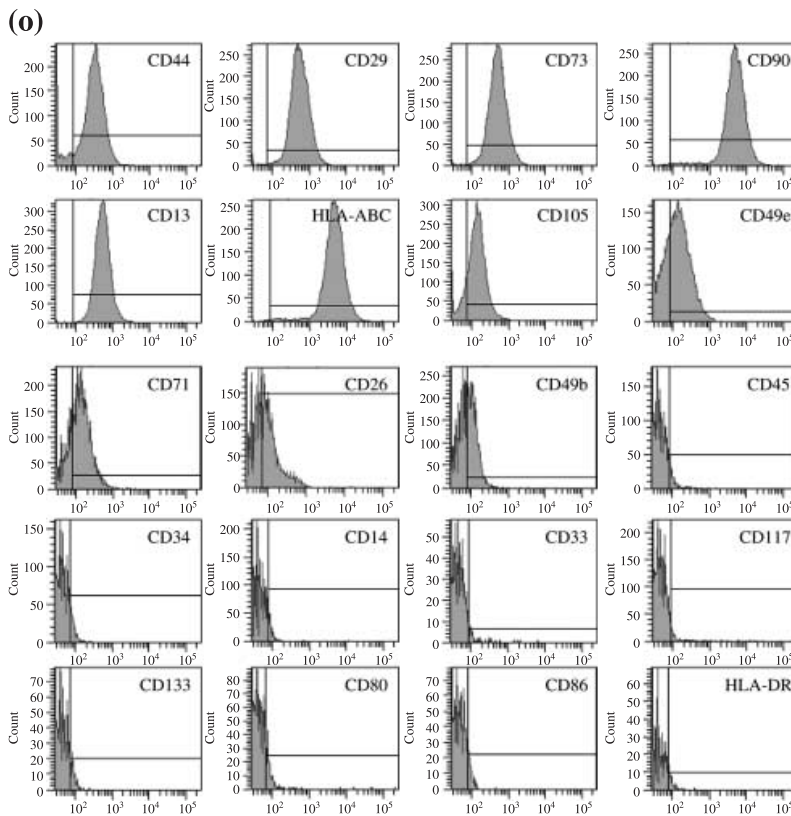
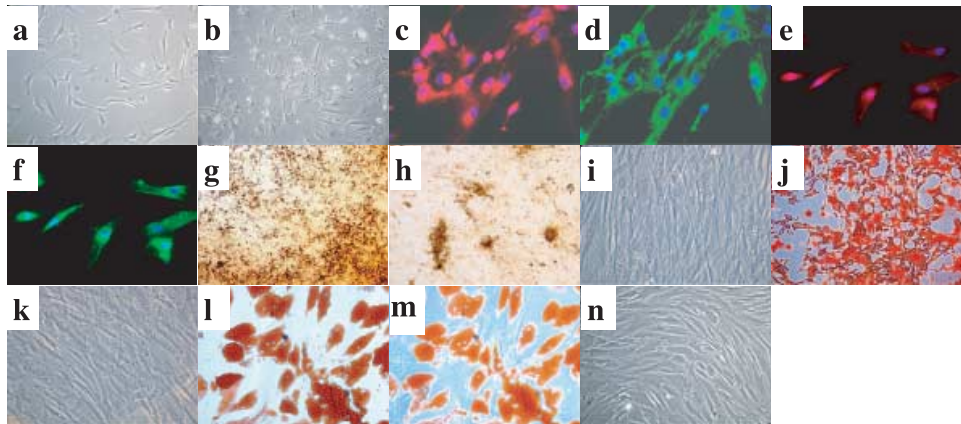


Figure 1. Characterization of MSCs. Example of MSCs morphology at passage 1, day 15 (a) that was altered at passage 8, day 99 (b). Expression of mesodermal antigens in MSCs at passage 2 as revealed by immunofluorescence [vimentin (c), fibronectin (d), ASMA (e), laminin (f), $n = 3$ each]. Staining of extracellular calcium matrix deposition by von Kossa (g, h) and alizarin red (j) colorations in MSCs after osseous differentiation. Accumulation of intracytoplasmic lipid-rich droplets revealed by oil red O staining (l, m) after adipocyte differentiation of MSCs. Respective controls are provided with undifferentiated MSCs (i, k, n). Example of representative immunophenotype of MSCs at passage 2 (o). Analyzed epitopes are indicated in the histograms. Bars indicate the fluorescence level of corresponding isotype. Pictures magnifications are: $\times 100$ (b, g), $\times 200$ (j), $\times 300$ (c–f) and $\times 400$ (a, i, k–n).

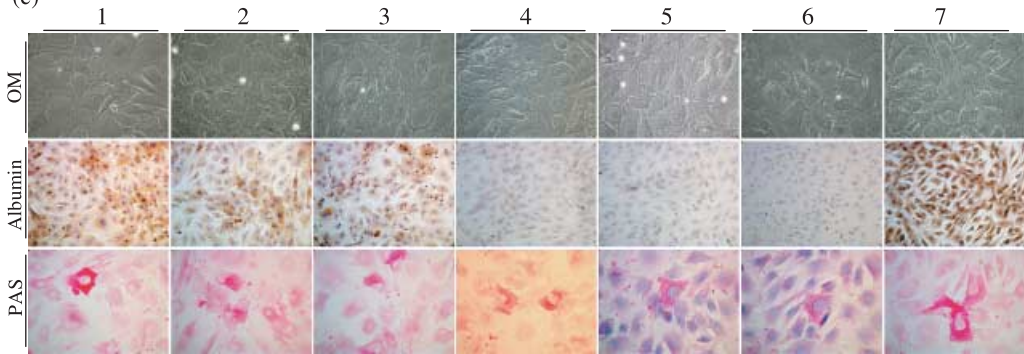
(a)

	A		B
Step 1	HGF	±	aFGF
			bFGF
			FGF4
			EGF
			OSM
			IL6
			DMSO
			dexa
			ascorbate
			ITS
Step 2	OSM	±	HGF
			dexa
			ITS

(b)

