LIVER

Functional integration of hepatocytes derived from human mesenchymal stem cells into mouse livers

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Aims: At present, clinical success of hepatocyte transplantation as an alternative to whole liver transplantation is hampered by the limited availability of suitable donor organs for the isolation of transplantable hepatocytes. Hence, novel cell sources are required to deliver hepatocytes of adequate quality for clinical use. Mesenchymal stem cells (MSCs) from human bone marrow may have the potential to differentiate into hepatocytes in vitro and in vivo.

Methods: Isolated MSCs were selected by density gradient centrifugation and plastic adherence, differentiated in the presence of human hepatocyte growth medium and transplanted in immunodeficient Pfp/Rag2 mice.

Results: Here, we demonstrate that human MSCs gain in vitro the characteristic morphology and function of hepatocytes in response to specified growth factors. Specifically, preconditioned MSCs store glycogen, synthesise urea and feature the active hepatocyte-specific gene promoter of phosphoenolpyruvate carboxykinase (PCK1). After transplantation into livers of immunodeficient mice, preconditioned MSCs engraft predominantly in the periportal portion of the liver lobule. In situ, the cells continue to store glycogen and express PCK1, connexin32, albumin and the human hepatocyte-specific antigen HepPar1, indicating that the transplanted cells retain prominent qualities of hepatocytes after their regional integration.

Conclusion: MSCs derived from human bone marrow may serve as a novel source for the propagation of hepatocyte-like cells suitable for cell therapy in liver diseases.

Today, hepatocyte transplantation has become a tangible alternative to liver organ transplantation for the treatment of diseases of human liver. Yet, hepatocytes are only available from a restricted number of donor organs, usually marginal livers not allocated for organ transplantation.[1–3] Adult human hepatocytes, however, have a poor proliferative potential, which probably does not suffice to effectively repopulate the host liver.[4] Thus, novel sources for transplantable cells will have to be established to counter the increasing shortage of donor organs and the limited suitability of mature hepatocytes.

Adult haematopoietic and non-haematopoietic stem cells can differentiate into hepatocyte-like cells. In a mouse model of human liver failure (fumarylacetoacetate hydrolase (FAH) deficiency), the systemic injection of adult bone marrow cells[5] or bone marrow transplantation into lethally irradiated mice rescued the diseased phenotype[6] by providing the wild-type FAH in hepatocytes derived from bone marrow haematopoietic stem cells (HSCs). There is, however, ongoing controversy about whether HSC-derived hepatocytes originate from hepatocyte differentiation[7 8] or fusion of stem cells with host hepatocytes.[9–12]

Apart from HSC, however, bone marrow harbours a population of multipotent mesenchymal CD34- and CD45-negative stem cells,[13] which can be propagated in vitro and differentiate into cells with a mesodermal phenotype. After transplantation these cells engraft in different organs and differentiate into the respective organ-specific cell types.[14] This ability, and their easy accessibility and expansion potential in vitro, make mesenchymal stem cells (MSCs) an attractive resource for clinical use.[15–17] There is now evidence that a subpopulation of MSCs, the multipotent adult progenitor cells (MAPCs), are capable of hepatogenic and biliary differentiation in vitro, although proof of their functional transformation in vivo is lacking.[18 19] The hepatogenic differentiation potential of MSCs derived from bone marrow of rat, mouse and humans has recently been corroborated by the expression of distinct hepatocyte markers after conditioning in vitro.[20–22] Hepatogenic differentiation of MSCs from other sources such as adipose tissue[23] or umbilical cord blood[24] was achieved in vitro, too, again without sufficient proof of their phenotype in vivo. However, injection of human MSCs directly into livers of allylalcohol-intoxicated rats resulted in the transient engraftment of hepatocyte-like cells expressing specific human hepatocyte markers.[25]

Here, we report that mesenchymal stem cells (hBM-MSCs) derived from human bone marrow can be preconditioned in vitro to feature a functional hepatocyte phenotype. After partial hepatectomy, the intrasplenic delivery of preconditioned hBM-MSCs in immunodeficient mice resulted in the regional integration and repopulation of the host liver by hBM-MSC-derived cells in a mouse model of liver regeneration. Engrafted cells continued to display hepatocyte-specific functions in vivo. Thus, hBM-MSCs may have the potential to functionally regenerate a host liver long-term and may become an alternative source for transplantable hepatocytes.

Abbreviations: FAH, fumarylacetoacetate hydrolase; HSCs, haematopoietic stem cells; MSCs, mesenchymal stem cells; MAPCs, multipotent adult progenitor cells; hBM-MSCs, human bone marrow-derived mesenchymal stem cells; hBM-MNCs, human bone marrow-derived mononuclear cells; ODM, osteogenic differentiation medium; ADM, adipogenic differentiation medium; HHMM, human hepatocyte maintenance medium; CFU-Fs, colony forming unit-fibroblasts; BM-MSC, bone marrow-derived mesenchymal stem cells; PPAR-γ2, peroxisome proliferator-activated receptor gamma 2; LPL, lipoprotein lipase

MATERIALS AND METHODS

Isolation, propagation and single-cell-derived cultures of hBM-MSCs

hBM-MSCs were isolated from human bone marrow (hBM) aspirates of voluntary donors as approved by the Institutional Ethics Review Board. Written informed consent was obtained from all donors of hMSCs; signed consent forms exist with the researchers (LPM). Citrate-hBM (20 ml) aspirated from the iliac crest was centrifuged (10 min, 500×*g*), the pellet resuspended in 1×phosphate-buffered saline (PBS), applied to a density gradient (Biocoll, 1.047 g/ml; Biochrom) and centrifuged again (30 min, 500×*g*). The interface containing hBM mononuclear cells (hBM-MNCs) was resuspended in growth medium, centrifuged (5 min, 500×*g*), and the single cells were plated in growth medium on fibronectin-coated tissue culture flasks at a density of 1×10^5 hBM-MNCs/cm^2. On reaching confluence, cells were detached by trypsin/EDTA and replated at a density of 10 to 100 cells/cm^2 or stored by cryopreservation.

To study characteristics of MSCs under clonal conditions, single-cell-derived cultures were obtained by limiting dilution. A final suspension of 100 cells in 20 ml of growth medium was plated in a 96-well plate in 200 µl aliquots. Growth of single cells was detected by light microscopy.

Growth medium: 60% DMEM, 40% MCDB, 5 µg/ml apotransferrin, 5 ng/ml selenous acid, 5 µg/ml linoleic acid, 5 µg/ml bovine insulin, 100 µM ascorbic acid 2-phosphate, 1 nM dexamethason, 10 ng/ml PDGF-BB, 10 ng/ml EGF (all Sigma), 100 U/ml penicillin/10 µg/ml streptomycin and 2% fetal calf serum (FCS) (all Invitrogen).

Flow cytometry

Flow cytometry was performed using the following mouse anti-human antibodies: anti-CD11 PE, anti-CD13 PE, anti-CD14 FITC, anti-CD45 FITC, anti-HLA-DR FITC (all Beckton Dickinson), anti-CD29 PE, anti-CD34 FITC, anti-CD44 PE, anti-CD166 PE, anti-glycophorin PE (all BD PharMingen), anti-CD105 FITC (Serotec). Cells were incubated with the respective antibody for 15 minutes at 4℃, then washed with PBS and analysed with a FACS-Calibur with CellQuest software (Becton Dickinson).

