Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential

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ABSTRACT

Background and aim: Mesenchymal stem cells from bone marrow (MSCs) may have the potential to differentiate *in vitro* and *in vivo* into hepatocytes. We investigated whether transplanted human MSCs (hMSCs) may engraft the liver of non-obese diabetic severe combined immuno-deficient (NOD/SCID) mice and differentiate into cells of hepatic lineage.

Methods: *Ex vivo* expanded, highly purified and functionally active hMSCs from bone marrow were transplanted (caudal vein) in sublethally irradiated NOD/SCID mice that were either exposed or not to acute liver injury or submitted to a protocol of chronic injury (single or chronic intraperitoneal injection of CCl_4, respectively). Chimeric livers were analysed for expression of human transcripts and antigens.

Results: Liver engraftment of cells of human origin was very low in normal and acutely injured NOD/SCID mice with significantly higher numbers found in chronically injured livers. However, hepatocellular differentiation was relatively rare, limited to a low number of cells (ranging from less than 0.1% to 0.23%) as confirmed by very low or not detectable levels of human transcripts for α-fetoprotein, CK18, CK19 and albumin in either normal or injured livers. Finally, a significant number of cells of human origin exhibited a myofibroblast-like morphology.

Conclusions: Transplanted hMSCs have the potential to migrate into normal and injured liver parenchyma, particularly under conditions of chronic injury, but differentiation into hepatocyte-like cells is a rare event and pro-fibrogenic potential of hMSC transplant should be not under-evaluated.

Evidence from experimental studies in rodents and retrospective studies in humans, receiving allogeneic bone marrow (BM) or liver transplants, indicate that BM-derived pluripotent haemopoietic stem cells (HSCs) have the potential to engraft the liver from the circulation and to give rise to cells expressing markers of hepatocellular differentiation.[1–10] However, a review of literature data suggests that the latter event occurs at a very low frequency (lower than 0.05%) often involving fusion with host cells.[11] Only few studies have reported a relatively higher hepatocyte yield including mainly experiments in which cells derived from the adherent fraction of cultured adult or fetal BM have been used.[12–16]

Along these lines, a still controversial and incompletely resolved issue is whether pluripotent, BM mesenchymal stem cells (MSCs) may engraft *in vivo* the liver and differentiate into cells of hepatic lineage. MSCs are stem cells with enormous plasticity[17] being able, when transplanted, to engraft different organ and tissues, including bone,[18 19] cartilage,[18 20] adipose tissue[21] and muscle,[22] as well as to differentiate into correspondent specific cell types. MSCs from human, rat and mouse BM, as well as from other sources, can be induced *in vitro* to express markers of hepatocyte differentiation[12 23–28] and administration of MSCs has been shown to improve liver injury in chronic models of fibrogenesis.[24 29] However, unequivocal evidence supporting *in vivo* ability of transplanted human MSCs to enter liver parenchyma by circulation and to acquire markers of hepatocyte-like differentiation (i.e. a goal of potential relevance) is still lacking.

The aim of the present study has been to establish whether intravenously transplanted highly purified human MSCs (hMSCs), obtained from BM donors and expanded *ex vivo*,[30] were able to engraft in normal as well as injured liver of non-obese diabetic severe combined immuno-deficient (NOD/SCID) mice and to show detectable signs of differentiation into cells of hepatic lineage.

MATERIALS AND METHODS

hMSCs from bone marrow and related experiments

Bone marrow cells were harvested from the iliac crest of 11 different human donors undergoing bone marrow collection for a related patient, after informed consent. Isolation, purification, *ex vivo* expansion, cytofluorimetric characterisation of hMSCs for CD antigens[30] as well as analysis of osteogenic, chondrogenic and adipogenic differentiation potential[31] have been described in detail elsewhere. hMSCs, collected for transplantation (Tx) from passage 3 to 5 in culture, were also checked for their *in vitro* hepatocytic differentiation potential as described by Lee *et al*.[23]

Western blot analysis on hMSC cell lysates, cell migration (wound healing assay) and proliferation analysis were as previously described.[32–35]

Transplantation of hMSCs

Acute liver injury

NOD/LtSz scid/scid NOD/SCID mice, obtained from The Jackson Laboratories (Bay Harbor, ME) through Charles River Italia (Calco, Italy) and maintained in the animal facilities of the Centro di

Table 1 Antibodies to human antigens used for immunofluorescence studies on cryostat liver sections of NOD/SCID mice or on cultured hMSCs

Antibody	Species	Manufacturer	Catalogue number	Dilution
α-FP	Goat	Santa Cruz	sc-8108	1:100
Albumin	Mouse	Cedarlane Lab.	CL2513A	1:1000
α-SMA	Mouse	Sigma	A2547	1:200
CK-7	Mouse	Dako	M7018	1:50
CK-18	Mouse	Dako	M7010	1:50
CK-19	Mouse	Dako	M0888	1:50
GFAP	Mouse	Chemicon	MAB3402	1:200
GFAP	Rabbit	Dako	Z033401	1:500
HLA class I	Mouse	Novelli *et al*[36]	–	1:100
c-Kit	Rabbit	Santa Cruz	sc-168	1:100
PDGF-βR	Rabbit	Upstate Biotech.	06–498	1:1000
VEGF-A	Rabbit	Santa Cruz	sc-152	1:100
Vimentin	Mouse	Dako	M0725	1:100
Nanog	Goat	Santa Cruz	sc-33759	1:50
Oct-4	Rabbit	Sigma	P 0873	1:100
CXCR4	Rabbit	Santa Cruz	sc-9046	1:50

Immunopositivity was revealed by the appropriate anti-mouse or anti-rabbit Cy3-conjugated secondary antibody (Amersham, Milan, Italy; final dilution 1:1000) or (Nanog or α-FP detection) by using an anti-goat FITC-conjugated secondary antibody (Santa Cruz Biotech., S. Cruz, CA, USA; final dilution 1:200).

Immunologia e Oncologia Sperimentale (CIOS, Turin, Italy), received humane care according to National and Local guidelines and were handled under sterile conditions and maintained in cage microisolators. A total of 46 NOD/SCID mice were used in the following protocols.

Protocol 1

Nineteen NOD/SCID mice (6–8 weeks old) were sublethally irradiated (350 cGy of total-body irradiation from a ^{137}Cs source) and then, after 24 h, received a single intravenous (tail vein) injection of 1×10^6 human MSCs (collected between passages 3–5 in culture). NOD/SCID mice then received either a single dose of the hepatotoxic agent, carbon tetrachloride (0.4 ml/kg b.w., intraperitoneal administration), or an equivalent volume of the diluent (i.e. control, non-injured, animals) and then sacrificed after an additional 4 weeks.