Hepatocyte differentiation protocols							
	1	2	3	4	5	6	7
Step 1	HGF aFGF bFGF	HGF EGF	HGF OSM	HGF IL6	HGF DMSO	OSM	HGF FGF4
Step 2	OSM	OSM	OSM HGF	OSM	OSM	OSM	OSM

(c)



(d)

Hepatocyte differentiation protocols							
	1	2	3	4	5	6	7
% Morphology	59 ± 2.7 (n = 942)	58.5 ± 1.4 (n = 899)	58.8 ± 3.2 (n = 913)	58 ± 1.6 (n = 920)	57.5 ± 1.9 (n = 889)	57 ± 2.1 (n = 908)	61.1 ± 1.1 (n = 910)
% Albumin positivity	53.5 ± 2.1 (n = 1005)	47.2 ± 1.7 (n = 952)	46.6 ± 1.8 (n = 892)	5.2 ± 3.2 (n = 922)	3.7 ± 2.1 (n = 932)	0.8 ± 0.2 (n = 1001)	65.5 ± 1.5 (n = 940)
% PAS positivity	15.7 ± 4.8 (n = 912)	14.1 ± 4.1 (n = 1012)	12.8 ± 3.6 (n = 960)	6.7 ± 3.8 (n = 911)	3.2 ± 2.5 (n = 875)	1.9 ± 1.5 (n = 942)	21.1 ± 5.3 (n = 1041)

(e)

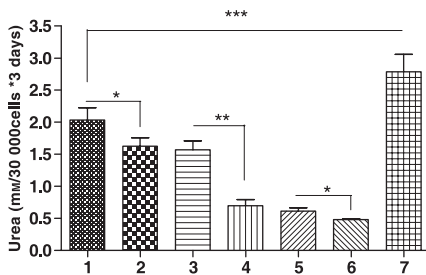


Figure 2. Description of the data collected for the elaboration of the complete hepatocyte differentiation protocol. (a) Description of the cytokines and factors tested in combinations ($n = 30$) following a two-step procedure. Column A describes the factors invariably used in the protocols. Column B lists the factors that were added independently or in a combined manner (for step 1). (b) Description of the most representative hepatocyte differentiation protocols designed by combination of the cytokines/factors described in table A. Factors as dexamethasone, ascorbate (ascorbic acid 2-phosphate), ITS and nicotinamide were not considered in the description. (c) Pictures showing the results obtained after completion of the protocols described in table B (30 days procedures) in terms of morphology analysed by optic microscopy (OM), albumin content assayed by immunocytochemistry and glycogen storage depicted by periodic acid-Schiff (PAS) staining. Morphology and PAS staining pictures were taken at magnification $\times 400$. Albumin staining pictures were taken at magnification $\times 200$. (d) Calculation, for each group of differentiation, of the amount of cells displaying a hepatocyte-like morphology or a positive staining for albumin or glycogen. Whereas similar morphological changes were observed in each procedure, the highest albumin and glycogen-positive cell content was noticed in protocol no. 7. Values are presented as mean \pm SD. Total cell count for each experiment is provided between brackets. (e) Values of urea secretion for each differentiation protocol at 30 days ($n = 6$ for each experiment). Urea production of the protocol no. 7 was significantly higher than all of the other protocols. P values are indicated in the graph ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

Table 3. Influence of BM-MSCs culture characteristics on hepatic markers expression

		BM samples					
		1	2	3	4	5	6
BM data	BM donor age	21 years	47 years	8 years	34 years	54 years	28 years
	Passage	P1	P2	P1	P3	P3	P2
	Growth	34 PD	23 PD	31 PD	28 PD	29 PD	26 PD
RT-PCR markers	Albumin	-	-	+	+	+	ND
	α FP	-	+	+	+	+	+
	CK-8	+	-	-	+	ND	-
	CK-18	ND	-	-	-	-	-
	α 1-AT	+	+	-	-	+	ND
	G6Pase	-	ND	+	-	-	+
	CYP 3A4	-	-	ND	-	-	-
	CYP 2B6	-	-	ND	-	-	-
	TAT	+	+	-	+	ND	-
	TDO	ND	+	+	-	-	ND
	HNF4	ND	-	-	-	-	-
	c-Met	-	-	-	ND	+	+
	PEPCK	+	ND	-	-	-	+
ICC markers	Albumin	-	-	+	+	+	+
	α FP	-	+	-	-	+	+
	DPPIV	+	+	+	+	+	+
	CK8	-	-	-	-	-	-
	CK18	-	-	-	-	-	-
	HepPar-1	-	-	-	-	-	-
	Connexin-32	+	ND	ND	+	+	+
E-cadherin	ND	+	+	ND	+	ND	

α 1-AT, α 1-antitrypsin; α FP, α -foetoprotein; BM, bone marrow; CK, cytokeratin; CYP, cytochrome P450; DPPIV, dipeptidylpeptidase IV; G6Pase, glucose-6-phosphatase; HNF4, hepatic nuclear factor 4; ICC, immunocytochemistry; ND, not determined; P, passage; PD, population doublings; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; TDO, tryptophan-2,3-dioxygenase.