In vitro differentiation

Fresh or cryopreserved cells were seeded at a density of 100 to 200 cells/cm^2. Undifferentiated cell samples were harvested or fixed at 50% confluence for further analysis. Cells for differentiation were grown to 100% confluence.

Osteogenic differentiation medium (ODM): DMEM containing 200 µM ascorbic acid 2-phosphate, 1 µM dexamethason, 10 mM glycerol-3 phosphate (all Sigma) and 10% FCS (Invitrogen). The medium was changed every 4 to 5 days over a period of 10 to 20 days.

Adipogenic differentiation medium (ADM): DMEM containing 50 µM dexamethason, 10 µg/ml bovine insulin, 100 µM indomethacin, 500 µM 3-iso-butyl 1-methylxanthine (all Sigma), 5 µM rosiglitazone (Alexis) and 10% FCS (Invitrogen). The medium was changed every 3 days over a period of 14 days.

Hepatogenic differentiation: cells were treated with 20 µM of 5'azacytidine for 24 hours. Thereafter, the growth medium was replaced by human hepatocyte maintenance medium (HHMM) supplemented with 2% FCS, HGF and EGF as described previously.[26] Cells were cultured for the times indicated and the medium changed every four days.

Cytochemistry of hMSC

Osteogenic and adipogenic differentiation was verified by staining for calcium depositions with alizarin-red-S pH 4 or intracellular lipid droplets with Oil-Red stain (all Sigma), respectively. Formalin-fixed cells were washed with PBS and then stained for 5 to 15 minutes followed by two to three washing steps with distilled water.

Polymerase chain reaction with reverse transcription (RT–PCR)

Cells were lysed with 1.2 ml TRIzol (Gibco BRL Life Technologies) and total RNA isolated according to manufacturer's instructions. RT–PCR for osteogenic and adipogenic transcripts was performed using the GeneAmp RNA PCR kit (Applied Biosystems). RT–PCR for hepatocyte transcripts was conducted using M-MLV reverse transcriptase (H-) (Promega). Subsequent PCR reactions were performed using cDNA, primer pairs and PCR Master Mix (MBI Fermentas) according to manufacturer's instructions. The primers used are listed in Table 1.

Transplantation of hMSC into Pfp/Rag2 mice

All animal experimentation procedures were in accordance with German legislation on animal protection. Pfp/Rag2$^{-/-}$ mice (malfunction of NK cell, T- and B-cell depletion) were obtained at 8 months of age from Taconic, Europe. Liver regeneration was temporarily inhibited by treating the mice with 0.1 mg propanolol hydrochloride (Schwarz Pharma) per millilitre drinking water for 24 hours before transplantation. After partial hepatectomy, 1×10^6 hBM-MSCs preconditioned in HHMM for 14 days as described were administered by intrasplenic injection. Livers were removed after 7 and 12 weeks and either frozen in isopropanol and stored at −80℃ or fixed in 3% formaldehyde for further analysis.

Immunocytochemistry

Cells were fixed with acetone/methanol (v/v, 1:1) for 20 min at −20℃. After blocking for 20 min in PBS containing 5% BSA and 0.5% Tween20, cells were incubated with primary antibodies overnight and with secondary antibodies for 1 hour at 37℃. Between each step, cells were washed with 1% BSA in PBS. The following primary antibodies were used: anti-CK18 antibody (1:100, Progen Biotechnik), anti-CX32 (1:5000, Sigma) and anti-HepPar1 (1:50, DakoCytomation). The rabbit polyclonal antibody against phosphoenolpyruvate carboxykinase (PCK1) was directed against a peptide comprising the amino acids 385 to 399 of the rat cytosolic form of the enzyme (Eurogentec). Alexa Fluor488 goat anti-mouse Ab (1:400, Molecular Probes) and anti-rabbit FITC-conjugated Ab (1:860, Sigma) were used as secondary antibodies.

Immunohistochemistry

After removal of paraffin from 1 µm serial sections of the formalin-fixed livers, the sections were incubated in 10 mM citrate buffer, pH 6.0, for 10 min at 96℃; endogenous peroxidase was inactivated with 0.3% hydrogen peroxide in methanol for 30 min at room temperature and blocked in 5% BSA and 0.1% Tween 20 in phosphate buffer for 1 hour at 37℃. The anti-HepPar1 (1:50), anti-PCK1 (1:100), anti-CX32 (1:500) and the anti-human serum albumin (1:1000; Abcam, UK) antibodies were added in blocking medium and sections incubated for 2 hours at 37℃. Labelled secondary antibody (HRP-labelled antimouse, 1:400, BD Transduction Laboratories or HRP-labelled antirabbit, 1:860, BD Transduction Laboratories) dissolved in blocking buffer was added for 1 hour at 37℃ followed by DAB Chromogen (DAKO) incubation for 10 min at room temperature. To stain human albumin, Vectastain Elite ABC Kit (anti-rabbit) (Vectorlabs) was used.

Table 1 Primer pairs used for PCR reactions to detect hepatocyte-, adipocyte- and osteocyte-specific gene expression in differentiated hBM-MSCs

Gene of interest	Primer pair	Product length (base pairs)	Annealing temperature (℃)
CK 18	5′-GAAGGAGACCATGCAAAGCCTG-3′ 5′-CATGAAGAGCAGCTCCTCCTTG-3′	400	62
CK 19	5′-CGAACCAAGTTTGAGACGGAAC-3′ 5′-CCGCTGGTACTCCTGATTCTGC-3′	600	62
CK 7	5′-TCATCGACAAGGTGCGGTTTC-3′ 5′-CTGCAGCTCTGTCAACTCCGTC-3′	400	62
CX 32	5′-GGCGTGAACCGGCATTCTAC-3′ 5′-ACAACAGCCGGAACACCACG-3′	400	61
CX 43	5′-TGGCCTTCTTGCTGATCCAG-3′ 5′-TTGCGGCAAGAAGAATTGTTTC-3′	400	58
AFP	5′-TTGCCCAGTTTGTTCAAGAAGC-3′ 5′-CGAGCAGCCCAAAGAAGAATTG-3′	400	59
Transferrin	5′-GTGGCCTTTGTCAAGCA-3′ 5′-CTCCATCCAAGCTCATG-3′	565	52
Albumin	5′-GATGTCTTCCTGGGCA-3′ 5′-CTTGGGCTTGTGTTTCAC-3′	645	52
PCK1	5′-GGCCGCACCATGTATG-3′ 5′-AGGATCAGCATGTGCTC-3′	398	58
CPS	5′-CTGTAAAAGCCATGAAGG-3′ 5′-CAATGGTGTCTGCTGCC-3′	399	60
CYP3A4	5′-TCTCATCCCAGACTTGGCCAT-3′ 5′-GGAGACAGAATAACATTCTT-3′	300	55
GAPDH	5′-CCA TGG AGA AGG CTG GGG-3′ 5′-CAA AGT TGT CAT GGA TGA CC	195	63
LPL	5′-ATG GAG AGC AAA GCC CTG CTC-3′ 5′-TAC AGG GCG GCC ACA AGT TTT-3′	299	63
PPAR-γ2	5′-GCT GTT ATG GGT GAA ACT CTG-3′ 5′-ATA AGG TGG AGA TGC AGG CTC-3′	351	63
Osteocalcin	5′-GTG CAG AGT CCA GCA AAG GT-3′ 5′-CTG GAG AGG AGC AGA ACT GG-3′	274	60
Osteonectin	5′-GTG CAG AGG AAA CCG AAG AG-3′ 5′-AAG TGG CAG GAA GAG TCG AA-3′	202	63