Protocol 2

Twenty-four NOD/SCID mice received first either CCl_4 or an equal volume of diluent and then from 24 h later were submitted to the same experimental procedure (irradiation and hMSC Tx) as for Protocol 1, but mice were sacrificed 1 week (n = 8) or 4 weeks (n = 16) after Tx.

A few animals (n = 3) were neither transplanted nor irradiated nor injured, and served as absolute, non-chimeric controls.

Protocol 3 for chronic liver injury

Fifteen NOD/SCID mice (6–8 weeks old) were irradiated and transplanted intravenously with hMSC as described for Protocol 1. After 4 weeks of recovery mice were then submitted to the protocol of chronic liver injury described by Russo *et al*[48] requiring chronic administration of the hepatotoxin carbon tetrachloride for an additional 4–6 weeks. Animals were sacrificed 72 h after the last administration of the toxin. A few animals (n = 3) were neither transplanted nor irradiated nor injured, and served as absolute, non-chimeric controls.

Morphological analysis

Indirect immunofluorescence was performed on liver cryostat sections (6 μm) or on cultured cells as previously described.[32–34] Antibodies and final dilutions used are provided in table 1. Nuclei were stained with the fluorescent dye 4,6-diamidine-2-phenylindole di-hydrochloride (DAPI).[35]

Direct fluorescence analysis was also performed on liver cryostat sections obtained from NOD/SCID mice receiving hMSC pre-labelled (before Tx) with the DNA fluorescent tracer Hoechst-33258.

Analysis of gene expression

Isolation of total liver RNA was performed using silica membrane filters (Macherey-Nagel, Düren, Germany) as described.[33]

Table 2 Primers and annealing temperatures used for RT-PCR

Gene	Sequence	Annealing temperature (°C)
Albumin	F 5′- TGC TTG AAT GTG CTG ATG ACA GGG -3′	56[23]
	R 5′- AAG GCA AGT CAG CAG GCA TCT CAT C -3′	
	F 5′- AGT GGG CAG CAA ATG TTG TAA AC -3′	55
	R 5′- TCA GGA CCA CGG ATA GAT AGT CTT C -3′	
	F 5′- AGC TTT TCT CTT CTG TCA ACC CCA CAC GCC TT -3′	65
	R 5′- GAG CAA AGG CAA TCA ACA CCA AGG CTT TGA AAT TTC -3′	
	F 5′- GTA CAA ATT CCA GAA TGC GCT ATT AGT TCG T -3′	61
	R 5′- TGC ACA GGG CAT TCT TTT TGC TTC AGG ATG TT -3′	
CK 19	F 5′- AGG TGG ATT CCG CTC CGG GCA -3′	55
	R 5′- ATC TTC CTG TCC CTC GAG CA -3′	
α-FP	F 5′- GAG GGA GCG GCT GAC ATT ATT -3′	55
	R 5′- TGG CCA ACA CCA GGG TTT -3′	
CK-18	F 5′- GAG GCT GAG ATC GCC ACC TA -3′	57
	R 5′- CCA AGG CAT CAC CAA GAT TAA AG -3′	
h GAPDH	F 5′- GTC GGA GTC AAC GGA TTT GG -3′	57
	R 5′- GGG TGG AAT CAT ATT GGA ACA TG -3′	
m GAPDH	F 5′-CGT GTT CCT ACC CCC AAT GT-3′	57
	R 5′-ATG TCA TCA TAC TTG GCA GGT TTC-3′	

Abbreviations: CK-19, cytokeratin 19; α-FP, α-fetoprotein; CK-18, cytokeratin 18; hGAPDH, human glyceraldehyde-3-phosphate dehydrogenase; mGAPDH, mouse glyceraldehyde-3-phosphate dehydrogenase. Note: for RT-PCR evaluation of human albumin four different primers were used. Primer sequences for either conventional and real-time PCR were designed using Primer Express Software (Applied Biosystems, Monza, Italy).

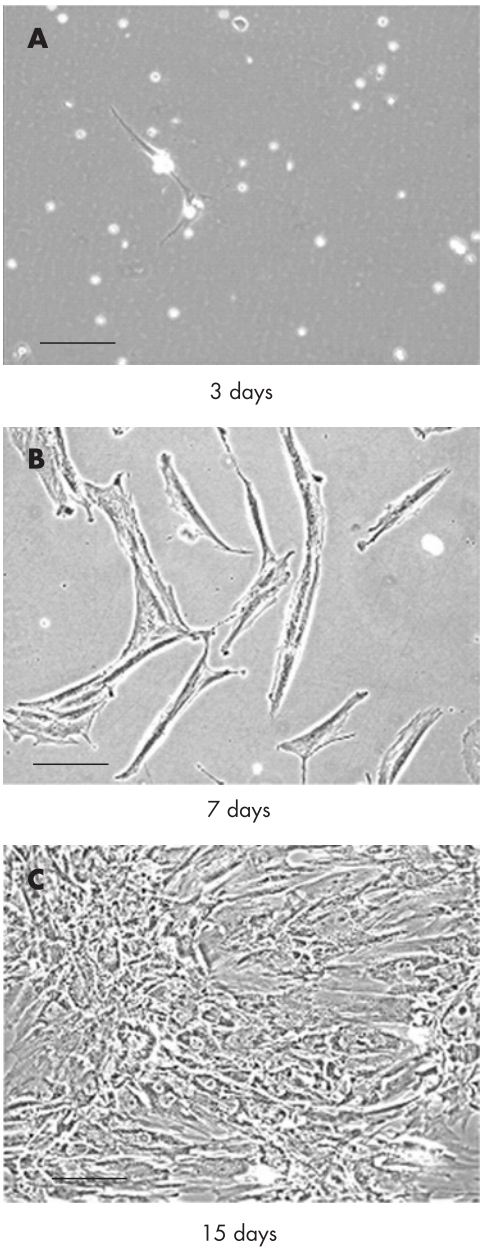

Figure 1 Micrographs of BM-hMSCs at different times from seeding rapidly acquiring the typical fibroblastoid phenotype. Bar = 100 μm.

