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# Hepatocyte Differentiation Potential of Mesenchymal Cell Lineages for Liver Regenerative Medicine

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*A Julie,*

*A mes parents,*

*A mes proches*

« La vérité, elle, demeure égocentriquement nôtre. »

Paul Veyne,

*Les Grecs ont-ils cru à leurs mythes ?*

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**Abbreviations**

| | |
|---|---|
| $\alpha_1$-AT: | α-1-antitrypsin |
| αFP: | α-1-fetoprotein |
| αMEM: | α-minimal essential medium |
| ADHLSCs: | Adult-derived hepatic liver stem cells |
| αSMA: | α-smooth muscle actin |
| BM: | Bone marrow |
| BSA: | Bovine serum albumin |
| CB: | Cord blood |
| CFU-F: | Colony forming unit-fibroblast |
| CK: | Cytokeratin |
| CN: | Crigler-Najjar |
| CX-32: | Connexin-32 |
| CYP: | Cytochrome P450 |
| DFs: | Dedifferentiated fibroblasts |
| DPPIV: | Dipeptidylpeptidase IV |
| EMT: | Epithelial-mesenchymal transition |
| ESCs: | Embryonic stem cells |
| FDAs: | Fibroblast-derived adipocytes |
| FDHLCs: | Fibroblast-derived hepatocyte-like cells |
| FDOs: | Fibroblast-derived osteocytes |
| FCS: | Fetal calf serum |
| G6Pase: | Glucose-6-phosphatase |
| G6Pate: | Glucose-6-phosphate |
| GAPDH: | Glyceraldehyde-3-phosphate dehydrogenase |

HAs: Human dermal adipocytes

HHs: Human primary hepatocytes

HNF4: Hepatic nuclear factor 4

HTM: Hepatectomy

I.S.: Immunosuppression

ISH: In situ hybridization

ITS: Insulin-transferrin-selenium

LIF: Leukemia inhibitory factor

LCS: Human liver cell suspension

LCT: Liver cell transplantation

MCF7: Human breast adenocarcinoma cell line

MDAs: Mesenchymal-derived adipocytes

MDHLCs: Mesenchymal-derived hepatocyte-like cells

MDOs: Mesenchymal-derived osteocytes

MET: Mesenchymal-epithelial transition

MNCs: Mononuclear cells

MSCs: Mesenchymal stem cells

OLT: Orthotopic liver transplantation

OSM: Oncostatin M

PBMCs: Peripheral blood mononuclear cells

PD: Population doublings

PAS: Periodic acid schiff

PEPCK: Phosphoenolpyruvate carboxykinase

PROD: Pentoxyresorufin-0-dealkylase

P/S: Penicillin/streptomycin

R-AA: Retrorsine-allyl alcohol

RT: Room temperature

RT-PCR: Reverse transcription-polymerase chain reaction

TAT: Tyrosine aminotransferase

TDO: Tryptophan-2,3-dioxygenase

TEM: Transmission electron microscopy

SaOs: Osteosarcoma cells

SCID: Severe combined immune deficiency

UCB: Umbilical cord blood

UCMSCs: Umbilical cord matrix stem cells

UFs: Undifferentiated fibroblasts

# CHAPTER I: INTRODUCTION

## 1. Liver metabolic diseases: a place for cell therapy

Liver-based inborn errors of metabolism (1/900 live births) are a group of diseases whose treatment is shared between physicians and surgeons. Conservative treatment remains challenging and requires continuous management and education of the patient and its family. Quality of life is often impaired because of stringent diets, high intake of medications, heavy out- or inpatient follow-up and poor socialization. This delicate medical situation is burden by life-threatening events and long term risk for mental or organ impairment. Orthotopic liver transplantation (OLT) cures these diseases and lead to a long-term survival of 90% (1). However, this radical treatment can look disproportionate to correct one single missing enzymatic function in an otherwise normal liver. The procedure is facing with non-negligible co-morbidity: up to 15% of OLT patients require re-transplantation, and progressive fibrosis of the graft is a subject of deep concern at long term (2). About 15% of patients die waiting for a donor graft (3). Controversy remains because patients and physicians are often reluctant to undertake such irreversible procedure and are seeking less invasive alternative options.

Auxiliary liver transplantation is another curative approach which advantage is to be reversible, while it remains associated to major pitfalls: besides invasive surgical procedure, the technique is awkward mainly because of perilous anastomosis that can lead to graft atrophy/ischemia or vascular thrombosis. The diagnosis of rejection is not easy because of minimal enzyme elevation.

Gene therapy met successful long-term achievements in the Gunn rat model (4;5). This technique has been described to depend on vector serotypes and to allow a reduction of serum bilirubin up to 64% after one year (6). Globally the technique is still facing with production of

antibodies in the host organism, which impedes perpetuation of the metabolic effect (7). Although encouraging, *ex vivo* gene transfer and cell injection is closely related to the quality of the cell preparation (8;9).

Since the first report by Fox et al. (10), liver cell transplantation (LCT) appeared as a new alternative treatment, intermediate between whole organ transplantation and gene therapy. Cells can be infused safely in the diseased liver, and are expected to bring sufficient enzyme activity to restore the metabolic defficiency, setting the patient within safer metabolic limits and increasing his quality of life. Advantages of the procedure are its lower invasiveness and cost and the possibility to program elective injections (11). LCT has been shown to be able to improve metabolic function in Crigler-Najjar patients (12), or in disorders of ammonium metabolism (13;14), glucose metabolism (15), clotting factor deficiencies (16), and even complex enzyme systems such as Refsum disease (17) (Table 1).

However, the technique remains limited by impaired donor cell quality and partial metabolic control due to low repopulation rates and time-restricted engraftment (18). Future challenges for this technique will be the improvement of cryopreservation/thawing technique altering cell quality mainly by intracellular ice formation (19), but also the enhancement of cell engraftment by acting on immunological interactions (12) and on procoagulant cell activity (20), or by considering co-transplantation procedures with cells owning permissive immunological properties.

**Table 1: Summary of clinical liver cell transplantation procedures for liver-based inborn errors of metabolism.**

| Indication | Patients (*n*) | Patient age | Cell amount (% liver cell mass) | Follow-up | Ref. |
|---|---|---|---|---|---|
| Familial Hypercholesterole-mia | 5 | 7-41 years | | .Partial reduction of LDL (3/5 patients)<br>.Donor hepatocytes detected by ISH at 4 months | (21) |
| CN Disease Type I | 1 | 10 years | $7.5 \times 10^9$ (5%) | .Decrease of bilirubin levels up to 11 months<br>.Detection of UGT1A1 enzyme activity and of glucurono-conjugates in bile | (10) |
| | 1 | 9 years | $7.5 \times 10^9$ (5%) | .50-65% reduction of bilirubin up to 3 months<br>.Donor hepatocytes not detected by short tandem repeat analysis at 40 days | (22) |
| | 2 | 18 months/3 years | ND | .50%/30% reduction of serum bilirubin over 7 months/ND follow-up<br>.Donor hepatocytes detected in one case by short tandem repeat analysis at 8 months | (23) |
| | 2 | 9 years/6 months | $6.1 \times 10^9$/$2.6 \times 10^9$ (4%/8%) | .Up to 35%/65% reduction of serum bilirubin over 6/4 mo follow-up | (12) |
| Infantile Refsum Disease | 1 | 4 years | $2 \times 10^9$ (5%) | .Donor Y-chromosomes detected by PCR at 7 days | (17) |
| Inherited Coagulation Factor VII Deficiency | 2 | 3 months/2 years | $1.1 \times 10^9$/$2.2 \times 10^9$ (4%/3%) | .Decrease in the factor VII requirements | (16) |
| PFIC 2 | 2 | ND | $0.3 \times 10^9$ | .No improvement | (33) |
| Glycogen Storage Disease Type Ia | 1 | 47 years | $2 \times 10^9$ (1%) | .Fasting tolerance: up to 7 hours<br>.Increase of glycemia<br>.Improvement of diete<br>.G6Pase activity detected | (15) |
| Urea Cycle Disease | 1 (OTC) | 5 years | $1 \times 10^9$ | .Improvement of ammonia levels<br>.Detection of enzyme activity | (24) |
| | 1 (OTC) | Day 0 | $10.5 \times 10^9$ | .Transient metabolic improvement between 20 and 31 days of life | (25) |
| | 1 (OTC) | 1 day | $1.9 \times 10^9$ | .Improvement of ammonia levels<br>.Increased urea synthesis | (26) |
| | 1 (OTC) | 14 months | $2.4 \times 10^9$ (6%) | .Improvement of psychomotor development and of ammonia levels<br>.Urea neo-synthesis | (13) |
| | 1 (ASL) | 3.5 years | $4.7 \times 10^9$ (9%) | .Improvement of psychomotor development and of ammonia levels<br>.Donor hepatocytes detected by FISH at 12 months and by enzyme activity at 8 months | (14) |

ASL, arginino-succinate lyase; CN, Crigler-Najjar; (F)ISH, (fluorescent) in situ hybridization; LDL, low density lipoproteins; ND, not documented; OTC, ornithine transcarbamylase; PCR, polymerase chain reaction; PFIC, progressive familial intrahepatic cholestasis; UGT1A1, bilirubine-uridine diphosphate glucuronosyltransferase

## 2. Looking for stem cells

In this context, stem cells were regarded as alternative sources for liver cell therapy after that their plasticity had been illustrated (27;28). These cells have beneficial properties compared to hepatocytes as most of them are easy to harvest and possess major proliferation potential making large amounts of cells available. For being considered for clinical use, stem cells should be readily storable. Some of these cells have been demonstrated to keep their differentiation potential after cryopreservation/thawing (29-32) while others are already used in clinics after cryopreservation/thawing (33;34). In the liver therapy field, many stem cells were considered as candidates for differentiation and *in vivo* procedures (35;36).

Embryonic stem cells (ESCs) constitute currently the best model for studying hepatocyte differentiation (37), while identification of the obtained populations remains controversial (38), and ethical (39;40) or safety (41) concerns limit their use in the clinical setting.

Haematopoietic stem cells (HSCs) can be isolated from bone marrow (BM), umbilical cord blood (UCB) or peripheral blood (42), making them highly available while they are still difficult to expand *in vitro* (43). Cell identity and phenotype of these cells are mainly determined by selection procedure whose criteria remain debated (44). HSCs have recently aroused much enthusiasm in the hepatology field since their hepatic differentiation potential has been evidenced (Table 2). However, this potential is increasingly discussed (45-51) and lack of precision concerning identification of cell populations involved in differentiation process or possibility of *in vivo* cell fusion make these cells less considerable for clinical use (52;53).

**Table 2: Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of HSCs.**

| Cell source | *In vitro* differentiation | *In vivo* differentiation | Ref |
|---|---|---|---|
| Mouse BM | ND | .*Host:* irradiated $FAH^{-/-}$ mice<br>.*Injury:* NTBC withdrawal<br>.*TRP:* 10 to 1000 cells; retro-orbital plexus<br>.*Cell characterization:* β-gal$^+$, Alb$^+$, DPPIV$^+$, E-cadherin$^+$, FISH Y$^+$, liver function, FAH activity<br>.*Repopulation rate:* 30%-50% β-gal$^+$ cells | (54) |
| Mouse BM | ND | .*Host:* irradiated CD45.1$^+$ mice<br>.*Injury:* hepatect or anti-Fas Ab treatment<br>.*TRP:* $10^6$ cells; vein<br>.*Cell characterization:* CK8$^+$, CK18$^+$, CK19$^+$, CD45.2$^+$, FISH Y$^+$, PCR Bcl-2$^+$<br>.*Repopulation rate:* up to 0.05%-0.8% with anti-Fas Ab treatment | (55) |
| Human UCB & BM | ND | .*Host:* irradiated NOD/SCID mice ± HGF<br>.*Injury:* $CCl_4$<br>.*TRP:* 2000 to $10^5$ cells; vein<br>.*Cell characterization:* Alb$^+$, CD45$^-$, human alu$^+$, CK19$^+$, Alb mRNA$^+$, serum Alb$^+$<br>.*Repopulation rate:* <1% | (56) |
| Human UCB & PB | ND | .*Host:* irradiated NOD/SCID mice<br>.*Injury:* $CCl_4$<br>.*TRP:* 2 to 6 × $10^5$ CD34$^+$ cells; tail vein<br>.*Cell characterization:* CD45$^+$, Alb$^+$, $\alpha_1$-AT$^+$, hum DNA$^+$, hum Alb mRNA$^+$ & prot$^+$<br>.*Repopulation rate:* ≤0.01% hum Alb mRNA in mice vs human liver | (57) |
| Mouse BM | Cocktail: co-culture with damaged liver tissue<br>Characterization: RT-PCR (8), ICC (10), FISH Alb mRNA | .*Host:* mice ± irradiation<br>.*Injury:* $CCl_4$<br>.*TRP:* $10^5$ cells; tail vein<br>.*Cell characterization:* Alb$^+$, FISH Y$^+$, E-cadherin$^+$, PKH26$^+$, serum liver function<br>.*Repopulation rate:* up to 7.6% at 2d<br>.*Fusion:* FISH X-Y: - | (58) |
| Mouse BM | ND | .*Host:* mice ± irradiation ± rhG-CSF<br>.*Injury:* partial hepatect or $CCl_4$<br>.*TRP:* 5 × $10^3$ selected cells or 5 × $10^5$ MNCs; spleen<br>.*Cell characterization:* no co-eGFP/DPPIV staining, PCR eGFP$^-$<br>.*Repopulation rate:* 0/6.9 × $10^7$ host hepatocytes | (45) |

$\alpha_1$-AT, α-1-antitrypsin; Ab, antibody; Alb, albumin; β-gal, β-galactosidase; BM, bone marrow; $CCl_4$, carbon tetrachloride; CK, cytokeratin; d, days; dexa, dexamethasone; DPPIV, dipeptidylpeptidase IV; eGFP, enhanced green fluorescent protein; FAH, fumarylacetoacetate hydrolase; hepatect, hepatectomy; HGF, hepatocyte growth factor; hum, human; MNCs, mononuclear cells; ND, not determined; NTBC, 2-2nitro-4-trifluoromethylbezoyle-1,3-cyclohexamedione; PB, peripheral blood; SCID, severe combined immune deficiency; UCB, umbilical cord blood.

Although identity and *in vivo* function of liver stem cells are controversed, different types of hepatic progenitors are described: oval cells, small hepatocytes and liver epithelial cells (59). Conditions for using these cell populations in therapy are a high proliferation rate and/or a robust cell banking capacity. Handling cultures of primary hepatocytes isolated from human adult liver, we isolated a mesenchymal-like cell population displaying high level of proliferation and hepatocyte-like phenotype acquisition (Chapter VI). Characterization of these cells and related pluripotency is ongoing.

Mesenchymal stem cells (MSCs) are currently the best candidate for liver tissue engineering as they own advantageous characteristics. First described in 1970 by Friedenstein et al. from BM isolates (60), their embryonic location has been identified in the aorta-gonad-mesonephros (61) while their *in vivo* function in adult organism remains enigmatic. MSCs can theoretically be obtained from all tissues (62) while greatest isolation yields are achieved using BM, cord blood (CB) or adipose tissue. These cells have extensive proliferation abilities (63) making them suitable for cell banking. In addition to this, MSCs' characterization is based on the following *in vitro* properties: spindle-shaped morphology, adherence to plastic, immunophenotype composed by a panel of non-specific markers in which CD105, CD90 and CD73 co-expression is most representative (Fig. 1), and the ability to differentiate into mesodermal lineages, classically osteocytes, adipocytes and chondrocytes (64). MSCs are easily transfected and as such useful for gene therapy procedures (65).

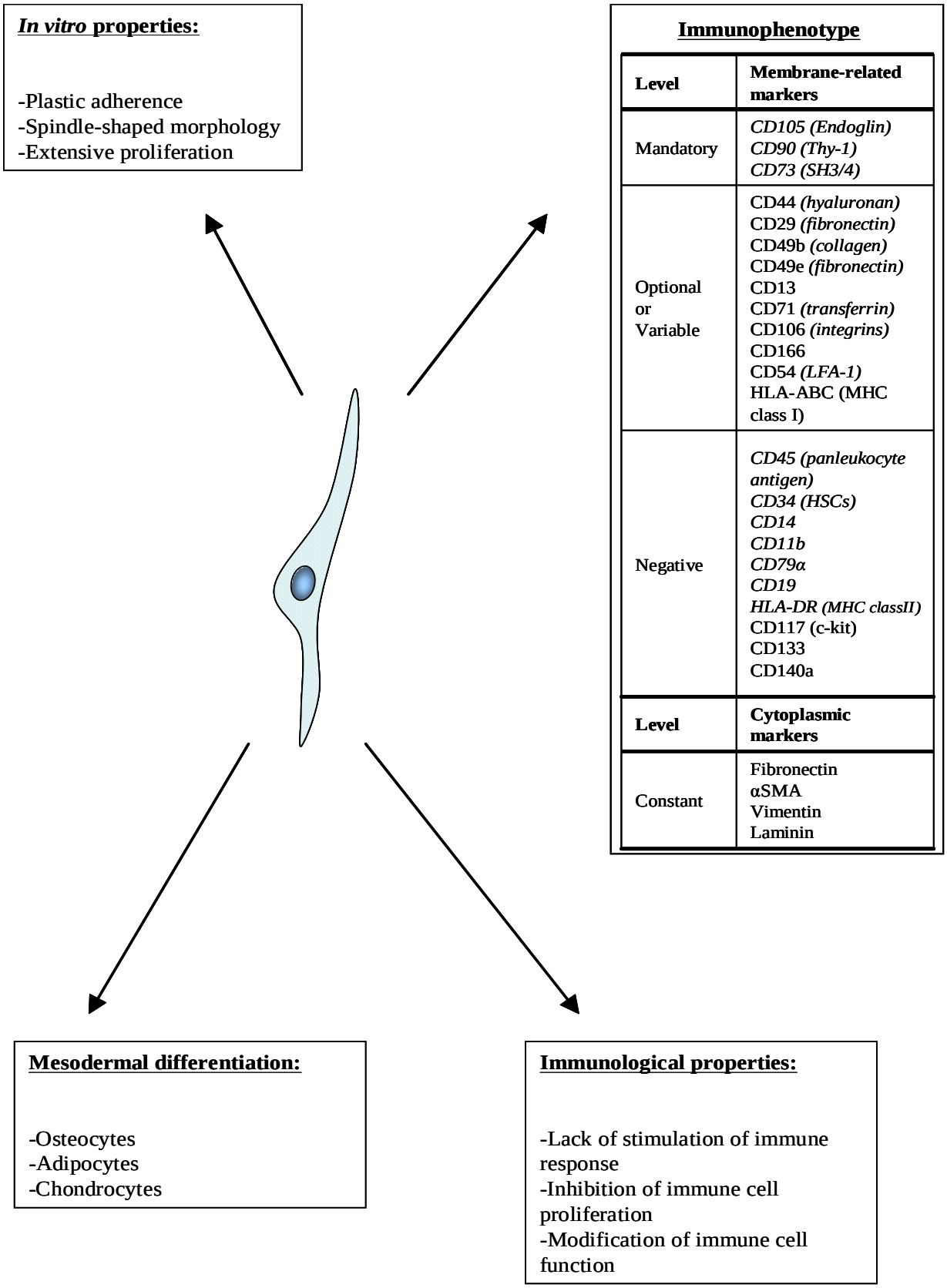


**Figure 1: Characteristics of mesenchymal stem cells.** Markers indicated in italic are these suggested by the International Society for Cellular Therapy. Others are proposed based on literature data.

Since recently, MSCs were studied in the immunology field for their unique properties among stem cell populations. These cells have the particularity to lack expression of human leukocyte antigen (HLA) major histocompatibility complex (MHC) class II (HLA-DR) and express intermediate levels of MHC class I antigen (HLA-ABC). They do not express co-stimulatory molecules as B7-1 (CD80), B7-2 (CD86), CD40 or CD40 ligand. MSCs have been described *in vitro* to suppress proliferation of T and B lymphocytes, natural killer or dendritic cells (66-68) and to modulate immune cell behavior (69). These effects have been attributed to cell-to-cell interactions and also to secretion of various factors as transforming growth factor-β1 (70), indoleamine 2,3-dioxygenase (71) or prostaglandin E2 (69;72). Great enthusiasm has been raised after the pioneer work of Le Blanc and colleagues concerning MSCs' potential to improve graft-versus-host reaction after BM transplantation (73), leading to consider MSCs as a tool for immunosuppression (68). Another clinical application of MSCs is their use in co-transplantation with allogeneic HSCs as these cells have been demonstrated to support hematopoiesis *in vitro* and to enhance HSCs' engraftment (74). MSCs possess a huge differentiation potential (63;75;76), and encouraging preclinical studies conducted to ongoing clinical trials (77) (Fig. 2).

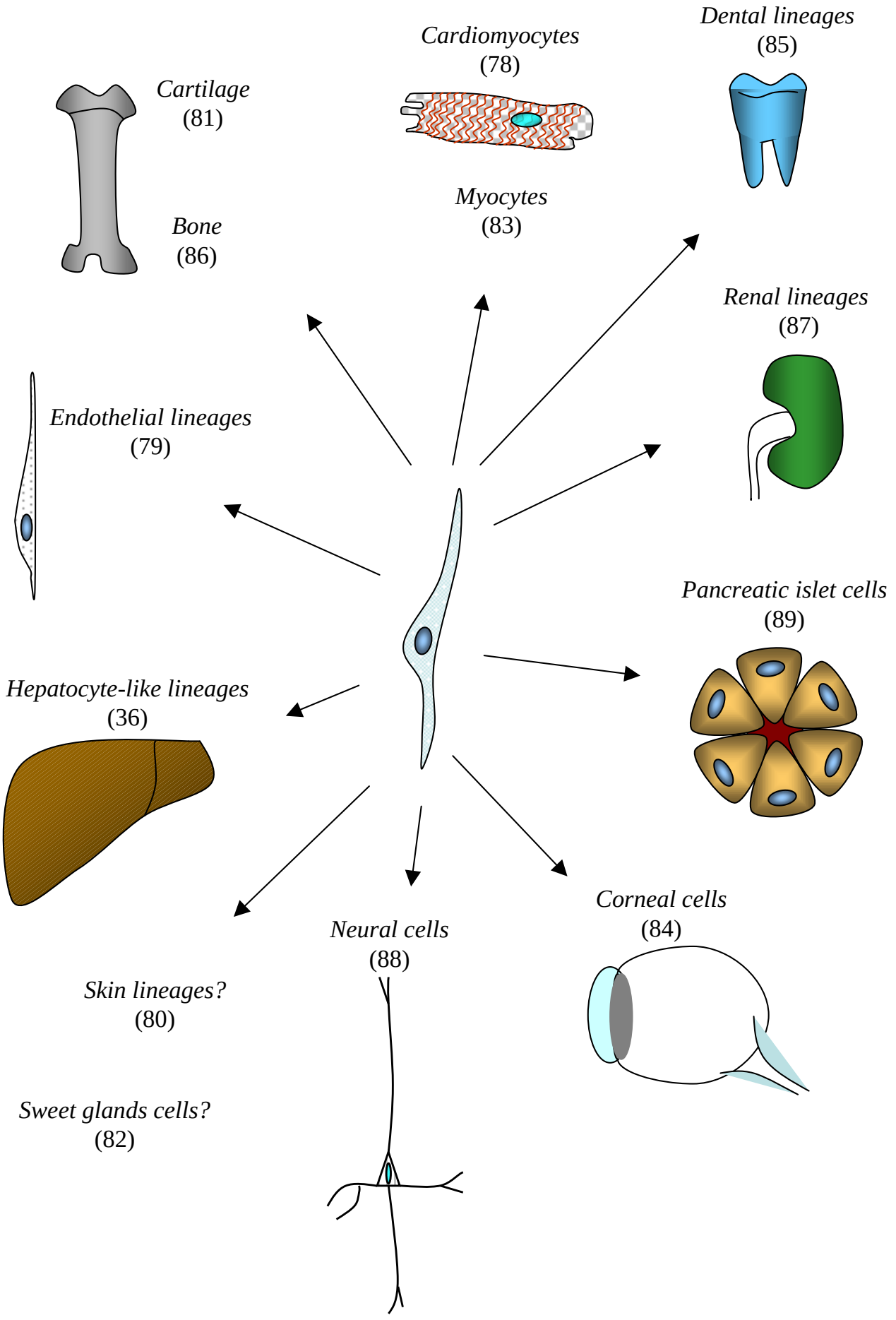


**Figure 2: Multipotency of MSCs.** Figure shows documented and controversed differentiation potentiality of MSCs. References indicate critical review on corresponding subject.

The first demonstration of hepatocyte differentiation potential of MSCs was provided by Lee et al. using UCB-MSCs (91). This potential has been illustrated *in vitro* and *in vivo* by other groups, totalizing 44 reports on MSCs isolated from BM (92-101), UCB (102-107), placenta (108;109), adipose tissue (110-113), or amniotic membranes (114;115). Most representative studies are summarized in table 3. In these studies, *in vitro* characterization of conditioned cells was based on acquisition of hepatocyte-like phenotype (morphological changes, protein and gene expression) and functions (albumin or urea secretion, glycogen storage, pentoxyresorufin-0-dealkylase (PROD) assay, low-density lipoprotein uptake). *In vivo*, efficient engraftment of native or pre-conditioned MSCs was described in mice and rat models with cells expressing some hepatocyte-specific markers *in situ*.

Although all these data indicate the potential of MSCs to acquire elective hepatocyte features *in vitro*, these were achieved using disparate methods and results are thus difficult to overlap. Globally, authors documented differentiation potential rather on phenotype (morphology or marker expression) than on true hepatocyte-like functionality. The overall literature did not provide data about quantitative acquisition of hepatocyte features or on MSCs' differentiation yield. Whether MSCs' phenotype evolves during differentiation procedure is not known. However, these informations are required before considering stem cells-derived hepatocyte-like cells in the clinical setting. It is mandatory for a clinician to precisely know the level of hepatocyte-like differentiation he can expect using stem cells. Although this concern has not been investigated yet, current differentiation procedures are based on exposure to single or restricted amount of factors and data regarding combined use of cytokines are lacking. *In vivo*, MSCs' engraftment level was documented only by Sato et al. in wild-type rats (96) and again there is no data that could be extrapolated for a clinical use. In this way, transplantation protocols are not uniform and there is a need for comparative studies.

**Table 3: Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of MSCs.**

| Cell Source | *In vitro* differentiation | *In vivo* differentiation | References |
|---|---|---|---|
| .Human BM & UCB<br>.Immunoselection | .*Cocktail:* EGF-bFGF (2d), HGF-bFGF-nicot (7d), OSM-dexa-ITS<br>.*Characterization:* RT-PCR (8), ICC (2), urea production, LDL uptake, PROD | ND | **(93)** |
| .Human UCB | ND | .*Host:* preimmune fetal sheep<br>.*Injury:* -<br>.*Cell characterization:* $Alb^+$, $HepPar1^+$<br>.*Fusion:* microdissection + PCR: -<br>.*Repopulation rate:* >20% | (106) |
| .Human adipose tissue | .*Cocktail:* DMSO – HGF – OSM<br>.*Characterization:* RT-PCR (4), ICC (1), urea production, LDL uptake | .*Host:* NOD/SCID mice<br>.*Injury:* $CCl_4$<br>.*TRP:* $1 \times 10^6$ cells, tail vein<br>.*Cell characterization:* $Alb^+$/CM-$Dil^+$<br>.*Repopulation rate:* up to 0.13% $Alb^+$ at 10d | (110) |
| .Human BM<br>.MSCs, $CD34^+$cells, non MSCs/$CD34^-$ cells | ND | .*Host:* WT rats (+CsA)<br>.*Injury:* AA<br>.*TRP:* $1 \times 10^6$ cells, liver<br>.*Cell characterization:* $Alb^+$, $CK18^+$, $CK19^+$, $\alpha FP^+$, $AGPR^+$, serum $Alb^+$<br>.*Fusion:* FISH hum Y/rat chr 12: - | (96) |
| .Human BM | .*Cocktail:* 5'aza (1d) - EGF-EGF (14d)<br>.*Characterization:* RT-PCR (11), ICC (4), WB (4), urea production, PAS, reporter gene construct for PEPCK activity | .*Host:* $Pfp/Rag2^{-/-}$ mice<br>.*Injury:* propanolol, partial hepatect<br>.*TRP:* $1 \times 10^6$ cells, liver<br>.*Cell characterization:* $Alb^+$, HepPar-$1^+$, $PEPCK^+$, $CX32^+$, $PAS^+$<br>.*Fusion:* FISH hum alu/mouse satellite: - | (92) |
| Human adipose tissue | .*Cocktail:* HGF, FGF1, FGF4 (3w) and + OSM, dexa (2w)<br>.*Characterization:* RT-PCR (6), WB (4), ICC (4), PAS, albumin production, LDL uptake, $NH_4$ detoxification | .*Host:* BALB/c nude mice (*n*=3)<br>.*Injury:* $CCl_4$<br>.*TRP:* $5 \times 10^5$ cells, tail vein<br>.*Cell characterization:* $Alb^+$ | (113). |

Alb, albumin; AA, allyl alcohol; αFP, α-fetoprotein; $CCl_4$, carbon tetrachloride; CB, cord blood; Chr, chromosome; CK, cytokeratin; CsA, ciclosporin A; d, days; dexa, dexamethasone; FGF, fibroblastic growth factor; G6Pase, glucose-6-phosphatase; hepatect, hepatectomy; HGF, hepatocyte growth factor; hum, human; ITS, insulin-transferrin-selenium; nicot, nicotinamide; OSM, oncostatin M, PAS, periodic acid-schiff; PEPCK, phosphoenolpyruvate carboxykinase; PROD, pentoxyresorufin-O-dealkylase; WT, wild-type.

## 3. Objectives of the study

To evaluate the putative role of mesenchymal cell lineages in liver regenerative medicine, we first studied the efficacy of hepatocyte differentiation of human BM (hBM)-MSCs by elaborating a differentiation protocol based on numerous *in vitro* assays. Cells obtained were identified as mesenchymal-derived hepatocyte-like cells (MDHLCs). We then explored the differentiation yield of MDHLCs in terms of reproducibility, quantitative acquisition of hepatocyte-like features and functionality. To examine MSCs' commitment in the endodermal lineage, we investigated the evolution of the native MSC phenotype throughout differentiation and dedifferentiation processes. Based on the collected data, we designed methods for optimizing differentiation yield: exposure to combinations of factors or cytokines and induction of hepatocyte-like functionality. The capacity of MSCs to engraft and differentiate into a diseased liver was analyzed *in vivo* by comparing various transplantation protocols on severe combined immune deficiency (SCID) mice but also in a wild-type mouse model, with an aim to investigate their advantageous immunological properties. The hepatocyte differentiation of hBM-MSCs was compared with that of other mesenchymal lineages: umbilical cord matrix stem cells (UCMSCs), human skin fibroblasts and adult-derived hepatic liver stem cells (ADHLSCs).

# CHAPTER II: STUDY OF THE EFFICACY OF HEPATOCYTE DIFFERENTIATION OF hBM-MSCS

## 1. Introduction

Taking into consideration current literature, no consensus exists on stem cell hepatocyte differentiation protocol. Although a majority of studies used 2-step protocols based on an induction and a maturation step, these are shaped using variable cytokines whereas OSM is commonly accepted as a maturing factor. Other reports used continuous exposure to diverse cytokines in which hepatocyte growth factor (HGF) is mostly represented.

Concerning data on hepatocyte differentiation of MSCs, characterization of the obtained cells is globally limited, except in a few reports (Table 4) while even there overlap of the results is arduous. Descriptions are often based on positive features expression and therefore no differentiation yield can be extrapolated from these reports. Screening of hepatocyte-like functionality is conducted with commercially available assays (enzyme-linked immunosorbent assays, colorimetric or PROD/EROD assays), whose handling is weakly reproducible and difficult to interpret, or with tests owning low specificity (low-density lipoprotein uptake).

Using Lee's hepatocyte differentiation protocol (93), we did not obtain reliable results and tested serial combinations of factors/cytokines for shaping protocol allowing highest differentiation. We screened a large panel of hepatocyte-specific markers both on gene and protein levels (Table 4) and studied yield of differentiation based on acquisition rate of specific features and on their expression level. Hepatocyte-like functionality was screened using commercial and home-made assays. We studied the fate of native MSCs' phenotype to

completely examine their involvement in the differentiation process. To explore the influence of cell heterogeneity on differentiation yield, we differentiated clonally-expanded MSCs and screened their marker expression.

Because epithelial-mesenchymal transition (EMT) has been recently described to occur in pathological processes (116;117) but also as a constitutive cellular mechanism of liver turnover (118), we examined the robustness of differentiation process by evaluating de-differentiation of MDHLCs incubated into their expansion medium.

Considering recent findings on permissive immunological properties of MSCs, we explored their potential of hepatocyte differentiation and engraftment in a stringent mouse model regarding immunological pressure.

**Table 4: Comparison of representative studies on hepatocyte marker expression of MSCs-derived hepatocyte-like cells.**

| | (92) | (91) | (112) | (100) | Present work |
|---|---|---|---|---|---|
| **mRNA markers** | | | | | |
| **Albumin** | + | + | + | + | + |
| **G6Pase** | + | ND | ND | + | + |
| **CK-8** | ND | ND | ND | ND | + |
| **CK-18** | + | + | ND | ND | - |
| **$\alpha_1$-AT** | ND | ND | ND | ND | + |
| **α-FP** | + | + | + | ND | + |
| **CYP3A4** | ND | + | ND | ND | - |
| **CYP2B6** | + | ND | ND | ND | - |
| **TAT** | + | ND | ND | + | + |
| **TDO** | + | ND | + | + | + |
| **HNF4** | + | ND | + | ND | - |
| **GS** | ND | ND | ND | ND | + |
| **c-Met** | ND | ND | ND | ND | + |
| **PEPCK** | ND | + | ND | + | + |
| **Others** | | CX-43 & 32, transferrin, carbamylphosphate synthetase | TTR, CYP7A1 | CYP1A1/2$^+$ | |
| **Protein markers** | | | | | |
| **Albumin** | + | ND | ND | + | + |
| **CK18** | ND | + | ND | ND | - |
| **HepPar-1** | ND | + | ND | ND | - |
| **CX-32** | ND | + | ND | ND | + |
| **Others** | | PEPCK$^+$ | CYP1A1$^+$, CYP3A4$^+$, CYP2C9$^+$, NADPH reductase$^+$ | HNF1/4α$^+$ | αFP$^+$, DPPIV$^+$, CK8$^-$ |

$\alpha_1$-AT, α-1-antitrypsin; α-FP, α-fetoprotein; CK, cytokeratin; CX, connexin; CYP, cytochrome P450; DPPIV, dipeptidylpeptidase IV; G6Pase, glucose-6-phosphatase; GS, glutamine synthetase; HNF, hepatic nuclear factor; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosin aminotransferase; TDO, tryptophan-2,3-dioxygenase.

## 2. Persistence of a chimerical phenotype after hepatocyte differentiation of hBM-MSCs

a. Article 1: Persistence of a chimerical phenotype after hepatocyte differentiation of human bone marrow mesenchymal stem cells. *Cell Proliferation* 2008 Feb;41(1):36-58.

*Cell Prolif.* 2008, **41**, 36–58 doi: 10.1111/j.1365-2184.2007.00507.x

# 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, pentoxyresorufin-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/cm$^2$ 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% $CO_2$.

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When reaching 70% confluence, cells were detached using 0.05% trypsin–0.53 mM EDTA4Na (Invitrogen) and were re-plated at $0.5 \times 10^4$ cells/cm$^2$. Colony forming unit-fibroblast assays were performed by plating cells at 0.5–10 cells/cm$^2$ 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 $1.5 \times 10^4$ cells/cm$^2$ in uncoated six-well dishes. To obtain osteogenic differentiation, they were incubated with MSC medium containing 0.1 μM 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 μM dexamethasone, 0.5 mM isobutyl-methylxanthine, 0.2 mM indomethacin (all from Sigma) and 10 μg/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 $1.5 \times 10^4$ cells/cm$^2$ 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 μM 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 $0.5–1 \times 10^3$/μL 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 (Immunokontact, 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, Utretch, 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: $2 \times 10^5$ cells were incubated in 200 μL Cytofix/Cytoperm (Becton Dickinson) for 20 min at room temperature, were washed with 1 mL 1 × Perm/Wash solution (Becton Dickinson) and were centrifuged for 5 min at 805 ***g***. Cell pellets were re-suspended in 50 μL Perm/Wash and were incubated with 1 μL FITC-labelled rabbit antihuman albumin antibody (Cedarlane Laboratories, Ontario, Canada) or 1 μL FITC-labelled IgG1 control isotype (BD Pharmingen), for 25 min at 4 °C. After washing

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cells with 1 mL PBS and centrifuging for 5 min at 2000 r.p.m., cells were fixed in 250 μL 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 (DPPIV) (Ancell, 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 (αFP) (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 μL. 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., Oberkochem, 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

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

| Primer | Sequence | Size (bp) | Cycle |
|---|---|---|---|
| Albumin | S: 5′-CCTTGGTGTTGATTGCCTTTGCTC-3′<br>AS: 5′- CATCACATCAACCTCTGGTCTCACC-3′ | 308 | 94 °C/1 min; 55 °C/1 min; 72 °C/2 min |
| G6Pase | S: 5′-TCAGCTCAGGTGGTCCTCTT-3′<br>AS: 5′-CCTCCTTAGGCAGCCTTCTT-3′ | 291 | 94 °C/1 min; 55 °C/1 min; 72 °C/2 min |
| CK-8 | S: 5′-AAGGGCTGACCGACGAGATC-3′<br>AS: 5′-GATCGCCACCTACAGGAAGC-3′ | 537 | 94 °C/30 s; 50 °C/30 s; 72 °C/60 s |
| CK18 | S: 5′-CCATGCGCCAGTCTGTGGAG-3′<br>AS: 5′-AGCAGATTGAGGAGAGCACCAC-3′ | 322 | 94 °C/30 s; 65 °C/45 s; 72 °C/45 s |
| α1-AT | S: 5′-TCGCTACAGCCTTTGCAATG-3′<br>AS: 5′-TTGAGGGTACGGAGGAGTTCC-3′ | 142 | 94 °C/30 s; 55 °C/1 min; 72 °C/2 min |
| αFP | S: 5′-TGAAATGACTCCAGTAAACCC-3′<br>AS: 5′-AATGAGAAACTCTTGCTTCATC-3′ | 199 | 94 °C/30 s; 55 °C/1 min; 72 °C/2 min |
| CYP 3A4 | S: 5′-TGCTGTCTCCAACCTTCACC-3′<br>AS: 5′-GGTGGTGATGATTCCAAGCTA-3′ | 802 | 94 °C/120 s; 50 °C/60 s; 72 °C/60 s |
| CYP 2B6 | S: 5′-CCTCTTCCAGTCCATTACCG-3′<br>AS: 5′-GCCATACACAGAGGCAGTCA-3′ | 551 | 94 °C/120 s; 50 °C/60 s; 72 °C/60 s |
| TAT | S: 5′-TGAGCAGTCTGTCCACTGCCT-3′<br>AS: 5′-CTCAGATCCTCCTCATTCACAT-3′ | 358 | 94 °C/40 s; 56 °C/50 s; 72 °C 60 s |
| TDO | S: 5′-ATACAGAGCACTTCAGGGAGC-3′<br>AS: 5′-GATACCGAAGATGAACCCAACCA-3′ | 316 | 94 °C/40 s; 56 °C/50 s; 72 °C/60 s |
| HNF4 | S: 5′-CCAAGTACATCCCAGCTTTC-3′<br>AS: 5′-CTTTGACCCAGATGCCAA-3′ | 295 | 94 °C/40 s; 56 °C/50 s; 72 °C/60 s |
| c-met | S: 5′-CAATGTGAGATGTCTCCAGC-3′<br>AS: 5′-TCTGCCTGCAATCTACAAGG-3′ | 560 | 94 °C/40 s; 55 °C/40 s; 72 °C 60 S |
| PEPCK | S: 5′-TTAGATGGGACAAAGCCTG-3′<br>AS: 5′-GCAAGACGGTGATTGTAACTC-3′ | 226 | 94 °C/40 s; 55 °C/40 s; 72 °C/60 s |
| Vimentin | S: 5′-CTACATCGACAAGGTGCGCTT-3′<br>AS: 5′-TGCCAGAGACGCATTGTCAA -3′ | 301 | 94 °C/30 s; 58 °C/30 s; 72 °C/60 s |
| ASMA | S: 5′-ATGTGTGAAGAAGAGGACAG-3′<br>AS: 5′-ACTCGTAACTCTTCTCAAGG-3′ | 730 | 94 °C/30 s; 55 °C/30 s; 72 °C/30 s |
| Fibronectin | S: 5′-ACGGGAGCCTCGAAGAGCAA-3′<br>AS: 5′-TGCAAGTGATGCGTCCGTG-3′ | 607 | 94 °C/30 s; 55 °C/30 s; 72 °C/30 s |
| GAPDH | S: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′<br>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.

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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–allylalcohol-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. Retrosine–allylalcohol 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 allylalcohol the day before cell injection (Yavorkovsky *et al*. 1995; Laconi *et al*. 2001; Menthena *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 $1 \times 10^6$ 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

| | Transplantation procedures | | |
|---|---|---|---|
| Groups | 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–allylalcohol.

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**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× 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., Peterbourough, 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 ×200) of each three different culture samples. Total cell counts are provided for each experiment.

**Statistics**

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

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# 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 ± 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 and 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), αFP (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 ± 14.5% by flow cytometry ($n = 5$) in MDHLC populations while no staining was noted in MSCs (2.6 ± 2.0%, $n = 3$) (Fig. 3c).

Analysis of hepatocyte-specific marker expression by RT-PCR revealed that MDHLCs expressed albumin (3/5), αFP (5/6), α1-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).

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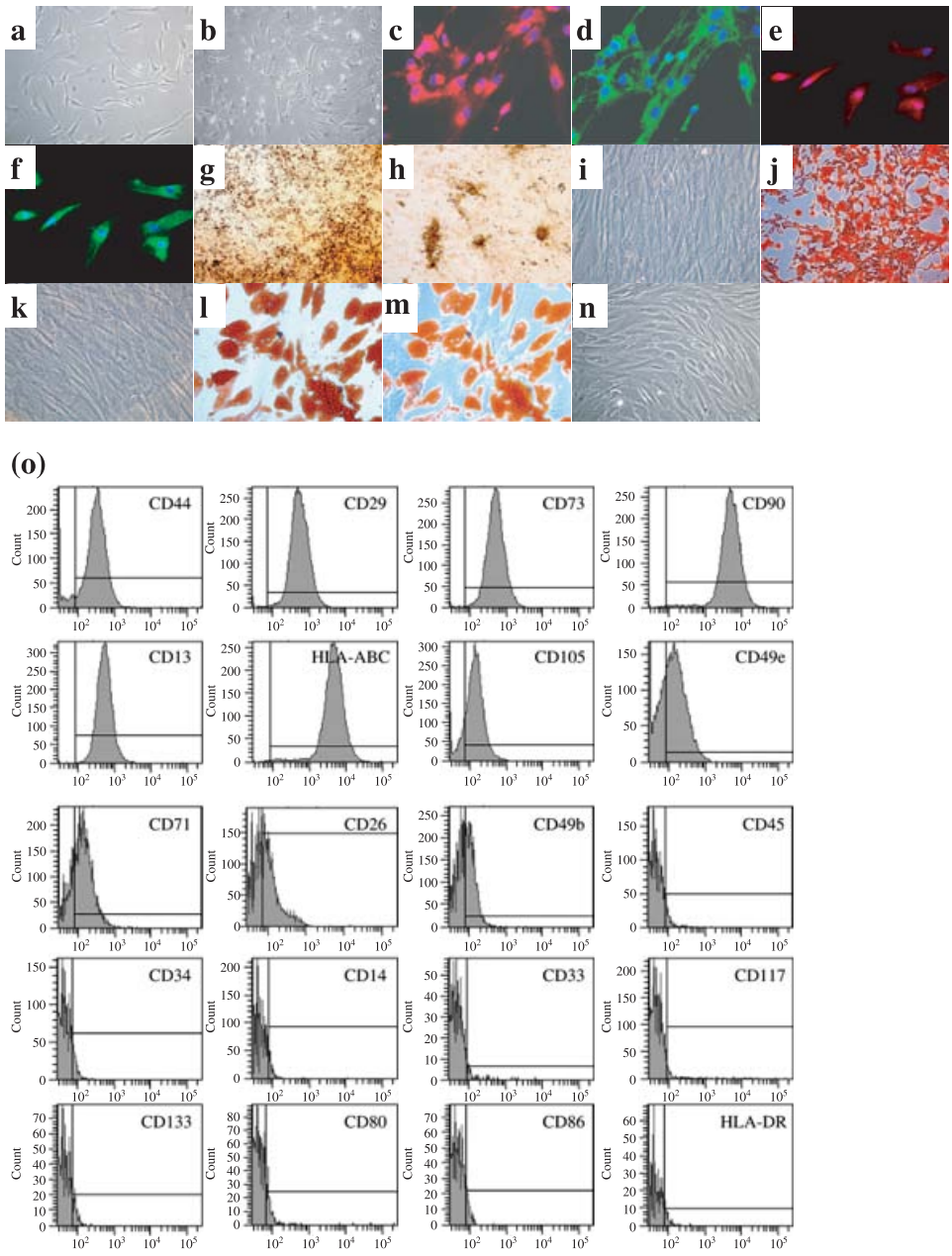


**Figure 1. Characterization of MSCs.** Example of MSCs morphology at passage 1, day 15 (a) that was altered at passage 8, day 99 (b). Expression of mesodermal antigens in MSCs at passage 2 as revealed by immunofluorescence [vimentin (c), fibronectin (d), ASMA (e), laminin (f), $n = 3$ each]. Staining 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: ×100 (b, g), ×200 (j), ×300 (c–f) and ×400 (a, i, k–n).

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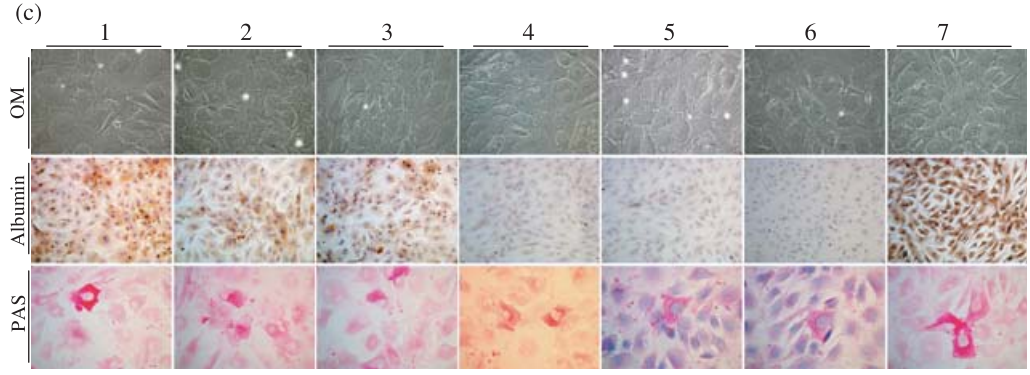
(a)

| | A | | B |
|---|---|---|---|
| **Step 1** | HGF | ± | aFGF |
| | | | bFGF |
| | | | FGF4 |
| | | | EGF |
| | | | OSM |
| | | | IL6 |
| | | | DMSO |
| | | | dexa |
| | | | ascorbate |
| | | | ITS |
| | | | nicot |
| **Step 2** | OSM<br>dexa<br>ITS | ± | HGF |

(b)

| | **Hepatocyte differentiation protocols** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** | **6** | **7** |
| **Step 1** | HGF<br>aFGF<br>bFGF | HGF<br>EGF | HGF<br>OSM | HGF<br>IL6 | HGF<br>DMSO | OSM | HGF<br>FGF4 |
| **Step 2** | OSM | OSM | OSM<br>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)

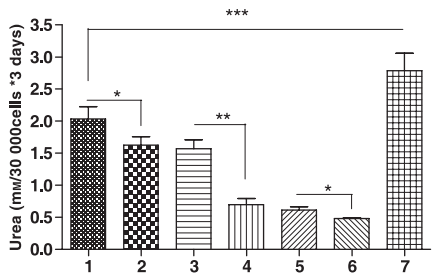



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**Table 3.** Influence of BM-MSCs culture characteristics on hepatic markers expression

| | | 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 |
| | αFP | – | + | + | + | + | + |
| | CK-8 | + | – | – | + | ND | – |
| | CK-18 | ND | – | – | – | – | – |
| | α1-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 | – | – | + | + | + | + |
| | αFP | – | + | – | – | + | + |
| | DPPIV | + | + | + | + | + | + |
| | CK8 | – | – | – | – | – | – |
| | CK18 | – | – | – | – | – | – |
| | HepPar-1 | – | – | – | – | – | – |
| | Connexin-32 | + | ND | ND | + | + | + |
| | E-cadherin | ND | + | + | ND | + | ND |

α1-AT, α1-antitrypsin; αFP, 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 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 dexa (dexamethasone), ascorbate (ascorbid acid 2-phosphate), ITS and nicot (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 ×400. Albumin staining pictures were taken at magnification ×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 ± 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 ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

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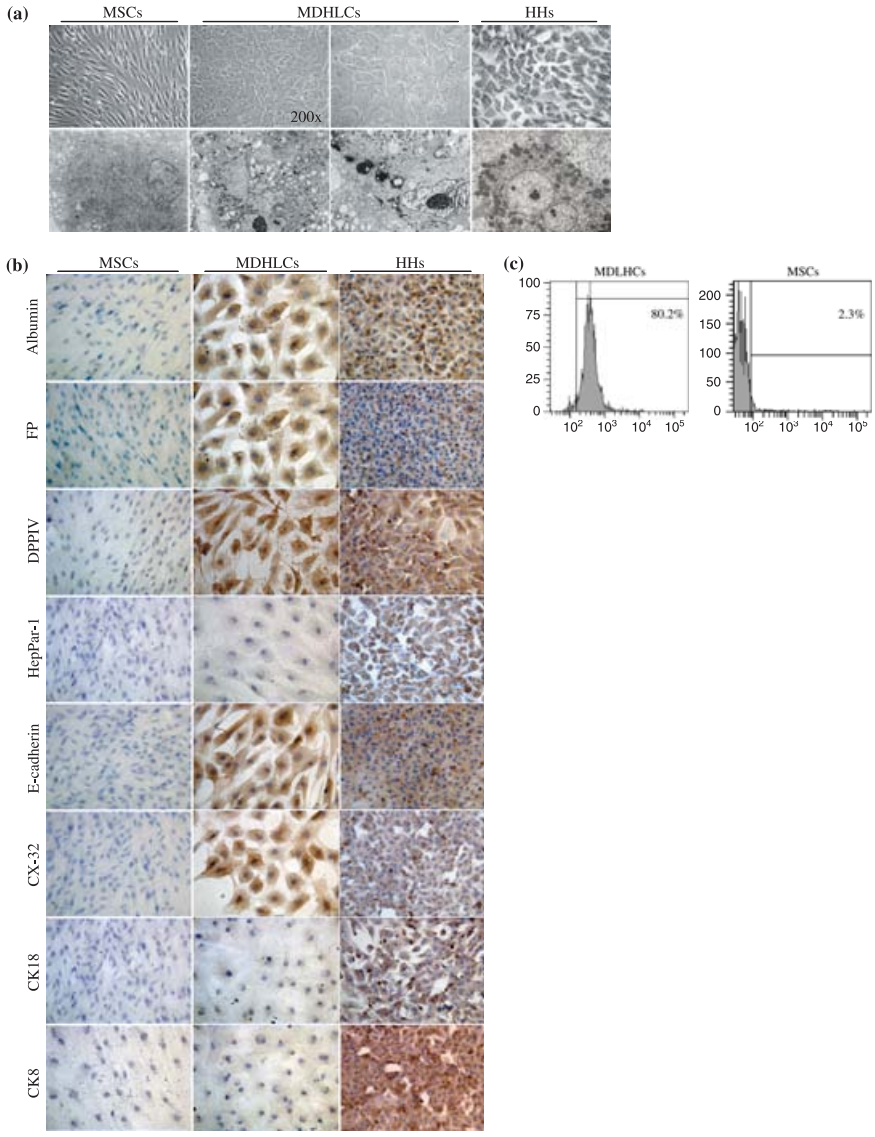


**Figure 3.** ***In vitro*** **hepatocyte differentiation of human bone marrow-MSCs.** (a) Comparative aspect of MSCs, MDHLCs and HHs as observed in optic (OM) and electron (EM) microscopy. Pictures show that MDHLCs acquire round or polygonal-shaped cells reduced in size and containing cytoplasmic granulations 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 ×400 unless indicated and EM pictures magnification was ×4000. (b) MDHLCs immunostaining for hepatocyte markers showing positive expression of albumin, αFP, 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 ×400. (c) Representative examples of albumin staining by flow cytometry in MDHLCs and MSCs. Bars indicate the fluorescence level of the corresponding isotype. αFP, 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.

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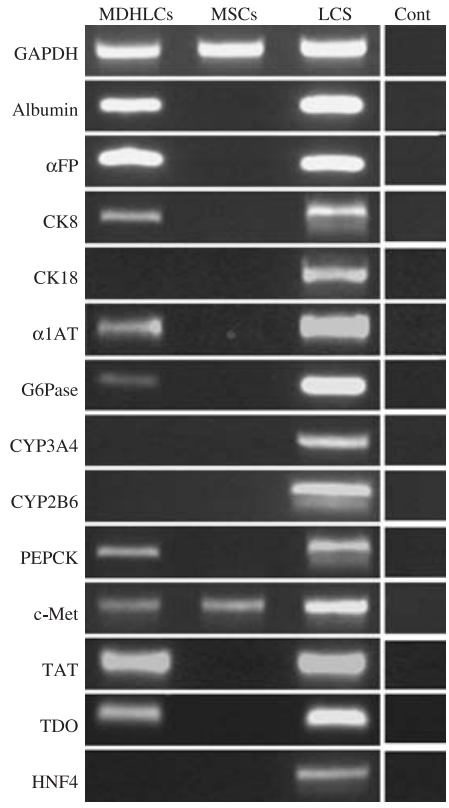


**Figure 4. Analysis of hepatocyte and mesodermal markers expression by RT-PCR in MDHLCs, MSCs (both on passage 1 to 3) and freshly isolated human liver cell suspension (LCS).** The figure shows concomitant immature (αFP, CK8) and mature (albumin, α1AT, G6Pase, PEPCK, TAT, TDO) hepatocyte gene expression 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 αFP 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: $84.2 \pm 7.5$ versus $98.8 \pm 0.8\%$ ($P < 0.05$); CD29: $89.3 \pm 6.6$ versus $98.2 \pm 1.7\%$ ($P < 0.05$);

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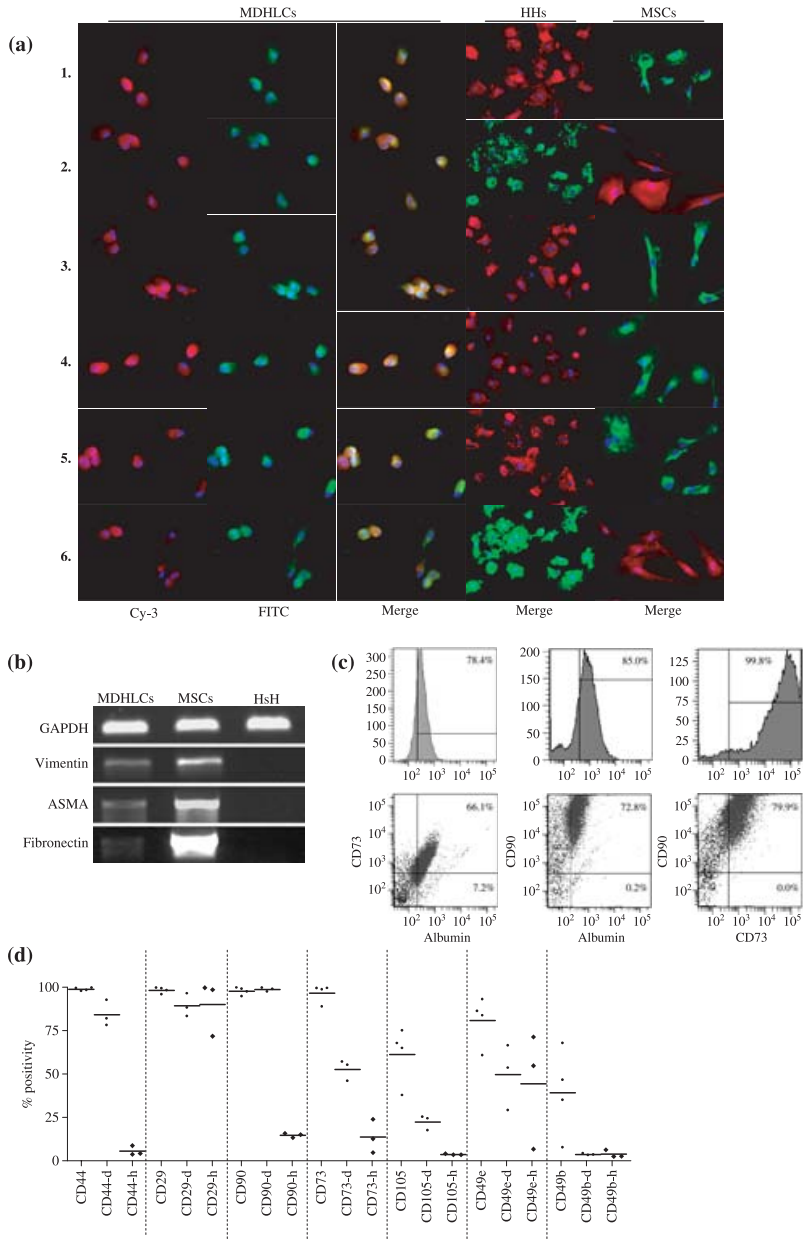


**Figure 5. Characterization of the chimerical phenotype of MDHLCs.** (a) Immunofluorescence assay showing co-staining of MDHLCs, HHs and MSCs for albumin/fibronectin (1), ASMA/αFP (2), DPPIV/fibronectin (3), E-cadherin/fibronectin (4), CX-32/fibronectin (5) and vimentin/albumin (6). Pictures were taken at magnification ×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 ($*P < 0.05$, $**P < 0.01$, $***P < 0.001$). αFP, α-foetoprotein; ASMA, α-smooth muscle actin; CX-32, connexin-32; DPPIV, dipeptidylpeptidase IV; HHs, human primary hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells.