Fluorescence in situ hybridization of human Alu sequences and mouse major satellite DNA

Sections were deparaffinised in xylene and rehydrated in graded ethanol series. The epitope retrieval was performed by using 0.01 M citrate buffer, pH 6.0, in a microwave oven. After cooling, fluorescein-labelled Alu (BioGenex, San Ramon, CA, USA) and DIG-labelled mouse major satellite DNA (a gift from Dr O Brüstle) probes were added simultaneously, the slides covered with coverslips and sealed with rubber cement. Sections were denaturated on a Hybridizer (DakoCytomation, Denmark) at 95℃ for 10 min followed by hybridisation at 30℃ overnight. The rubber cement and the coverslips were removed and the sections washed stringently using SSC with 0.1% sodium dodecyl sulphate (SDS): 2×SSC/0.1% SDS for 2×5 min at room temperature, 0.1×SSC for 10 min at 40℃, 2×SSC/0.1% SDS for 5 min at room temperature. Slides were blocked with 3% BSA/1×PBS/0.1%Tween 20 for 1 h at room temperature, followed by an avidin-biotin-block (Vector Lab., Burlingame, USA; SP-2001) as described by the manufacturer's protocol. The human probe was detected by using a biotinylated anti-flourescein antibody (Vector Lab.; BA-0601; dilution 1:100) followed by streptavidin-Cy2 (Jackson ImmunoResearch Baltimore, MD, USA; 016-220-084; dilution 1:100). The mouse-specific probe was visualised by using Cy3-conjugated secondary antibody against DIG (Jackson ImmunoResearch; 200-162-156; dilution 1:250). After incubation of each antibody, the slides were washed three times with PBS, pH 7.4. Nuclei were counterstained using 4′,6′-diamidino-2-phenylindole (DAPI) (Molecular Probes) at a concentration of 2.3 μg/ml. Immunofluorescence was detected by laser scanning confocal microscopy (LSM 510; Zeiss, Oberkochen, Germany) at an excitation wavelength of 543 nm (helium/neon, red Cy3 immunofluorescence) and 488 nm (argon, yellow-green Cy2 immunofluorescence).

Tools and Assays

Western blot analysis was conducted after electrophoretic separation of 40 μg of soluble protein by 10% SDS – polacrylamide gel elctrophoresis (PAGE) and transfer to PVDF membranes using the following antibody dilutions: anti-HepPar1 (1:300), anti-CX32 (1:500), anti-CK18 (1:200, Santa Cruz) and anti-PCK1 (1:500). After overnight incubation at 4℃, immunocomplexes were detected with a horseradish-peroxidase-conjugated secondary antibody (BD Biosciences Pharmingen) and the ECL detection system (Amersham Bioscience).

Glycogen deposits were visualised in cells fixed in acetone/methanol by conventional periodic acid-Schiff (PAS) staining.

Urea concentration was determined in the culture medium by using the colorimetric diacetyl monoxim method according to Wybenga *et al*.[27]

Transfection of hMSC and primary human hepatocytes

Differentiated hBM-MSCs and human hepatocytes cultured for 24 hours were transiently transfected with Effectene (Qiagen) according to the instruction manual. At a cell density of 2×10^4 cells/cm^2, 0.8 μg of the reporter plasmid (pDsRed2-N1, Invitrogen/Clontech Laboratories) expressing red fluorescent protein (RFP) under the control of the human PCK1 promoter[28] was added. After 17 hours, medium was changed to fresh HHMM; thereafter the medium was changed every four days.

RESULTS

Characterisation of MSCs derived from human bone marrow (hBM-MSCs)

Cells selected from the human bone-marrow mononuclear cell fraction by plastic adherence displayed typical features of MSCs, in particular the respective growth pattern in vitro, the

phenotype as assessed by flow cytometry and the multiple differentiation potential.

After 5 days of culture, the isolated single cells started to grow as colonies of fibroblast-like cells, thus presenting as the typical colony-forming unit fibroblasts (CFU-Fs). After 12 days of culture, flow cytometry revealed a homogeneous cell population with a characteristic, non-haematopoietic phenotype. The CFU-Fs expressed CD13, CD29, CD44, CD105 and CD166, but were negative for CD11c, CD14, CD45, CD34, glycophorin-A and HLA-DR (Fig 1A).

The multiple differentiation potential of CFU-F was demonstrated by inducing osteogenic and adipogenic differentiation in non-clonal cell cultures and single cell cultures. Adipogenic differentiation resulted in the accumulation of intracellular lipid droplets as revealed by Oil-Red staining. Osteogenic differentiation yielded an extracellular precipitate, which was identified as calcium deposits by alizarin stain (Fig 1B).

hBM-MSC populations derived from single cells were generated to prove the multiple differentiation potential of clonal cell cultures. As in non-clonal differentiated hBM-MSC populations, lipid droplet accumulation and calcium depositions were observed upon induction of the adipogenic and osteogenic differentiation, respectively (unpublished data). In addition, differentiated cells expressed genes specific for adipocytes such as PPAR-γ2 and LPL, or for osteoblasts such as osteocalcin. As previously reported,[13] gene transcripts of early osteogenic differentiation like osteonectin were detectable in undifferentiated cultures of single-cell-derived hBM-MSCs as well as osteogenic or adipogenic differentiated cell cultures (Fig 1C).

