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World J Gastroenterol 2006 September 28; 12(36): 5834-5845
World Journal of Gastroenterology ISSN 1007-9327

CLINICAL RESEARCH

Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells

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Supported by the ALIVE Foundation, the FIS from Instituto de Salud Carlos III, Spain, No. 03/0339, and the European Commission, No. LSHB-CT-2004-504761
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Received: 2006-04-27 Accepted: 2006-07-24

Abstract

AIM: To investigate and compare the hepatogenic transdifferentiation of adipose tissue-derived stem cells (ADSC) and bone marrow-derived mesenchymal stem cells (BMSC) *in vitro*. Transdifferentiation of BMSC into hepatic cells *in vivo* has been described. Adipose tissue represents an accessible source of ADSC, with similar characteristics to BMSC.

METHODS: BMSCs were obtained from patients undergoing total hip arthroplasty and ADSC from human adipose tissue obtained from lipectomy. Cells were grown in medium containing 15% human serum. Cultures were serum deprived for 2 d before cultivating under similar pro-hepatogenic conditions to those of liver development using a 2-step protocol with sequential addition of growth factors, cytokines and hormones. Hepatic differentiation was RT-PCR-assessed and liver-marker genes were immunohistochemically analysed.

RESULTS: BMSC and ADSC exhibited a fibroblastic morphology that changed to a polygonal shape when cells differentiated. Expression of stem cell marker Thy1 decreased in differentiated ADSC and BMSC. However, the expression of the hepatic markers, albumin and CYPs increased to a similar extent in differentiated BMSC and ADSC. Hepatic gene activation could be attributed to increased liver-enriched transcription factors (C/EBPβ and HNF4α), as demonstrated by adenoviral expression vectors.

CONCLUSION: Mesenchymal stem cells can be induced to hepatogenic transdifferentiation *in vitro*. ADSCs have a similar hepatogenic differentiation potential to BMSC, but a longer culture period and higher proliferation capacity. Therefore, adipose tissue may be an ideal source of large amounts of autologous stem cells, and may become an alternative for hepatocyte regeneration, liver cell transplantation or preclinical drug testing.

Key words: Mesenchymal stem cells; Bone marrow; Adipose tissue; Transdifferentiation; Hepatic lineage; Liver cell transplantation.

Taléns-Visconti R, Bonora A, Jover R, Mirabet V, Carbonell F, Castell JV, Gómez-Lechón MJ. Hepatogenic differentiation of human mesenchymal stem cells from adipose tissue in comparison with bone marrow mesenchymal stem cells. *World J Gastroenterol* 2006; 12(36): 5834-5845

http://www.wjgnet.com/1007-9327/12/5834.asp

INTRODUCTION

Most liver diseases lead to hepatocyte dysfunction with the possibility of eventual organ failure. The replacement of diseased hepatocytes and the stimulation of endogenous or exogenous regeneration by stem cells are the main aims of liver-directed cell therapy. There is growing evidence to suggest that reservoirs of stem cells may reside in several types of adult tissue[1,2]. These cells may retain the potential to transdifferentiate from one phenotype to another, presenting exciting possibilities for cellular therapies. One of

the important findings is that liver stem cells might be derived from bone marrow. Petersen *et al*[3] first identified this phenomenon in a rat model of liver injury. Subsequently, many researchers have reported similar *in vivo* and *in vitro* findings[4-8]. Bone marrow cells have been hypothesized as the third recruitment source in liver regeneration besides hepatocytes and endogenous liver stem cells[9].

Human bone marrow, derived from the embryonic mesoderm, is a complex tissue formed by a population of hematopoietic stem cells (HSC), supported by a mesenchymal stroma. The bone marrow stroma is heterogeneous in composition and is a reservoir of several stem cell populations, such as mesenchymal stem cells (MSC), and multi-potent adult progenitor stem cells (MAPC)[10]. It has been reported that MSC contributes to the regeneration of a variety of mesenchymal tissues. Furthermore, it retains the ability to differentiate into cells of the mesoderm lineage, such as osteoblasts, chondrocytes, adipocytes, myoblasts and cardiomyocytes, and into various types of tissue cells derived from other embryonic layers, including neural and liver cells[11-16]. The transdifferentiation of BMSC into hepatic cells *in vivo* was described in rats[3,17], mice[7] and humans[8,18]. This has brought new hope to cell therapy using autologous bone marrow cells as these present few ethical problems and could be applied to severe liver disease. MSC can easily be obtained following a simple bone marrow aspiration procedure, and may be subsequently cultured and expanded *in vitro* without losing their stem cell potential, making them an attractive target for cell therapy. In addition to long-term self-renewal capability, MSC possess versatile differentiation potential ranging from mesenchyme-related multipotency to neuroectodermal and endodermal competency. However, traditional bone marrow procurement procedures may be distressful for the patient and may yield low numbers of MSCs upon processing, which has recently led to investigate alternative MSC sources outside the bone marrow microenvironment.

Although bone marrow was the first source reported to contain MSC, it has been reported that MSC can also be isolated from human umbilical cord blood, synovium, placenta, periosteum, skeletal muscle, and adipose tissue[1,19-24]. Adult adipose tissue, like bone marrow, is derived from the embryonic mesenchyme, and a putative stem cell population within the adipose stromal compartment has been identified. Adipose tissue represents a rich source of mesenchymal stem cells, and provides an abundant and accessible source of adult stem cells with minimal patient discomfort. These cells have been termed adipose tissue-derived stem cells (ADSC). The characterization of these ADSC has defined a population similar to bone marrow-derived and skeletal muscle-derived stem cells. This cell population can be isolated from human lipoaspirates, and can be differentiated toward the osteogenic, adipogenic, neurogenic, myogenic and chondrogenic lineages[25-29] like bone marrow BMSC. In fact, some works have shown that human ADSC have similar characteristics to BMSC *in vitro* and *in vivo*[22,23,30,31]. In addition, ADSC compared with MSC from other sources possessed the longest culture period and the highest proliferation capacity[23]. Thus, adipose tissue may be an ideal source of large amounts of autologous stem cells attainable by a less invasive method than BMSC.

The success seen in differentiating ADSC into various mesenchymal lineages generates great interest in the use of ADSC for hepatic differentiation. To our knowledge, however, only a very recent report has shown the hepatogenic differentiation potential of ADSC[32] by using a differentiation protocol showing significant differences to the one used in this study. In addition, a comparative study of the hepatogenic differentiation potential *in vitro* of both types of MSC has not yet been performed.

Based on these previous findings, the aim of this study was to investigate and compare the hepatic differentiation of human MSC from bone marrow (BMSC) and adipose tissue (ADSC) obtained from healthy donors. To this end, these cells were isolated and cultured under similar pro-hepatogenic conditions to those of liver development to define the different capacities of hepatic differentiation of the two subsets *in vitro*. Briefly, BMSC and ADSC cultures were serum deprived for 2 d and pre-cultured with EGF and bFGF (conditioning step). Then a 2-step differentiation protocol followed with a sequential addition of growth factors, cytokines and hormones (step-1 differentiation: HGF and bFGF for 7 d and step-2 differentiation: OMS, dexamethasone, and ITS + up to d 21). Moreover, the response to inductive extracellular signals and the role of key liver-enriched transcription factors (LEFTs) in the differentiation process *in vitro* have been revealed.