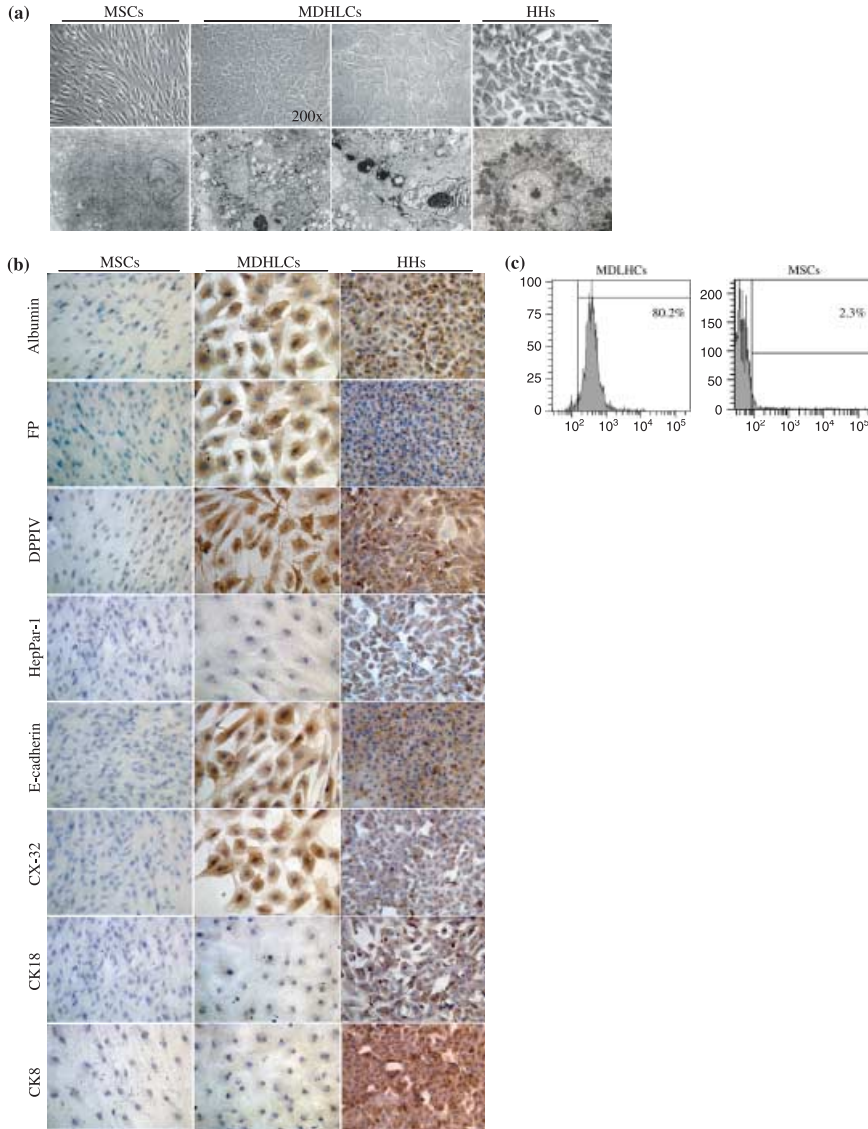


Figure 3. *In vitro* hepatocyte differentiation of human bone marrow-MSCs. (a) Comparative aspect of MSCs, MDHLCs and HHs as observed in optic (OM) and electron (EM) microscopy. Pictures show that MDHLCs acquire round or polygonal-shaped cells reduced in size and containing cytoplasmic granulations and central nucleus with prominent nucleolus. HHs comparative cell size was smaller. EM reveals that MDHLCs exhibited a higher content in cytoplasmic organites (endoplasmic reticulum, mitochondria) and a prominent nucleolus suggesting their enhanced metabolic activity. They produced intracytoplasmic glycogen vacuoles that are a hallmark of hepatocyte-like functionality. Comparison with MSCs and HHs is provided. OM pictures were taken at magnification $\times 400$ unless indicated and EM pictures magnification was $\times 4000$. (b) MDHLCs immunostaining for hepatocyte markers showing positive expression of albumin, α FP, DPPIV and some epithelial markers (E-cadherin, CX-32) but not of HepPar-1, CK8 and CK18. MSCs and HHs stainings were provided as negative and positive controls, respectively. Pictures were taken at magnification $\times 400$. (c) Representative examples of albumin staining by flow cytometry in MDHLCs and MSCs. Bars indicate the fluorescence level of the corresponding isotype. α FP, α -foetoprotein; CK18, cytokeratin 18; CX-32, connexin-32; DPPIV, dipeptidylpeptidase IV; HHs, human primary hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells.

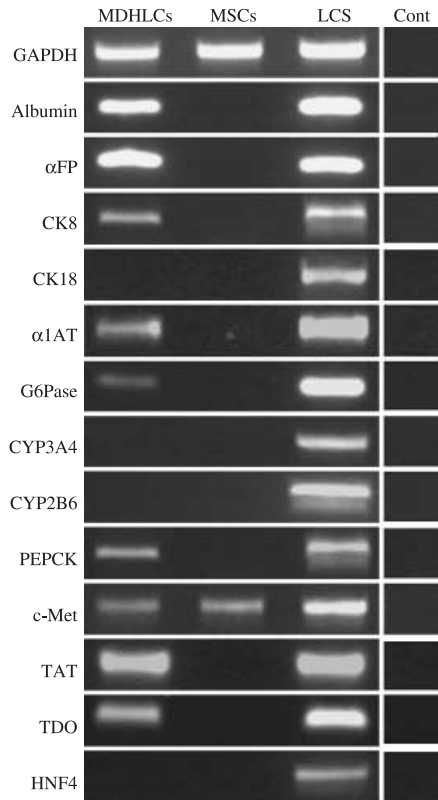


Figure 4. Analysis of hepatocyte and mesodermal markers expression by RT-PCR in MDHLCs, MSCs (both on passage 1 to 3) and freshly isolated human liver cell suspension (LCS). The figure shows concomitant immature (α FP, CK8) and mature (albumin, α 1AT, G6Pase, PEPCK, TAT, TDO) hepatocyte gene expression in MDHLCs while other markers are absent (CK18, CYP2A3, CYP2B6, HNF4). Both MSCs and MDHLCs expressed HGF receptor c-met. Controls without reverse transcriptase (Cont) are shown for each analysed primer. Abbreviations of oligonucleotides are listed in Table 1.

