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Original Article

Human Bone Marrow Mesenchymal Stem Cells Can Express Insulin and Key Transcription Factors of the Endocrine Pancreas Developmental Pathway upon Genetic and/or Microenvironmental Manipulation In Vitro

CHRISTINE MORISCOT,[a,b] FLORENCE DE FRAIPONT,[a,b] MARIE-JEANNE RICHARD,[b] MÉLANIE MARCHAND,[c,d]
PIERRE SAVATIER,[c,d] DOMENICO BOSCO,[e] MARIE FAVROT,[a,b] PIERRE-YVES BENHAMOU[a,b,f]

[a]Institut National de la Santé et de la Recherche Médicale (INSERM), Unité 578, Grenoble, France; [b]Centre d'Investigation Biologique, Centre Hospitalier Universitaire, Grenoble, France; [c]Institut National de la Santé et de la Recherche Médicale (Inserm), Unité 371, Institut Fédératif des Neurosciences de Lyon, Bron, France; [d]Laboratoire de Biologie Moléculaire de la Cellule, CNRS UMR 5161, Ecole Normale Supérieure de Lyon, France; [e]Cell Isolation and Transplantation Center, University of Geneva Medical Center, Geneva, Switzerland; [f]Département d'Endocrinologie, Centre Hospitalier Universitaire, Grenoble, France

ABSTRACT

Multipotential stem cells can be selected from the bone marrow by plastic adhesion, expanded, and cultured. They are able to differentiate not only into multiple cell types, including cartilage, bone, adipose and fibrous tissues, and myelosupportive stroma, but also into mesodermal (endothelium), neuroectodermal, or endodermal (hepatocytes) lineages. Our goal was to characterize the multipotential capacities of human mesenchymal stem cells (hMSCs) and to evaluate their ability to differentiate into insulin-secreting cells in vitro. hMSCs were obtained from healthy donors, selected by plastic adhesion, and phenotyped by fluorescence-activated cell sorter and reverse transcription–polymerase chain reaction analysis before and after infection with adenoviruses coding for mouse *IPF1, HLXB9,* and *FOXA2* transcription factors involved early in the endocrine developmental pathway. We found that native hMSCs have a pluripotent phenotype (OCT4 expression and high telomere length) and constitutively express *NKX6-1* at a low level but lack all other transcription factors implicated in beta-cell differentiation. In all hMSCs, we detected mRNA of cytokeratin 18 and 19, epithelial markers present in pancreatic ductal cells, whereas proconvertase 1/3 mRNA expression was detected only in some hMSCs. Ectopic expression of *IPF1, HLXB9,* and *FOXA2* with or without islet coculture or islet-conditioned medium results in insulin gene expression. In conclusion, our results demonstrated that in vitro human bone marrow stem cells are able to differentiate into insulin-expressing cells by a mechanism involving several transcription factors of the beta-cell developmental pathway when cultured in an appropriate microenvironment. STEM CELLS *2005;23:594–604*

INTRODUCTION

An important application for cell therapy is diabetes mellitus. By restoring normal endogenous insulin secretion in patients with diabetes, cell therapy may challenge the actual treatment by exog-

enous insulin. Transplantation of pancreatic islet cells as a potential cure for diabetes has become the subject of intense interest and activity over the past two decades [1, 2]. However, the limited supply of human islet tissue available for transplantation prevents this therapy from being used to treat the thousands of patients with type 1 diabetes. In vitro expansion of human beta cells or genetic engineering of human insulin-secreting cells may represent one approach, but the clinical use is limited by the difficulties in achieving prolonged or physiologically regulated insulin secretion [3–5]. One way to overcome these problems and obtain functional glucose-sensitive insulin-secreting cells for transplantation is to derive islet cells from other sources such as embryonic stem cells [6–8] and intestinal [9], hepatic [10, 11], ductal, or pancreatic stem cells [12, 13]. These studies have opened fascinating perspectives. An easily accessible, expandable, glucose-responsive, and autologous stem cell would offer obvious advantages in a clinical setting.