MATERIALS AND METHODS

Materials and reagents

Medium Dulbecco's Modified Eagle's medium (DMEM-low glucose), Ham's F-12 and Leibovitz L-15 were purchased from Gibco (Paisley, UK), gentamicine from Normon (Madrid, Spain), phosphate-buffered saline (PBS) and dexamethasone from Merck Pharma (Mollet del Vallés, Spain) and trypsin-EDTA and newborn calf serum were obtained from Biochrom AG (Berlin, Germany), ITS + premix from BD Biosciencies (Madrid, Spain). Basic fibroblast growth factor (bFGF) was purchased from Invitrogen (Barcelona, Spain), epidermal growth factor (EGF) and nicotinamide were from Sigma-Aldrich (Madrid, Spain). Oncostatin M (OMS) was purchased from PeproTech EC (London, UK). Hepatocyte growth factor (HGF) was a kind gift from Dr. T. Nakamura (Tokohu University, Sendai, Japan). Trizol and DNase I Amplification Grade were purchased from Invitrogen, Life Technologies (Barcelona, Spain), 3,3'-diaminobenzidine from Sigma-Aldrich (Madrid, Spain) and tolouidine blue from BDH chemicals (Poole, UK). Monoclonal antibodies against human antigens CD13-PE, CD34-APC, CD45-FITC, CD90-APC, were purchased from Becton Dickinson (Mountain View, CA); CD105-PE from Serotec (Oxford, UK) and 7-Amino-actinomycin D (7-AAD) from Sigma-Aldrich (Madrid, Spain). Mouse monoclonal anti-human alphafetoprotein was purchased from Santa Cruz Biotechnology (Madrid, Spain) and polyclonal rabbit anti-human albumin, alphafetoprotein, polyclonal goat anti-mouse HRP and polyclonal goat anti-rabbit HRP were from DakoCytomation (Barcelona, Spain).

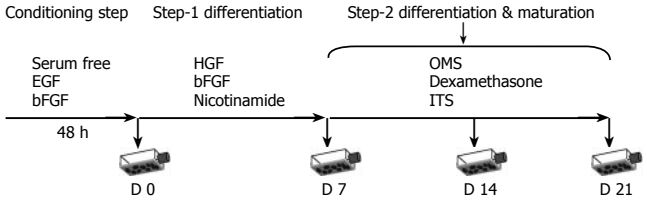

Figure 1 Hepatic differentiation protocol by sequential addition of exogenous factors according to embryogenesis. Passage 2 cultures at 85% of confluency were used for differentiation assays. Cells were pre-cultured in serum-free medium supplemented with EGF and bFGF for 2 d (Conditioning step). Then cells were cultured in medium supplemented with HGF, bFGF and nicotinamide, for 7 d (Step-1 differentiation). Finally cells were cultured in maturation medium supplemented with OMS, dexamethasone, and ITS + premix (insulin, transferrin, selenious acid, BSA and linoleic acid) up to 21 d (Step-2 differentiation; maturation). Media were changed twice a week and hepatic differentiation was assessed at different time points.

Cell cultures

Cancellous bone was obtained from the femoral heads of five patients (aged between 60 and 69 yr) undergoing total hip arthroplasty. Human adipose tissue was obtained by suction-assisted lipectomy (i.e. liposuction) as discarded tissue from surgical interventions from six patients (aged between 38 and 49 years). These protocols were approved by the institutional review board. Tissues were obtained in conformity with the rules of the Hospital's Ethics Committee and after obtaining an informed consent from patients.

Both bone and fat were dissected into small pieces. Trabecular bone fragments were digested with collagenase II (3 mg/mL) in Hank's balanced salt solution (HBSS) at 37℃ for two hours with intermittent shaking. Adipose tissue fragments were digested with collagenase I (1 mg/mL) in HBSS at 37℃ for 60 min with intermittent shaking. Thereafter, the resulting suspensions (bone and fat) were filtered using two layers of cotton gauze to remove debris and then centrifuged at 400 *g* for 10 min. Supernatants were discarded and pellets were resuspended in 160 mmol/L NH_4Cl at room temperature for 10 min to lyse the remaining red blood cells. Cells were collected by centrifugation, as detailed above, resuspended in culture medium (DMEM-low glucose supplemented with 15% AB human serum stock (pooled from 10 donors) from our regional transfusion centre and 50 μg/mL of gentamicine). These primary cells were plated in tissue culture flasks in a humidified atmosphere at 37℃ with 50 mL/L CO_2 for 48 h. Thereafter, the cultures were washed to remove non-adherent cells. Media were changed twice a week, maintaining cells at subconfluent levels. The cells were subcultured with 0.4% trypsin/0.2% EDTA solution, seeded at a density of 5-10 × 10^3 cells/cm^2 and cultured in the same conditions used for the primary culture (passage 1). Five to seven days later, subconfluent monolayers were subcultured (passage 2) and cells were seeded at the same density as for passage 1 to be used for differentiation assays.

Cell lines

HepG2 cells were plated in Ham´s F-12/Leibovitz L-15 (1:1 v/v) supplemented with 7% newborn calf serum, 50 U penicillin/mL and 50 μg streptomycin/mL. Cells were subcultured with 0.25% trypsin/0.02% EDTA at 37℃.

Table 1 Sequence of primers used for RT-PCR and length of fragments

Primer	Sequences	Product
ALB	Upper 5' TGAGAAAACGCCAGTAAGTGAC 3' Lower 5' TGCGAAATCATCCATAACAGC 3'	265 bp
AFP	Upper 5′ GCTTGGTGGTGGATGAAACA 3′ Lower 5′ TCCTCTGTTATTTGTGGCTTTTG 3′	157 bp
Thy1	Upper 5′ CACACATACCGCTCCCGAACC 3′ Lower 5′ GCTGATGCCCTCACACTTGACC 3′	189 bp
KRT 18	Upper 5′ CCCGTCACGCCCTACAGAT 3′ Lower 5′ ACCACTTTGCCATCCACTATCC 3′	271 bp
KRT 19	Upper 5′ TCCAGATGAGCAGGTCCGAGGTTA 3′ Lower 5′ GCTGCGGTAGGTGGCAATCTCC 3′	281 bp
CYP3A4	Upper 5′ CCTTACAT TACACACCCTTTGGAAGT 3′ Lower 5′ AGCTCAATGCATGTACAGAATCCCCGGTTA 3′	382 bp
CYP2E1	Upper 5′ ACAGAGACCACCAGCACAACT 3′ Lower 5′ ATGAGCGGGGAATGACACAGA 3′	580 bp
C/EBPβ	Upper 5′ CTCGCAGGTCAAGAGCAAG 3′ Lower 5′ CTAGCAGTGGCCGGAGGCGAGC 3′	271bp
HNF4α	Upper 5′ GCCTACCTCAAAGCCATCAT 3′ Lower 5′ GACCCTCCCAGCAGCATCTC 3′	275 bp
hPBGD	Upper 5′ CGGAAGAAAACAGCCCAAAGA 3′ Lower 5′ TGAAGCCAGGAGGAAGCACAGT 3′	294 bp

Hepatic differentiation protocol

Passage 2 BMSC and ADSC cultures at 85% confluency were used for differentiation assays. Cells were serum deprived for 2 d and pre-cultured in DMEM supplemented with 20 ng/mL EGF and 10 ng/mL bFGF (conditioning step) to stop cell proliferation, prior to induction of differentiation toward a hepatic phenotype. Then a 2-step differentiation protocol was performed, followed by a sequential addition of growth factors, cytokines and hormones (Figure 1). Step-1 differentiation medium, consisting of DMEM supplemented with 20 ng/mL HGF, 10 ng/mL bFGF and nicotinamide 4.9 mmol/L, for 7 d, followed by step-2 differentiation medium, consisting of DMEM supplemented with 20 ng/mL OMS, 1 μmol/L dexamethasone, and 10 μL/mL ITS + premix (final concentration: 100 μmol/L insulin, 6.25 μg/mL transferrin, 3.6 μmol/L selenious acid, 1.25 mg/mL BSA and 190 μmol/L linoleic acid) to achieve cell maturation up to D21. Media were changed twice weekly and hepatic differentiation was assessed at different time points by RT-PCR for liver-associated genes, as listed in Table 1. The protocol was applied to BMSC and ADSC from five and six different donors, respectively, and identical results were obtained.