For conventional RT-PCR, cDNA synthesis was performed with 360 ng of total RNA in a reaction containing 50 ng of Random Examers (Random Primers, Invitrogen, CA, USA), 200 μmol dATP, dGTP, dCTP, dTTP (Amersham Pharmacia Biotech Inc.), 13 U of RNA guard (Amersham Pharmacia Biotech Inc.), 4 μl of 5X first-strand buffer, 100 U of M-MULV reverse transcriptase (Invitrogen, CA, USA). Samples were incubated for 1 h at 37°C and the reaction was stopped by heating for 5 min at 95°C. PCR were performed in a PTC-200 Peltier Thermal Cycler (MJ Research), with 1 μl of cDNA (18 ng), 250 μmol dNTPs (Amersham Pharmacia Biotech Inc.), 200 nmol 5′- and 3′-primer and 0.625 U of Ampli Taq gold DNA polymerase (Applied Biosystem). The amplification program consisted of 10 min at 95°C for Taq gold activation, a denaturing step (40 s at 95°C), an annealing step (1 min at primer annealing temperature), an extension step (1 min at 72°C) for 35 cycles. The amplified products underwent electrophoresis in 2% agarose gel and bands were visualised with ethidium bromide.

For quantitative SYBR Green real-time PCR, 18 ng of cDNA was used per reaction. Each 25 μl SYBR Green reaction consisted of 1 μl of cDNA (18 ng/μl), 12.5 μl of 1X SYBR Green PCR Master Mix (Applied Biosystem, Monza, Italy), 1.5 μl of 300 nmol CK-18 or 200 nmol α-FP forward and reverse primers or 0.75 μl of 150 nmol mouse GAPDH or 200 nmol human GAPDH forward and reverse primers.

Quantitative PCR was performed on an ABI PRISM®7700 Sequence Detection System (Applied Biosystems) according to the following schedule: 2 min at 50°C, 10 min at 95°C and then 40 cycles of 15 s at 95°C, 40 s at the annealing temperature and 40 s at 72°C. A summary of the conditions used for RT-PCR is given in table 2.

Table 3 Characterisation of *ex vivo* expanded human MSC isolated from bone marrow

Marker	X ± SD (% value)	Marker designation
CD 45	4.5±5.6	Leukocyte common antigen (L-CA)
CD 14	6.6±7.8	Monocyte differentiation antigen
CD 90	92.6±7.9	Thy-1 membrane glycoprotein
CD106	32.5±29.5	Vascular cell adhesion molecule 1
CD166	40.5±35.6	Activated leukocyte adhesion molecule
CD 29	66.9±28.9	Integrin β1 (Fibronectin receptor β subunit)
CD 44	94.6±6.3	Phagocytic glycoprotein 1 (PGP-1)
CD105	90.9±9.6	Endoglin

Human MSCs expanded *ex vivo* were characterised by cytofluorimetric analysis: 2×10^5 to 5×10^5 cells were immunolabelled for 20 min using mouse anti-human antibodies conjugated with either fluorescein isothiocyanate (FITC) or phycoerythrin (PE) provided by Becton Dickinson (San Jose, CA, USA) and raised against human CD antigens: CD45–FITC, CD14–PE, CD90–FITC, CD106–PE, CD29–FITC, CD44–PE, CD105–PE, CD166–FITC (see Mareschi *et al*[30]). Data are expressed as % values and as X ± SD of different bone marrow isolates (n = 11).

Statistical analysis

Data in bar graphs represents means ± SEM and were obtained from average data of at least three independent experiments. Luminograms and morphological images are representative of at least three experiments with similar results. Statistical analysis was performed using the Student t test or ANOVA for analysis of variance when appropriate ($p<0.05$ was considered significant).

RESULTS

Characteristics of hMSCs

BM-derived hMSCs used in the present study showed a typical fibroblastoid phenotype within 1 week (fig. 1) when expanded *ex vivo* in culture. Contamination of haemopoietic cells was very low at first passage and became negligible later on. Cytofluorimetric analysis (table 3) indicated that human MSC preparations expressed low levels of CD45 and CD14, high levels of CD90, CD29, CD44 and CD105, as well as variable levels of CD166 and CD106.

hMSCs also expressed (fig. 2A,B) HLA type I class antigens, α-smooth muscle actin (α-SMA), glial fibrillar acidic protein (GFAP), vimentin, PDGF-β receptor subunits (PDFG-βR), vascular endothelial growth factor-A (VEGF) and, unexpectedly, cytokeratin 7 (CK-7), but not Nanog and Oct-4 or albumin, α-fetoprotein (α-FP), cytokeratin 18 and 19 (CK-18, CK19) or c-Kit. The latter negative data, potentially relevant for differentiation

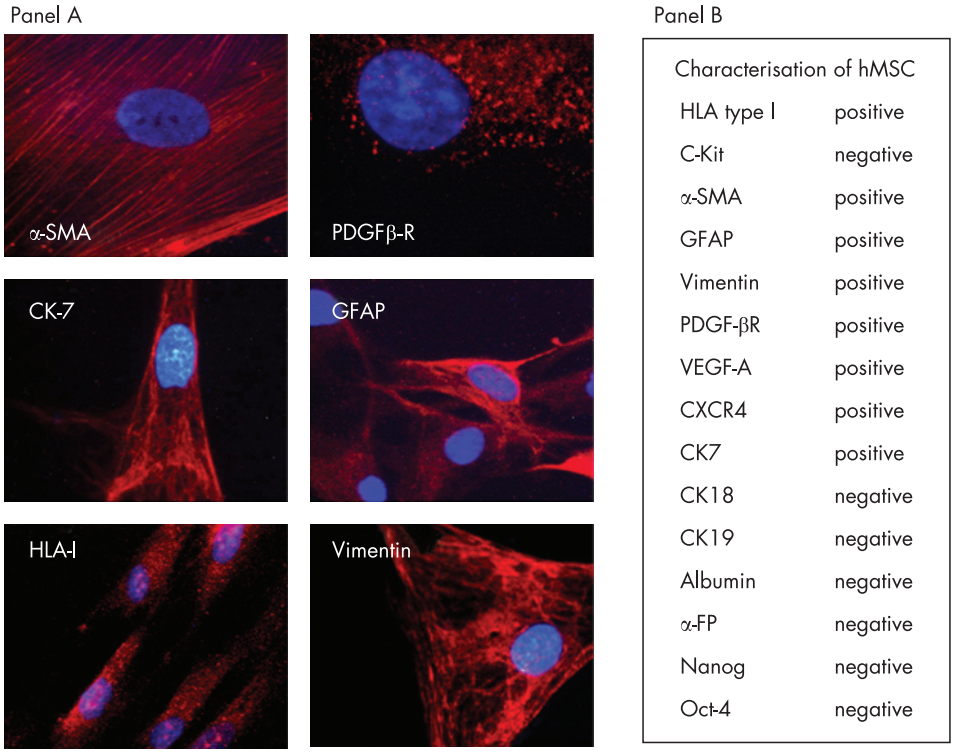

Figure 2 Characterisation of cultured hMSCs (passages 3–5). (A) Merging of indirect immunofluorescence (IIF) images for the indicated human antigens (red fluorescence) and nuclei (blue fluorescence). Original magnification: ×200 (HLA class I, GFAP), ×400 (CK-7, vimentin) or ×1000 (α-SMA, PDGFβR). (B) Summary of IIF results.