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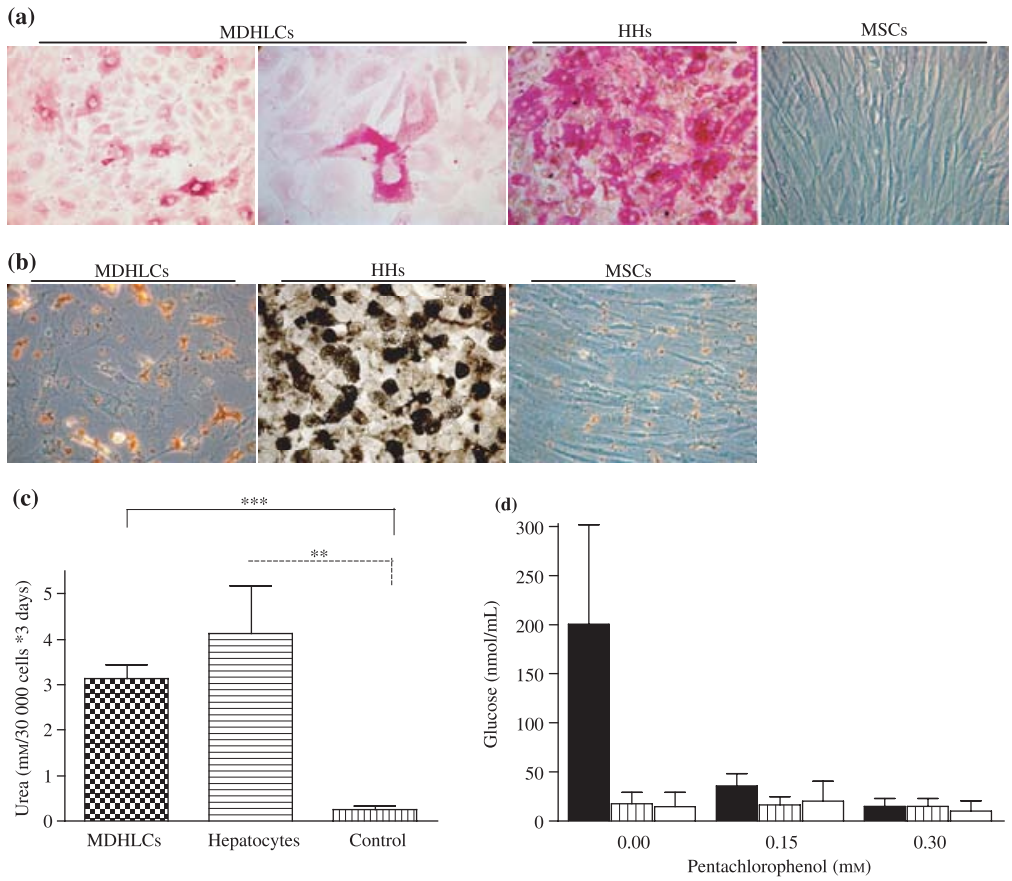


**Figure 6. Functional characterization of MDHLCs.** (a) Periodic acid-Schiff (PAS) staining showing cytoplasmic glycogen deposition in MDHLCs as compared to HHs and MSCs. (b) G6Pase assay ($n = 5$) showing lack of enzyme activity in MDHLCs revealed by absence of brownish lead sulphide cytoplasmic precipitate. Positive and negative controls were performed respectively on HHs and MSCs. Similar results were obtained with overnight 5 mM G6Pate incubation. Pictures were taken at magnification ×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 ± 5.2% ($P < 0.001$); CD105: 22.4 ± 4.2 versus 61.3 ± 16.3% ($P < 0.05$). Down-regulation of CD49e (49.8 ± 18.9 versus 80.9 ± 14.0%) and CD49b (3.7 ± 0.5 versus 39.2 ± 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 ± 5.3% (calculation in three different random fields of each three samples at magnification ×200, total *n* cells = 1041) (Fig. 6a). Although G6Pase was expressed at the mRNA level,

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we could not detect enzyme activity in MDHLCs by cytochemical assay, despite 24 h incubation with 5 mM G6Pate (Fig. 6b). However, we detected significant urea production and noticed that this secretion was comparable to that of mouse hepatocytes (respectively $3.1 \pm 0.5$ and $4.1 \pm 2.1$ mM/30 000 cells *3 days compared to $0.2 \pm 0.2$ 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 αFP 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–allylalcohol 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 αFP) (Fig. 7a). When we differentiated MSCs prior to transplantation (groups D and E), cells expressed albumin and αFP 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 i*n 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

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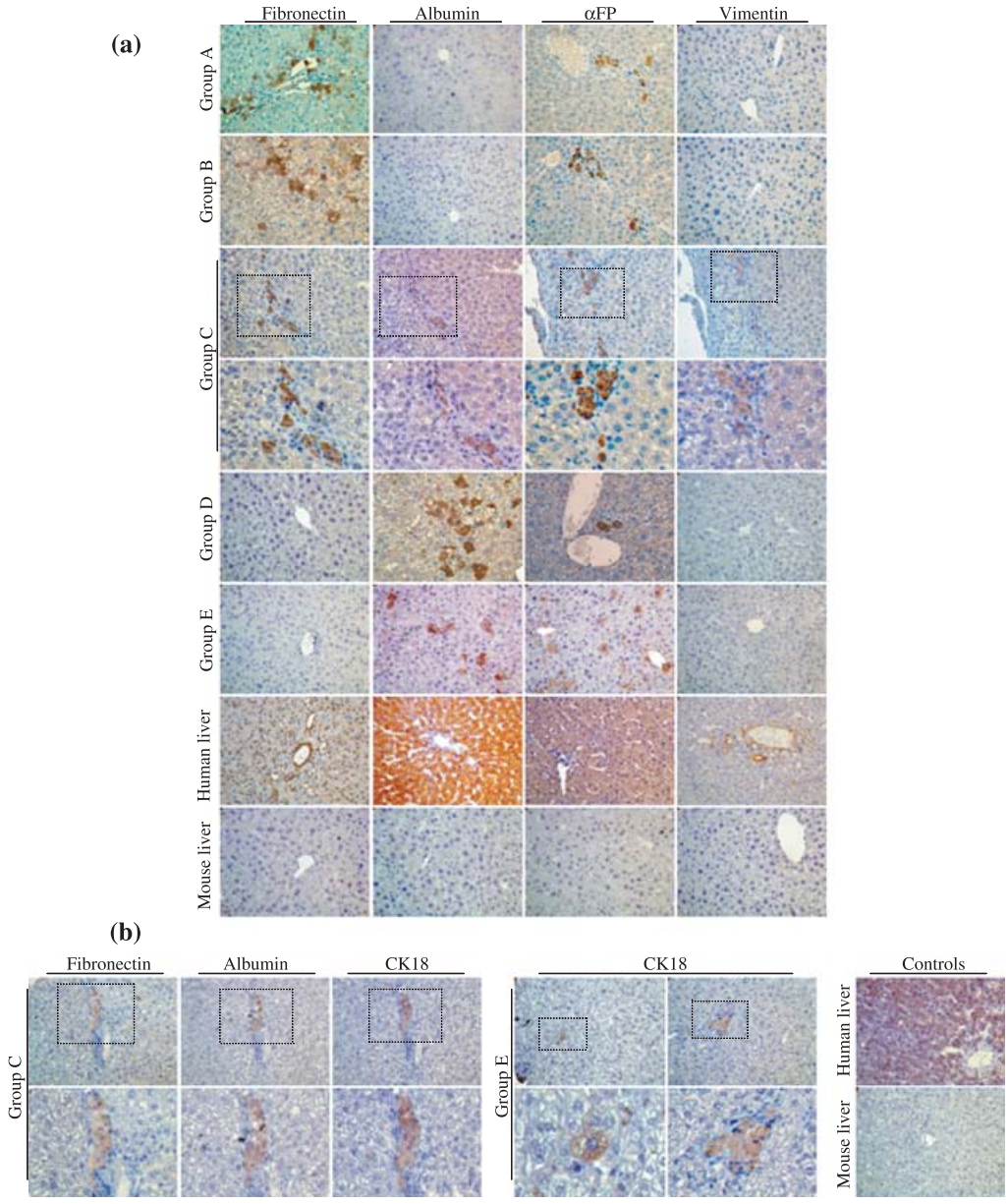


**Figure 7.** ***In vivo*** **characterization of MSCs and MDHLCs after transplantation into SCID mice.** (a) Analysis of *in vivo* differentiation potential of MSCs and MDHLCs. Pictures show immunostaining for human mesodermal (fibronectin, vimentin) and hepatocyte (albumin, αFP) antigens. In groups A and B, the engrafted cells formed clusters presenting staining for fibronectin or αFP 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 ×400. αFP, α-foetoprotein.

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**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.

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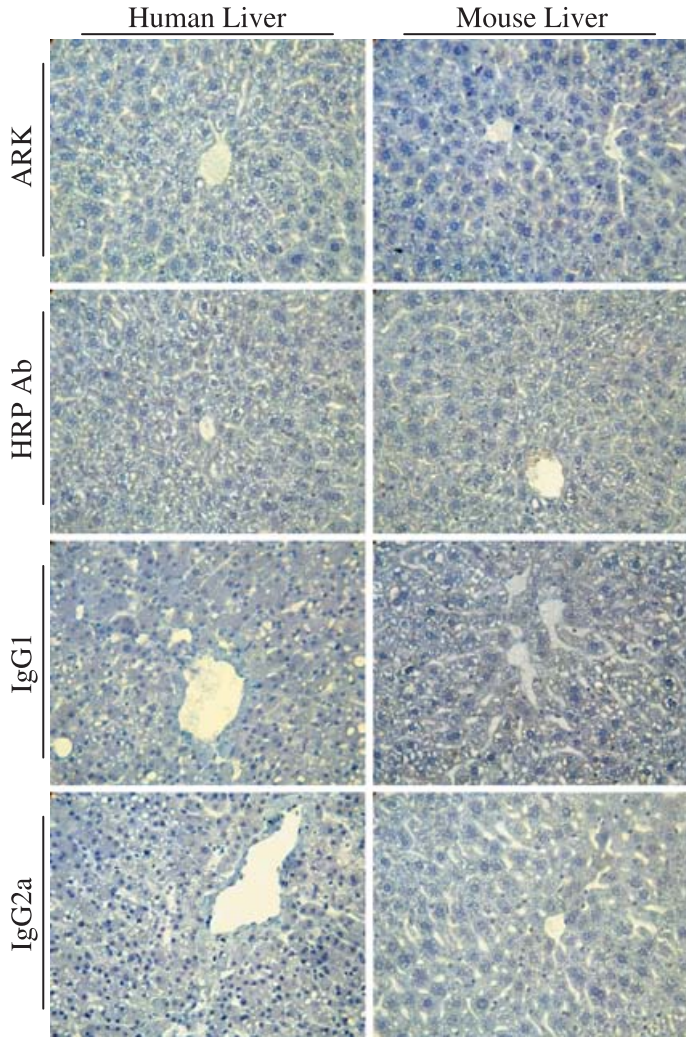


**Figure 8. Control immunohistochemistry.** Pictures show staining assays performed without primary antibodies using ARK kit (ARK) or HRP-conjugated anti-rabbit antibody (HRP Ab). For monoclonal antibodies, stains with corresponding isotypes were performed (IgG1 for anti-albumin and CK18 antibodies,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

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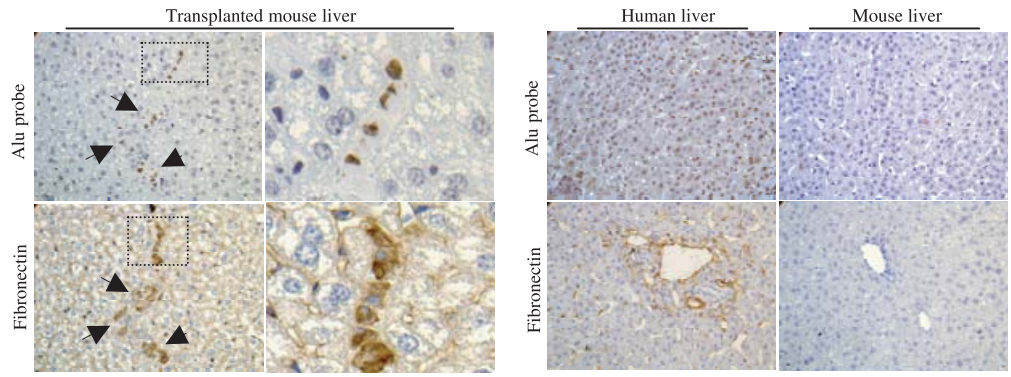


**Figure 9.** ***In vivo*** **tracking of human cells.** Pictures show that engrafted human cells were co-stained at the nuclear level with Alu probe by *in situ* hybridization technique and at the cytoplasmic level with antihuman fibronectin antibody by immunohistochemical assay on serial sections.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

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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 differentiational capability. 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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## 3. Estimation of hepatocyte differentiation of purified populations of human BM-MSCs

### a. Introduction

In the article presented in II, 2, we described a variable hepatic marker expression into MDHLCs obtained from one BM sample to another. No correlation could be performed with BM sample's characteristics (donor's age, growth capacities of MSCs, passage of MSCs used for differentiation). For this study, we handled a global population of MSCs that were obtained after a classical cell subculturing, and which contained several colonies of MSCs. Variability of expression intensity between samples could therefore reflect the heterogeneity of the cell populations used. To investigate differentiation of more purified populations, we applied hepatocyte differentiation protocol on MSCs obtained after clonal assays.

### b. Material and Methods

*Cell Culture*

Colony forming unit-fibroblast (CFU-F) cultures were performed by plating hBM-MSCs (*n*= 5) at 10 cells/cm² in α-minimal essential medium (αMEM) containing 10% fetal calf serum (FCS), into 6-well uncoated dishes (Greiner). After 10 days of culture, CFU-Fs appeared and were marked. One CFU-F per well was subjected to selective trypsinization: 5 µL of trypsin-EDTA were loaded on a marked CFU-F and incubated for 3 minutes, and then the well was washed with phosphate-buffered saline (PBS). After centrifugation, cells from one well were plated on a well of a new 6-well plate. When reaching 100% confluency, cells were detached and plated in 24-well dishes for hepatocyte differentiation. The technique is illustrated in figure 3.

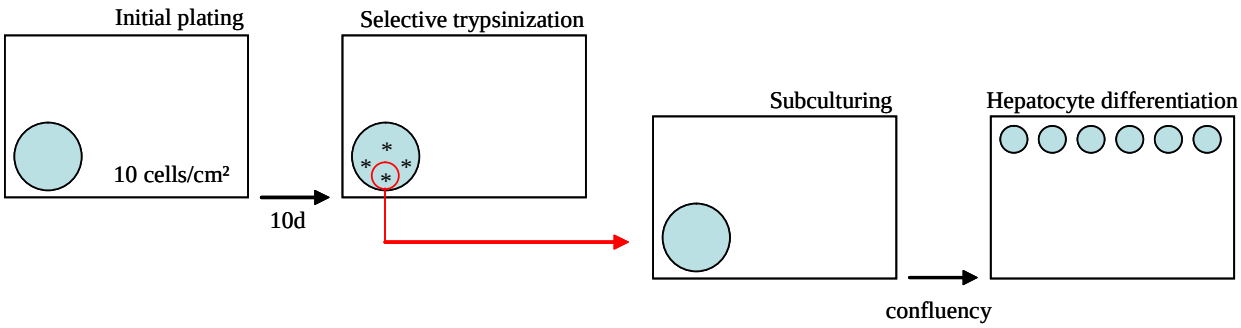


**Figure 3: Description of clonal culture of MSCs.** D, days.

*Hepatocyte differentiation*

The protocol for hepatocyte differentiation is described in the Material and Methods section of article 1, paragraph II-2.

*Immunocytochemistry*

The protocol for immunoperoxidase staining is described in the Material and Methods section of article 1, paragraph II-2. Monoclonal antibodies were directed against human albumin, connexin-32 (CX-32) and E-cadherin; polyclonal antibody was directed against human α-fetoprotein (αFP). Cell counting was performed as described in article 1, paragraph II-2.

c. Results

Analysis of staining rates for hepatocyte markers in clonal MDHLCs showed albumin positivity ranging from 86.9 to 97.6%, αFP from 72.7 to 93.5%, CX-32 from 18 to 79.3% and E-cadherin from 39.2 to 93.2% (Table 5). Staining intensity was also variable as demonstrated in figure 4. However, all analyzed fields showed positively stained cells.

**Table 5: Expression rates of hepatocyte markers in clonal MDHLCs**

| | Samples | | | | |
|---|---|---|---|---|---|
| | **1** | **2** | **3** | **4** | **5** |
| **Albumin** | 88.1 ± 3.8 (188) | 91.1 ± 3.6 (130) | 89.2 ± 5.7 (127) | 97.6 ± 1.6 (131) $^{*}$ | 86.9 ± 1.3 (133) $^{¥¥}$ |
| **αFP** | 87.5 ± 5.5 (156) | 88.8 ± 4.8 (164) | 93.5 ± 4.3 (188) | 72.7 ± 4.9 (119) $^{*\,\#\,‡‡}$ | 81.6 ± 2 (174) $^{¥}$ |
| **CX-32** | 18 ± 2.2 (145) | 72.9 ± 3.2 (116) $^{***}$ | 76.4 ± 4.1 (114) $^{***}$ | 33.9 ± 8.9 (103) $^{*\,\#\#\,‡‡}$ | 79.3 ± 4.9 (103) $^{***\,¥¥}$ |
| **E-cadherin** | 39.2 ± 5 (169) | 80.9 ± 5.5 (131) $^{**}$ | 93.2 ± 4.4 (114) $^{***}$ | 73.4 ± 5.4 (178) $^{**\,‡}$ | 72.3 ± 3.3 (161) $^{**\,‡‡}$ |

Positivity rates are expressed as percentages ± SEM. Total cell counts are provided between brackets. P values are indicated (**P*<0.05, ***P*<0.01, ****P*<0.001). ($^{*}$) compared with sample 1, ($^{\#}$) with 2, ($^{‡}$) with 3 and ($^{¥}$) with 4.

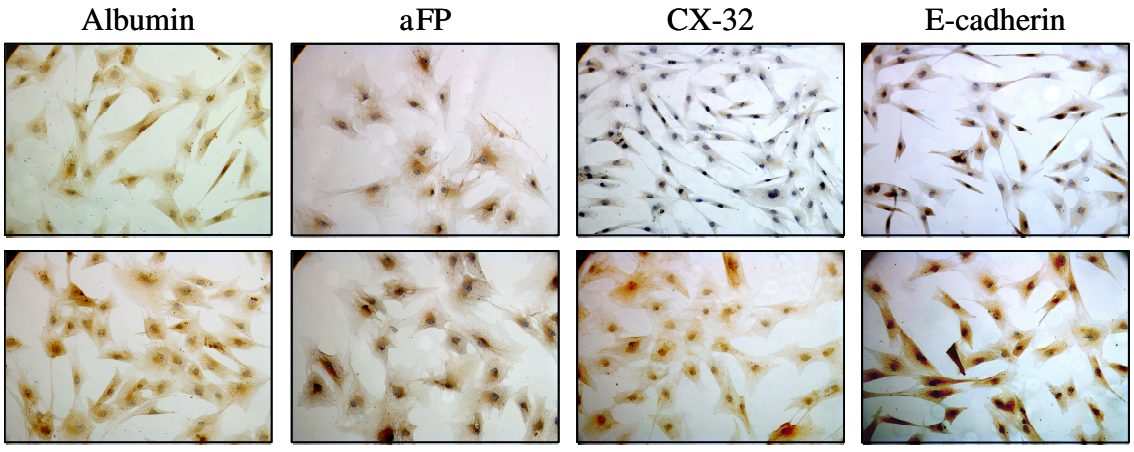


**Figure 4: Variability of staining for hepatic markers in clonal MDHLCs.** Top and bottom pictures corresponded to two different MDHLC samples. αFP, α-1-fetoprotein; CX-32, connexin-32. Pictures were taken at magnification ×400.

d. Conclusion

As we compared positivy rates exposed here with these obtained elsewhere with non clonally-expanded MSCs (Chapter II, 2 and chapter III, 2), we observed that clonal MDHLCs enhanced their expression rates of albumin (mean 90.6%), αFP (mean 84.8%) and CX-32 (mean 56.1%). However, as positivity rates were variable, some clonal MDHLC samples showed lower expression levels (sample 1 for example). Staining patterns were heterogeneous independently from positivity rates and were globally comparable or even lower than what we observed with non clonal MDHLCs (Chapter II, 2, Fig. 3).

In conclusion, clonal expansion of MSCs remained associated with variable staining rates and patterns while comparing distinct BM samples. As we compare with literature data, only few studies designed characterization based on clonally expanded MSCs (91, 93, 101) and no data about expression yield could be extrapolated from these reports. Nevertheless, in our hands clonal MDHLCs appeared to enhance quantitatively but not qualitatively their marker expression, and whether these cells could display higher hepatocyte-like functionality or lower expression of mesodermal markers should be further attested.

## 4. Study of the reversibility of hepatocyte differentiation of hBM-MSCs

### a. Introduction

As we demonstrated that MDHLCs remained chimerical in their phenotype (Chapter II, 2), with persistent expression of native MSC markers, we investigated whether these cells could regain, after incubation into growth medium, their initial properties in terms of morphology, marker expression and growth potential. We also investigated gene expression of EMT validated markers as *snail*, *slug* and *notch-1*.

### b. Material and Methods

#### *Cell Culture*

Cultures of MDHLCs (*n*= 3) were lifted using trypsin-EDTA and replated at 1000 cells/cm² in αMEM containing 10% FCS (growth medium). Cells were grown until confluency, detached and processed for further analyses or replated at 1000 cells/cm² for subculture.

#### *Immunocytochemistry*

For immunostaining, monoclonal antibodies were directed against human albumin, αFP, α-smooth muscle actin (αSMA), CX-32, CK8, CK18, CK19, DDPIV, E-cadherin, HepPar-1 and vimentin; polyclonal antibody was directed against human fibronectin.

#### *Reverse Transcription Polymerase Chain Reaction (RT-PCR)*

The protocol for RT-PCR is described in the Material and Methods section of article 1, paragraph II-2. Primers and cycle specifications are listed in table 6.

**Table 6: Primers for RT-PCR characterization.**

| Primer | Sequence | Size (bp) | Cycle |
|---|---|---|---|
| Albumin | S: 5'-CCTTGGTGTTGATTGCCTTTGCTC-3' | 308 bp | 94°C/1 min; 55°C/1 |
| | AS: 5'- CATCACATCAACCTCTGGTCTCACC-3' | | min; 72°C/2 min |
| α-FP | S: 5'-TGAAATGACTCCAGTAAACCC-3' | 199 bp | 94°C/30 sec; 55°C/1 |
| | AS: 5'-AATGAGAAACTCTTGCTTCATC-3' | | min; 72°C/2 min |
| Snail | S: 5'-GCGAGCTGCAGGACTCTAAT-3' | 836 bp | 94°C/30sec; 60°C/30 |
| | AS: 5'-ATTCCATGGCAGTGAGAAGG-3' | | sec; 72°/30 sec |
| Slug | S: 5'-AACAGCCCATTTTGAACCAG-3' | 926 bp | 94°C/30sec; 60°C/30 |
| | AS: 5'-GCACGGAGCTATAGGTGCTC-3' | | sec; 72°/30 sec |
| Notch-1 | S: 5'-CTTCAATGACCCCTGGAAGA-3' | 806 bp | 94°C/30sec; 60°C/30 |
| | AS: 5'-CCACGAAGAACAGAAGCACA-3' | | sec; 72°/30 sec |
| GAPDH | S: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' | 307 bp | 94°C/1 min; 58°C/1 |
| | AS: 5'-AGC CTT CTC CAT GGT GGT-3' | | min; 72°C/2 min |

α-FP, α-fetoprotein; GAPDH, glyceraldehyde dehydrogenase.

c. Results

After being incubated into growth medium, MDHLCs regained a spindle-shaped morphology in one week (Fig. 5A, 5B). When replated at low density, they restarted to proliferate and reached at second passage the proliferation level of native MSCs (Fig. 6).

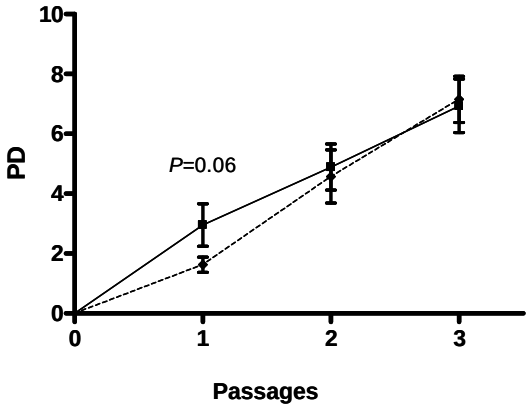


**Figure 6: Compared proliferation activity of MSCs and dedifferentiated MDHLCs.** Graph represents cumulative population doublings (PD) of MSCs (plain line) and dedifferentiated MDHLCs (hatched line). Data represents mean ± SEM of 4 independent experiments. P value is indicated in the graph.

Analysis of mesodermal marker expression showed that dedifferentiated cells lost in one week the expression of albumin, αFP, DPPIV, CX-32 or E-cadherin (Fig. 5C-F) that had been acquired after 30 day-procedure (paragraph II-2). No change in the expression of CK8, CK18, CK19 or HepPar-1 could be achieved (Fig. 5H-K). Immunocytochemical assay also showed a

robust staining for fibronectin and αSMA (Fig. 5D, 5G). Dedifferentitated MDHLCs lost the expression of albumin and αFP while they neo-expressed EMT genes as *snail*, *slug* and *notch-1*, as shown by RT-PCR (Fig. 5L).

d. Conclusion

In this paragraph, we demonstrated that hepatocyte differentiation of hBM-MSCs is dependent on exposition to a specific medium and reverses while cells are exposed to growth culture condition. Cells then dedifferentiate, acquire the expression of EMT genes and restart to proliferate in a similar fashion than native MSCs.

These results underline the robustness of the remaining MSC phenotype in MDHLCs as these latter cells could loose their hepatic features in one week. It strengthens the need for culture conditions allowing deeper involvement of MSCs in the epithelial phenotype and it also questions the long-term *in vivo* behavior of these cells after transplantation as microenvironment and factor exposition are thereby changed.

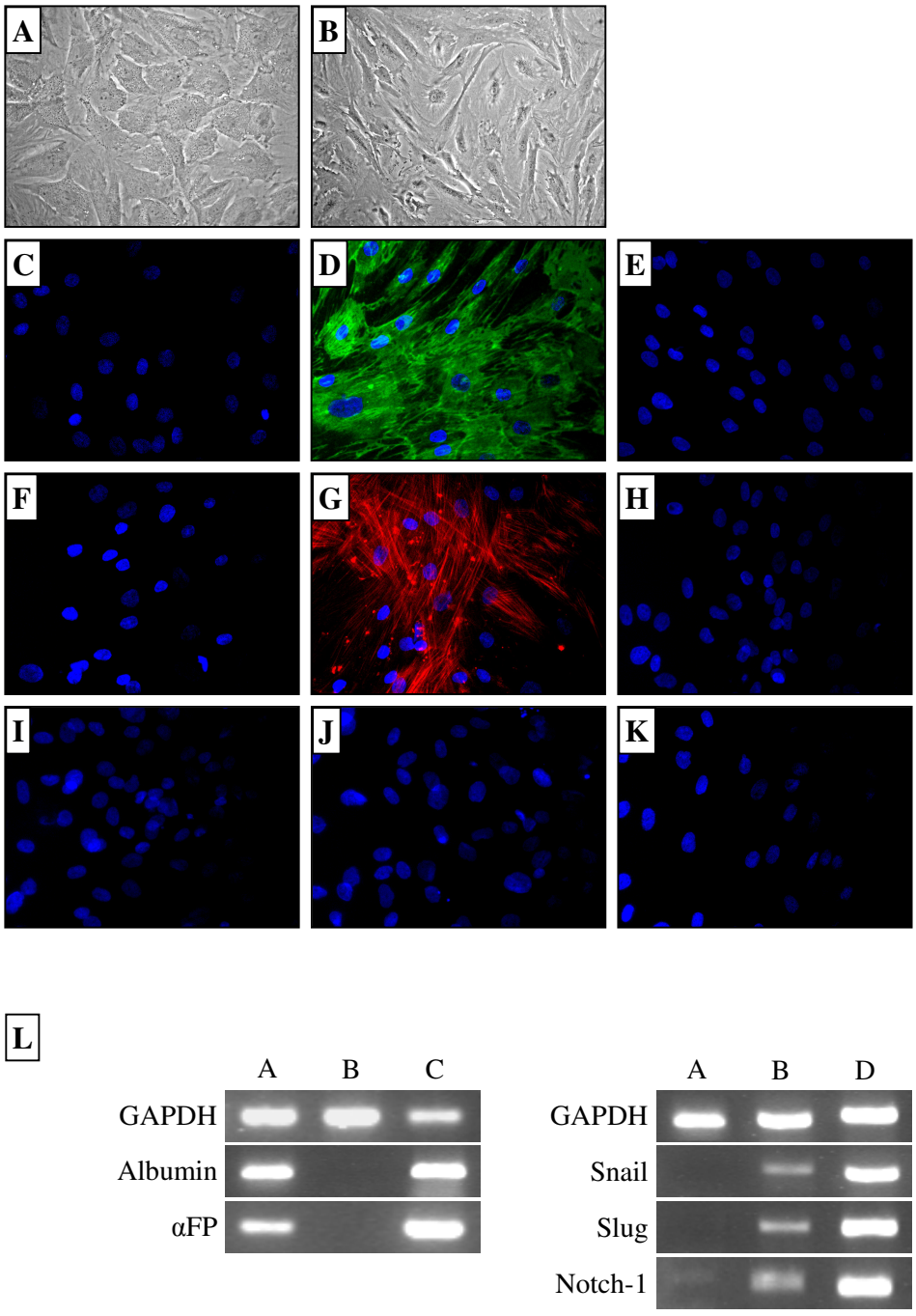


**Figure 5: Analysis of morphology and immunophenotype of MSCs through EMT.** Aspect of morphology of MDHLCs before (A) and after one week incubation into growth medium (B) showing cells regaining a spindle-shaped form. These dedifferentiated cells did not stain for albumin or αFP (C, merged picture), DPPIV (D, merged picture), CX-32 (E) or E-cadherin (F) while they continuously expressed αSMA (G) and fibronectin (D). No expression of CK18 (H), CK8 (I), CK19 (J) or HepPar-1 (K) was observed in these cells. Pictures were taken at magnifications ×300 (C-J) and ×400 (A, B). (L) RT-PCR analysis showing lost of albumin and αFP mRNA expression in dedifferentiated cells (B) compared to MDHLCs (A) or human liver cells (C) and the neo-expression of *snail*, *slug* and *notch-1* genes after EMT, as compared to MCF-7 human breast adenocarcinoma cells (D).

## 5. Liver engraftment and differentiation of hBM-MSCs in a wild-type mouse model

### a. Introduction

Human BM-MSCs have been described as cells owning favorable immunological properties and a permissive phenotype making them less immunogeneic (119). Based on these assumptions, we explored whether hBM-MSCs could engraft or differentiate in immunosuppressed wild-type mice, thus when these cells are subjected to stringent immunological pressure. Our investigations were motivated by the work of Sato et al. (96) who showed engraftment and hepatocyte differentiation of hBM-MSCs after intrahepatic injection into ciclosporin A-treated wild-type rats.

### b. Material and Methods

#### *Cell Transplantation Procedures*

Cell transplantations were performed on 8-12-week-old female NMRI mice as summarized in table 7. Nine transplantation groups were designed based on the nature of infused cells (MSCs or MDHLCs), injection site (spleen or liver), hepatic regeneration stimulus performed (partial hepatectomy, retrorsine-allyl alcohol (R-AA) treatment) and use of immunosuppression. Animals were immunosuppressed or not using daily subcutaneous injections of 5 mg/kg FK506 (Tacrolimus) performed at a fixed hour. Each group consisted of 6 mice receiving injections of $1 \times 10^6$ cells suspended in PBS. Positive controls were performed by injecting $2 \times 10^6$ allogeneic C57BL/6 mouse hepatocytes into spleens of FK506-treated hepatectomized mice. Negative controls consisted of 100 µL PBS-injected mice. Description of transplantation methods is presented in article 1, paragraph II-2.

In group G and in positive control group, cells were stained with a CellTracker before injection to allow their tracing *in vivo*. For this purpose, trypsinized cells were incubated for

45 minutes with 50 µM CellTracker Orange CMTMR tetramethylrhodamine (C2927, Molecular Probes), at 37°C. Cells were then washed and incubated in their respective medium for 30 minutes at 37°C. After two washes, viability was controlled with trypan blue exclusion test and cells were incubated in PBS for injection.

To validate the immunosuppression protocol, control NMRI female mice aged 8 weeks were injected subcutaneously with a single dose of 5 mg/kg FK506, and blood sampling was performed after 8 hours (*n*= 3 mice) and 24 hours (*n*= 3 mice). This was compared with a group of mice (*n*= 3) receiving single injection of 2 mg/kg FK506 that was analyzed at 24 hours. FK506 concentrations were screened routinely (Laboratory of Biochemistry, UCL, Prof. Wallemacq).

**Table 7: Description of transplantation procedures into wild-type mice.**

| **Mice** | | **Procedure** | | | **Engraftment analysis** | | |
|---|---|---|---|---|---|---|---|
| **Group** | **I.S.** | **Cells** | **Injection** | **Hepatic Stimulus** | **Sacrifice** | **Fibronectin** | **Albumin** |
| **A** | - | MSCs | spleen | HTM | 2w | - | - |
| **B** | - | MSCs | spleen | no | 2w | - | - |
| **C** | + | MSCs | spleen | HTM | 2w | + | - |
| **D** | + | MSCs | spleen | no | 2w | - | - |
| **E** | + | MSCs | liver | HTM | 2w | + | - |
| **F** | + | MSCs | liver | no | 2w | + | - |
| **G** | + | MSCs | spleen | R-AA | 3w | + | - |
| **H** | + | MDHLCs | spleen | HTM | 2w | + | + |

HTM, hepatectomy; I.S., immunosuppression; R-AA, retrorsine-allyl alcohol; w, weeks.

*Immunohistochemistry*

Protocol for immunohistochemistry is described in article 1, paragraph II-2. Monoclonal antibody was directed against human albumin, and polyclonal antibodies were directed against human fibronectin and αFP. To analyze cell tracing by CellTracker, liver sections were analyzed under a fluorescence microscope.

c. Results

The immunosuppression protocol was valid as we found reliable plasma concentrations at 8 and 24 hours post-injection (90.2 ± 30.4 ng/mL and 6.8 ± 2.9 ng/mL, respectively). The expected value ranged between 5-14 ng/mL. By comparison, plasma concentrations were measured at 4.3 ± 1.2 ng/mL at 24 hours after injection of 2 mg/kg FK506.

No engraftment could be observed in groups without immunosuppression. In FK506-treated mice, we found cells dispersed throughout the liver parenchyma of mice of groups C, E and F (Table 7 and Fig. 7A). However, no human albumin-positive cells could be observed in these groups. No cells engrafted when they were injected into spleens of FK506-treated mice without hepatic injury (data not shown). In group G, where R-AA protocol was applied, engrafted cells were rather organized as clusters staining for human fibronectin but also for αFP (Fig. 7B). The human origin of these cell clusters was further attested by tracing them with a fluorescent dye (Fig. 7C). When we injected MDHLCs into spleens of hepatectomized mice (group H), we noticed cells engrafting as single cells or as clusters expressing both fibronectin and albumin (Fig. 7D). Allogeneic hepatocytes were also able to engraft in FK506-treated mice, and organized as cell clusters expressing fluorescent CellTracker (Fig. 7E).

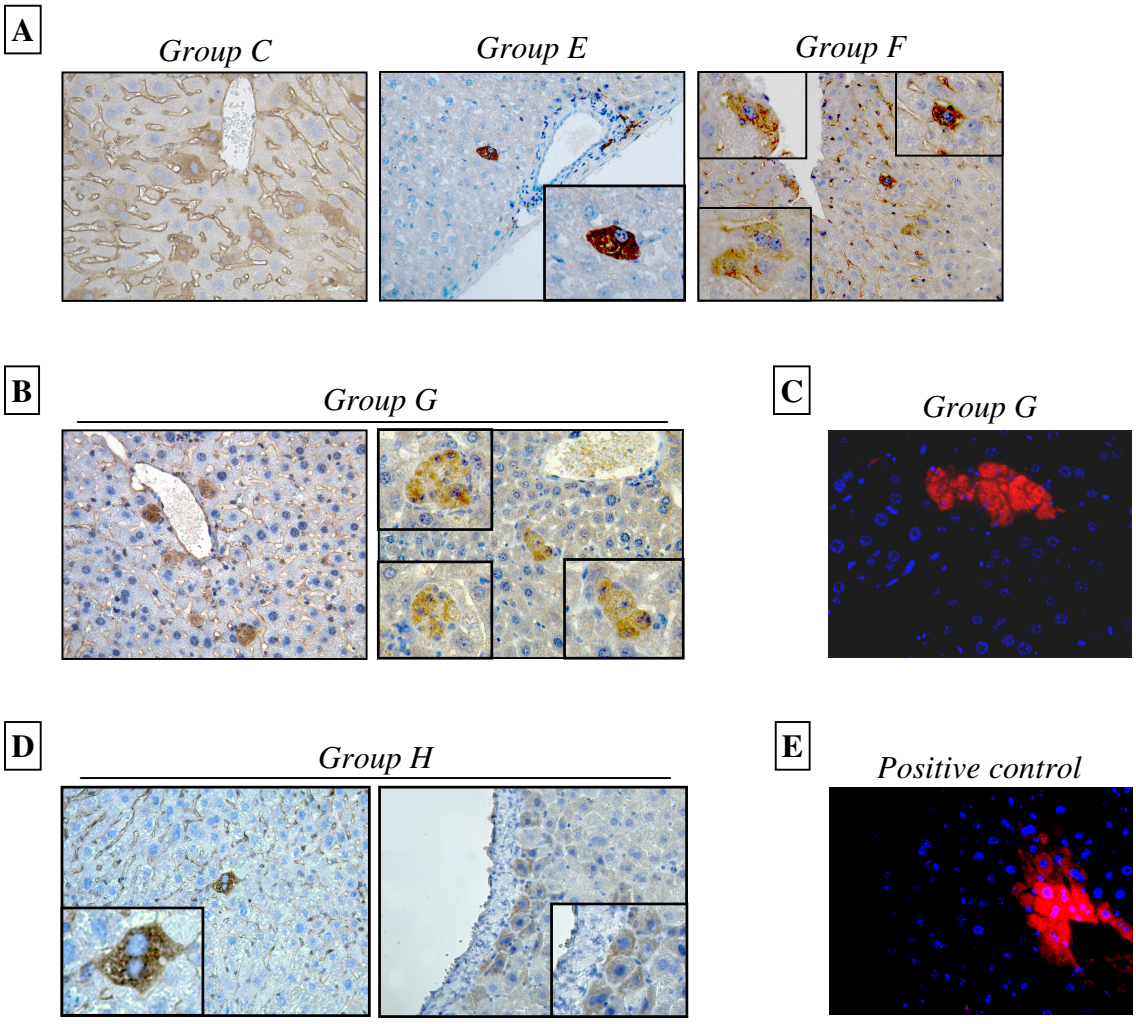


**Figure 7: *In vivo* analysis of cell engraftment after transplantation into wild-type mice.** (A): Staining for human fibronectin in groups C, E and F revealed engrafted cells dispersed throughout the liver parenchyma. Boxes represent enlargements of positive cells presented in the back picture. (B): Staining for human fibronectin (left) and human αFP (right) showing, in group G, cells organized as single cells or as clusters, and able to express one hepatic marker. Boxes in the right picture show representative examples of cell clusters staining for anti-αFP. (C): Analysis of red fluorescence in group G revealed cell clusters, as visualized in (B), maintaining the CellTracker coloration which confirmed their human origin. (D): Pictures showing anti-human fibronectin (left) and human albumin (right) staining in group H. Boxes show enlargement of positive cells presented in the back picture. (D): Red fluorescence of a cell cluster observed after injection of allogeneic hepatocytes in immunosuppressed wild-type mice. Pictures were all taken at magnification ×400.

d. Conclusion

This is, to our knowledge, the first demonstration of the ability of hBM-MSCs to engraft in wild-type mice under FK506 monotherapy. This immunosuppression protocol was supported by a plasmatic dose-response analysis but also by efficient engraftment of allogeneic hepatocytes using this model. However, these cells engrafted only when permissive proceedings were applied. Indeed, MSCs where observed in the spleen-injected mouse livers only when a hepatic injury was applied. Moreover, when the cells were injected directly into the liver parenchyma, no hepatic injury was necessary, suggesting a beneficial role of the liver microenvironment, as previously described with SCID mice in article 1 (paragraph II-2). MSCs were organized as clusters only in group G, evoking advantage of R-AA treatment for cell engraftment in these procedures. However, whereas cells engrafted, they were not able to express human albumin, which implies a lack of differentiation capacity that could be explained by stringent immunological pressure occurring in this model, compared with our results obtained with SCID mice. Although Sato et al. (96) presented broader *in vivo* differentiation yield, in addition to the fact that our model was different, we applied a minimal immunosuppression protocol more linked to the OLT clinical setting and our major goal was to evaluate MSCs' engraftment potential within high immunological restraints. Indeed, our results confirmed indirectly the poor immunogeneicity of MSCs in sight of their potential to efficiently engraft in a xenogeneic transplantation model, and this constitutes an advantage on human hepatocytes that were not able to engraft, in our hands, in immunosuppressed wild-type mice subjected to partial hepatectomy (data not shown).

## 6. Discussion

In this section, we demonstrated that hepatocyte differentiation of hBM-MSCs is limited in terms of specific features acquisition and is not fully reproducible as BM samples do not provide stable endodermal potential regardless of BM characteristics. Our data about variability of hepatocyte features acquisition were in accordance with a contemporaneous work showing variable alkaline phosphatase expression irrespective to donor samples' characteristics (120). Using CFU-F-derived MDHLC samples, we demonstrated that the expression of hepatocyte markers overally increased while staining rate and intensity remained variable between populations. Intriguingly we could not classify differentiation state of our MDHLCs as they presented both immature and mature markers, or as they acquired sophisticated hepatic functions while lacking more basic ones (albumin secretion). These data suggest a narrow influence of cytokines/factors considered here, whatever combination tested, allowing only elective induction of hepatocyte-like features. In our chimerical MDHLCs, the native phenotype of MSCs was much strongly represented than the hepatocyte-like one as it remains through differentiation procedure and reappeared unchanged when the exposition to complete protocol ended. However, MSCs' native phenotype was down-expressed in MDHLCs as suggested by flow cytometric data showing that their immunophenotype approached this of human hepatocytes. This indicates the opportunity to lessen mesenchymal phenotype of MDHLCs via *in vitro* conditioning. However, further investigations are necessary to elucidate whether persistence of MSC phenotype could impair mature hepatocyte differentiation (for example by using siRNA inhibition). Because these data were not evidenced elsewhere, our work underlines crucial interest of using combined methods and broad marker panel to achieve hepatocyte differentiation.

Our *in vivo* procedures in stringent xenogeneic mouse model add arguments about low immunogenicity of MSCs as these cells could engraft or even partially differentiate in the

mice livers. As we also transplanted MDHLCs that engrafted and maintained part of their markers, we can also suggest that MSCs retain at least partially their immunological properties after *in vitro* differentiation.

Based on these results, we elaborated tools for improving MDHLCs differentiation yield: we first studied the effect of new growth factors and varied timing of their exposition; we then designed methods to enhance functionality of MDHLCs.

# CHAPTER III: ATTEMPTS FOR IMPROVING HEPATOCYTE DIFFERENTIATION OF hBM-MSCS

## 1. Introduction

Implements for improving hepatocyte differentiation of hBM-MSCs were designed at the level of culture microenvironment and of functionality. We elaborated additional test protocols containing cytokines or factors whose influence on hepatocyte differentiation was described or not. One cytokine, leukemia inhibitory factor (LIF), whose action on differentiation was poorly documented, showed unexpected influence on MSCs' fate *in vitro*. As data about kinetics of biological activity of the tested cytokines/factors were lacking, influence of exposure timing was also studied. At functional level, as our MDHLCs were poorly metabolically active or lacked mature hepatocyte functions, we tried to improve the activity of urea cycle and of gluconeogenesis by incubating the cells with graded concentrations of substrate or by inducing enzyme expression and/or activity with glucagon hormone, respectively.

**2. Leukemia inhibitory factor contributes to hepatocyte-like differentiation of hBM-MSCs**

a. Article 2: Leukemia inhibitory factor contributes to hepatocyte-like differentiation of huma bone marrow mesenchymal stem cells. *Differentiation* 2008, in press.

**Leukemia Inhibitory Factor Contributes to Hepatocyte-Like Differentiation of Human Bone-Marrow Mesenchymal Stem Cells**

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**Short title for running head:** LIF allows hepatic differentiation of MSCs

**Number of text pages:** 22**; tables:** 1**; figures:** 6**.**

**Abstract words count:** 178**.**
**Total words count:** 3004**.**

**List of abbreviations:** $\alpha_1$-AT, α-1-antitrypsin; αFP, α-1-fetoprotein; BSA, bovine serum albumin; CX-32, connexin-32; CYP, cytochrome P450; CK, cytokeratin; DPPIV, dipeptidylpeptidase IV; ESCs, embryonic stem cells; FCS, fetal calf serum; G6Pase, glucose-6-phosphatase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBM, human bone marrow; IL, interleukin; ITS, insulin-transferrin-selenium; LIF, leukemia inhibitory factor; LIFR, leukemia inhibitory factor receptor; MDHLCs, mesenchymal-derived hepatocyte-like cells; MNCs, mononuclear cells; MSCs, mesenchymal stem cells; OSM, oncostatin M; OSMR, oncostatin M receptor; P/S, penicillin/streptomycin; PAS, periodic acid schiff; PBMCs, peripheral blood mononuclear cells; PEPCK, phosphoenolpyruvate carboxykinase; RT, room temperature; RT-PCR, reverse transcription-polymerase chain reaction; TAT, tyrosine aminotransferase; TDO, tryptophan-2,3-dioxygenase.

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**Abstract**

The current majority of protocols for hepatocyte differentiation of mesenchymal stem cells (MSCs) are conducted using oncostatin M (OSM) as an inducer of hepatocyte-like maturation. As leukemia inhibitory factor (LIF) and OSM share similar signaling pathways, we examined whether LIF could play a role in the hepatocyte differentiation process. A differentiation protocol was designed using LIF as a maturation cytokine and this was compared to standard and control protocols applied to human MSCs of bone marrow origin. We observed that mesenchymal-derived hepatocyte-like cells (MDHLCs) acquired similar morphological changes when exposed to LIF or to OSM. Using protein and gene expression assays, we noticed a comparable hepatic marker expression in both differentiation conditions. Furthermore, LIF and OSM allowed the acquisition of equivalent levels of hepatocyte-like functionality as attested by evaluation of urea secretion and glycogen deposition. However, no increase in the expression of hepatocyte-like features could be observed in MDHLCs after a combined exposition to LIF and OSM. In conclusion, we demonstrated that LIF can play a similar role than OSM in the hepatocyte differentiation process of human MSCs.

**Key words:** hepatocyte differentiation, leukemia inhibitory factor, mesenchymal stem cells, oncostatin M

**Introduction**

Hepatocyte differentiation potential of human bone marrow (hBM) MSCs has been recently described (Lee et al. 2004;Wang et al. 2004;Aurich et al. 2006;Lysy et al. 2007) and critically discussed (Hengstler et al. 2005;Nussler et al. 2006). To achieve this differentiation, numerous studies conducted on MSCs (Hong et al. 2005;Lee et al. 2004;Lysy et al. 2007;Seo et al. 2005) but also on embryonic stem cells (ESCs) (Teratani et al. 2005;Cho et al. 2007) used a two-step procedure based on a induction step mainly dependent on hepatocyte growth factor (HGF) and a maturation step in which oncostatin M (OSM) is the essential cytokine. The use of OSM was opted according to the description of its role in liver development (Kinoshita et al. 1999;Suzuki et al. 2003) or in maturation of fetal cell lineages (Kamiya et al. 2002).

OSM is a pleiotropic cytokine playing a role in several biological or pathological conditions as liver or neuronal development, hematopoiesis, inflammation or tumor progression (Morikawa 2005;Tanaka and Miyajima 2003). OSM belongs to the interleukin (IL)-6 subfamily and as such its activity is conducted through the interaction with a membrane receptor complex that includes gp 130 as a common signal transducer. The binding to the receptor activates pathways involving Jak/STAT (Signal Transducers and Activators of Transcription) transcription factors or MAP (Mitogen-Activated Protein) kinases, as described elsewhere (Heinrich et al. 2003). Through its structure and function, OSM is closely similar to another IL-6 subfamily member, leukemia inhibitory factor (LIF). Although these cytokines possess their own specific receptor, OSM can act via the interaction with LIF receptor (LIFR) (Nakashima and Taga 1998) and by the way these cytokines share biological actions.

LIF is a cytokine owning many functions including blastocyst implantation, neuronal development, bone formation or acute phase protein production by hepatocytes (Metcalf 2003). In the differentiation area, LIF is crucial for maintaining totipotency of murine ESCs

and has been described as an inducer of proliferation of hematopoetic stem cells (Escary et al. 1993) and murine progenitor cells (Jiang et al. 2002), and hence has a preventing action on their differentiation. At the contrary, LIF has been implicated in the differentiation of macrophages (Zhang et al. 1998) and was recently suggested to increase adipocyte differentiation of mesenchymal progenitor cells (Falconi, Oizumi, and Aubin 2007).

In this work we investigated the role of LIF in the maturation of hepatocyte-like differentiation of hBM-MSCs, by comparison or in combination with OSM. This effect was analyzed at protein and gene expression levels, together with the screening of hepatocyte-specific functions.

## Material and Methods

*Ethical committee*
The present study was approved by the institutional human and animal ethical committee.

*Cell Isolation and Culture*
For this study, we handled 10 BM samples obtained by aspiration of the vertebras of post-mortem donors aged 18 to 62 years old. BM aspirates were collected into heparinized syringes containing 10% HBSS (Invitrogen) and processed within 48 hours. Mononuclear cells (MNCs) were separated by centrifugation at 805 × g for 20 minutes at room temperature (RT) after carefully loading BM on Ficoll-Paque™ PLUS (GE Healthcare). Cells were then washed twice with PBS (Lonza). Cell viability was always greater than 95% as assessed by trypan blue exclusion test. MNCs were seeded in culture flasks at 1 to 6 × 106 cells/cm² in α-minimal essential medium (MEM) (Invitrogen) containing 10% fetal calf serum (FCS) (Perbio) and 1% penicillin/streptomycin (P/S) (Invitrogen). Colonies of MSCs appeared spontaneously in the flasks and first medium change was performed after 48 to 72 hours and then twice weekly. Cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. When reaching 70% confluence, cells were lifted using 0.05% trypsin-0.53 mM EDTA (Invitrogen) and replated at 0.1 × 104 cells/cm².

*Cell Differentiation*
For mesodermal differentiation assays, cells were plated at 1.5 × 104 cells/cm² in uncoated 6-well dishes. To obtain osteogenic differentiation, cells were incubated in 10% FCS αMEM containing 0.1 µM dexamethasone, 0.05 mM ascorbate and 10 mM β-glycerophosphate (all from Sigma), and changed twice a week. After four weeks, calcium deposition was evaluated by von Kossa and Alizarin Red stains. For adipogenic differentiation, cells were incubated in 10% FCS αMEM containing 1 µM dexamethasone, 0.5 mM isobutyl-methylxanthin, 0.2 mM indomethacin (all from Sigma) and 10 µg/mL insulin (Lilly) with medium change twice a week. After four weeks, lipid vesicles were revealed by Oil Red-O staining.
For hepatocyte differentiation, we plated MSCs from passages 1 to 4 at a density of 1.5 × 104 cells/cm² on dishes coated with rat tail collagen type I (BD Biosciences). The cells were incubated for 10 days in 1% P/S IMDM (Invitrogen) containing 20 ng/mL HGF, 10 ng/mL FGF4 (all from Peprotech), 1% insulin-transferrin-selenium (ITS) premix (Invitrogen), and 0.61 g/L nicotinamide (Sigma) (induction medium); and for 20 days in 1% P/S IMDM containing 20 ng/mL OSM and/or 50 ng/mL LIF (Peprotech), 1 µM dexamethasone and 1% ITS premix (maturation medium). The protocols whose maturation step contained OSM, LIF or OSM and LIF were identified as test protocols. The negative control protocol differed as its maturation medium contained only dexamethasone and ITS premix.

*Flow Cytometry*
Cells were trypsinized and suspended at a concentration of 0.5 to 1 × 105/mL in PBS containing 0.5% bovine serum albumin (BSA, Sigma) and then incubated for 30 minutes at 4°C with the following anti-human antibodies: CD45-PE-Cy7, CD34-PE, CD13-APC, CD90-APC, CD73-PE, CD44-FITC, CD29-APC, CD49b-FITC, CD49e-PE, HLA-ABC-FITC, HLA-DR-PerCP (Becton Dickinson); CD105 Endoglin-FITC, CD26-PE (Ancell); LIFRα-PE (R&D Systems) and OSMRβ-FITC (Santa Cruz Biotechnology). Controls were made by incubating the cells with corresponding isotypes: IgG1 coupled with FITC, PE, APC, PerCP or PE-Cy7 (Becton Dickinson). Cells were then washed and suspended in PBS. Intracytoplasmic human albumin staining was performed as follows: 2 × 105 cells were incubated in 200 µL Cytofix/Cytoperm (BD Bioscience) for 20 minutes at RT and washed

with 1× Perm/Wash solution (BD Bioscience). Cells were incubated with 1/50 FITC-labeled rabbit anti-human albumin antibody (Cedarlane), 1/10 FITC-labeled anti-pancytokeratin antibody (Miltenyi) or FITC-labeled IgG1 control isotype (BD) for 25 minutes at 4°C. After washing with PBS, cells were fixed in 250 µL Cellfix (BD Bioscience). Cells were analyzed on a FACSCantoII flow cytometer (BD) and at least 10.000 events were acquired for each sample.

*Immunocytochemistry*
After fixation with 4% formaldehyde for 15 minutes, cells were incubated with 3% hydrogen peroxide (Sigma) for 3 minutes, washed with distilled water and then permeabilized with 1% Triton X-100. After 1 hour blockade in 1% BSA, cells were incubated 1 hour at RT with primary mouse anti-human monoclonal antibodies in 0.1% BSA as follows: 1/50 albumin (Sigma); 1/50 HepPar1, 1/50 cytokeratin (CK) 19 (Dako Cytomation); 1/50 CK18 (Progen); 1/200 connexin-32 (CX-32), 1/50 CK8 (Chemicon); 1/20 dipeptidylpeptidase-IV (DPPIV) (Ancell); 1/100 E-cadherin (BD Pharmingen) and rabbit polyclonal anti-human antibodies in 0.1% BSA as follows: 1/100 fibronectin, 1/100 α-1-fetoprotein (αFP) (Dako Cytomation). Cells were washed 5 times with PBS and then exposed for 30 minutes with anti-rabbit or anti-mouse Dako Envision HRP antibody (Dako Cytomation). Staining was performed by 3 minutes exposition to diaminobenzidine (Sigma). After rinsing, nuclei were colored with Mayer's Hematoxylin (10 minutes) and cells were washed and then mounted for microscopic examination (DMIL, Leica). Each antibody was tested on fresh (< 24 hours) cultures of human hepatocytes as positive control and MSCs as negative control.

*Reverse-Transcription Polymerase Chain Reaction*
Total RNA was extracted from cells (n~ 1 x 106) using the TriPure isolation reagent (Roche) and cDNA was generated using a reverse transcription (RT) 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 µL. All PCR experiments were performed at 30 cycles. Samples were electrophoresed on a 1.5% agarose gel and nucleic acids were visualized by ethidium bromide staining.

*Real-Time Quantitative Polymerase Chain Reaction*
Total RNA was extracted and reverse transcribed using a similar protocol than for RT-PCR. Complementary DNA made from 10 ng of RNA was used for each Real-Time Quantitative Polymerase Chain Reaction (qPCR). The qPCR experiments were performed using an iQ5 real-time PCR detection system (Bio-Rad Laboratories) according to the manufacturer's instructions. Human-specific primers used were as follows: albumin (S: 5'-cgcctttggcacaatga-3', AS: 5'-acgaaacacacccctgga-3'); CX-32 (S: 5'-aatgtggccgaggtggt-3', AS: 5'-ttgcgggaaggtggatt-3'); GAPDH (S: 5'-atgcccccatgttcgtc-3', AS: 5'- tggtgcaggaggcattg-3'). Primers were custom made and were provided by Invitrogen. Reactions were carried out using iQ SYBR Green Supermix (Bio-Rad) in a final volume of 25 µL with 0.2 µM of each primer. The qPCR reactions were performed in triplicate for each gene. Negative controls were performed as no template cDNA-reactions and melting curves were used to confirm the results. The results were normalized using GAPDH concentrations of each sample.

*Glycogen Staining*
After 4% formaldehyde fixation, cells were incubated for 10 minutes in 1% periodic acid (Sigma), washed with distilled water and incubated with Schiff's reagent (Sigma) for 15 minutes. After rinsing 10 minutes with tap water, nuclei were stained 10 minutes with

Karazi's Hematoxylin. Cells were then washed with tap water and mounted. Freshly cultured (< 24 hours) human hepatocytes and MSCs served respectively as positive and negative controls. Inner control consisted of MDHLCs treated with 5g/L amylase (Sigma) for 15 minutes at 37°C prior to PAS staining.

*Urea Assay*

Urea production was evaluated in the supernatants of cultures submitted to a 24 hours incubation with 5 mM $NH_4Cl$ (Sigma) added in the corresponding medium. Supernatants were collected and urea concentration was measured using a colorimetric assay (Gentaur), handled as per the manufacturer's instructions and analyzed with a Multiskan EX absorbance reader (Thermo). Serum-free IMDM incubated with freshly cultured (<24 hours) mouse hepatocytes and serum-free IMDM served respectively as positive and negative controls.

*Cell Counting*

Cell numbers were obtained by counting positive cells in 3 different random fields (magnification 200×) of each 3 different culture samples. Total cell counts are provided for each experiment.

*Statistics*

Statistical analyses were performed using GraphPad PRISM 4.01 Software. Statistically significant differences (*$P<0.05$, **$P<0.01$, ***$P<0.001$) were estimated using the unpaired Student's t-test and results are expressed as mean ± S.E.M.

**Results**

*Characterization of Human Bone Marrow Mesenchymal Stem Cells*

In growth culture medium, hBM-MSCs presented a spindle-shaped morphology (Fig. 1B-1C) and their proliferation rate reached 35 cumulative population doublings in 8 passages. Their phenotype was demonstrated first by flow cytometric analysis showing a high expression of CD90, CD73, CD44, CD29, CD13, HLA-ABC, a moderate expression of C105, CD49e, CD49b and absence of CD45, CD34 and HLA-DR expression (Fig. 1A). Confirmation of their identity was provided by successful differentiation of these cells into osseous and adipose lineages, as revealed by histochemical stains (Fig. 1D-1G).

*Induction of Hepatocyte-Specific Marker Expression by Leukemia Inhibitory Factor*

Sensitivity of hBM-MSCs to LIF and OSM was verified by detecting mRNA expression of their respective cognate receptors, which was comparable to that of peripheral blood mononuclear cells (PBMCs) (Fig 2A). We also noticed that MSCs and PBMCs expressed LIF. Membrane expression of LIFR and OSMR by hBM-MSCs was attested using flow cytometry (Fig. 2B). We then subjected these cells to hepatocyte differentiation protocols containing or not (control medium) LIF and OSM separately or in combination. All test protocols allowed MSCs to adopt hepatocyte-like morphology, defined by the acquisition of a round or a polygonal shape, granular cytoplasmic inclusions suggesting an enhanced content of organites and a central nucleus with prominent nucleolus (Fig. 2C). Similar changes did not occur in cells exposed to control medium.

Hepatic gene expression profile of MDHLCs obtained by RT-PCR after LIF or OSM exposition was analogous and consisted of co-expression of early (αFP, albumin, CK8, CK19) and late markers (α1AT, CX32, G6Pase, TDO, TAT) while other markers of mature hepatocytes were lacking (CK18, CYP3A4) (Fig. 3A). By comparison, MSCs exposed to

control medium and native MSCs did not express any of analyzed markers. We observed that the adjunction of LIF and OSM in differentiation protocol did not improve the expression profile of MDHLCs (Fig. 3B).

Handling qPCR experiments (Fig 3C), we observed that MDHLCs exposed to OSM or LIF expressed similar levels of albumin and CX-32, and that albumin expression levels were only about 5-fold lower than which of human hepatocytes. Contrarily, MDHLCs did not show comparable CX-32 expression levels than these measured in human hepatocytes (13.9 ± 2.9-fold GAPDH level).

We analyzed the expression of hepatocyte-specific markers on protein level by immunocytochemistry, and observed an equivalent profile after exposition of MSCs to protocols containing LIF or OSM (Fig. 4). Indeed, these MDHLCs were all positively stained for albumin, αFP, DPPIV, CX-32, E-cadherin but were unable to express other epithelial markers as CK8, CK18 and HepPar-1. However, these cells slightly stained for the biliary marker CK19. According to results obtained at gene level, co-exposition of MDHLCs to OSM and LIF did not improve hepatic marker expression profile (Fig. 4). Staining patterns for CX32, E-cadherin and DDPIV were cytoplasmic and not membranous in both MDHLCs and human hepatocytes cultures whereas some foci of inter-membranous staining for E-cadherin could be identified in the hepatocytes population (Fig. 4). Although MSCs exposed to control medium expressed fibronectin, no staining for hepatic markers could be observed nor in these cells (Fig. 4) nor in native hBM-MSCs (data not shown).