Flow cytometry analysis

Flow cytometric analysis was performed with a FACScalibur flow cytometer (Becton Dickinson, Mountain View, CA) after labelling 1 × 10^6 cells with respective directly conjugated antibodies, using isotype-matched controls. 7-Aminoactinomicyn D was used in order to exclude non-viable cells from the analysis, and nonspecific staining was performed as previously described[33]. This analysis was carried out after the initial sample was obtained and later in the following culture stage.

Immunohistochemistry

MSC in monolayer were washed twice with phosphate buffered saline (PBS), fixed with ethanol 70% for 10 min, and with ethanol 100% for 10 min thereafter. Plates were washed three times with PBS. Then, plates were incubated for 30 min at room temperature with a solution of methanol with 1.7% H_2O_2, and washed again with PBS. Plates were then incubated for 30 min at 37℃ with sheep serum diluted 1/10 in PBS, and washed with PBS tween (0.05%) four times. Subsequently, the plates were incubated for 2 h at 37℃ with the primary antibody diluted in PBS tween 0.05%: anti-human alphafetoprotein (1/50) and anti-human albumin (1/2000). After incubation, plates were washed with PBS tween (0.05%) four times and incubated for 1 hour at 37℃ with peroxidase labelled goat anti-mouse IgG diluted 1/100 in PBS tween 0.05% for alphafetoprotein, and with peroxidase labelled goat anti-rabbit IgG diluted 1/1000 in PBS tween 0.05% for albumin. After repeated washes, plates were incubated with the substrate (3,3´-diaminobenzidine diluted in PBS with 0.1% H_2O_2). H_2O was used to stop the reaction, and tolouidine blue was employed as contrast staining. Finally, plates were examined under the microscope.

RNA extraction and real-time quantitative PCR

Total RNA was extracted from undifferentiated (used as control), differentiating and differentiated cells using Trizol reagent, following the manufacturer's recommendations; contaminating genomic DNA was removed by incubation with DNase I Amplification Grade. The amount of purified RNA was estimated by ribogreen fluorescence (Molecular Probes), and its purity was assessed by the absorbance ratio 260/280 nm. RNA integrity was examined by agarose gel electrophoresis. RNA (1 μg) was reverse transcribed as described[34,35]. Diluted cDNA (3 μL) was amplified with a rapid thermal cycler (LightCycler Instrument, Roche Diagnostics) in 15 μL of LightCycler DNA Master SYBR Green I (Roche Molecular Biochemicals), 5 mmol/L $MgCl_2$ and 0.3 μmol/L of each oligonucleotide. Optimal $MgCl_2$ concentration was empirically determined for each set of primers. Gene-specific primers were designed using OLIGO software. The sequence of both the forward and reverse primers and the expected sizes of the PCR-amplified DNA are listed in Table 1. In parallel, we analyzed the mRNA concentration of the human housekeeping porphobilinogen deaminase (PBGD, hydroxymethylbilane synthase) as an internal control for normalization. A no-template negative control (H_2O) was also run for every cDNA-specific primer set. Each sample cDNA was measured in duplicate. PCR amplicons were confirmed to be specific by size and melting curve analysis. After denaturing for 30 s at 95℃, amplification was performed with 40 cycles of 1 s at 94℃, 5 s at 62℃ and 20 s at 72℃. The real-time monitoring of the PCR reaction, the precise quantification of the products in the exponential phase of the amplification and the melting curve analysis were performed with the LightCycler quantification software, as recommended by the manufacturer.

Transduction with adenoviral vectors encoding C/EBPα and β

CCAAT/enhancer-binding proteins (C/EBP) are a family of liver-enriched transcription factors, which play an important role in regulating the transcription of multiple hepatic genes, including CYPs. Recombinant adenoviruses encoding the C/EBPα and C/EBPβ full-length proteins were prepared as described elsewere[36,37]. Briefly, recombinant shuttle pAC/CMVpLpA plasmids containing full-length C/EBPα and C/EBPβ cDNAs were cotransfected into 293 cells with pJM17 (AdE1A-transformed human embryonic kidney cells) by calcium phosphate/DNA coprecipitation. The expression cassette of pAC/CMVpLpA is located between the sequences representing 0-1.3 mu and 9.2-16 mu of adenovirus type 5, whereas pJM17 encodes a full length adenovirus-5 genome (dl309) interrupted by the insertion of the bacterial plasmid pBRX at position 3.7 mu, thereby exceeding the packaging limit for the adenovirus. A homologous recombination between adenovirus sequences in the shuttle vector and in the pJM17 plasmid results in the substitution of the pBRX sequences in pJM17 by the chimeric gene. This generates a genome of a packageable size in which most of the adenovirus early region 1 is lacking, thus rendering the recombinant virus replication defective. The resulting viruses (called Ad-C/EBPα, Ad-C/EBPβ) were plaque-purified, expanded into a high-concentration stock and titrated by plaque assay, as previously described[38]. ADSCs and BMSCs were infected with recombinant adenoviruses for 120 min at a MOI (multiplicity of infection) ranging from 3 to 15 PFUs (plaque forming units) per cell (MOI). Thereafter, cells were washed with PBS and fresh medium was added. Forty-eight hours post-transduction cells were analyzed or harvested for analysis and frozen in liquid N_2. MSCs were also transduced with a control adenoviral vector expressing GFP to confirm a highly efficient transduction of adenovirus exposed human cells and to demonstrate that adenoviral transduction *per se* does not cause unspecific alterations in differentiation marker genes.

Statistical analysis

Data were expressed as mean ± SE. Results were analysed by the Student's *t* test. $P < 0.05$ was considered statistically significant.

RESULTS

Differentiation of human BMSC and ADSC to hepatic phenotype

Analysis by flow cytometry of cell surface markers of BMSC and ASDSC after isolation was performed.

Homogeneity and reproducibility of the isolation procedure of BMSC and ADSC were demonstrated by flow cytometry. Two analytical regions were established combining R1 region, which selects viable cells, and R2 in forward and side characteristics of both MSC types (Figures 2A, B and 3A, B). The surface antigen markers of human mesenchymal stem cells CD13, CD45, CD90 and CD105 expression on BMSC and ADSC were analysed after isolation