into cells of hepatic lineage, were also confirmed by RT-PCR (data not shown). Concerning CK-7 positivity, this is an unusual finding since cells of mesenchymal origin *in vivo* are negative for this marker; however, hMSCsat the time of first plating were negative and this finding is likely to represent an adaptation to culture conditions on a plastic substrate.

hMSCs were functionally active, as shown by stimulation of Ras/Erk and PI 3-K signalling (fig. 3A) by PDGF-BB and IP-10.

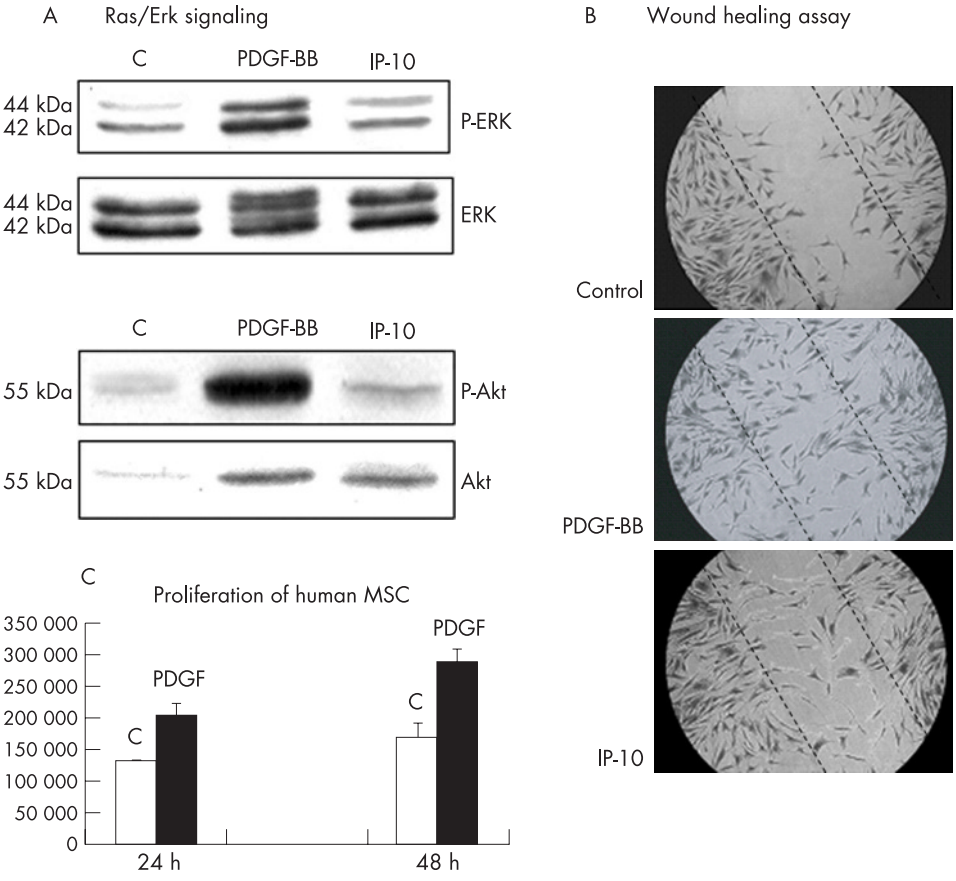

Figure 3 BM-hMSCs are functionally active. (A) Total cell lysates were obtained from control hMSCs or from cells after 15 min exposure to PDGF-BB (10 ng/ml) or IP-10 (100 ng/ml). Western blot analysis of the state of phosphorylation of ERK1/2 (p42 and p44) and c-Akt sample loading was evaluated by reblotting membranes with antibodies raised against unphosphorylated proteins. (B) Representative micrographs of hMSC migration in a wound healing assay performed on cells seeded on 24 wells coated with collagen type I, grown to confluence in complete medium and then left for 24 h in serum-free medium. hMSCs were then exposed for 20 h to PDGF-BB (10 ng/ml) or IP-10 (100 ng/ml). (C) Analysis of proliferation in 24 h serum-deprived and 60% confluent hMSCs in the absence or presence of PDGF-BB (10 ng/ml). Data represent mean ± SD of cells counted at 24 and 48 h in different experiments (n = 4).