After having noticed variability in acquisition of hepatocyte markers in MDHLCs, we further evaluated maturation levels of these cells by studying their expression of mesenchymal markers. We observed that MDHLCs maintained MSC marker expression as assessed by immunofluorescence analysis, showing co-expression of fibronectin and albumin, E-cadherin and CX-32, ASMA and α FP and vimentin plus albumin (Fig. 5a). No immunohistochemical staining was detected for these mesodermal antigens in primary human hepatocytes. These data were confirmed at mRNA level by demonstration of persistence of vimentin, ASMA and fibronectin expression in MDHLCs (Fig. 5b). We estimated the ratio of hepatocyte/mesenchymal marker coexpression by analysing co-staining for albumin, CD73 and CD90 in MDHLCs (Fig. 5c) and observed that the majority of albumin-positive cells co-expressed the mesenchymal markers (for the albumin-positive population, mean CD73 positivity was $56.5 \pm 8.4\%$ and mean CD90 positivity was $62.7 \pm 10.7\%$) whereas $76.7 \pm 14.1\%$ of MDHLCs co-expressed CD73 and CD90. Besides showing persistence of MSC markers in MDHLCs, flow cytometric analysis indicated that some markers were significantly down-regulated in these cells compared to MSCs (Fig. 5d): CD44: 84.2 ± 7.5 versus $98.8 \pm 0.8\%$ ($P < 0.05$); CD29: 89.3 ± 6.6 versus $98.2 \pm 1.7\%$ ($P < 0.05$);

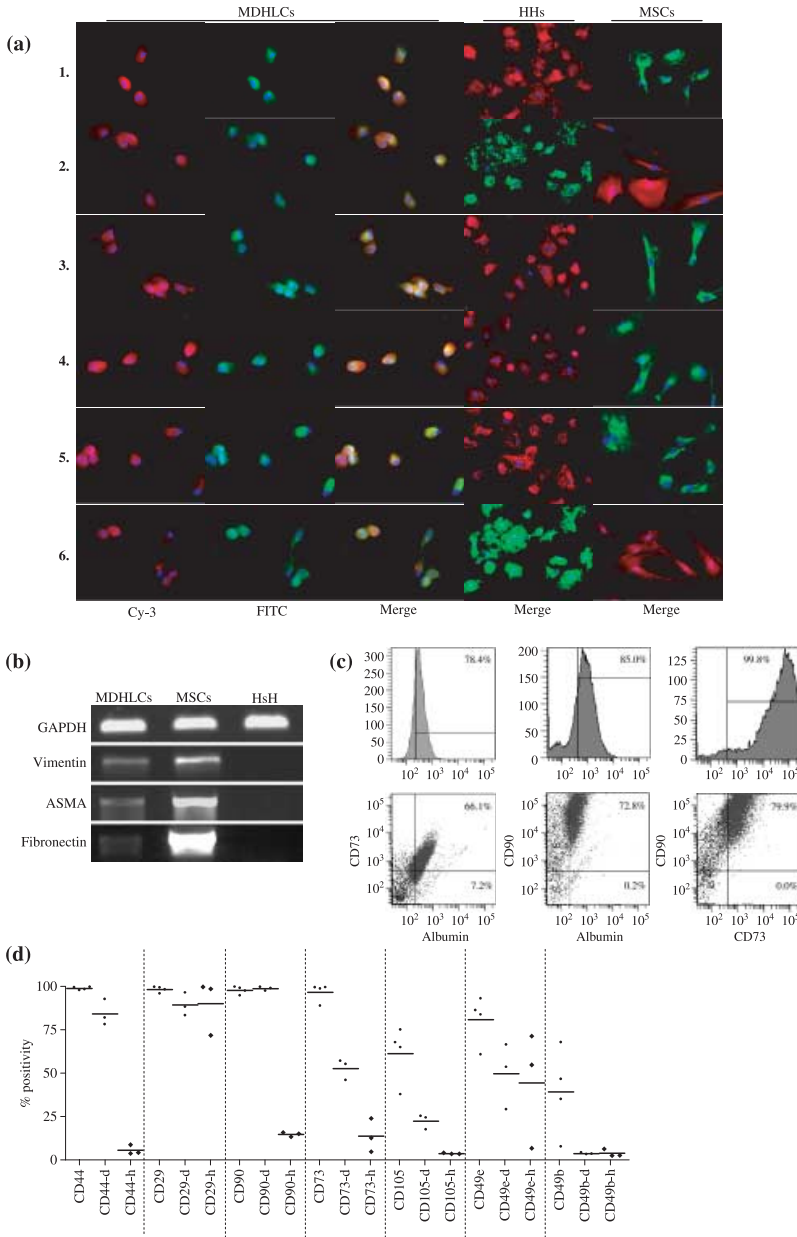


Figure 5. Characterization of the chimeral phenotype of MDHLCs. (a) Immunofluorescence assay showing co-staining of MDHLCs, HHs and MSCs for albumin/fibronectin (1), ASMA/ α FP (2), DPPIV/fibronectin (3), E-cadherin/fibronectin (4), CX-32/fibronectin (5) and vimentin/albumin (6). Pictures were taken at magnification $\times 300$. (b) RT-PCR analysis revealing the persistence of vimentin, ASMA and fibronectin expression in MDHLCs population. (c) Flow cytometry assay showing a representative example of albumin, CD73 and CD90 co-staining on MDHLCs. (d) Comparative flow cytometric phenotype of MSCs (passage 2 to 4), MDHLCs (passage 1 to 3), and human hepatocytes. MDHLCs and hepatocytes are specified respectively by (-d) and (-h) suffixes. P values are indicated below graph (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). α FP, α -foetoprotein; ASMA, α -smooth muscle actin; CX-32, connexin-32; DPPIV, dipetidylpeptidase IV; HHs, human primary hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells.