Hepatogenic preconditioning of hBM-MSCs in vitro

Major determinants of hepatogenic differentiation of hBM-MSC in vitro were growing cells to confluence and changing culture conditions from propagation to differentiation with a culture medium containing HGF and EGF. Over a period of 15 days of culture, cell morphology changed from a spindel to a rather polygonal shape typically associated with adult hepatocytes (Fig 2A). Linked with the transformation, the expression of cytokeratin 18 (CK18), an intermediary filament protein predominantly expressed in epithelial cells, was upregulated as demonstrated by immunocytochemistry and Western blot analysis (Fig 2B). The hepatocytic phenotype of the cells was further substantiated by the appearance of functional in vitro markers barely expressed in undifferentiated cells. By weeks 2 to 3 post-induction, differentiated cells expressed the hepatic gap junction protein connexin 32 (CX32), the hepatocyte-specific antigen HepPar1 and the cytosolic key control enzyme of gluconeogenesis, phosphoenolpyruvate carboxykinase (PCK1) (Fig 2B). The process of differentiation from MSCs to hepatocyte-like cells was also analysed by RT–PCR over a period of 22 days of culture (Fig 2C). With the exception of the mRNA expression for the liver stem-cell-specific gap junction protein CX43,[29] which was already highly expressed in undifferentiated cells, mRNA for markers of early hepatogenic differentiation such as alpha-fetoprotein (AFP), CK19 and CK7[29] was upregulated within 7 days of culture, slowly diminishing or disappearing thereafter. In contrast, transcripts for markers of well-differentiated hepatocytes such as the xenobiotic metabolism enzyme cytochrome P450 subtype 3A4 (CYP3A4), the gluconeogenic PCK1, the urea cycle enzyme carbamylphosphate synthetase (CPS), the hepatocyte-specific gap junction protein CX32, the epithelial marker CK18 and the secretory plasma proteins albumin and transferrin (TFN) showed a time-dependent upregulation and were highly expressed at weeks 2 and 3 post-induction. Thus, hBM-MSCs developed an epithelial cell- and hepatocyte-specific phenotype using the culture conditions described, a process that we term hepatocyte preconditioning of hBM-MSCs. The in vitro differentiation, however, of the cells was dependent on cell-to-cell contact. Although cultured in medium HHMM, MSCs did not acquire hepatocyte-specific features in cultures not grown to confluence (unpublished data).

To show whether the induction of hBM-MSC hepatocyte differentiation depended on the presence of specific factors in the HHMM and was not a default mechanism, cells were grown in growth medium for 3 weeks. Expression of specific markers was detected by RT–PCR. Cells grew to confluence and expressed CK7 but not CK19 and AFP (hepatocyte progenitor markers). They did not express albumin and CYP3A4 either (differentiated hepatocyte markers). Thus, the hepatocyte differentiation of hBM-MSCs was not a default mechanism but rather depended on the provision of factors specifically inducing the hepatocyte phenotype (data not shown). In addition, neither growth to confluence nor treatment with HHMM alone (see above) was sufficient to differentiate hBM-MSCs to cells with hepatocyte-specific features.

Metabolic function of hepatocyte preconditioned hBM-MSCs in vitro

Hepatocyte-specific metabolic functions—namely glycogen storage, urea synthesis and activation of the hepatocyte-specific promoter of PCK1—were determined in differentiated hBM-MSCs. Although only low levels of glycogen were detectable by PAS staining in undifferentiated cells – comparable with the staining in fibroblasts (Fig 3A, I vs. Fig 3A, III) – differentiated hBM-MSCs after 21 days of culture were highly positive for glycogen with an equivalent staining pattern to primary human hepatocytes used as positive control (Fig 3A, II vs Fig 3A, IV).

Another major metabolic function of hepatocytes, the detoxification of ammonia through the synthesis of urea in the urea cycle, was assessed by measuring urea secretion in the supernatant. Urea production was minimal in undifferentiated hBM-MSCs, but increased 10-fold by 35 days of culture corroborating the time-dependent hepatocyte differentiation of hBM-MSCs (Fig 3B).

The formation of glucose by gluconeogenesis is regulated in the liver by the upregulation of the cytosolic form of the PCK gene (PCK1). At 15 days post-induction, hBM-MSCs were transfected with a reporter gene construct expressing RFP under the control of the human PCK1 gene promoter. Two days later, red fluorescent cells were detected microscopically demonstrating activation of the PCK1 gene promoter in the respective cells. Although the transfection efficiency was 0.1% as monitored by RFP expression from a CMV promoter, only 10% of transfected cells expressed RFP with the activated PCK1 gene promoter (Fig 3C, II). However, transfection of primary human hepatocytes with the same constructs had a similar transfection efficiency of 0.1%, and again only 10% of transfected hepatocytes expressed RFP with the PCK1 gene promoter (Fig 3C, I).

Functional engraftment of hepatocyte preconditioned hBM-MSCs in mouse livers

Partial hepatectomy provokes liver regeneration by stimulating the proliferation of residual hepatocytes.[30] To provide transplanted hepatocyte preconditioned hMB-MSCs with a growth advantage over host hepatocytes, immunodeficient Pfp/Rag2$^{-/-}$ mice were treated with the β-receptor antagonist propranolol before partial hepatectomy to impede proliferation of residual hepatocytes.[31 32] Hepatocyte preconditioned hBM-MSCs were delivered to mouse livers through the portal vein after intrasplenic administration. Twelve weeks later, animals were killed and the expression of hepatocyte-specific proteins assessed