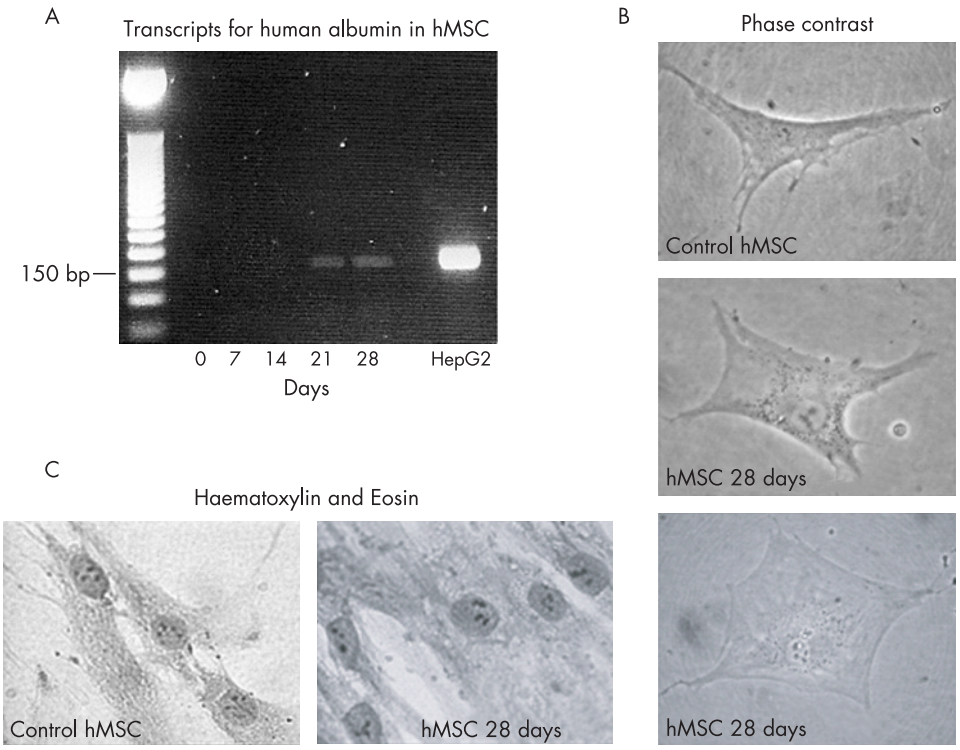

Figure 4 hMSCs cultured *in vitro* with the differentiation protocol of Lee *et al*[23] start to express at days 21 and 28 albumin mRNA (RT-PCR analysis, Panel A) as well as to modify their original fibroblastoid phenotype to a polygonal, hepatocyte-like one (Panel B, phase contrast; Panel C, haematoxylin and eosin staining).

PDGF-BB stimulated either non-oriented migration in the wound healing assay (fig. 3B) as well as proliferation (fig. 3C), whereas the chemokine IP-10 selectively induced only migration (fig. 3B).

Finally, when analysed for their *in vitro* potential to differentiate into hepatocyte-like cells, hMSCs were exposed to the protocol described by Lee *et al*[23] and found to express transcripts for human albumin (fig. 4A) and to modify their phenotype into hepatocyte-like cells (fig. 4B,C) as observed by phase contrast or after haematoxylin/eosin stain, in agreement with data previously described by the same authors.[23]

Engraftment and differentiation of hMSCs in normal liver and acutely injured liver of NOD/SCID mice

Hepatic engraftment of human cells and the presence of human hepatocyte-like cells was evaluated on NOD/SCID liver sections by either (1) immunofluorescence, using monoclonal antibodies against human HLA class I antigens (fig. 5)[36] that do not cross-react with mouse antigens (data not shown) or (2) by detecting nuclear fluorescence in experiments in which hMSCs, before Tx, were pre-labelled with the fluorescent DNA tracer Hoechst-33258 (fig. 6).

Morphological analysis performed on sections from chimeric livers provided similar qualitative results in both normal and CCl_4-injured livers (whatever the protocol used). As a rule, a slightly higher number of human cells was found in injured livers. With both protocols human cells were detected mostly as single cells placed around portal tracts in both injured and non-injured chimeric livers (fig. 5, images A,B); very rarely aggregates of few cells (see also nuclei in fig. 6C–E) were detected. Most human-derived cells had a rather undefined phenotype although, particularly 4 weeks after Tx and in the protocols

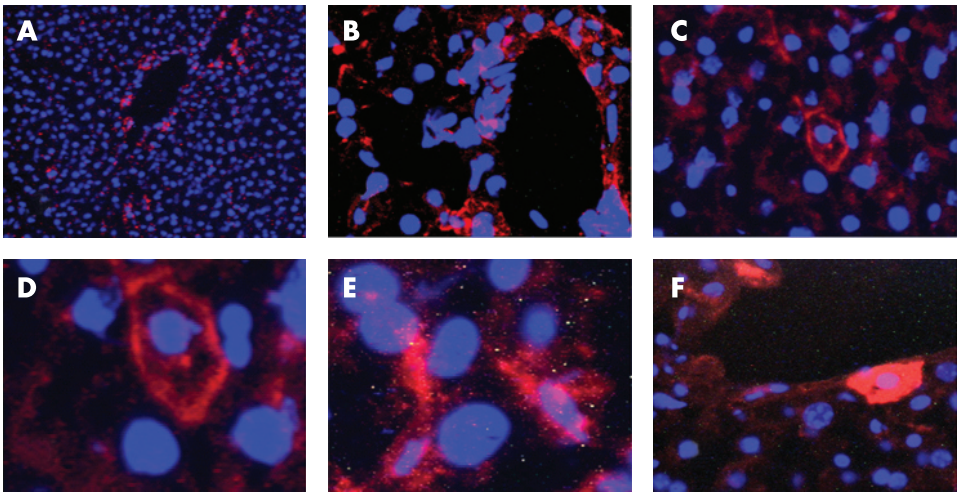

Figure 5 Immunofluorescence analysis of liver cryostat sections from NOD/SCID mice transplanted with hMSCs. Representative micrographs (see also numerical data in table 4) are from mice submitted to Protocol 1 plus liver injury and sacrificed 4 weeks after Tx. Merging of images (red fluorescence) for human HLA class I antigens (panels A–E) or human albumin (panel F) and nuclei (blue fluorescence). Original magnification ×200 (panel A) or ×400. Panels D and E represent digital magnification of ×400 images.

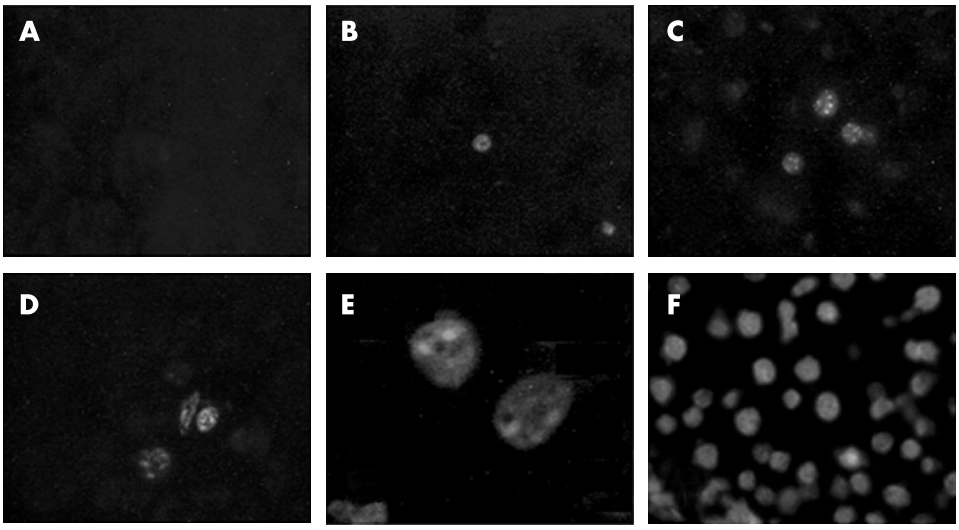

Figure 6 Analysis of nuclear fluorescence in cryostat liver sections from non-transplanted (panel A) or transplanted NOD/SCID mice at 4 weeks (panels B–E, Protocol 2) after Tx with hMSC pre-labelled with DNA tracer Hoechst-33258. Panel F is an example of DAPI staining on serial section of NOD/SCID livers serving to evaluate numerically auto-fluorescent (i.e. human) nuclei versus host nuclei. Original magnification ×200 (panels A,B), ×400 (panels C,D,F) or ×1000 (panel E).

involving liver injury, we detected some cells with a (myo)fibroblast-like shape (fig. 5E), few isolated cells showing a more regular, hepatocyte-like, phenotype (fig. 5C,D) and, very rarely, cells positive for human albumin (fig. 5F).