Recent work suggests that adult stem cells from one tissue or organ can differentiate into cells of other organs, either in vitro or in vivo [14]. Among them, bone marrow–derived stem cells (hematopoietic or mesenchymal) carry the more significant implications for possible clinical development, because they are easily accessible for an autograft and routinely collected from adults without ethical concern inherent to fetal embryonic tissues [14–19].

Based on their ability to adhere to plastic support [15, 19], multipotential stem cells can be isolated from the bone marrow, expanded, and cultured. Under appropriate experimental conditions, they differentiate into multiple mesenchymal cell types, including cartilage, bone, adipose and fibrous tissues, and myelosupportive stroma [15, 19]. Moreover, treatment with growth factors such as epithelial growth factor and brain-derived neurotrophic factor or chemical products such as dimethyl sulfoxide and butylated hydroxyanisole induced the bone marrow stroma cells to exhibit a neuronal phenotype [20, 21].

A more likely candidate may be the multipotential adult progenitor cells (MAPCs) derived from adult bone marrow [22]. These cells exhibit a remarkable plasticity, with the ability to differentiate into cells with mesodermal, neuroectodermal, and endodermal characteristics in vitro [23]. Furthermore, upon transplantation, MAPCs can differentiate into epithelium of the liver, lung, and gut. MAPCs express the *OCT4* and *REX1* transcription factors, two specific markers of undifferentiated embryonic stem (ES) cells. Recently, the marrow-isolated adult multilineage-inducible (MIAMI) cells capable of differentiating in vitro into cell lineages from all three germ layers have been described [24].

During development, the formation of the pancreas and its subsequent differentiation into the different exocrine and endocrine cell types and mature adult beta cells result from the orderly activation and extinction of a large number of genes. Experiments with transgenic mice have identified a hierarchy in the transcription key factors, such as *HLXB9*, *FOXA2* (formerly named *HNF3β*), *IPF1* (*PDX1*), *NEUROG3* (*NGN3*), *NEUROD1*, *NKX2-2*, *PAX4*, *NKX6-1*, and *PAX6*, that control embryonic formation of pancreatic islets [25]. Among these factors, *FOXA2* [26, 27] and *IPF1* [27–30] play a central role in initiating the differentiation of the islet cells.

If human mesenchymal stem cells (hMSCs) could form new beta cells, they would become a particularly useful target for therapies that aim at beta-cell replacement in diabetic patients, because they are abundantly available in the human bone marrow. In this study, we first confirm that hMSCs express the phenotypic surface marker characteristics of multipotent cells. We second provide evidence, by reverse transcription–polymerase chain reaction (RT-PCR), for the presence of some factors implicated in pancreatic development and function. Their phenotype as well as their adipogenic differentiation ability were not modified by adenoviral infection. Finally, we show that genetic manipulations or appropriate culture conditions allow hMSCs to express insulin mRNA.

MATERIALS AND METHODS

Isolation and Culture of hMSCs

hMSCs were obtained from 5- to 10-ml aspirates from the iliac crest of normal donors after informed consent was given. Cells were plated at a density of 5.10^6 per 25-cm^2 flask in 5 ml of CMRL 1066 (Invitrogen, Carlsbad, CA) supplemented with 100 μg/ml penicillin, 100 μg/ml streptomycin (Invitrogen), 2 mmol/l L-glutamine (Invitrogen), and 10% fetal calf serum (Invitrogen), incubated at 37°C in a humidified atmosphere containing 5% CO_2. After 72 hours, the nonadherent cells were discarded and adherent cells were washed gently with medium and cultured for approximately 21 days. Fresh complete medium was replaced twice a week. Upon reaching near confluence, cells were detached with a solution of 0.25% trypsin and 1 mmol/l EDTA (Invitrogen) for 2–3 minutes at 37°C and plated at 1,000 cells/cm^2 with medium replacement twice a week.

Colony-forming efficiency was determined by plating the cells at 10 cells per cm^2 in 60-mm dishes (BD Biosciences, Le Pont de Claix, France). After 14 days of culture, the number of visible colonies was counted after staining the dishes with Giemsa. Colony-forming efficiency is calculated as the percentage of the number of cells initially plated that give rise to visible colonies (>50 cells).