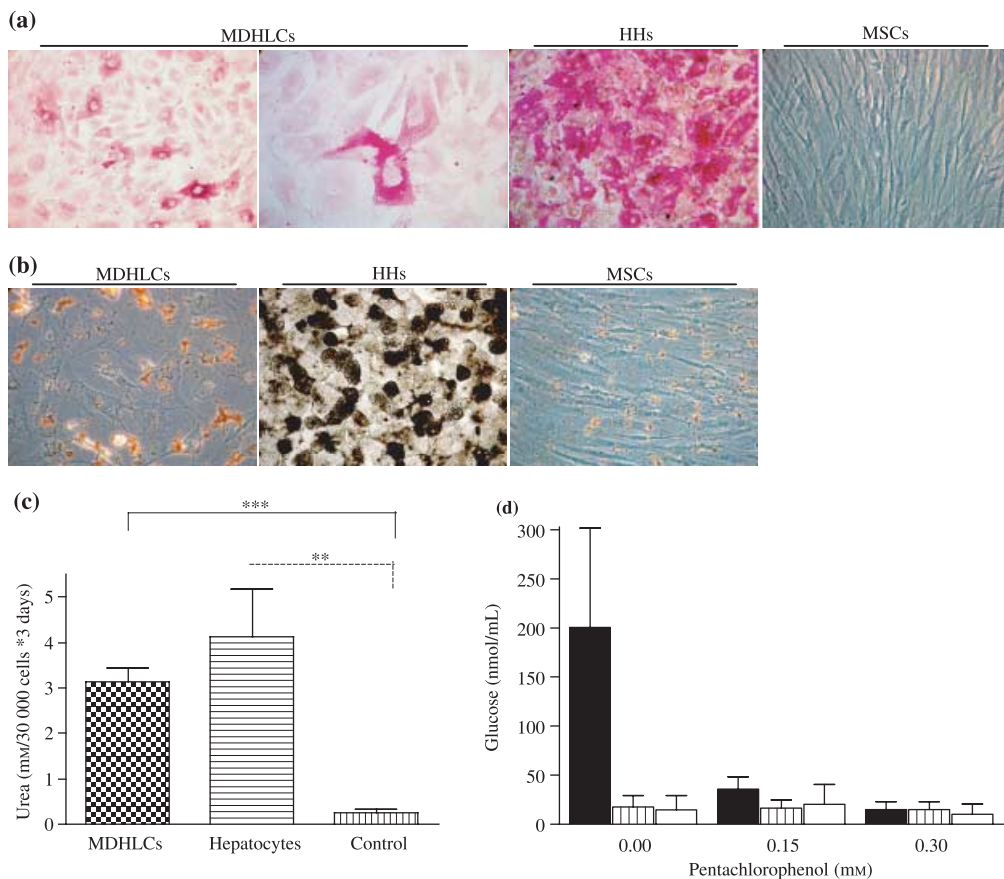


Figure 6. Functional characterization of MDHLCs. (a) Periodic acid-Schiff (PAS) staining showing cytoplasmic glycogen deposition in MDHLCs as compared to HHs and MSCs. (b) G6Pase assay ($n = 5$) showing lack of enzyme activity in MDHLCs revealed by absence of brownish lead sulphide cytoplasmic precipitate. Positive and negative controls were performed respectively on HHs and MSCs. Similar results were obtained with overnight 5 mm G6Pase incubation. Pictures were taken at magnification $\times 400$. (c) Colorimetric assay showing significant urea production in MDHLCs ($n = 4$) as compared to MSCs ($n = 4$). By comparison, no difference could be observed between the urea production of MDHLCs and mouse hepatocytes. (d) Gluconeogenesis assay showing the decrease of glucose neoformation in mouse hepatocytes (plain bars) after exposure of growing concentrations of pentachlorophenol. No glucose production was observed in MDHLCs (shaded bars) or in MSCs (empty bars) ($n = 3$ each). HHs, human primary hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells.

CD73: 52.7 ± 5.9 versus $96.5 \pm 5.2\%$ ($P < 0.001$); CD105: 22.4 ± 4.2 versus $61.3 \pm 16.3\%$ ($P < 0.05$). Down-regulation of CD49e (49.8 ± 18.9 versus $80.9 \pm 14.0\%$) and CD49b (3.7 ± 0.5 versus $39.2 \pm 25.0\%$) was not significant. MDHLCs did not modify the expression of CD90 (Thy-1) (Fig. 5b) or HLA-DR, HLA-ABC, CD80 and CD86 (data not shown). Comparison of immunophenotype of MDHLCs with human hepatocytes demonstrated that the down-regulation occurring in MDHLCs tended these cells to resemble to mature hepatocytes (Fig. 5d).

We then studied the hepatocyte-like functional activity of MDHLCs. We first evaluated glycogen production which was noticed in all analyzed samples, with a mean positivity of $21.1 \pm 5.3\%$ (calculation in three different random fields of each three samples at magnification $\times 200$, total n cells = 1041) (Fig. 6a). Although G6Pase was expressed at the mRNA level,

we could not detect enzyme activity in MDHLCs by cytochemical assay, despite 24 h incubation with 5 mM G6Pase (Fig. 6b). However, we detected significant urea production and noticed that this secretion was comparable to that of mouse hepatocytes (respectively 3.1 ± 0.5 and 4.1 ± 2.1 mM/30 000 cells *3 days compared to 0.2 ± 0.2 mM/30 000 cells *3 days for negative control) (Fig. 6c). Although this production appeared within 3 days of culture, it did not increase significantly but was maintained throughout the differentiation procedure (30 days). We also analysed MDHLC supernatants and did not detect any albumin secretion levels as assessed by two different ELISA assays. Compared to G6Pase, whereas MDHLCs displayed phosphoenolpyruvate carboxykinase (PEPCK) mRNA, we did not observe glucose neoformation after exposure to gluconeogenesis substrates (Fig. 6d).

***In vivo* hepatocyte differentiation of mesenchymal stem cells**

To investigate hepatocyte differentiation potential of MSCs *in vivo*, we transplanted these cells into spleens or livers of SCID mice subjected to a liver regeneration stimulus (Fig. 7). When they were injected into spleens of liver-injured mice (groups A and B), we observed engrafted cells organized as single cells or as clusters and expressing fibronectin and α FP but not vimentin or albumin (Fig. 7a). We did not observe any difference in engraftment level or the marker expression between groups A and B, suggesting that equivalent hepatic regeneration stimulus was provided by hepatectomy or retrorsine-allyl alcohol conditioning, in our experimental conditions (Table 4). Noticeable improvement of engraftment was obtained when MSCs were infused into the mouse livers (group C). In this group, engrafted cells analysed on serial sections displayed combined expression of mesodermal (fibronectin and vimentin) and hepatocyte markers (albumin and α FP) (Fig. 7a). When we differentiated MSCs prior to transplantation (groups D and E), cells expressed albumin and α FP but lost the expression of fibronectin and vimentin (Fig. 7a). We then explored CK18 expression in groups A, C and E and observed positive staining in cells engrafted after intrahepatic infusion (groups C and E) (Fig. 7b). Moreover, in group C, we noticed cell clusters co-expressing CK18 together with albumin and fibronectin, confirming the chimaeric phenotype described above. No anti-CK18 staining could be detected in group A (data not shown). Engraftment level was similar between MSC and MDHLC groups when comparing same injection site (Table 4). Specificity of each type of staining was ensured using appropriate controls (Fig. 8). We did not detect human albumin in mouse sera of any transplant groups (data not shown).