Expression of albumin, cytokeratins and DPPIV (CD26) by MDHLCs obtained after exposition to test and control protocols was quantified by flow cytometry (Fig. 5A-5B). We obtained similar expression levels for albumin in test groups (64.4 ± 10.7% for OSM+LIF group, 62.2 ± 9.3% for OSM group, 63.7 ± 6.1% for LIF group) whereas control group showed negligible amounts of staining (6.5 ± 0.6%). Slight staining for cytokeratins was

observed in OSM- and LIF- matured MDHLCs (12.7 ± 7.7% and 11.0 ± 6.5 % respectively) and was assumed to reflect the small CK19 expression revealed by the immunocytochemistry assay. However, no statistically significant differences could be observed between these groups, control group (3.8 ± 0.9%) and OSM+LIF-matured MDHLC group (4.3 ± 0.5%). The expression of DPPIV (CD26) was enhanced in all the test groups as compared to control group (44.0 ± 4.6% for OSM+LIF group, 72.8 ± 10.6 for OSM group, 70.0 ± 9.6 for LIF group and 23.8 ± 4.6% for control group). We also observed a strong CD90 expression in all analyzed groups.

*Induction of Hepatocyte-Like Functionality by Leukemia Inhibitory Factor*

PAS assays revealed that MDHLCs obtained after OSM and/or LIF maturation were able to store glycogen and that the quantification of glycogen-positive cells was not different between these groups (29.1 ± 3.4% for OSM-matured MDHLCs (n= 1415); 24.3 ± 2.5% for LIF-matured MDHLCs (n= 1812) and 24.8 ± 3.1% for OSM+LIF-matured MDHLCs (n= 1120)) (Fig. 6A-6C). However, the content of glycogen-positive cells was significantly higher in the human hepatocytes cell population (61.8 ± 1.8%, n= 1126) than in MDHLC groups. No glycogen-positive cells were noticed in control group. PAS staining was demonstrated to be specific as the signal disappeared after exposition to amylase (Fig. 6B).

After completion of differentiation protocols, urea production was quantified and found to be similar in OSM, LIF and OSM+LIF-matured MDHLCs with respective rates of 1.9 ± 0.4, 1.8 ± 0.2 and 2.4 ± 0.5 mM/30000 cells*day (Fig. 6D). Mouse hepatocytes displayed a significantly higher production of urea (5.7 ± 0.7 mM/30000 cells*day) whereas negligible amounts were observed in control group (0.4 ± 0.03 mM/30000 cells*day).

**Discussion**

Here we describe for the first time the role of LIF in hepatocyte differentiation of hBM-MSCs. We demonstrated that LIF, as a key-cytokine of hepatocyte differentiation protocol, allows the acquisition of hepatocyte features in hBM-MSCs, as epithelial morphology, protein and gene expression of hepatic-specific markers but also liver functions as secretion of urea or glycogen storage. Quantification of the acquired features permitted us to conclude that LIF's actions on MSCs were not different from OSM which is largely used in hepatocyte differentiation protocols. LIF was presumed to act via his receptor expressed by hBM-MSCs as there is no evidence of cross-reaction with OSM receptor (OSMR) (Tanaka and Miyajima 2003). We demonstrated specificity of LIF's effects as its removal from protocol did not allow MSCs to acquire any hepatocyte features. This observation suggests the incapacity of induction step, mainly dependent on HGF action, to strongly and permanently induce acquisition of hepatocyte traits, and this is reinforced by the fact that the morphological changes are occurring during the maturation step and therefore depend on OSM or LIF action. However, one can argue that MDHLCs could loose some of their acquired characteristics during exposition to a maturation medium not containing OSM or LIF. We also analyzed the synergic action of OSM and LIF in differentiation process but did not observe any improvement neither of MDHLCs hepatocyte-like features, nor of expression of markers lacking in MDHLCs, as HepPar-1, CK18 for protein markers and CK18 and CYP3A4 for mRNA markers. Moreover, OSM and LIF combination did not significantly enhance the urea secretion or glycogen storage potential of MDHLCs. Whereas OSM was described to inhibit CYP3A4 synthesis and activity in human hepatocytes at a higher level than LIF (Guillen et al. 1998), we found no positive effect of OSM replacement by LIF on the CYP3A4 expression. However, the same team suggested a negative effect of HGF on CYP3A4 expression and function (Donato et al. 1998) while HGF and OSM were documented to induce CYP3A4

activity in MSCs (Oyagi et al. 2006;Campard et al. 2007). As described elsewhere (Lysy et al. 2007), we confirmed that MDHLCs maintain the expression of CD90 stem cell marker and noticed that this chimerical phenotype was not modified by LIF adjunction to protocol.

In conclusion, LIF is -like OSM- a pleiotropic cytokine owning various actions that are difficult to overlap and which can even be contradictory, as for example LIF's role on stem cell differentiation. In parallel to the documented structural and functional homology between LIF and OSM, we herein detailed the potential of LIF to induce hepatocyte differentiation of hBM-MSCs with a similar efficacy than OSM. We therefore provided a new tool of investigation about LIF's potential roles in the hepatology field.

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**Table 1: Primers used for RT-PCR characterization.**

| Primer | Sequence | Size | Cycle |
|---|---|---|---|
| Albumin | S: 5'-CCTTGGTGTTGATTGCCTTTGCTC-3' | 308 bp | 94°C/60 sec; 55°C/60 |
| | AS: 5'- CATCACATCAACCTCTGGTCTCACC-3' | | sec; 72°C/120 sec |
| G6Pase | S: 5'-TCAGCTCAGGTGGTCCTCTT-3' | 291 bp | 94°C/60 sec; 55°C/60 |
| | AS: 5'-CCTCCTTAGGCAGCCTTCTT-3' | | sec; 72°C/120 sec |
| CK-8 | S: 5'-AAGGGCTGACCGACGAGATC-3' | 537 bp | 94°C/30 sec; 50°C/30 |
| | AS: 5'-GATCGCCACCTACAGGAAGC-3' | | sec; 72°C/60 sec |
| CK18 | S: 5'-CCATGCGCCAGTCTGTGGAG-3' | 322 bp | 94°C/30 sec; 65°C/45 |
| | AS: 5'-AGCAGATTGAGGAGAGCACCAC-3' | | sec; 72°C/45 sec |
| $\alpha_1$-AT | S: 5'-TCGCTACAGCCTTTGCAATG-3' | 142 bp | 94°C/30 sec; 55°C/60 |
| | AS: 5'-TTGAGGGTACGGAGGAGTTCC-3' | | sec; 72°C/120 sec |
| α-FP | S: 5'-TGAAATGACTCCAGTAAACCC-3' | 199 bp | 94°C/30 sec; 55°C/60 |
| | AS: 5'-AATGAGAAACTCTTGCTTCATC-3' | | sec; 72°C/120 sec |
| CYP 3A4 | S: 5'-TGCTGTCTCCAACCTTCACC-3' | 802 bp | 94°C/1 min; 50°C/60 |
| | AS: 5'-GGTGGTGATGATTCCAAGCTA-3' | | sec; 72°C/60 sec |
| TAT | S: 5'-TGAGCAGTCTGTCCACTGCCT-3' | 358 bp | 94°C/40 sec; 56°C/50 |
| | AS: 5'-CTCAGATCCTCCTCATTCACAT-3' | | sec; 72°C 60 sec |
| TDO | S: 5'-ATACAGAGCACTTCAGGGAGC-3' | 316 bp | 94°C/40 sec; 56°C/50 |
| | AS: 5'-GATACCGAAGATGAACCCAACCA-3' | | sec; 72°C/60 sec |
| PEPCK | S: 5'-TTAGATGGGACAAAGCCTG-3' | 226 bp | 94°C/40 sec; 55°C/40 |
| | AS: 5'-GCAAGACGGTGATTGTAACTC-3' | | sec; 72°C/60 sec |
| LIF | S : 5'-GAATGGGGAGGCCTGAGT-3' | 988 bp | 94°C/30 sec; 60°C/60 |
| | AS: 5'-GCTAAGCCGGATGAAGCA-3' | | sec; 72°C/60 sec |
| LIFR | S : 5'-CTCTAAACGGCGCTAACCTG-3' | 763 bp | 94°C/30 sec; 60°C/60 |
| | AS : 5'-ACCAATGACTGGGCTTTCAC-3' | | sec; 72°C/60 sec |
| OSMR | S:5'-TGGCTCTATTTGCAGTCTTTCA-3' | 676 bp | 94°C/30 sec; 60°C/60 |
| | AS: 5'-ATGCCTTCACTGACATTTCCTT-3' | | sec; 72°C/60 sec |
| GAPDH | S: 5'-CGGAGTCAACGGATTTGGTCGTAT-3' | 307 bp | 94°C/1 min; 58°C/1 |
| | AS: 5'-AGC CTT CTC CAT GGT GGT-3' | | min; 72°C/2 min |

Abbreviations: A, antisense; $\alpha_1$-AT, alpha-$_1$-antitrypsin; α-FP, alpha-fetoprotein; bp, base pair; CK, cytokeratin; CYP, cytochrome p450; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; G6Pase, glucose-6-phosphatase; LIFR, leukemia inhibitory factor receptor; OSMR, oncostatin M receptor; PEPCK, phosphoenolpyruvate carboxykinase; TAT, tyrosine aminotransferase; TDO, tryptophan-2,3-dioxygenase; S, sense.

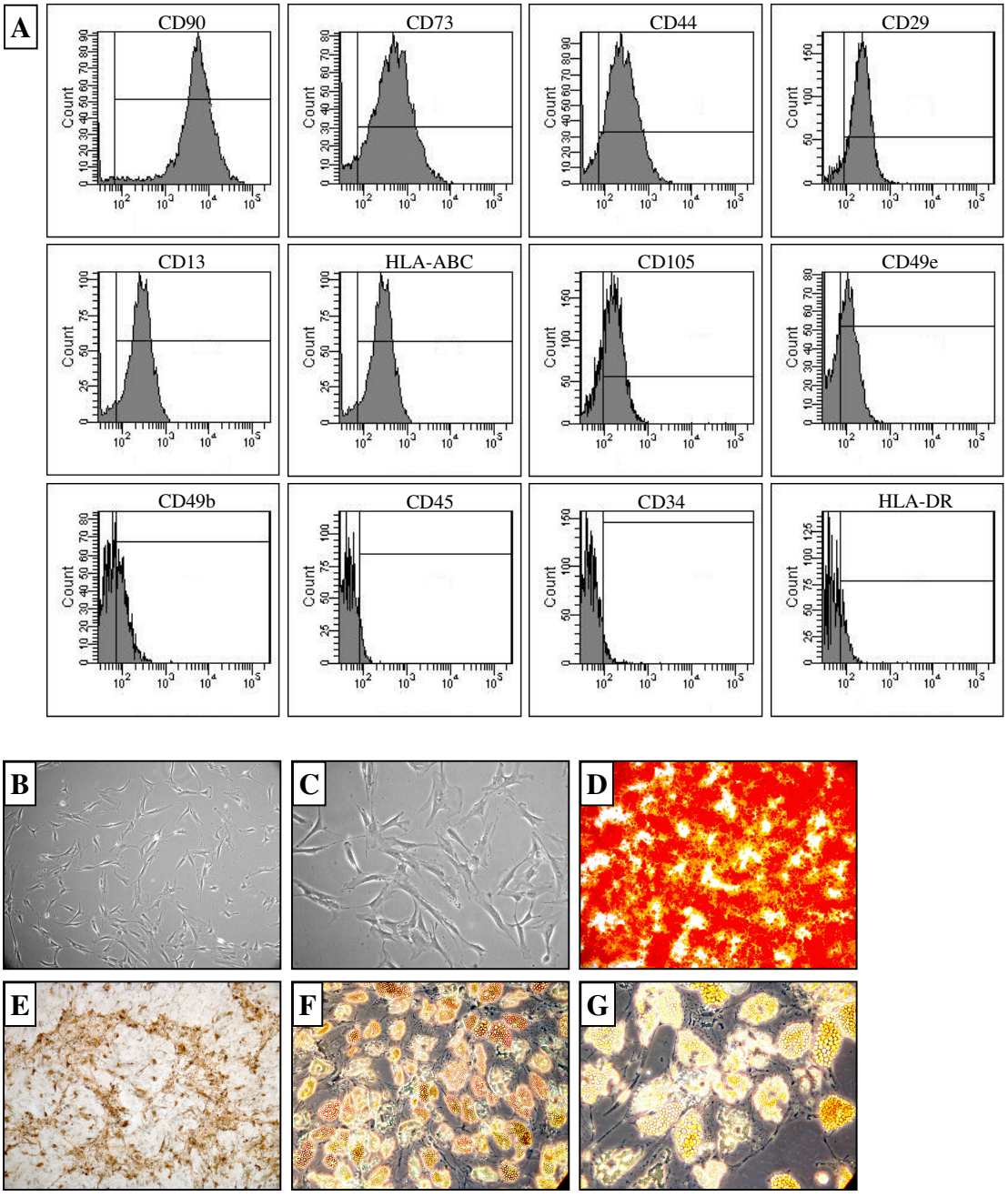



**Figure 1: Characterization of hBM-MSCs.** (A): Representative example of the flow cytometric expression profile of MSCs suggesting their mesenchymal phenotype. The analyzed epitopes are indicated in the histograms. Bars represent the fluorescence level of corresponding isotype. (B, C): Morphology of MSCs in growth culture at passage 1. (D, E): Extracellular calcium matrix deposition obtained after osseous differentiation of MSCs as revealed respectively by alizarin red and von Kossa stains. (F, G): Accumulation of lipid-rich droplets in the cytoplasm of MSCs subjected to adipose differentiation shown after Oil Red-O staining. Pictures were taken at 100× (D), 200× (B, E, F) and 400× magnifications (C, G).

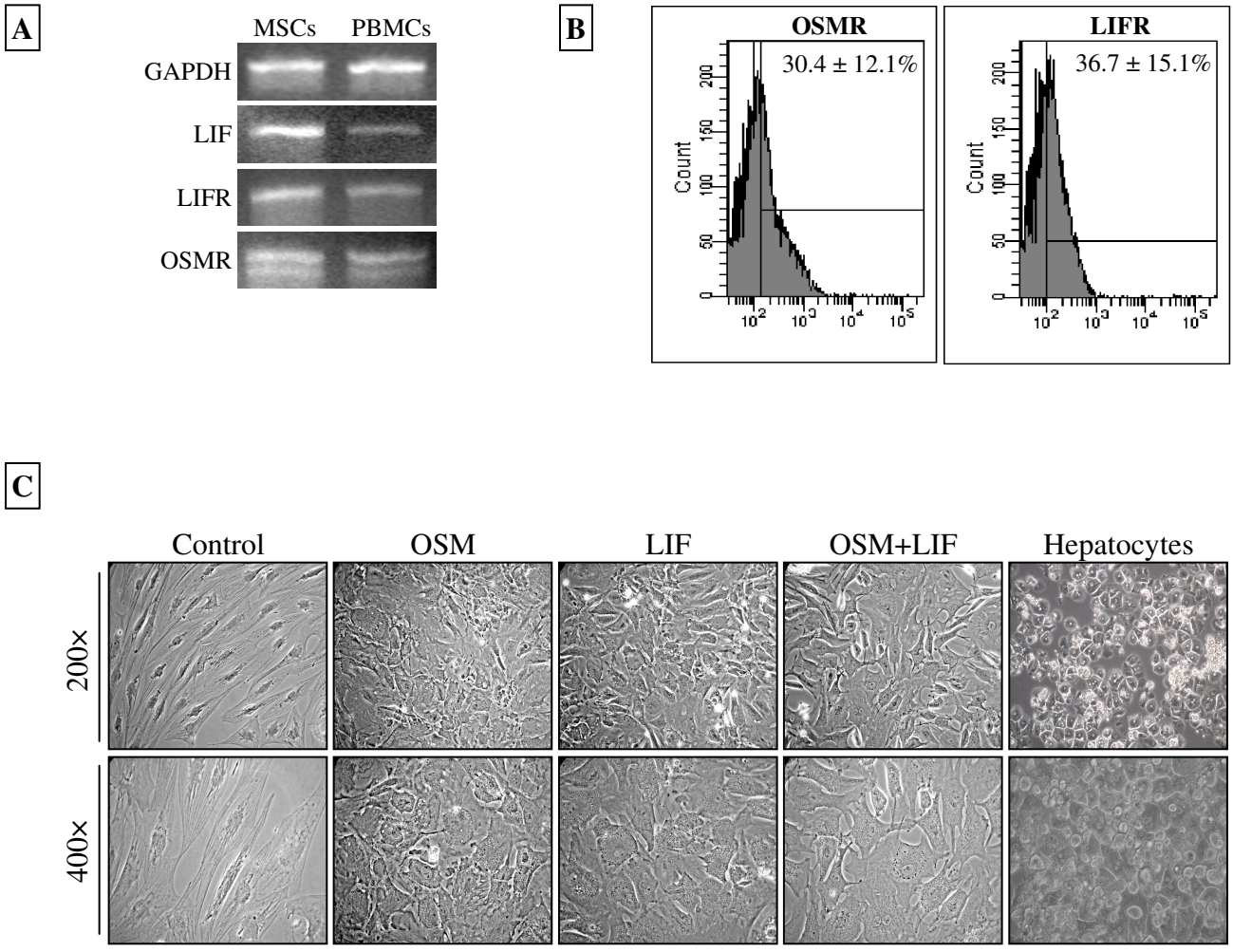



**Figure 2: Analysis of LIF, LIFR and OSMR expression and of comparative hepatocyte-like morphology.** (A): RT-PCR assay showing expression of LIF, LIFR and OSMR mRNAs by MSCs and PBMCs. (B): Membrane protein expression of OSMR and LIFR in MSCs as shown by flow cytometry. Indicated values represent mean ± SEM of 3 independent experiments. Bars represent fluorescence value of corresponding isotype. (B): Pictures show a similar acquisition of hepatocyte-like morphology in MDHLCs obtained after incubation with test protocols. These cells acquired a round or polygonal shape, cytoplasmic granular inclusions suggesting an enhanced content of organites and a nucleus containing prominent nucleolus. No changes were observed in cells incubated with control protocol (without OSM). By comparison, morphology of freshly isolated human hepatocytes is provided. Magnifications are indicated in the figure.

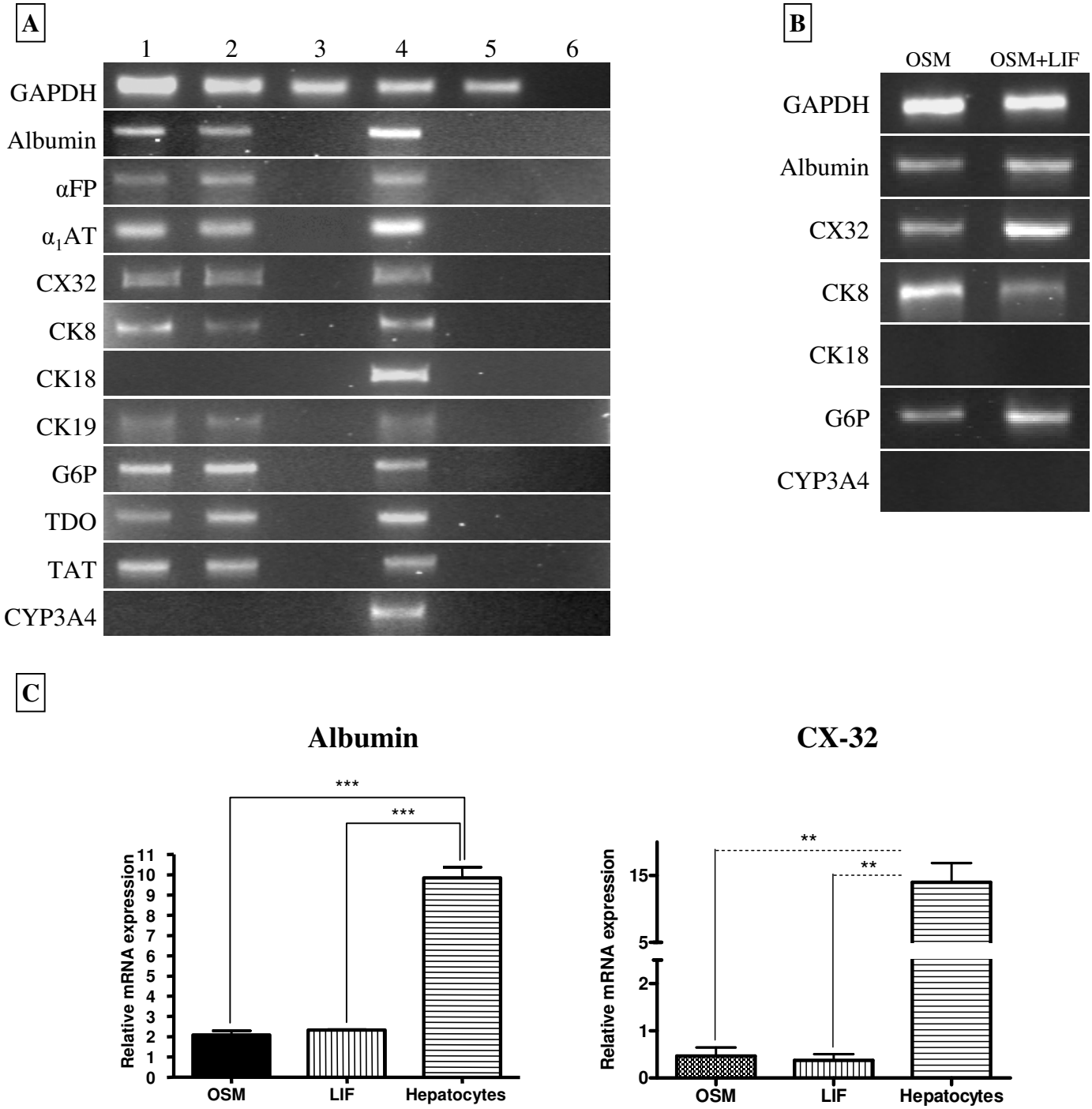



**Figure 3: Compared hepatic-specific gene expression analysis.** (A): RT-PCR analysis showing comparative expression profile of hepatic markers in OSM- and LIF-matured MDHLCs (respectively 1 and 2), control group without OSM (3), freshly isolated liver cell suspension (4) and native MSCs (5). The assay reveals in test groups (1 & 2) a comparable co-expression of early (αFP, albumin, CK8, CK19) and late hepatocyte markers ($\alpha_1$AT, CX32, G6Pase, TDO, TAT) while other markers of mature hepatocytes are lacking (CK18, CYP3A4). No expression of hepatic markers could be observed in the control group (3) or in MSCs (5). Controls without reverse transcriptase are shown for each analyzed primer (6). (B): Comparison of the expression of albumin, CX32, CK8, CK18, G6Pase and CYP3A4 in OSM- and OSM+LIF-matured MDHLCs showing a comparable profile. Abbreviations of oligonucleotides are listed in table 1. (C): Graphs of qPCR experiments showing similar normalized expression of albumin and CX-32 by OSM- and LIF-matured MDHLCs. By comparison, human hepatocytes expressed only a 5-fold higher level of albumin whereas their CX-32 expression was about 35-fold greater. Data represent means ± SEM of three independent experiments. P values are indicated in the graphs (*$P$<0.05, **$P$<0.01, ***$P$<0.001).

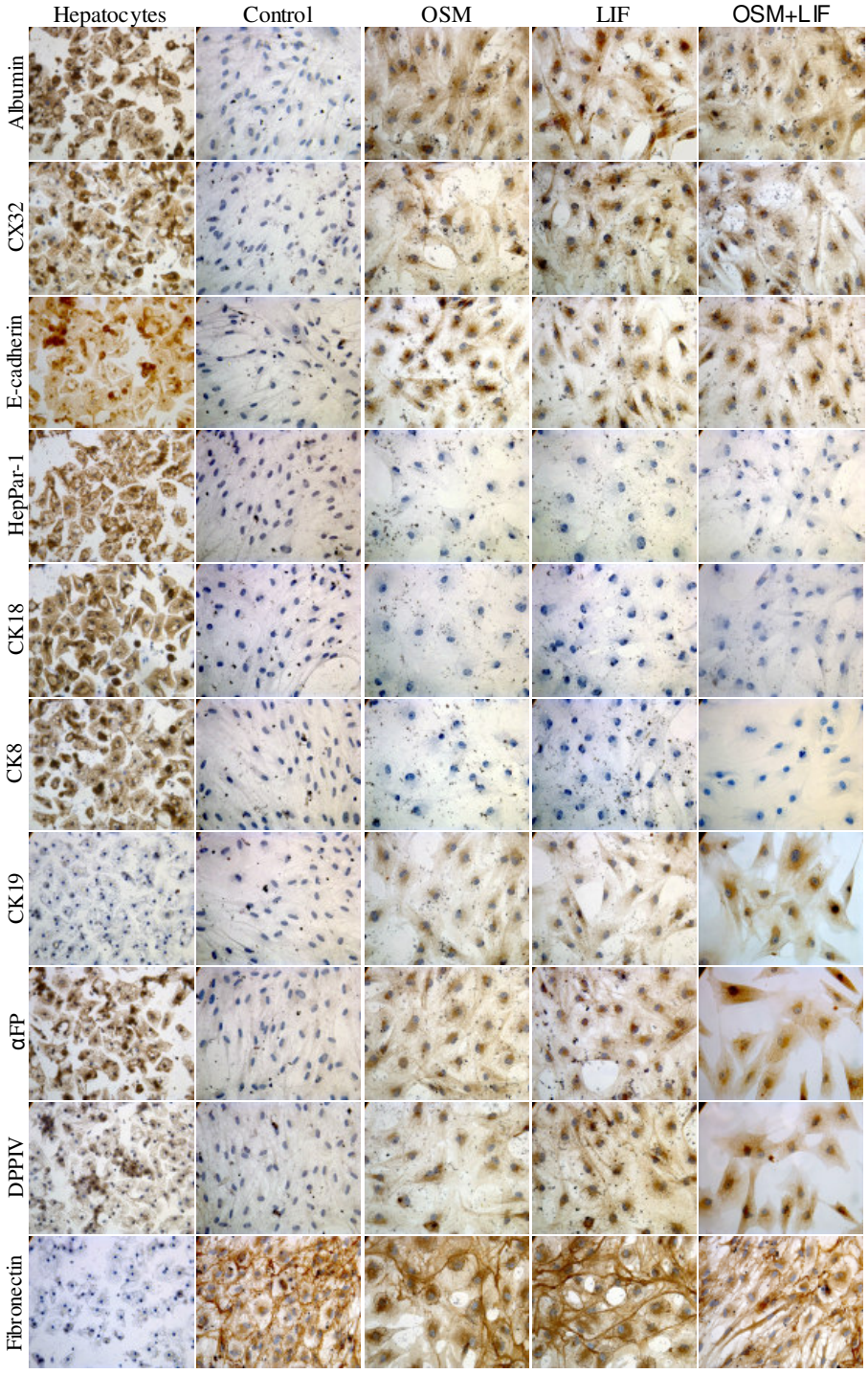



**Figure 4: Analysis of hepatocyte-specific markers expression by immunocytochemistry.** (A): Immunocytochemistry assay showing hepatocyte-specific protein expression in MDHLCs induced with test protocols or in MSCs exposed to control medium, compared with expression profile of freshly isolated human hepatocytes. Pictures show that OSM- and LIF-matured MDHLCs are positively stained for hepatocyte markers albumin, αFP, DPPIV, CX32, E-cadherin, slightly stained for biliary marker CK19 and are positive for mesodermal marker fibronectin. No expression of epithelial markers HepPar-1, CK8 and CK18 could be observed in these cells. Similar expression profile was observed in OSM+LIF-matured MDHLCs. MSCs exposed to control protocol were negative for all hepatocyte markers but stained for fibronectin. Hepatocytes showed an overall positivity except for CK19 and fibronectin. Data are representative of three independent experiments. All pictures were taken at magnification ×400. Data are representative of three independent experiments. Pictures are shown at magnification ×400.

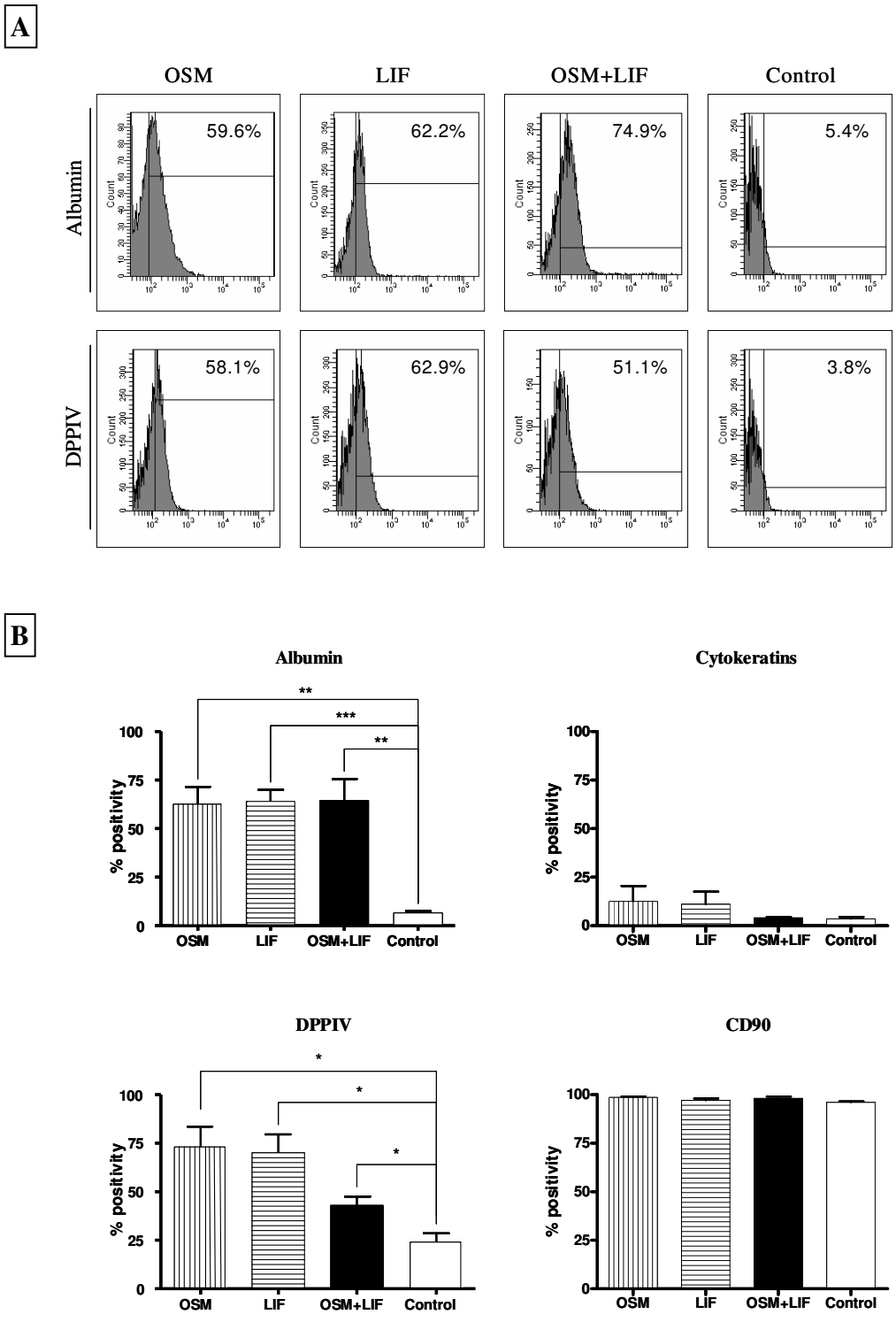



**Figure 5: Comparative flow cytometric analysis of hepatic and mesenchymal marker expression.** (A): Histograms showing representative stains for albumin and DPPIV (CD26) in test and control groups. Numbers indicate staining values of the exposed histograms. Bars represent fluorescence level of corresponding isotype. (B): Graphs showing the dispersion of values obtained after staining for albumin, cytokeratins, DPPIV (CD26) and CD90 in test and control groups. P values are indicated in the graphs (*$P$<0.05, **$P$<0.01, ***$P$<0.001).

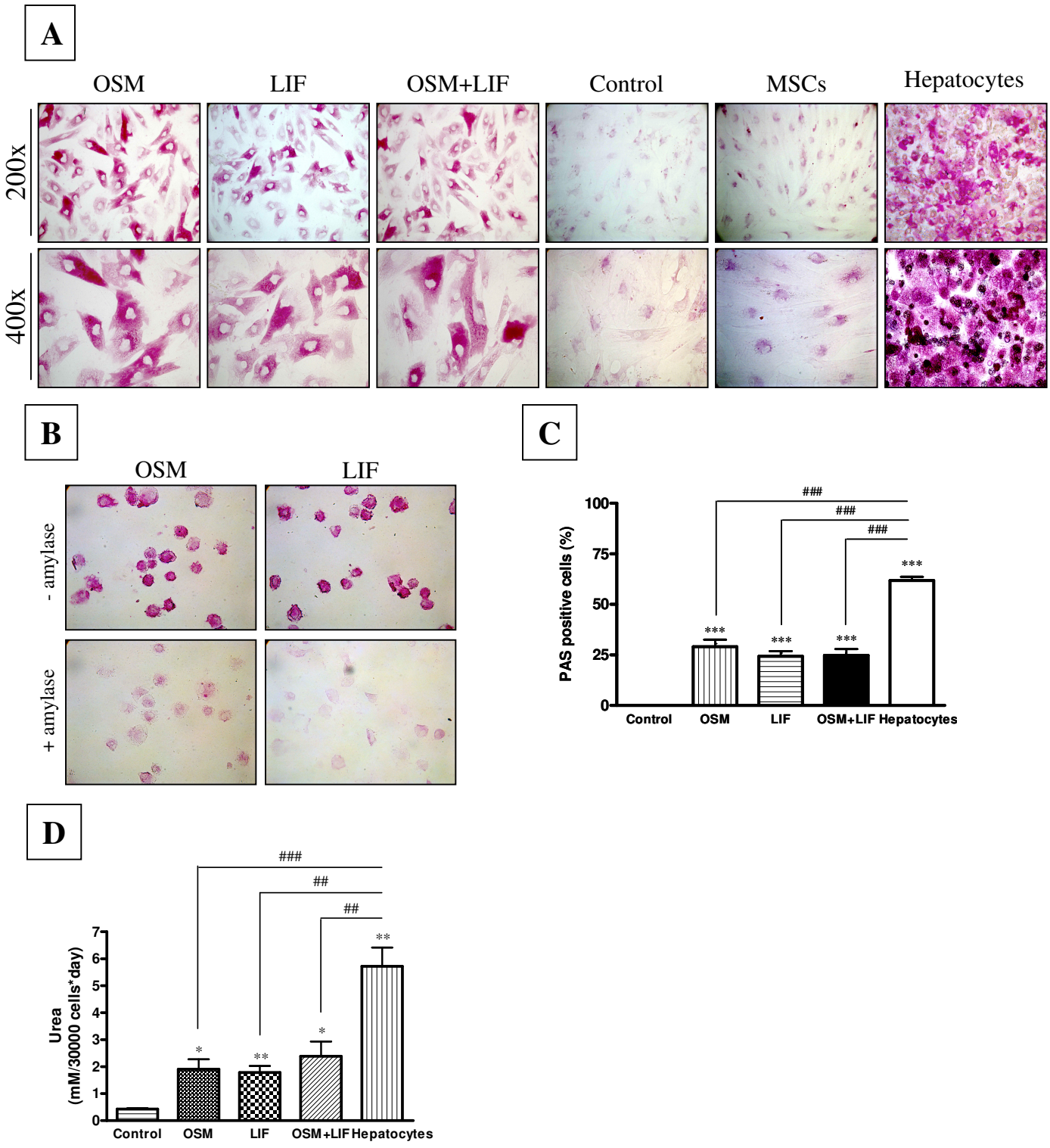



**Figure 6: Comparative analysis of hepatocyte-like functionality.** (A): PAS assays showing glycogen-positive cells in test groups, as compared to staining levels of control groups (control medium without OSM and MSCs) or freshly isolated primary human hepatocytes. Magnifications are indicated in the figure. (B): Pictures showing specific inhibition of PAS staining by prior incubation of cells with amylase in cytospined OSM- and LIF-matured MDHLCs. Pictures were shown at magnification ×400 (C): Levels of glycogen-positive cell content in test or control groups and in human hepatocytes. (*) compared with control whereas (#) compared with human hepatocytes. (D): Graph showing urea secretion levels in supernatants of test or control groups and of primary human hepatocytes. (*) compared with control whereas (#) compared with human hepatocytes. P values are indicated in the graphs (*$P$<0.05, **$P$<0.01, ***$P$<0.001).

## 3. Acting on differentiation cocktail

### a. Introduction

With the aim of improving MSCs' hepatocyte differentiation, we designed differentiation test protocols based on the modification of two variables: timing of factor exposure and introduction of cytokines involved in liver development or regeneration. We assumed that the limited acquisition of hepatocyte-like phenotype by MDHLCs could be related to an inadequate time-dependent exposition to factors. Based on the data obtained by Snykers et al. (121), who described an enhanced differentiation yield after sequential exposure of BM stem cells to cytokines, we elaborated a test protocol involving action of several cytokines whose contribution to the acquisition of epithelial-like phenotype has been documented. Activin A, a member of the TGFβ superfamily, is an inhibitor of hepatocyte proliferation and as such has been implicated in the regulation of their number during liver development (122). This cytokine has been recently described to be useful as an inducer of hepatocyte differentiation of ESCs (123;124). Follistatin, an activin inhibitor, has been demonstrated as a promoter of liver regeneration (125). BMP4 is involved in the early stages of the liver ontogeny (126;127) and is efficient in differentiating ESCs towards hepatocyte lineage (128). FGFs, also implicated in primitive liver development (129), have been widely used in hepatocyte differentiation protocols applied on stem or progenitor cells (for review, see (36)). This protocol was compared to another conserving a non-sequential shape.

Because, in our hands, MDHLCs did not express liver epithelium markers CK8 or CK18 at the protein level, we tested whether the exposition to keratinocyte growth factor (KGF) could induce their expression. KGF, a cytokine belonging to the family of the FGFs, is essential for the culture (130) and the regeneration (131) of keratinocytes and other cell types (132) and has been recently used in a differentiation cocktail of hBM-MSCs (133). As our MDHLCs

express CK8 but not CK18 mRNA, we evaluated the upregulation of these markers both at protein and gene expression levels, after KGF exposition.

b. Material and Methods

*Cell Differentiation*

For differentiation, hBM-MSCs were plated at 15000 cells/cm² in dishes coated with rat tail collagen type I. Cells were incubated in 1% penicillin/streptomycin (P/S) IMDM containing 50 ng/mL activin A, 50 ng/mL BMP4 (R&D Systems), 50 ng/mL aFGF, 10 ng/mL bFGF (both from Peprotech EC), 10 ng/mL FGF4, 100 ng/mL follistatin or 20 ng/mL HGF. Protocols test 1 (T1) and test 2 (T2) were designed by combination of these factors, as described in figure 8. As a reminder, induction medium was constituted of 1% P/S IMDM added with 20 ng/mL HGF, 10 ng/mL FGF4, 1% insulin-transferrin-selenium (ITS) premix and 0.61 mg/mL nicotinamide. Maturation medium consisted of 20 ng/mL OSM, 1% ITS premix and 1µM dexamethasone in same basal medium. KGF protocol was elaborated by adding 50 ng/mL KGF (Peprotech EC) to all steps of complete protocol. Complete protocol, described elsewhere (paragraph II-2), was used here as a control protocol.

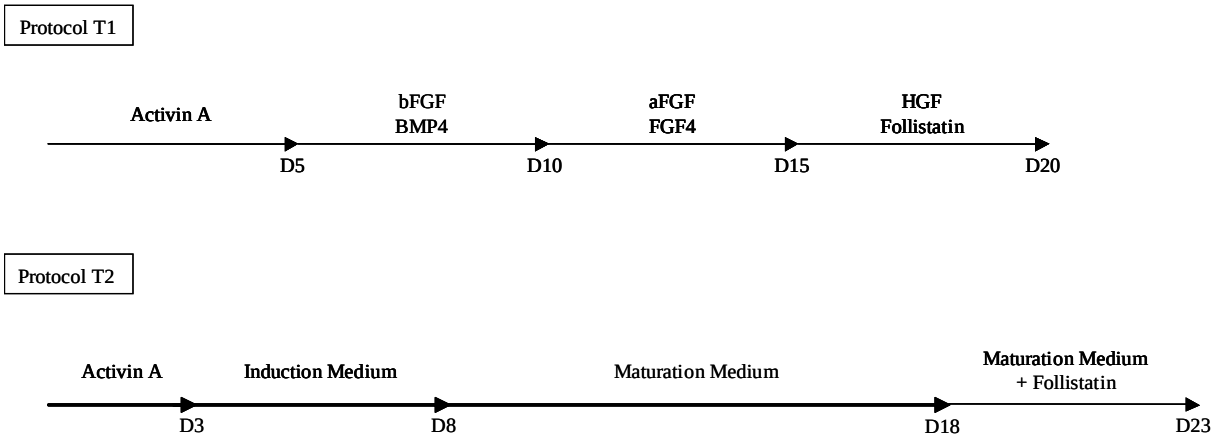



**Figure 8: Description of test protocols T1 and T2.** D, days.

*Immunocytofluorescence*

Protocol is detailed in article 1, paragraph II-2. Monoclonal antibodies were: anti-human albumin, CK8, CK18, CX-32 and HepPar-1; polyclonal antibodies were: anti-human αFP and fibronectin.

*Flow cytometry*

Protocol is described in article 1. Antibodies were: anti-human albumin and pan-cytokeratin.

*Metabolic assays*

Protocols for glycogen staining and urea secretion assays are provided in article 1.

*Reverse-Transcription Polymerase Chain Reaction Assays*

Reverse-Transcription Polymerase Chain Reaction (RT-PCR) protocol is detailed in article 1. Real-time quantitative polymerase chain reaction (qPCR) protocol is described in article 2, chapter III, paragraph 2. Human-specific primers used were as follows: CK18 (S: 5'-cgcctggcctcttacct-3', AS: 5'-cccggattttgctctcc-3'), GAPDH (S: 5'-atgcccccatgttcgtc-3', AS: 5'- tggtgcaggaggcattg-3'). The qPCR reactions were performed in triplicate for each gene. Negative controls were performed as no template cDNA-reactions and melting curves were used to confirm the results. The results were normalized using the GAPDH concentrations of each sample.

c. Results

We first analyzed the morphology of cells obtained after incubation with control and T1, T2 or KGF test protocols (Fig. 9). MDHLCs of the control group showed typical hepatocyte-like morphology defined as round or polygonal shaped cells reduced in size, containing

cytoplasmic granulations and a central nucleus with prominent nucleolus. By comparison, MDHLCs of T2 and KGF groups showed more disparate morphology with higher content of spindle-shaped cells. No cells presenting hepatocyte-like morphology were noticed in T1 group.

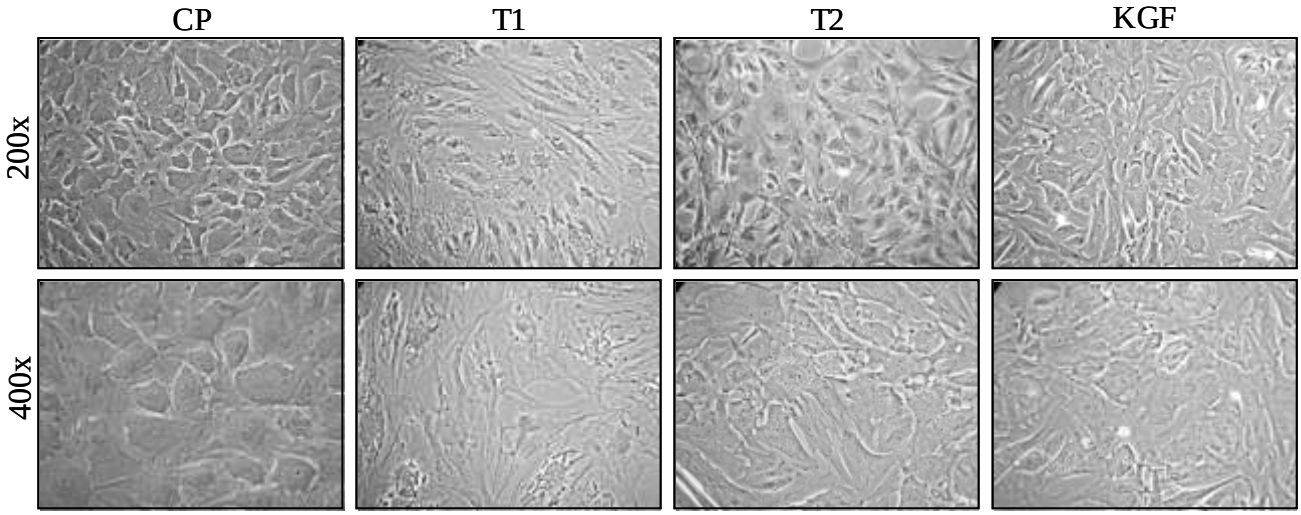
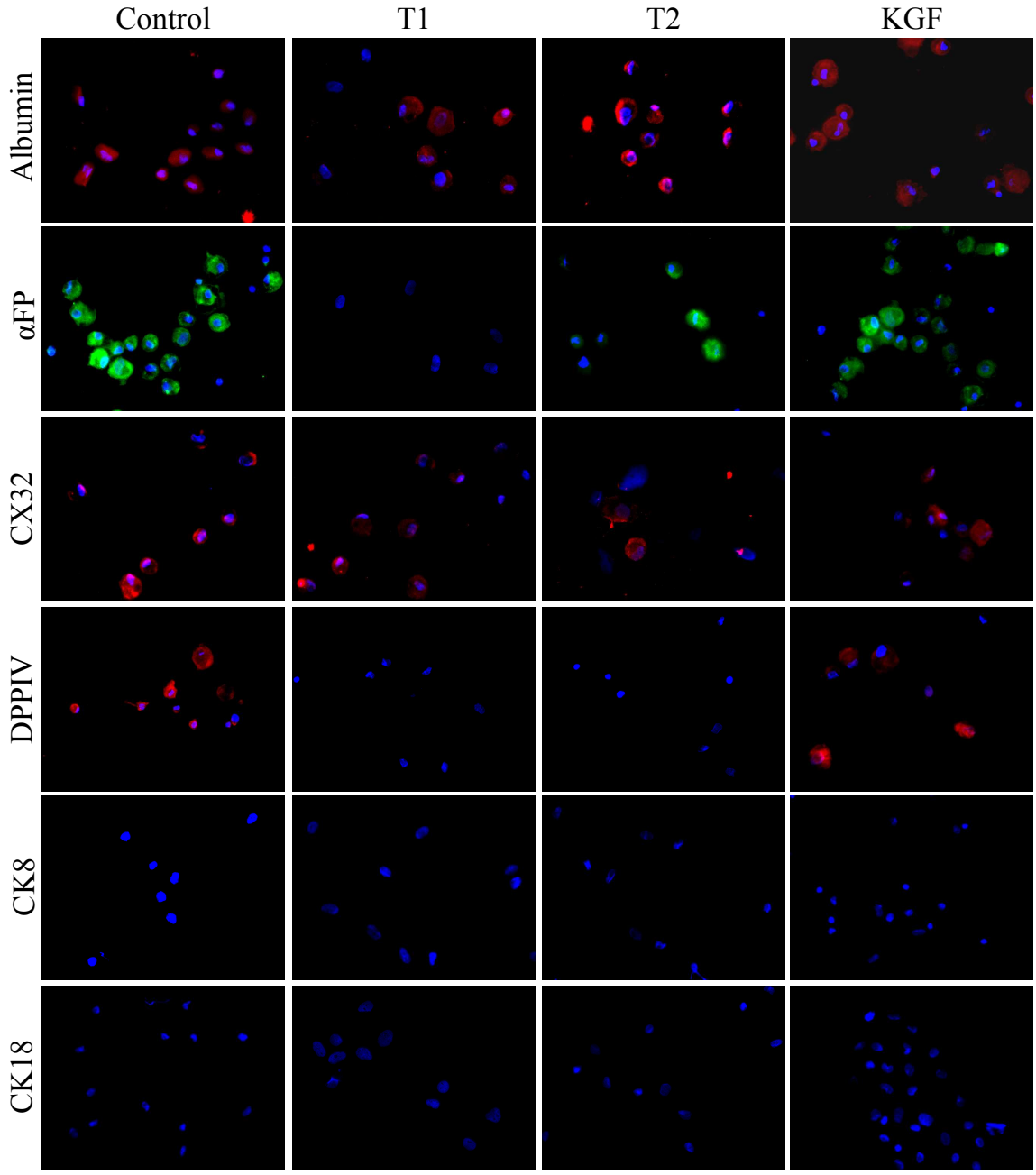



**Figure 9: Morphology of MDHLCs obtained after completion of complete, T1, T2 and KGF protocols.** Pictures show a heterogeneous cell population obtained after incubation of MSCs with T2 and KGF protocols compared to typical hepatocyte-like morphology of MDHLCs obtained after complete protocol (CP) incubation. No cells exhibiting hepatocyte-like morphology were noticed in the T1 group. Magnifications are provided.

Examination of hepatocyte-specific protein expression (Fig. 10) confirmed the profile of control MDHLCs that stained for albumin, αFP, CX-32, DPPIV but not for CK8 and CK18. Comparable expression profile was noticed in KGF group with a lesser level in the overall stains. In T2 group, DPPIV staining was absent while T1 group lacked DPPIV and αFP expression. Except for albumin in T2 group, staining level was markedly decreased in both T1 and T2 groups compared to control MDHLCs.

We demonstrated a similar albumin expression in control, T2 and KGF groups (62.2 ± 9.3%, 59.4 ± 2.7% and 60.3 ± 17.3% respectively) using flow cytometry (Fig. 11A, 11B). Comparatively, albumin staining of cells of T1 group was evaluated at 32.7 ± 1.7%.



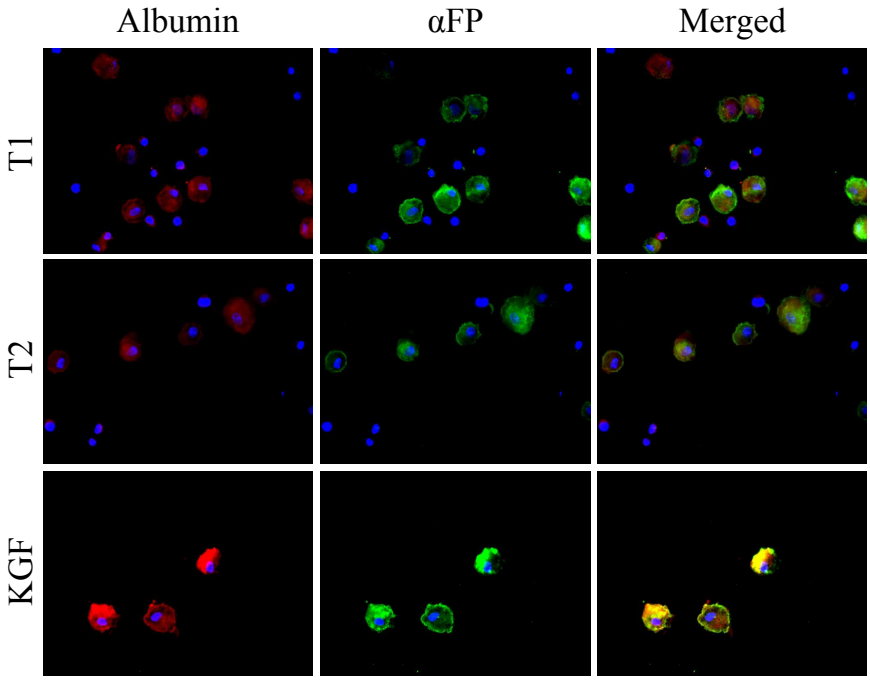




**Figure 10: Immunocytochemistry of MDHLCs obtained after completion of complete, T1, T2 and KGF protocols.** Pictures were taken at magnification ×300.

No significant difference could be observed in the expression of cytokeratins in any differentiation group (10.1 ± 9.4% for complete protocol, 6.7 ± 0.6% for T1, 5.4 ± 1.2% for T2 and 5.8 ± 2.6% for KGF) (Fig. 11A-11B). As a control, HepG2 cells submitted or not to a 72h-exposition to KGF were analyzed by flow cytometry and showed a significant increase in their expression of cytokeratins growing from 77.6 ± 7.1% to 93.3 ± 4.4% positivity before and after KGF induction, respectively ($P<0.01$, $n=5$) (Fig. 11C).

We observed a slight neo-expression of CK18 in KGF-induced MDHLCs compared to control MDHLCs whereas in both cell types we detected CK8 mRNA (Fig. 12). We confirmed by qPCR experiments a slight enhancement of CK18 mRNA concentration in the KGF-induced group compared to control group or to HepG2 cells (respectively 0.42 ± 0.03, 0.32 ± 0.02 and 1.48 ± 0.05 CK18/GAPDH concentrations).

MDHLCs obtained after exposition to control, T2 and KGF protocols were able to store glycogen whereas proportion of positive cells was significantly higher in control than in other groups (Fig. 13A-13B). Cells of these groups produced also similar amounts of urea in their supernatants (Fig. 13B). Inversely, neither glycogen-positive cells nor urea secretion could be detected in cells of T1 group.

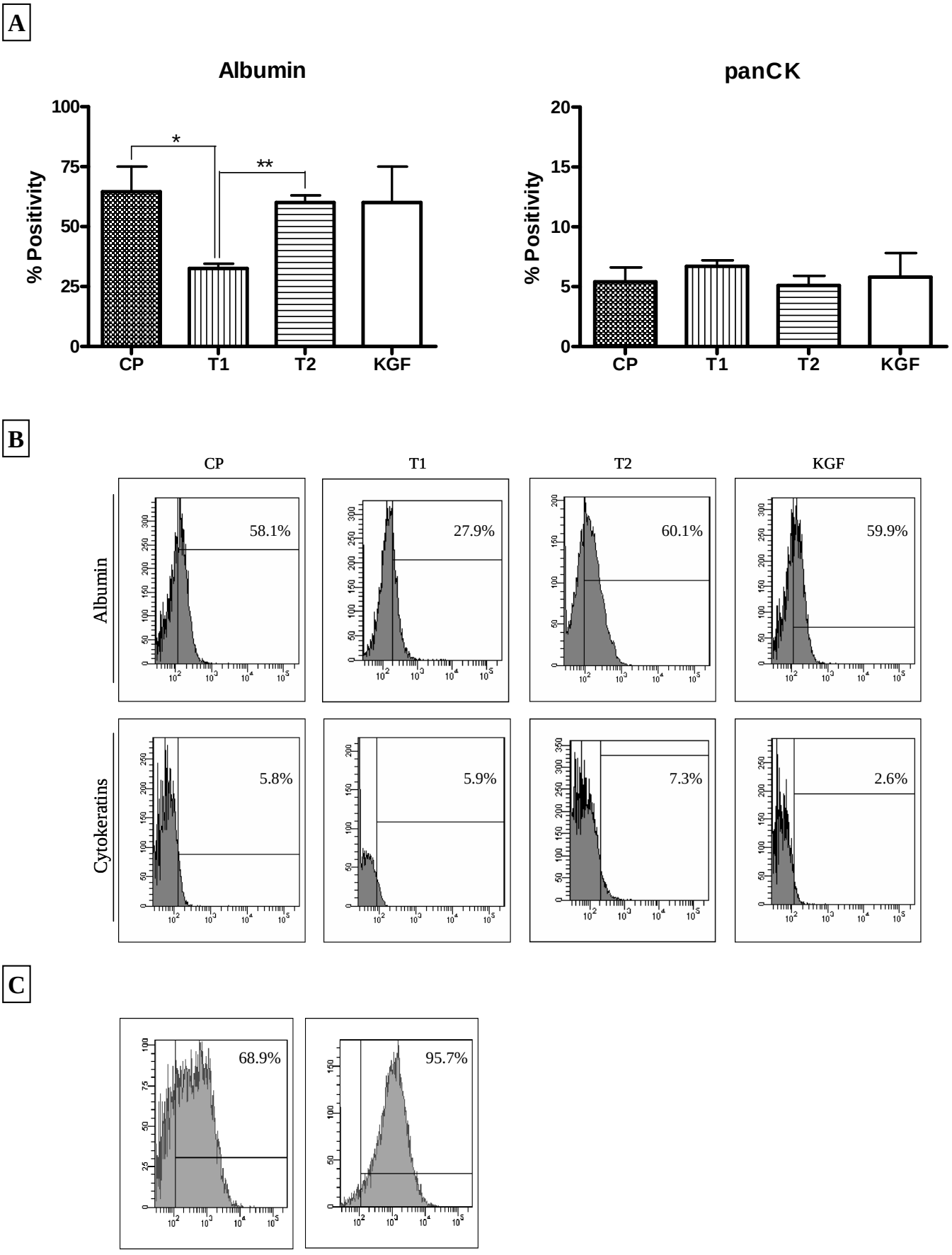



**Figure 11: Flow cytometric expression profile of control and test protocols.** (A): Comparative dispersion values of albumin and cytokeratin staining. P values are indicated in the graphs (**P*<0.05, ***P*<0.01). (B): Representative example of staining for albumin and cytokeratin. Values of the exposed histograms are indicated. Bars represent level of corresponding control isotype. (C): Staining pattern for cytokeratin in HepG2 cells before (left) and after (right) a 72h-exposition of KGF. Bars indicate level of control isotype. CP, complete protocol; T, test protocol.

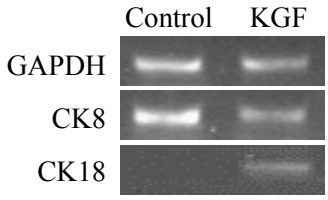
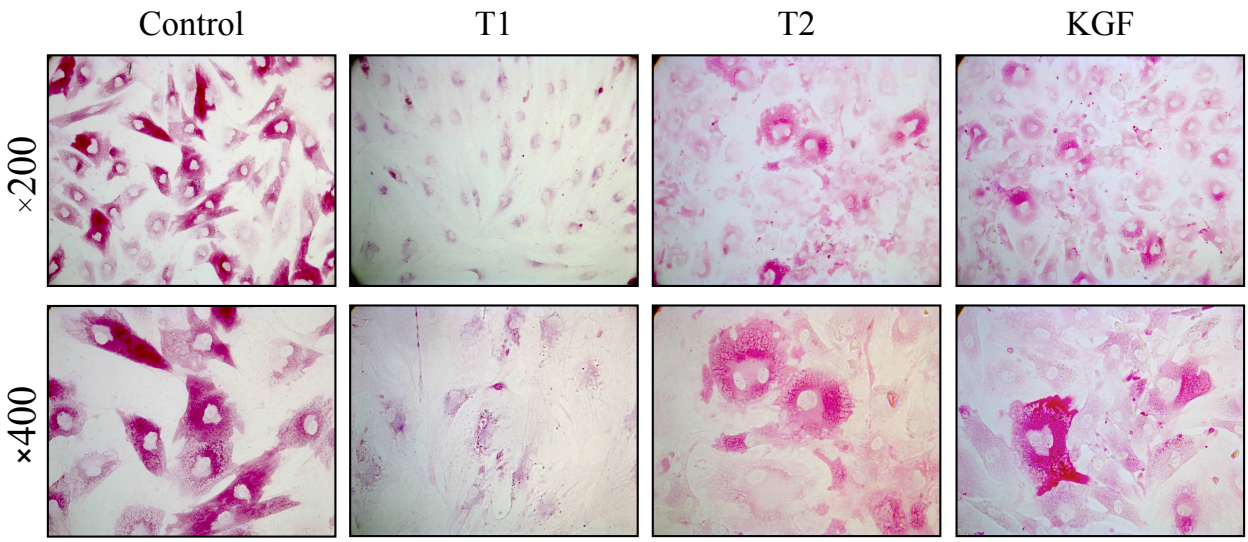



**Figure 12: Comparison of CK8 and CK18 expression in control and KGF-induced MDHLCs by RT-PCR.**

**A**




**B**

| | Control | T2 | KGF |
|---|---|---|---|
| PAS-positive cells *(%)* | 29.1 ± 3.4% | 13.4 ± 1.2% * | 14.6 ± 1.0% * |
| Urea secretion amounts *(mM/30000cells*day)* | 1.9 ± 0.4 | 1.7 ± 1.9 | 1.6 ± 0.4 |

**Figure 13: Glycogen synthesis and urea production of control and test groups.** (A): Pictures show glycogen storage in MDHLCs obtained after control, T2 and KGF protocols. Compared T1 group did not exhibit any glycogen-positive cells. Magnifications are provided. (B): Values of glycogen-positive cells and amounts of urea secretion as observed in MDHLCs of control, T2 and KGF groups. (*) compared with control group with a $P$ value < 0.05.