To confirm that hMSCs did not possess malignant properties, in vitro colony formation assay in a soft-agar medium was conducted. A total of 5,000 cells were mixed with CMRL 1066 containing 0.35% (wt/vol) agar and placed over a layer of identical composition but of higher agar concentration (0.5%, wt/vol)

in 35-mm Petri dishes and then incubated at 37°C in humidified incubator for 14 days. After this period, dishes were stained with 0.5% crystal violet for 1 hour.

In Vitro Differentiation of hMSCs into Adipocytes

To induce differentiation into adipocytes, hMSCs were plated at 20,000 cells per cm^2 in 12-well plates in CMRL 1066 containing 10% fetal calf serum, 1 µmol/l dexamethasone, 10 µg/ml insulin, 0.5 mmol/l 3-isobutyl-1-methylxanthine, and 100 µmol/l indomethacin (Sigma, Saint Quentin Fallavier, France) [15]. Medium was changed twice a week. After 3 weeks of adipogenic stimulation, cells were rinsed once with phosphate-buffered saline (PBS), fixed with methanol at −20°C for 2 minutes, and then rinsed with ethanol 50% and incubated for 10 minutes with Oil Red-O to stain lipid vacuoles and rinsed again with methanol 50%.

Pancreatic Human Islets

Human pancreas was procured from heart-beating cadaveric donors according to the French regulations (1994 Bioethic Act allowing procurement with presumed consent). Islets were isolated in the Cell Isolation and Transplantation Center, University of Geneva Medical Center, Geneva, as previously reported in collaboration with P. Morel and Dr. T. Berney [1, 2]. Briefly, islets were isolated by ductal distension of the organ and digestion of the tissue at 37°C for approximately 20 minutes with Liberase (Roche Diagnostics, Meylan, France). Digested islets were purified by a Biocoll (VWR International S.A.S., Limonest, France) density-gradient centrifugation using a COBE cell processor (Denver).

After an overnight shipping to Grenoble at 20°C in culture medium, islets were plated in nonadherent six-well plates (Greiner Bio-One, Poitiers, France) at 37°C in 95% air and 5% CO$_2$ until use. Medium was replaced twice a week. Islet culture medium was filtered through a 0.22-µM membrane and used as a conditioned medium. In coculture experiments, pancreatic islets were placed in culture inserts (Millicell Culture Plate Inserts, Millipore, Billerica, MA) without any contact with hMSCs. Culture medium was CMRL 1066 containing 10% fetal bovine serum, 25 mmol/l HEPES, 24 mmol/l sodium bicarbonate, 1 mmol/l sodium pyruvate, and penicillin-streptomycin solution. Islet number was determined on a sample after dithizone staining and expressed as equivalent number of islets (the number of islets if all were 150 µm in diameter).

hMSC Phenotype Analysis

hMSCs were immunophenotyped by fluorescence-activated cell sorting (FACS). Cells were detached with trypsin-EDTA, washed in PBS, and immediately stained with the following labeled antibodies: *CD10*-PE, *CD11b*-FITC, *CD31*-FITC, *CD34*-cychrome, *CD44*-FITC, *CD45*-cychrome, *CD49b*-PE, *CD73*-PE, *CD90*-PE, *CD105*-PE, *CD117*-PE, and *CD147*-FITC (BD Pharmingen, Immunotech, and Amcell) and then analyzed using a FACSCalibur (Becton-Dickinson).

Telomerase Activity

Telomerase activity in hMSCs at different passages was measured by means of the LightCycler *T*elo *TAGGG*hTERT quantification kit (Roche) according to the manufacturer's recommendations. Telomere length was determined using Telo TAGGG telomere length assay (Roche, Meylan, France). Briefly, genomic DNA was digested by HinfI and RsaI enzymes, and DNA fragments were separated on agarose gel, transferred, and hybridized with a telomere-specific digoxigenin-labeled hybridization probe. After chemiluminescence detection, results were compared with DIG molecular weight markers and two control DNAs (low and high telomere length).