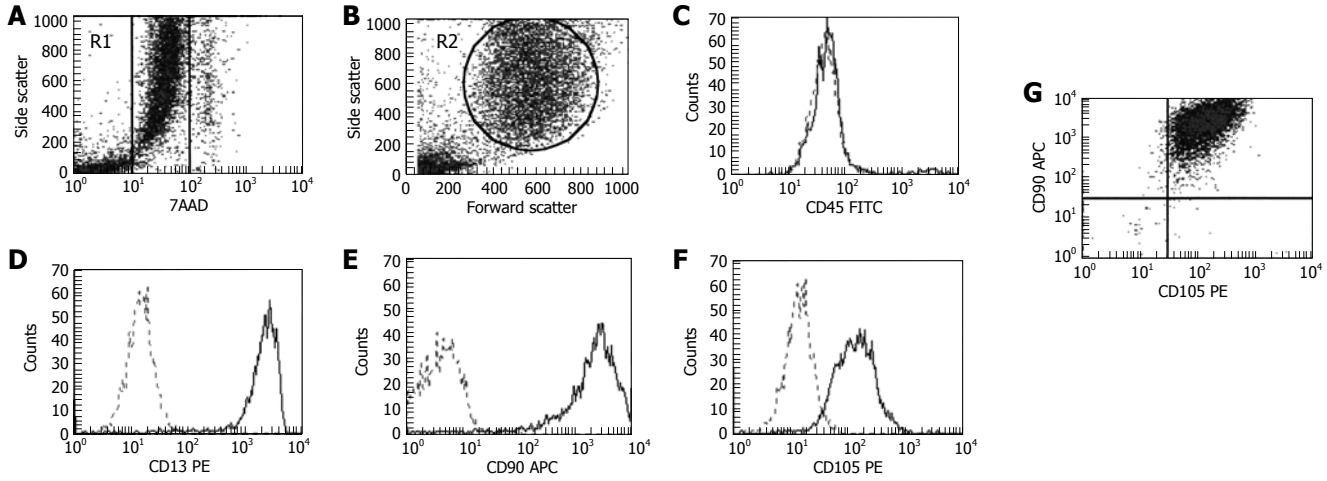

Figure 2 Flow cytometry analysis of surface protein markers of BMSC. Two analytical regions were established combining R1 region (**A**), which selects viable cells, and R2 (**B**) in forward and side characteristics of BMSC. Surface antigen markers were positive vis-à-vis isotypic controls for CD13 (**D**), CD90 (**E**) and CD105 (**F**) and negative for CD45 (**C**). Co-expression of CD90-CD105 is shown in dot-plots (**G**). 7ADD: 7-Aminoactinomicyn D.

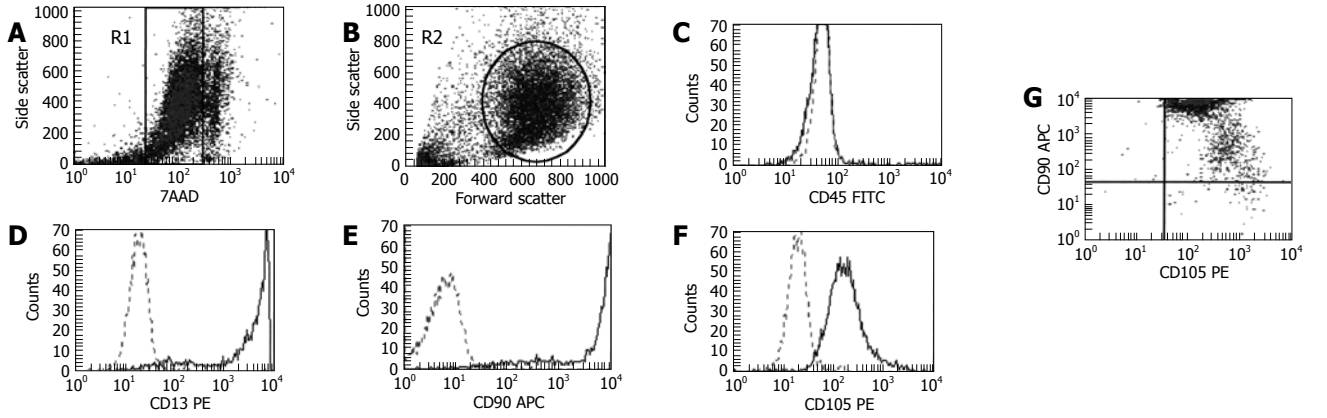

Figure 3 Flow cytometry analysis of surface protein markers of ADSC. Two analytical regions were established combining R1 region (**A**), which selects viable cells, and R2 (**B**) in forward and side characteristics of ADSC. Surface antigen markers were positive vis-à-vis isotypic controls for CD13 (**D**), CD90 (**E**) and CD105 (**F**) and negative for CD45 (**C**). Co-expression of CD90-CD105 is shown in dot-plots (**G**). 7ADD: 7-Aminoactinomicyn D.

and at different steps of the culture. Representative histograms for BMSC and ADSC are shown in Figures 2 and 3 respectively. Both types of undifferentiated MSC were positive for the mesenchymal markers CD13 (Figures 2D and 3D), CD90 (Thy1) (Figures 2E and 3E) and CD105 (Figures 2F and 3F), but did not express the hematopoietic marker CD45 (Figures 2C and 3C). As shown in Figures 2G and 3G, the coexpression of both antigens CD90 (Thy1) and CD105 is found in ADSC and BMSC. ADSC showed an identical pattern of surface protein expression as BMSC, and no significant variation of surface marker expression was observed among cells from different donors. Table 2 shows that the percentage of positive cells after the primary culture of BMSC and ADSC is similar. Data for the mean fluorescence intensity for the markers CD90 and CD105 show differences between individual donors with a mean fluorescence greater for both markers in ADSC than BDSC. Nevertheless these last data may take into account the variations in the photomultiplier settings of the cytometer for the timing of the different cultures.

Morphologic changes in cultured ADSC and BMSC

We analysed the morphological changes of BMSC and ADSC at the various differentiation protocol stages in order to evaluate the effect of growth factors, hormones and cytokines. Both MSC obtained from human adipose tissue and human bone marrow expanded easily *in vitro*. However, ADSC showed a higher proliferation rate and longer survival in culture than BMSC. When cells were precultured for 2 d in serum-free medium supplemented with EGF and bFGF, cell proliferation stopped. Cells before differentiation (D0) exhibited a fibroblast-like morphology (Figure 4A and B). Cell morphology of both BMSC (Figure 4C) and ADSC (Figure 4D) did not change significantly during differentiation step-1, when cultures were treated with HGF, although the fibroblastic morphology was lost and cells developed a broadened flattened shape. However, a polygonal shape developed during differentiation step-2 when cells were exposed to media containing OMS and hormones (Figure 4E and F). The protocol used includes the sequential addition of exogenous factors that have

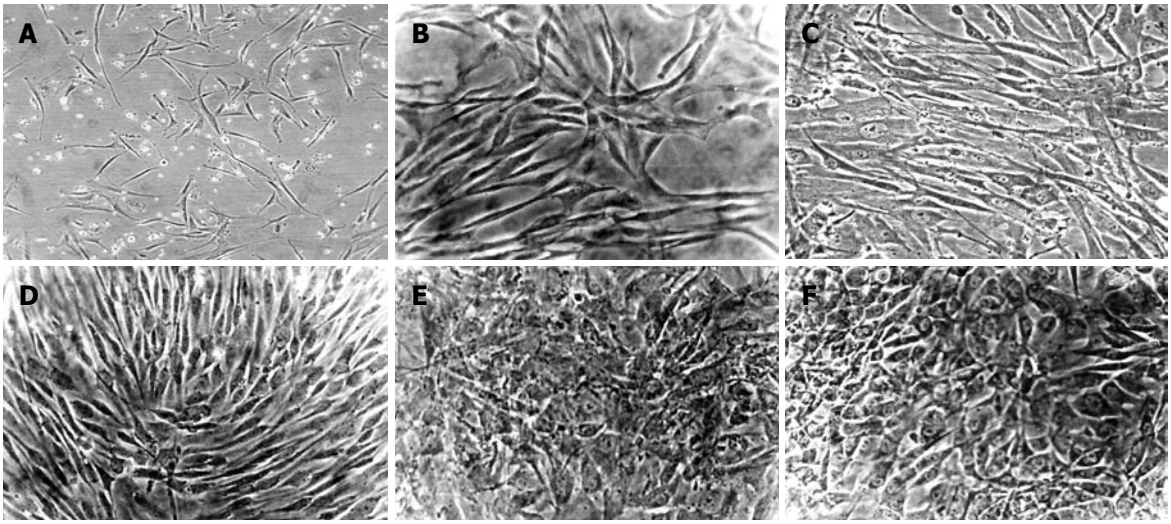

Figure 4 Morphology of human mesenchymal stem cells from bone marrow and adipose tissue during the differentiation protocol. Cells were induced to differentiate by using a sequential addition of growth factors, cytokines and hormones. Morphology of passage 2 BMSC (**A**) and ADSC (**B**) cells. No significant morphological changes were observed in BMSC (**C**) nor in ADSC (**D**) cells during the step-1 differentiation. However, both BMSC (**E**) and ADSC (**F**) cells significantly changed the morphology, and developed a polygonal shape during step-2 differentiation (magnification 20 x for all pictures).