Other protocols (identified A to D) were designed (Fig. 14) and analyzed on morphological and protein expression levels (Figs. 15 and 16). MDHLCs obtained after incubation with protocols A to D showed a similar acquisition of hepatocyte-like morphology than control MDHLCs (Fig. 15). Test protocols-induced MDHLCs were all able to express human albumin, αFP and CX-32 but not HepPar-1 (Fig. 16A). However, control MDHLCs showed highest amounts of expression as confirmed by cell counting (Fig. 16B). Furthermore, groups C and D had globally a higher content of positive cells than group A, except for CX-32 staining in group D. Analysis of albumin staining by flow cytometry confirmed these data by showing highest and lowest positivity rates respectively in control group and in group A (Fig. 16C).

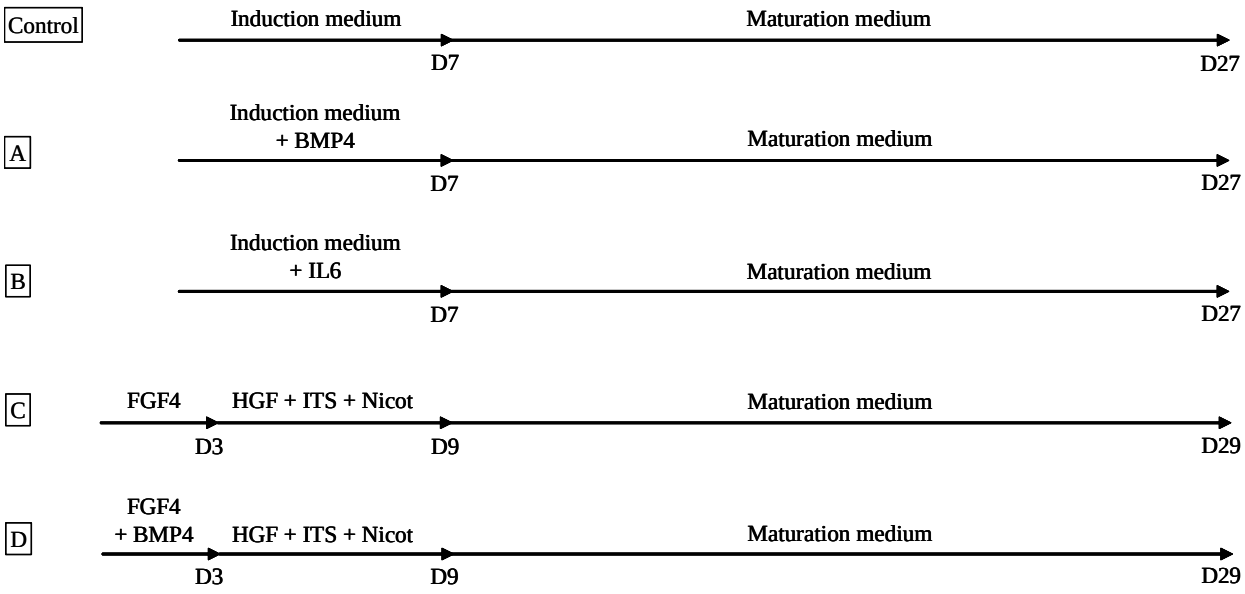



**Figure 14: Description of test protocols A, B, C and D.** D, days.

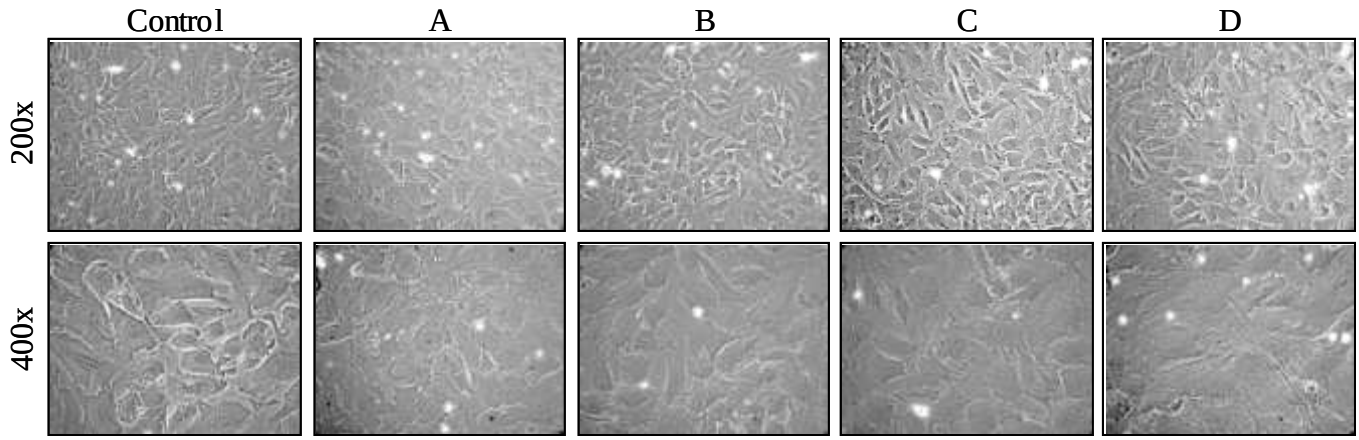
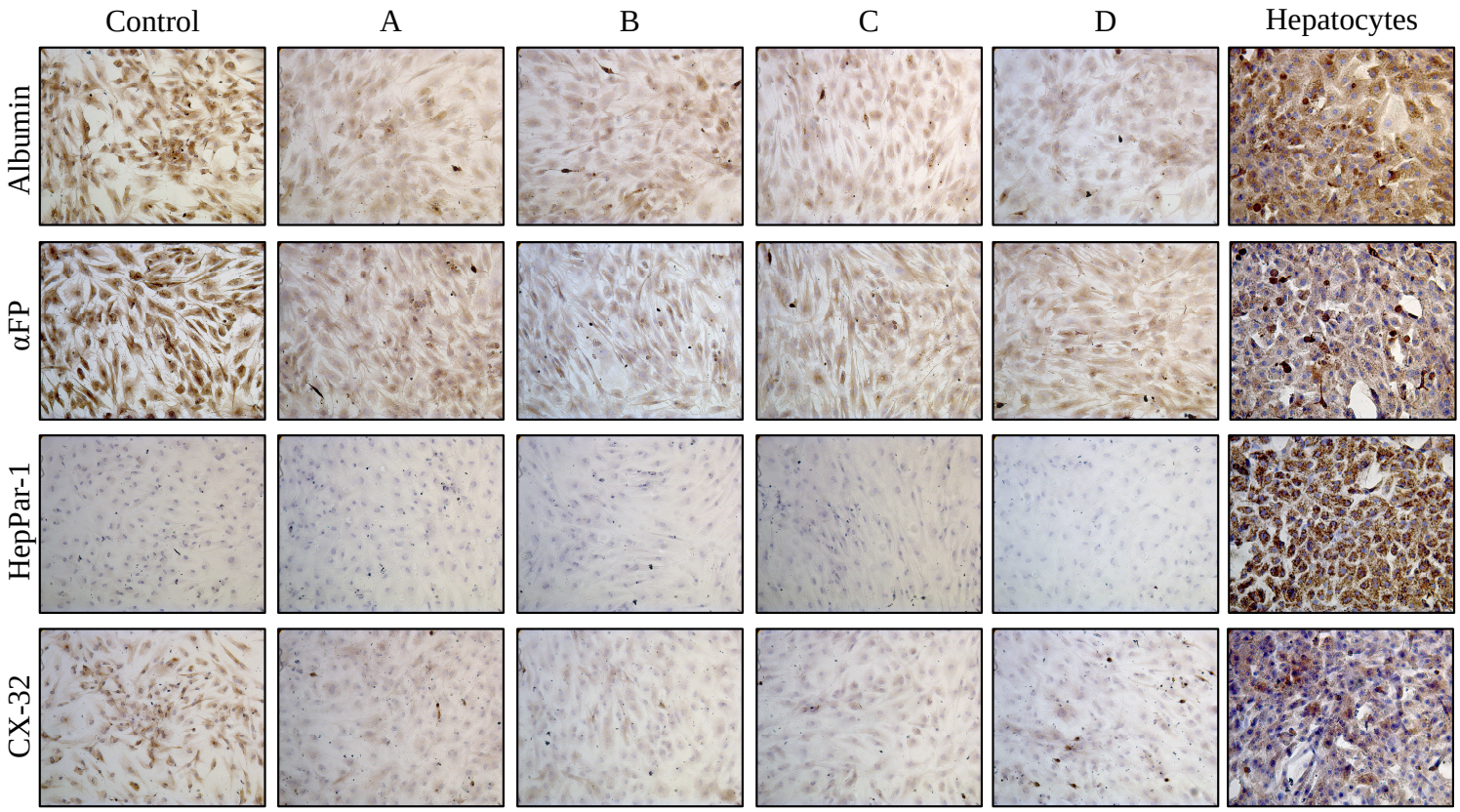



**Figure 15: Morphology of MDHLCs obtained after incubation with test protocols A-D.** Similar morphological changes occurred in MDHLCs of any test conditions compared to control MDHLCs. Picture magnifications are indicated.

**A**




**B**

| | Control | A | B | C | D |
|---|---|---|---|---|---|
| Albumin | 67.1 ± 0.07 | 41.7 ± 0.05 ** | 50.0 ± 0.07 * | 55.8 ± 0.01 * # | 54.1 ± 0.02 * # |
| αFP | 87.2 ± 0.01 | 45.7 ± 0.04 *** | 51.2 ± 0.04 *** | 62.5 ± 0.05 ** # | 71.3 ± 0.05 * ## ‡ |
| CX-32 | 37.6 ± 0.02 | 12.5 ± 0.02 *** | 20.5 ± 0.06 * | 29.5 ± 0.07 # | 31.0 ± 0.16 |

**C**

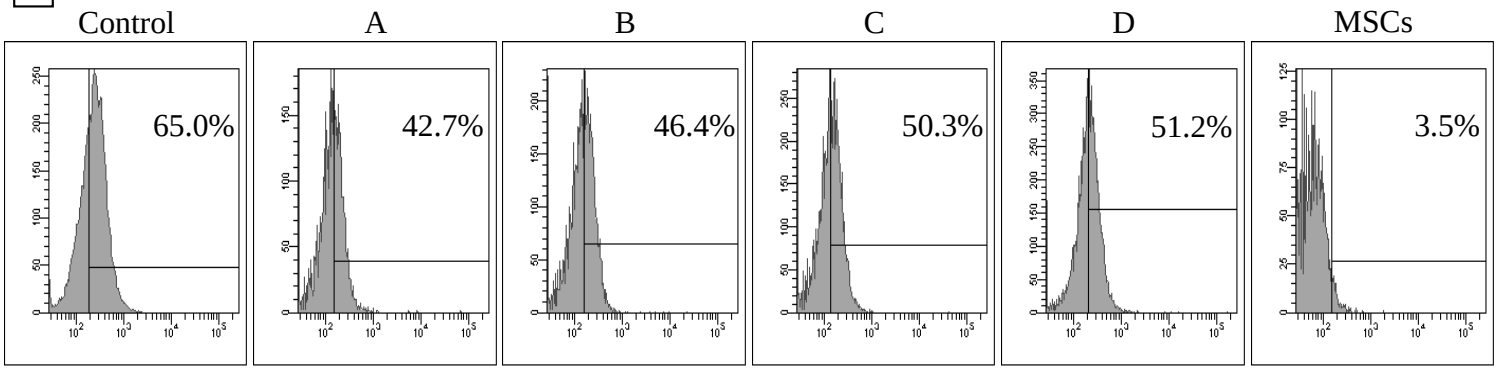



**Figure 16: Analysis of hepatocyte-specific protein expression in MDHLCs obtained after incubation with protocols A-D.** (A): Expression of human albumin, αFP, HepPar-1 and CX-32 in test protocol-induced MDHLCs, compared with expression profile of control MDHLCs and human primary hepatocytes. Pictures were taken at magnification ×200 except albumin staining of hepatocytes taken at 400×. (B): Dispersion values of albumin, αFP and CX-32 staining in control and test protocol-induced MDHLCs. P values are indicated in the graphs (*$P$<0.05, **$P$<0.01, ***$P$<0.001). (*) compared with control, (#) compared with group A and (‡) compared with group B. (C): Flow cytometric albumin staining analysis on control and test protocol-induced MDHLCs, compared to MSCs. Staining values are indicated in histograms. Bars indicate fluorescence level of corresponding isotype.

d. Conclusion

Using sequential exposures to factors supposed to mimic liver ontogeny (protocols T1, C and D), we were not able to improve hepatocyte differentiation efficacy of MSCs, but rather observed a decrease in their acquisition of hepatocyte-like features. These results were in contradiction with the work of Snykers et al. (121), although we designed additional sequential protocols. Low efficiency of sequential exposures could be linked to the duration of incubation with each cytokine, leading to protocols of 20 days for T1 and 23 days for T2, which are shorter than complete protocol (minimum 27 days). Another explanation is a lack of factors indispensable for differentiation to occur, as OSM in protocol T1. This was further illustrated when we omitted OSM from complete protocol (see paragraph III-2). Based on this assumption, we designed procedures whose complete protocol was added with cytokines such as BMP4, IL6 or others described in paragraph II-2. However, any of these protocols increased differentiation yield but were rather less efficient than related sequential exposure (cfr protocol A *versus* D). This might reflect deleterious effect of such cytokines whose use should be restricted in time. Protocol T2 was designed as a compromise between a sequential and a 2-step exposure by adding activin A before induction step and follistatin at the end of maturation step. Again, whereas these cytokines were described to play a role in liver parenchyma development, their supplementation in our complete protocol decreased its efficiency.

A recent brief report showed CK18 mRNA expression in MSCs exposed to HGF, EGF, insulin growth factor-2 and KGF, while authors did not demonstrate the acquisition of a corresponding protein expression or of an epithelial-like morphology (133). In our work, KGF exposition did not induce protein expression of either CK8 or CK18 in MDHLCs. However, we showed that this cytokine induced CK18 mRNA expression in these cells, and confirmed this induction by qPCR. This suggests the opportunity to improve, at least partially, the

epithelial-like phenotype of MDHLCs. However, this was not associated with an enhancement of hepatocyte-like functionality in MDHLCs as demonstrated by glycogen or urea assays.

The data exposed here suggest that, in our hands, MDHLCs are limited in their acquisition of hepatocyte-like phenotype and functionality, and that this limitation is difficult to improve by acting on differentiation cocktail. Based on this assumption, we wondered if specific actions on metabolic pathways could improve MDHLCs' functionality.

## 4. Methods for improvement of hepatocyte-like functionality

### a. Introduction

Urea cycle functionality has been studied in stem cells-derived hepatocyte-like cells while quantification was variously presented rendering comparison perilous (Table 8). Globally, urea secretion levels remained low and, in our hands, were always inferior to these of mature hepatocytes. It has been demonstrated that rats could increase their excretion of urea after ingestion of $NH_4Cl$ (134) whereas rat livers perfused with $NH_4Cl$ solution had an enhanced ureagenesis (135), suggesting a stimulation of urea cycle activity. We therefore tested the influence of graded concentrations of $NH_4Cl$ on urea cycle of MDHLCs.

We noticed previously that the gluconeogenesis activity of MDHLCs was negligible (paragraph II-2). As previous reports showed induction of PEPCK expression in primary hepatocytes by incubation with glucagon (136;137), we tested the effects of this hormone on MDHLCs' ability to synthesize glucose.

**Table 8: Representative examples of urea assays performed on stem cells-derived hepatocyte-like cells.**

| References | Cell Type | HLCs | Timing | HHs |
|---|---|---|---|---|
| (138) | Differentiated MAPCs | ~15 pg/cell/h | 25 days | ND |
| (93) | MDHLCs | ~22 pg/cell/h | 10 weeks | ND |
| (139) | NeoHep cells | 23.87 ± 9.9 µmol/day/mg protein | 15 days | 24.93 ± 9.19 µmol/day/mg protein |
| (140) | Differentiated ESCs | ~17.9 µg/48h/50000cells | 28 days | ND |
| (141) | Differentiated UCMSCs | ~0.8 mM/10000 cells/day | 22 days | ~0.85 mM/ 10000 cells/day |

HHs, human hepatocytes; HLCs, stem cells-derived hepatocyte-like cells; ND, not documented.

b. Material and Methods

*Urea assay*

Urea production was evaluated in supernatants of cultures submitted to 4 or 24 hour-incubation with 0.5, 5 and 10 mM $NH_4Cl$ (Sigma) added in serum-free culture media of freshly cultured (<24 hours) mouse hepatocytes and MDHLCs. Supernatants were centrifuged and urea concentration was measured using a colorimetric assay (Gentaur) handled as per manufacturer's instructions and analyzed on a Thermo absorbance reader. Serum-free IMDM and 10 mM $NH_4Cl$ served as negative controls.

*Gluconeogenesis assay*

Functional gluconeogenesis assay was performed on freshly isolated mouse hepatocytes from fasted mice and MDHLCs. Cells were first incubated or not with 200 nM glucagon (Glucagon Hypokit, Novo Nordisk Pharma) overnight in their corresponding medium. Insulin was removed from medium supplemented with glucagon. After 2 washes with PBS, cells were

incubated 4 hours in PBS or in a Krebs glucose-free solution (pH 7.4) containing lactate (10 mM) and pyruvate (1 mM) (Sigma). Gluconeogenesis was evaluated via glucose formation measured with glucose oxidase method as reported by Lefebvre et al. (142).

*Real-Time RT-PCR experiments*

Reat-time PCR assay is described in paragraph III-2. Primer used was PEPCK (S: 5'-cagcctgggcaacagag-3', AS: 5'-ccggggaagacaggttc-3'). Control experiments were performed using HepG2 cells.

c. Results

No significant difference could be observed in urea secretion of hepatocytes exposed 4h to graded concentrations of $NH_4Cl$ (Fig. 18A). When we increased the exposition to 24h, urea levels were significantly improved with highest values obtained after 5 mM $NH_4Cl$ induction. MDHLCs enhanced significantly their urea secretion after induction with 5 mM $NH_4Cl$, with values growing from 1.3 ± 0.1 to 2.0 ± 0.1 mM/30000 cells*day (Fig. 18B). Sensitivity of urea test was ensured by showing residual detection levels in $NH_4Cl$ control group.

Concerning gluconeogenesis, we observed by qPCR that MDHLCs significantly increased their PEPCK expression after an overnight exposition to 200 nM glucagon (0.10 ± 0.00 vs 0.08 ± 0.01 PEPCK/GAPDH concentration) (Fig. 19). By comparison, increase of PEPCK expression in HepG2 cells was not significant (0.09 vs 0.08 PEPCK/GAPDH concentration). We then measured the glucose production of MDHLCs and hepatocytes cultures before and after induction with glucagon. We observed a significant increase of glucose levels detected in the culture supernatants of MDHLCs after induction compared to non-induced MDHLCs or to MSCs. However, the increase of glucose production in primary hepatocytes after induction was not significant. No glucose could be detected in MDHLCs cultures exposed to PBS.

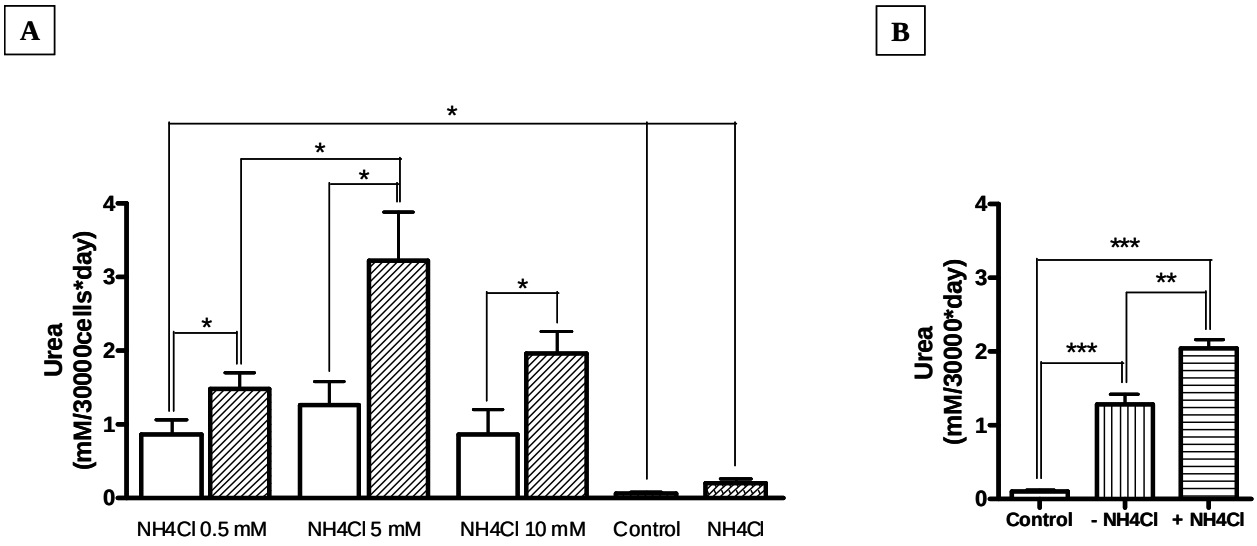



**Figure 18: Urea secretion induction tests.** (A): Values of urea secretion in mouse hepatocytes after a 4 h (white columns) or a 24 h (shaded columns) incubation with $NH_4Cl$ at indicated concentrations, compared to negative control IMDM and 10 mM $NH_4Cl$. (B): Urea secretion levels of MDHLCs with (+) or without (-) 5 mM $NH_4Cl$ 24 h-incubation, compared to control MSCs. Data represent mean of at least 3 independent experiments. P values are indicated in the graphs (**P*<0.05, ***P*<0.01).

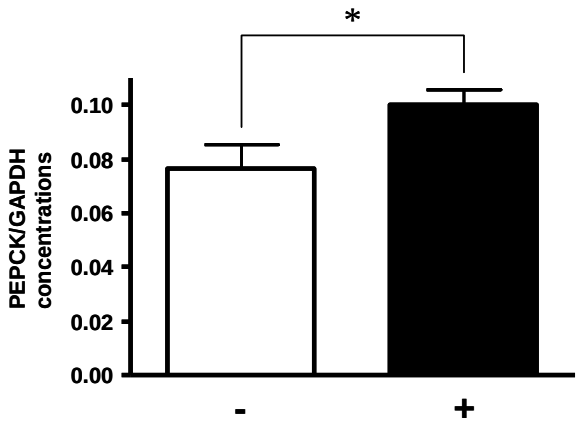



**Figure 19: PEPCK expression in MDHLCs before (-) and after (+) 200 nM glucagon overnight induction.** Data represent mean ± SEM values of 3 independent experiments. P values are indicated in the graph (**P*<0.05, ***P*<0.01).

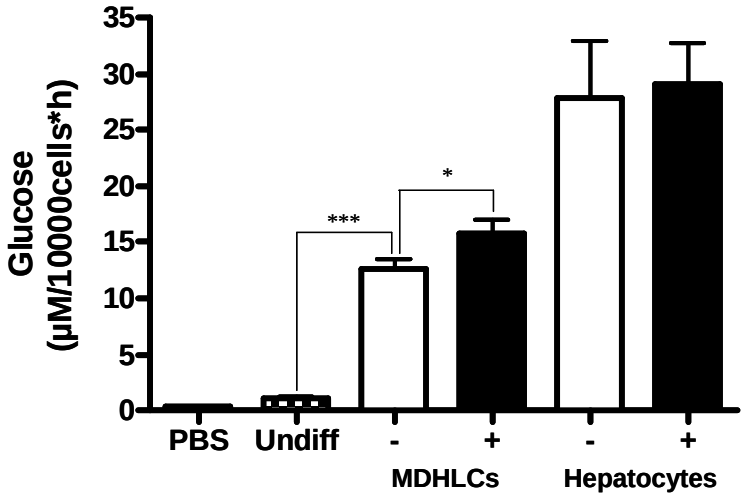



**Figure 20: Gluconeogenesis assay with or without glucagon induction.** Graph showing glucose production of MDHLCs and hepatocytes with (+) or without (-) glucagon induction, compared to glucose levels of MSC cultures or of MDHLCs incubated with PBS. Data represent mean ± SEM of 4 independent experiments. P values are indicated (**P*<0.05, ***P*<0.01, ****P*<0.001).

d. Conclusion

Here we showed that $NH_4Cl$ incubation increased urea production of MDHLCs and of hepatocytes. Best results were obtained with 5 mM $NH_4Cl$, whereas with 10 mM $NH_4Cl$, urea levels were diminished, suggesting an adverse effect on urea cycle or perhaps a global toxicity against hepatocytes. We did not observe any improvement of urea secretion after incubation with $NH_4Cl$ of less than 0.5 mM. Although our experiments showed significant impact of $NH_4Cl$ induction on urea cycle, levels of urea obtained here were comparable with other data (paragraph III-2). This was assumed to reflect culture heterogeneity, disparate differentiation potential or cell viability. With these assays, we demonstrated enhancement of urea cycle functionality but did not screen corresponding enzyme expression. Argininosuccinate synthetase has been reported to be upregulated in primary hepatocytes by incubation with glutamine (143). Whether this substrate could enhance similar enzymatic activity in MDHLCs should be tested.

Confirming previous works on human hepatocytes (137), we reported the induction of PEPCK expression after incubation with glucagon in MDHLCs and at lower level in HepG2 cells. Using gluconeogenesis assay, we previously did not detect glucose neo-formation in MDHLCs (paragraph II-2). This production appeared as we enhanced incubation time with lactate/pyruvate solution and as we removed insulin from culture medium. We observed a beneficial effect of glucagon on glucose production by MDHLCs, whereas no significant difference could be noticed after induction of mouse hepatocytes. Although qPCR experiments revealed low contents of PEPCK mRNA in all analyzed cells, relevant increase of glucose concentrations could be detected in MDHLCs. Discrepancies between mRNA levels and related protein production or function were described elsewhere (121;139;144). However, in our assays, this could reflect a positive effect on PEPCK enzyme activity. Another explanation is that, while PEPCK is the restricting enzyme of gluconeogenesis pathway, the positive influence of glucagon on G6Pase activity (145) could counterbalance low PEPCK levels.

## 5. Conclusion

Although we handled several factor combinations (*n*= 41), we did not succeed in improving differentiation yield obtained with complete protocol and this suggests three hypothesis. First, this involves that best differentiation yield can be obtained by a 2-step procedure using HGF, FGF4 and OSM as main factors. This was suggested by data obtained with protocols lacking these factors (paragraph II-2 and III-2). Second, it implies low efficiency of other factors as hepatocyte phenotype inducers, at least in the conditions we tested. Third, global point of view reveals a limitation in achieving mature hepatocyte differentiation of hBM-MSCs via *in vitro* factor exposition on 2D monolayer cultures.

In this section, we also provided evidence for a possibility to partially improve some hepatocyte-like metabolic functions in MDHLCs by adding substrate or inducing hormone in the differentiation cocktail. Nevertheless, data showed mild global functionality remaining lower than primary hepatocytes. As we do not consider ESCs, the only demonstration of a functionality similar to mature human hepatocytes was provided using NeoHep cells that showed aspartate transaminase, urea, albumin and uridine diphosphate production in comparable or even higher levels than hepatocytes (139). However, even in these conditions, authors observed a ~80 times lower CYP3A4 functionality in NeoHep cells than in human hepatocytes.

# CHAPTER IV: HEPATOCYTE DIFFERENTIATION OF HUMAN UMBILICAL MSCS

## 1. Isolation and culture of Umbilical Cord Blood-MSCs

### a. Introduction

UCB was regarded according to its easiness of harvest, absence of ethical limitations and lower risk of viral transmission than BM (146). Great enthusiasm arouses about UCB's potential for MSCs supply after pioneer works (147-149), hence this cell source was further analyzed. Besides amazing description about unexpected properties of UCB lineages (106), some authors were more prudent and proposed parameters for MSC isolation (150). Other iconoclastic reports questioned UCB as an accurate source of MSCs (151;152). However, as hepatocyte differentiation potential of UCB-MSCs has been described (91) and further confirmed (104), we handled and characterized UCB isolates.

### b. Material and Methods

Fresh (*n*= 58) and cryopreserved (*n*= 8) UCB units were collected at full term delivery with the informed consent of the mother. Samples were collected into anticoagulant-containing bags at room temperature until processing. MNCs were separated and cultured as for BM samples. Several conditions were tested for initiating MSCs' growth, as described in table 9. Midi MACS (Miltenyi) cell selections were performed as per manufacturer's instructions.

Rat UCB units (volume: 336.7 ± 7.8 µL, *n*= 27) were collected from female Wistar rats at 21-22 days gestation using 24G syringe and processed as for human UCB.

**Table 9: Description of conditions tested for initiation of UCB-MSCs growth.**

| Variables | Test conditions |
|---|---|
| Cell processing | 1 to 31 hours |
| Dishes coating | FBS, rat tail collagen type I |
| Cell selection | $CD34^-$, $CD14^-$, $CD133^+$ |
| Plating at passage 0 | 0,01 to $6 \times 10^6/cm^2$ |
| Medium | αMEM, DMEM, IMDM, William's E containing 2.5-20% FBS or cord blood serum |
| Growth factors | EGF, bFGF, dexamethasone |
| First medium change | day 1, day 3, day 7 |

c. Results

From UCB volumes of 99.7 ± 17.8 mL, we collected 88.3 ± 64.0 × $10^6$ MNCs whose immunophenotype was analyzed by flow cytometry (Table 10). Contaminating hematopoietic cells remained in first passage cultures and consisted of cells displaying typical 'fried-egg' morphology, supposed to be monocytes or granulocytes (Fig. 21A-21B), whereas some more fusiform cells were observed in these cultures, owning low proliferation rates, and assumed to belong to the endothelial lineage (Fig. 21A-21B). Cell population of first passage was analyzed by flow cytometry and contained predominantly hematopoietic cells staining at 98.8 ± 1.5 % for CD45, corresponding to a majority of granulocytes (48.1 ± 10.4% $CD31^+$ cells) and monocytes (33.5 ± 15.3% $CD14^+$ cells) (Fig. 22A-22B). Whereas CD44 and CD29 were expressed at high levels (Fig. 22A-22B), CD73 staining was low, suggesting a poor content of MSCs in these cultures. We did not succeed in removing hematopoietic cells from early passages, neither by pre-coating of culture dishes with FBS, as described elsewhere (150), nor

by magnetic beads selection of cell populations or any of test conditions applied to UCB cultures presented in table 9. Early (<4 h) isolation of UCB units enhanced hematopoietic cell content in first passage culture.

Four populations of MSCs were isolated from fresh UCB units and these cells showed typical spindle-shaped morphology (Fig. 21C-21F). Their mesenchymal origin was attested using flow cytometry demonstrating staining for CD73, CD44, CD29 but not for CD45 (Fig. 22B). These cells also expressed CD49e, CD49b and HLA-ABC but not HLA-DR. Immunocytochemical assay revealed their expression of mesodermal markers, as αSMA, fibronectin, vimentin or laminin (Fig. 21G-21J). However, these cells did not grow up to passage 3 and could not be further analyzed. We did not succeed in isolating MSCs from rat UCB.

**Table 10: Immunophenotype of UCB-MNCs.**

| Epitopes | UCB-MNCs (%) |
|---|---|
| CD45 | 96.5 ± 0.5 |
| CD3 | 20.5 ± 0.5 |
| CD19 | 27 ± 2 |
| CD14 | 16 ± 1 |
| CD34 | 2.3 ± 0.4 |
| CD44 | 91.5 ± 0.5 |
| CD73 | 7.5 ± 2.5 |

Data are expressed as mean ± SEM of 3 independent experiments.

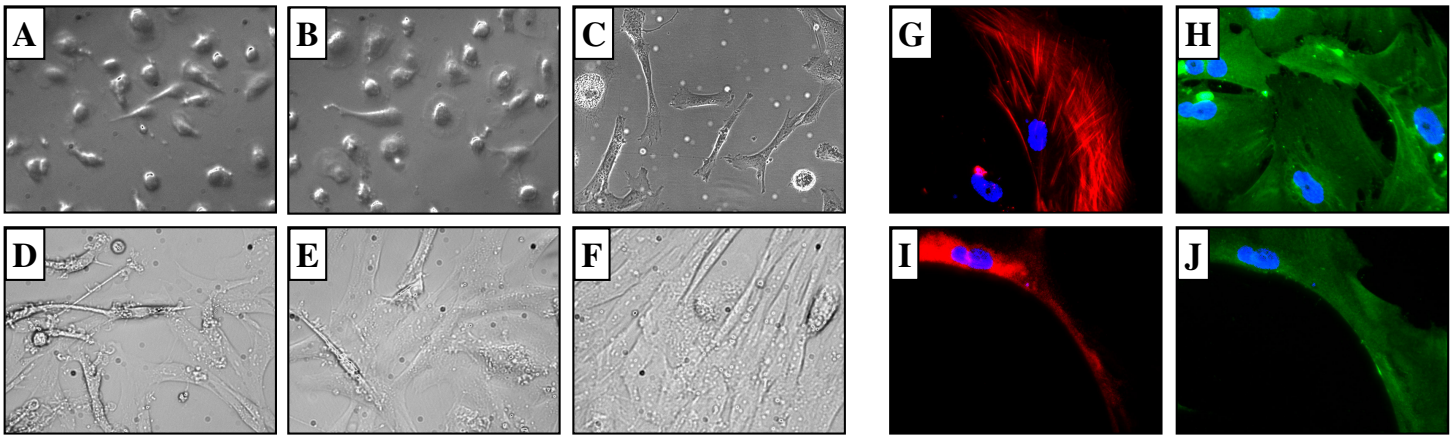



**Figure 21: Evolutive morphology of UCB cultures and mesodermal marker expression analysis of UCB-MSCs.** Morphology of UCB cultures at day 10 (A, B), day 21 (C), day 25 (D, E) and day 30 (F) showing progressive apparition of spindle-shaped cells. Immunofluorescence assay showing expression of human αSMA (G), laminin (H), vimentin (I) and fibronectin (J) in MSCs. Pictures were taken at magnification 400×.

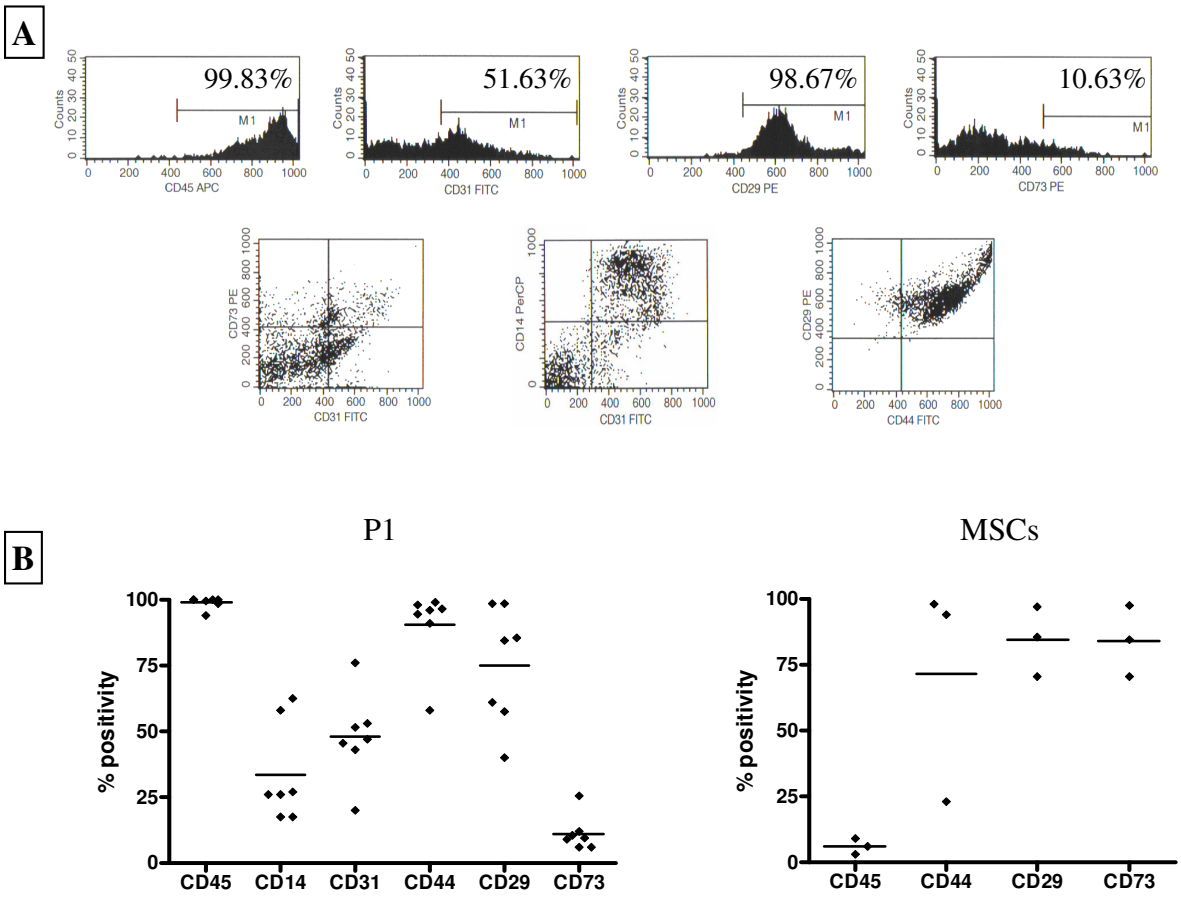



**Figure 22: Flow cytometric analysis of UCB cultures.** (A): Representative example of immunophenotype of UCB cells at first passage. Analyzed epitopes and related values are indicated in histograms. Bars represent fluorescence level of corresponding isotype. (B): Graphs showing flow cytometric expression profiles of first passage (P1) UCB cultures and of UCB-MSCs.

d. Conclusion

Our work first described the immunophenotype of UCB-MNCs which has been documented elsewhere with other markers (153). We demonstrated the difficulty to reliably obtain MSCs from UCB, with an isolation yield of 7%. This yield was parallel to some investigations describing 6% isolation rate (154;155) or isolation of 2.2% MSCs having relevant growth capacities (156). The weak growth capacity of UCB-MSCs that we observed was confirmed elsewhere (154;157). Concerning MSCs characterization, our data questioned the use of CD44 and CD29 as MSC markers as their expression remained high whatever the examined population, suggesting their poor reliability. At the contrary, CD73 expression was low in MNCs but grew in MSCs, underlying its specificity. Although it was recently described (158), we could not isolate growing MSCs using magnetic bead sorting. All other tested conditions failed to improve isolation yield. Cryopreserved UCB units did not provide MSCs as reported (159). We never found MSCs after culturing rat UCB-MNCs, although it was described by Naruse et al. (160), which is the only related report in the literature, to our knowledge.

It arises from data comparing characteristics of MSCs isolated from various tissue sources (148;152;161-163) that UCB-MSCs own the lowest isolation yield and differentiation potential. As umbilical cord was recently described as an accurate source of expandable MSCs owning differentiation capacities, we handled this tissue source for hepatocyte differentiation.

## 2. Hepatocyte Differentiation of Umbilical Cord Matrix Stem Cells

a. Article 3: Native Umbilical Cord Matrix Stem Cells express hepatic markers and differentiate into hepatocyte-like cells. *Gastroenterology* 2008, in press.

# Native Umbilical Cord Matrix Stem Cells Express Hepatic Markers and Differentiate Into Hepatocyte-like Cells

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**See editorial on page 878.**

***Background & Aims:*** **Umbilical cord matrix stem cells (UCMSCs) are able to differentiate into mesodermal and ectodermal lineages. The present study investigates the differentiation potential of human UCMSCs into hepatic lineage.** ***Methods:*** **We isolated human UCMSCs and characterized them in vitro by measuring their expansion potential, by assessing expression of mesenchymal stem cell (MSC) markers, and by evaluating their ability to differentiate into adipocytes and osteocytes. UCMSCs were thereafter subjected to a hepatogenic differentiation protocol. Expression of hepatic and MSC markers in differentiated cells was analyzed by reverse-transcription polymerase chain reaction, flow cytometry, and immunocytochemical assays and compared with undifferentiated UCMSCs and freshly isolated liver cells. UCMSCs were transplanted into livers of hepatectomized-SCID mice, and engraftment capacity was investigated by detection of human nucleus and mitochondria and human hepatic-specific proteins.** ***Results:*** **In vitro expanded UCMSCs constitutively expressed markers of hepatic lineage, including albumin, α-fetoprotein, cytokeratin-19, connexin-32, and dipeptidyl peptidase IV. In vitro-differentiated UCMSCs exhibited hepatocyte-like morphology, upregulated several hepatic markers, stored glycogen, produced urea, and exhibited an inducible CYP 3A4 activity. However, absence of some hepatic markers in differentiated UCMSCs, as HepPar1 or hepatocyte nuclear factor 4 (HNF-4), implied that their differentiation did not reach the level of mature hepatocytes. We also noticed that differentiated UCMSCs partially preserved MSC markers. Engraftment capacity of UCMSCs was observed, and expression of human albumin and α-fetoprotein was detected 2, 4, and 6 weeks after transplantation in mice livers, while cytokeratin 19 was completely down-regulated.** ***Conclusions:*** **We conclude that UCMSCs, with a newly demonstrated endodermic differentiation potential, might be an alternative source for liver-directed cell therapies.**

Liver cell transplantation is an emerging technique next to whole organ transplantation for the treatment of liver-based inborn errors of metabolism.[1–6] However, organ shortage remains a major limiting step of this procedure. More recent developments in stem cell technology have brought hope to identify new expandable sources of liver cells to be used for regenerative medicine. Embryonic stem cells can be considered the best model of multipotency, but their use remains subject to moral/ethical debates as well as safety concerns.[7] Adult stem cells are largely explored as a more acceptable source of cells. Recently we showed that stem-like cells isolated from the adult liver can differentiate into hepatocytes, although this source is also dependent on organ availability.[8,9] Several investigators have demonstrated the capacity of mesodermal cells such as multipotent adult progenitor cells,[10] mesenchymal stem cells (MSCs),[11,12] and fibroblasts[13] to differentiate into cells with features of hepatocytes, and the majority was isolated from bone marrow. However, the technique of bone marrow collection remains invasive and is not exempt of risks, even more so as bone marrow/MSCs are more easily obtained from young donors.[11] MSCs with hepatogenic capacities have also been isolated from umbilical cord blood,[14,15] but the yield of isolated cells is highly dependent on blood quality and remains limited compared with bone marrow.[16] Recently a bundle of data focused on a new promising source of MSCs derived from the matrix of the umbilical cord (Wharton's jelly).[17–23] Multipotency of umbilical cord matrix stem cells (UCMSCs) has widely been shown by conventional in vitro assays, inducing differentiation into adipocytes, osteocytes, and chondrocytes.[19,22] Furthermore, UCMSCs are also able to differentiate into neurons[19,20,22,24] and cardiomyocytes.[22,25,26] Moreover, in vivo experiments showed that transplanted

*Abbreviations used in this paper:* APC, Allophycocyanine; α1AT, $\alpha_1$-antitrypsin; CK, cytokeratin; CYP, cytochrome P450; Cy7, Cyanin-7; DPPIV, dipeptidyl peptidase IV; FITC, fluorescein isothiocyanate; G6P, glucose-6-phosphatase; IMDM, Iscove's modified Dulbecco's medium; MSC, mesenchymal stem cell; PE, Phycoerythrin; PerCP, Peridinin-chlorophyll-protein complex; RT-PCR, reverse-transcription polymerase chain reaction; TAT, tyrosine aminotransferase; TDO, tryptophan 2,3-dioxygenase; UCMSC, umbilical cord matrix stem cell.

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UCMSCs were able to restore functional defects in rats with brain injury or retinal disease.[18,23,27] So far, differentiation of human UCMSCs into hepatic lineage has not been reported.

In the current study, we isolated MSCs from Wharton's jelly and characterized these cells for their morphology, growth potency, phenotypic profile, and capacity to differentiate into various lineages. We then evaluated the ability of UCMSCs to differentiate into hepatocytes, and differentiated cells were compared with native UCMSCs and adult human liver cells for expression of early and late hepatic markers and some hepatocyte-specific activities. The capacity of transplanted UCMSCs to engraft durably into the liver of hepatectomized SCID mice was also investigated.

## Materials and Methods

### *Isolation and Expansion of UCMSCs*

The present study was approved by the ethical committee of the Hospital and Faculty of Medicine of Université Catholique of Louvain. Umbilical cords were obtained from consenting patients delivering full-term infants (n = 15). Cords of 9.15–51.77 g were collected in 0.9% NaCl sterile solution and immediately transferred for cell isolation. Cords were cut in pieces of 3–4 cm, and sections were incised along their length to expose underlying Wharton's jelly. The umbilical vein and the 2 arteries were pulled away, and the remaining mesenchymal tissue was scraped off with a scalpel and centrifuged at 250*g* for 5 minutes at room temperature. The pellet was suspended in Hank's balanced salt solution with calcium and magnesium (Invitrogen, Merelbeke, Belgium) supplemented with 1 mg/mL collagenase type I (C-0130; Sigma, Bornem, Belgium), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin (Invitrogen) and transferred to a 75-cm$^2$ flask with ventilated cap (Greiner Bio-One, Wemmel, Belgium). Tissues were digested at 37°C and 5% $CO_2$ atmosphere for 18–24 hours. The homogenate was diluted in Dulbecco's phosphate-buffered saline (Cambrex Bio Sciences, Verviers, Belgium) and centrifuged at 600*g* for 15 minutes. The cell pellet was resuspended in expansion medium composed of Dulbecco's modified Eagle medium low glucose (1 g/L D-glucose) with sodium pyruvate and L-glutamine (Invitrogen) supplemented with 10% fetal bovine serum (A15-101; PAA Laboratories GmbH, Pasching, Austria), 100 U/mL penicillin, and 100 $\mu$g/mL streptomycin (Invitrogen). Viable cells were counted using trypan blue exclusion assay. The total number of viable isolated cells ranged between $1.8 \times 10^5$ and $3.6 \times 10^6$ (mean, $1.22 \times 10^6 \pm 1.09 \times 10^6$), and these were seeded in a single 25- or 75-cm$^2$ tissue culture flask (Greiner Bio-One) at a density of $4.8 \times 10^3$ to $1 \times 10^4$ cells/cm$^2$.

Plated cells were cultured in expansion medium at 37°C and 5% $CO_2$ in a fully humidified atmosphere. At 80%–90% of confluence, cells were detached from the flask using 0.05% trypsin/EDTA solution (Invitrogen). Cells were replated at a density of 8000 cells/cm$^2$ in expansion medium, and medium was changed twice a week.

### *Differentiation Procedures*

**Adipogenic and osteogenic differentiation.** UCMSCs at passage 2 were plated at a density of $1 \times 10^4$ cells/cm$^2$ in expansion medium containing 1 $\mu$mol/L dexamethasone, 0.5 mmol/L isobutyl methylxanthine, 0.2 mmol/L indomethacin (all from Sigma) and 10 $\mu$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.

UCMSCs at passage 2 were plated at a density of $3 \times 10^4$ cells/cm$^2$ in expansion medium containing 0.1 mol/L dexamethasone, 0.1 mmol/L ascorbate, and 10 mmol/L $\beta$-glycerophosphate (all from Sigma) with medium change twice a week. After 4 weeks, calcium deposition was evaluated by von Kossa and Alizarin Red stains. Tetracycline-labeled bone nodules were observed by inverted fluorescence microscopy (Axiovert S100; Carl Zeiss SA, Zaventem, Belgium) 1 day after refeeding cultures once with osteogenic medium containing 10 $\mu$g/mL tetracycline (7660; Sigma).

**Hepatogenic differentiation.** UCMSCs (from passage 2 to passage 6) were seeded at a density of $1.5 \times 10^4$ cells/cm$^2$ in 6- or 24-well plates coated with 5 $\mu$g/cm$^2$ of rat tail collagen type I (BD Biosciences, Bedford, MA) in Dulbecco's modified Eagle medium low glucose supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 $\mu$g/mL streptomycin. Culture medium was switched 24 hours later to Iscove's modified Dulbecco's medium (IMDM; Invitrogen) containing 20 ng/mL epidermal growth factor (Peprotech EC Ltd, London, England) and 10 ng/mL basic fibroblast growth factor (Peprotech EC Ltd) for 2 days. Thereafter, induction of differentiation was triggered by culturing UCMSCs for 10 days with IMDM containing 20 ng/mL hepatocyte growth factor (Peprotech EC Ltd), 10 ng/mL basic fibroblast growth factor, 0.61 g/L nicotinamide (Sigma), and 1% insulin-transferrin-selenium premix (Invitrogen). The final maturation step consisted of treatment with IMDM containing 20 ng/mL oncostatin M (Peprotech EC Ltd), 1 $\mu$mol/L dexamethasone (Sigma), and 1% insulin-transferrin-selenium premix for 10 days. For each step, medium was changed every 3 days.

### *Flow Cytometry*

Undifferentiated UCMSCs (at passage 4), differentiated UCMSCs, and liver cells were trypsinized and suspended at a concentration of $1 \times 10^6$ cells/mL in phosphate-buffered saline (PBS) 0.5% bovine serum albumin (Sigma) and then incubated at 4°C with antibodies against hematopoietic cell markers (CD45-phycoerythrin-

cyanin7 [PE-Cy7], CD14-fluorescein isothiocyanate [FITC], CD34-PE [phycoerythrin], CD117-allophycocyanin [APC]), mesenchymal stem cell markers (CD90-APC, CD73-PE, CD105-FITC, CD13-APC, CD54-APC, CD166-PE, CD140a-PE), integrins, and receptors for some elements of extracellular matrix (CD29-APC, CD49b-FITC, CD49e-PE, CD49f-FITC, CD44) and major histocompatibility complex I and II (HLA-ABC-FITC and HLA-DR-PerCP, respectively) (all provided by Becton Dickinson CDC, Erembodegem, Belgium, except CD105-FITC and CD26-FITC provided by Ancell Corp, Bayport, MN). After 20 minutes, cells were washed and resuspended in 300 μL Cell Fix (BD Biosciences). For intracellular staining of human albumin (albumin-FITC; Cedarlane Labs Ltd, Burlington, Ontario, Canada) and cytokeratins (pan-cytokeratin-FITC; Miltenyi Biotec, Utrecht, The Netherlands), cells were permeabilized with 200 μL Cytofix/Cytoperm reagent (BD Biosciences) for 20 minutes, washed with PermWash reagent (BD Biosciences), and incubated for 20 minutes at 4°C with FITC rabbit anti-human albumin at 1:50 (vol/vol) in 50 μL PermWash, finally being washed in PBS and fixed. Negative controls included unstained cells and cells incubated with respective isotypes coupled with FITC, PE, APC, PerCP, and PE-Cy7 (BD Biosciences). Cells were analyzed on a FACSCanto II flow cytometer (BD Biosciences), and at least 10,000 events were acquired for each sample.

### *Immunocytochemistry*

UCMSCs cultured on collagen type I–coated 12-mm round glass coverslips were fixed with 4% formaldehyde (vol/vol) for 15 minutes at room temperature and permeabilized thereafter with 1% Triton X-100 (vol/vol) in Tris-buffered saline (Tris-HCl 50 mmol/L, NaCl 150 mmol/L, pH 7.4) for 15 minutes. Nonspecific immunostaining was prevented by 1-hour incubation in a Tris-buffered saline solution containing 3% nonfat dry milk at 37°C. Cells were then incubated in the same solution for 1 hour at room temperature with primary antibodies as follows: albumin (1:70, A6684; Sigma, Bornem, Belgium), cytokeratin (CK)-7 (1:50, MAB3226; Chemicon, Hants, England), CK-8 (1:50, CBL195; Chemicon), CK-19 (1:50, M0888; DakoCytomation, Heverlee, Belgium), CK-18 (1:50, 61028; Progen Biotechnik, Heidelberg, Germany), dipeptidyl peptidase IV (DPPIV) (1:20, 175-020; Ancell), vimentin (1:50, 10515; Progen Biotechnik), fibronectin (1:100, A0245; DakoCytomation), α-smooth muscle actin (1:50, M0851; DakoCytomation), α-fetoprotein (1:100, A0008; DakoCytomation), HepPar1 (1:50, M7158; DakoCytomation), and connexin 32 (1:200, MAB3069; Chemicon). Cells were washed 3 times with Tris-buffered saline and incubated for 1 hour at room temperature with the secondary antibodies: Cy3-coupled anti-mouse immunoglobulin G (Jackson Immunoresearch Laboratories Inc, Newmarket, Suffolk, England) or FITC-coupled anti-rabbit immunoglobulin G (Sigma). Nuclei were revealed by 30 minutes of staining with the nuclear dye 4′,6-diamidino-2-phenylindole (1:5000; Sigma). After 3 washes, preparations were mounted with Fluoprep (BioMerieux, Brussels, Belgium) and examined with a fluorescence microscope (Axioskop 40; Carl Zeiss SA).

For immunoperoxidase staining, cells were plated directly in 24-well collagen type I–coated plates. Cells were fixed and endogenous peroxidase activity was prevented by 3% hydrogen peroxide for 3 minutes. Cells were washed with distilled water and then permeabilized before 1 hour of blocking in PBS 1% bovine serum albumin. Cells were incubated for 1 hour at room temperature with primary antibodies diluted in PBS 0.1% bovine serum albumin, washed with PBS, and exposed for 30 minutes with EnVision+ System–labeled polymer horseradish peroxidase anti-rabbit or anti-mouse (DakoCytomation). Peroxidase activity was revealed by 5 minutes of exposure to diaminobenzidine (Sigma). After washes, Mayer's hematoxylin counterstain was performed for 10 minutes, and then preparations were washed and mounted for microscopic examination (DMIL; Leica, Brussels, Belgium).

### *Reverse-Transcription Polymerase Chain Reaction*

Total RNA was extracted from cells grown in 6-well plates using the TriPure isolation reagent (Roche Diagnostics, Vilvoorde, Belgium). Quantity and purity of RNA was estimated after reading absorbance at 260 and 280 nm. For each sample, complementary DNA was generated from 3.5 μg RNA using the Thermoscript RT kit (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 μL. Cycle specifications are indicated in Table 1. The amplified PCR products were electrophoresed on a 2% agarose gel containing 0.5 μg/mL ethidium bromide for nucleic acid visualization under UV light.

### *Periodic Acid–Schiff Staining for Glycogen Accumulation*

After 4% formaldehyde fixation, cells were incubated for 10 minutes in 1% periodic acid (Sigma), washed with distilled water, and incubated with Schiff's reagent (Sigma) for 15 minutes. After a 10-minute wash in tap water, Karazi's hematoxylin counterstain was performed for 10 minutes, and then preparations were washed and mounted. Freshly cultured (<24 hours) human hepatocytes and UCMSCs cultured in differentiation medium without hepatogenic growth factors served as positive and negative controls, respectively. Inner control consisted of hepatocyte-differentiated UCMSCs treated with 5 g/L amylase (A3176; Sigma) for 15 minutes at 37°C before periodic acid–Schiff staining.

**Table 1.** Primers for RT-PCR Characterization of Native and Hepatocyte Differentiated UCMSCs

| Primer | Sequence | Size (*base pairs*) | Cycle |
|---|---|---|---|
| Albumin | | | |
| S: | 5′-CCTTGGTGTTGATTGCCTTTGCTC-3′ | 308 | 94°C/1 min; 55°C/1 min; 68°C/2 min |
| AS: | 5′-CATCACATCAACCTCTGGTCTCACC-3′ | | |
| α1AT | | | |
| S: | 5′-TCGCTACAGCCTTTGCAATG-3′ | 142 | 94°C/30 s; 55°C/1 min; 68°C/2 min |
| AS: | 5′-TTGAGGGTACGGAGGAGTTCC-3′ | | |
| α-Fetoprotein | | | |
| S: | 5′-TGAAATGACTCCAGTAAACCC-3′ | 199 | 94°C/30 s; 55°C/1 min; 68°C/2 min |
| AS: | 5′-AATGAGAAACTCTTGCTTCATC-3′ | | |
| Connexin 32 | | | |
| S: | 5′-ATGAACTGGACAGGTTTGTA-3′ | 734 | 94°C/1 min; 55°C/30 s; 68°C/1 min |
| AS: | 5′-TGCTTGTATTCAGGTGAGAG-3′ | | |
| CK-8 | | | |
| S: | 5′-AAGGGCTGACCGACGAGATC-3′ | 537 | 94°C/30 s; 50°C/30 s; 68°C/1 min |
| AS: | 5′-GATCGCCACCTACAGGAAGC-3′ | | |
| CK-18 | | | |
| S: | 5′-CCATGCGCCAGTCTGTGGAG-3′ | 322 | 94°C/1 min; 50°C/30 s; 68°C/1 min |
| AS: | 5′-AGCAGATTGAGGAGAGCACCAC-3′ | | |
| CK-19 | | | |
| S: | 5′-TTTGAGACGGAACAGGCTCT-3′ | 426 | 94°C/30 s; 55°C/1 min; 68°C/2 min |
| AS: | 5′-CAGCTCAATCTCAAGACCCTG-3′ | | |
| CYP3A4 | | | |
| S: | 5′-TGCTGTCTCCAACCTTCACC-3′ | 802 | 94°C/120 s; 50°C/60 s; 68°C/1 min |
| AS: | 5′-GGTGGTGATGATTCCAAGCTA-3′ | | |
| CYP2B6 | | | |
| S: | 5′-CCTCTTCCAGTCCATTACCG-3′ | 551 | 94°C/120 s; 50°C/60 s; 68°C/1 min |
| AS: | 5′-GCCATACACAGAGGCAGTCA-3′ | | |
| G6P | | | |
| S: | 5′-TCAGCTCAGGTGGTCCTCTT-3′ | 291 | 94°C/1 min; 55°C/1 min; 68°C/2 min |
| AS: | 5′-CCTCCTTAGGCAGCCTTCTT-3′ | | |
| Hepatocyte nuclear factor 4 | | | |
| S: | 5′-CCAAGTACATCCCAGCTTTC-3′ | 295 | 94°C/40 s; 56°C/50 s; 68°C/1 min |
| AS: | 5′-CTTTGACCCAGATGCCAA-3′ | | |
| c-met | | | |
| S: | 5′-CAATGTGAGATGTCTCCAGC-3′ | 560 | 94°C/40 s; 55°C/40 s; 68°C/1 min |
| AS: | 5′-TCTGCCTGCAATCTACAAGG-3′ | | |
| Phosphenolpyruvate carboxykinase | | | |
| S: | 5′-TTAGATGGGACAAAGCCTG-3′ | 226 | 94°C/40 s; 55°C/40 s; 68°C/1 min |
| AS: | 5′-GCAAGACGGTGATTGTAACTC-3′ | | |
| TAT | | | |
| S: | 5′-TGAGCAGTCTGTCCACTGCCT-3′ | 358 | 94°C/40 s; 56°C/50 s; 68°C/1 min |
| AS: | 5′-CTCAGATCCTCCTCATTCACAT-3′ | | |
| TDO | | | |
| S: | 5′-ATACAGAGCACTTCAGGGAGC-3′ | 316 | 94°C/40 s; 56°C/50 s; 68°C/1 min |
| AS: | 5′-GATACCGAAGATGAACCCAACCA-3′ | | |
| Glyceraldehyde-3-phosphate dehydrogenase | | | |
| S: | 5′-CGGAGTCAACGGATTTGGTCGTAT-3′ | 307 | 94°C/1 min; 58°C/1 min; 68°C/2 min |
| AS: | 5′-AGC CTT CTC CAT GGT GGT-3′ | | |

S, sense; AS, antisense.



### *Glucose-6-Phosphatase Activity Assay*

Assay for revelation of the glucose-6-phosphatase activity (G6P) was performed on UCMSCs cultured in differentiation medium with or without hepatogenic growth factors as previously described.[28]

### *Urea Assay*

Fresh hepatocytes and differentiated and undifferentiated UCMSCs were cultured for 24 hours into Williams' medium E (Invitrogen) supplemented or not with 0.3 mmol/L $NH_4Cl$ (Sigma). Supernatants were collected, centrifuged, and conserved at –20°C in view of further investigation. Urea concentration was measured by a colorimetric assay (Gentaur, Brussels, Belgium), handled according to the manufacturer's instructions, and analyzed with an HTS7000PLUS absorbance reader (PerkinElmer NV/SA, Zaventem, Belgium). Serum-free Williams' medium E served as negative control.