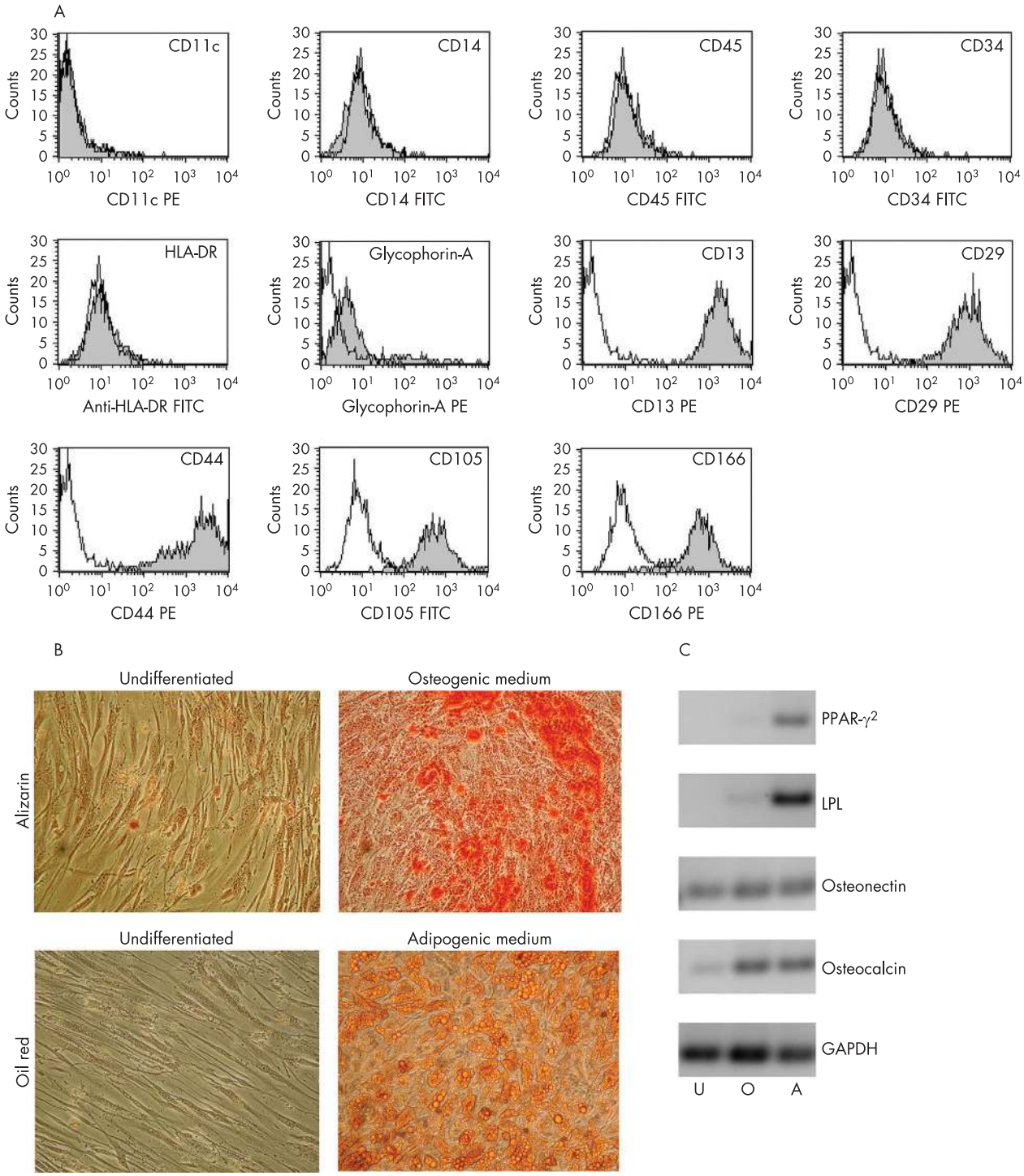

Figure 1 Characterisation of hBM-MSCs (A) Phenotype of hBM-MSCs from first passage. Cells were incubated with the respective FITC- or PE-labelled antibodies and analysed by flow cytometry (mean channel fluorescence histograms). Data are from one analysis representative of 10 independent experiments. Filled curve-specific antibody, unfilled curve-isotype control. (B) Multiple differentiation potential of non-clonal hBM-MSCs. After one passage, confluent cells were stained with alizarin and Oil Red before (undifferentiated) or after incubation with osteogenic or adipogenic differentiation medium. Micrographs (original magnification ×100) represent results of one out of 10 independent experiments. (C) Multiple differentiation potential of clonal hBM-MSCs. Total RNA of confluent hBM-MSC cultures, grown from one single cell, was harvested before (U) and after osteogenic (O) or adipogenic (A) differentiation and analysed by RT–PCR for genes of the respective lineage differentiation or for genes expressed constitutively (GAPDH). Data shown are representative of three independent experiments with similar results.

by immunohistochemistry. Human cells were detected in the background of the mouse liver by staining with anti-human HepPar1 antibody, which showed no relevant crossreactivity for murine antigens. In corresponding serial sections, PCK1, CX32, human albumin and glycogen deposition were used as markers of the functional activity of the transplanted hepatogenic hMB-MSCs. HepPar1 co-localised with PCK1, a periportal marker enzyme,[33] thus demonstrating at lower magnification a

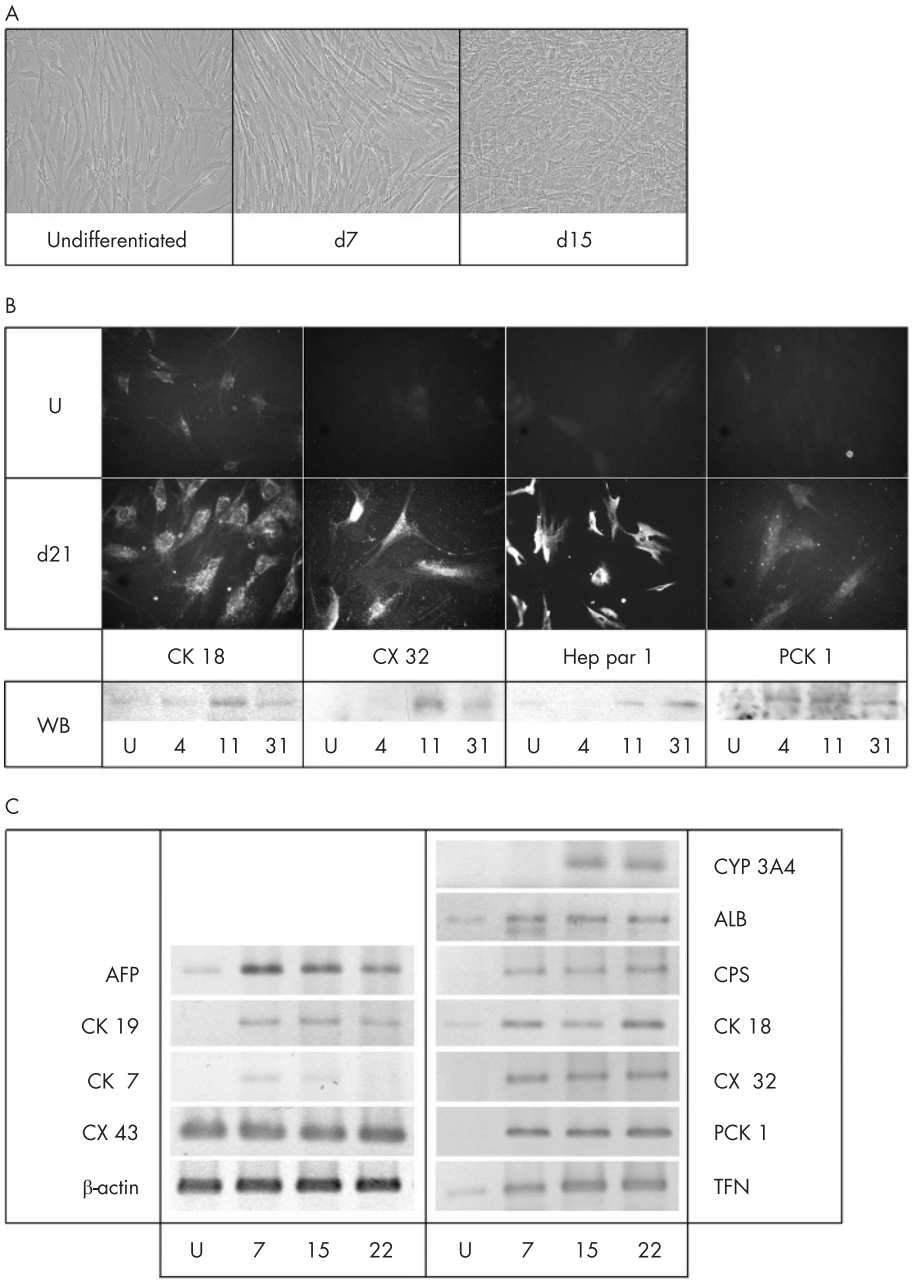

Figure 2 Hepatocyte preconditioning of hBM-MSC in vitro. (A) Morphological changes. During differentiation, hBM-MSCs change from a fibroblast-like spindle shape (undifferentiated) to a hepatocyte-like polygonal shape as culture proceeds (day 7 and day 15). (B) Detection of hepatocyte-specific proteins. Immunocytochemical staining (upper panels) and detection by Western blot (bottom panels) of CK18, CX32, HepPar1 and PCK1 in cells before differentiation (u) and after different days of culture in differentiation medium (day 21 (upper) and days 4, 11, 31 (bottom), respectively). (C) Expression of hepatocyte-specific RNA transcripts. Total RNA was extracted from hBM-MSCs before differentiation (u) and after different times of culture in differentiation medium (days 7, 15, 22). RNA transcripts typically expressed in early- (AFP, CK19, CK7, CX43) and late- (CYP3A4, ALB, CPS, CK18, CX32, PCK1, TFN) differentiated hepatocytes were detected by RT–PCR after normalisation to the amount of GAPDH transcripts as an internal standard (not shown). WB, Western blot.