Table 2 Expression of CD90 and CD105 in ADSC and BMSC after the initial culture

	ADSC				BMSC			
	CD90$^+$		CD105$^+$		CD90$^+$		CD105$^+$	
Donor	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence	Cell population %	Mean channel fluorescence
1	99.88	8623.91	93.92	211.55	98.83	8347.11	94.85	338.36
2	99.83	5467.01	99.83	1118.22	87.69	1980.05	92.20	175.20
3	99.82	6157.88	99.08	1089.24	99.80	8538.72	96.27	129.80
4	99.89	6830.06	97.79	758.79	98.17	8700.00	89.48	622.80
5	99.78	7495.60	99.70	2025.79	98.91	8562.77	95.53	602.95
6	99.81	8052.66	99.54	1510.95	76.74	1088.24	98.70	139.73
7	98.83	8878.06	99.35	1298.27	76.42	234.83	89.20	648.32
8	91.14	5123.70	73.52	203.77	98.98	6274.51	94.90	549.53
Mean	98.62	7078.61	95.34	1027.07	91.94	5465.78	93.89	400.84
SD	3.04	1418.10	9.03	624.75	10.26	3724.95	3.33	229.88

been reported to be implicated in liver development and proved to be effective to induce the hepatic differentiation of human MSC from human bone marrow and umbilical cord blood[39].

RT-PCR analysis of hepatic gene expression of BMSC and ADSC differentiated cells

To determine whether differentiated cells show the characteristic expression of hepatic phenotype markers, total RNA from BMSC and ADSC was isolated at D7, 14 and 21 of the differentiation protocol and the mRNA levels of several hepatic genes were examined by RT-PCR. Undifferentiated cells were used as controls (D0 of the differentiation protocol). The CK-18 and CK-19 expression did not significantly change by induction of differentiation (data not shown). Expression of HNF4α, C/EBPβ (liver-enriched transcription factors that play important roles in regulating the expression of hepatic genes) and albumin were significantly increased with the differentiation time in both cell types, BMSC and ADSC. However, the expression of hepatocyte specific markers in ADSC occurs predominantly in the first differentiation step, whereas the second differentiation step is required in BMSC. It is unclear whether this fact could imply functional differences between both cell populations, and further research is needed to clarify this point. The time-course induction of C/EBPβ correlated well with albumin upregulation (Figure 5A-C), while HNF4α induction showed a different profile (Figure 5D). The increase in key liver-enriched transcription factors suggests that our differentiation protocol was effective in driving MSC toward a hepatic phenotype.

Then, we went on to compare the levels of the Thy1 (CD90) and alphafetoprotein (AFP) expressions in ADSC and BMSC with that of adult human liver tissue from a liver bank (pooled liver from 10 donors). To this aim, we performed quantitative RT-PCR relative to housekeeping hPBGD at the initial and final times of the differentiation

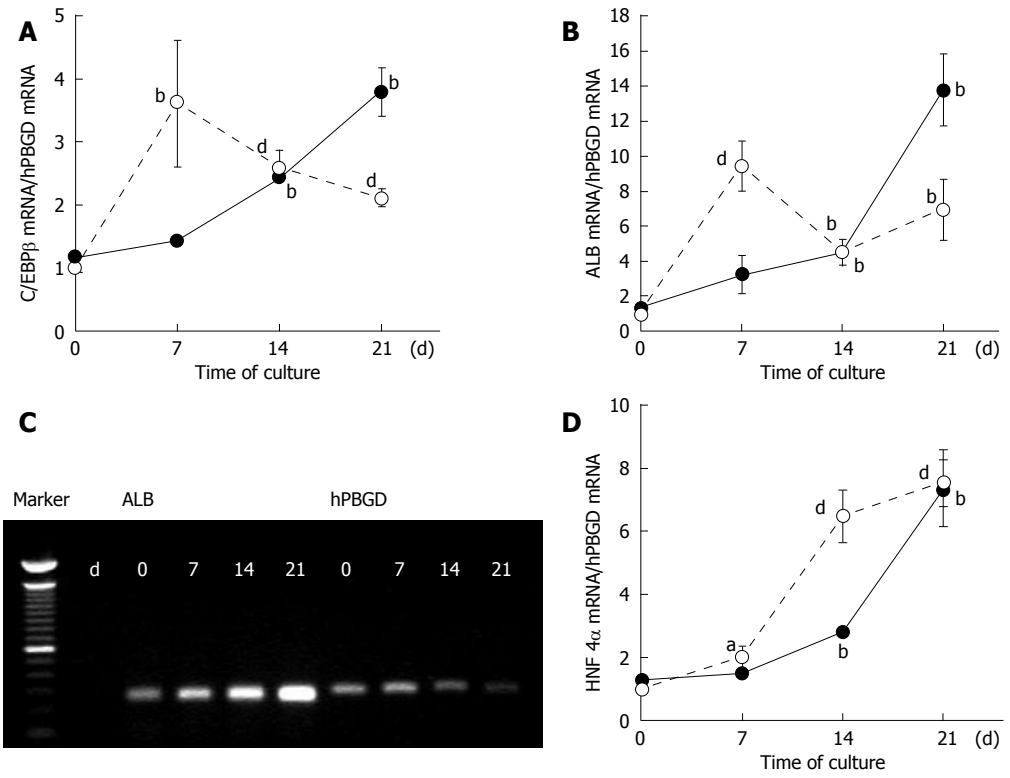

Figure 5 Real-time PCR analysis of the expression of mRNA for liver markers determined at established times in the transdifferentiation protocol of BMSC and ADSC. Expression of C/EBPβ (**A**) (liver-enriched transcription factor, that plays important roles in regulating the expression of hepatic genes), albumin (**B** and **C**) and HNF4α (**D**), were analysed in BMSC (bold symbols) and ADSC (clear symbols) at different time points of the differentiation protocol. Data are shown as the fold increase in the mRNA level compared to the undifferentiated cells (d 0 of the differentiation protocol), and were normalized by hPBGD. The agarose gel shows the ALB expression of BMSC (**C**). HNF4α: hepatocyte nuclear factor 4 alpha; C/EBPβ: CCAAT / enhancer binding protein beta; ALB: albumin. Data are the mean ± SE of 5 and 6 different cultures of BMSC and ADSC respectively. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$.