### *Cytochrome P450 3A4 Metabolic Activity Assay*

Cytochrome P450 (CYP) 3A4 enzyme activity assay was conducted directly in UCMSCs cultured in collagen type I–coated 48-well plates. After culture in control or hepatogenic differentiation medium, cells were cultured in IMDM supplemented or not with 1 mmol/L phenobarbital (Sigma) for 48 hours with a daily medium change. Metabolism was assessed by measurement of luciferase activity with the P450-Glo CYP3A4 assay (V8901; Promega Benelux BV, Leiden, The Netherlands) according to the manufacturer's instructions. Briefly, cells were incubated at 37°C in IMDM supplemented with 50 μmol/L luciferin PFBE (150 μL/well). After 3 hours of incubation, 50 μL of medium was transferred in a 96-well opaque white plate (3912; Corning Life Science, Lowell, MA) and mixed with 50 μL of luciferin detection reagent to initiate luminescent reaction. After 20-minute incubation at room temperature, luminescence was measured with a Victor3 luminometer (PerkinElmer).

### *Albumin Assay*

Human albumin concentration of culture supernatants and plasma of mice were screened by enzyme-linked immunosorbent assay (Cygnus Technologies, Inc, Southport, NC) according to the manufacturer's instructions and analyzed with a Multiskan EX absorbance reader (ThermoFisher Scientific, Brussels, Belgium). Human albumin and serum-free IMDM served as positive and negative controls, respectively.

### *UCMSC Transplantation*

All animal experiments were performed in compliance with Belgian law for animal protection and approved by the local ethical review board. One million UCMSCs at passage 3 were injected into the spleens of 8-week-old SCID female mice. Left liver lobe hepatectomy was performed before injection of UCMSCs. Mice receiving intrasplenic injection of 100 μL of 0.9% NaCl physiologic serum (Braun, Melsungen, Germany) served as negative controls. Livers were harvested for immunohistochemistry 2, 4, and 6 weeks posttransplantation.

### *Immunohistochemistry*

Livers were fixed in formaldehyde 4% for 24 hours and embedded in paraffin. Five-micrometer-thick liver sections were deparaffinized and fixed. Endogenous peroxidase activity was prevented by a bath of methanol 0.3% hydrogen peroxide for 15 minutes. Antigen retrieval was performed by incubating the section in warm (97°C) 0.02 mol/L citrate buffer (pH 5.8). After washing, nonspecific immunostaining was prevented by 1-hour incubation in PBS 1% bovine serum albumin. Tissues were incubated overnight at 4°C with rabbit anti-human antibodies against fibronectin (1:3000, A0245; DakoCytomation), α-fetoprotein (1:1000, A0008; DakoCytomation), and albumin (1:2.000, 126584; Calbiochem), with guinea pig anti-human CK-19 antibody (1:200, GP-CK19; Progen Biotechnik), or with mouse anti-human mitochondria antibody (1:75, MAB1273; Chemicon). Detection was performed after incubating the sections with horseradish peroxidase–conjugated anti-rabbit, anti-mouse (EnVision+ System; DakoCytomation), or goat anti–guinea pig (1:500, 106-035-003; Jackson Immunoresearch Laboratories Inc) antibodies. Peroxidase activity was revealed by 5-minute exposure to diaminobenzidine (Sigma). After washing, preparations were counterstained with Mayer's hematoxylin for 10 minutes and mounted for analysis.

### *Detection of Human Alu Sequences by In Situ Hybridization*

Three-micrometer-thick liver sections were deparaffinized and rehydrated in graded alcohol series. Antigen retrieval was performed by incubating the sections in citric acid monohydrate solution (pH 6.0) and exposed twice to a microwave oven for 10 minutes. After washing in cold water and in PBS, sections were treated with 3% hydrogen peroxide in 70% ethanol for 20 minutes. Slides were immersed in water for 10 minutes and dehydrated in increasing grades of ethanol. After air drying for 15 minutes, sections were incubated with human Alu-DNA-Probe (PR-1001-01; Innogenex, San Ramon, CA) and sealed with coverslips. Slides were warmed to 95°C for 5 minutes to denature DNA, and hybridization was performed at 37°C. After overnight incubation, coverslips were removed and slides were placed in a first bath of 2× standard saline citrate (pH 7.0) for 10 minutes, a second bath at 56°C for 10 minutes, and a third bath at room temperature for 15 minutes. Nonspecific binding was prevented by incubation in PBS 3% bovine serum albumin for 1 minute and a 2-step avidin/biotin block (SP-2001; Vector Laboratories Ltd, Peterborough, England). Sections were incubated for 1 hour with biotinylated goat anti-fluorescent antibody (1:100, BA-0601; Vector Laboratories Ltd), washed in PBS, and exposed for 15 minutes to streptavidin/horseradish peroxidase (DakoCytomation). Development was performed by a short incubation with diaminobenzidine. After washing, sections were counterstained with Mayer's hematoxylin and dehydrated before being mounted.

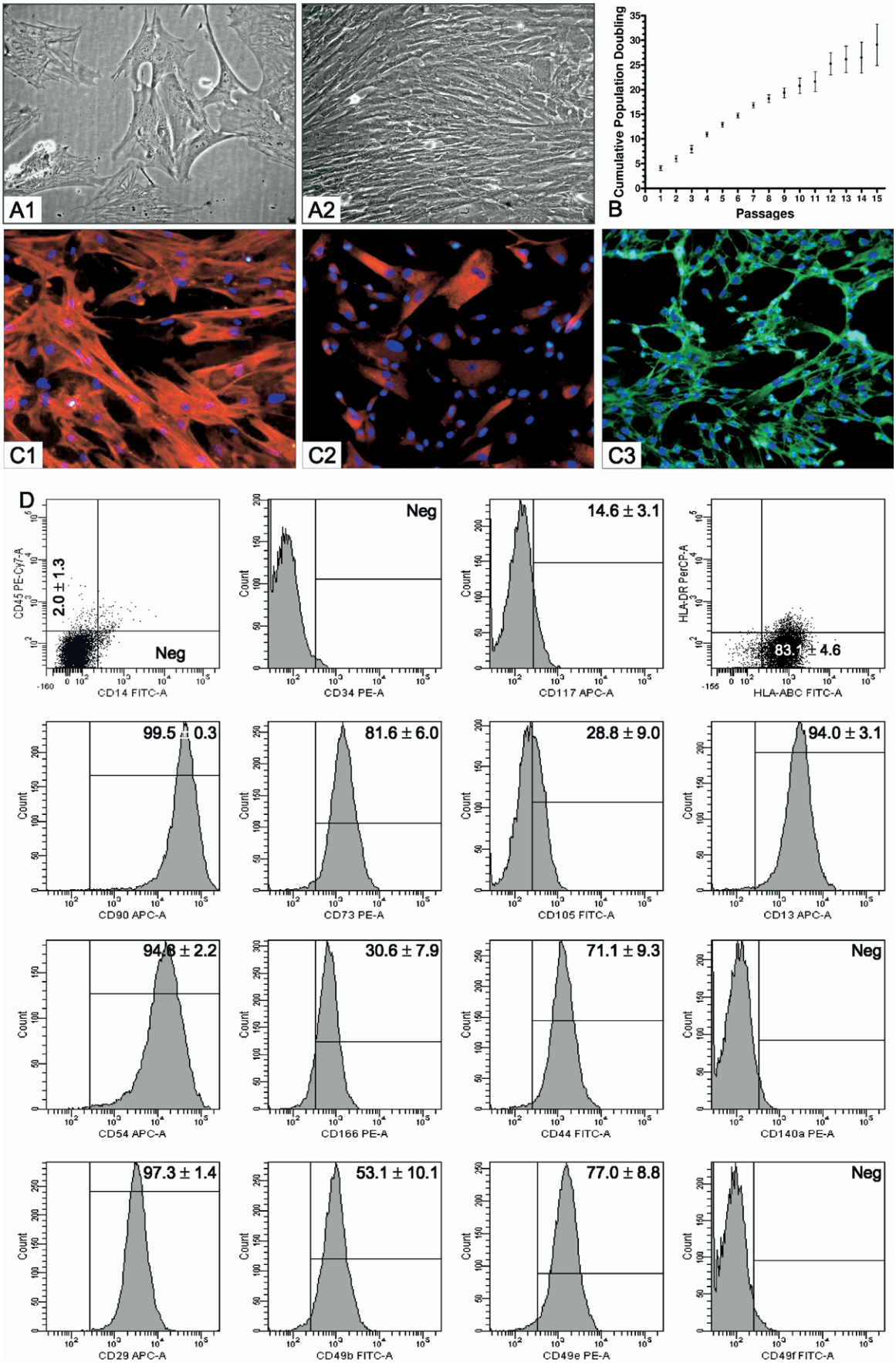
### *Statistical Analysis*

Statistical analyses were performed using PRISM 4.01 (GraphPad Software, Inc, San Diego, CA). The probability of a significant difference between groups was determined using the Mann–Whitney *U* test. Results are expressed as means ± SEM.

## Results

### *Characterization of UCMSCs*

Isolated cells obtained after enzymatic treatment of Wharton's jelly displayed a fibroblast-like appearance during the first 3 days of culture (Figure 1*A1*). The


A1
A2
B
Cumulative Population Doubling
Passages
C1
C2
C3
D
CD45 PE-Cy7-A
2.0 ± 1.3
Neg
CD14 FITC-A
Neg
CD34 PE-A
14.6 ± 3.1
CD117 APC-A
HLA-DR PerCP-A
83.1 ± 4.6
HLA-ABC FITC-A
99.5 ± 0.3
CD90 APC-A
81.6 ± 6.0
CD73 PE-A
28.8 ± 9.0
CD105 FITC-A
94.0 ± 3.1
CD13 APC-A
94.8 ± 2.2
CD54 APC-A
30.6 ± 7.9
CD166 PE-A
71.1 ± 9.3
CD44 FITC-A
Neg
CD140a PE-A
97.3 ± 1.4
CD29 APC-A
53.1 ± 10.1
CD49b FITC-A
77.0 ± 8.8
CD49e PE-A
Neg
CD49f FITC-A
Count

majority of cells were flat with a wide cytoplasm within stress fiber patterns inside. Morphology of the cells changed with a higher level of confluence; the cytoplasm became narrower, and stress fibers were less apparent (Figure 1*A2*).

The growth rate between the first and seventh passages was relatively homogeneous through the different cultures (Figure 1*B*) with a doubling time of 5.4 ± 0.9 days (n = 9). The population reached 20.4 ± 1.5 cumulative doublings at the 10th passage. Between the 10th and 12th passages, growth slowed down and stopped, whereas some cultures (2 of 9) continued to grow until the 20th passage to reach 37 population doublings (data not shown).

Flow cytometry confirmed MSC signature of UCMSCs at the fourth passage with a high expression of CD90, CD73, CD13, and CD54 (Figure 1*D*). However, CD105 was expressed only in 28.8% ± 9.0% of the population. Contamination of culture with hematopoietic cells was excluded by absence of CD45, CD14, and CD34, but a small subpopulation of UCMSCs expressed the receptor of stem cell factor (CD117). Affinity of UCMSCs for extracellular matrix elements was suggested by a high expression of the integrin for fibronectin (CD29/CD49e) and for the receptor of hyaluronic acid (CD44), which is a major component of umbilical cord matrix. UCMSCs also moderately expressed CD29b and CD166. UCMSCs did not express the receptor of platelet-derived growth factor (CD140a). The presence of major histocompatibility complex I on the membrane of UCMSCs and the absence of major histocompatibility complex II was consistent with the general description of MSCs. Immunocytochemical detection of α–smooth music actin, vimentin, and fibronectin confirmed the mesodermal origin of UCMSCs (Figure 1*C1*, *C2*, and *C3*, respectively).

UCMSC adipogenic differentiation was shown by formation of intracytoplasmic lipid droplets (Figure 2*A2*) stained with Oil Red O (Figure 2*A4*). Nevertheless, the UCMSC response to adipogenic induction was heterogeneous because cells did not all display this phenotype and because of the disparity in number and size of lipid droplets per cell. Osteogenic differentiation was also achieved by formation of clusters of small polygonal cells (Figure 2*B2*). These bone nodules were a site of calcium mineralization, as shown by Alizarin Red and von Kossa stains (Figure 2*B6* and *B8*, respectively) and tetracycline incorporation (Figure 2*B4*).

Interestingly, we observed that UCMSCs constitutively express some hepatic markers. Indeed, albumin expression was detected both at the gene and protein levels in UCMSCs cultured in expansion medium (Figure 3*A* and *C1*). Flow cytometry analysis confirmed that 48.2% ± 10.3% of the UCMSC population was positive for this marker (Figure 3*B*). Other hepatic markers such as α-fetoprotein and connexin 32 were also constitutively expressed (Figure 3*A*, *C2*, and *C3*, respectively). CK-8, CK-18, and CK-19 were also expressed at different levels (Figure 3*C6*, *C7*, and *C4*, respectively), while CK-7 was not detected (Figure 3*C8*). Concerning enzymes involved in hepatic metabolism, G6P, phosphoenolpyruvate carboxykinase, tryptophan 2,3-dioxygenase (TDO), and $\alpha_1$-antitrypsin (α1AT) were detected at the gene level, as shown by reverse-transcription (RT)-PCR (Figure 3*A*). DPPIV protein was also detected (Figure 3*C9*) and was present in the same proportion as albumin (Figure 3*B*). On the other hand, gene expression of CYP450 2B6 and 3A4 isoforms and hepatocyte nuclear factor 4 (Figure 3*A*) and protein expression of HepPar1 (Figure 3*C9*) were not detected in UCMSCs.

### *In Vitro Differentiation of UCMSCs Into Hepatocyte-Like Lineage*

Partial hepatocyte identity of UCMSCs suggested hepatogenic potency of UCMSCs. Hence, cells were submitted to an in vitro differentiation protocol based on successive exposure to growth factors, cytokines, and chemicals that mimicked liver development. After 32 days of culture in hepatogenic medium, morphologic changes were observed in all cultures (n = 14); cell spreading was reduced, and UCMSCs adopted a polygonal shape with a granular cytoplasm. Immunocytochemical and RT-PCR analyses of differentiated UCMSCs showed persistence of albumin, α-fetoprotein, CK-18, CK-19, and connexin 32 (Figures 4 and 5). The rate of cells expressing albumin and DPPIV was not modified (Table 2), but the presence of small clusters in DPPIV immunostaining of differentiated UCMSCs suggested cell polarization of the protein (Figure 5, *white arrowheads*). No modulation of α1AT and G6P gene expression was noticed between expanding UCMSCs (Figure 3*A*) and differentiated cells (Figure 4). Expression of tyrosine aminotransferase (TAT) and TDO expression was up-regulated (Figure 4), and a small but consistent signal for CYP3A4 messenger RNA was detected in some cultures of differentiated cells. Neither CYP2B6 nor hepatocyte nuclear factor 4 was up-regulated in differentiated cells (Figure 4). Immunocytochemical analysis showed that UCMSCs cultured in the absence or presence of hepato-



**Figure 1.** Characterization of UCMSCs. (*A*) Morphology of UCMSCs at the third day of culture (*A1*, original magnification 400×) and at the 11th day when cells reached confluence (*A2*, original magnification 200×). (*B*) Expansion of UCMSCs from passage 0 to passage 15. (*C*) Immunocytochemical analysis of expanding UCMSCs for α–smooth muscle actin (*red*, *C1*), vimentin (*red*, *C2*) and fibronectin (*green*, *C3*) (nuclei were stained *blue* with 4′,6-diamidino-2-phenylindole; original magnification 200×). (*D*) A representative flow cytometry analysis of UCMSCs at passage 4. Results are means of rate of positive cells (%) for 8 independent experiments ± SEM.

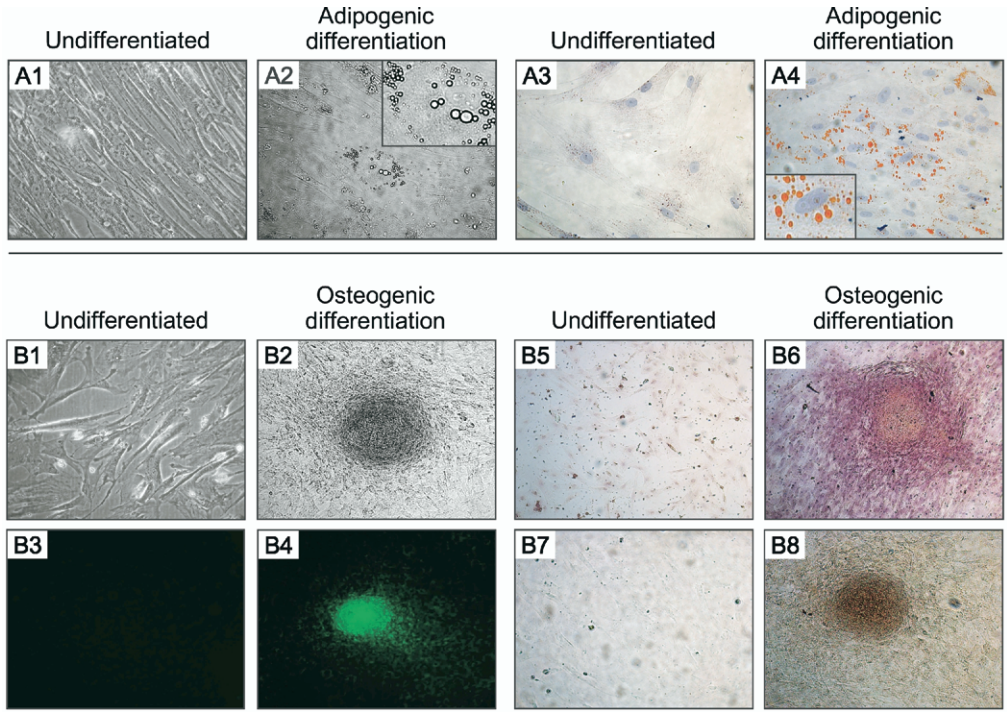



**Figure 2.** Osteogenic and adipogenic differentiation of UCMSCs. Adipogenic potential of UCMSCs was shown by formation of intracytoplasmic lipid droplets of different sizes that were observed by phase-contrast microscopy (*A2*) and were positively stained with Oil Red O (*A4*) (original magnification 400×; *inset* 1000×). UCMSCs cultured in control medium (*A1* and *A3*, respectively) did not exhibit such adipocytic features. Osteogenic potential of UCMSCs was shown by microscopic observation of bone nodules (*B2*, original magnification 200×) that incorporated tetracycline (*B4*, original magnification 100×) and by calcium mineralization as revealed by Alizarin Red (*B6*, original magnification 100×) and von Kossa stains (*B8*, original magnification 200×). Respective control cultures of UCMSCs (*B1*, *B3*, *B5*, and *B7*) did not exhibit such osteocytic features.



genic inducers were not recognized by a specific antibody against human hepatocyte (HepPar1) (Figure 5).

In parallel, the analysis of mesenchymal marker expression showed that CD73 was down-regulated after hepatogenic differentiation and reached levels similar to human liver cells (Table 2). However, the rate of CD90-positive UCMSCs remained higher (Table 2) and the expression of cytoplasmic markers such as α–smooth muscle actin, vimentin, and fibronectin was maintained in differentiated UCMSCs (Figure 5).

Differentiation efficiency was also evaluated at the functional level. Periodic acid–Schiff staining showed that UCMSCs submitted to hepatogenic differentiation protocol were able to specifically store glycogen (Figure 6*A2*) as compared with undifferentiated UCMSCs (Figure 6*A1*). Activity of G6P was revealed in few undifferentiated UCMSCs and was up-regulated in differentiated cells (Figure 6*B1* and *B2*, respectively). This result corroborated data obtained with RT-PCR experiments (Figure 4). Urea production was significantly increased in cultures of differentiated UCMSCs exposed for 24 hours to 0.3 mmol/L ammonium chloride and reached the level of hepatocytes in same culture conditions (Figure 6*C*). Activity of the CYP3A4 isoenzyme has been detected in differentiated UCMSCs and was significantly enhanced when cells were cultured with 1 mmol/L phenobarbital compared with undifferentiated UCMSCs (Figure 6*D*). Albumin was not detected in supernatants of differentiated UCMSC cultures (data not shown).

### *UCMSCs Engrafted in Livers of SCID Mice*

To investigate the engraftment capacity of UCMSCs into liver, $1 \times 10^6$ undifferentiated UCMSCs were injected into the spleen of 8-week-old SCID mice after a partial hepatectomy. Two, 4, and 6 weeks later, in situ hybridization for human Alu sequences was performed on livers of mice that had undergone transplantation. Isolated and small groups of Alu-positive nuclei were observed in recipient liver parenchyma and perivascular areas 2 and 4 weeks posttransplantation, concluding that human cells engrafted into recipients' livers (Figure 7*B2* and *B3*). Tracing of human cells into recipient mouse livers has been confirmed by immunohistochemical detection of human mitochondria (Figure 7*A2* and *A3*). Human mitochondria and Alu probe staining 6 weeks posttransplantation indicate an increase of the number of human cells per cluster (Figure 7*A4* and *B4*, respectively).

Immunohistochemical analysis of mice 2 and 4 weeks after transplantation revealed the presence of human albumin in small clusters of polygonal cells located near portal veins, confirming engraftment of UCMSCs and maintenance of a constitutively expressed hepatic marker (Figure 7*C2* and *C3*, respectively). The albumin staining

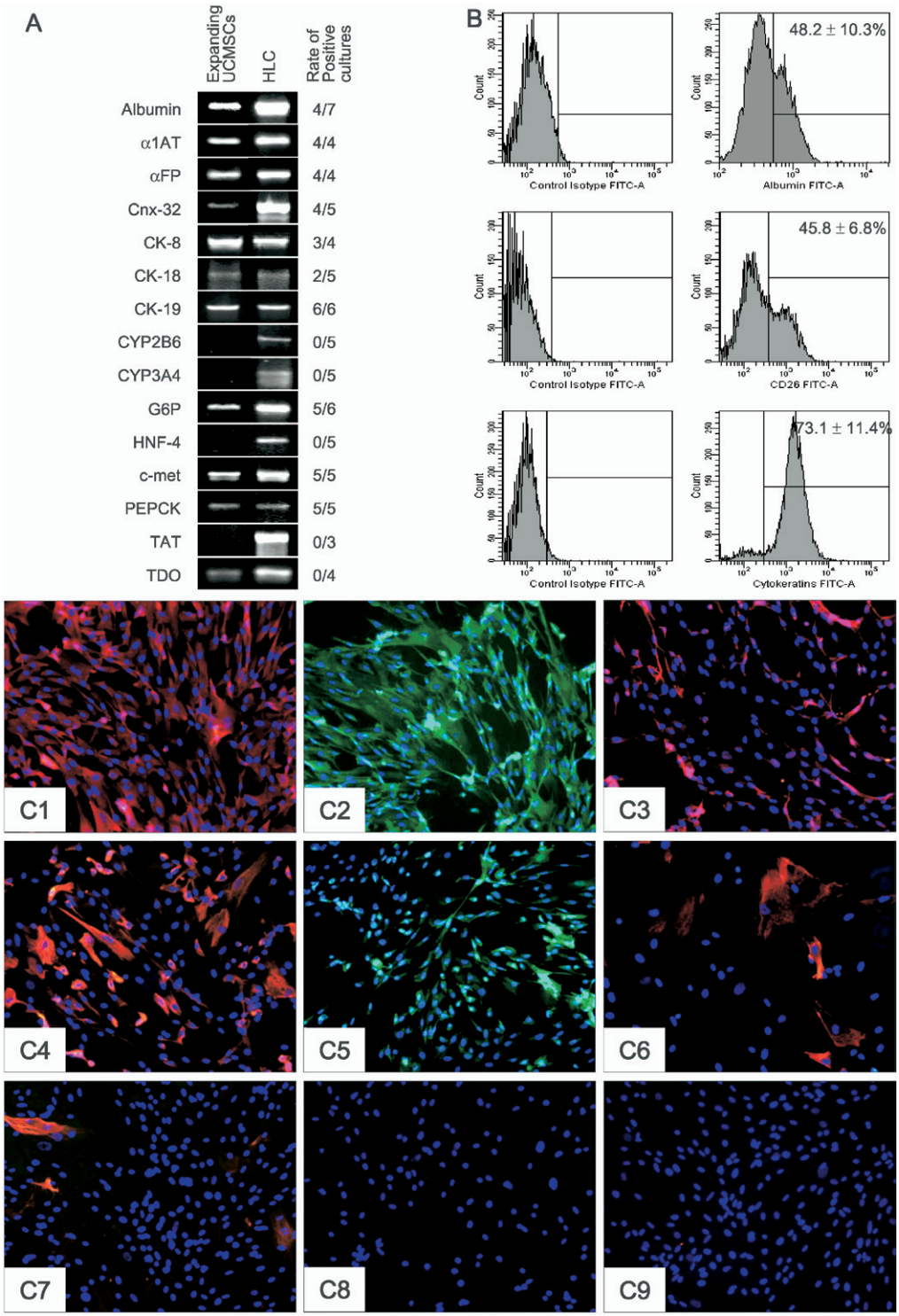



**Figure 3.** Expression of hepatic markers by native UCMSCs. (*A*) A representative RT-PCR analysis of UCMSCs cultured in expansion medium for late and early hepatic markers compared with human liver cells (HLC). Heterogeneity from culture to culture of UCMSCs was represented by the rate of gene-expressing cultures indicated in the *right column*. αFP, α-fetoprotein; Cnx-32, connexin 32; HNF-4, hepatocyte nuclear factor 4; PEPCK, phosphoenol pyruvate carboxykinase. (*B*) Flow cytometry quantification of albumin-, DPPIV- or cytokeratin-positive UCMSCs cultured in expansion medium. Respective isotype control antibody curves are shown in the *left panels*. Results are means of rate of positive cells (%) for 7–10 independent experiments ± SEM. (*C*) Immunocytochemical analysis of expanding UCMSCs (n = 4) showed that UCMSCs strongly expressed albumin (*red*, *C1*), α-fetoprotein (*green*, *C2*), connexin 32 (*red*, *C3*), CK-19 (*red*, *C4*), and DPPIV (*green*, *C5*). Filaments of CK-8 (*red*, *C6*) and CK-18 (*red*, *C7*) were observed in few cells, while CK-7 (*red*, *C8*) and HepPar1 (*red*, *C9*) were not expressed. Nuclei were stained *blue* with 4′,6-diamidino-2-phenylindole (original magnification 200×).

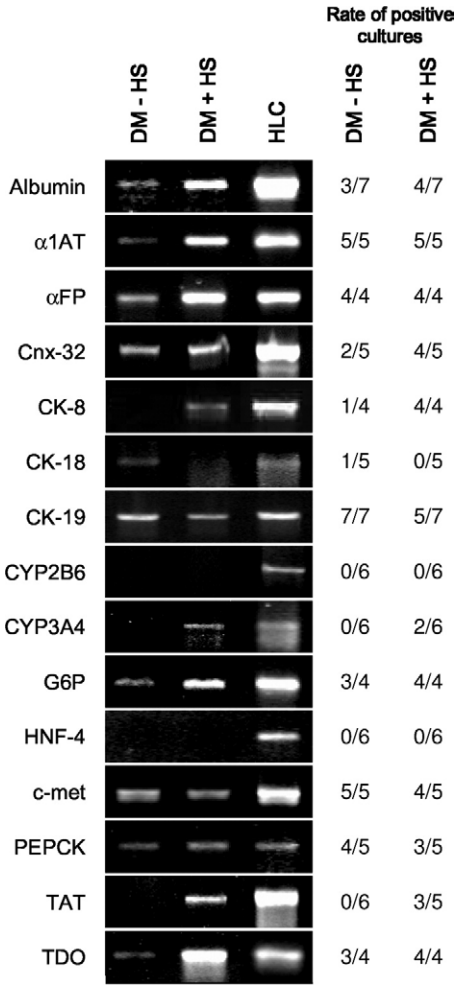



**Figure 4.** Effect of hepatogenic differentiation protocol on expression of hepatic specific genes. UCMSCs were cultured in differentiation medium with hepatogenic supplements (DM + HS) and were examined for their messenger RNA content for hepatic markers. Gene expression profiles were compared with undifferentiated UCMSCs cultured in differentiation medium without hepatogenic supplements (DM − HS) or human liver cells (HLC). A representative profile is presented, and the rate of gene-expressing cultures for DM − HS and DM + HS conditions is indicated in the *2 right columns*. Depending on the culture, expression of a gene was modified or not, suggesting heterogeneity of UCMSCs in response to hepatogenic induction. αFP, α-fetoprotein; Cnx-32, connexin 32; HNF-4, hepatocyte nuclear factor 4; PEPCK, phosphoenol pyruvate carboxykinase.

pattern after 6 weeks corroborated human mitochondria and nuclei stains with few isolated cells scattered in hepatic parenchyma and rare but large clusters of cells. Human α-fetoprotein has also been detected in few isolated cells, and this pattern was similar in all analyzed groups (Figure 7*D2*, *D3*, and *D4*). Fibronectin was expressed in several engrafting cells 2 weeks posttransplantation but was not even detected after 6 weeks, indicating that this mesodermal marker was down-regulated in vivo (Figure 7*F2*, *F3*, and *F4*). CK-19, which was constitutively and widely expressed in native UCMSCs in vitro, was not detected as of 2 weeks posttransplantation (Figure 7*E2*, *E3*, and *E4*).

## Discussion

In the present study, we showed that human umbilical cord matrix from full-term neonates can be considered as a source of MSCs with an in vitro hepatogenic potential. In vivo, the cells are able to engraft in the recipient liver parenchyma and to maintain hepatocyte lineage markers over 6 weeks posttransplantation. The phenotypic profile of UCMSCs isolated in our hands corroborates results from previous studies and the documented expression of a consensus marker set of MSCs. Indeed, UCMSCs were positive for CD90, CD73, CD44, CD29, and CD13. The percentage rate of CD105-expressing UCMSCs was in our hands relatively lower than bone marrow or umbilical cord blood MSCs. The expression of CD105 by UCMSCs fluctuated between different studies and might depend on the precise location (perivascular vs intervascular) in the cord where the cells are extracted.[21,23] We also noticed the expression of the receptor of stem cell factor (CD117, c-kit) in a small subpopulation of UCMSCs. This result confirmed previously presented data[20,21] and conferred a particular identity to these cells because neither umbilical cord blood MSCs[15] nor multipotent adult progenitor cells[29] expressed CD117. Moreover, UCMSCs slightly expressed CD166 in contrast to umbilical cord blood MSCs[30] and did not express CD140a in contrast to multipotent adult progenitor cells. Typical characterization of UCMSCs was achieved by demonstration of their ability to differentiate into adipocytes and osteocytes. As previously described by Karahuseyinoglu et al, UCMSCs can differentiate into adipocytes, but this differentiation seemed

**Figure 5.** Effect of hepatogenic differentiation protocol on expression of hepatic specific proteins. Morphology and immunocytochemical analysis of undifferentiated or hepatocytic differentiated UCMSCs cultured in medium without (DM − HS) or with hepatogenic supplements (DM + HS), respectively. Immunocytochemical analysis of differentiated UCMSCs showed maintenance of constitutively expressed hepatic markers compared with UCMSCs cultured in control medium (DM + HS vs DM − HS, positive culture/total of tested culture): albumin (11/11 vs 11/11), α-fetoprotein (αFP; 7/7 vs 6/6), CK-19 (8/8 vs 9/9), and connexin 32 (Cnx32; 6/7 vs 6/7). Small variations have been observed for CK-18 (7/10 vs 5/9) and CK-8 (5/9 vs 4/9), but hepatogenic induction failed to up-regulate mature hepatocyte marker HepPar1 (0/7 vs 0/7). DPPIV was detected in similar levels in both culture conditions (8/8 vs 8/8), but fluorescence spots (*white arrowheads*) suggest polarization of the protein. Finally, persistence of mesenchymal markers α–smooth muscle actin (αSMA), vimentin, and fibronectin was observed in differentiated UCMSCs (7/7 vs 7/7 for all markers). Original magnification 200× except for DPPIV (100×; *inset* 200×).

incomplete because morphology of the cells was not critically modified and intracytoplasmic lipid droplets were smaller and fewer as compared with differentiated bone marrow MSCs.[19] UCMSCs under osteogenic induction aggregated into clusters of small polygonal cells and elaborated bone matrix. These bone nodules have been previously described[21] and reproduced differentiation of osteoprogenitors to a mature phenotype.[31]

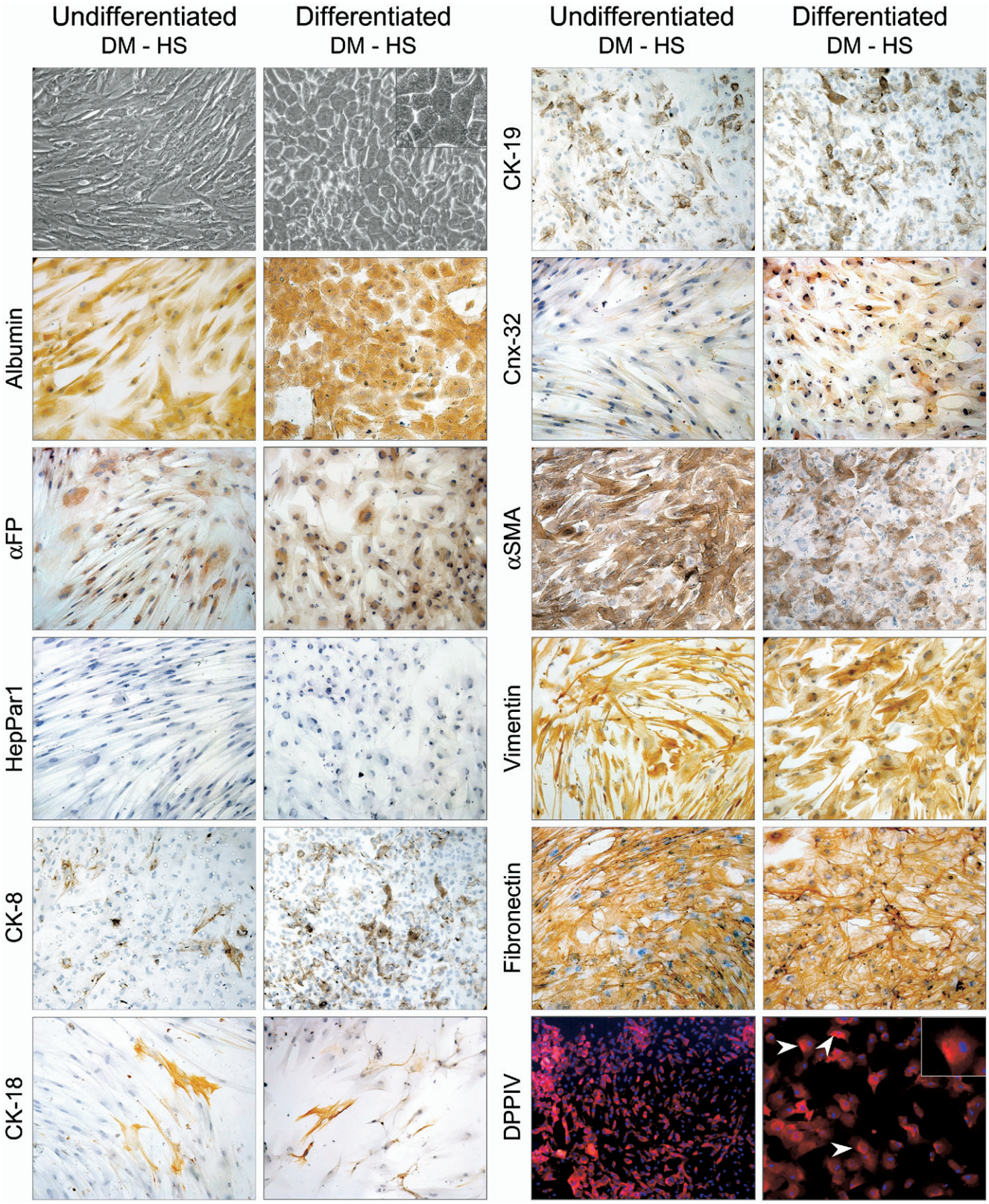


**Table 2.** Flow Cytometry Characterization of Native and Hepatocyte Differentiated UCMSCs

| | DM − HS | DM + HS | HLC |
|---|---|---|---|
| MSC | | | |
| CD90 | 99.4 ± 0.1[a] | 99.8 ± 0.1[b] | 13.7 ± 2.5 |
| CD73 | 51.5 ± 4.9[a] | 16.8 ± 3.6[c] | 15.4 ± 6.3 |
| CD105 | 11.7 ± 2.1 | 6.5 ± 1.1 | 6.5 ± 2.3 |
| CD13 | 69.6 ± 8.4 | 97.6 ± 0.6[d] | 84.9 ± 1.3 |
| CD44 | 23.2 ± 14.7 | 8.8 ± 2.5 | 7.1 ± 1.9 |
| CD29 | 82.8 ± 3.6 | 85.3 ± 5.0 | 79.8 ± 5.9 |
| CD49[b] | 14.6 ± 3.3[a] | 3.5 ± 0.4[e] | 2.9 ± 0.3 |
| CD49[e] | 25.5 ± 6.2 | 66.5 ± 5.3[c] | 64.1 ± 10.0 |
| CD49[f] | 3.4 ± 0.6 | 2.9 ± 0.2 | 4.8 ± 1.6 |
| CD54 | 79.2 ± 8.2 | 62.5 ± 8.4[f] | 85.4 ± 1.3 |
| CD166 | 5.2 ± 1.0[a] | 13.3 ± 2.6[d] | 19.7 ± 17.6 |
| CD140[a] | 4.3 ± 2.1 | 5.0 ± 1.4 | 1.9 ± 0.6 |
| MHC | | | |
| HLA-ABC | 18.4 ± 6.9 | 16.6 ± 4.3 | 20.8 ± 13.7 |
| HLA-DR | 2.2 ± 0.1[a] | 2.2 ± 0.2[b] | 4.5 ± 0.9 |
| Hematopoietic | | | |
| CD45 | 4.9 ± 0.4[f] | 2.7 ± 0.2[b] | 16.7 ± 12.4 |
| CD14 | 3.1 ± 0.1[a] | 2.7 ± 0.2[b] | 23.0 ± 8.6 |
| CD34 | 3.3 ± 1.0[f] | 2.2 ± 0.1[a] | 11.7 ± 3.9 |
| CD117 | 11.7 ± 8.4 | 5.0 ± 1.9 | 10.7 ± 3.5 |
| Hepatic | | | |
| Albumin | 29.4 ± 6.3[a] | 24.4 ± 6.3[f] | 51.9 ± 8.2 |
| Cytokeratins | 26.2 ± 1.8[f] | 20.7 ± 4.2[f] | 96.5 ± 0.9 |
| CD26 | 53.0 ± 7.3 | 34.2 ± 6.5 | 43.0 ± 8.3 |

NOTE. UCMSCs were cultured in differentiation medium with or without hepatogenic supplements (DM + HS and DM − HS, respectively, n = 9) and were analyzed for expression of class I and class II major histocompatibility complex (MHC), MSC, hematopoietic, and hepatic markers. Expression profiles were compared with human liver cells (HLC, n = 7). MSC marker CD73 and integrin for collagen CD29b were down-regulated after hepatogenic differentiation and reached levels similar to HLC. On the contrary, MSC marker CD90 remained stable and CD166 and integrin for fibronectin CD29e were up-regulated. Results are means of rates of positive cells (%) ± SEM. [a].001 ≤ $P$ < .01; [b]$P$ <.001; [c]$P$ < .001: DM + HS vs. DM − HS; [d].001 ≤ $P$ < .01; [e].01 ≤ $P$ < .05: DM + HS vs DM − HS; [f].01 ≤ $P$ < .05: DM + HS or DM − HS vs HLC.



Our study showed for the first time constitutive expression of markers generally assigned to hepatic lineage. Indeed, almost half of the UCMSC population expressed albumin and DPPIV. Moreover, high levels of α-fetoprotein and CK-19 were detected by RT-PCR and by immunocytochemical analysis, emphasizing previous results.[19] In a recent review, Hengstler et al pointed out that the expression of few hepatic markers by stem cell–derived hepatocytes does not make them mature hepatocytes.[32] Taken individually, markers attributed to hepatic lineage are not limited to the liver. Studies showed that expression of cytokeratin was detected in gastric cardia and skin[33] and in the umbilical cord.[19] More specifically, CK-18 was observed in the lung[34] and CK-8 was one of the most expressed genes in UCMSCs.[23] Moreover, messenger RNAs for albumin, α-fetoprotein, and α1AT were detected in human pancreas.[35] Focusing on bone marrow stem cell populations, $\beta_2m^-$/Thy-$1^+$ cells consistently expressed α-fetoprotein, CK-8, and CK-18,[36] and low levels of albumin, CK-18, and TDO transcripts were detected in MSCs.[14] Hepatic markers were found in other stem cell compartments because unrestricted somatic stem cells isolated from placental cord blood expressed CK-8 and CK-18[37] and human adipose tissue–derived stromal cells expressed CK-18 and CK-19.[38]

The UCMSCs displayed a combination of several hepatic markers that could confer hepatic identity to these cells. Based on this assumption, we suggested definition of a maturation level to these UCMSCs. Low expression levels of c-kit and phosphoenolpyruvate carboxykinase, combined with a moderate expression of albumin, α-fetoprotein, connexin 32, and CK-19, could confer a status of hepatoblast to UCMSCs.[39] We draw attention to the expression of CK-19 by UCMSCs because this protein is expressed by a bipotential progenitor (immature hepatocyte/biliary epithelial lineage) in the fetal liver.[39–42] Assumption of biliary lineage of UCMSCs suggested by the expression of CK-19 was excluded by expression of hepatocyte markers and absence of CK-7. Constitutive expression of mature hepatocyte genes (α1AT, TDO) in UCMSCs distinguished the cells from the immature hepatocyte.[41] However, absence of HepPar1, TAT, and P450 isoenzymes suggested an intermediate phenotype between the hepatoblast and the mature hepatocyte.

Morphology of UCMSCs submitted to the protocol of differentiation reached a hepatocyte-like phenotype. In several cultures, some genes coding for enzymes involved in liver metabolism, such as TAT and TDO, were up-regulated and transcripts of CYP3A4 were detected by RT-PCR. The modification of gene and protein expression pattern was not consistent from culture to culture and should reflect the heterogeneity of the UCMSC population. Indeed, we reported differences in the expression levels of hepatic-associated markers in isolated UCMSCs that could be associated with their precise location in the umbilical cord stroma. Variation in digestion efficiency during isolation procedure could be a source of this heterogeneity. As previously described for neurogenic differentiation,[19,21,43] a portion of stromal cells may be responsive to hepatogenic induction. The number of albumin- or DPPIV-positive cells did not increase after hepatogenic stimulation. However, immunocytochemical analysis revealed clustering of DPPIV staining, suggesting a cell polarization phenomenon. The expression of HepPar1, CYP2B6, and hepatocyte nuclear factor 4, not detected in native UCMSCs, was not triggered by culture with hepatogenic supplements. However, despite this partial hepatocyte phenotype acquisition, cells acquired some degree of hepatocyte functionality with active CYP3A4 and G6P enzymes and a capacity to store glycogen and to produce urea. Differentiated UCMSCs preserved specific markers of MSCs either at an initial

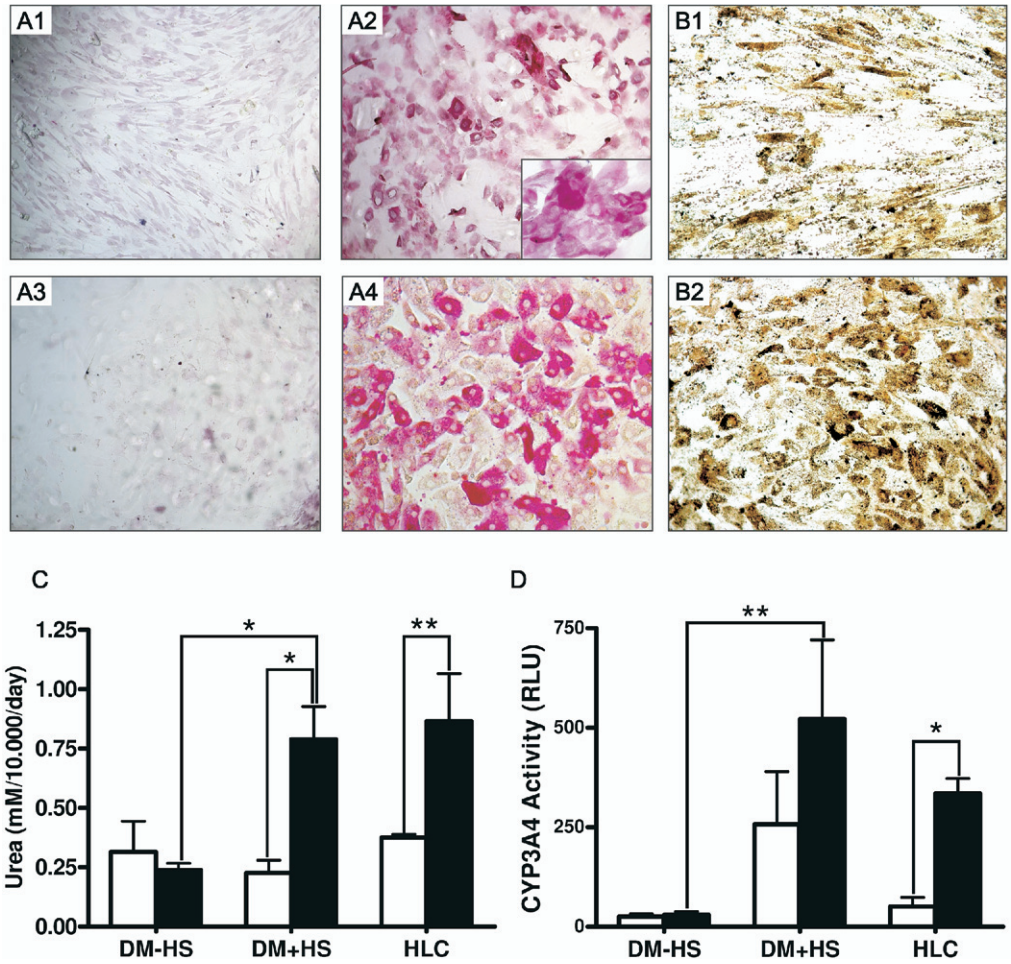



**Figure 6.** Functionality of hepatocyte-like cells derived from UCMSCs. UCMSCs cultured in medium without hepatogenic supplements (*A1*, control medium) did not display a positive reaction to periodic acid–Schiff staining. However, UCMSCs cultured in hepatogenic differentiation medium were positive, revealing accumulation of glycogen in their cytoplasm (*A2*). Staining was specific for glycogen because treatment of differentiated UCMSCs with amylase prevented a positive reaction (*A3*). Efficiency of periodic acid–Schiff staining was tested on fresh hepatocytes as positive control (*A4*). Original magnification: *A1–A3*, 200×; *A4* and *inset* in *A2*, 400×. (*B*) G6P activity was detected in undifferentiated UCMSCs (*B1*) and was enhanced after the differentiation process (*B2*) (n = 4). (*C*) UCMSCs were cultured in differentiation medium (DM) with (+, n = 5) or without (−, n = 5) hepatogenic supplements (HS) and were stimulated for 24 hours with 0.3 mmol/L $NH_4Cl$ before measuring urea concentration in culture supernatants. Urea production was compared with cultures of fresh hepatocytes (HLC) (n = 5). Data are means ± SEM. **.001 ≤ $P$ < .01; *.01 ≤ $P$ < .05. (*D*) Primary culture of human liver cells (HLC) and undifferentiated (DM − HS) or hepatocytic differentiated UCMSCs (DM + HS) were cultured for 48 hours in medium with or without 1 mmol/L phenobarbital (*black* and *white bars*, respectively). Following exposure, catalytic activity of CYP3A4 was determined using 50 μmol/L luciferin substrate (luciferin PFBE). After 3-hour incubation with the substrate, luciferase activity was assessed. Results are expressed as luciferase activity in relative luminescence unit (RLU) and are mean ± SEM of 5 independent experiments.



level, such as CD90, or partially down-regulated, such as for CD73, CD105, CD44, and CD54. Moreover, expression of CK-19 in hepatocyte-like cells derived from UCMSCs suggested the persistence of initial lineage markers, as was recently reported in bone marrow MSCs.[28]

The hepatogenic capacity of stem cells was further illustrated by engraftment and long-term maintenance of cells into recipient livers. Undifferentiated UCMSCs were injected into the spleen of partially hepatectomized SCID mice. Human cells were detected using human Alu probe and anti-human mitochondria antibody in the recipients' livers 2 weeks posttransplantation and were maintained over 6 weeks. Clusters of engrafted cells immunopositive for human albumin and scattered isolated cells immunopositive for α-fetoprotein were observed in recipient mouse livers 2 to 6 weeks posttransplantation. CK-19 was no longer detected after 2 weeks in vivo, even in perivascular areas, whereas injected cells expressed this protein before transplantation. Loss of this intermediate filament protein suggested that engrafted cells might restrict their potency to hepatocyte lineage. However, this assumption must be carefully considered because the animal model can influence the phenotype of engrafted cells. Indeed, Wang et al showed that CK-19 was found in NOD/SCID/$\beta_2$-null mice but not in NOD/SCID mice, whereas the injected cells were the same.[44] Finally, fibronectin, which was maintained in vitro in differentiated cells, was down-regulated in engrafted cells,

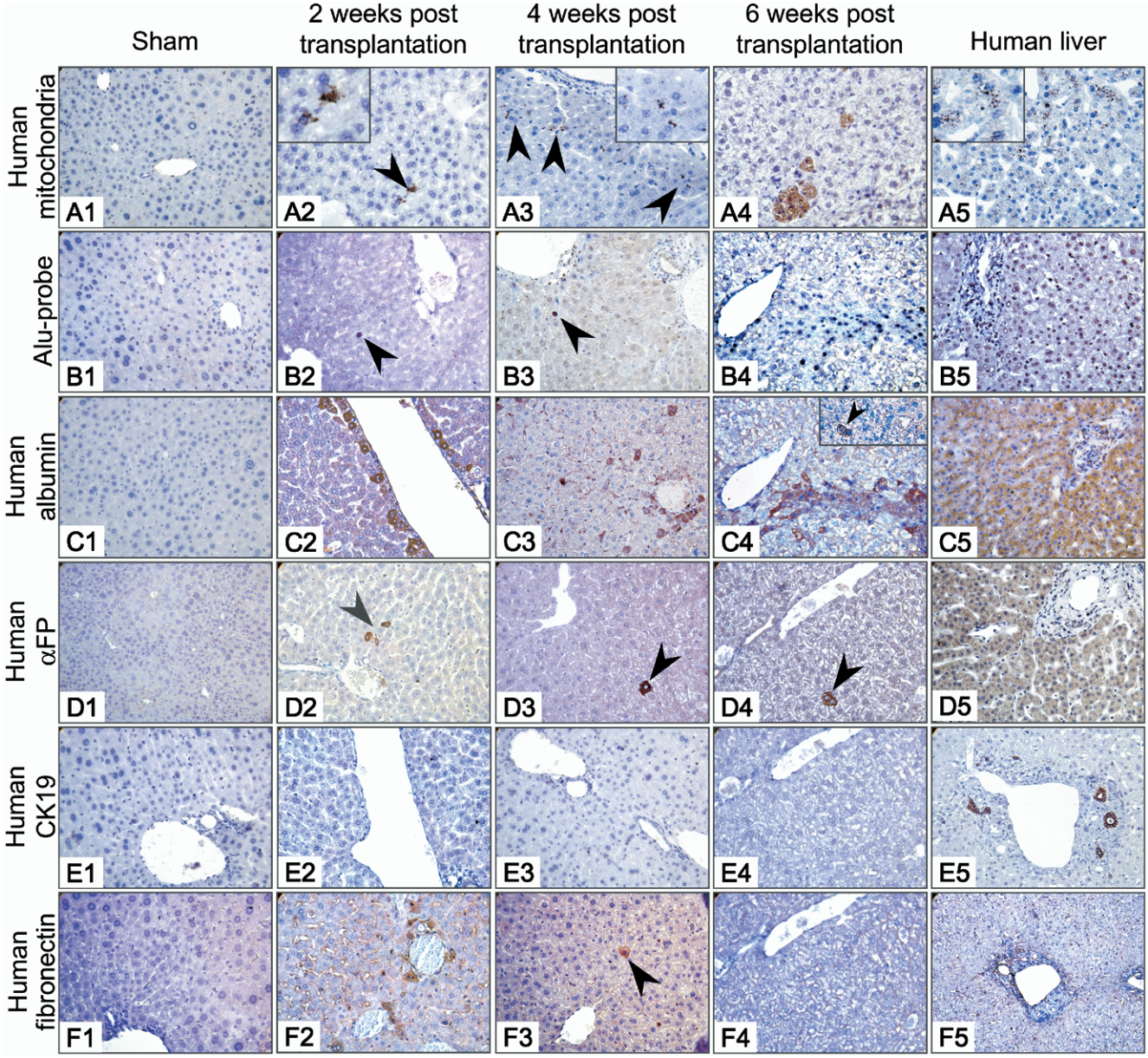



**Figure 7.** Undifferentiated UCMSCs engrafted livers of partially hepatectomized SCID mice. One million human UCMSCs were injected into the spleen of hepatectomized SCID mice and were tracked into liver recipients 2 (n = 3), 4 (n = 3), and 6 (n = 4) weeks later by in situ hybridization with human Alu probe. Immunostaining with anti-human mitochondria confirmed engraftment of human UCMSCs into SCID mice (*black arrowheads*). Immunohistochemical analysis included detection of human albumin, α-fetoprotein (αFP), CK-19, and fibronectin. Small clusters of cells and isolated cells stained expressed albumin in mice that had undergone transplantation. However, only isolated cells stained positive for α-fetoprotein or fibronectin. CK-19 was not detected in livers of mice that had undergone transplantation. PBS-injected mice served as negative controls, and sections of human livers served as positive controls. Original magnification 400× except for anti-mitochondria (530×; *inset* 1000×).

suggesting the decrease of the mesodermal identity in an appropriate in vivo environment.

All together, these properties of UCMSCs represent good arguments for their use in regenerative medicine. Indeed, the isolation method is robust, the efficiency of expansion allows large-scale production, and UCMSCs can restart to expand after freezing/thawing (data not shown; Karahuseyinoglu et al[19]). Differentiation of UCMSCs into different lineages[19,20,22,24–26] and the gene expression profile[23] bring evidence for their multipotency. The absence of acute rejection in experiments of xenotransplantation on non–immune-suppressed animals suggests low immunogene potency of UCMSCs.[45] Moreover, unlike embryonic stem cells, UCMSCs did not induce teratomas or death after intravenous or subcutaneous transplantation into SCID beige mice.[46] Finally, cells are isolated from a noncontroversial and inexhaustive source. In conclusion, UCMSCs appear to be a technically and ethically appropriated extrahepatic source for generation of hepatocyte-like cells.

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# CHAPTER V: HEPATOCYTE DIFFERENTIATION OF HUMAN SKIN FIBROBLASTS

## 1. Introduction

The phenotypic homology between MSCs and fibroblasts has been recently demonstrated and because standard immunophenotype lacks accuracy for distinguish both cell types, some authors used specific methods (as microarray or proteoma) to identify differences in corresponding expression pattern (164;165). Although fibroblasts had already been demonstrated as a potential source for osteocyte-like cell engineering (166), these cells are currently considered as unable to differentiate and are used as control for differentiation assays (162;164). Recent advances suggested further their differentiation potential into mesodermal lineages (167;168). In our hands human skin fibroblasts were highly similar to MSCs in their phenotype and mesodermal differentiation potential. We thus exposed these cells to a hepatocyte differentiation protocol and observed their endodermal potential which was highly similar to this of MSCs.

2. **Article 4:** Human Skin Fibroblasts: From Mesodermal to Hepatocyte-Like Differentiation. *Hepatology* 2007 Nov;46(5):1574-85.

# Human Skin Fibroblasts: From Mesodermal to Hepatocyte-Like Differentiation

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**The phenotypic homology of fibroblasts and mesenchymal stem cells (MSCs) has been recently described. Our study investigated the in vitro potential of human skin fibroblasts to differentiate into mesodermal (osteocyte and adipocyte) and endodermal (hepatocyte) cell lineages by comparison with human bone marrow (hBM) MSCs. The endodermal potential of fibroblasts was then explored in vivo in a mouse model of liver injury. Fibroblasts were able to acquire osteocyte and adipocyte phenotypes as assessed by cytochemistry and gene expression analyses. After exposure to a specific differentiation cocktail, these cells presented hepatocyte-like morphology and acquired liver-specific markers on protein and gene expression levels. Furthermore, these fibroblast-derived hepatocyte-like cells (FDHLCs) displayed the ability to store glycogen and synthesize small amounts of urea. By gene expression analysis, we observed that fibroblasts remained in a mesenchymal-epithelial transition state after hepatocyte differentiation. Moreover, FDHLCs lost their hepatocyte-like phenotype after dedifferentiation. In vivo, human fibroblasts infused directly into the liver of hepatectomized severe combined immunodeficient (SCID) mice engrafted in situ and expressed hepatocyte markers (albumin, alpha-fetoprotein, and cytokeratin 18) together with the mesodermal marker fibronectin. Despite lower liver-specific marker expression, the in vitro and in vivo differentiation profile of fibroblasts was comparable to that of mesenchymal-derived hepatocyte-like cells (MDHLCs). In conclusion, our work demonstrates that human skin fibroblasts are able to display mesodermal and endodermal differentiation capacities and provides arguments that these cells share MSCs features both on the phenotypic and functional levels. (HEPATOLOGY 2007;46:1574-1585.)**

Fibroblasts and mesenchymal stem cells (MSCs) are mesodermal in status and have been isolated from various tissues. However, unlike fibroblasts, MSCs were recently demonstrated to possess broad differentiation capacities.[1-3] Recent studies suggested the difficulty discriminating fibroblasts from MSCs based on phenotype (morphology and specific protein markers) or growth capacity.[4-6] These works proposed pluripotency as an exclusive feature of MSCs or identified relationships between cell phenotype and gene expression profile. Conversely, previous data reported the ability of fibroblasts to differentiate into osteocytes,[7-14] or chondrocytes.[15,16] While adipose differentiation was thought to be reserved for embryonic fibroblasts or 3T3-L1 cell line,[17,18] Feldon et al. recently obtained adipocytes from human orbital fibroblasts.[19] However, there is to date no evidence that fibroblasts possess endodermal differentiation potential. In this work, we analyzed the in vitro mesodermal (osteocyte and adipocyte) and endodermal (hepatocyte) differentiation capacities of human skin fibroblasts. We then confirmed the endodermal differentiation potential of these cells in vivo after transplantation into hepatectomized severe combined immunodeficient (SCID) mice. We used human bone marrow (hBM) MSCs as a control for all experiments.

In parallel, we explored whether fibroblasts present an epithelial-mesenchymal transition (EMT) expression profile throughout differentiation and dedifferentiation processes. As reported for hepatic stellate cells,[20] we observed that fibroblast-derived hepatocyte-like cells (FDHLCs) still expressed mesodermal marker $\alpha$-smooth

*Abbreviations: αFP, alpha-fetoprotein; ASMA, α-smooth muscle actin; BM, bone marrow; BSA, bovine serum albumin; CK18, cytokeratin 18; FDHLC, fibroblast-derived hepatocyte-like cell; MSC, mesenchymal stem cell; hBM, human bone marrow; MDHLC, mesenchymal-derived hepatocyte-like cell; EMT, epithelial-mesenchymal transition; DF, dedifferentiated fibroblast; FBS, fetal bovine serum; MNC, mononuclear cell; RT, room temperature; CX-32, connexin-32; DPPIV, dipeptidylpeptidase IV; PD, population doublings; FDO, fibroblast-derived osteocyte; MDO, mesenchymal-derived osteocyte; FDA, fibroblast-derived adipocyte; MDA, mesenchymal-derived adipocyte; PAS, periodic acid schiff; HH, human primary hepatocyte; LCS, human liver cell suspension; SaOs, osteosarcoma cells; SCID, severe combined immunodeficient; HA, human dermal adipocyte; MCF7, human breast adenocarcinoma cell line; UF, undifferentiated fibroblast.*

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muscle actin (ASMA) whereas dedifferentiated fibroblasts (DFs) lost the acquired hepatocyte-like phenotype and neo-expressed slug gene, confirming that in both processes cells remained in an EMT state.