In parallel, we tracked engrafted human cells by staining nuclei with human Alu probe using *in situ* hybridization technique (Fig. 9). By analysis of co-expression on serial sections, we demonstrated that these human Alu-positive cells contained human protein fibronectin in their cytoplasm (Fig. 9).

DISCUSSION

In this work, we provide clear demonstration of persistence of initial lineage phenotype, in MDHLCs, associated with incomplete acquisition of hepatocyte-like features. We have produced evidence that *in vitro*, hepatocyte differentiation of MSCs is restricted to the phenotype level as depicted by morphological changes and specific marker expression. Because this expression associates both early and late differentiation markers, the MDHLC phenotype does not correspond to a defined differentiated state. Incomplete expression of hepatocyte markers indicated the partiality of the differentiation procedure. Besides positive staining for E-cadherin

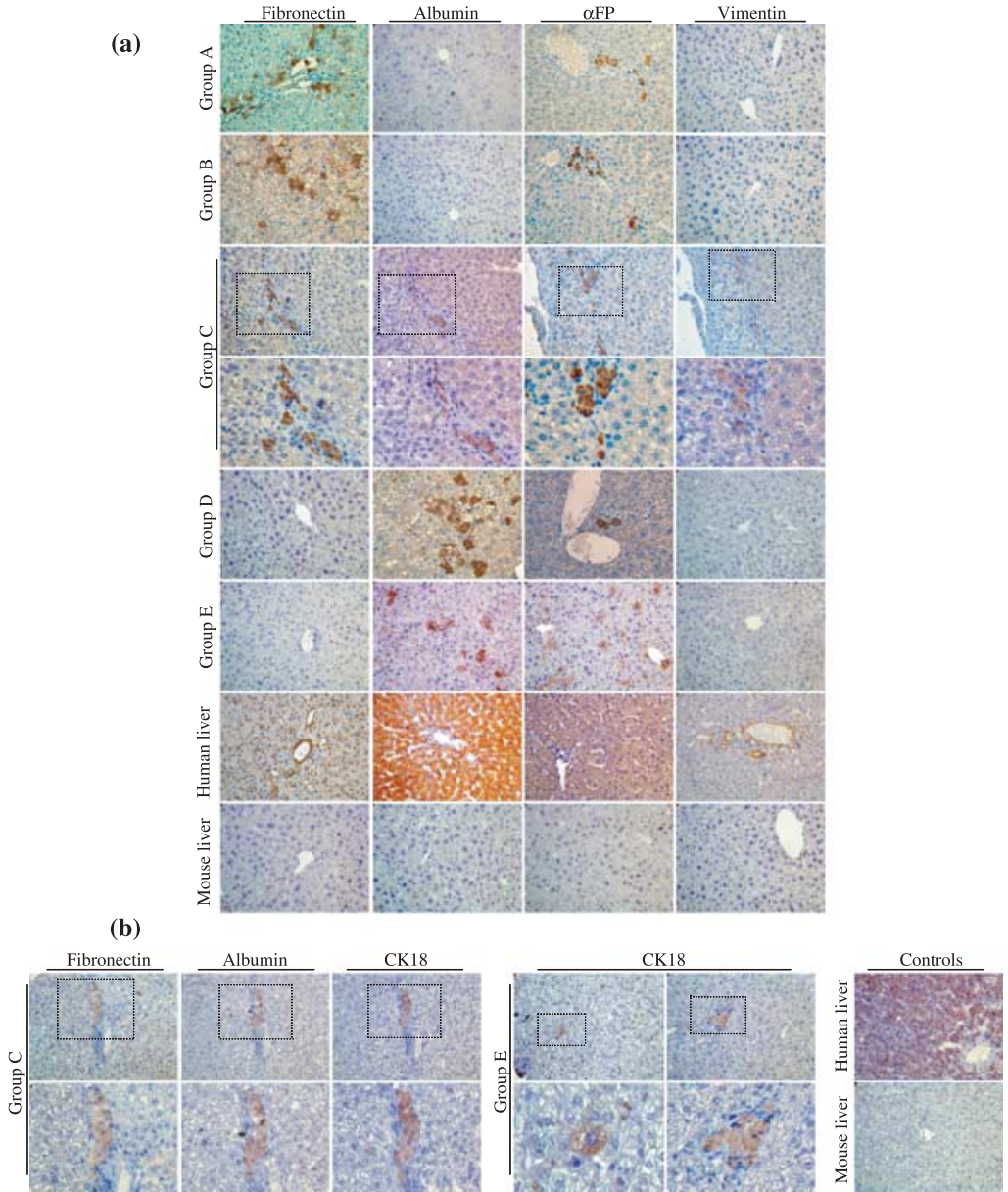


Figure 7. *In vivo* characterization of MSCs and MDHLCs after transplantation into SCID mice. (a) Analysis of *in vivo* differentiation potential of MSCs and MDHLCs. Pictures show immunostaining for human mesodermal (fibronectin, vimentin) and hepatocyte (albumin, α FP) antigens. In groups A and B, the engrafted cells formed clusters presenting staining for fibronectin or α FP separately. In group C, cell clusters co-expressed mesodermal and hepatocyte markers as assessed by serial sections. In groups D and E, where MDHLCs were injected respectively into the spleen or the liver, the engrafted cell clusters expressed only hepatocyte markers. Stains were performed on human and mouse livers respectively as positive and negative controls. (b) Analysis of CK18 expression in groups C and E mice. In group C, the engrafted cells displayed co-expression of fibronectin, albumin and CK18 whereas in group E, isolated staining for CK18 was observed in single or clustering cells. Control stains for CK18 were performed on human and mouse livers. Pictures were taken at magnification $\times 400$. α FP, α -foetoprotein.