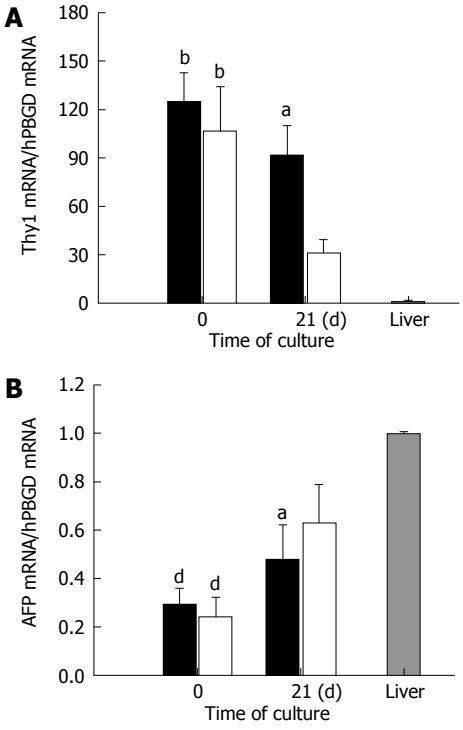

Figure 6 Real-time PCR analysis of the expressions of mRNA for Thy1 and alphafetoprotein in BMSC and ADSC. The levels of Thy1 (CD90) (**A**) and the alphafetoprotein (**B**) expression in differentiated BMSC (black bars) and ADSC (clear bars) were quantified by RT-PCR at the initial and final times of the differentiation protocol, and compared with that of human pooled liver tissue from a liver bank. Data are shown as a fold increase in the mRNA level compared to the liver tissue and normalized by hPBGD. AFP, alphafetoprotein. Data are the mean ± SE of 4 different cultures of BMSC and ADSC. $^{a}P < 0.05$; $^{b}P < 0.01$; $^{d}P < 0.001$.

protocol. The relative mRNA levels of the mesenchymal marker Thy1 in undifferentiated ADSC and BMSC was significantly higher ($P < 0.01$), than in human liver, as expected (Figure 6A). However, after 21 d of the differentiation protocol ADSC significantly reduced the Thy1 expression level, while a more modest decrease was observed in BMSC ($P < 0.05$). AFP is considered a characteristic fetal hepatic marker. AFP expression levels in fetal mouse liver steadily increases 1000-fold from day 9.5 to 15.5 of gestation and drops dramatically in adult liver. At low levels however, AFP is still reproducibly detected in multiple adult liver samples by real-time RT-PCR. Regarding AFP, a similar level of expression was observed in undifferentiated ADSC and BMSC, where it was significantly lower than that in liver ($P < 0.001$). However, differences with liver tissue were reduced in ADSC (NS) and BMSC ($P < 0.05$) after the step-2 protocol (Figure 6B). In the less differentiated human hepatoma HepG2 cells, AFP expression was much higher than in differentiated MSCs or liver tissue (data not shown).

We also determined the expression of CYPs (CYP2E1 and CYP3A4) as well as the differentiated markers of adult hepatic phenotype in ADSC and BMSC. We compared expression levels by RT-PCR at the initial and final times of the differentiation procedure (Figure 7). Levels of CYP2E1 in the undifferentiated MSC were not significantly different from that of the human hepatoma HepG2, a cell line showing characteristics of fetal hepatocytes. However, a significant increase of CYP2E1 expression was observed at the final differentiation time in both ADSC ($P < 0.01$) and BMSC ($P < 0.001$) (Figure 7A). Levels of CYP3A4 at the initial time were significantly lower in MSC when compared with HepG2 ($P < 0.001$), but these levels increased in the differentiated cells: BMSC showed a similar mRNA level while ADSC reached a higher expression level than the HepG2 cells ($P < 0.001$) (Figure 7B and C). The expression level of CYP2E1 and CYP3A4 in differentiated MSCs was still lower than in human hepatocytes (data not shown), which suggests that

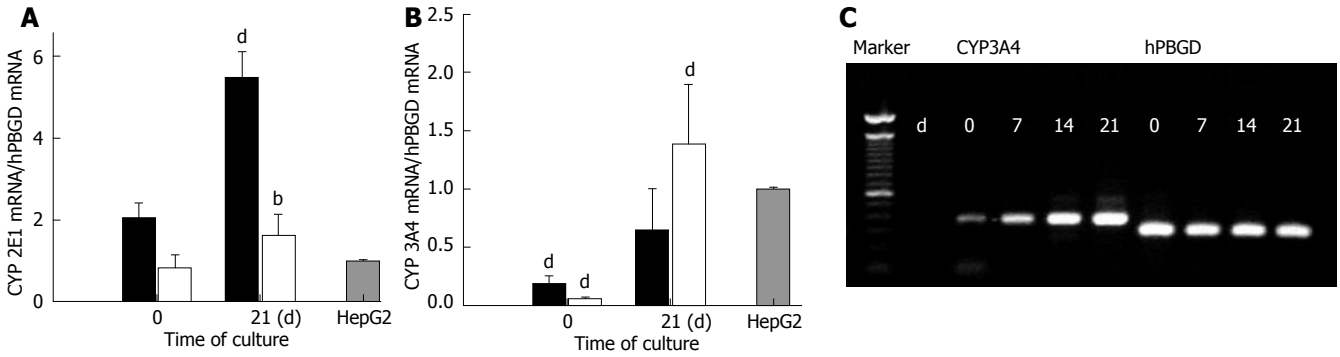

Figure 7 Real-time PCR analysis of the expression of mRNA for CYP2E1 and CYP3A4 in BMSC and ADSC. The expression of two major drug metabolising enzymes, CYP2E1 (**A**) and CYP3A4 (**B**), in BMSC (black bars) and ADSC (clear bars) were quantified by RT-PCR at the initial and final times of the differentiation protocol, and compared with that of the human hepatoma cells HepG2. The agarose gel shows the CYP3A4 expression of ADSC (**C**). Data are shown as a fold increase in the mRNA level compared to HepG2, and were normalized by hPBGD. Data are the mean ± SE of 3 different cultures of BMSC and ADSC. $^{b}P < 0.01$; $^{d}P < 0.001$.

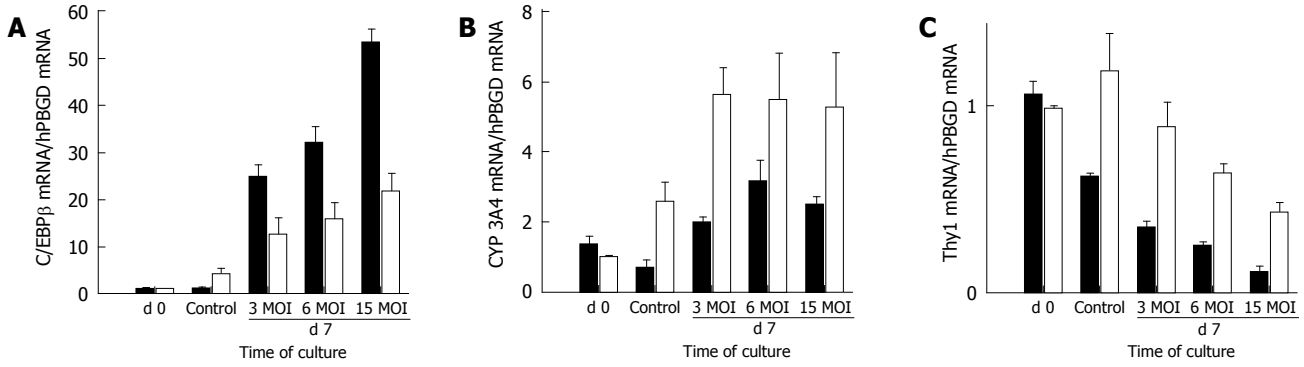

Figure 8 Evaluation of the role of C/EBP transcription factors in the differentiation of BMSC and ADSC cells. Adenoviral transduction caused a dose-dependent increase in the level of C/EBPβ mRNA, as assessed by real-time PCR analysis (**A**). Concomitantly, an up-regulation of CYP3A4 was observed which was more significant in ADSCs (**B**), and was paralleled by a significant down-regulation of the mesenchymal cells marker Thy1 in both BMSC and ADSC (**C**). Controls were not transduced. Data are shown as a fold increase in the mRNA level compared to the control and normalized by hPBGD. Data are the mean ± SE of 3 different cultures of BMSC and ADSC.