predominantly periportal engraftment of the transplanted human cells (Fig 4A). Higher magnification confirmed the co-expression of HepPar1 as a marker of differentiated hepatocytes with PCK1, the key control enzyme of gluconeogenesis, and with the gap junction protein CX32 (Fig 4B). Storage of human albumin within the hepatogenic hMB-MSCs was specifically recognised with an anti-human antibody without cross-reactivity (Fig 4C), whereas glycogen deposition could be detected in HepPar1-positive cells by PAS-staining (Fig 4D). After transplantation into a mouse liver, hepatocyte preconditioned hBM-MSCs thus continued to feature a human hepatocytic phenotype and associated capabilities, indicating a functional integration of the transplanted cells within the mouse liver.

Cell fusion has been demonstrated to be the major mechanism of transplanted haematopoietic stem cells acquiring hepatocyte features in the FAH-deficient mouse model.[9] In the study presented, no co-localisation of human Alu sequences

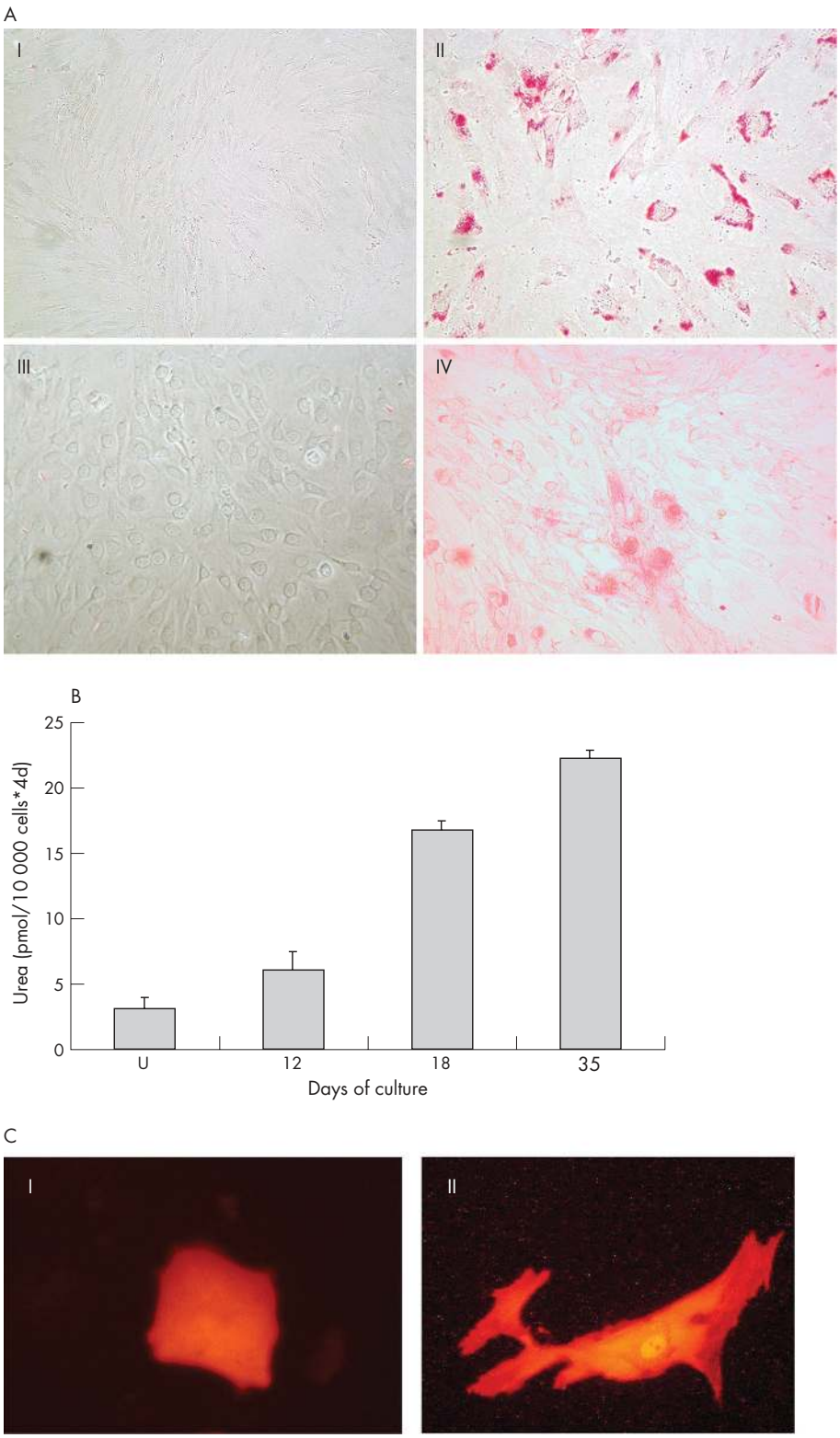

Figure 3 Hepatocyte-specific functions in hBM-MSCs after preconditioning in vitro. (A) Glycogen deposition. Glycogen was detected by PAS staining in cells before differentiation (I) and after 21 days of culture in differentiation medium (II). For comparison, PAS staining was conducted with 3T3 fibroblasts (III) and with cultured primary human hepatocytes (IV). (B) Urea synthesis. Urea was determined in medium supernatants of cultured hBM-MSCs before differentiation (u) and at different timepoints of culture in differentiation medium as indicated. (C) Activation of the PCK1 gene promoter. Preconditioned hBM-MSCs after 15 days of culture in differentiation medium were transfected with a reporter gene plasmid expressing RFP from the hepatocyte-specifc PCK1 promoter. Red fluorescence was detected after another 24 h of culture (II). For comparison, cultured primary human hepatocytes were transfected (I). Transfection efficiency was 0.1% in both experiments.

and mouse major satellite DNA was observed by fluorescence in situ hybridization (FISH). Evidence of fusion between transplanted hepatocyte preconditioned hBM-MSCs and mouse host hepatocytes was not seen in the nine liver sections analysed in three animals, which suggests that cell fusion was not a common event (Fig 5).