## Material and Methods

***Ethical Committee.*** This study was approved by the local institutional human ethical committee.

***Cell Isolation and Culture.*** Human fibroblasts (n = 5) were collected by skin biopsy (medio-anterior side of the forearm) of 8-year-old to 35-year-old volunteers after written informed consent. Cells were grown on culture dishes with AmnioMax-C100 Basal Medium containing C100 Supplement (Invitrogen). After reaching confluence, cells were detached using 0.05% trypsin–1 mM ethylene diamine tetraacetic acid (EDTA) (Invitrogen) and replated at $0.5 \times 10^4$ cells/cm$^2$ in $\alpha$MEM medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Perbio) and 1% penicillin/streptomycin (Invitrogen) (defined as growth medium). Supernumerary cells were suspended in FBS containing 10% dimethyl sulfoxide (VWR) and frozen in 1-mL vials in liquid nitrogen. When necessary, frozen aliquots were thawed, plated in culture flasks at $1 \times 10^3$ cells/cm$^2$ and handled as fresh cultures. Bone marrow (BM) samples (n = 13) were collected by aspiration of vertebrae of postmortem donors. BM aspirates were collected into heparinized syringes containing 10% Hank's balanced salt solution (Invitrogen) and processed within 48 hours. Mononuclear cells (MNCs) were separated by centrifugation at 400*g* for 20 minutes at 20°C after carefully loading BM on Ficoll-Paque PLUS (Amersham Biosciences). Cells were then washed twice with phosphate-buffered saline (PBS, Invitrogen). MNCs were seeded in culture flasks at 1 to $6 \times 10^6$ cells/cm$^2$ in growth medium. First medium change was performed after 48 to 72 hours and then twice weekly. Passaging of both cell types was performed at 70% confluence with 0.05% trypsin–EDTA and cells were replated at $0.5 \times 10^4$ cells/cm$^2$ in growth medium. Cells were cultured at 37°C in a humidified atmosphere containing 5% $CO_2$. Fibroblast colony forming unit (CFU-F) assays were performed by plating 0.5 to 10 cells/cm$^2$ in growth medium. Plates were controlled after 10 days for presence of colonies. 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.[21]

***Transmission Electron Microscopy.*** Skin biopsy samples were fixed with 2.5% electron microscopy grade glutaraldehyde (Agar Scientific) buffered in 0.1 M sodium cacodylate for 48 hours, postfixed in 1% osmium tetroxide (Agar Scientific), and embedded in Epoxy Embedding Medium (Fluka Chemie, Buchs, Switzerland). Semithin sections were contrasted with uranyl acetate and lead citrate before examination in transmission electron microscopy with a Zeiss EM109 (Carl Zeiss Inc., Oberkochen, Germany).

***Mesodermal Differentiation.*** Cells were plated at $1.5 \times 10^4$ cells/cm$^2$ in uncoated 6-well dishes. To obtain osteogenic differentiation, cells were incubated with growth medium containing 0.1 $\mu$M dexamethasone, 0.05 mM ascorbate and 10 mM $\beta$-glycerophosphate (all from Sigma), and changed twice a week. After 4 weeks, calcium deposition was evaluated by von Kossa and alizarin red stainings. For adipogenic differentiation, cells were incubated with growth medium containing 1 $\mu$M dexamethasone, 0.5 mM isobutyl-methylxanthin, 0.2 mM indomethacin (all from Sigma) and 10 $\mu$g/mL insulin (Lilly) with medium change twice a week. After 4 weeks, lipid vesicles were revealed by Oil Red-O staining.

***Hepatocyte Differentiation.*** For hepatocyte differentiation, cells were plated at $1.5 \times 10^4$ cells/cm$^2$ on collagen type I coated dishes. Cells at passage 1 to 3 were incubated with 20 ng/mL HGF (Peprotech), 10 ng/mL FGF4 (Peprotech), 1% insulin-transferrin-selenium (ITS premix, Invitrogen) and 0.61 g/L nicotinamide (Sigma) in serum-free Iscove's modified Dulbecco's medium (IMDM, Invitrogen) for 10 days (referred as induction medium), and then with 20 ng/mL OSM (Peprotech), 1 $\mu$M dexamethasone (Sigma) and 1% ITS premix in the same basal medium for $\geq$ 20 days (maturation medium). For negative control, cells were incubated with serum-free IMDM.

***Flow Cytometry.*** Trypsinized cells were suspended at a concentration of 0.5 to $1 \times 10^3/\mu$L in PBS and then incubated for 30 minutes at 4°C with fluorescence-conjugated monoclonal antibodies and control mouse isotypes as specified in Table 1. Cells were then washed and resuspended in Isoton (Beckman Coulter) for reading with a FACS Calibur Flow Cytometer (Becton Dickinson). Intracytoplasmic human albumin staining was performed as follows: $2 \times 10^5$ cells were incubated in 200 $\mu$L Cytofix (BD Bioscience) for 20 minutes at room temperature (RT), washed with 1 mL 1× Perm/Wash solution (BD Bioscience) and centrifugated for 5 minutes at 805 g. Cell pellets were suspended in 50 $\mu$L Perm/Wash and incubated with 1 $\mu$L fluorescein isothiocyanate (FITC)-labeled rabbit anti-human albumin antibody (Cedarlane) or 1 $\mu$L FITC-labeled IgG1 control isotype (BD Pharmingen) for 25 minutes at RT. After washing cells with 1 mL PBS and centrifugating for 5 minutes at 805 g, cells

**Table 1. Description of Fluorescence-Conjugated Monoclonal Antibodies Used for Flow Cytometry**

| Antigen specificity | Fluorescence | Isotype | Provenance (MoAb – isotypes) |
|---|---|---|---|
| **Mesenchymal lineage markers** | | | |
| CD105 | FITC | IgM | 2 – 2 |
| CD73 | PE | IgG1 | 1 – 1 |
| CD90 | PE-Cy5 | IgG1 | 1 – 6 |
| CD29 | PE | IgG1 | 1 – 1 |
| CD44 | PE | IgG2b | 3 – 5 |
| CD49b | FITC | IgG1 | 3 – 5 |
| CD49e | FITC | IgG2b | 3 – 5 |
| CD71 | PE | IgG1 | 3 – 1 |
| **Hematopoietic lineage markers** | | | |
| CD45 | APC | IgG1 | 1 – 1 |
| CD34 | APC | IgG1 | 1 – 1 |
| CD14 | PE-Cy5 | IgG2a | 1 – 5 |
| CD13 | PE | IgG1 | 4 – 1 |
| CD117 | PE-Cy5 | IgG1 | 3 – 6 |
| **Immunoreactivity markers** | | | |
| CD80 | PE | IgG1 | 3 – 1 |
| CD86 | PE | IgG1 | 1 – 1 |
| HLA-DR | FITC | IgG2a | 1 – 6 |
| HLA-ABC | PE | IgG2a | 4 – 5 |
| **Hepatocyte markers** | | | |
| CD26 | PE | IgG1 | 3 – 1 |
| Albumin | FITC | IgG1 | 7 – 1 |

Abbreviations: APC, allophycocyanin; FITC, fluorescein isothiocyanate; MoAb, monoclonal antibody; PE, phycoerythrin; PE-Cy5, phycoerythrin-cyanin 5. Provenance of monoclonal antibodies and control isotypes is specified as follows: (1) Becton Dickinson, (2) Immunotech, (3) Dako Systems, (4) Beckman Coulter, (5) Serotec, (6) BD Pharmingen, (7) Cedarlane.

were fixed in 250 $\mu$L Cellfix (BD Bioscience) and read on a FACSCalibur flow cytometer.

***Immunocytochemistry.*** Cells were fixed with 3.5% formaldehyde for 15 minutes, then permeabilized with 1% Triton X-100 (Sigma) in tris-buffered saline (TBS) buffer (pH 7.5) for 15 minutes and rinsed with TBS. After 1 hour incubation with blocking solution (3% dry milk in TBS), cells were rinsed with TBS and then incubated for 1 hour at RT with primary mouse anti-human monoclonal antibodies as follows: 1/50 vimentin, 1/50 albumin (Sigma); 1/50 ASMA, 1/50 HepPar1 (Dako Cytomation); 1/50 CK18 (Progen); 1/50 CK8, 1/200 connexin-32 (CX-32) (Chemicon); 1/20 dipeptidylpeptidase-IV (DPPIV) (Ancell); 1/50 CD200 (GeneTex); 1/100 E-cadherin (BD Pharmingen) and rabbit polyclonal anti-human antibodies as follows: 1/50 laminin, 1/100 fibronectin, 1/100 $\alpha$FP (Dako Cytomation). Cells were washed 5 times and primary antibodies were revealed with 1 hour incubation with Cy3-coupled anti-mouse IgG (Jackson Immunoresearch Laboratories) and FITC-coupled anti-rabbit IgG (Sigma). After rinsing, nuclei were revealed by 30 minutes staining with 4,6-diamidino-2-phenylindol (DAPI, Sigma). For immunoperoxydase staining, cells were incubated after formaldehyde fixation with 30% hydrogen peroxide (Sigma) for 3 minutes. They were washed with distilled water and then permeabilized with 1% Triton X-100. After 1 hour blockade in 1% bovine serum albumin (BSA, Sigma), cells were incubated for 1 hour at RT with primary antibodies in 0.1% BSA, washed with PBS, and exposed for 30 minutes to anti-rabbit or anti-mouse Dako Envision Horseradish Peroxidase (HRP) antibody (Dako Cytomation). Staining was performed by 3 minutes exposition to diaminobenzidine (Sigma). After rinsing, nuclei were colored with Mayer's hematoxylin (10 minutes) and cells were mounted. Each antibody was tested on fresh (<24 hours) cultures of human primary hepatocytes (HHs) as positive control and MSCs or fibroblasts as negative controls.

***Reverse-Transcription Polymerase Chain Reaction.*** Total RNA was extracted from cultured cells using the TriPure isolation reagent (Roche) and complementary DNA 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 (Supplementary Table 1) and polymerase elongase (Invitrogen) in a final volume of 25 $\mu$L. Amplifications are specified in Supplementary Table 1. All PCR experiments were performed at 30 cycles. Samples were electrophoresed on a 1% agarose gel and nucleic acids were visualized by ethidium bromide staining.

***Glycogen Staining.*** After 3.5% formaldehyde fixation, cells were incubated for 10 minutes in 1% periodic acid (Sigma), washed with distilled water and incubated with Schiff's reagent (Sigma) for 15 minutes. After rinsing 10 minutes with tap water, nuclei were stained 10 minutes with Karazi's hematoxylin. Cells were then washed with tap water and mounted. Freshly cultured (<24 hours) mouse hepatocytes served as positive control. Fibroblasts and MSCs were used as negative controls.

***Urea Assay.*** Urea concentrations of culture supernatants were screened by a colorimetric assay (Gentaur), handled as per manufacturer's instructions and analyzed with a HTS7000PLUS absorbance reader (PerkinElmer). Serum-free IMDM incubated with freshly cultured (<24 hours) mouse hepatocytes served as positive control. Serum-free IMDM served as negative control.

***Albumin Assay.*** Human albumin concentrations of culture supernatants were screened by an enzyme-linked immunosorbent assay (ELISA, Cygnus Technologies) per the manufacturer's instructions and analyzed with a Multiskan EX absorbance reader (Thermo). Serum-free IMDM incubated with freshly cultured (<24 hours) HHs served as positive control. Serum-free IMDM served as negative control.

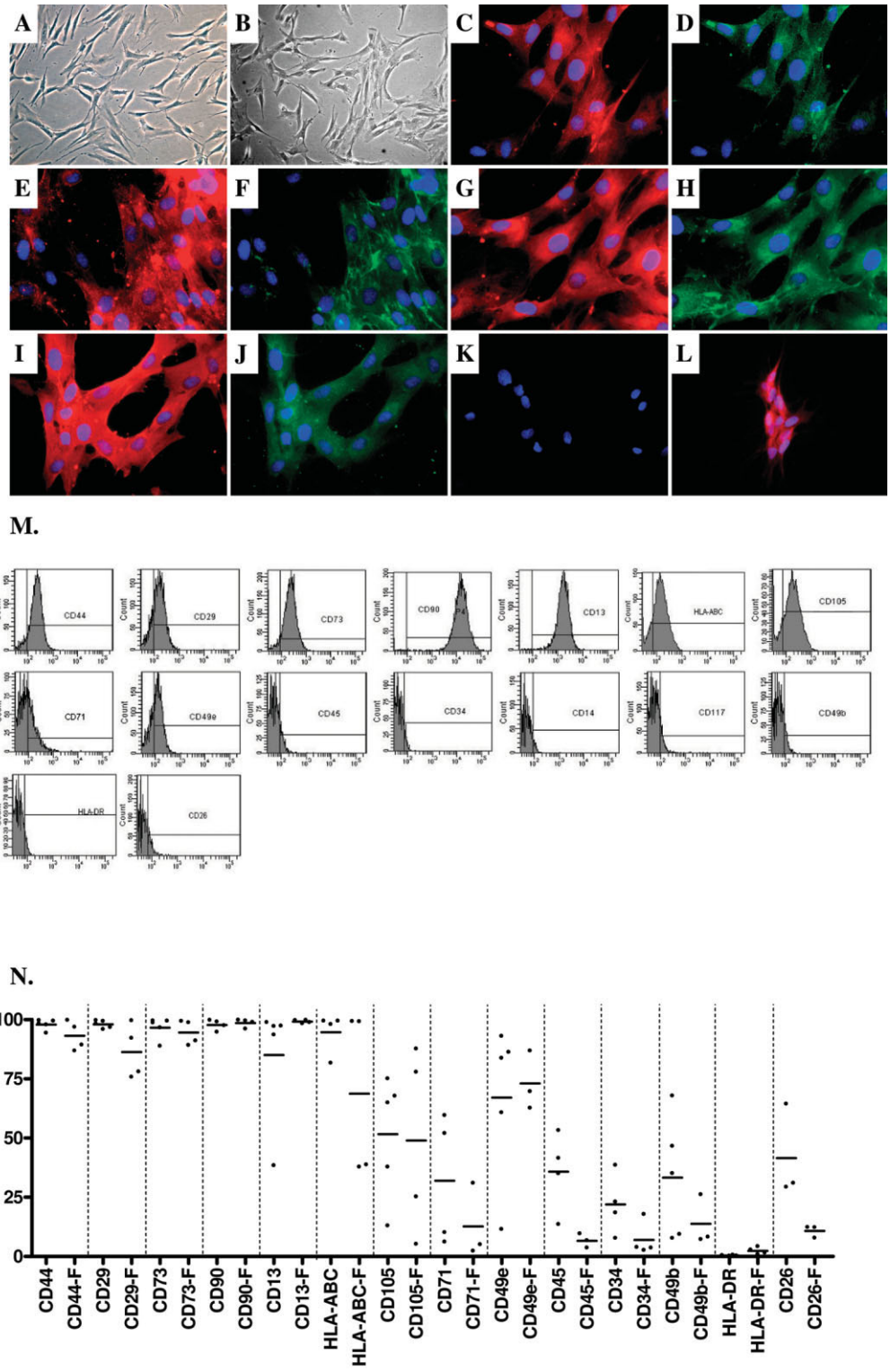


Fig. 1. Comparative fibroblasts and MSCs characterization. Morphological aspect of fibroblasts (A, day 13) and MSCs (B, day 15) in first passage growth culture. Immunofluorescence assay showing stainings of fibroblasts (C-F) and MSCs (G-J) for vimentin (C,G), fibronectin (D, H), ASMA (E,I) and laminin (F,J) (n =3 each). Absence of CD200 expression in fibroblasts (K) as shown by immunocytofluorescence assay whereas this marker was positively detected in human neuroblastoma SH-SY5Y cells (n = 3). Nuclei were revealed by DAPI staining. Pictures magnifications are (A-J) 400× and (K,L) 200×. (M) Example of representative flow cytometric profile of fibroblasts at first passage. Analyzed epitopes are indicated in the histograms. Bars indicate the fluorescence level of corresponding isotype. (N) Comparative cytometric phenotype of MSCs and human skin fibroblasts (both cells from passages 2 to 4). Fibroblasts are indicated by (-f) suffixes.

***Estimation of Cell Rates.*** Cell rates estimation was performed by counting positive cells (for hepatocyte-like morphology or glycogen storage) in 3 different random fields (200× magnification) of each 3 different culture samples. Total cell counts are provided for each experiment.

***Cell Transplantation.*** Cell transplantations were performed on 8-week-old to 12-week-old female SCID mice. The animals were randomly assigned into 2 groups. In the first group, undifferentiated fibroblasts (UFs) were injected into the spleen of hepatectomized mice. In the second group, fibroblasts were injected into the liver of hepatectomized mice. Hepatectomy was performed on left or anterior liver lobes when associated respectively to spleen or liver infusion and cells were infused after the surgical procedure. Each group of mice was constituted of 3 control mice receiving 100 $\mu$L PBS and 6 sample mice receiving injections of $1 \times 10^6$ cells suspended in PBS. After 3 weeks, serum was collected and tissues (liver and spleen) were removed and paraffin enrobed for further analyses.

***Immunohistochemistry.*** Liver sections (5 $\mu$m) were deparaffinized with Histosafe, isopropanol, and methanol solutions. Epitope retrieval was performed by incubating sections for 75 minutes in 0.02 M citrate buffer (pH 5.8) heated to 97°C. Endogenous peroxydase was inactivated with 0.3% hydrogen peroxide in methanol for 15 minutes at RT. Unspecific binding was blocked by incubating sections for 30 minutes at RT in PBS with 1% BSA. Tissues were stained with mouse monoclonal anti-human albumin (1/250) antibody using avidin-biotin technique with DakoCytomation ARK as per manufacturer's instructions. Polyclonal anti-human antibodies were incubated overnight at RT at the following concentrations: 1/3000 rabbit anti-human fibronectin, 1/1000 rabbit anti-human $\alpha$FP and 1/200 guinea pig anti-human CK18 (Progen). Sections were rinsed with PBS and then incubated for 30 minutes at RT with anti-rabbit Dako Envision HRP antibody or for 45 minutes with HRP-conjugated goat anti-guinea pig antibody (Progen). Sections were washed and immunoreactivity was revealed by 3 minutes exposition with diaminobenzidine. After rinsing, nuclei were counterstained with Mayer's hematoxylin for 10 minutes and then sections were mounted. Controls for albumin and fibronectin staining were performed on human or mice liver sections.

***Statistics.*** Results are expressed as mean ± standard deviation (SD) and statistically ($^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$) significant differences were assessed by unpaired Student *t* test.

## Results

We first compared fibroblasts and MSCs at the phenotype level. Although both cell types displayed similar in vitro spindle-shaped morphology (Fig. 1A,B), fibroblasts

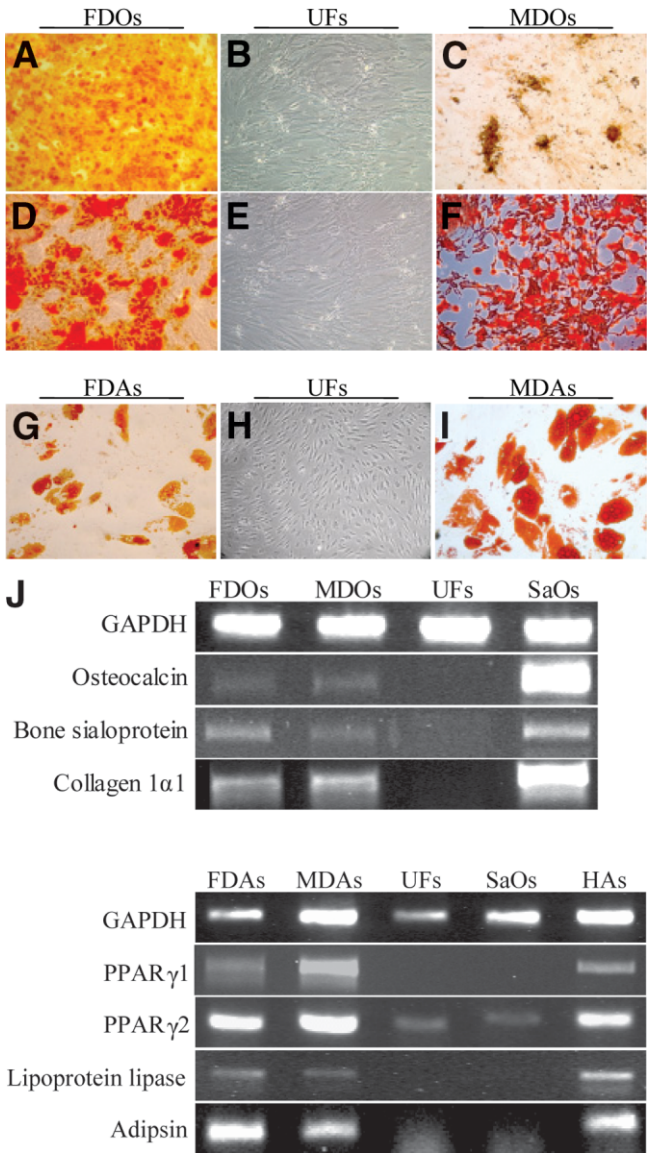


Fig. 2. Comparative mesodermal differentiation of fibroblasts and MSCs. Aspect of extracellular calcium mineralization in (A,D) fibroblasts and (C,F) MSCs after osteocyte differentiation revealed by (A-C) von Kossa and (D-F) alizarin red stains. Intracytoplasmic lipid-rich droplets stained by Oil Red O assay in (G) adipocyte differentiated fibroblasts and (I) MSCs. No staining could be observed in undifferentiated fibroblasts (B,E,H). (J) RT-PCR analysis showing comparable gene expression of osteocyte (osteocalcin, bone sialoprotein and collagen type I-$\alpha$1) and adipocyte (PPAR$\gamma$1, PPAR$\gamma$2, lipoprotein lipase, and adipsin) markers in differentiated fibroblasts and MSCs. Whereas PPAR$\gamma$2 was slightly expressed in UFs and SaOs, all analyzed markers were neo-expressed in differentiated cells. Abbreviations: FDAs, fibroblasts-derived adipocytes; FDOs, fibroblasts-derived osteocytes; HAs, human adipocytes; MDAs, mesenchymal-derived adipocytes; MDOs, mesenchymal-derived osteocytes; SaOs, osteosarcoma cells; UFs, undifferentiated fibroblasts. Pictures were taken at (A-F, H) 100× and (G,I) 400× magnifications.

had greatest proliferation capacity with significantly superior growth rate (doubling time of 3.8 ± 1.5 days for fibroblasts (n = 3) versus 8.0 ± 3.7 days for MSCs (n = 3) within the first 10 passages, $^*P < 0.05$). Furthermore, fibroblast cultures performed up to 52 population doublings (PD) within 15 passages whereas MSCs stopped proliferating at passage 8 and grewed up to 34 PD. As mesodermal lineages, fibroblasts and MSCs displayed comparable expression profile of mesodermal antigens (vimentin, fibronectin, ASMA, laminin) as demonstrated by immunofluorescence (Fig. 1C-J). Similarly, equivalent immunophenotype was observed in both cell types by flow cytometry with high expression of CD44, CD29, CD73, CD90, CD13 and HLA-ABC, moderate expression of CD105, CD71, CD49e and low expression of CD45, CD34, CD14, CD117, CD49b, HLA-DR and CD26 (Fig. 1M,N). Both cell types were negative for CD80 and CD86 (data not shown). Fibroblasts cultures were ascertained for the absence of contaminating epidermal stem cells by sampling biopsies in follicle-free skin region (medio-anterior side of the forearm) and by analysing these biopsies using optic and electron microscopy which confirmed the lack of follicula structures in these samples (Supplementary Fig. 1). These data were corroborated by showing lack of CD200 expression in the cultured cells (Fig. 1K).[22]

After having observed phenotypic homology of fibroblasts and MSCs, we compared mesodermal differentiation capacity of these cells to further delineate their pluripotency. By classical mesodermal differentiation assays, we observed that fibroblasts up to passage 3 were able to acquire morphological characteristics of osteocytes as assessed by von Kossa and alizarin red stains revealing deposition of mineral matrix (Fig. 2A,D). Fibroblasts could also accumulate intracytoplasmic lipid-rich droplets, a feature of adipocytes demonstrated by Oil Red O staining (Fig. 2G). These features were acquired in a similar fashion than MSCs. We confirmed these results by analysing the gene expression profile of fibroblasts-derived mesodermal lineages by RT-PCR (Fig. 2J). Fibroblast-derived osteocytes (FDOs) neo-expressed osteocyte-specific genes (osteocalcin, bone sialoprotein, collagen type 1-α1) that were not detected in UFs. Up-regulation of PPAR-γ2 was observed in fibroblasts after adipocyte differentiation whereas this marker was slightly expressed after osteocyte differentiation and in UFs sug-

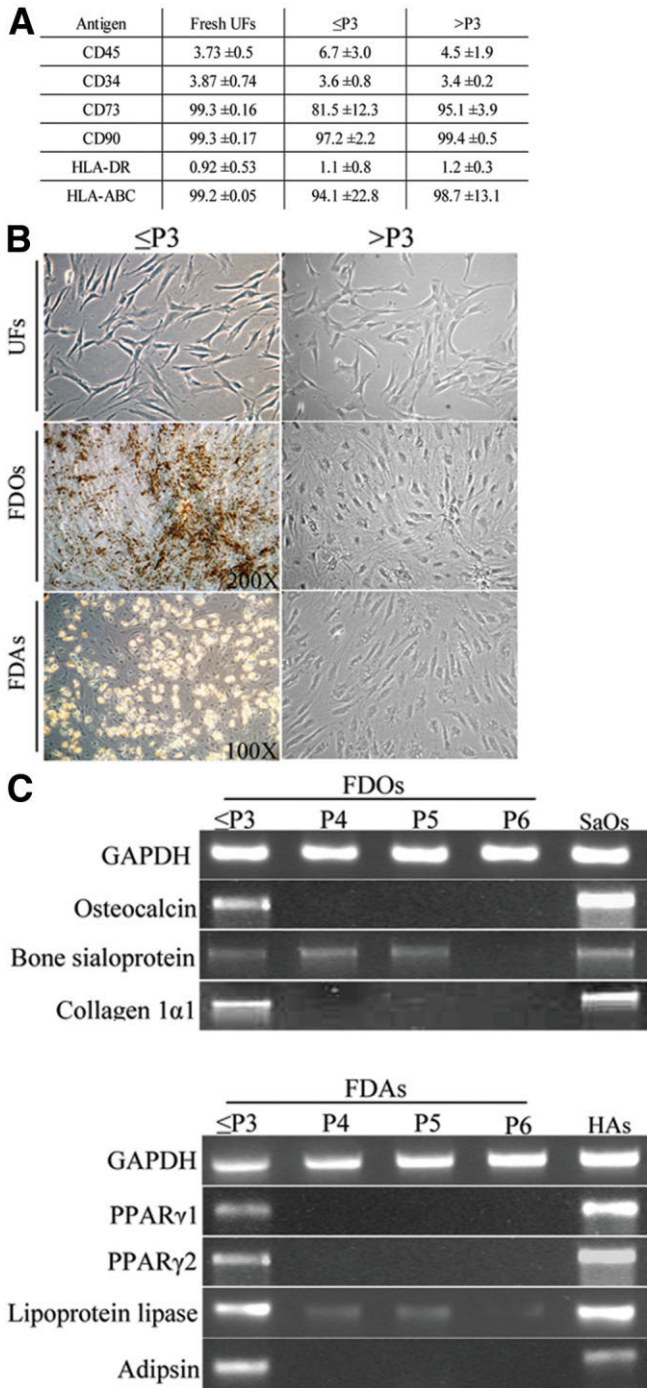


Fig. 3. Comparison of mesodermal differentiation potential of fresh, cryopreserved (≤P3) and subcultured (>P3) cryopreserved fibroblasts. (A) Analysis of cell phenotype (hematopoietic, mesenchymal and immunological markers) by flow cytometry revealing no statistically significant difference between fresh, cryopreserved and subcultured (>P3) fibroblasts populations. (B) Pictures show comparable morphology of undifferentiated fibroblasts cultivated less or more than 3 passages and loss of mesodermal differentiation (calcium matrix deposition or intracytoplasmic lipid-rich droplets) potential of subcultured (>P3) cryopreserved fibroblasts. (C) RT-PCR analysis confirmed the lack of mesodermal differentiation of >P3 fibroblasts as these cells did not express most of the osteocyte-specific and adipocyte-specific genes. For each group, data are representative of 3 independent experiments. Abbreviations: FDAs, fibroblast-derived adipocytes; FDOs, fibroblast-derived osteocytes; HAs, human adipocytes; UFs, undifferentiated fibroblasts; SaOs, osteosarcoma cells. Unless indicated, pictures were taken at 400× magnification.

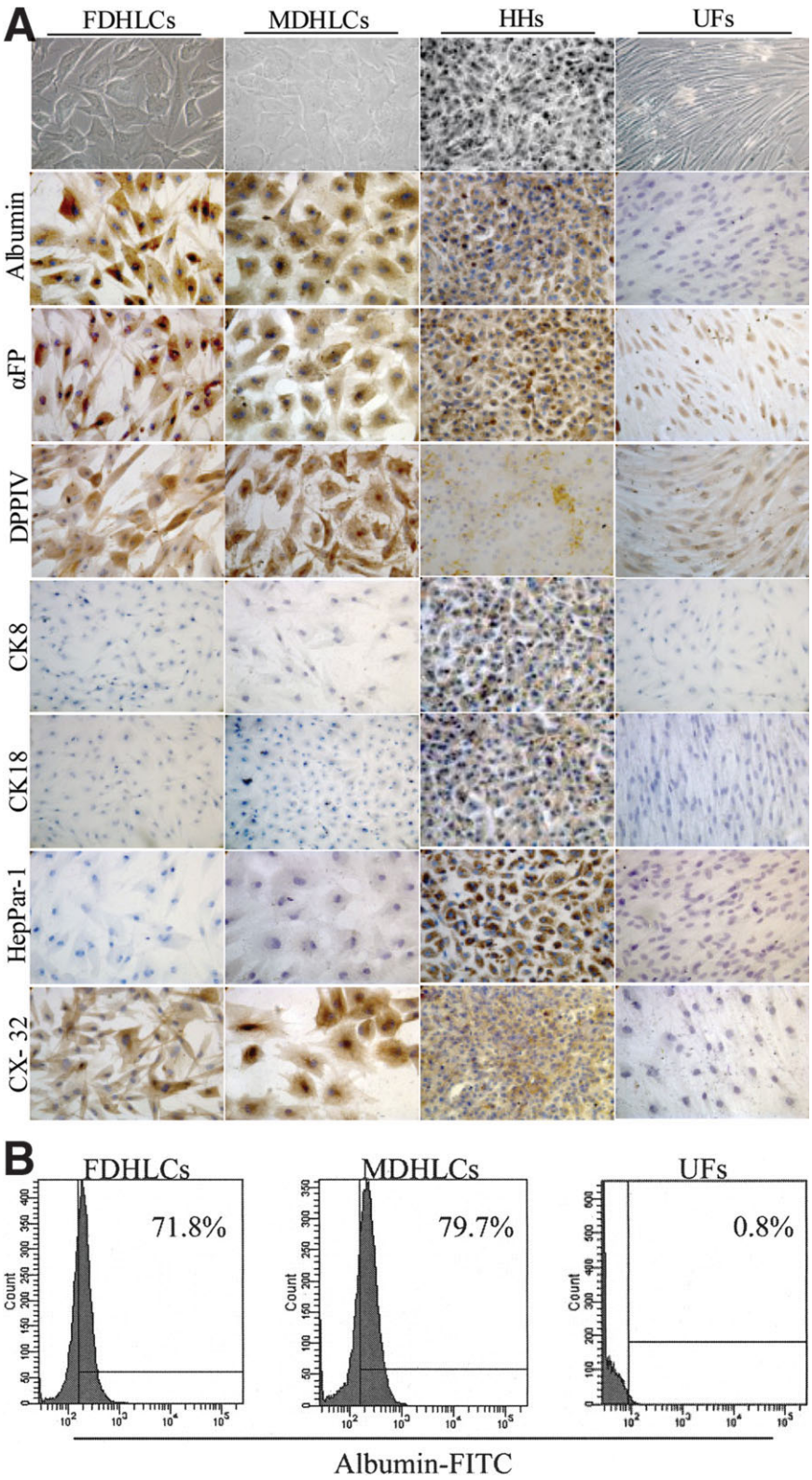


Fig. 4. Comparative immunostaining of hepatocyte-specific antigens expression in FDHLCs and MDHLCs. (A) Pictures show that after 35 days of in vitro hepatocyte differentiation, FDHLCs and MDHLCs acquired a polygonal-shaped morphology with intracytoplasmic granulations. The comparison of hepatocyte marker expression of both cell types revealed similar positivity for albumin, αFP, DPPIV, and CX-32 whereas those cells were negative for CK8, CK18, and HepPar-1 epithelial markers. Positive and negative controls of corresponding immunomarking were performed respectively on human liver cells and UFs. Pictures were taken at 400× magnification. (B) Representative examples of flow cytometric albumin immunostaining in FDHLCs, MDHLCs, and UFs. For each sample, positivity value is indicated in the corresponding histogram. Abbreviations: αFP, alpha-1-fetoprotein; CX-32, connexin-32; CK, cytokeratin; DPPIV, dipeptidylpeptidase IV; FDHLCs, fibroblast-derived hepatocyte-like cells; HHs, human hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells; UFs, undifferentiated fibroblasts.

gesting less specificity of this marker, as confirmed elsewhere.[23] However, fibroblast-derived adipocytes (FDAs) displayed neo-expression of PPAR-γ1, lipoprotein lipase and adipsin. We obtained similar results on cytochemistry and RNA expression assays after mesodermal differentiation of MSCs (data not shown). However, we were not able to differentiate fibroblasts aged more than 3 passages as described in Fig. 3. To compare differentiation potential of fibroblasts cultured less or more than 3 passages, we used fresh (<P3)

cells and thawed cryopreserved fibroblasts for subculture (>P3). We noticed that cell phenotype of the 3 cell populations (fresh and ≤P3 or >P3 cryopreserved fibroblasts) was comparable as assessed by flow cytometry (Fig. 3A) and cell morphology (Fig. 3B). However, subcultured (>P3) fibroblasts were not able to synthesize mineral matrix or intracytoplasmic lipid-rich droplets after mesodermal differentiation as confirmed by cytochemistry and RT-PCR assays (Fig. 3B,C).

After having assessed the mesodermal differentiation potential of fibroblasts, we assayed these cells for endodermal hepatocyte differentiation. We observed that in vitro conditioned fibroblasts acquired a morphology defined as round or polygonal shaped cells reduced in size and containing cytoplasmic granulations and central nucleus with prominent nucleolus (Fig. 4A). FDHLCs morphology appeared in the maturation step as for mesenchymal-derived hepatocyte-like cells (MDHLCs) (Fig. 4A). Similar counts of hepatocyte-like cells were estimated in FDHLCs and MDHLCs populations (respectively 61.6 ± 7.6% [n = 675] and 61.3 ± 1.2% [n = 921]). This phenotype was observed on each sample induced to differentiate and could be maintained in culture up to 10 weeks. Using immunocytochemistry, we performed comparative analysis of hepatocyte-specific antigen expression in FDHLCs and MDHLCs and observed highly similar profiles. Both cell types positively stained for albumin, αFP, DPPIV and epithelial marker CX-32 but were negative for CK8, 18 and HepPar-1 (Fig. 4A). All these markers were expressed by HHs whereas undifferentiated fibroblasts did not stain positive for hepatocyte-specific protein markers except for DPPIV in small proportion (Fig. 4A) as confirmed by flow cytometry analysis of CD26 expression. By comparison, DPPIV staining was strongly enhanced in FDHLCs (Fig. 4A). The average of albumin-positive cells in the FDHLCs population was estimated by flow cytometry at 65.2 ± 13.3% (Fig. 4B) while MDHLCs population contained 73.4 ± 17.1% albumin positive cells and UFs were negative for albumin staining (0.9 ± 0.4 %) (n = 3 for each cell type).

Hepatocyte-specific markers expression analysis by RT-PCR revealed that FDHLCs expressed albumin, αFP, α1-antitrypsin, G6Pase, TDO, GS and HGF receptor c-met (Fig. 5). However, FDHLCs lacked expression of some other immature or mature hepatocyte markers as HNF4, PEPCK, TAT, CYP3A4, CYP2B6 and also epithelial markers as CK8 and CK18. Comparatively, MDHLCs expressed larger amount of hepatocyte-specific markers and also the epithelial marker CK8 but not CK18. While MSCs naturally expressed HGF receptor c-met, fibroblasts lineages needed in vitro conditioning to express this marker. Both MDHLCs and FDHLCs displayed persistent expression of mesodermal marker vimentin. According to what we observed after mesodermal differentiation of subcultured (>P3) fibroblasts, we were not able to induce the expression of hepatocyte-specific markers within these cells on the protein or mRNA level as depicted in Fig. 6.

At the functional level, FDHLCs displayed ability to store glycogen and we estimated 21.7 ± 5.1% of Periodic Acid Schiff (PAS) positive cells in these populations (n = 804) versus 21.1 ± 5.3% PAS-positive cells in the MDHLCs population (n = 1041) (Fig. 7). Comparatively, 75.5 ± 6.9% of the HHs population (n = 1134)

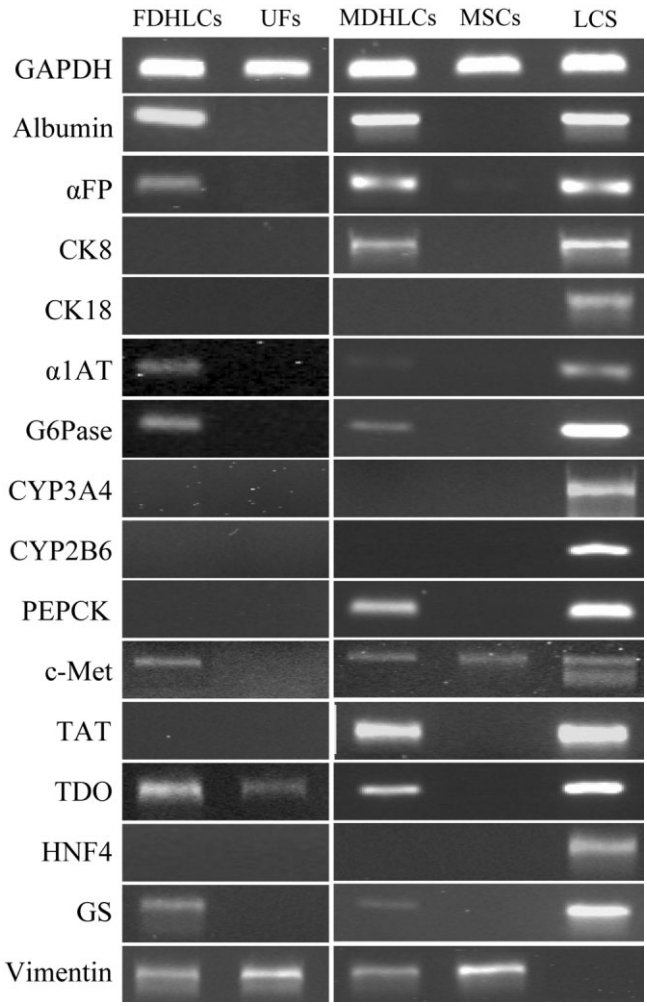


Fig. 5. Compared gene expression analysis in FDHLCs and MDHLCs by RT-PCR. The figure shows concomitant immature (αFP) and mature (α1AT, G6Pase, TDO, and GS) hepatocyte marker expression in both FDHLCs and MDHLCs whereas other markers are not expressed (HNF4, CYP2A3, CYP2B6). MDHLCs expressed some liver-specific markers lacking in FDHLCs (CK8, PEPCK, TAT). Although MSCs constitutively expressed HGF receptor c-met, fibroblasts needed in vitro conditioning to express this transcript. Both MDHLCs and FDHLCs displayed persistence of mesodermal marker vimentin. Abbreviations: FDHLCs, fibroblasts-derived hepatocyte-like cells; LCS, human liver cell suspension; MDHLCs, mesenchymal-derived hepatocyte-like cells; MSCs, mesenchymal stem cells; UFs, undifferentiated fibroblasts. Abbreviations for oligonucleotides are listed in Table 1.

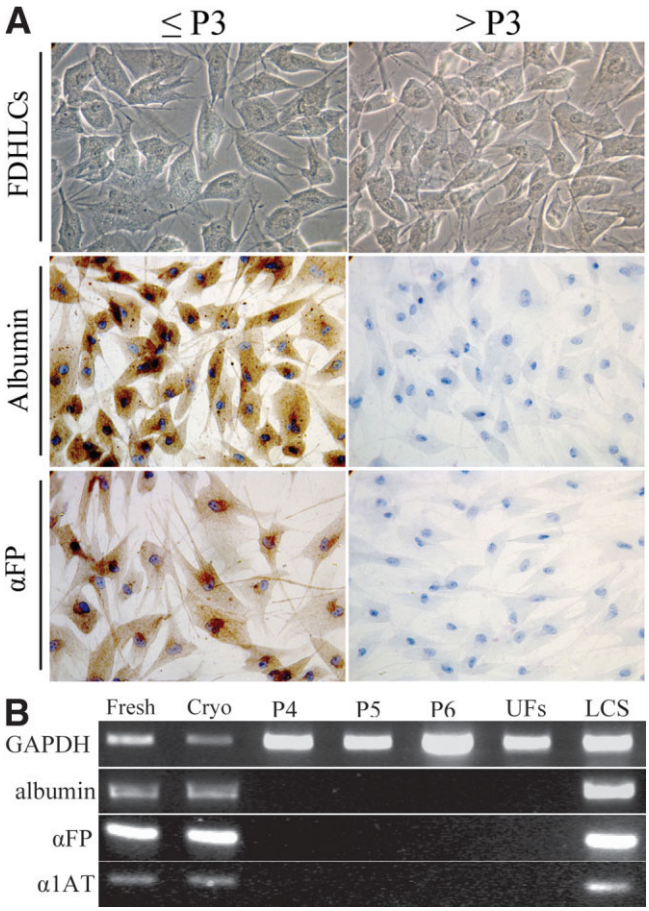


Fig. 6. Comparison of hepatocyte differentiation potential of fresh, cryopreserved (≤P3) and subcultured (>P3) cryopreserved fibroblasts. (A) Although hepatocyte-like morphology was similarly acquired in ≤P3 and >P3 cultured fibroblasts, these latter cells were not able to express albumin and αFP as shown by immunocytochemistry assay. (B) RT-PCR confirmed that no difference could be observed in albumin, αFP, and α1AT expression after hepatocyte differentiation of fresh or cryopreserved fibroblasts. However, differentiation of >P3 cryopreserved fibroblasts did not allow the expression of these hepatocyte-specific markers. For each group, data are representative of 3 independent experiments. Abbreviations: α1AT, alpha-1 antitrypsin; αFP, alpha-fetoprotein; Cryo, cryopreserved (<P3) fibroblasts; FDHLCs, fibroblast-derived hepatocyte-like cells; Fresh, fresh (<P3) fibroblasts; LCS, liver cell suspension; UFs, undifferentiated fibroblasts. Pictures were taken at 400× magnification.

presented glycogen storage (Fig. 7C) while no staining was noticed in the MSCs population (Fig. 7D). Supernatant analysis reported that urea secretion level of FDHLCs was not significantly different from MDHLCs or human hepatocytes (respectively 0.62 ± 0.31, 1.09 ± 0.23 and 1.37 ± 0.68 mM/30,000 cells*day) but that these levels were significantly higher to control urea level (0.08 ± 0.06 mM/30,000 cells*day) (Fig. 7). Human albumin was not detected by ELISA assay in the supernatants of FDHLCs or MDHLCs suggesting an negligeable amount of albumin producing cells in these populations.

To investigate whether human fibroblasts were able to engraft and differentiate into hepatocyte-like cells in vivo, we injected $1 \times 10^6$ UFs into the spleen or the liver of hepatectomized SCID-mice. Three weeks later, livers were harvested and analysed by immunohistochemistry for human mesodermal and endodermal proteins. No engraftment of human fibroblast could be observed when these cells were injected into the spleen (data not shown). However, when cells were transplanted directly into the left liver lobe of SCID mice, we observed that fibroblasts engrafted in mice livers as small cell clusters expressing

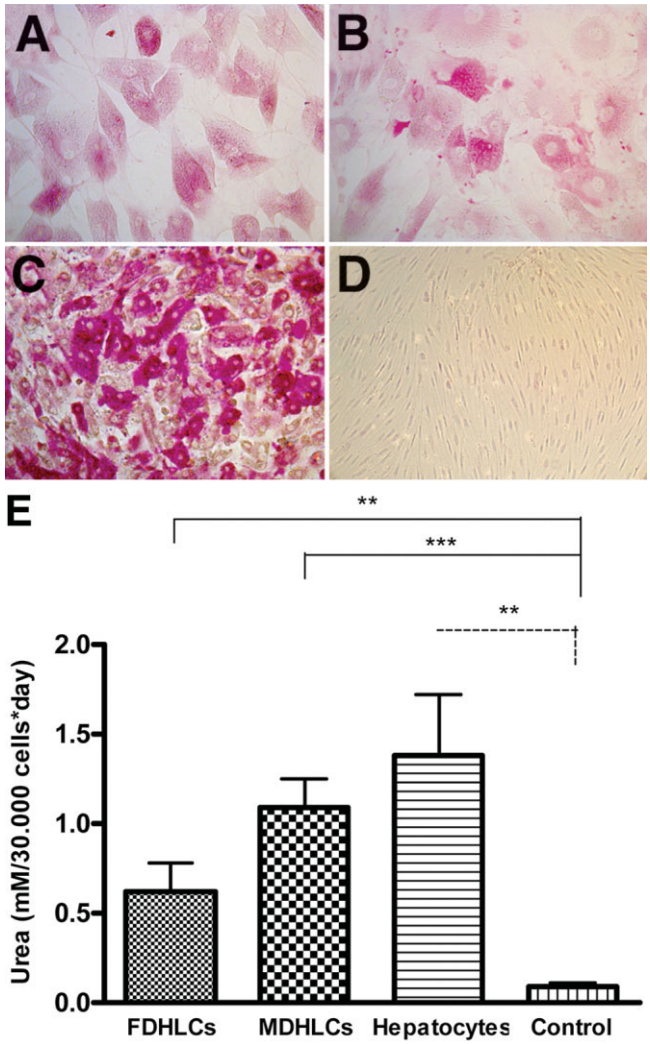


Fig. 7. Comparison of hepatocyte-like functionality of FDHLCs and MDHLCs. (A-D) Comparable glycogen cytoplasmic deposition was noticed in (A) FDHLCs and (B) MDHLCs by PAS staining. Positive and negative control stainings are presented respectively by (C) HHs and (D) UFs. (E) Urea production levels of FDHLCs (n = 4), MDHLCs (n = 4), and mouse hepatocytes (n = 4) were not statistically different whereas urea concentrations were significantly higher in FDHLCs, MDHLCs, and mouse hepatocyte supernatants than in IMDM controls (n = 6). Data represent mean ± SD of n independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ compared with control. Abbreviations: FDHLCs, fibroblast-derived hepatocyte-like cells; HHs, human hepatocytes; MDHLCs, mesenchymal-derived hepatocyte-like cells. Pictures were taken at 400× magnification.

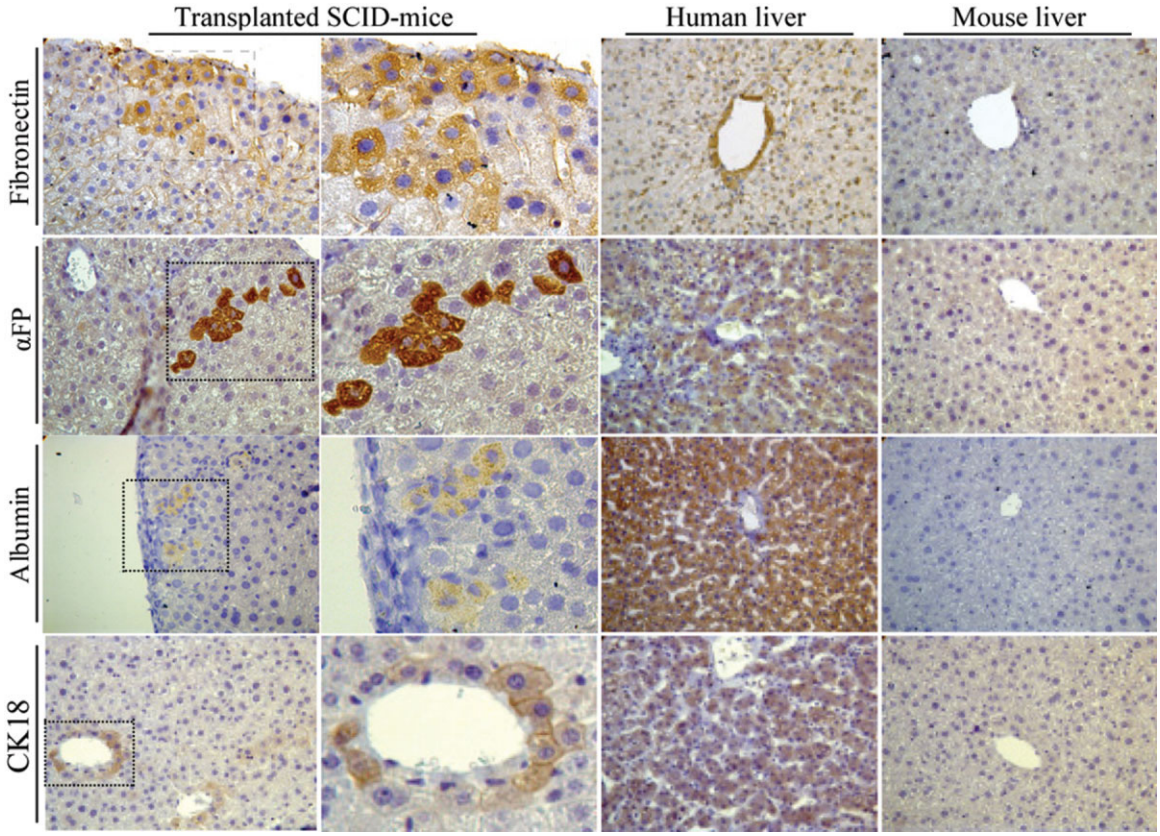


Fig. 8. In vivo hepatocyte differentiation of human skin fibroblasts. Pictures show immunohistochemical stains for human markers fibronectin, αFP, albumin, and CK18. Analysis of livers of transplanted SCID-mice revealed that fibroblasts engrafted as small clusters of cells presenting combined expression of mesenchymal marker fibronectin and liver-specific markers αFP, albumin, and CK18. Positive and negative control stains are presented, respectively, on human and mouse livers. Abbreviations: αFP, alpha-1-fetoprotein. Pictures were taken at 400× magnification.

both mesodermal marker fibronectin and hepatocyte markers albumin, αFP and CK18 (Fig. 8). When analyzed on adjacent serial sections, these cell clusters showed co-expression of human albumin, αFP and CK18 or of fibronectin and albumin (Supplementary Fig. 2). Although there was a low repopulation rate, the in vivo engraftment and cell expression profile was comparable to what we observed with hBM-MSCs transplanted into SCID mice.[24] Estimation of the engrafted cell number is provided in Supplementary Table 2. In order to seek out the hepatocyte-like functionality of in vivo engrafted cells, we evaluated the production of human albumin in mice sera by ELISA but did not detect this protein. We noticed analogous albumin ELISA results in sera of mice transplanted with hBM-MSCs.

Finally, we explored whether fibroblasts presented an EMT status during hepatocyte differentiation or dedifferentiation processes. For that purpose, we studied the expression of EMT markers (as snail, slug, notch-1, and e-cadherin) on UFs, FDHLCs, and dedifferentiated fibroblasts (DFs) by comparison with the expression profile of MCF-7 cells (human breast adenocarcinoma cell line) and human hepatocytes. DFs were obtained after incubating FDHLCs for 1 week with growth medium. These cells lost hepatocyte-like morphology and restructured their cytoskeleton to regain a spindle-shaped form (Fig. 9A,B). They restarted to proliferate and reached confluency in one week after being plated at $1 \times 10^6/cm^2$. Furthermore, DFs lost the expression of hepatocyte-specific mRNA markers albumin, αFP and α1AT (Fig. 9C). We observed that UFs expressed snail, notch-1 and e-cadherin which expression was down-regulated after hepatocyte differentiation (Fig. 9D). Slight neo-expression of slug and e-cadherin was observed in DFs whereas FDHLCs expressed none of these markers. Moreover, fibroblasts maintained ASMA expression throughout differentiation/dedifferentation processes (Fig. 9D). E-cadherin protein expression assay confirmed the RT-PCR results as UFs and DFs but not FDHLCs positively stained for this marker (Fig. 9E-H).

## Discussion

In this work, we present for the first time the endodermal differentiation potential of human skin fibroblasts. We initially demonstrated the phenotypic homology between fi-

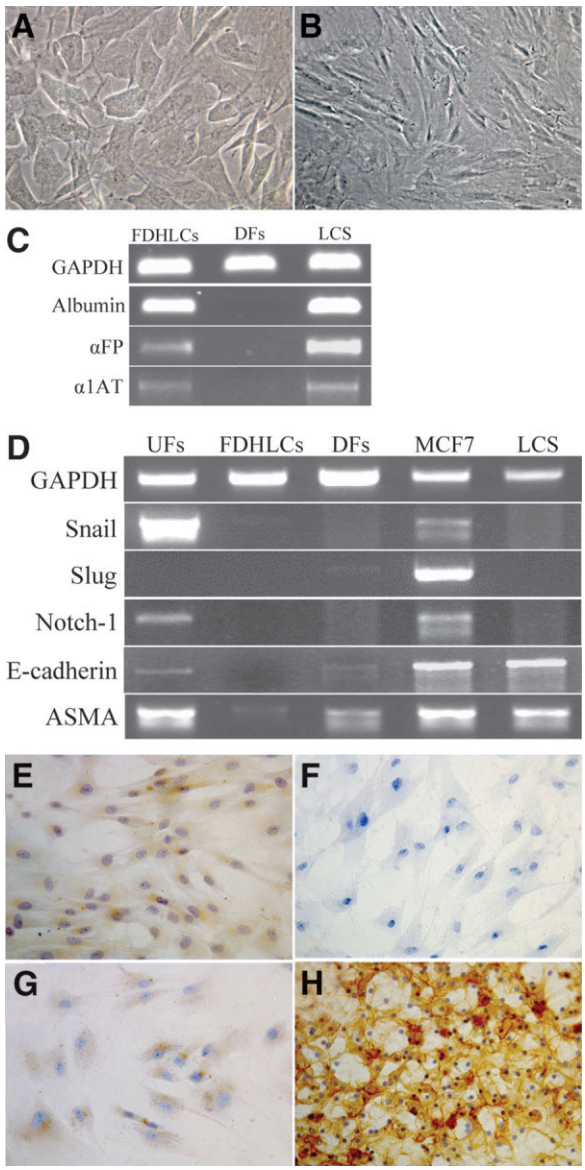


Fig. 9. Epithelial-mesenchymal transition analysis in differentiating fibroblasts. Comparison of morphological aspect of (A) FDHLCs and (B) DFs. Panel (C) shows the loss of albumin, αFP, and α1AT mRNAs expression in DFs. (D) RT-PCR showing snail, notch-1 and e-cadherin expression in UFs while these markers were down-regulated after hepatocyte differentiation. DFs neo-expressed low levels of slug and e-cadherin. Panels (E–H) show immunocytochemical staining for human e-cadherin in (E) UFs, (F) FDHLCs, (G) DFs, and (H) HHs. It confirms that epithelial marker e-cadherin was expressed in UFs, DFs, and HHs but not in FDHLCs. Abbreviations: DFs, dedifferentiated fibroblasts; FDHLCs, fibroblast-derived hepatocyte-like cells; HHs, human hepatocytes; LCS, liver cells suspension; MCF7, human breast adenocarcinoma cell line; UFs, undifferentiated fibroblasts. Pictures were taken at (A,B,E-G) 400× and (H) 200× magnifications.

broblasts and MSCs evoked elsewhere,[5] and then compared both cell types for their potential to differentiate into mesodermal lineages. Classically, this differentiation potential is considered as a constitutive feature of MSCs and ultimately warrants for their phenotype.[3] Distinction between MSCs and fibroblasts is usually based on this MSCs-specific potential although recent reports identified lineage-specific gene expression profiles.[4,6] Our data were consistent with previous works describing the potential of fibroblasts to differentiate into osteocytes[7-14] and adipocytes.[19] Discrepancies within the literature regarding mesodermal differentiation ability of fibroblasts could be explained by cell diversity depending on tissue source, donor age, culture passaging or in situ cell diversity.[25] Accordingly, we were not able to differentiate fibroblasts aged more than 3 passages (Fig. 3). Together, these data attest that fibroblasts and MSCs share equivalent phenotypic features and mesodermal differentiation capacities and suggest that the corresponding assays are inefficient in distinguishing both these cell types. Our observations raise caution about the MSCs identity and have vast implications as tissue sampling for MSCs and fibroblasts are largely equivalent. It underlines the critical need for development of cell lineage characterization based on other features than phenotype (for example, gene array or metabolic assays).

Because MSCs were recently shown to possess hepatocyte differentiation capacity,[26,27] we studied the endodermal potential of fibroblasts. The data reported that even if MDHLCs displayed slightly broader marker expression, both cell types acquired similar morphological changes and liver-like functional features (urea secretion, glycogen storage). Conversely, whereas MDHLCs expressed CK8 RNA marker, none of these F/MDHLCs were able to acquire cytokeratin 8 or cytokeratin 18 expression on the protein level. However, both cell types positively stained for CX-32 suggesting a partial acquisition of the liver epithelial phenotype. The loss of pluripotency of fibroblasts after passage 3 which was also observed with MSCs after passage 8 (data not shown) reflects probably the deleterious effect of in vitro cell proliferation on native cell biology and functionality. Differences in the occurrence of this loss of functionality could depend on the robustness of these cell types.

We confirmed the hepatocyte differentiation potential of fibroblasts in vivo in a model of liver-injured SCID mice. These cells were able to engraft and express hepatic markers as albumin and αFP together with mesodermal markers. Furthermore, the engrafted cells displayed the expression of CK18 which evoked a positive role of the murine microenvironement on the acquisition of an epithelial phenotype. However, no human albumin could be detected in mice serum suggesting that the functionality of differentiated fibroblasts did not improve in vivo in our experimental conditions. Nevertheless, the in vivo data were similar to what could be observed with MSCs confirming the homology of both cell types.

Because EMT is a phenomenon of growing interest that has been described in normal organogenesis and in various

physiopathological situations like fibrosis or neocarcinogenesis,[28] we studied the EMT status of fibroblasts after endodermal differentiation and dedifferentiation. Our results suggested that FDHLCs behave like some described liver progenitors (for example, hepatic stellate cells) as they co-express mesodermal and endodermal markers.[20] We confirmed this result after in vivo differentiation by showing coexpression of fibronectin and albumin in engrafted fibroblasts (Fig. 8B). Furthermore, we showed that fibroblasts are susceptible to lose the phenotype they acquired after differentiation when they are replaced in a growth factor-free medium. Together these data confirm that fibroblasts remain in a EMT status throughout differentiation/dedifferentiation processes by co-expressing mesodermal and endodermal markers and by presenting EMT-specific markers while recovering an undifferentiated state.

In conclusion, whereas fibroblasts were recently described as phenotypicaly similar to hBM-MSCs, our observations suggest that both cell types also share in vitro and in vivo differentiation properties. The comparison of hepatocyte potential of fibroblasts and hBM-MSCs argues that these latter cells possess more robust differentiation capability as fibroblasts could only be differentiated at early passages. As recently reported,[29,30] we confirmed the limitation of hepatocyte differentiation on the functional level in both cell types and the need for in vitro developments of cell culture conditions to improve the hepatocyte-like phenotype. However, regarding the ease of sampling and the advantageous proliferation capacities of fibroblasts, these cells should be further considered in the hepatocyte differentiation field.

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# CHAPTER VI: HEPATOCYTE DIFFERENTIATION OF ADULT- DERIVED HEPATIC LIVER STEM CELLS

## 1. Introduction

Growing data are arising about hepatic progenitor cells isolated from adult or fetal liver parenchyma subjected or not to specific conditioning regiment (59). In this section, we describe a new cell line isolated from healthy adult liver under specific culture conditions. The cells, called adult-derived hepatic liver stem cells (ADHLSCs), displayed a mesenchymal-like phenotype with spindle-shaped morphology and proliferation potential together with constitutive expression of hepatocyte markers. Under differentiation conditions, ADHLSCs improved their hepatocyte-like phenotype and were demonstrated to own important *in vivo* engraftment and differentiation potential. Whether further assessments are mandatory to explore *in vivo* origin of these cells and *in vitro* hepatocyte-like functionality, our data provide arguments about usefulness of using ADHLSCs for *in vivo* pre-clinical assays.