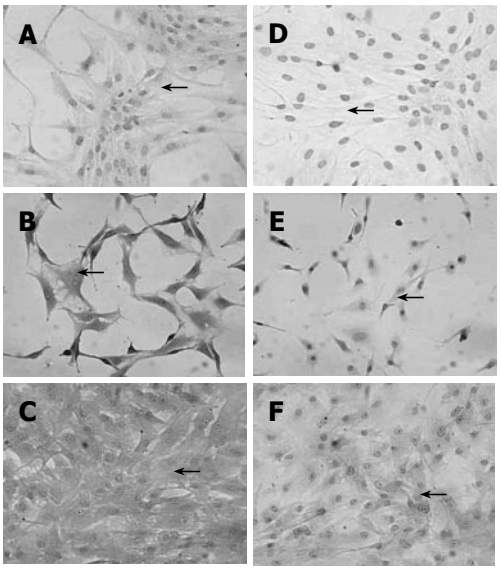

Figure 9 Immunohistochemical analysis of albumin and alphafetoprotein in ADSC cells. Homogeneous expression of albumin (**A-C**) and AFP (**D-F**) in ADSC cells was further confirmed by immunocytochemistry. Staining for both albumin and AFP was negative in undifferentiated cells (**A**,**D**). ADSC cells homogeneously increase their protein expression levels in response to the differentiation protocol. Positive staining was shown after 14 d (**B**, **E**) and 21 d (**C**, **F**) of the 2-step differentiation protocol. AFP; alphafetoprotein.

additional stimuli may be needed to reach adult physiologic expression levels of CYP genes. By taking these results together it is difficult to clarify whether ADSC and BMSC are in fact equally potent hepatic progenitor cells, and further functional studies are required.

Role of C/EBP transcription factors in mesenchymal to hepatic transition

We observed a significant increase in the expression of key liver transcription factors during the transdifferentiation of MSC into the hepatic phenotype. To investigate the relevance of this finding, we transduced undifferentiated cells with adenoviral vectors encoding full-length C/EBP proteins and analyzed their effects by driving MSC towards the hepatic phenotype. The most important results were found with C/EBPβ, while C/EBPα appeared to play a minor role.

Cells were transduced after seven d of culture with increasing doses of adenoviruses, ranging from 3-15 MOI. Infection of MSC with a control adenovirus expressing the GFP demonstrated an 85%-95% transduction efficiency. Moreover, transduction of MSC with Ad-GFP or Ad-PAC (insertless adenoviral vector) up to 15 MOI neither modified the morphology nor the expression of

differentiation marker genes, demonstrating that all the effects observed in Ad-C/EBP transduced cells can be attributed to the increased expression of the adenoviral transgene (data not shown). As expected, adenoviral transduction caused a dose-dependent increase in the level of C/EBPβ mRNA (Figure 8A). Concomitantly, we observed an up-regulation of CYP3A4, which was more significant in ADSCs (Figure 8B), and expression levels above those found in well-differentiated hepatoma cells were reached. In parallel, we observed a significant down-regulation of the mesenchymal marker Thy1 (Figure 8C), suggesting that the C/EBPβ up-regulation is an important event in triggering the mesenchymal to hepatogenic transition.

Immunohistochemistry

To further confirm the homogeneous expression of albumin and AFP in our cell populations, we examined differentiated ADSC by immunocytochemistry. This analysis showed that undifferentiated cells stain negative for albumin and AFP (Figure 9A and D). Positive staining was detected at the end of step-1 differentiation when cells had been exposed to HGF (Figure 9B and E), and a more intense positive staining was found at d 21 at the end of step-2 differentiation, when cells were cultured with OSM and hormones (Figure 9C and F). Our results demonstrate that the levels of hepatic protein markers increased homogeneously in most of the ADSC cells in response to the differentiation protocol, correlating well with mRNA expression analysis.

DISCUSSION

Over recent years, stem cells have generated great interest given their potential therapeutic use. Bone marrow has been the best characterized tissue, and has been considered as the major source of multipotent adult MSC since it is able to differentiate into mesodermal and ectodermal lineages[11-16]. Several studies have indeed shown the potential of BMSC to differentiate into hepatocyte-like cells[3,7,8,17,18]. However, the presence of uncommitted MSC has been found in many other tissues, such as adipose tissue, thus representing an alternative source of multipotent stromal cells[1,19-22]. In fact, human ADSC have similar characteristics to BMSC[22,23,30,31]. In addition, Zuk *et al*[22] reported that ADSC are capable of differentiating *in vitro* into several cell lineages. Moreover, it has been recently shown for the first time that human ADSC can differentiate towards hepatic lineage *in vitro* under appropriate culture conditions[22]. Therefore, adipose tissue may be readily accessible and a good candidate as a source of stem cells with a therapeutic potential for hepatic cell therapy and tissue engineering. Although this is a controversial area, some studies clearly show that transplanted bone-marrow-derived hepatocytes can colonise a wide variety of tissues in the body of a host due to cell fusion[40,41], while others do not report cell fusion[42].

Although the stem cell differentiation mechanism remains unclear to date, transdifferentiation might either be induced by stimulating with suitable media/substrates/factors, or by genetic reprogramming *in vitro*[43-45]. Liver development is known to proceed *via* several distinct steps in which growth factors and cytokines are involved. Among the factors implied in the embryonic liver development, fibroblast growth factors (FGFs), produced by cardiac mesodermal cells, are involved at an initial stage of endodermal patterning to induce hepatic fate[46,47]; OSM, a member of the interleukin-6 cytokine family produced by hematopoietic cells, is required from the mid-fetal to the neonatal stages[48] and apparently coordinates liver development and hematopoiesis in the fetus[49]; finally, several extracellular signals including EGF, HGF, OSM, FGFs, glucocorticoids and insulin are involved in the late maturation stage leading to an increase in liver-specific gene expressions, and their effects on differentiation vary as a function of gestation age[50]. Corticosteroids, HGF and EGF play important roles in hepatic biology[51]. HGF is a more potent proliferating factor for human hepatocytes in culture than EGF[52,53], and plays an important role in liver development and regeneration in humans[54,55]. The differentiation of BMSCs into hepatocyte-like phenotypes *in vitro* by induction with HGF has been reported[55,56]. Other reports showed differentiation of bone marrow-derived MAPC toward hepatocyte-like cells induced by FGF-4, however the degree of differentiation was higher when cells were also treated with HGF[6]. This is consistent with the fact that FGF-4 may play a role in endoderm specification[47], and that HGF induces differentiation of hepatocytes that are not actively proliferating[55]. Bone marrow cells cultured with HGF and EGF showed morphologic and phenotypic characteristics of mature hepatocytes[54]. OSM has been shown to have a specific differentiation-inducing effect on primary fetal hepatic cells towards mature hepatocytes, and the presence of glucocorticoids is required for OSM effects[49,58].

In this study we have investigated the induction to the hepatogenic differentiation of human ADSC in comparison with BMSC. Seo *et al*[32] first showed that ADSC can be differentiated into hepatocyte-like cells by treatment with cytokine mixtures (HGF and OSM) and DMSO in serum-free medium. However, we have used a 2-step differentiation protocol with a sequential addition of growth factors (bFGF), cytokines (OSM and HGF), hormones (dexamethasone and insulin) and nicotinamide, which have been reported to be involved in the development and differentiation of hepatocytes[57,59]. The choice of exogenous factors and the time course to induce hepatogenic transdifferentiation are based on previous reports on BMSC differentiation[6,39]. As previously mentioned, HGF plays an essential role in the development and regeneration of the liver[57]; bFGF is required to induce a hepatic fate in the foregut endoderm[6]; OSM increases hepatocyte size and enhances hepatic differentiation[57], whereas nicotinamide significantly enhances the *in vitro* maturation of fetal liver cells[59].