DISCUSSION

Hepatocytes from hBM-MSCs

MSCs derived from human bone marrow display two prominent features: (I) in culture, they grow clonally on plastic supports, which allows selection of the cells without contamination by haematopoietic cells; and (II) they differentiate into

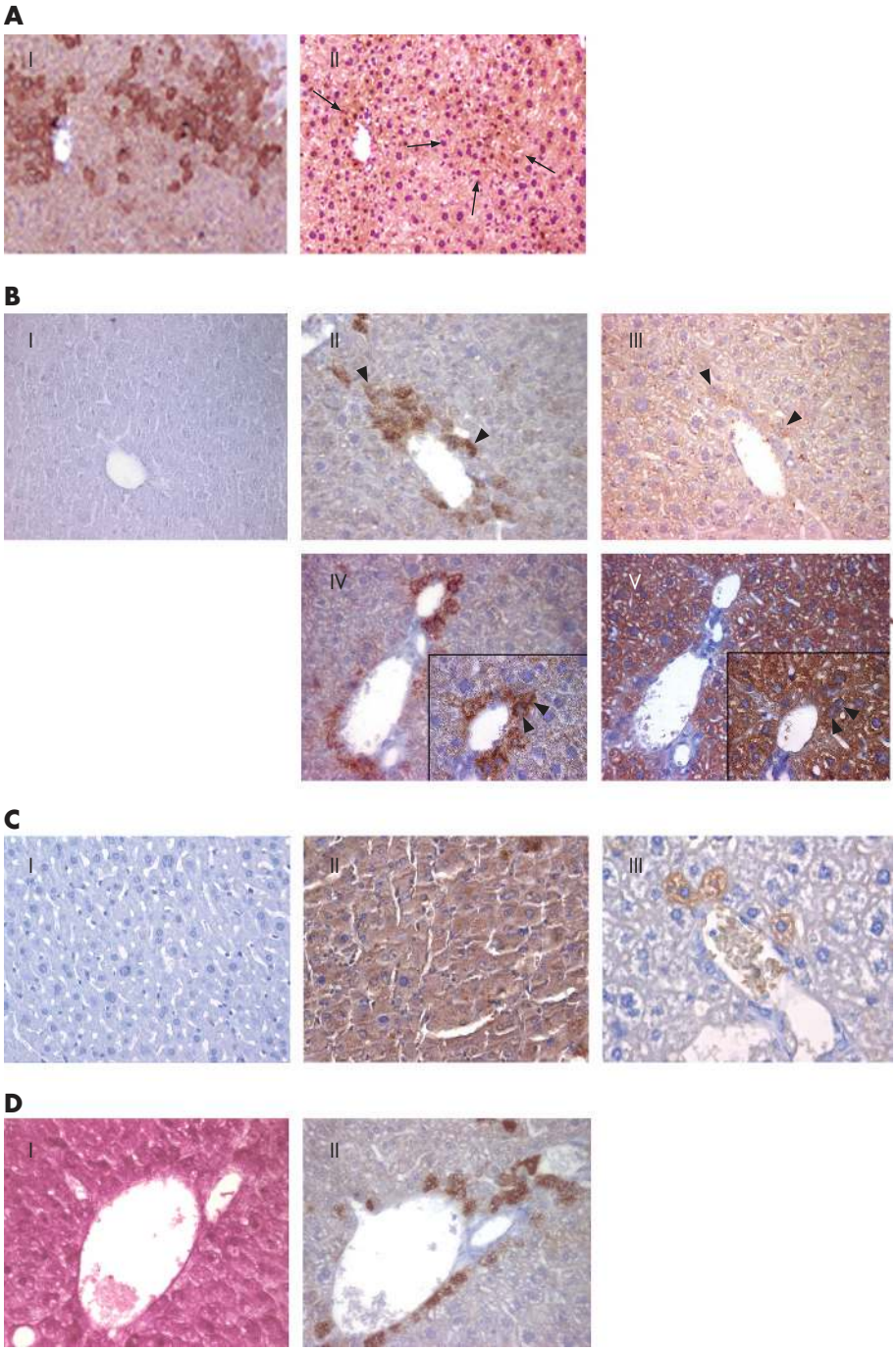

Figure 4 Hepatocyte-specific functions in transplanted hBM-MSCs after preconditioning in vitro. (A) Predominant periportal engraftment. hBM-MSCs were preconditioned in vitro for 14 days. Differentiated hBM-MSCs were then detached from the culture dish and 106 cells delivered to the livers of Pfp/Rag2$^{-/-}$ mice by intrasplenic application. Animals were killed 12 weeks after transplantation. Transplanted human cells were detected immunohistochemically by the expression of hepatocyte-specific HepPar1 (I). The expression of PCK1 was detected in a serial section to identify periportal regions of the liver (II, arrows) (original magnification ×100). (B) Expression of hepatocyte-specific proteins. Transplanted cells were identified by the expression of human hepatocyte-specific HepPar1 (II, IV), which was co-localised in serial sections by immunohistochemistry with the expression of PCK1 (III) and CX32 (V) (arrowheads, original magnification ×400). In (B I), a negative control omitting primary antibody is shown. (C) Expression of albumin. Transplanted cells were identified by the expression of albumin detected by an anti-human albumin-specific antibody (III). In (I), a mouse liver section of a non-transplanted animal, and in (II) a human liver section is shown as a negative or positive control, respectively, stained with the human anti-human albumin-specific antibody. (D) Glycogen deposition. Transplanted cells were identified by the expression of HepPar1 (I), which was co-localised in serial sections with glycogen (II) stained by the PAS method (original magnification ×400).

multiple cellular lineages in vitro including osteoblasts, chondrocytes, adipocytes, neuronal cells and hepatocytes (for review, see[34 35]). More recently, the hepatogenic differentiation of MSCs derived from other sources such as adipose tissue[23] and umbilical cord blood[8 24] has been demonstrated in vitro and in vivo, too. Our data presented here support and build on these

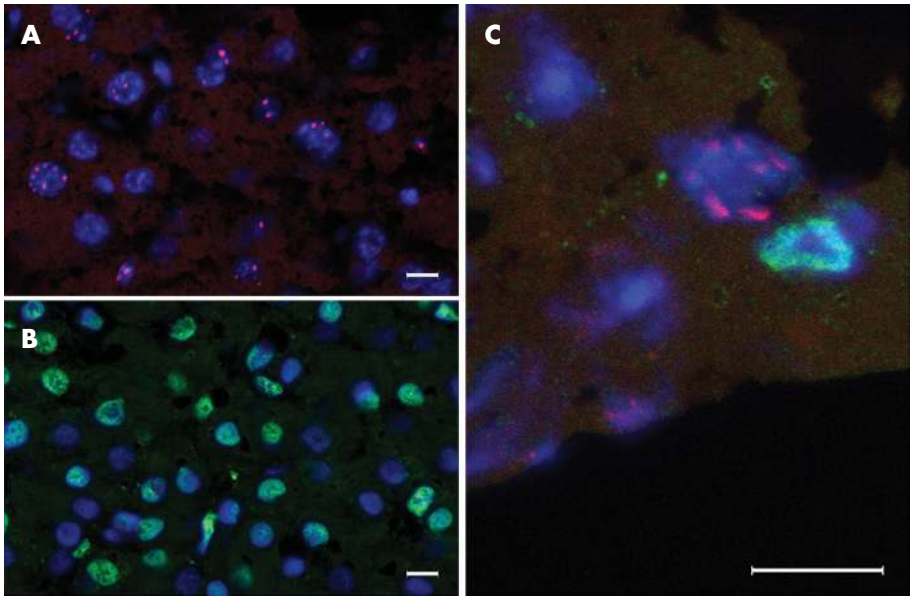

Figure 5 Engraftment of transplanted preconditioned hBM-MSCs in the mouse liver without cellular fusion. Mouse major satellite DNA and human Alu sequences were detected by FISH 3 weeks after transplantation. Murine liver from non-transplanted animals (A) and human liver (B), respectively, were incubated with both the Alu (green fluorescence) and mouse major satellite DNA (pink fluorescence) probe to demonstrate specific hybridisation to the respective DNA sequences without cross-hybridisation. When probes were applied simultaneously to tissue sections from transplanted animals, mouse and human nuclei were identified by their specific hybridisation signals from the mouse major satellite DNA (pink signals) and human Alu sequences (green signals) (C). No co-localisation of signals was observed, indicating that cell fusion was a rare event. (Bar 10 μm)