2. **Article 5:** Adult-derived human liver mesenchymal-like cells as a potential progenitors reservoir of hepatocytes? *Cell Transplantation* 2007;16(7):717-28.

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# Adult-Derived Human Liver Mesenchymal-Like Cells as a Potential Progenitor Reservoir of Hepatocytes?

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It is currently accepted that adult tissues may develop and maintain their own stem cell pools. Because of their higher safety profile, adult stem cells may represent an ideal candidate cell source to be used for liver cell therapies. We therefore evaluated the differentiation potential of mesenchymal-like cells isolated from adult human livers. Mesenchymal-like cells were isolated from enzymatically digested adult human liver and expanded in vitro. Cell characterization was performed using flow cytometry, RT-PCR, and immunofluorescence, whereas the differentiation potential was evaluated both in vitro after incubation with specific media and in vivo after intrasplenic transplantation of uPA$^{+/+}$-SCID and SCID mice. Adult-derived human liver mesenchymal-like cells expressed both hepatic and mesenchymal markers among which albumin, CYP3A4, vimentin, and α-smooth muscle actin. In vitro differentiation studies demonstrated that these mesenchymal-like cells are preferentially determined to differentiate into hepatocyte-like cells. Ten weeks following intrasplenic transplantation into uPA$^{+/+}$-SCID mice, recipient livers showed the presence of human hepatocytic cell nodules positive for human albumin, prealbumin, and α-fetoprotein. In SCID transplanted liver mice, human hepatocyte-like cells were mostly found near vascular structures 56 days posttransplantation. In conclusion, the ability of isolated adult-derived liver mesenchymal stem-like cells to proliferate and differentiate into hepatocyte-like cells both in vitro and in vivo leads to propose them as an attractive expandable cell source for stem cell therapy in human liver diseases.

Key words: Stem cells; Transplantation; SCID mice; Differentiation; Cell therapy

## INTRODUCTION

Liver cell transplantation (LCT) has emerged as a promising alternative to whole liver transplantation (17). We and others have demonstrated that LCT can be beneficial in patients with inborn errors of liver metabolism (6,8,16,30,32,34). Recently, we demonstrated in an arginino-succinate lyase deficiency patient a correlation between metabolic improvement, recovery of the deficient enzyme activity, and engraftment of donor hepatocytes in the recipient's liver (33). However, the improvement of the technique remains closely related to the amount and the quality of transplanted hepatocytes. Organ shortage, proliferation inability of cultured hepatocytes, and alteration of mature hepatocytes observed after cryopreservation have led to direct research towards the isolation of other cell sources. Hepatocyte progenitors or stem cells recently received much more attention because of their proliferative potential, their ability to (trans)differentiate into mature functional hepatocytes, and their potentially lower immunogenicity.

Beside fetal liver, evidence has been accumulated to point out the presence of stem cells into the mammalian adult liver. Data obtained from injured animal models have led to identify different cell types, some of them candidate progenitor cells for liver regeneration. Studies regarding adult liver stem cells have mostly focused on oval cells as well as hematopoietic and mesenchymal cells from bone marrow origin. Oval cells are localized in the canal of Hering, the smallest branches of the intrahepatic biliary tree, and are able to bipotentially differentiate into hepatocytes and biliary epithelial cells and hence participate in liver parenchyma regeneration (7, 19,29). These progenitor cells express immature hepatocytes (α-fetoprotein) and biliary duct cells markers (cytokeratins 7 and 19) as well as hematopoietic markers

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as, for instance, the stem cell factor receptor c-kit and CD34 (10,12,18,20,31). A different liver stem cells pool has been proposed in the periductular/intraportal zone. These periductal cells are one of the earliest cell populations that proliferate after hepatectomy, being probably capable of differentiating in all hepatic lineages (28). These not yet characterized reactive ductular cells have been proposed to be from an extrahepatic origin. Bone marrow represents the widely studied extrahepatic source of stem cells able to trans-differentiate into mature hepatocytes both in vitro and in vivo. Hematopoietic bone marrow stem cells have been shown to trans-differentiate into mature hepatocytes in both rodent and human livers (21,35). Nevertheless, Sato et al. have demonstrated that the mesenchymal cell type is the most potent component of bone marrow in hepatic differentiation (25). The contribution of these bone marrow stem cells in liver regeneration may happen or not by fusion between host and donor cells (25,37).

In the current study, we have isolated a mesenchymal-like progenitor cell type from adult human liver after primary hepatocyte culture. These cells are able to proliferate and differentiate, both in vitro and in vivo, into adult hepatocyte-like cells and are hence an attractive cell source to be proposed for human liver cell transplantation as an alternative to hepatocytes isolated from post mortem livers. Because these cells are neither from fetal nor embryonic origin, fewer ethical or safety concerns are to be expected for their use in human medicine.

## MATERIALS AND METHODS

### *Liver Cell Isolation Procedure*

Human liver cells were obtained from the left liver segment originating from healthy cadaveric donors. The protocol and experiments were approved by the institutional ethical review board. Cells were isolated between 6 and 12 h after the gross clamp time while livers were kept on ice in a University of Wisconsin medium until perfusion. Hepatocytes were isolated using a classic two-step perfusion technique (27). Liver segments were sequentially perfused by the apparent blood vessels with an EGTA solution (Earl's balanced salt solution without $Ca^{2+}$ and $Mg^{2+}$, 0.5 mM EGTA, 5 mM HEPES, 2 mg/L gentamicin, and 100,000 IU/L penicillin G) and a digestion enzyme solution for 9–12 min each at 37°C. The digestion solution (EBSS with $Ca^{2+}$ and $Mg^{2+}$, 5 mM HEPES, 2 mg/L gentamicin, and 100,000 IU/L penicillin G) included 0.9 mg/ml of collagenase P and 0.03 mg/ml of soybean trypsin inhibitor. The liver capsule was incised and the hepatocytes were released by gentle shaking. Digestion was stopped with ice-cold wash medium (medium M199, 5 mM HEPES, 2 mg/L gentamicin, and 100,000 IU/L penicillin G), containing 0.03 mg/ml of soybean trypsin inhibitor and 100 ml/L of human plasma. The cells were filtered and rinsed through four metal sieves of 4.5, 1, 0.5, and 0.25 mm of pore size, respectively. Cells were washed three times by centrifugation at 1200 rpm for 3 min in a cold M199 wash medium. According to several assays performed after liver isolation, the obtained cell suspension was predominantly constituted by mature hepatocytes (≥95%). A small fraction of the obtained cell suspension was used for cell culture.

### *Primary Cell Culture*

Single cell suspensions were resuspended in Williams' E medium (Invitrogen) supplemented with 10% fetal calf serum (FCS) (Perbio, Hyclone), 25 ng/ml EGF (Peprotech), 10 μg/ml insulin, 1 μM dexamethasone, and 1% penicillin/streptomycin (P/S) (Invitrogen). The cells were plated on six-well rat tail collagen I-coated plates (Greiner Bio-one) and cultured at 37°C in a fully humidified atmosphere containing 5% $CO_2$. After 24 h, medium was changed in order to eliminate the nonadherent cells and thereafter renewed every 3 days whereas the culture was microscopically followed every day. Culture medium was then switched to DMEM with high glucose concentrations (Invitrogen) supplemented with 10% FCS and 1% P/S in order to accelerate the elimination of hepatocytes. A cell type with mesenchymal-like morphology then spontaneously emerged, proliferated, and filled the empty space in the well plate as observed by phase contrast microscopy. When reaching 70% confluence, cells were lifted with 0.25% trypsin and 1 mM EDTA and replated at a density of $1 \times 10^4$ cells/cm$^2$. For each passage, cell suspension was also analyzed using RT-PCR and immunofluorescence.

### *Characterization of Adult-Derived Human Liver Stem Cells (ADHLSC)*

*Hepatogenic Differentiation.* Cells (from third to eighth passages) were seeded at a density of $1 \times 10^4$/cm$^2$ in six-well plates coated with rat tail collagen type I in DMEM supplemented with FCS and P/S. Culture medium was switched 24 h later to Iscove's modified Dulbecco's medium (IMDM) (Invitrogen). Cells were differentiated according to Lee et al. (13) with some modifications. Cells were incubated with IMDM containing 20 ng/ml epidermal growth factor (Peprotech) and 10 ng/ml basic fibroblast growth factor (bFGF) (Peprotech) for 2 days. Thereafter, cells were subjected to differentiation induction after their incubation for 10 days with IMDM containing 20 ng/ml hepatocyte growth factor (HGF) (Peprotech, UK), 10 ng/ml bFGF, nicotinamide 0.61 g/L (Sigma), and 1% insulin-transfer-

rin-selenium (ITS) (Invitrogen) premix. Subsequent maturation step consisted of treatment with IMDM containing 20ng/ml oncostatin M (Peprotech), 1 μM dexamethasone (Sigma), and 1% ITS premix for 10 days. For each step, medium was changed every 3 days.

*Immunofluorescence.* Cells grown on collagen type 1-coated 12-mm-round glass coverslips were fixed with 4% paraformaldehyde (v/v) for 15 min at room temperature and permeabilized thereafter with 1% Triton X-100 (v/v) in TBS (Tris-HCl 50 mM, NaCl 150 mM, pH 7.4) for 15 min. Nonspecific immunostaining was prevented by 1-h incubation in a TBS solution containing 3% nonfat dry milk at 37°C. Cells were then successively incubated in the same solution for 1 h at room temperature with primary antibodies, rinsed five times with TBS and for 1 h with secondary antibodies. Nuclei were stained for 30 min with the nuclear dye DAPI (1/5,000). After three rinses, preparations were mounted in Fluoprep medium (BioMerieux, Brussels, Belgium) and examined using an Olympus IX70 inverted microscope coupled to a CCD camera (T.I.L.L. Photonics, Martinsried, Germany). Excitation light was obtained from a Xenon lamp coupled to a monochromator (T.I.L.L. Photonics). Digital images were acquired using appropriate filters and combined using the TILLvision software.

*Flow Cytometry.* Cells were collected after centrifugation at 1200 rpm for 5 min and resuspended at a concentration of 500–1,000/μl in PBS. Cells were then incubated for 30 min at 4°C with the following antibodies: CD45-APC, CD34-APC, CD49f-PE (Becton Dickinson); CD90-PC5, HLA-DR-FITC, CD73-PE, CD29-PE (BD Pharmingen); CD105 Endoglin-FITC (Immunokontact); CD44-FITC, CD117-PC5, CD133-APC, CD49b-FITC, CD49e-FITC (Immunotech); HLA-ABC-PE (Dako Systems), CD13-PE (Beckham Coulter). The corresponding isotypes [IgG1-FITC, IgG2a-FITC, IgG2b-FITC (Serotec); IgM-FITC (Immunotech); IgG1-PC5 (BD Pharmingen); IgG2a-PE, IgG1-PE, IgG1-APC (Becton Dickinson)] were used for evaluation of nonspecific binding of monoclonal antibodies. The corresponding control isotypes were used for evaluation of nonspecific binding of monoclonal antibodies. Cells were then washed and resuspended in Isoton® (Beckham Coulter) for reading with a Beckham Coulter Flow cytometer.

*RT-PCR.* Total RNA was extracted from cells grown in six-well plates using the TriPure isolation reagent and cDNA was generated using the reverse transcription kit, according to the manufacturer's instructions. PCR amplifications were performed using polymerase elongase in a final volume of 25 μl and appropriate primers (Table 1). Samples were thereafter electrophoresed on a 1% agarose gel and nucleic acids were visualized by ethidium bromide staining.

*Mice Transplantation.* All animal experiments were carried out in compliance with Belgian law on animal protection and approved by the local ethical review board. One million ADHLSC were injected into the spleen of 14-day-old $uPA^{+/+}$-SCID mice and 6–8-week-old SCID mice. At selected times posttransplantation, animals were bled and plasma was stored at −70°C for human albumin assay. Livers were harvested for immunohistochemistry and RT-PCR analyses.

*Immunohistochemistry.* Liver specimens were fixed in formalin for 24 h and embedded in paraffin. Immunohistochemistry was then performed on 5-μm-thick liver sections of mice transplanted with human cells that were incubated overnight with primary polyclonal rabbit anti-human albumin (Calbiochem), anti-human prealbumin (Dakocytomation), anti-α-fetoprotein (Dakocytomation), and anti-Ki-67 (Dakocytomation) antibodies at 4°C. Detection was performed after incubating the slices with peroxidase-labeled polymer and substrate chromogen (Envision-DAB system, Dako, Carpinteria, CA). Counterstaining was performed using hematoxylin.

*Quantification of Human Albumin.* Human albumin in mouse plasma was measured according to the protocol described elsewhere (15). Briefly, after coating the Maxisorp immunoplates (Nunc, Roskilde, Denmark) with goat anti-human albumin antibody (Bethyl Laboratories, Montgomery, TX), blocking, and washing, diluted samples or calibrators were added. Human albumin bound to the plate was detected with a HRP-conjugated goat anti-human albumin antibody (Bethyl Laboratories). The human albumin quantitative ELISA kit uses antibodies that specifically react with human albumin. Cross-reaction with mouse albumin is undetectable as confirmed using appropriate controls for each assay. The kit is optimized for 400–6.25 ng/ml dilutions and can be adapted for higher concentrations.

## RESULTS

### *Establishment and Characterization of Adult-Derived Human Liver Stem-Like Cells*

Cell suspension of the parenchymal fraction obtained after human liver isolation was plated on type I collagen-coated flasks with viability of 90% as estimated by the trypan blue exclusion test. In our experimental conditions, after 10–15 days of routine culture with a serum containing medium, the hepatocytes did not divide (upper layer) and started to detach from the dish, whereas emerging morphologically different cell types started spreading and proliferating (lower layer). One predominant cell type presented a mesenchymal-like morphol-

**Table 1.** Primers Used

| Primer | Sequence | PCR Product Size |
|---|---|---|
| AAT | F: 5′-TCGCTACAGCCTTTGCAATG-3′<br>R: 5′-GGAACTCCTCCGTACCCTCAA-3′ | 142 bps |
| Albumin | F: 5′-CCTTGGTGTTGATTGCCTTTGCTC-3′<br>R: 5′-CATCACATCAACCTCTGGTCTCACC-3′ | 308 bps |
| AFP | F: 5′-TGAAATGACTCCAGTAAACCC-3′<br>R: 5′-GATGAAGCAAGAGTTTCTCATT-3′ | 199 bps |
| HNF4 | F: 5′-CCAAGTACATCCCAGCTTTC-3′<br>R: 5′-TTGGCATCTGGGTCAAAG-3′ | 295 bps |
| Vimentin | F: 5′-CTACATCGACAAGGTGCGCTT-3′<br>R: 5′-TGCCAGAGACGCATTGTCAA-3′ | 301 bps |
| CK-8 | F: 5′-AAGGGCTGACCGACGAGATC-3′<br>R: 5′-GCTTCCTGTAGGTGGCGATC-3′ | 537 bps |
| CK-19 | F: 5′-TTTGAGACGGAACAGGCTCT-3′<br>R: 5′-CAGCTCAATCTCAAGACCCTG-3′ | 426 bps |
| G6P | F: 5′-TCAGCTCAGGTGGTCCTCTT-3′<br>R: 5′-CCTCCTTAGGCAGCCTTCTT-3′ | 291 bps |
| GS | F: 5′-GTCAAGATTGCGGGGACTAA-3′<br>R: 5′-TACGATTGGCTACACCACCA-3′ | 396 bps |
| GGT | F: 5′-GACGACTTCAGCTCTCCCAG-3′<br>R: 5′-CTTGTCCCTGGATTGCTTGT-3′ | 489 bps |
| MRP2 | F: 5′-ACACCAACCAGAAATGTGTC-3′<br>R: 5′-CCAAGGCCTTCCAAATCTC-3′ | 660 bps |
| Oct-4 | F: 5′-CGACCATCTGCCGCTTTGAG-3′<br>R: 5′-CCCCCTGTCCCCCATTCCTA-3′ | 573 bps |
| TDO | F: 5′-ATACAGAGCACTTCAGGGAGC-3′<br>R: 5′-GATACCGAAGATGAACCCAACCA-3′ | 316 bps |
| TAT | F: 5′-TGAGCAGTCTGTCCACTGCCT-3′<br>R: 5′-ATGTGAATGAGGAGGATCTGAG-3′ | 358 bps |
| CYP1B1 | F: 5′-GAGAACGTACCGGCCACTATCACT-3′<br>R: 5′-GTTAGGCCACTTCACTGGGTCATGAT-3′ | 357 bps |
| CYP3A4 | F: 5′-TGCTGTCTCCAACCTTCACC-3′<br>R: 5′-TAGCTTGGAATCATCACCACC-3′ | 802 bps |
| CYP2B6 | F: 5′-CCTCTTCCAGTCCATTACCG-3′<br>R: 5′-TGACTGCCTCTGTGTATGGC-3′ | 551 bps |
| GAPDH | F: 5′-CGGAGTCAACGGATTTGGTCGTAT-3′<br>R: 5′-AGCCTTCTCCATGGTGGTGAAGAC-3′ | 307 bps |

ogy, flattened form, broad cytoplasm, and ovoid nuclei with one or two nucleoli and showed microscopically the highest proliferative potential (Fig. 1). These cells that were reproducibly isolated from 11 different healthy livers and that we termed adult-derived human liver stem-like cells (ADHLSC), according to the data presented below, remained predominant after trypsin application and replating. This led to their purification after the second passage as demonstrated using flow cytometry analysis. Indeed, we demonstrate that $99 \pm 0.3\%$ and $92 \pm 8\%$ of ADHLSC population was positive for CD90 (Thy-1) and CD73 (SH-3, SH-4), respectively, markers of mesenchymal lineage (Fig. 2). The cell population was also positive for CD29 ($88 \pm 8\%$), CD44 ($92 \pm 7\%$),

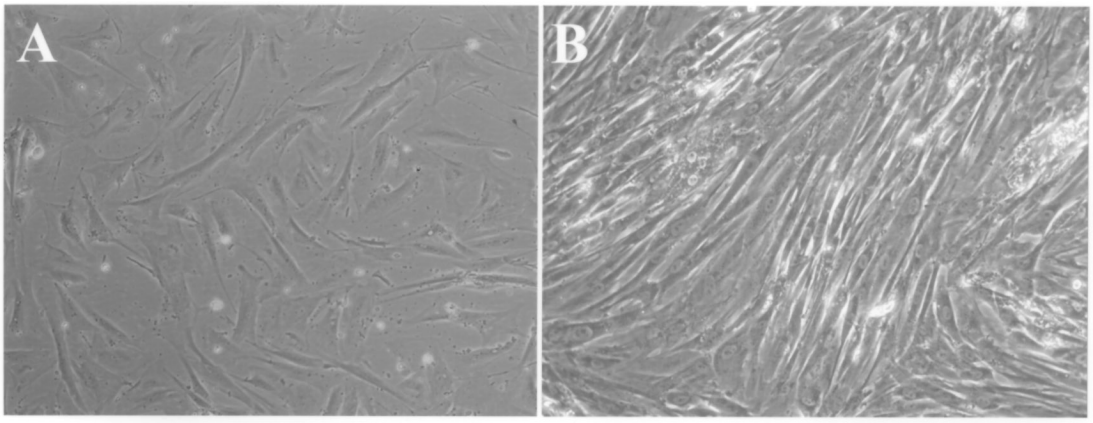


**Figure 1.** Morphology of ADHLSC. Phase contrast microscopic view of ADHLSC obtained after enzymatic liver digestion and plating in collagen type I-coated dishes after the second passage. (A) Lower confluence and (B) higher confluence. Original magnification 100×.

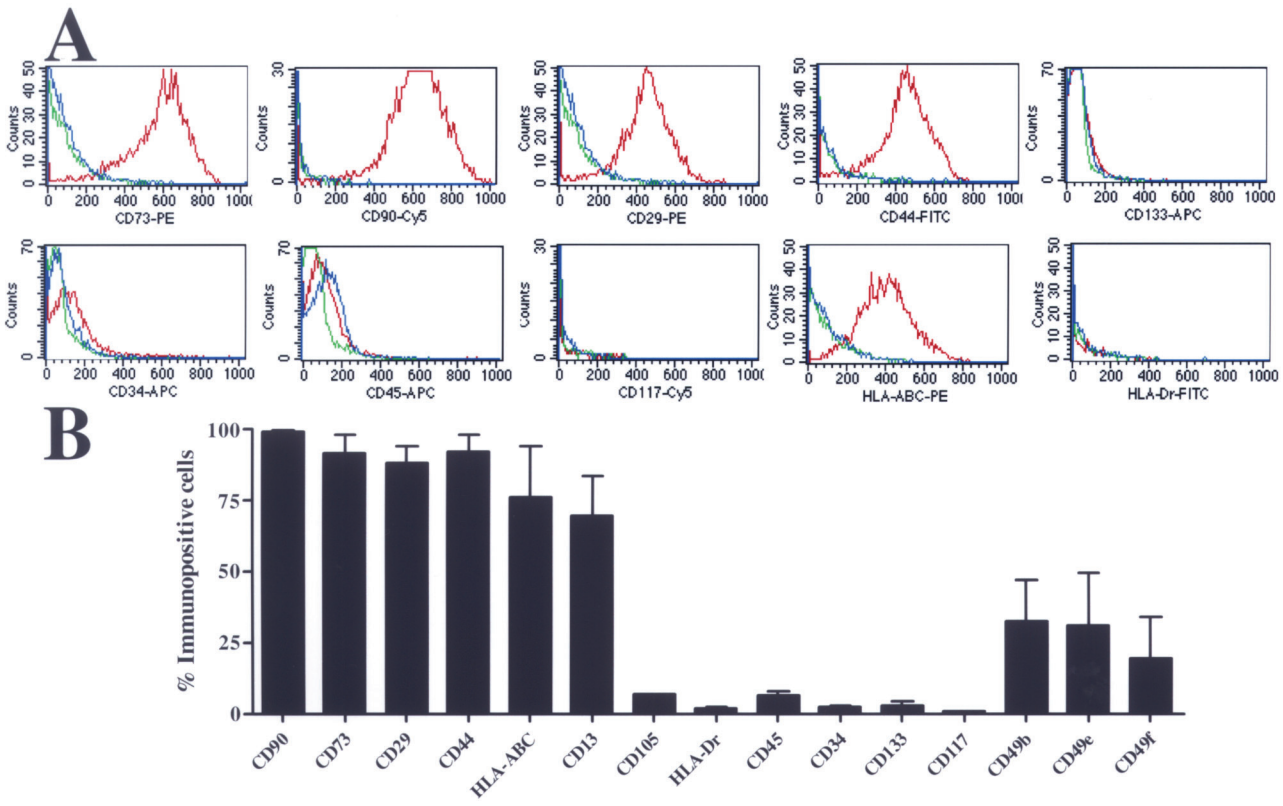


**Figure 2.** Cell surface markers profile of ADHLSC. Selected cell surface molecules were analyzed using flow cytometry. Their expression is shown as histograms (red) and compared to corresponding control isotypes IgG (green and blue). (A) Flow cytometry histograms representative of at least four cell populations isolated from different livers. (B) Mean expression percentage of analyzed cell markers obtained from three different cell populations after the second passage.

HLA-Class I (76 ± 22%), and CD13 (69 ± 18%). ADH LSC population did not express CD105 (SH2) (7%), CD133 (3.1 ± 1.5%), CD117 (0.8 ± 0.2 %), CD45 (2.2 ± 0.7%), CD34 (6.4 ± 1.4%), and HLA-DR (2.0 ± 0.4%) (Fig. 2). Regarding the extracellular matrix interaction, ADHLSC population was slightly positive for CD49b (32 ± 18%) and CD49e (31 ± 23%) but negative for CD49f (19 ± 17%) (Fig. 2).

These data are in good agreement with the cell surface expression profile of the documented mesenchymal stem cells except for the CD105 marker. The analysis of ADHLSC using immunofluorescence and RT-PCR confirmed their mesenchymal phenotype by the demonstration of vimentin and ASMA-positive expression at both the protein and mRNA levels (Fig. 3) but not of cytokeratins 8 and 18 (data not shown) or 7 and 19 (Fig. 3B). In order to demonstrate that ADHLSC may constitute a cell population different from the ones already described in the liver, RT-PCR analysis was performed on control mRNAs extracted from cell suspension containing isolated human hepatocytes (parenchymal cell fraction directly obtained after liver isolation), human isolated stellate cells (mesenchymal lineage), and human hepatoblastoma cell line HepG2. The data revealed that ADHLSC expressed mRNA of specific hepatic markers, such as albumin and α-fetoprotein (Fig. 3B). Interestingly, immunofluorescence staining confirmed the expression of albumin in ADHLSC (Fig. 3A) in accordance with their hepatic origin, whereas α-fetoprotein was weakly expressed (data not shown). The albumin staining was investigated using monoclonal and polyclonal antibodies after the evaluation of several rigorous controls related to the technique (use of human cells from different origins) and to the fluorescence microscope used for that study.

RT-PCR analysis also revealed that other liver-specific markers were at least expressed at the mRNA level, such as glucose 6-phopshatase, α-antitrypsin, glutamine synthase, γ-glutamyl transpeptidase, MRP2 transporter, and hepatocyte nuclear factor-4 (Fig. 3B). ADHLSC did not express the POU-domain transcription factor Octamer-4 (Oct-4) transcript, confirming their adult liver origin. Interestingly, ADHLSC expressed adult liver-specific transcripts such as CYP3A4 and CYP1B1 but not CYP2B6, tyrosine aminotransferase (TAT), or tryptophan 2,3-dioxygenase (TDO). Together these data demonstrate that ADHLSC, beside mesenchymal properties, express hepatic markers confirming their liver origin.

### *Characterization of ADHLSC In Vitro Differentiation*

Because of their mesenchymal phenotype, we evaluated the behavior of ADHLSC in presence of growth factors and hormones-defined media and accordingly their potency to differentiate into tissue-specific cells. As clearly evidenced in the literature, one of the characteristic of mesenchymal cells is their ability to adopt adipocytic and osseous tissue phenotype under specific culture conditions (22,39). In our study, we never succeeded to induce osteogenic nor adipocytic differentiation of ADHLSC after 5 weeks of culture while such differentiation could be easily obtained with human bone marrow mesenchymal cells that were used as positive controls (data not shown). Because of their liver origin, we then studied if ADHLSC were able to respond to chemical signals that induce hepatocytic differentiation. For that purpose, $1 \times 10^4$ cells/cm$^2$ were used and treated according to a specific protocol. ADHLSC exposed to differentiation cocktails in a serum-free medium started to lose their sharp edges, became progressively smaller, and lost their initial morphology to adopt a polygonal shape with a granular cytoplasm (Fig. 4A). We then assessed whether the morphological changes were correlated to genetic expression of mature hepatocyte markers and demonstrated that the expression of the analyzed liver-associated genes was increased in the differentiated cells compared to IMDM serum-free cultivated cells (Fig. 4B). Glycogen production is one of the specific metabolic functions of the mature hepatocyte. Hence, the storage of glycogen was analyzed using periodic acid Schiff staining and showed significant increase in differentiated cells compared to undifferentiated ones (Fig. 4C). Specificity of the staining was checked and confirmed using amylase (data not shown).

### *Characterization of ADHLSC In Vivo Differentiation*

In order to investigate their undergoing behavior in the hepatic environment in vivo, 1 million undifferentiated ADHLSC were transplanted into the spleen of two 14-day-old uPA$^{+/+}$-SCID mice, a transplantation animal model widely used for the study of liver regeneration. The purity of our ADHLSC suspension (>97%) was checked by means of phase contrast microscopy, flow cytometry, and immunofluorescence of vimentin and cytokeratins 8 and 19. Livers of the transplanted mice were histologically analyzed 10 weeks posttransplantation and showed nodules containing cells with larger size and empty appearing cytoplasm compared to recipient parenchymal cells (Fig. 5A). Immunohistochemical staining was performed to check their human origin. Some of the nodules positive for human albumin (Fig. 5B, C) presented cells with a morphology resembling the documented putative progenitors (24). The polyclonal anti-human albumin antibody used in our study did not stain the murine uPA$^{+/+}$-SCID proliferating cells of the red foci (cells that have lost the transgene expression) (data not shown). Positive immunostaining for human prealbumin and α-fetoprotein was also observed in the trans-

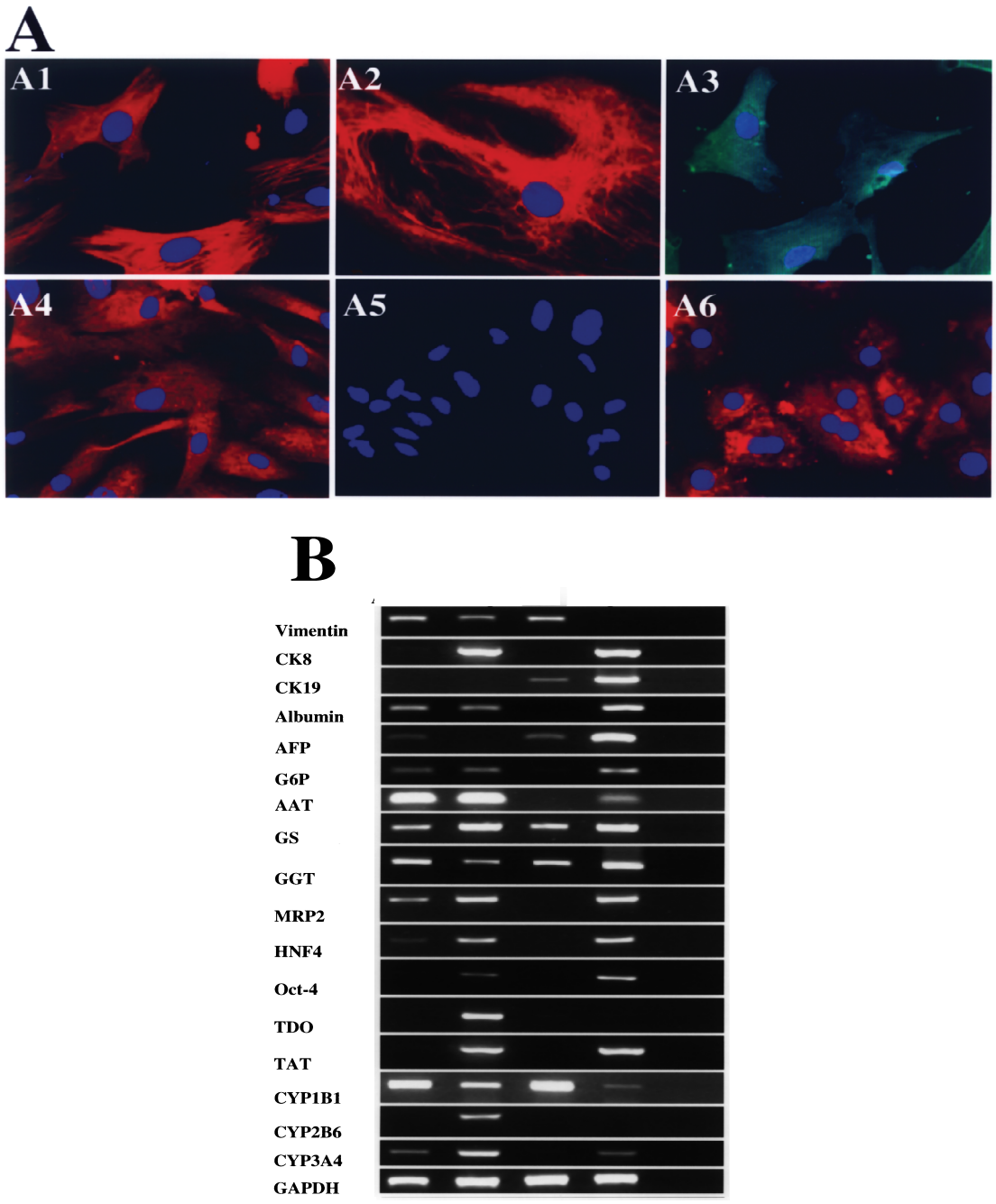


**Figure 3.** Expression of mesenchymal and hepatic markers in ADHLSC. (A) Mesenchymal markers ASMA and vimentin and albumin immunostaining were analyzed by fluorescent microscopy. ADHLSC plated on collagen type I-coated coverslips were fixed and incubated with corresponding primary antibodies. Immunoreactivity was visualized using a cyanine-3-conjugated goat anti-mouse antibody for ASMA (A1), vimentin (A2), and albumin (A4), and fluorescein isothiocyanate-conjugated goat anti-rabbit antibody for albumin (A3). Human hepatocytes were used as positive control for albumin staining (A6) whereas negative control consisted of incubating ADHLSC only with the secondary anti-mouse Cy-3 antibody (A5). Cell nuclei are stained using DAPI (blue). Images are representative of several fields examined from at least five cell populations. (B) Genetic expression of mesenchymal and hepatic markers was assessed by RT-PCR performed on mRNA extracted from undifferentiated ADHLSC at the second passage. Data shown are agarose gel electrophoresis of amplification products corresponding to mesenchymal and hepatic markers. AFP, α-fetoprotein; G6P, glucose 6-phosphatase; AAT, α1-antitrypsin; GS, glutamine synthase; GGT, γ-glutamyl transpeptidase; MRP2, multidrug resisting protein-2; HNF-4, hepatocyte nuclear factor-4; Oct-4, POU-domain transcription factor Octamer-4; TDO, tryptophan 2,3-dioxygenase; TAT, tyrosine aminotransferase; CYP1B1, cytochrome P450, family 1, subfamily B, polypeptide 1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

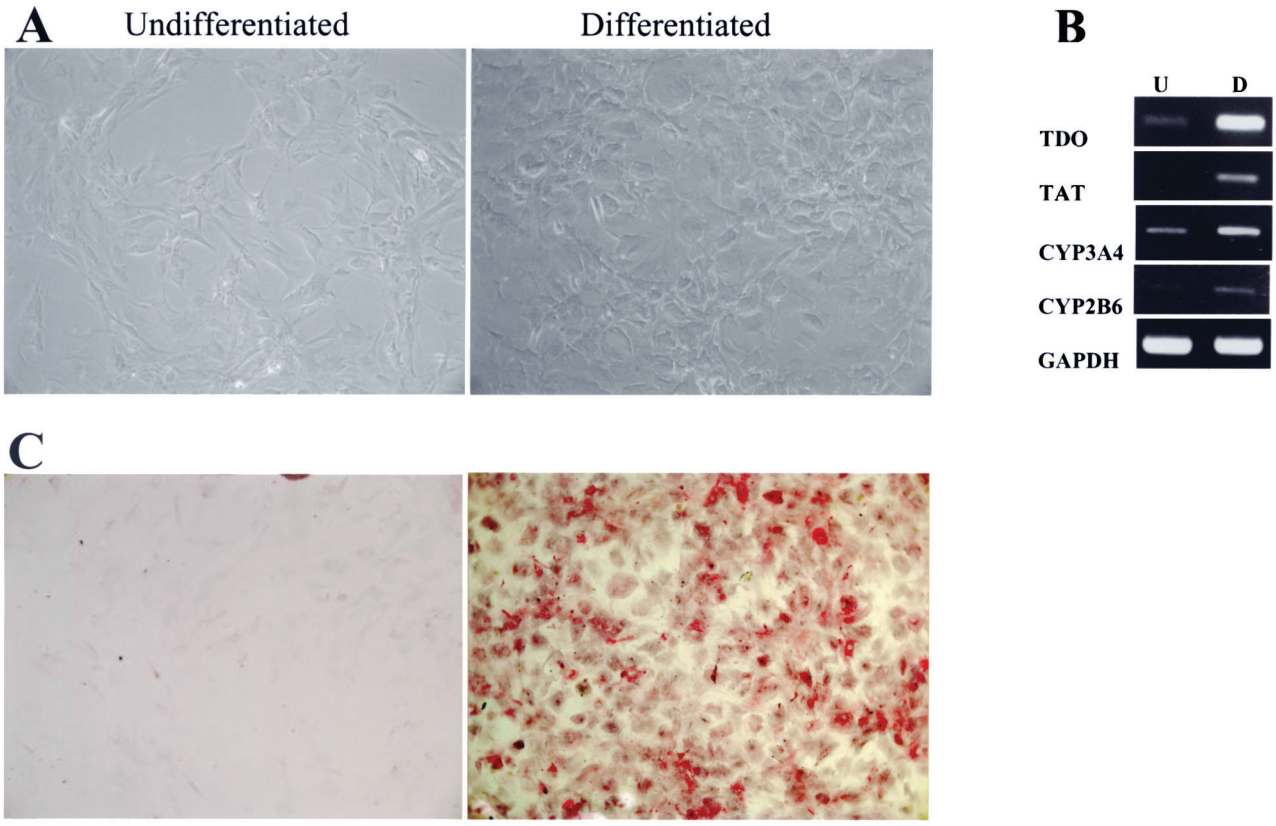


**Figure 4.** Evaluation of in vitro ADHLSC hepatic differentiation potential. (A) Undifferentiated ADHLSC were able to change their morphology by adopting a polygonal shape with a granular cytoplasm after 4 weeks of treatment with specific growth factors and cytokines. (B) Gene expression profile was analyzed using RT-PCR and demonstrated the expression of mature hepatocyte-specific marker genes by the differentiated ADLHSC compared to undifferentiated IMDM serum-free cultivated ones in correlation with morphological changes. (C) Differentiated ADHLSC produced glycogen, a specific function of mature hepatocyte. Storage was revealed after fixation of the cells and PAS staining (magenta color). Presented images are representative of at least five different experiments.

planted cells (Fig. 5E, F). Ki-67 staining revealed that some of the human hepatocyte-like cells were proliferating (Fig. 5G). Human albumin and α-fetoprotein levels were also analyzed in the recipient mouse plasma. Three weeks posttransplantation, albumin was detected in the first mouse at a concentration of 2 μg/ml whereas no circulating albumin was detected in the second transplanted mouse. Seven weeks later (10 weeks posttransplantation), albumin levels increased to 11 and 2 μg/ml in the first and the second mouse, respectively. Interestingly, no human serum α-fetoprotein was detected 3 and 10 weeks posttransplantation (data not shown).

Thereafter, the behavior of ADHLSC was analyzed in another transplantation animal model. We intrasplenically transplanted 6–8-week-old SCID mice ($n = 6$) with 1 million undifferentiated cells with or without 70% hepatectomy. Liver specimens were taken 56 days later and analyzed for the expression of human albumin. As shown in Figure 6A, analysis of albumin gene expression was evaluated using RT-PCR. Human albumin transcript was found in total mRNA extracted from three mice. Immunohistochemistry on liver samples confirmed the presence of human hepatocyte-like cells in all the mouse livers, mostly near the portal areas and the portal veins. We did not observe a specific organization of the transplanted cells in this model, as individual and foci containing 2–10 cells were observed (Fig. 6B).

## DISCUSSION

In the current study, we convincingly demonstrated the presence of a potential mesenchymal-like progenitor cells pool within adult human liver. These cells are able to differentiate in vitro into hepatocyte-like cells after treatment with cocktails of specific growth factors and cytokines. After transplantation in two animal models of LCT, the undifferentiated cells have been shown to

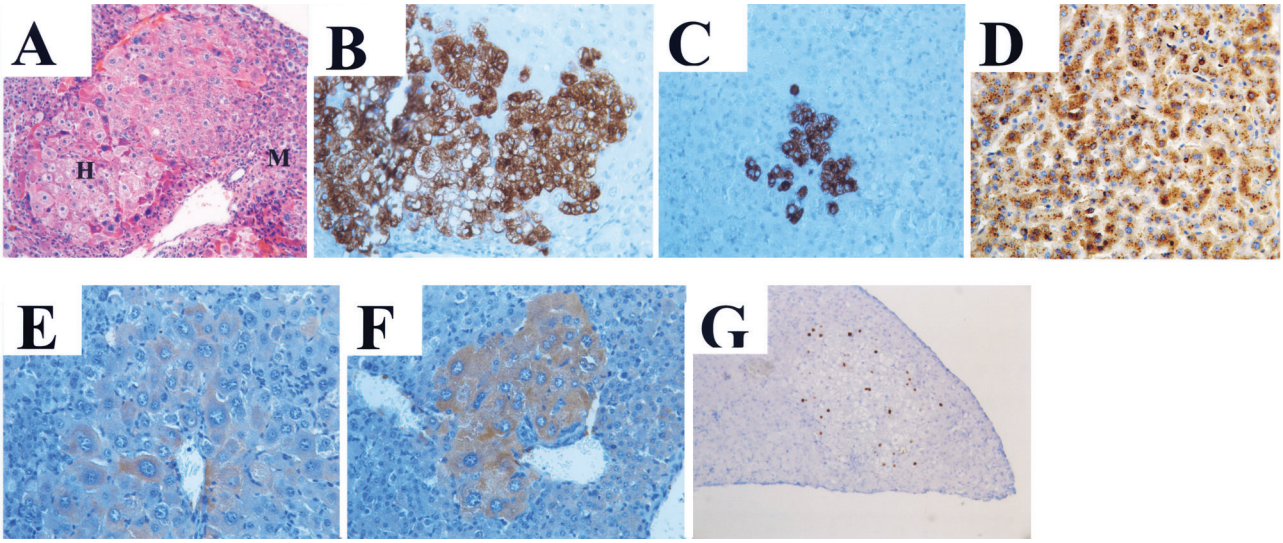


**Figure 5.** Immunohistochemical evaluation of ADHLSC differentiation potential in transplanted uPA$^{+/+}$-SCID mice. (A) Representative H&E staining of uPA$^{+/+}$-SCID liver transplanted mice. Human cells (H) were organized in nodules containing bigger size and pale cytoplasm cells and surrounded by murine hepatocytes (M). (B) Liver section of transplanted uPA$^{+/+}$-SCID mouse, obtained 10 weeks after intrasplenic injection of undifferentiated ADHLSC suspension, was stained with polyclonal antibody specific for human albumin and counterstained with hematoxylin (blue). Positive cells that integrated into the mouse parenchyma show polygonal morphology. (C) Positive cells with intermediate-like morphology and human origin that are also seen in the mouse liver parenchyma. (D) Staining of human liver tissue with the human albumin polyclonal antibody. Nodules of human hepatocyte-like cells could also be revealed using antibodies against human prealbumin (E) and α-fetoprotein (F). Some of the nodules contained proliferating cells as demonstrated by immunostaining using an anti-human Ki-67 (G).

be able to migrate, engraft, and differentiate within the heterologous recipient liver parenchyma.

ADHLSC have been obtained thanks to enzymatic liver cell disaggregation and primary culture after the elimination of hepatocytes and other liver cell types. Our protocol used for liver cell isolation leads to recover a cell suspension predominantly containing mature hepatocytes as demonstrated using immunocytochemistry (>95%). We confirmed, after plating the isolated cell suspension in collagen I-coated plates, that hepatocytes lose their differentiated properties after 7 days in culture (morphology, albumin and urea levels), as reported elsewhere (3,36). Because they were not able to divide, the hepatocyte layer started disaggregating, leading to empty spaces in the plate for other emerging cells of the lower layer. Among the evidenced cell populations, cells with mesenchymal-like morphology showed the highest proliferative potential and remained predominant after the first trypsin application and replating.

We demonstrated, thanks to flow cytometry, that ADHLSC were positive for CD90, CD73, CD29, and CD44, documented markers for mesenchymal lineage, and negative for CD117 and CD105 (Fig. 2). The data also revealed that cell surface immunophenotype of ADHLSC was different from the that of our isolated adult human hepatocytes and multipotent adult progenitor cell (MAPC) (23). Nevertheless, ADHLSC express similar cell markers to extrahepatic mesenchymal cells originated from umbilical cord blood, placenta, and human pancreatic ductal epithelium with one exception for CD105 (1,13,14,26,40). Contamination by hematopoietic stem cells was excluded because ADHLSC suspension was negative for CD117 and CD133. In addition, the mesenchymal phenotype of ADHLSC was confirmed by positive immunofluorescence staining for vimentin and ASMA. Vimentin is an intermediate filament that has also been documented to be upregulated in primary dedifferentiated hepatocyte long-term culture on collagen I as well as biliary cytokeratins 7 and 19 (3). In our study, although positive for vimentin, none of the analyzed cytokeratins (hepatocyte and biliary) was detected in ADHLSC, suggesting that our cell line was different from differentiated and dedifferentiated primary hepatocytes. This was confirmed by microscopic observation of the primary cultures that clearly revealed two different cell layers with different morphologies and proliferation potential, the upper one representing dying hepatocytes and the lower one containing mesenchymal-like cells.

Many evidences support our data demonstrating that

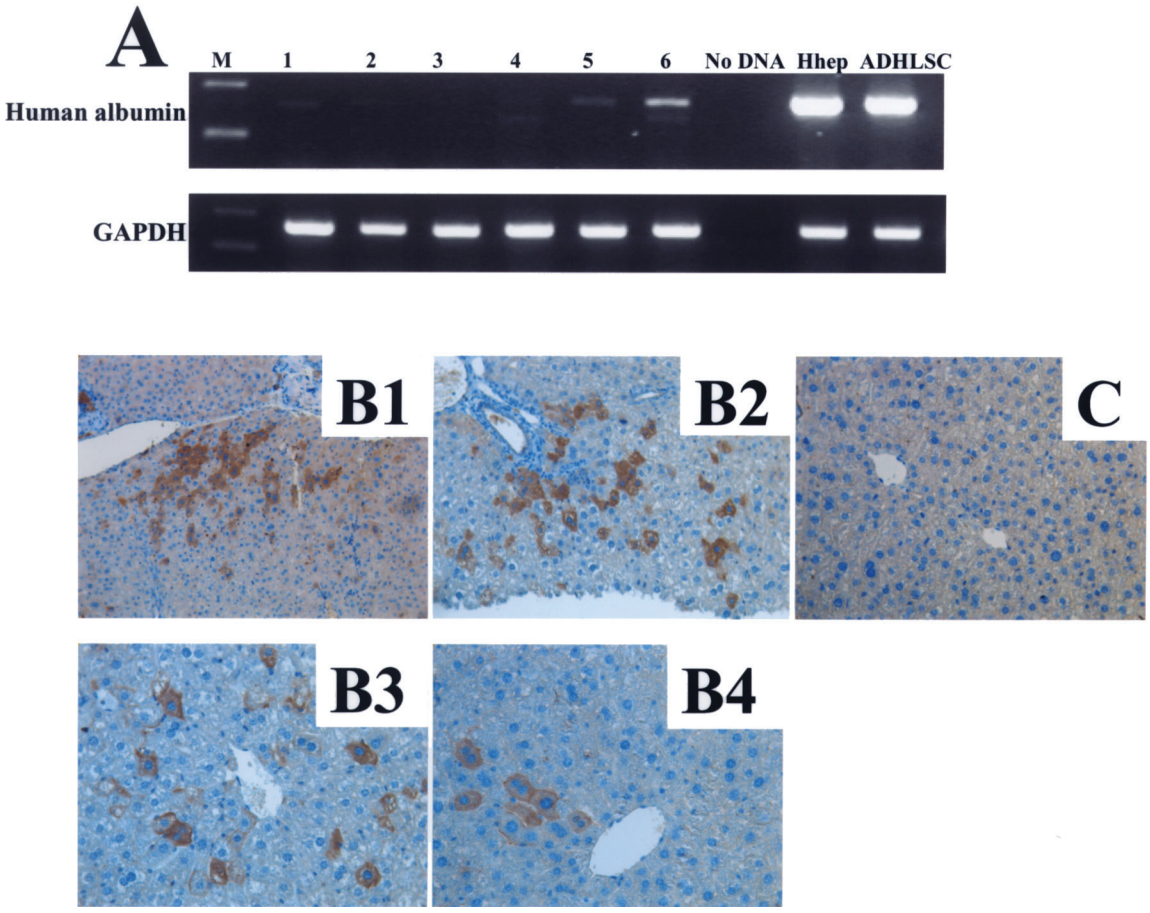


**Figure 6.** Evaluation of ADHLSC differentiation potential in transplanted SCID mice. (A) RT-PCR analysis of human albumin 56 days after intrasplenic injection of undifferentiated ADHLSC suspension in SCID mice. Total mRNA was extracted from transplanted mouse liver (lanes 1–3: nonhepatectomized, lanes 4–6: with 70% hepatectomy) and analyzed using specific probes to detect human albumin. Isolated human hepatocytes (Hhep) and in vitro cultured undifferentiated ADHLSC were used as controls. Three of the six animals analyzed showed the presence of human albumin gene into the mouse liver. M, molecular weight marker. (B) Human albumin immunohistochemistry in liver sections from transplanted SCID mice with undifferentiated ADHLSC (56 days posttransplantation). (B1–2) Organized cells. Original magnification 100×. (B3–4) Isolated human cells into the mouse liver parenchyma. Original magnification 200×. (C) Staining of PBS-injected SCID mouse liver tissue with the polyclonal anti-human albumin used in this study. Original magnification 200×.

ADHLSC are also different from human liver stellate cells. First, stellate cells have been shown to express both hepatocytic and biliary cytokeratins in the first passages of in vitro culture and lose completely this expression after the 10th passage (14). Second, stellate cells express HLA- class II membrane markers (38) that are negative for ADHLSC. Third, the genetic profile of ADHLSC, which is different from that of human liver stellate cells, mature adult hepatocytes, and hepatoblastoma cell line HepG2, is maintained up to the eighth passage.

Furthermore, our data corroborate results of a recent study demonstrating the isolation and the presence of mesenchymal cell population in the adult mouse liver (5). These latter cells do share with other described mesenchymal cells the ability to differentiate into mesodermal and endodermal cells. On the contrary, an important characteristic of the ADHLSC cell line is the inability to differentiate into osteocytes or adipocytes using the classical differentiation protocols (bone marrow mesenchymal cells were used as positive experiment controls). This suggests that the ADHLSC are already engaged in the liver lineage as it has been documented for other adult stem cells from other tissues (4).

In our study, we demonstrated that ADHLSC can differentiate into hepatocyte-like cells in vitro. Differentiation of ADHLSC was displayed in both the morphological, gene expression pattern and glycogen storage, a

specific function related to mature hepatocytes. Differentiation studies also revealed that ADHLSC were quite stable even after the eighth passage. We then assessed the in vivo outcome of undifferentiated cells after intrasplenic transplantation in animal models of LCT. We first transplanted two uPA$^{+/+}$-SCID mice and evaluated engraftment after 10 weeks posttransplantation. Liver samples were analyzed by immunohistochemistry and compared to nontransplanted heterozygote mice with "red foci," nodules that are formed by proliferating murine hepatocytes in which the transgene was excised (15). These nodules have also been observed in transplanted mice and hence were used as additional internal negative controls for immunostaining studies within the same tissue. Our data demonstrated the presence of nodules with cells of human origin, some of which were proliferating (Fig. 6). In parallel, analysis of human markers in the plasma of transplanted cells revealed the presence of human albumin, suggesting the functional differentiation of ADHLSC after engraftment.

We also transplanted SCID mice, half of them being subjected to 2/3 hepatectomy to induce a regenerative stimulus, with undifferentiated ADHLSC. Human albumin mRNA was detected after 56 days posttransplantation in three mice. The absence of human albumin mRNA in the other samples could be due to sampling phenomenon. Indeed, using immunohistochemistry, donor immunopositive cells for human albumin were also detected within the recipient transplanted liver. The engrafted human cells were scattered throughout the recipient parenchyma, especially near vascular structures, and appeared mostly as isolated cells or small foci. The transplantation efficiency could be impaired in this animal model or may be linked to the low ability of the ADHLSC to respond to murine differentiation signals. Nevertheless, the data obtained in vivo revealed the migration and engraftment potential of ADHLSC and confirmed their ability to differentiate into hepatocyte-like cells. In the current study, we did not investigate the karyotype of engrafted cells in order to determine if differentiation was the result of cell fusion between transplanted human and recipient mouse hepatocytes, as demonstrated for other mesenchymal stem cells (2,9, 11,37).

One important aspect of our cell line is the higher safety profile to be expected from adult-derived stem cells compared to embryonic or fetal candidates, and also the lesser ethical dilemma if these cells were to be used in the future for human therapeutic purpose.

In conclusion, we describe here an original mesenchymal-like cell population from adult human liver able to expand in vitro. These cells have the potential to differentiate towards hepatocyte-like cells both in vitro and in vivo. Hence, ADHLSC should be viewed as an interesting candidate for liver cell-based therapy.

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# CHAPTER VII: GENERAL DISCUSSION

## 1. Conclusions

### a. ‘Stem cell’ label

*In vitro characterization*

It is noteworthy whether current knowledge on most stem cell lineages is based on *in vitro* properties. As growing interest is carried to this amazing field, abundant data are unceasingly appearing concerning the isolation of new cell types. For example, with regard to ‘mesenchymal’ lineages, in the last years appeared chronologically stromal cells (60), MSCs (169), MAPCs (170), USSCs (106), marrow-isolated adult multilineage inducible (MIAMI) cells (171) or more soberly termed cells as multipotent adult stem cells (MASCs) (172). Besides USSCs, all these lineages were isolated from bone marrow. All these cells share phenotypic and differentiation features while they own some specificities allowing distinct labelling and which can be deducted from specific isolation and culture methods (for review, refer to Phinney et al. (173)).

In the present work, we isolated cell lineages from various sources (BM, umbilical cord, skin and adult liver) that all displayed specific phenotypic and differentiation charateristics (Table 11). Surprisingly, classical charaterization methods conducted us to two unexpected observations: phenotypic homology between validated stem cells (MSCs) and so-called quiescent cells (fibroblasts), and existence of a chimerical phenotype in extrahepatic MSCs (UCMSCs). Contrarily to many works using fibroblasts as control for differentiation experiments, we demonstrated that these cells were highly similar to MSCs and could only been distinguished by their *in vitro* proliferation properties and inability to differentiate after

being subcultured more than three passages. Although a recent report suggested the presence of MSCs into fibroblast cultures (174), the authors did not propose tools for identifying these contaminating populations. Furthermore, their hypothesis could be inverted by suggesting the presence of fibroblastic colonies in MSC cultures, what is more likely regarding the heterogeneity of differentiation potential in MSC populations. Nevertheless, this kind of suggestions underlines the difficulty to objectively state about a real 'MSC' or 'fibroblast' phenotype.

**Table 11: Comparison of *in vitro* characterisitcs of mesenchymal cell lineages.**

| | | | Native phenotype | | |
|---|---|---|---|---|---|
| | ***In vitro* Morphology** | **Growth** | **Flow cytometry** | **ICC** | **Mesodermal Differentiation** |
| **hBM-MSCs** | Spindle-shaped | .Doubling time: 8.0 ± 3.7 days .Cumulative PD: up to 34 ($8^{th}$ P) | $CD13^{+}$, $CD71^{+/-}$, $CD26^{+/-}$, $CD117^{-}$, $CD133^{-}$ | αSMA, fibronectin, vimentin, laminin | $Osteo^{+}$, $Adipo^{+}$ |
| **UCMSCs** | Spindle-shaped with cytoplasmic stress fibers | .Doubling time: 5.4 ± 0.9 days .Cumulative PD: up to 20.4 ± 1.5 ($10^{th}$ P) | $CD13^{+}$, $CD54^{+}$, $CD117^{+}$, $CD166^{+/-}$ | $Alb^{+}$, $\alpha FP^{+}$, $CX\text{-}32^{+}$, $CK8^{+}$, $CK18^{+}$, $CK19^{+}$, $CK7^{-}$, αSMA, fibronectin, vimentin | $Osteo^{+}$, $Adipo^{+/-}$ |
| **Human Fibroblasts** | Spindle-shaped | .Doubling time: 3.8 ± 1.5 days .Cumulative PD: up to 52 ($15^{th}$ P) | $CD13^{+}$, $CD71^{+/-}$, $CD117^{-}$ | αSMA, fibronectin, vimentin, laminin | $Osteo^{+}$, $Adipo^{+}$ (+related mRNAs) |
| **ADHLSCs** | Spindle-shaped with broad cytoplasm | .Doubling time: 6.6 ± 4.0 days .Cumulative PD: 17.9 ± 2.8 ($11^{th}$ P) | $CD13^{+}$, $CD49f^{+/-}$, $CD117^{-}$, $CD133^{-}$, $HLA\text{-}ABC^{+/-}$ | αSMA, vimentin, albumin | $Osteo^{-}$, $Adipo^{-}$ |

Adipo, adipocyte; osteo, osteocytes; P, passage.

More astonishing is our description of a hybrid phenotype in native UCMSCs, expressing both mesenchymal and hepatocyte markers. Whether these cells could share hepatocyte's features according to similar embryological origin (vitelline vesicle) or represent artificial *ex vivo* phenotype is enigmatic. Ectopic marker expression can also be questioned because hepatocyte-like features were not correlated with specific functions (175). However, our work echoes this of Avital and colleagues who have described native hepatic marker expression in $\beta_2m^-$/Thy-$1^+$ cells isolated from bone marrow (176). Hybrid identity was also observed in our ADHLSC cell line whose phenotype is more likely to depend on an EMT process. This is suggested by several characteristics that are shared by cell lineages obtained after dedifferentiation of primary hepatocyte cultures: acquisition of proliferation capacities, morphological changes to more elongated architecture and expression of mesenchymal markers (c-kit, Thy-1 and CD49f) (177-179). In this case, EMT process could start during liver cell isolation while intercellular connexions are disrupted. Heterogeneity of expression patterns of EMT cell lines compared to ADHLSCs could reflect differences of culture media. However, MSCs were recently isolated from healthy livers (180-182) and mesenchymal lineages were localized in periportal area of adult porcine livers (183), suggesting the possible existence of a mesenchymal progenitor cell reservoir in healthy adult liver.

Even if publication and patency laws are tough commanders of researchers' vocation, there is a need for consensual state-of-the-art about defining 'mesenchymal' stem cell identity and behavior. Characterizing new cell lines, cell culturists should consider the possibility of artificial creation of *in vitro* phenotypes, *a fortiori* because correlations with similar *in vivo* cell lineages are lacking. In that way it has been demonstrated that stem cell plasticity and marker expression can vary depending on cell cycle transit or on external stimuli (184). Efforts are being made now to elicit more restricted panel of criteria to identify and classify

mesenchymal cells (64). However, as demonstrated in the present work, current phenotypic characterization techniques are limited and do not strictly allow distinction between some lineages of mesodermal origin (MSCs *vs* fibroblasts).

*Pluripotency and metaplasia*

Recent developments in stem cell field overcome old paradigms on cell differentiation. Whereas a cell was supposed to be restricted to its germinal origin, evidence is now provided about stem cell potential to overcross these barriers and generate cells from all three germ layers (mesoderm, endoderm and ectoderm). Although 'transdifferentiation' is a commonly used term for defining this property, it suggests the change of a fully differentiated cell to another fully differentiated and mature cell, and 'metaplasia' has been suggested as a more generic term owning plethoric examples in humans (185).

Besides growing interest on stem cell differentiation potential (for review, see (63;75)), more revolutionary reports on true transdifferentiation from highly committed lineages aroused: peripheral blood monocytes have been shown to be pluripotent (139;186;187) and we demonstrated mesodermal and endodermal differentiation potential of human skin fibroblasts (Chapter VI). Works on monocytes, whether original, could be extrapolated from *in vivo* demonstration of the hepatic fate of myelomonocytic lineages after HSC transplantation (188;189). Fibroblastic differentiation potential could also find its correlative with *in vivo* models. Indeed, mesenchymal-epithelial transition (MET) occurs *in vivo* during embryological development through several cycles (primary mesenchyme -> secondary epithelia -> secondary mesenchyme -> tertiary epithelial structures) and MET has been involved in the ontogeny of the kidney, the gastrointestinal tract, the lungs and skin (190).

All these data suggest the need for revising the stem cell definition as a property of a cell to possess specific markers, to be able of self-renewing or to differentiate into a pre-defined variety of lineages. Differentiation is more likely to be a less restricted event depending on culture or *in vivo* microenvironment and on cell properties, as suggested by Blau et al. (191), reporting that 'rather than referring to a discrete cellular entity, a stem cell most accurately refers to a biological function that can be induced in many distinct types of cells, even differentiated cells'. This was highlighted by the demonstration of differentiation potential of epithelial cells both *in vitro* (192) and *in vivo* (193).

b. Assigning hepatocyte-like identity *in vitro*

*Phenotypic characterization as a reflect of hepatocyte-like commitment*

Our work reports the potential to induce or enhance the expression of hepatocyte features in mesenchymal cell lineages *in vitro*. These features were revealed at the level of morphology, gene and protein expression and specific functionality. Table 12 compares hepatocyte features observed after differentiation of each lineage. As described, higher levels of differentiation were obtained using UCMSCs and ADHLSCs while hybrid phenotype of their native relatives makes these cells poorly comparable to classical MSCs. By comparison, after conditioning of hBM-MSCs *in vitro* we obtained chimerical products highly comparable to native UCMSCs and ADHLSCs. We showed that the differentiation yield of hBM-MSCs was variable and procured cells with undefined differentiation state as they co-expressed early (as αFP, albumin or CK8) and late hepatic markers (as TAT, PEPCK) and as they acquired some mature functionality while lacking other ones. In MDHLCs, we observed dissociation between marker expression and related function (ex: G6Pase activity, albumin secretion). These cells behaved as their features were electively stimulated by exposure to cytokines/factors.