Concerning engraftment, we could not show (Protocol 2) major differences in the number of human cells between the two experimental time points (1 and 4 week after transplant, see table 4). However, at the earliest time point we could not detect human cells showing an hepatocyte-like phenotype.

Human transcripts in chymeric livers were then evaluated by either conventional semi-quantitative RT-PCR or, for some genes, quantitative real-time PCR. Data indicate that, whatever the protocol and the presence or absence of liver injury, after intravenous transplant of hMSCs detectable levels of human transcripts were detected only in some chymeric livers and limited essentially to α-FP and CK-18 (fig. 7), being usually at the lower limit of analytical detection when detectable. Moreover, whatever the protocol and the probes used, human albumin transcripts were undetectable in the liver of NOD/SCID animals transplanted with hMSCs in spite of perhaps extremely rare morphological images of cells positive for albumin.

Engraftment and differentiation of hMSCs in the chronically injured liver of NOD/SCID mice

In order to complete the study we analysed chronically injured chimeric livers (4 and 6 weeks) of NOD/SCID mice to document whether intravenously transplanted hMSCs, isolated from bone marrow, may be able to engraft damaged liver parenchyma and differentiate into cells of hepatic lineage.

In chronically injured chimeric livers, in which the expected changes (i.e. progressive development of fibrotic septa, see fig. 8A,C,I) were detected, the number of cells positive for human HLA-I antigens was significantly higher than in previous experiments (table 5) with a mean apparent figure of 3.02% and of 7.02% positive cells at 4 and at 6 weeks of treatment, respectively. By analysing the morphology of cells of human origin (table 5, fig. 8) we found a rather complex scenario. The number of human cells having a hepatocyte-like morphology (fig. 8F) was still relatively low although the apparent numbers in this chronic model were slightly increased ranging from 0.18% (4 weeks) to 0.23% (6 weeks); however, albumin positive cells were almost undetectable (data not shown) and the levels of human transcripts related to hepatocellular differentiation (fig. 7) were again very low (α-FP, CK-18) or undetectable (human albumin).

A significant number of cells positive for human HLA-I antigens had a characteristic morphology (fig. 8C,D,E) closely resembling the one of myofibroblast-like cells (see α-SMA or GFAP positive cells in fig. 8, images I–J and K–L, respectively) ranging from 1.06% (4 weeks) to 3.33% (6 weeks). Most, but not all, of these cells were both positive for human HLA-I antigens as well as GFAP and were mostly located within or around fibrotic septa (fig. 8M–N). Finally, we also found a consistent number of small cells positive for human HLA-I antigens that were characterised by a low nucleus/cytoplasm ratio as well as by a more undefinable morphology, sometimes resembling either endothelial cells or, rarely, macrophages (fig. 8G,H), with bile duct cells being always apparently negative (fig. 8B) as also confirmed by absence of any trace of human transcripts for CK-19 (data not shown).

Table 4 Morphological analysis of hepatic engraftment and hepatocyte-like differentiation (Protocols 1 and 2)

Experimental condition	Human cells	Hepatocyte-like cells
Control (no injury)		
4 weeks (Protocol 1, n = 7)	49 (0.021)	12 (0.0053)
1 week (Protocol 2, n = 3)	61 (0.033)	0 (0)
4 weeks (Protocol 2, n = 3)	71 (0.045)	15 (0.0097)
Protocol 1 (IR + Tx + acute injury)		
4 weeks (n = 7)	78 (0.089)	8 (0.0091)
Protocol 2 (acute injury + IR + Tx)		
1 week (n = 6)	87 (0.041)	4 (0.0016)
4 weeks (n = 17)	102 (0.078)	6 (0.0047)

Morphological detection of human cells and of hepatocyte-like cells of human origin (based on positivity for HLA class I antigens or detection of fluorescent pre-labelled nuclei) in chimeric livers of NOD/SCID mice. Data are expressed as mean number of human cells or human hepatocyte-like cells found per mouse liver cryostat sections as well as (in brackets) % value of host cells.

DISCUSSION

The original aim of this study was to establish whether transplanted hMSCs, obtained from a readily available and ethically acceptable multipotent cells source, may differentiate *in vivo* into hepatocytes, an event already described *in vitro*.[23–27] Indeed, MSC also express HGF and its related receptor c-met[37] as well as functionally active CXCR4 and CX3CR1 (receptors for SDF-1 or CXCL12 and fractalkine or CX3CL1, respectively)[38] or the multiligand receptors CCR1 and CCR7: this may favour