Table 4. Estimation of liver engraftment level after cell transplantation into SCID mice

Groups	Fibronectin				Albumin				αFP				CK18			
	Cell clusters	Cells/ cluster	Isolated cells	Total cells	Cell clusters	Cells/ cluster	Isolated cells	Total cells	Cell clusters	Cells/ cluster	Isolated cells	Total cells	Cell clusters	Cells/ cluster	Isolated cells	Total cells
A	0.7 (0–2)	28 (10–41)	3.7 (0–15)	16.5 (0–65)	0	0	0	0	0.2 (0–1)	9 (5–9)	2 (0–8)	4.2 (0–17)	0	0	0	0
B	2.2 (0–5)	11 (3–24)	31.2 (0–67)	50.5 (0–109)	0	0	0	0	0	0	4.2 (0–17)	4.2 (0–17)	ND	ND	ND	ND
C	5 (0–12)	11.8 (2–33)	6.7 (0–17)	65.7 (0–201)	6.7 (0–16)	8.7 (2–32)	2.7 (0–12)	60.8 (0–163)	5.3 (0–12)	8.7 (2–38)	8.7 (0–26)	55 (0–195)	2.2 (0–6)	9.6 (3–24)	4.3 (0–19)	26.7 (0–51)
D	0	0	0	0	3.5 (0–8)	4.8 (2–12)	17 (0–39)	33.7 (0–70)	1.2 (0–3)	6.8 (3–12)	5.2 (3–7)	13.7 (5–22)	ND	ND	ND	ND
E	0	0	0	0	5.7 (0–9)	5.6 (2–23)	42.7 (3–70)	74.3 (3–109)	7.7 (3–15)	8.3 (2–35)	6.3 (0–15)	70.3 (34–133)	3.3 (0–7)	7.5 (2–31)	4.7 (3–7)	28.3 (4–71)

Values present the number of cell clusters, cell content of clusters, amount of isolated staining cells and total amount of cells per section for each group of staining performed in the mouse livers (minimum sections per group = 6). Data are provided as mean with range value in brackets. αFP, α-foetoprotein; CK18, cytokeratin 18; ND, not documented; SCID, severe combined immune deficiency.

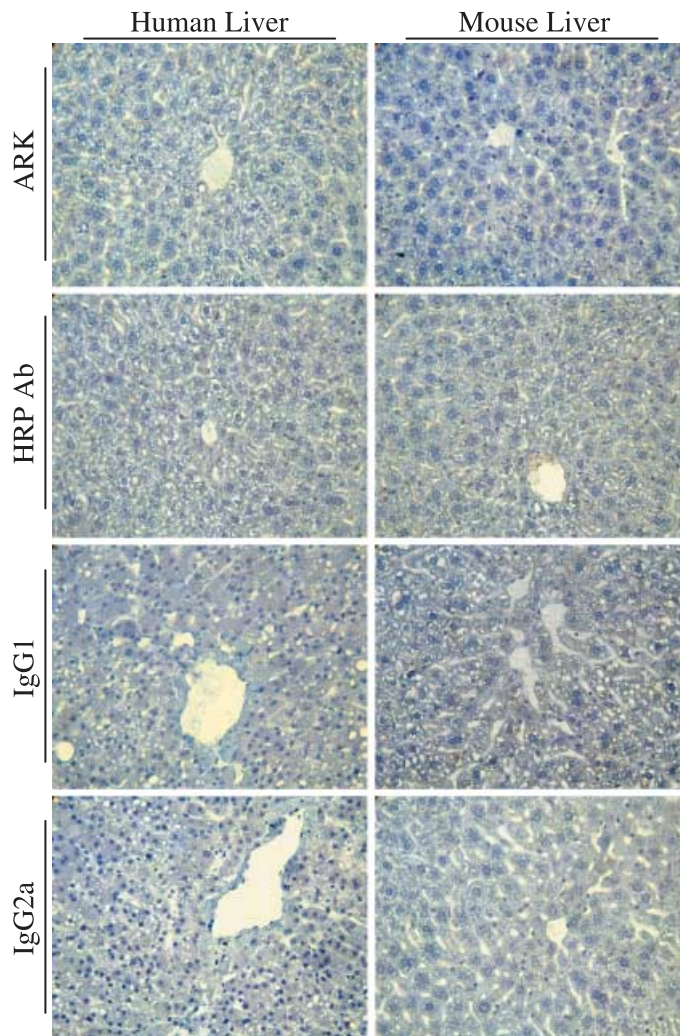


Figure 8. Control immunohistochemistry. Pictures show staining assays performed without primary antibodies using ARK kit (ARK) or HRP-conjugated anti-rabbit antibody (HRP Ab). For monoclonal antibodies, stains with corresponding isotypes were performed (IgG1 for anti-albumin and CK18 antibodies, IgG2a for anti-vimentin antibody). No cross-reactivity was observed in any of these conditions. Pictures were taken at magnification $\times 400$.

and CX-32, lack of CK8 and 18 protein expression *in vitro* suggested inability of MDHLCs to fully acquire a mature epithelial phenotype. Whereas this expression was rarely seen *in vitro* in differentiated MSCs (Hong *et al.* 2005; Oyagi *et al.* 2006), Sharma *et al.* have demonstrated lack of these parameters after differentiation of human cord blood cells *in vivo* (Sharma *et al.* 2005). Moreover, while MDHLCs expressed *G6Pase* and *PEPCK* genes, corresponding metabolic activities were not detected here. However, these cells were able to secrete small amounts of urea and to store glycogen as demonstrated in previous studies (Schwartz *et al.* 2002; Lee *et al.* 2004; Aurich *et al.* 2007; Chien *et al.* 2006). At cell population level, acquisition rate estimation of the observed hepatocyte-specific features revealed that only a proportion of MDHLCs were engaged in the differentiation process. Furthermore, some of these features were not always associated