**Table 12: Comparison of hepatocyte feature acquisition in mesenchymal cell lineages.**

| | hBM-MSCs | UCMSCs | Human Fibroblasts | ADHLSCs |
|---|---|---|---|---|
| **mRNA markers** | | | | |
| **Albumin** | + | + | + | + |
| **G6Pase** | + | + | + | + |
| **CK-8** | + | + | - | - |
| **CK-18** | - | + | - | - |
| **α1-AT** | + | + | + | + |
| **α-FP** | + | + | + | + |
| **CYP 3A4** | - | + | - | + |
| **CYP 2B6** | - | - | - | + |
| **TAT** | + | + | - | + |
| **TDO** | + | + | + | + |
| **HNF4** | - | - | - | + |
| **GS** | + | ND | + | + |
| **c-Met** | + | + | + | ND |
| **PEPCK** | + | + | - | ND |
| **Others** | | $CK19^+$ | | $CYP1B1^+$, $GGT^+$, $MRP2^+$, $Oct4^-$ |
| **Protein markers** | | | | |
| **Albumin** | + | + | + | + |
| **CK18** | - | + | - | ND |
| **HepPar-1** | - | - | - | - |
| **CX-32** | + | + | + | + |
| **Others** | $\alpha FP^+$, $DPPIV^+$, E-$cadh^+$, $CK8^-$, $CK18^-$ | $\alpha FP^+$, $DPPIV^+$, $CK8^+$, $CK19^+$ | $\alpha FP^+$, $DPPIV^+$, $CK8^-$, $CK18^-$ | |
| **Functionality** | | | | |
| **Urea cycle** | + | + | + | ND |
| **Albumin** | - | - | - | - |
| **Glycogen storage** | + | + | + | + |
| **G6Pase activity** | - | + | ND | + |
| **Gluconeogenesis** | + | ND | ND | + |
| **CYP3A4 activity** | ND | + | ND | ND |

ND, not documented

Efforts to enhance *in vitro* accuracy and effectiveness of differentiation were performed by studying the effect of 41 combinations of *in vitro* conditioning regimen. We tested majority of cytokines known to be involved in liver biology or reported in differentiation protocols (Table 13, (194;195)). Nevertheless, we were not able to enhance rate of hepatocyte feature acquisition.

**Table 13: Comparative use of compounds for hepatocyte differentiation.**

| | **Documented (195)** | **Present work** |
|---|---|---|
| **Cytokines/growth factors** | Activin A, BMP2, BMP4, EGF, FGF1, FGF2, FGF4, HGF, IGF, TGFβ, OSM | Activin A, BMP4, EGF, FGF1, FGF2, FGF4, follistatin, HGF, IL-6, IL-9, KGF, LIF, OSM |
| **Chemical compounds** | Ascorbic acid, dexamethasone, DMSO, linoleic acid, nicotinamide, norepinephrine, retinoic acid, sodium butyrate, ITS | Ascorbic acid, dexamethasone, DMSO, nicotinamide, ITS |
| **Extracellular matrix** | Collagen type I, fibronectin, Matrigel | Collagen type I |

Documented use of compounds on ESCs and on MSCs is compared to our present work. DMSO, dimethylsulfoxide; IGF, insulin-like growth factor; ITS, insulin-transferrin-selenium; TGF, transforming growth factor.

Because of weak accuracy and relevance of expression patterns observed after conditioning of mesenchymal lineages, we assumed that marker expression analysis brings only relative information about cell differentiation potential. This is also evoked by the fact that many hepatocyte markers lack specificity. For example, expression of albumin, αFP and $\alpha_1$AT was detected in human pancreas (102), cytokeratins were observed in gastric cardia and skin (196) or in lungs (195;197), DPPIV is expressed in the kidney (198) and $\alpha_1$AT is a common enzyme

of the lungs (199). Strikingly, several studies demonstrated the expression of hepatic markers on stem cells before differentiation (Table 14).

**Table 14: Ectopic expression of hepatocyte markers in undifferentiated stem/progenitor cells.**

| Cell type | Detection method | Markers | References |
|---|---|---|---|
| **hBM-MSCs** | RT-PCR | Albumin, CK18, TDO | (93) |
| **hUCB-MSCs** | RT-PCR | Albumin, CK18, GS | (104) |
| **hUSSCs** | Flow cytometry | CK8/18 | (106) |
| **hADSCs** | RT-PCR | αFP | (110) |
| **hHPCs** | RT-PCR | Albumin, αFP, CK19, HNF1, TAT, | (47) |
| **hUCB-MSCs** | ICC | Albumin, C/EBPα, CYP1A1/2 | (144). |

ADSC, adipose tissue-derived stromal cells; HPC, hematopoietic progenitor cells.

*Functional characterization as a proof of hepatocyte-like identity*

As described by Hengstler et al. in a recent review (200), the demonstration of numerous functional activities is mandatory before considering a cell as 'hepatocyte-like'. In that way, we handled several assays and showed the potential of mesenchymal lineages to acquire hepatocyte-specific metabolic functions after differentiation (Table 12). As for marker expression, differentiated UCMSCs displayed highest level of hepatocyte-like functionality as these cells were able to produce urea, store glycogen and acquire G6Pase and CYP3A4 activities. We never succeeded to detect secreted albumin in any differentiated cell type although we handled two different ELISA commercial kits with detection sensitivity up to 200 pg/mL. This secretion was shown elsewhere on differentiated stem cells using same assays (138;201) but was missing in the characterization from other reports of reference (92;93). Globally our results about acquisition of hepatocyte-like functionality were difficult to compare with literature data because of the absence of unit standardization (Table 8) or

even of normalization. Implementation of results from literature is rendered hardous because of these discrepancies but also due to overall lack of control experiments or correlation with standard hepatocytes. We showed that the functionality can be improved by inducting activity of enzymes of urea cycle (with $NH_4Cl$), gluconeogenesis (with glucagon), or of G6Pase (with G6Pate) and CYP3A4 (with phenobarbital). However, except for UCMSCs, functional activity of differentiated cells was each time lower than which of hepatocytes, even after induction.

Put together our *in vitro* investigations demonstrate the current limitations in establishing fully matured stem cell-derived hepatocytes using classical differentiation strategies as factors exposition on 2D-cultures.

c. *In vivo* studies

We demonstrated the potential of mesenchymal lineages to engraft into several mouse models up to 10 weeks (Table 15). The engrafted cells showed acquisition of hepatocyte markers and in some experiments their human origin was confirmed using combined techniques (as *in situ* hybridization and immunohistochemistry). For hBM-MSCs, we showed that the *in vivo* expression profile depended on the transplant procedure. Indeed, for these cells, intrahepatic injection allowed further differentiation and stronger engraftment while *in vitro* pre-conditioning of the cells lead to down-regulation of MDHLCs' chimerical phenotype. In a wild-type mouse model, we showed the potential of hBM-MSCs to engraft and partially differentiate under stringent conditions, emphasizing their advantageous immunological properties. Fibroblasts displayed similar *in vivo* expression and engraftment potential than BM-MSCs. UCMSCs engrafted at lower levels than BM-MSCs (data not shown) and changed their phenotype *in vivo* as they lost their strong expression of CK19. Best *in vivo*

performances were obtained using ADHLSCs as their engraftment was not dependent on hepatic injury and as they formed large cell clusters and secreted albumin in mice serum.

**Table 15: *In vivo* investigations with mesenchymal lineages.**

| Cell type | Mouse model | Hepatic injury | Timing | Expression |
|---|---|---|---|---|
| BM-MSCs | SCID | Hepatectomy, R-AA | 4 w | Albumin, αFP, CK18, fibronectin, human alu, vimentin |
| | Wild-type | Hepatectomy, R-AA | 2, 3 w | Albumin, αFP, fibronectin |
| UCMSCs | SCID | Hepatectomy | 2, 4 & 6 w | Albumin, αFP, fibronectin, human alu human mitochondria |
| Fibroblasts | SCID | Hepatectomy, propanolol | 4 w | Albumin, αFP, CK18, fibronectin |
| ADHLSCs | uPA-SCID or SCID | Hepatectomy | 10 w | Albumin, αFP, Ki-67, prealbumin, serum albumin |

αFP, alpha-1-fetoprotein; CK, cytokeratin; R-AA, retrorsine-allyl alcohol; uPA, urokinase plasminogen activator.

All these data suggest the potential of mesenchymal lineages to produce hepatocyte-like cells *in vivo*. However, characterization of these differentiated cells remains limited by antibodies availability and specificity. For example, even if HepPar-1 is largely used in staining assays, its cross-reactivity hampered objective *in vivo* cell detection in our hands and elsewhere (202). Also limited are demonstrations of *in vivo* cell functionality. In our experiments, only ADHLSCs were able to secrete detectable amounts of albumin after transplantation. To our knowledge, only two reports were described about MSCs potential to reduce liver fibrosis (203) or mortality rate (204). These data corroborated investigations on similar models using BM cells or HSCs (205-210). Nevertheless, such reports only provide indirect concordance

between cell injection and improvement of the disease without determination of cell functionality. Proof of concept was provided only using fumarylacetoacetate hydrolase $(FAH)^{-/-}$ mouse model (54;211;212).

As for *in vitro* functional assays, comparison with literature data concerning MSCs engraftment rates was hazardous due to lack of achievement and discrepancies of methods. The only report quantifying hBM-MSCs engraftment was provided in a wild-type rat model using FISH and immunohistochemistry assays (96). Although these authors could co-localize human Y $chromosome^{+}$ nuclei and human $albumin^{+}$ cells in rat livers, the rate of albumin-positive cells was higher than this of human cells and this latter was up to two times higher than that of rat 12-$chromosome^{+}$ cells, underlying lack of accuracy of the techniques used. However, overall transplantation procedures with stem cells are associated with low repopulation rates as reviewed with HSCs (53) and other cell linages (35), showing also a high diversity of normalization methods.

Compared to what has been described with hematopoietic (stem) cells, up to now no evidence of cell fusion events could be detected after MSCs transplantation for liver therapy (92;96). In our work, we showed a colocalization of human markers both in the nucleus and the cytoplasm of engrafted MSCs which, whether not excluding fusion events, corroborated literature data.

Because of the difficulty to objectivelly achieve stem-cell derived hepatocyte-like phenotype *in vivo* without using combined techniques, and considering current repopulation rates, it remains questionable how rare engraftment events can be viewed as true differentiation or as involvement of paracrine factors. The question has been suggested indirectly by a recent study of *in vivo* liver differentiation of stem cell lineages (103). In this work, authors described the existence of two disctinct types of engrafting cells: one with a human nucleus and lacking epithelial-like morphology (type 1) and another with a true hepatocyte shape containing a

mouse nucleus while staining for human albumin (type 2). Although authors concluded about a possible horizontal gene transfer from human cells to type 2 cells, one may ask whether human albumin immunohistochemistry assay is accurate enough to allow validation of the results without confrontation with another technique. Paracrine mechanisms could warrant usefulness of MSCs by stimulating regeneration *via* inhibition of apoptosis, by suppressing immune reactions or by increasing angiogenesis. For example these cells have been described to be beneficial for endogeneous neuronal regeneration by a transfer of mitochondria or mitochondrial DNA to defective cells (213). The hypothesis was also recently reviewed (173;214) and suggested to be related to the biochemical heterogeneity of MSCs.

## 2. Perspectives

### a. *In vitro* culture management

Because of the wide phenotypic heterogeneity of mesenchymal cell lineages, there is a need for cell biologists to identify and use standardized techniques for cell isolation, culture and characterization, allowing confrontation of the data. New cell lineages should be each time validated by other teams because confusion is growing for the non specialist or for clinicians: collaborations should be invited, as programs designed by the European Commission.

The potential of mesenchymal ‘stem’ cells for hepatocyte differentiation has been suggested here and elsewhere. It is time now to provide further arguments about their usefullness for liver tissue engineering. As *in vitro* differentiation remains the first step for concept validation and for programming costly *in vivo* assays, it should be further analyzed whether 2D-cultures are sufficiently efficacious. In our hands, factors exposition bring interesting but limited results while new methods of combinations, using broader amounts of factors and studying time-depedency, should be designed. Assays on 3D-cultures, mimicking better the liver microenvironment, are probably the next step of investigations in stem cell-derived liver tissue engineering. Also co-culture experiments should be developed according to paracrine beneficial mechanisms of MSCs which could lead to faster validated application.

As we demonstrated the persistence of native mesenchymal phenotype in MDHLCs, it should be of interest to study whether related MSCs advantageous properties are remaining (as resistance to cryopreservation, easiness of transfectability or immunomodulation properties). At the contrary, in which proportion the remaining mesenchymal phenotype of MDHLCs could hamper hepatocyte differentiation to occur should also be analyzed.

For accurate characterization of the hepatocyte-like cell phenotype, we recommend to use the broadest panel of markers with combined techniques in order to alleviate data

misinterpretation. Quantifying hepatocyte-like functionality, one should prefer quantitative and comparative functional assays rather than commercially available kits which are lesser reproducible. Non specific assays as LDL uptake should be avoided from protocols and data should always be normalized to allow comparison of the performances. Functionality is the corner stone of *in vitro* assays: as a cell should display one specific hepatic function, it will be further considered for cell therapy. Although specialized functions have been described in hepatocyte-like cells, these are far from reaching global hepatocyte functionality while this is mandatory before considering these cells for replacement therapy in the context of global liver dysfunction. With the aim to enhance specialized functions in hepatocyte-like cells, some induction experiments are available while others should be evaluated (Table 16).

**Table 16: Expression and modulation of hepatocyte-like metabolic pathways after *in vitro* differentiation.**

| Metabolic pathway | Gene expression | Induction |
|---|---|---|
| Glutamine synthase | + | Glutamine |
| Urea cycle | ? | Glutamine |
| G6Pase | + | Glucagon |
| TAT | + | Tyrosine? |
| CYP | - | Phenobarbital, rifampicine |

b. *In vivo* investigation models

As for *in vitro* assays, hepatocyte commitment of mesenchymal lineages has been suggested *in vivo* whereas no proof of concept has been provided yet. In that way, SCID mice models only bring data about potentiality of engraftment and differentiation. However, it is still enigmatic how xenogeneic transplant procedures could bring transposable data (215). First guidelines concerning methods for transplantation and assessment of hepatocyte-like phenotype should be stated. Then should be identified accurate rodent models owning metabolic diseases comparable to the clinical setting (35;216). In these models, successful allogeneic cell transplantation will provide powerful arguments about *in vivo* stem cell functionality. Researchers are often reluctant to program rodent allogeneic protocols because of lesser breakthrough of data compared to human material but also to technical limitations, as hardous *in vivo* cell tracking and identification. This kind of studies is the next challenge to be established concerning *in vivo* studies. Other major step before considering stem cells for liver therapy will be the demonstration of their successful and safe transplantation into non-human primate models of liver regeneration.

c. On the road to clinic

MSCs are currently being regarded for their potential use in cell and gene therapy for various diseases (77). However, as stated by Prockop and Olson (217), essential precautions are omitted in some poorly designed clinical trials and this can make shade to new therapies as what happened with gene therapy (218). To date, no consensus exists about MSCs culturing for clinical purposes (219;220) whereas research is being performed to validate animal serum-free culture conditions (221;222). Another question is cell banking of derived phenotypes and although many laboratories are currently working on liver tissue engineering, there is up to now no data about the feasibility to reliably freeze and thaw stem cells-derived hepatocyte-

like cells. MSCs' role in liver therapy remains to be determined and is likely to evolve as besides differentiation studies, growing interest is given to these cells for their use in co-transplantation procedures. Because of their immunological properties, MSCs could be considered earlier as an adjuvant for LCT or OLT procedures than as a tool for direct cell therapy. However, besides demonstration of the concept, first line of battle is safety of the cell infusion procedure, and several related points deserve to be adressed. First is the possibility for bacterial and viral infections carried by donor cells and risk of life-threatening allergic reactions. Another point is that MSCs might compromise the host immune system. This purpose has many implications including the potential of stimulation of malignancies (223). This has been described using MSCs in some experimental conditions (224;225) and these cells have been demonstrated to become neoplastic after long-term culture (226;227). Moreover, there is a risk of emboli while using high concentrated cell suspension as aggregates are quickly generated (217). Nevertheless, as for many other therapies, clinical trials are conducted before having answered to all safety concerns and they will provide, parallel to more cautious pre-clinical assays, arguments concerning the real place for mesenchymal lineages in human cell therapy.

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**Lysy PA**, Smets F, Sibille C, Najimi M, and Sokal EM. Human Skin Fibroblasts: From Mesodermal to Hepatocyte-Like Differentiation. Hepatology 2007 Nov;46(5):1574-85.

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## Summary

Human MSCs are being largely studied for their differentiation potential and immunological properties. In the present study, we evaluated the ability to reliably differentiate mesenchymal lineages into hepatocyte-like cells both *in vitro* and *in vivo*. For this purpose, we handled several tissue sources and compared typical MSCs from BM or umbilical cord, to liver-derived mesenchymal-like cells and to fibroblasts. We observed that hepatocyte differentiation of BM-MSCs was incomplete and variable with elective expression of some specific markers. These mesenchymal-derived hepatocyte-like cells (MDHLCs) were also chimerical in their phenotype as they expressed mesenchymal markers while these were down-regulated. We therefore designed differentiation cocktails with an aim to improve MDHLC phenotype and some unexpected results were obtained with LIF cytokine whose action on stem cells for hepatocyte differentiation was not documented. Nevertheless, we observed a limitation in the acquisition yield of hepatic features. Furthermore, the hepatocyte-like phenotype of MDHLCs completely disappeared when the cells were incubated into growth medium. However, we showed that hepatic functionality of these cells, as urea secretion and gluconeogenesis, could be increased under specific conditions, suggesting the potential to improve MDHLC phenotype.

*In vivo*, MSCs were able to express hepatic markers into SCID-mice livers while their chimerical phenotype remained. In contrast, MDHLCs down-regulated their hybrid phenotype after transplantation suggesting a beneficial influence of *in vitro* differentiation step. MSCs were also able to engraft and even partially differentiate into wild-type mice which was a strong argument for their low immunogenicity.

Surprisingly, fibroblasts showed highly similar potential than MSCs to differentiate into hepatocyte-like cells both *in vitro* an *in vivo* and these results underlined the difficulty to accurately distinguish between both cell types using current techniques. UCMSCs and

ADHLSCs were different in nature and displayed a native hybrid phenotype while their differentiation allowed high levels of hepatocyte-like feature acquisition. Together all these data suggest the current possibility to engineer mesenchymal-derived hepatocyte-like cells owning specific features acquisition while remaining limited in their commitment. This highlights the need for further investigations to evidence the usefulness of these mesenchymal lineages for liver cell therapy.

## Résumé

Les cellules souches mésenchymateuses (CSM) sont de plus en plus étudiées pour leur important potentiel de différenciation et pour leurs capacités immunomodulatrices. Dans ce travail, nous avons étudié l'efficacité et le rendement de différenciation hépatocytaire des CSM en comparaison avec des fibroblastes et des cellules mésenchymateuses isolées à partir de foie adulte. Nous avons d'abord observé que la différenciation hépatocytaire des CSM leur permettait d'acquérir un phénotype hybride avec co-expression de marqueurs mésenchymateux et de marqueurs hépatocytaires dont la présence était variable. Des tests de différenciation *in vitro* ont dès lors été élaborés et ainsi l'influence inattendue du LIF sur le devenir hépatocytaire des CSM a été observée. Malgré cela, nos conditions expérimentales n'ont pas permis d'améliorer le phénotype hépatocytaire des cellules différenciées et, de plus, ce phénotype s'est avéré labile, disparaissant au sein de cellules exposées à un milieu de croissance standard. Il est possible d'améliorer la fonctionalité hépatocytaire des CSM différenciées vu que nous avons démontré une augmentation de leur sécrétion d'urée et de glucose dans certaines conditions.

Nous avons observé également que la transplantation des CSM dans des souris immunodéficientes leur permettait d'acquérir des marqueurs hépatocytaires et avons confirmé que ces cellules maintenaient leur caractère chimérique. Toutefois, les CSM pré-différenciées *in vitro* perdaient ce caractère *in vivo*, ce qui démontre l'importance d'une étape de pré-différenciation. Ces CSM étaient également capables de se greffer et d'exprimer quelques marqueurs hépatocytaires après transplantation dans des souris sauvages, ce qui souligne leur faible pouvoir immunogène.

Une découverte importante a été la démonstration de capacités similaires de différenciation hépatocytaire au sein de fibroblastes de peau, cellules considérées comme quiescentes, ce qui démontre la difficulté de distinguer de manière fiable des lignées d'origine mésodermique. En

comparaison, les CSM de cordon ombilical et les cellules mésenchymateuses dérivées du foie ont un phénotype naturellement hybride, et leur différenciation hépatocytaire a permis l'obtention de caractéristiques hépatocytaires plus avancées que celles observées après différenciation des CSM de moëlle osseuse. Les résultats décrits dans ce travail démontrent la possibilité actuelle de produire des lignées de type hépatocytaire à partir de cellules d'origine mésenchymateuse, dont les spécificités restent néanmoins limitées. Des programmes de différenciation plus élaborés et tenant mieux compte du microenvironement cellulaire seront nécessaires pour améliorer cet état de différenciation, tout comme des modèles animaux thérapeutiques viendront avantageusement agrémenter la place des cellules mésenchymateuses pour le traitement de maladies du foie.

# ANNEX

**Article 6:** Stem cells for liver tissue repair: Current knowledge and perspectives. World J Gastroenterol 2008 Feb 14;14(6):864-75.

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*TOPIC HIGHLIGHT*

**Etienne M Sokal,** *Series Editor*

# Stem cells for liver tissue repair: Current knowledge and perspectives

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## Abstract

Stem cells from extra- or intrahepatic sources have been recently characterized and their usefulness for the generation of hepatocyte-like lineages has been demonstrated. Therefore, they are being increasingly considered for future applications in liver cell therapy. In that field, liver cell transplantation is currently regarded as a possible alternative to whole organ transplantation, while stem cells possess theoretical advantages on hepatocytes as they display higher *in vitro* culture performances and could be used in autologous transplant procedures. However, the current research on the hepatic fate of stem cells is still facing difficulties to demonstrate the acquisition of a full mature hepatocyte phenotype, both *in vitro* and *in vivo*. Furthermore, the lack of obvious demonstration of *in vivo* hepatocyte-like cell functionality remains associated to low repopulation rates obtained after current transplantation procedures. The present review focuses on the current knowledge of the stem cell potential for liver therapy. We discuss the characteristics of the principal cell candidates and the methods to demonstrate their hepatic potential *in vitro* and *in vivo*. We finally address the question of the future clinical applications of stem cells for liver tissue repair and the technical aspects that remain to be investigated.

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**Key words:** Stem cells; Hepatocyte differentiation; Liver regeneration; Cell therapy

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## INTRODUCTION

Orthotopic liver transplantation (OLT) is the gold standard treatment for end-stage liver failure and for numerous liver based inborn errors of metabolism. However, organ shortage remains a major limiting factor and alternative solutions are being examined in the liver therapy field. Liver cell transplantation (LCT) is emerging with heartening success[1,2], but is still limited by cell viability, modest engraftment and limited tissue availability. Increasing interest is carried to stem cells regarding the recent demonstration of their plasticity[3]. Theoretical advantages of stem cells for tissue regenerative medicine are multiple: ease of harvest, proliferation capacity, efficiency of *in vitro* transfection and potential use of autologous cells. Different types of stem cells are eligible for liver cell therapy according to their hepatic potential, for instance mesenchymal stem cells (MSCs), hematopoietic stem cells (HSCs) and adult liver stem/progenitor cells. Despite encouraging results, key pitfalls remain while using stem cells-derived hepatocyte-like cells: lack of tissue-specific functionality and, up to now, no evidence of strong liver repopulation level in animal models. Moreover, the demonstration of the acquired hepatocyte-like phenotype is impaired by technical restraints. To reach clinical application, stem cell therapy requires further development to become competitive regarding LCT. Here, we present the current knowledge on the use of stem cells for hepatic tissue engineering and describe the future orientations in the field.

## IDENTIFICATION OF THE STEM CELL CANDIDATES

### *Mesenchymal stem cells*

Firstly described in 1970 from bone marrow (BM) isolates[4], these cells can currently be obtained from various tissue sources as BM, cord blood (CB), adipose tissue, umbilical cord blood (UCB). Their *in vivo* function and behavior remain largely unknown[5]. MSCs have the

**Table 1 Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of MSCs**

| Cell source | *In vitro* differentiation | *In vivo* differentiation | References |
|---|---|---|---|
| Human BM&UCB Immunoselection | Cocktail: EGF-bFGF (2 d), HGF-bFGF-nicot (7 d), OSM-dexa-ITS<br>Characterization: RT-PCR (8), ICC (2), urea production, LDL uptake, PROD | ND | Lee *et al*[8] |
| Human UCB | ND | Host: preimmune fetal sheep<br>Injury: -<br>Cell characterization: $Alb^+$, $HepPar1^+$<br>Fusion: microdissection + PCR: -<br>Repopulation rate: > 20% | Kögler *et al*[16] |
| Human adipose tissue | Cocktail: DMSO-HGF-OSM<br>Characterization: RT-PCR (4), ICC (1), urea production, LDL uptake | Host: NOD/SCID mice<br>Injury: $CCl_4$<br>TRP: $1.10^6$ cells, tail vein<br>Cell characterization: $Alb^+$/CM-$Dil^+$<br>Repopulation rate: up to 0.13% $Alb^+$ at 10 d | Seo *et al*[22] |
| Human BM<br>MSCs, $CD34^+$ cells,<br>non MSCs/$CD34^-$ cells | ND | Host: WT rats (+CsA)<br>Injury: AA<br>TRP: 1.106 cells, liver<br>Cell characterization: $Alb^+$, $CK18^+$, $CK19^+$, $\alpha FP^+$, $AGPR^+$, serum $Alb^+$<br>Fusion: FISH hum Y/rat chr 12: - | Sato *et al*[21] |
| Human BM | Cocktail: 5'aza (1 d) - EGF-EGF (14 d)<br>Characterization: RT-PCR (11), ICC (4), WB (4), urea production,<br>PAS, reporter gene construct for PEPCK activity | Host: $Pfp/Rag2^{-/-}$ mice<br>Injury: propanolol, partial hepatect<br>TRP: $1.10^6$ cells, liver<br>Cell characterization: $Alb^+$, HepPar-$1^+$, $PEPCK^+$, $CX32^+$, $PAS^+$<br>Fusion: FISH hum alu/mouse satellite: - | Aurich *et al*[10] |
| Human BM | Cocktail: HGF-FGF4-ITS-nicot (7 d), OSM-dexa-ITS (15 d)<br>Characterization: RT-PCR (17), ICC (8), flow cytometry, urea&Alb production, PAS, G6Pase activity, neoglucogenesis | Host: SCID mice<br>Injury: partial hepatect or retrorsine/AA<br>TRP: $1.10^6$ cells, spleen or liver<br>Cell characterization: $Alb^+$, $\alpha FP^+$, $CK18^+$, fibronectin$^+$, vimentin$^+$, hum $alu^+$ | Lysy *et al*[106] |

Alb: Albumin; AA: Allyl alcohol; αFP: Alpha-fetoprotein; $CCl_4$: Carbon tetrachloride; CB: Cord blood; CK: Cytokeratin; CsA: Ciclosporin A; d: Days; dexa: Dexamethasone; FGF: Fibroblastic growth factor; G6Pase: Glucose-6-phosphatase; hepatect: Hepatectomy; HGF: Hepatocyte growth factor; hum: Human; ITS: Insulin-transferrin-selenium; nicot: Nicotinamide; OSM: Oncostatin M; PAS: Periodic acid-schiff; PEPCK: Phosphoenolpyruvate carboxykinase; PROD: Pentoxyresorufin-O-dealkylase; WT: Wild-type; chr: Chromosome; FISH: Fluorescent *in situ* hybridization; ICC: Immunocytochemistry; ND: Not documented; RT-PCR: Reverse transcription-polymerase chain reaction; TRP: Transplantation; WB: Western blot. Amounts of analyzed markers are provided between brackets.

characteristics to be highly proliferative *in vitro*, easily transfectable, resistant to cryopreservation[6] and to display a large *in vitro* and *in vivo* differentiation potential[7]. The first description of their hepatic potential was provided by Lee *et al*[8] and confirmed by other studies[9-26] (Table 1). While some studies evidenced the *in vivo* potential of MSCs[10,21], this was recently questioned by other authors who did not detect engrafting cells after syngeneic transplantation[20]. However, experimental protocols were quite different rendering comparison hazardous. A key advantage of MSCs is their immunological properties making them lesser immunogenic and possibly able to induce tolerance as highlighted by promising *in vitro* studies and clinical trials[27-32].

### Hematopoietic stem cells

These cells constitute the paradigm of stem cells: cells capable of self-renewing, proliferation and production of a progeny of committed cell lineages, permitting the regeneration of the tissue, even after transplantation. These can be isolated from BM, UCB and peripheral blood[33] making them highly available while they are still difficult to expand *in vitro*[34,35]. In this cell population, cell identity and phenotype are mainly determined by a selection procedure whose criteria remain debated[36]. HSCs have recently aroused much enthusiasm in the hepatology field after the demonstration of their hepatic differentiation potential as described in Table 2. A majority of studies used mononuclear cell preparations[37-63], which hamper the identification of the cells involved in the differentiation process. For that reason, we included in Table 2 only studies using defined subpopulations[64-80]. However, HSCs' liver potential is increasingly debated[40,65,81-84] and the possibility of cell fusion as an explanation of 'plasticity' make these cells less considerable for a clinical use[85].

### Adult liver stem cells

As organ shortage is limiting the availability of this cell population, the conditions for their use in cell therapy are governed by two main characteristics, a high proliferation

**Table 2 Representative studies investigating *in vitro* and/or *in vivo* hepatocyte differentiation of HSCs**

| Cell source | *In vitro* differentiation | *In vivo* differentiation | References |
|---|---|---|---|
| Mouse BM | ND | Host: irradiated FAH$^{-/-}$ mice<br>Injury: NTBC withdrawal<br>TRP: 10 to 1000 cells; retroorbital plexus<br>Cell characterization: β-gal$^+$, Alb$^+$, DPPIV$^+$, e-cadherin$^+$, FISH Y$^+$, liver function, FAH activity<br>Repopulation rate: 30%-50% βgal$^+$ cells | Lagasse *et al*[73] |
| Mouse BM | ND | Host: irradiated CD45.1$^+$ mice<br>Injury: hepatect or anti-Fas Ab treatment<br>TRP: $10^6$ cells; vein<br>Cell characterization: CK8$^+$, CK18$^+$, CK19$^+$, CD45.2$^+$, FISH Y$^+$, PCR Bcl-2$^+$<br>Repopulation rate: up to 0.05%-0.8% with anti-Fas Ab treatment | Mallet *et al*[74] |
| Human UCB&BM | ND | Host: irradiated NOD/SCID mice ± HGF<br>Injury: $CCl_4$<br>TRP: 2000 to $10^5$ cells; vein<br>Cell characterization: Alb$^+$, CD45$^-$, human alu$^+$, CK19$^+$, Alb mRNA$^+$, serum Alb$^+$<br>Repopulation rate: < 1% | Wang *et al*[77] |
| Human UCB&PB | ND | Host: irradiated NOD/SCID mice<br>Injury: $CCl_4$<br>TRP: 2 to 6 × $10^5$ CD34$^+$ cells; tail vein<br>Cell characterization: CD45$^+$, Alb$^+$, α1-AT$^+$, hum DNA$^+$, hum Alb mRNA$^+$&prot$^+$<br>Repopulation rate: ⩽ 0.01% hum Alb mRNA in mice *vs* human liver | Kollet *et al*[70] |
| Mouse BM | Cocktail: co-culture with damaged liver tissue<br>Characterization: RT-PCR (8), ICC (10), FISH Alb mRNA | Host: mice ± irradiation<br>Injury: $CCl_4$<br>TRP: $10^5$ cells; tail vein<br>Cell characterization: Alb$^+$, FISH Y$^+$, E-cadh$^+$, PKH26$^+$, serum liver function<br>Repopulation rate: up to 7.6% at 2 d<br>Fusion: FISH X-Y: - | Jang *et al*[69] |
| Mouse BM | ND | Host: mice ± irradiation ± rhG-CSF<br>Injury: partial hepatect or $CCl_4$<br>TRP: 5 × $10^3$ selected cells or 5 × $10^5$ MNCs; spleen<br>Cell characterization: no co-eGFP/DPPIV staining, PCR eGFP$^-$<br>Repopulation rate: 0/6.9 × $10^7$ host hepatocytes | Cantz *et al*[65] |

α1-AT: Alpha-1-antitrypsin; Ab: Antibody, Alb: Albumin; β-gal: β-galactosidase; BM: Bone marrow; $CCl_4$: Carbon tetrachloride; CK: Cytokeratin; d: Days; dexa: Dexamethasone; DPPIV: Dipeptidylpeptidase IV; eGFP: Enhanced green fluorescent protein; FAH: Fumarylacetoacetate hydrolase; hepatect: Hepatectomy; HGF: Hepatocyte growth factor; hum: Human; MNCs: Mononuclear cells; ND: Not documented; NTBC: 2-2nitro-4-trifluoromethylbezoyle-1,3-cyclohexamedione; PB: Peripheral blood; UCB: Umbilical cord blood; E-cadh: E-cadherin; rhG-CSF: Recombinant human Granulocyte-Colony Stimulating Factor; TRP: Transplantation. Amounts of analyzed markers are provided between brackets.

rate and/or a robust cell banking capacity. Regarding their promising hepatocyte-like functionality, some cell compartments could be promptly considered for toxicological assays. Even if identity and *in vivo* function of this cell population are currently under controversy, four main types of hepatic progenitors are described: oval cells, small hepatocytes, liver epithelial cells and mesenchymal-like cells. Oval cells are generated from the biliary tree in response to hepatic injury. They display a bipotent differentiation potential (hepatic and biliary cells) and can be expanded *in vitro*. There is no consensus on whether these cells are originating in BM or not[48,86]. However, similar cell types were recently isolated from healthy liver[87]. The first description of small hepatocytes isolation was made by Mitaka *et al*[88] from a non-parenchymal fraction after centrifugation of isolated liver cells. These cells are smaller than hepatocytes, possess an *in vitro* proliferation capacity[89] and can differentiate into mature hepatocytes *in vitro*[90]. Liver epithelial cells is a population firstly described by Tsao *et al*[91] and more recently in healthy adult human liver[92]. Although different from oval cells, these cells are bipotential and able to differentiate into hepatocyte-like cells *in vivo*[92]. A mesenchymal-like cell population has also been isolated from adult human liver[93,94]. This population depicts high level of proliferation and, as MSCs, possesses a broad

differentiation potential. How these cells share MSCs' immunological properties has to be determined. Other cell types were also isolated from manipulated livers[95], but the possibility of culture artefacts[96,97] should always be considered before describing new 'liver progenitor cells'.

### *Other cell types*

Embryonic stem cells constitute, at present, the best *in vitro* model for hepatocyte differentiation. However, ethical restrictions[98] and the possibility of malignancies development[99] mainly limit their use in the clinical setting[100,101]. Other more committed cell lineages as monocytes[102,103] or fibroblasts[104] were evaluated for hepatic differentiation. In this context, recent data about nuclear reprogramming[105] provided possible explanation of the differentiation potential of cells thought to be restricted to a cell lineage.

## *IN VITRO* STUDY AS ARGUMENT FOR HEPATOCYTE COMMITMENT

Up to now, studies illustrating *in vitro* hepatic features of stem cells-derived hepatocyte-like cells were essentially confined at the phenotypic rather than the functional level. Acquisition of specific markers is a tool for evidencing a cell commitment while hepatocyte-like functionality is required to consider a cell for therapy.

### *Hepatocyte-like phenotype*

While a morphological change is common after *in vitro* cell conditioning, the morphology is rarely similar to mature hepatocytes and authors should rather talk about a 'hepatocyte-like morphology' in differentiating cells, more especially as ultrastructural data are mostly not provided. How the morphological changes are evocative of the acquisition of an epithelial phenotype is questionable as similar morphological changes can be obtained when incubating hBM-MSCs with a classical differentiation medium or with a control medium[106]. Using specific protein and mRNA marker expression for the description of a hepatocyte-like phenotype could also be questioned as many studies were based on restricted panel of markers and as some authors described the presence of hepatocyte-specific markers in undifferentiated cells[8,14,16,22,82,107,108]. The broadest amount of markers for phenotypic characterization should be used. In this manner, we observed an incomplete and erratic acquisition of hepatocyte-specific features in hBM-MSCs[106], cf Table 1. Moreover, these cells partially maintained the expression of native mesenchymal markers, suggesting a chimerical phenotype. This phenotype could be the result of the artificial *in vitro* differentiation process.

### *Hepatocyte-like functionality*

The functional characterization of stem cells-derived hepatocyte-like cells is, up to now, principally achieved by commercially available assays (ELISA, colorimetric tests, PROD or EROD assays) whose handling are often poorly reproducible and quite difficult to interpret, or by other tests (as lipoprotein uptake) with low specificity. As clearly stated by Hengstler *et al*[109] in a recent report, there is a crucial need for standardized and normalized techniques for characterizing hepatocyte-like functionality in differentiating cells. Regarding clinical application, one should provide the evidence of a specific functional activity after differentiation before considering stem cells for cell therapy.

## *IN VIVO* STUDY AS PROOF OF HEPATOCYTE DIFFERENTIATION POTENTIAL

### *Direct detection of differentiated donor cells in situ*

Most studies about stem cells *in vivo* hepatic repopulation and differentiation are performed in a xenogeneic setting (e.g. human cells transplanted into immunocompromised rodents). The impact of cell-to-cell interactions in an interspecies microenvironment on cell adhesion and differentiation[110] is poorly documented. Moreover, this model does not reflect clearly the clinical reality and data must be carefully interpreted. In the same way, a drawback of allogeneic cell transplantation in rodents is that stem cell behavior was described to be quite different between species making extrapolation to human perilous[111]. Besides a huge diversity in the studies design, almost all *in vivo* models are based on the analysis of a repopulation potential after cell infusion in a diseased liver. Putative mechanisms of hepatic repopulation and tools designed to enhance it are described in Figure 1. It arises from the literature that hepatocyte differentiation from extrahepatic stem cells does not occur (or at very low level) in a non-injury model, and that the injury has to be strong enough and give advantage to the donor cells to seed and proliferate into the liver parenchyma. This was elegantly suggested by Stadtfeld *et al*[83] who demonstrated in a transgenic mouse model the minor role of the hematopoietic compartment in the adult liver ontogeneic development. Drug-induced liver injury has to be considered only as a tool for studying *in vivo* hepatocyte differentiation potential and no consensual protocol exists to date. Many questions are still opened: type of liver injury, time dependency of liver stimulus (acute *vs* chronic liver failure), use of drugs blocking host cell regeneration, relevance of injury after cell transplantation (compatibility with donor cell viability), *etc*. Globally, liver repopulation after stem cell transplantation remains limited. It is noteworthy that these data were obtained after single cell injection while for clinical purpose one would consider serial injections. Engraftment level has to be determined in this situation. By comparison, LCT can potentially reach higher repopulation levels[112] and, for that reason, is currently more valuable for clinical purpose. Conversely, some authors reported amazing repopulation levels with HSCs[73] or with UCB stem cells[16], but in both cases animal models were highly permissive and not representative of common cell transplant procedures. Methods for evaluation of cell engraftment are multiple and deal each with proper pitfalls[113]. For example, fluorescent in situ hybridization for detecting Y chromosome in a sex-mismatched transplantation can be difficult to interpret and, at low positivity levels,

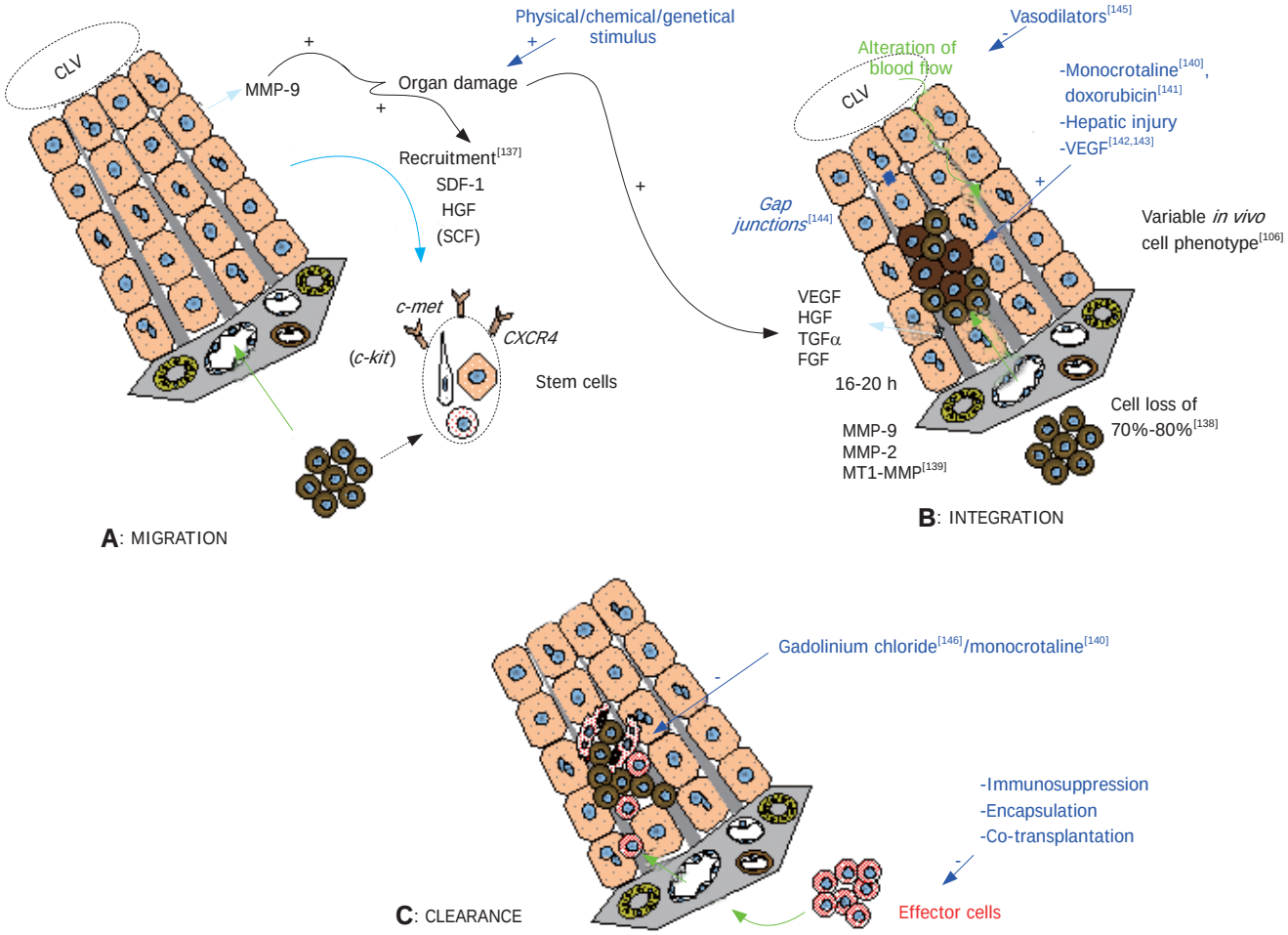



**Figure 1** Mechanisms of liver repopulation after stem cell transplantation. **A**: Stem cell migration into the liver parenchyma after spleen infusion is guided mainly by the splenic and the portal vein flow. Homing of the cells is tightly regulated by chemoattractive agents (as SDF-1, HGF and SCF) secreted by liver cells under liver damage conditions[136], and acting respectively through the interaction with CXCR4, c-met and c-kit receptors of stem cells. MMP-9, expressed by host hepatocytes after injury, is implicated in the homing process through its action on the extracellular matrix. This process can be triggered by various types of liver injury; **B**: While reaching the portal vein, 70% to 80% of the infused cells are lost and phagocytosis by macrophages/phagocytes can be observed in the portal areas[137]. After 16-20 h post-infusion, the remaining cells enter the liver sinusoid after having crossed over the endothelial barrier rendered permeable by a local secretion of VEGF[137], and by the combined action of various MMPs[138]. The permeabilization of the endothelium by specific drugs owning toxicity against endothelial cells (as monocrotaline[139] or doxorubicin[140]) or acting on the permeation (VEGF[141,142]) was described to enhance the repopulation rate of the infused cells. The penetration of the parenchyma is facilitated by the action of MMPs and the disruption of gap junctions[143]. The cell implantation creates an alteration of venous blood flow which can hamper the progression of the cells inside the parenchyma but can be alleviated by the use of vasodilators[144]. Globally, inducing liver injury facilitates cell integration by remodeling the liver architecture and by creating a local microenvironment favorable for the proliferation of the transplanted cells. This is mediated by the local secretion of cytokines/growth factors (HGF, FGF, TGFα). Cell implantation implicates the reformation of gap junctions that can be observed 3 to 7 d after transplantation. The implanted cells display variable cell phenotype parallel to the level of their hepatocyte differentiation; **C**: After integration, some cells will be cleared from the hepatic parenchyma. Dead cells lacking implantation stimulus or cell-to-cell interactions will be phagocyted by Kupffer cells. This can be inhibited by the use of gadolinium chloride[145] or by monocrotaline[139] which was demonstrated in animal models to improve the kinetics of cell implantation. Other cells will be rejected by the host immunological system (effector cells) and tools to alleviate this rejection are multiple (immunosuppression protocols, encapsulation of the infused cells, co-transplantation with cells owning immunosuppressive properties). FGF: fibroblastic growth factor; HGF: Hepatocyte growth factor; MMP: Matrix metalloproteinase; SCF: Stem cell factor; SDF: Stromal derived factor; TGF: Transforming growth factor; VEGF: Vascular endothelial growth factor.

could be hampered by female microchimerism occurring after several litters[114]. The evaluation of *in vivo* cell differentiation is prominently performed by objectifying protein markers expression whereas immunoassays are often technically limited by antibodies affinity or specificity. For instance, the demonstration of *in vivo* expression of HepPar-1 (OCHE15 clone) should be questioned because of the antibody's cross-reactivity between species. For that reason, the use of combined techniques is recommended for the identification of *in vivo* differentiating cells, as staining assays performed on serial sections and association of various techniques (ex: *in situ* hybridization, immunostaining, cell tracking with fluorescent molecules, flow cytometry, RT-PCR, cellular magnetic resonance) (Figure 2).

### *Demonstration of donor cell functionality through their metabolic activity*

The gold-standard for evaluating *in vivo* differentiation is the detection of cell functionality. To this end, some authors reported albumin secretion in transplanted mice serum. Other reports on '*in vivo* functionality' are argued on basis of the reduction of mortality rate, improvement of liver regeneration or of liver fibrosis[26,59,81,115-118]. It has to be determined how these findings are the fact of indirect cell-to-cell interactions or of direct cell functionality.

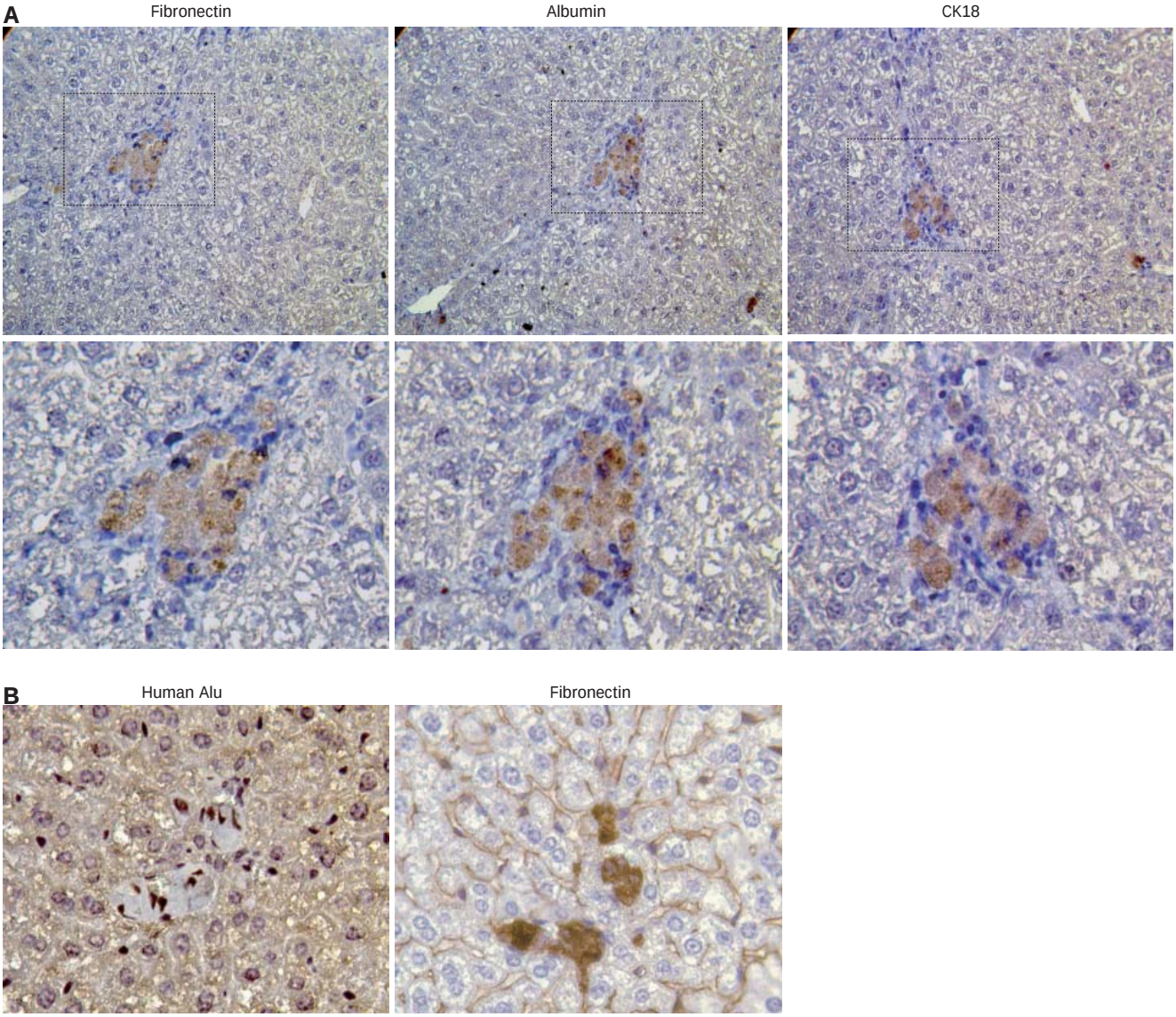



**Figure 2** Analysis of liver engraftment of transplanted stem cells. Human BM-MSCs were transplanted into the liver of partially hepatectomized SCID mice and livers were screened after one month for analysis. **A**: Staining on serial sections for fibronectin, albumin and CK18 by immunohistochemistry revealed a co-expression of mesodermal and hepatocyte markers and confirmed the chimerical phenotype of hepatocyte differentiating MSCs (400 ×); **B**: The use of combined techniques for revealing the expression of human alu probe by in situ hybridization and of fibronectin by immunohistochemistry in a cell cluster (800 ×).

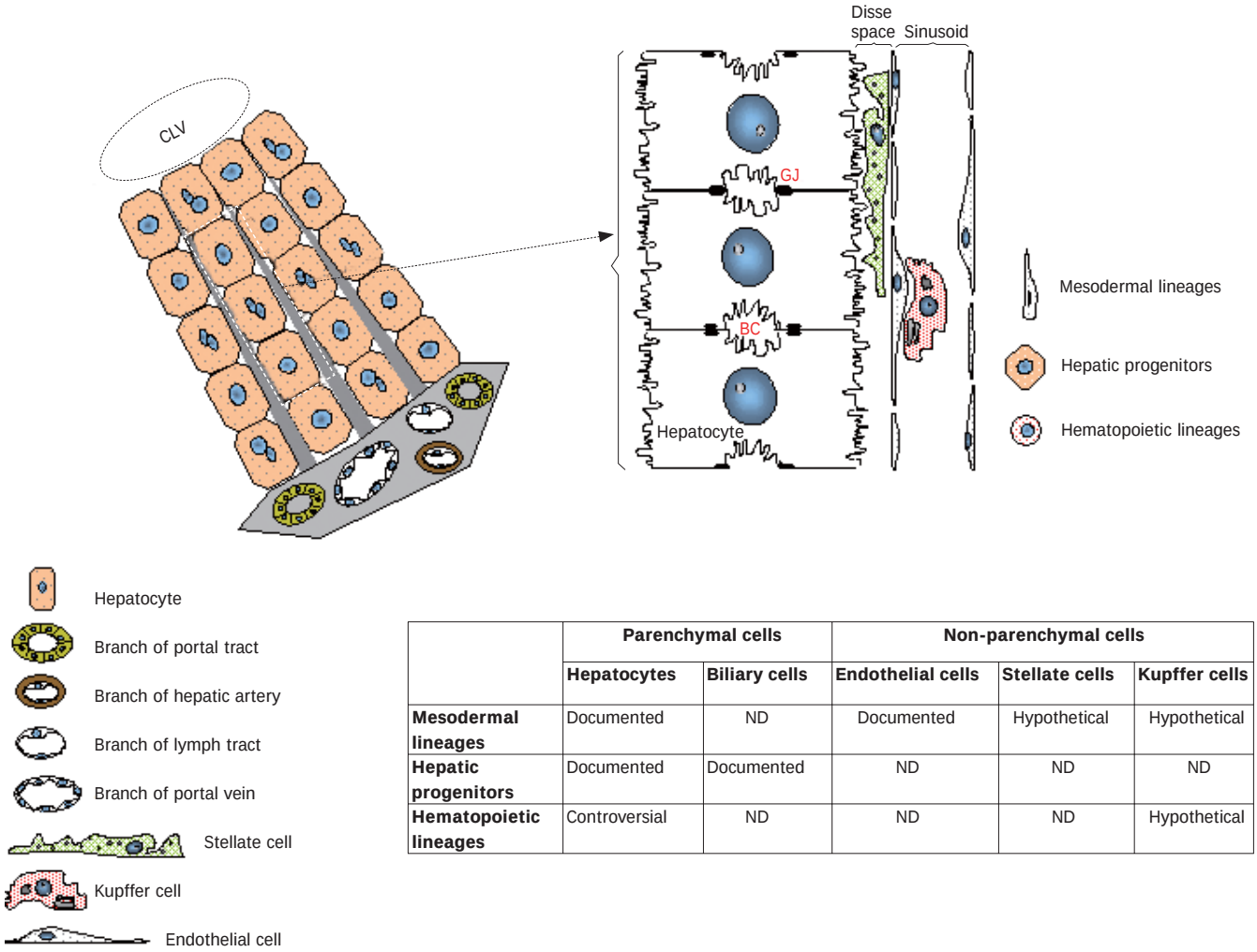
However, Lagasse *et al*[73] historically demonstrated the improvement of tyrosinemia disease in mouse after cell infusion whereas the selective pressure in this model is high and elicits fusion events.

### *Implication of cell fusion in the differentiation process*

The fusion process implies that a cell inserts its genetic content into another cell to form a resulting unit that acquire the 'host' phenotype. The resultant product creates a heterokaryon in which the nuclei do not always fuse. The concept of fusion has emerged after experiments on co-culture of BM cells with ESCs[119,120]. Fusion between hematopoietic cells and hepatocytes has been demonstrated[43,121-125] and invalidated[69,126,127]. This is explained by the conceptual diversity of these studies and perhaps the complexity of the process itself. For example, fusion events can be acted by HSCs[128] or require homing of these cells and implication of progeny, as highlighted by studies showing fusion between myelomonocytic lineages and hepatocytes[122,125]. Interestingly, studies providing strong data about fusion used the $FAH^{-/-}$ model which produces mitotic and chromosomal abnormalities[129] that could strengthen the fusion process. It appears that selective pressure is necessary to induce relevant fusion processes which happen rarely in a non-injury model. How the cell fusion and plasticity phenomenon are parts of the same process or vary in importance according to the population used (stem cells versus committed cells[130]), has to be explored by tracing the donor cells in their route and studying signalling pathways. Interestingly, fusion events have, to date, never been described with MSCs[10,21].

## FROM BENCH TO CLINICAL SIDE

Literature concerning the use of stem cells for clinical therapy is unceasingly growing in many fields (including




| | Parenchymal cells | | Non-parenchymal cells | | |
|---|---|---|---|---|---|
| | **Hepatocytes** | **Biliary cells** | **Endothelial cells** | **Stellate cells** | **Kupffer cells** |
| **Mesodermal lineages** | Documented | ND | Documented | Hypothetical | Hypothetical |
| **Hepatic progenitors** | Documented | Documented | ND | ND | ND |
| **Hematopoietic lineages** | Controversial | ND | ND | ND | Hypothetical |

**Figure 3** Involvement of stem cells in the regeneration of the liver parenchymal and non-parenchymal cell lineages. Schema shows the liver architecture and details the topography of the cell populations. In the table are resumed the cell fractions that could potentially be the targets of differentiation after transplantation of stem cells. Some of these differentiation potentials were documented with stem cells while other were contradicted and were thus exposed as controversial. Other differentiation pathways are purely hypothetical based on the embryonic origin of the stem cells. BC: Bile canalicule; CLV: Centrolobular vein; GJ: Gap junctions.

hematology, cardiology, neurology and orthopedics)[131]. However, to date, there is no clear documentation about long-term safety of such procedure[132,133]. Multi-center consensual state-of-the-art about several clinical aspects is mandatory. First is the election of the appropriate clinical indication. In the hepatology field, liver-based inborn errors of metabolism are the most attractive candidate because of the possibility for elective treatment and, in some cases, the absence of global liver function impairment making technical procedures more feasible. The lack of liver injury may limit stem cells engraftment and differentiation. However, stem cells therapy remains relevant since small increases of the deficient enzyme activity can improve patients' quality of life. In that case, the acquisition of a specific enzyme activity has to be demonstrated *in vitro* before cell transplantation. Liver cirrhosis/fibrosis has been considered: besides encouraging results, there is, to date, no evidence about a direct or a mediated cell involvement, nor about how the fibrosis could hamper cell implantation[116]. Before considering acute liver failure for stem cell therapy, it would be necessary to achieve a performing banking of functional hepatocyte-like cells. Globally, the possibility of repeated cell infusions could render cell therapy advantageous over OLT in patients whose disorder is not life-threatening (ex: Criggler-Najar patients). In this condition, whether stem cells could have an advantage on liver cells according to their proliferative capacity needs further assessment. Other points requiring consensus is the technique and route of cell delivery (surgically placed catheter *vs* transcutaneous injection), the amount of delivered cells and timing of infusions, and the protocol of immunosuppression.

## CONCLUSION

The first challenge for considering stem cells for therapy is the proof of their safety at long term by *in vitro* and *in vivo* assays. The other major goal is the determination of their *in vivo* functionality in small animal models combining a metabolic disorder allowing objective screening of cell function and a permissive immunological context (SCID or knock-out mice, immunosuppression therapy in allotransplantation). Large animal models would provide definitive arguments about safety and functionality and

allow an ideal setting for designing immunosuppression protocol and cell injection program. These models will be useful to attest the place of stem cells in liver cell therapy by comparison with LCT. Further research is also mandatory to examine the involvement of stem cells in the reconstitution of the non-parenchymal cell compartment after transplantation. Indeed, some cell populations have the potential to differentiate into endothelial cells[93,134,135] or could differentiate into mesodermal lineages similar to their phenotype (as hepatic stellate cells or fibroblasts) and as such interfere with the liver tissue architecture (Figure 3). Regarding the promising results obtained using MSCs as third-party for HSCs transplantation[29,31] or in immunological pathologies[30], the immunomodulatory role of these cells as adjuvant in LCT or in OLT should be determined.

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