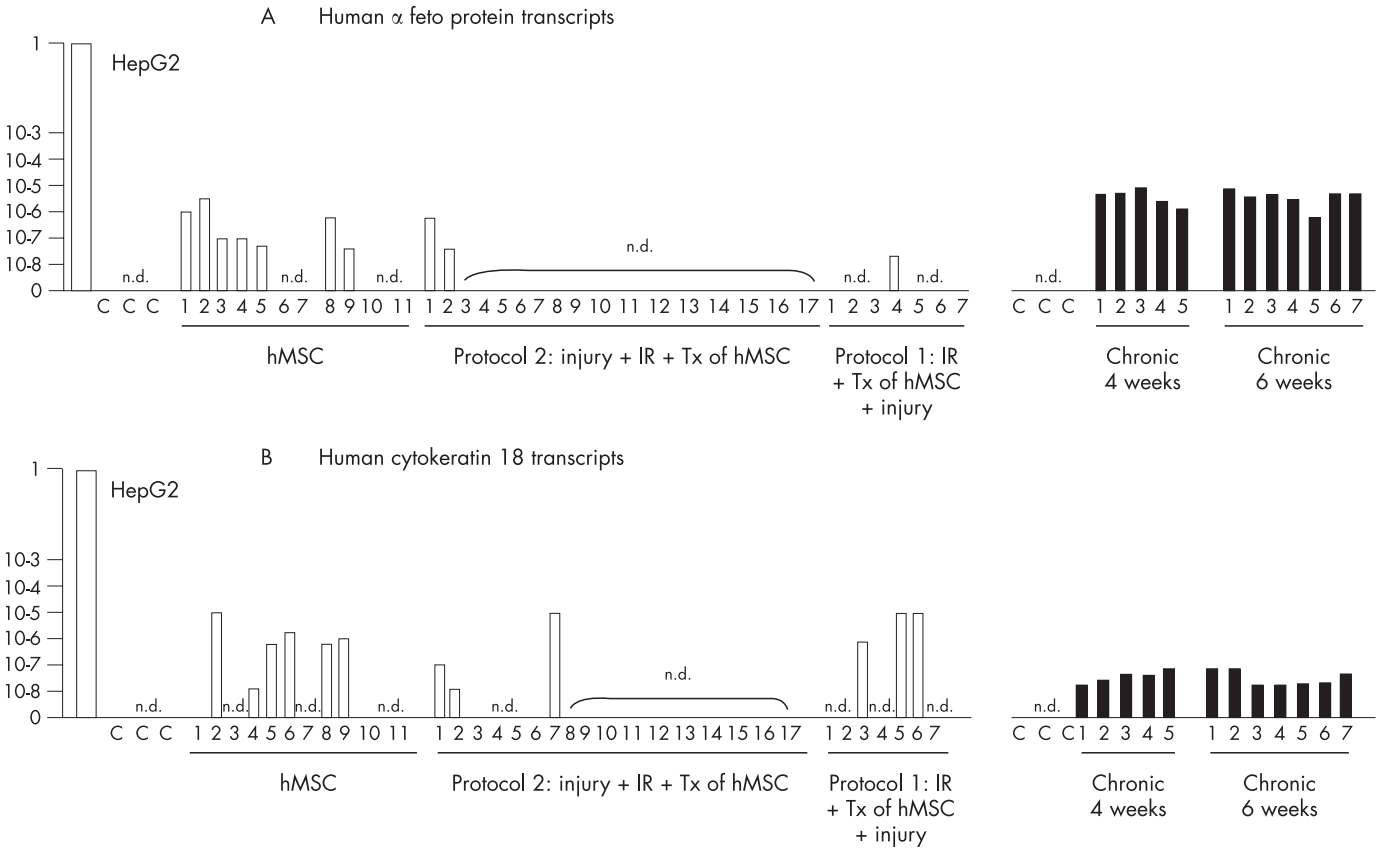

Figure 7 Real-time PCR analysis of human transcripts for α-FP and CK-18 in the liver of not transplanted (Control) NOD/SCID mice, or transplanted only with hMSCs (hMSC) or submitted to entire Protocol 1 or 2 (final time point 4 weeks) or to induction of chronic liver injury at 4 and 6 weeks. Data are referred (*y* axis) as signal intensities as compared to reference signal intensity for an equal loading of cDNA prepared from human HepG2 cells.

migration towards several injured tissues,[39] including the liver.[40 41]

However, only two and perhaps inconclusive *in vivo* studies have investigated the hepatic "fate" of transplanted MSCs. In the first study[16] hMSCs were directly injected into injured liver parenchyma of immunosuppressed rats and engraftment was rather obviously limited to the site of injection, with an estimated differentiation efficiency lower than 0.5% at 4 weeks after Tx, the standard time point in most research studies. In a second study, stromal cells derived from human adipose tissue, rather than hMSCs, were transplanted intravenously into NOD/SCID mice,[27] but engraftment/differentiation, perhaps again very limited, was documented only by morphological detection of human albumin and the analysis was not extended later than 10 days after Tx.

To overcome these limitations, here highly purified and functionally active MSCs isolated from human bone marrow, efficiently expanded *ex vivo* in culture, were transplanted intravenously into immune-deficient NOD/SCID mice in the presence or absence of either acute or chronic liver injury. Procedures able to minimise erroneous identification of transplanted human cells into chymeric liver (i.e. a relevant problem in these studies, see Thorgeirsson and Grisham[11] and Shackel and Rockey[42]) were adopted. Although liver engraftment of cells of human origin (negligible in the other experimental protocols) was definitively more significant under conditions of chronic injury, data from either normal liver and from all the three protocols of liver injury adopted indicate unequivocally that *in vivo* differentiation of intravenously transplanted hMSCs into hepatocyte-like cells represents a relatively rare and quantitatively unsatisfactory event. This is clearly documented by the very few human cells with an hepatocyte-like morphology and the very low or undetectable levels of human transcripts for α-FP, CK-18 and albumin in chymeric livers.

These unsatisfactory results are numerically in line with previous studies in the field and[11] the established rare generation of hepatocytes from multi-potent stem cells (either haemopoietic of stromal from BM or other sources) raise the question of why the efficiency of hepatocyte-like trans-differentiation is so low since even nuclei of differentiated cells can be reprogrammed to generate new cell lineages.[43 44] Along these lines, hMSC transplantation by either direct injection or by the intravenous route is unlikely to deposit them into the liver-specific stem cell niche.[45 46] Accordingly, most positive results in terms of liver repopulation have been obtained by transplanting partially and/or fully *in vitro* pre-differentiated hepatocytes from HSC.[11] A very recent study has indeed described *in vivo* engraftment and hepatocellular differentiation of *in vitro* pre-differentiated human MSCs into hepatocyte-like cells.[47] However, the study reported a low efficiency and the experimental protocol had a limit since, to provide a selective advantage for transplanted cells, it was designed to inhibit proliferation of host hepatocytes.[47] The use of MSCs in regenerative medicine applied to liver pathology, particularly during chronic injury, has also been questioned by studies which suggest two apparently opposite scenarios. In a study that did not require bone marrow ablation, it was shown that transplantation of *ex vivo* expanded and HGF-treated MSCs