Adenoviral Production and hMSC Infection

We checked the capacity of hMSCs to be infected by adenovirus vectors. hMSCs were exposed to a recombinant adenovirus expressing the *lacZ* gene driven off the cytomegalovirus (CMV) promoter. After a colorimetric reaction, we observed an efficient expression of the transgene 48 hours after the infection. A multiplicity of infection (MOI) ratio of 40:1 to 100:1 was shown to result in a proper balance between infection efficiency and cell survival with approximately 40%–60% infected cells (data not shown). Then we used E1/E3-deleted, replication-deficient recombinant adenovirus containing mouse *IPF1*, *HLXB9*, or *FOXA2* under the control of CMV promoter. Ad*HLXB9* and Ad*FOXA2* were generated in the Production and Control Department, Genethon III (Evry, France). Ad*IPF1* was generated in the Gene Therapy Laboratory (Nantes, France). The recombinant adenoviruses were propagated in 293 cells and were purified by CsCl density purification and chromatography and resuspended in PBS buffer. We used as a negative control a similar adenovirus containing no cDNA (adNull).

hMSCs were plated at an initial density of 4.10^3 cells per well in a 24-well plate and cocultured with or without human pancreatic islets 96 hours before adenoviral infection.

Adenoviruses were added at MOI ratios of 0:1, 20:1, 40:1, 50:1, or 100:1 in 100 µl culture medium containing 1% fetal calf serum, and cells were incubated at 37°C in 95% air and 5% CO$_2$ for 1 hour. Then medium containing adenovirus was removed and replaced with fresh culture medium and cells were cultured in 24-well plates for 7 days before recovery and mRNA analysis.

mRNA Analysis

Total RNA was isolated using the RNA+ extraction kit (Qbiogene, Illkirch, France) as described by the supplier.

The resulting RNA, was subjected to DNase treatment using RQ1 RNase-free DNase (Promega, Charbonnières-les-bains, France), phenol-chloroform extraction, and isopropanol precipitation. RNA 2 µg, was reverse transcribed using M-MLV reverse transcriptase (Promega) in a 20-µl volume containing 0.2-µg pdN6 primers (Amersham, Saclay, France), 400 µmol/l deoxynucleotide triphosphate, and buffers supplied by the manufacturer. cDNA 2-µl samples were subjected to PCR amplification using human primer pairs described in Table 1. PCR was performed using Taq DNA Polymerase (Promega) or FastStart Taq DNA polymerase (Roche) when indicated by a footnote in a 25-µl volume.

Table 1. Primers sequences used in polymerase chain reactions

Gene	Sequence (5'3')	Product size (bp)	Annealing condition (°C)	Cycle number
Hlxb9	forward: AAG ATG CCC GAC TTC AAC TCC C	233	55	40
	reverse: GCC TTT TTG CTG CGT TTC CAT TTC			
FoxA2	forward: ACT GGA GCA GCT ACT TAG CAG AGC	360	58[a]	40
	reverse: TCA TGG AGT TCA TGT TGG CGT AG		(5% DMSO)	
Pdx-1	forward: CCC ATG GAT GAA GTC TAC C	262	54[a]	40
	reverse: GTC CTC CTC CTT TTT CCA C			
Ngn3	forward: GTA GAA AGG ATG ACG CCT CAA CC	241	52[a]	40
	reverse: TCA GTG CCA ACT CGC TCT TAG G			
NeuroD1	forward: CAG AAC CAG GAC ATG CCC	216	60[a]	40
	reverse: ATC AAA GGA AGG GCT GGT G			
Nkx 2.2	forward: TTC TAC GAC AGC AGC GAC AAC C	393	56[a]	40
	reverse: CGT CAC CTC CAT ACC TTT CTC G		(3% DMSO)	
Pax 4	forward: TTT GTG CTG AAG GGC TTT GC	216	53[a]	35
	reverse: GGG AGA AGA TAG TCC GAT TCC G			
Nkx 6.1	forward: ACA CGA GAC CCA CTT TTT CCG	336	58	35
	reverse: TGC TGG ACT TGT GCT TCT TCA AC			
Isl 1	forward: ATT TCC CTA TGT GTT GGT TGC G	229	58	40
	reverse: CGT TCT TGC TGA AGC CGA TG			
Pax 6	forward: CGG CAG AAG ATT GTA GAG	290	58	35
	reverse: GAT GAC ACG CTT GGT ATG			
PC 1/3	forward: TTG GCT GAA AGA GAA CGG	457	58	40
	reverse: ACT TCT TTG GTG ATT GCT TTG			
Glut 2	forward: AGC TTT GCA GTT GGT GGA AT	300	57[a]	30
	reverse: AAT AAG AAT GCC CGT GAC GA			
GK	forward: TGG ACC AAG GGC TTC AAG GCC	207	56[a]	37
	reverse: CAT GTA GCA GGC ATT GCA GCC			
Insulin	forward: TGT GAA CCA ACA CCT GTG	262	56	35
	reverse: CGT CTA GTT GCA GTA GT			
CK 18	forward: CGT CTT GCT GCT GAT GAC TTT AGA G	269	56	35
	reverse: TTG GCG AGG TCC TGA GAT TTG			
CK 19	forward: ATG GCC GAG CAG AAC CGG AA	317	60	40
	reverse: CCA TGA GCC GCT GGT ACT CC			
CD 90	forward: TCG CTC TCC TGC TAA CAG TCT TG	377	58	35
	reverse: GCC CTC ACA CTT GAC CAG TTT G			
ß actin	forward: ATC ATG TTT GAG ACC TTC AA	316	58	23
	reverse: CAT CTC TTG CTC GAA GTC CA			
Oct 4	forward: CGA CCA TCT GCC GCT TTG AG	577	60	30
	reverse: CCC CCT GTC CCC CAT TCC TA			