The morphologic and phenotypic features and gene expression changes in both types of cells have been compared. Finally, the role of key hepatic transcription factors in the regulation of the transdifferentiation process has been investigated using adenoviral vectors. The results show that MSCs are capable of giving rise to a hepatogenic transdifferentiation in response to a sequential addition of

growth factors, assessed by an examination of morphology and hepatocyte-specific markers.

It should be highlighted that we have established the culture conditions of both human BMSC and ADSC, as well as the differentiation protocol under adequate conditions for a suitable supply of hepatocyte-like resources for the potential use of human cell transplantation therapy. To achieve this purpose, we have used only a human serum controlled stock from our blood bank instead of fetal calf serum for cell cultures. Cells were serum deprived for 2 d prior to inducing hepatic transdifferentiation, and were then cultured in serum-free conditions (Figure 1). Furthermore, we always used passage 2 cultures for differentiation assays, as it is convenient to differentiate cells for clinical use in low passages to avoid spontaneous differentiation.

Similar morphological changes were observed in BMSC and ADSC, as shown in Figure 4. During Step-1, cells developed a fibroblastic-like flattened shape, but changed to a polygonal morphology during Step-2 in the presence of OMS and hormones. These morphological changes, in parallel with the differentiation process, are coincident with those shown in previous reports either in BMSC[6,39] or ADSC[30,32]. BMSC and ADSC cells showed similar expression pattern surface protein markers, and no significant variations of surface marker expression were noted among different samples (Figures 2 and 3). We also found that both undifferentiated MSC cell types were positive for stem cell markers, such as Thy1, CD105 and CD13, but they did not express the hematopoietic marker CD45 in agreement with previous reports[30,32,58]. The relative amount of Thy1 in undifferentiated ADSC and BMSC cells was significantly higher than in liver cells, but a significant decrease was observed in the differentiated cells, particularly in ADSC. A similar pattern was observed in the amount of AFP in undifferentiated ADSC and BMSC, and it was lower than that in liver. The differentiation potential of BMSCs and ADSC toward the hepatic phenotype was supported by the expression of albumin and CYP isozymes (CYP2E1 and CYP3A4). Albumin expression increased similarly in differentiated BMSCs and ADSC. Homogeneous albumin expression in differentiated ADSC was further confirmed by immunocytochemical analysis, as also shown in BMSC by other authors[6,32,60]. Concerning the biotransformation capability of differentiated ADSC and BMSC assessed by the expression of CYP2E1 and CYP3A4, a significant increase in CYP2E1 levels was found in differentiated MSCs when compared with the HepG2 cells. The widely used human hepatoma cell line HepG2 expresses gene products that are characteristic of fetal hepatocytes, including serum albumin[61]. However, hepatoma cells of the fetal phenotype are deficient in the use of certain hepatic transcription factors and show low levels of well-characterized adult liver genes, such as CYP2E1 and CYP3A4. Differentiated ADSC showed higher CYP mRNA levels than HepG2 cells, although the gene expression was still lower than that in the adult liver. Thus, our results suggest that hepatogenic differentiation in ADSC cells was successful, although the hepatocyte adult phenotype was not reached and may require further differentiation steps.

CYP3A4 levels were significantly increased in both differentiated MSCs, although the increase of CYP3A4 levels in differentiated ADSC was consistently higher compared with both the initial time and with HepG2 cells levels. Biotransformation capability, assessed by PROD activity, has been reported in human and rat MAPC derived from bone marrow[6]. However, studies on ADSC differentiation into hepatic lineage are very scarce[30,32] and there are no previous results showing CYP expression in differentiated ADSC. The expression pattern and related activity profiles of CYP isoenzymes in the differentiated cells suggest that these cultures might be useful to study many aspects of drug metabolism and drug-related toxicity pathways[62].

The molecular mechanism, by which our 2-step protocol induces the transdifferentiation of ADSC and BMSC into hepatic lineage cells, seems to be dependent on the induction of several liver-enriched transcription factors, such as C/EBPβ and HNF4α. We opted to investigate these two transcription factors for several reasons. C/EBPβ is a key component that distinguishes the liver program of differentiation. The relevance of C/EBPβ has been emphasized in recent studies which reveal that pancreatic cells can be transdifferentiated into hepatocytes. In these studies, transdifferentiation was associated with both an elevation of expression of the transcription factor C/EBPβ and a reduction of the transcription factor Pdx-1 (pancreatic duodenal homeobox-1). Moreover, transfection of C/EBPβ into the cells can provoke transdifferentiation; while a dominant-negative form of C/EBPβ can inhibit the process[63,64]. Similarly, HNF4α seems to be a very important factor to establish hepatic lineage as HNF4α was shown to be responsible for the final commitment of oval cells to differentiate into hepatocytes, which regenerate the liver parenchyma[65]. Moreover, HNF4α is essential for the morphological and functional differentiation of hepatocytes and the generation of a hepatic epithelium. It has been shown that HNF4α is a dominant regulator of the epithelial phenotype because its ectopic expression in fibroblasts induces a mesenchymal-to-epithelial transition.

Other liver-enriched transcription factors have also been shown to be associated with the hepatic trans-differentiation of BMSC[60]. The expressions of HNF1α, HNF3α, HNF3β, HNF4α, GATA4 and GATA6 were increased during the transdifferentiation of BMSCs, suggesting that hepatic nuclear factors and the GATA family of proteins could also be major components to induce this transdifferentiation[60]. In this study however, C/EBPα and C/EBPβ did not change. Our results demonstrate the induction of C/EBPβ during the hepatic transdifferentiation of ADSC and BMSC. This discrepancy in the role of C/EBPβ could result from a divergent differentiation protocol. In the study by Saji *et al*[60], bFGF was used to induce BMSC transdifferentiation and bFGF may not induce C/EBPβ. However, HGF could well be a potent inducer of this transcription factor. It has been shown that HGF induced an early transition of albumin ALB-negative stem cells to ALB-positive hepatic precursors resembling hepatoblasts, and then OMS promoted their differentiation to mature

hepatocytes[66]. In the first step of stem cell differentiation induced by HGF, the expression of C/EBPβ was induced dramatically; when the C/EBP function was inhibited in stem cells, these no longer differentiated to hepatocyte-lineage cells[66].

In conclusion, the autologous nature of mesenchymal stem cells, together with their putative multipotentiality, may make these cells an excellent choice for many future tissue engineering strategies and cell-based therapies. Previous results indicating the great similarity of the gene expression between BMSC and ADSC by gene array analysis have been reported[30], supporting the hypothesis that both types of MSC cells originated from a common precursor. They both have a proliferation potential and similar expression pattern of surface markers.

The present study indicates that under certain defined inducing conditions, MSC, isolated from bone marrow and adipose tissue, can differentiate toward a hepatic phenotype *in vitro*. In addition to hepatic biochemical functions, as shown in a previous report[32], our results demonstrate for the first time that differentiated ADSC also express key drug metabolising enzymes. ADSC have a similar differentiation potential towards the hepatic lineage just as BMSC have, but their abundance and accessibility, their longer culture period and higher proliferation capacity differ from BMSC (as suggested by the present study and previous reports)[23,30,32]. Therefore, adipose tissue seems to be an ideal source of high large amounts of autologous multilineage mesodermal stem cells for tissue repair and cell therapy of hepatic tissues as well as for preclinical drug testing.

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