findings. The protocol used for the selection of MSCs yielded highly purified cells, uncontaminated by haematopoietic stem cells according to their phenotypic profile and capable of osteogenic and adipogenic differentiation. MSCs also differentiated into hepatocyte-like cells with relevant specific features for their topological integration (CK18) and cell–cell communication (CX32), as well as their metabolic (CYP3A4, PCK1, CPS) and secretory (ALB, TFN) functions. Indeed, in a time-dependent manner, the differentiated MSCs started to produce urea (which requires the complex metabolic network of the urea cycle) and activated the PCK1 gene promoter (which reflects the interplay of several transcription factors necessary to activate the promoter).[36 37] Additionally, the cells stored glycogen, a basic but important requirement for the hepatocytes' prominent role in glucose homeostasis.

The hepatocytic phenotype and function perpetuated after transplantation of the cells into livers of immunodeficient mice, as demonstrated by the expression of the specific antigens HepPar1, CX32, PCK1, albumin and the deposition of glycogen. So far, only two reports are available describing similar experiments of MSC transplantation, both relying on in vivo differentation of the stem cells. In the first study, human adipose stromal cells were injected into the tail vein of CCl_4-treated NOD/SCID mice.[23] Engrafted cells were localised in the liver 10 days later by the expression of human albumin, but only in intoxicated animals, which indicates that transplanted stem cells also require a growth advantage over host hepatocytes. In the second study, human CD34-negative MSCs were administered directly into the liver in an allogenic rat model after intoxication with allylalcohol. Again, the toxin was continuously given, even after transplantation to induce a high selective pressure for transplanted cells to engraft and differentiate.[25] In both experiments stem-cell-derived hepatocytes did not comprise more than 1% of the total liver mass. Although no quantitative analysis has been performed, it can be concluded from a roughly estimated percentage that this value is of the same order of magnitude as in our study using in vitro differentiated MSCs. Thus, in vivo stem-cell proliferation and differentiation does not appear to be advantageous for a successful cell engraftment. However, using stem-cell-derived hepatocytes preconditioned in vitro instead of undifferentiated cells may avoid the need for a high selection pressure, thus making the clinical transmission of the method more feasible.

Cell fusion with host hepatocytes has been reported to be the main mechanism of hepatogenic differentiation of transplanted haematopoietic stem cells from bone marrow.[9 38] However, it seems to be restricted to the FAH mouse model with its high selective pressure. Numerous studies have since shown hepatogenic differentiation from haematopoietic and mesenchymal stem cells without cellular fusion in a variety of different animal models.[7 8 25 39] Again in our study, no evidence for this process was apparent (Fig 5).

Differentiation of hepatocyte preconditioned hBM-MSCs

Initial experiments using human multipotent adult progenitor cells (MAPCs), a sub-population of MSCs, demonstrated that these cells required 35 population doublings to reach hepatogenic differentiation.[19] Less stringent conditions—that is, expansion in culture by five passages—have been used to accomplish hepatogenic differentiation of MSCs derived from adipose tissue and bone marrow.[23 25] In the present study, we used cells passaged only once. After more passages, MSCs started to express CK18, reflecting a transition from stem cell to an early differentiated epithelial cell (unpublished data). Plastic adherence alone or cell–cell contacts during prolonged culture might initiate such epithelial differentiation, and indeed hepatogenic differentiation was only achieved in confluent cultures, emphasising the relevance of intercellular communication on the differentiation process.[19]

Hepatogenic differentiation was initiated in our study using culture conditions and growth factor milieus (HGF, EGF) previously established to maintain the differentiated phenotype of human primary hepatocytes in long-term cultures.[26] The combination of HGF and EGF has also been shown to be an

effective stimulant of hepatic functions such as albumin and urea production in oval cell-derived hepatocytes.[40] This specific combination of growth factors appears therefore to be a key factor for the differentiation of precursor/stem cells into hepatocytes. Treated with the growth factors, hBM-MSCs differentiated in vitro in the obvious chronological order, first expressing hepatogenic lineage markers of early differentiation like AFP, CK7 and CK19. After 2 weeks of culture, these were replaced by markers of mature hepatocytes such as albumin, CK18 or the P450 enzyme CYP3A4. It is therefore open for speculation whether the hepatogenic differentiation of hBM-MSC in vitro followed the lineage of hepatocytes from precursor cells during liver organogenesis. Our own preliminary results have shown in fact that hBM-MSCs grown in the presence of FGF-4 instead of EGF expressed hepatic progenitor cell markers such as AFP, CK7 and CK19 more strongly and for a longer time-period (data not shown), consistent with the role of FGF-4 in the endodermal patterning of the primordial gut.[41] A chronological differentiation of hBM-MSCs after hepatogenesis was reported both in vitro and in vivo, [19 25] and was suggested for the in vitro differentiation of MSCs derived from umbilical cord blood to hepatocyte-like cells.[24]

Clinical implications of hBM-MSC-derived hepatocytes

Hepatocyte transplantation has been implicated as an alternative to whole-liver transplantation for the past 30 years.[42] A major obstacle has always been the poor engraftment of the available hepatocytes. Today, the increasing shortage of donor organs also heightens the shortage of transplantable hepatocytes and renders stem-cell-derived hepatocytes an attractive alternative resource. Because of clinical and ethical objections, adult stem cells are preferable to embryonic stem cells as the starting cell material. Mesenchymal stem cells from bone marrow, adipose tissue[23] or umbilical cord blood[43] are easily accessible, may be expanded in vitro and can be recovered without contamination. As demonstrated, they display the potential to differentiate into functional hepatocyte-like cells. However, there are only limited data available on the mechanism of their differentiation and repopulation of the liver. In CCl_4-treated fibrotic mice, the injection of murine $Flk1^+$ BM-MSCs ameliorated pathological serum parameters as well as liver histology.[21] In the study presented here, the model of partial hepatectomy was used to assess the participation of transplanted cells in liver regeneration. However, an effective repopulation of the regenerated organ by differentiated hBM-MSC was not achieved. At least 2.5 to 5% of a human liver needs to be replaced by healthy cells to reverse a pathological condition.[44] The low repopulation efficiency in our study might well be a consequence of the xenograft, with the murine liver failing to provide the proper cell–cell or cell–matrix communications required for efficient integration of the human cells. Nevertheless, the presented data demonstrate functional engraftment of MSC-derived hepatocytes. It will be the goal of future studies to improve the ability of MSCs to successfully replace diseased liver tissue.

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