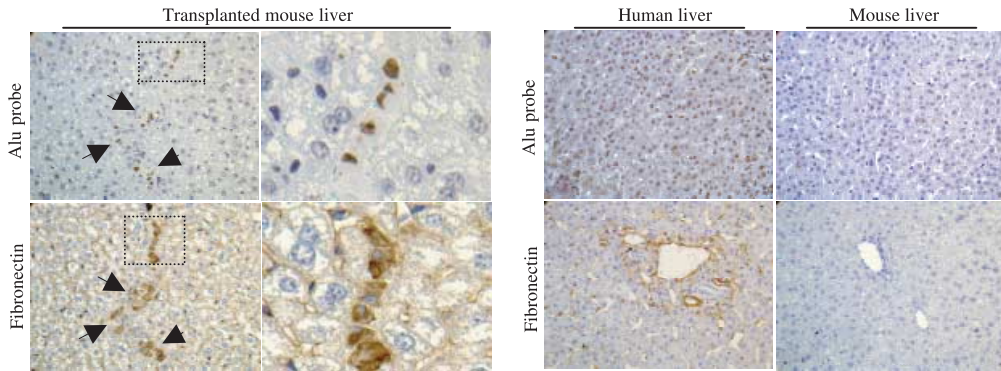


Figure 9. *In vivo* tracking of human cells. Pictures show that engrafted human cells were co-stained at the nuclear level with Alu probe by *in situ* hybridization technique and at the cytoplasmic level with antihuman fibronectin antibody by immunohistochemical assay on serial sections. Human and mouse livers are provided respectively as positive and negative controls. Arrows indicate human positive cells. Pictures were taken at magnification $\times 400$.

with efficient differentiation, as, for instance, hepatocyte-like morphological changes that were obtained with control medium (maturation medium). These data provided arguments concerning hepatocyte-like commitment of MDHLCs while further advance is mandatory to demonstrate their capacity to acquire mature hepatocyte functionality (for review, see Hengstler *et al.* 2005). Furthermore, the usefulness of hepatic markers for demonstrating efficiency of differentiation could also be questioned, as some authors have described expression of such markers in undifferentiated cells (Avital *et al.* 2001; Kogler *et al.* 2004; Lee *et al.* 2004; Hong *et al.* 2005; Seo *et al.* 2005).

We have confirmed the partial differentiation status of MDHLCs by demonstrating persistent expression of mesenchymal markers in these cells. As reported by protein and gene expression analyses, MDHLCs retained expression of vimentin, fibronectin, ASMA and laminin. This was confirmed by flow cytometric analysis of albumin, CD73 and CD90 co-expression. Nevertheless, flow cytometry identified some mesenchymal markers (CD44, CD29, CD73 and CD105) that were significantly down-regulated in MDHLCs, these cells tending to resemble mature hepatocytes. It is noteworthy that these markers are mostly useful to identify MSCs and the onset of their preferential down-regulation in MDHLCs could confirm that the cells represent a transitional state between MSCs and mature hepatocytes. This down-regulation could be related to the presence of HGF in the differentiation cocktail as demonstrated for CD105 marker expression in MSCs (Forte *et al.* 2006).

These *in vitro* findings were corroborated *in vivo* by showing that MSCs maintained mesodermal marker expression while acquiring liver-specific antigens. Spleen-infused MSCs displayed elective expression of fibronectin and α FP regardless of hepatic regeneration stimulus used. Intrahepatic injection of MSCs was performed to evaluate influence on the expression profile of direct implantation of these cells into the liver microenvironment. We observed that liver-infused MSCs had a complete pattern of mesenchymal and hepatocyte marker expression. Furthermore, we detected expression of the mature hepatocyte marker CK18, only in mice receiving intrahepatic cell injection indicating that direct *in situ* cell injection allowed further maturation of the hepatocyte-like phenotype to take place. Compared to MSCs, engrafted MDHLCs lost mesenchymal marker expression suggesting that pre-transplant conditioning was also an important factor for maturation of the cell phenotype *in vivo*. Further investigations will

be necessary to determine whether MSCs exposed to the liver micro-environment for longer term, would display similar maturation. However, no enhancement of repopulation rate could be observed with MDHLCs as compared to mice infused with MSCs. In the xenogeneic transplantation model, even if intrahepatic cell injection enhanced repopulation rates, these appeared to be weak over all procedures, reflecting a possibly inadequate environment such as immune reactions, cell-to-cell contacts (Bhatia *et al.* 1999), receptor factor interactions and more. We further assured human origin of the engrafted cells by showing co-expression of human markers at the nuclear and cytoplasmic levels. Globally, our data were consistent with recent work suggesting positive influence of pre-transplant HGF treatment in terms of MSCs engraftment *in vivo*. In contrast, Seo *et al.* obtained equivalent repopulation rates with pretreated or native adipose tissue-derived stromal cells. We also have corroborated what have been described as haematopoietic (stem) cells (Fiegel *et al.* 2003; Kakinuma *et al.* 2003; Yamazaki *et al.* 2003; Jang *et al.* 2004; Ruhnke *et al.* 2005; Yamada *et al.* 2006) and other cell lineages (Lysy *et al.* 2007; von Mach *et al.* 2004; Nussler *et al.* 2006; Najimi *et al.* 2007) that have been suggested to display hepatocyte differentiability. However, recent work has provided strong arguments concerning the difficulty in obtaining hepatocyte features from haematopoietic stem cells efficiently, both *in vitro* and *in vivo* (Lian *et al.* 2006; Thorgeirsson & Grisham 2006; Yamaguchi *et al.* 2006).

In conclusion, in our hands, hepatocyte differentiation of MSCs appeared to be effective in producing phenotype-restricted hepatocyte-like cells presenting partially down-regulated native mesenchymal markers. Absence of this chimaerism *in vivo* after MSC pre-transplantation differentiation underlies the importance of the *in vitro* step for improvement of the yield of differentiation of engrafted cells. However, more investigations are required to evaluate whether chimaeric status of MDHLCs is associated with preserved advantageous properties of MSCs [less immunogenicity and immunomodulatory properties (Le Blanc & Ringden 2005), resistance to cryopreservation, proliferation potential or easiness of transfection (Javazon *et al.* 2004)] or conversely, whether the remaining mesodermal phenotype of these cells hampers the differentiation process. Elaboration of *in vitro* conditions, that mimic the liver microenvironment and allow enhanced hepatocyte-specific metabolic activity of MDHLCs, is also mandatory.

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