[a] PCR was performed using FastStart Taq DNA polymerase (Roche).
Abbreviation: DMSO, dimethyl sulfoxide.

RESULTS

Phenotype Characteristics of Expanded Undifferentiated hMSCs

After plastic adherence selection, hMSCs were cultured over four passages. Growth was exponential over the studied period (60 days) with slight differences between donors (Fig. 1A). FACS analysis of hMSCs showed that these cells were negative for CD11b, CD31, CD34, CD45, CD49b, and CD117. They expressed high levels of CD44, CD73, CD90, CD105, and CD147 and low levels of CD10. CD34 and CD45 represent two of the major hematopoietic markers, whereas CD73, CD90, CD105, CD147, and CD10 are cell-surface markers characteristic of MSCs (Fig. 1B). The same phenotype was maintained for passages 0 through 3 and for all of the hMSCs analyzed. We showed that hMSCs express *OCT4*, a transcription factor important in maintaining undifferentiated ES cells (Fig. 2B). Furthermore, average telomere length of hMSCs cultured during 46 days (three passages) was at least as long as the high telomere length control of 10.2 kbp (Fig. 2A), even though no telomerase activity was detected by quantitative RT-PCR (data not shown).

Expression of Islet-Associated Transcription Factors in hMSCs

To evaluate the potential of hMSCs to differentiate into beta cells, we first examined the expression of islet-related transcription factors. As shown in Figure 3A, RT-PCR analysis revealed that hMSCs express *NKX6-1* at a low level but lacked all the other transcription factors involved in beta-cell differentiation without variation from passage 0 through 3. Additional experiments on different hMSCs ($n = 4$) confirmed this pattern of expression. hMSCs express the marker *CD90* (Fig. 3B) and present some similarities with pancreatic beta cells, as we detected mRNA of epithelial markers such as cytokeratin 18 and 19 as well as proconvertase 1/3. Cytokeratins 18 and 19 were expressed in all of the samples analyzed, whereas proconvertase expression seems to vary from one passage to the other and between donors. In addition, we failed to detect any gene implicated in glucose metabolism (glucose transporter 2, glucokinase, or insulin).