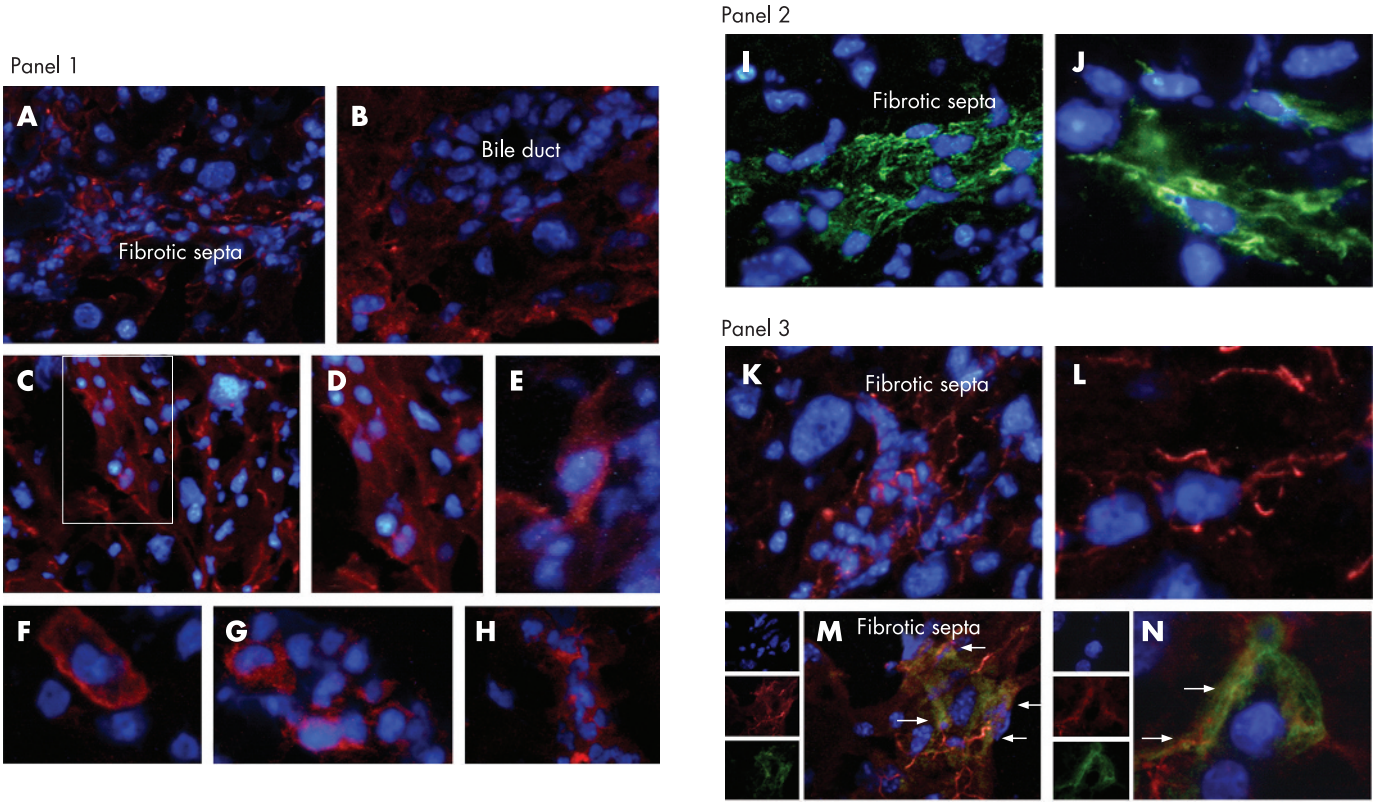

Figure 8 Immunofluorescence analysis of liver cryostat sections from NOD/SCID mice transplanted with hMSC and submitted to the protocol for the induction of chronic liver injury and fibrosis. Representative micrographs were taken at 6 weeks and are always presented as electronic merging of images for specific antigens with those for nuclei (blue fluorescence). Panel 1, images A–H: cells positive for human HLA-I antigens (red fluorescence). Panel 2, images I and J: cells positive for α-SMA (green fluorescence). Panel 3, images K and L: cells positive for GFAP (red fluorescence). Panel 3, images M and N: merging of images for GFAP (red fluorescence) and human HLA class I antigens (green fluorescence) and nuclei (blue fluorescence). Original magnification ×200 (images A,B,C,I,K), ×400 (images F,G,H,M) or ×1000 (images L,N). Panels D, E and J represent digital magnification of ×200 images.

from bone marrow was able to significantly reduce experimental chronic liver injury and fibrosis,[29] supporting the concept that this procedure may be of therapeutic efficacy.

On the other hand, in murine models involving bone marrow ablation, transplanted BM cells were reported to engraft the liver from bone marrow during chronic liver diseases and to significantly contribute to liver fibrosis by differentiating into pro-fibrogenic myofibroblast-like cells, with hepatocyte-like trans-differentiation again being a rare event.[48] In particular, these authors provided convincing evidence indicating that the predominant source of myofibroblasts in their models was represented by MSCs.[48] Indeed, our findings in chronically injured chimeric livers, in which a significant number of cells positive for human HLA-I antigens had morphological features of myofibroblasts and/or were positive for GFAP, conceptually complete and confirm data obtained in the murine models,[48] supporting the view, which should be not under-evaluated, that MSCs recruited from bone marrow to chronically injured liver may display a pro-fibrogenic potential. Accordingly, further studies are needed to definitively elucidate whether MSCs may behave as potentially "useful" or "dangerous" cells in the scenario of CLDs.

However, the fact that hMSCs were negative for fundamental markers of undifferentiated embryonic-like stem cells, such as Nanog and Oct-4, strengthen the message of a recent report suggesting that only a very small population of CXCR4+, Sca-1+, lin− and CD45− bone marrow cells (i.e. which are likely to be co-isolated with MSCs from the same source) expressing Nanog, Oct-4 and Rex-1 can differentiate into cells of all the three germ layers,[49] including cells of the hepatic lineage.

Table 5 Morphological analysis of hepatic engraftment differentiation of hMSCs at 4 and 6 weeks in the chronic model of liver injury

Experimental condition	4 weeks (n = 5)	6 weeks (n = 7)
Human HLA-I positive cells		
Total % of HLA-I positive cells	3.02%	7.02%
Hepatocyte-like cells	0.18%	0.23%
Cells with HSC/myofibroblast morphology	1.06%	3.33%
Small cells with low nucleus/cytoplasm ratio	1.64%	3.47%
Bile duct epithelial cells	Negative	Negative

Morphological detection of cells of human origin (based on positivity for HLA class I antigens). Data are expressed as mean % value of HLA-I positive cells versus total number of cells (nuclei) in mouse liver cryostat sections. In particular, data are referred to mean % of total human HLA-I positive cells as well as to mean % of human HLA-I positive cells showing the indicated morphological features in chronically injured chimeric livers of NOD/SCID mice.

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Human mesenchymal stem cells as a two-edged sword in hepatic regenerative medicine: engraftment and hepatocyte differentiation versus profibrogenic potential

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Notes