Effect of Adenoviral Infection on hMSC Differentiation and Transformation

To check that adenoviral infection had no effect on hMSC differentiation ability, we infected hMSCs with AdNull, and 7 days after infection, we cultured them in adipogenic differentiation medium. Nearly all of the cells, infected or not infected, showed adipose tissue-forming capacity and accumulated large amounts of triglycerides in their cytoplasm (Fig. 4). Moreover, soft agar assay did not show any colonies with either control or infected cells (data not shown).
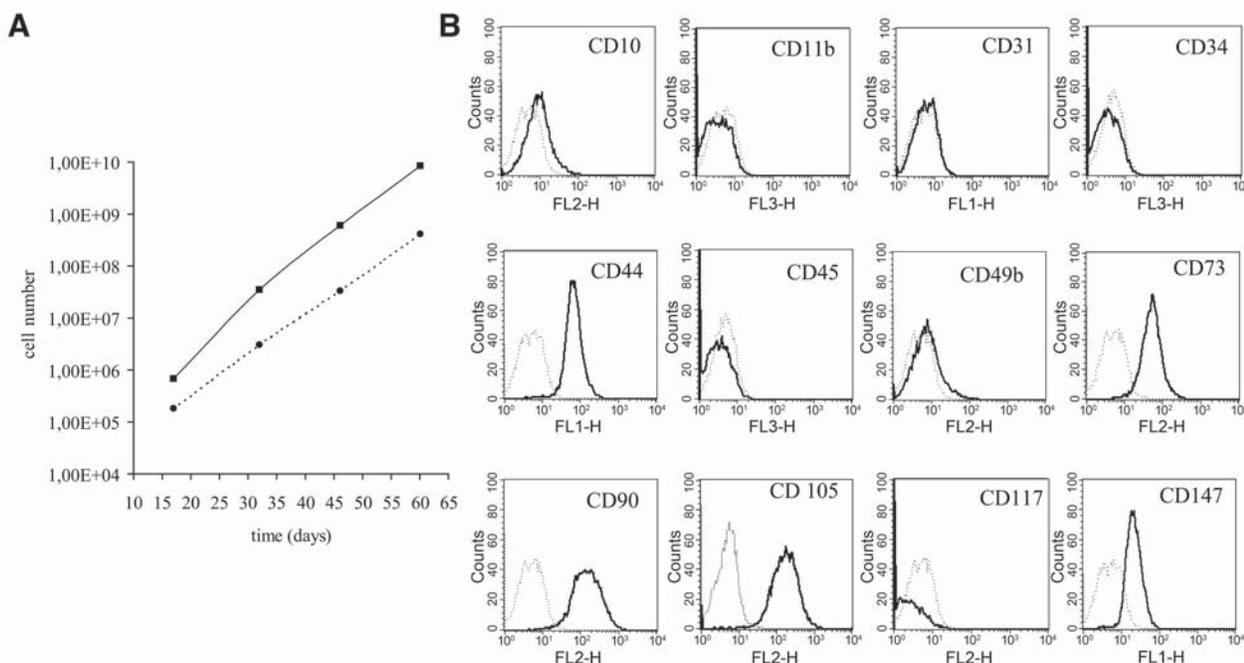
Figure 1. Culture of human mesenchymal stem cells (hMSCs). **(A):** Growth curves of hMSCs isolated from two donors, hMSC 54 (filled square) and hMSC 56 (filled circle), were determined by numeration at each passage. Cell number was plotted against time in days. **(B):** Phenotype of hMSCs. Cells were harvested and labeled with antibodies against CD10, CD11b, CD31, CD34, CD44, CD45, CD49b, CD73, CD90, CD105, CD117, and CD147 or control immunoglobulin G, as indicated and analyzed by fluorescence-activated cell sorting. Plots show isotype control immunoglobulin G staining profile (dotted line) versus specific antibody staining profile (thick line). A representative example of more than 20 hMSCs is shown.

hMSCs Express Insulin After Adenoviral Infection with Genes Coding for Transcription Factors of the Beta Endocrine Pathway or Specific Culture Conditions

Based on the hypothesis that hMSCs' ectopic expression of transcription factors involved in beta-endocrine pathway might favor their differentiation into insulin-expressing cells, we infected hMSCs 4 days after plating with adenoviruses coding for mouse *IPF1*, mouse *HLXB9*, or mouse *FOXA2* using various MOI ratios. hMSCs were concomitantly cultured either alone, in the presence of islet-conditioned medium, or in the presence of human islets placed in a culture insert. Cells were harvested and RNAs were extracted 7 days after infection and analyzed by RT-PCR (Fig. 5).

In the first experiment, hMSCs were infected with Adm*IPF1* using a high MOI ratio (100:1). We detected expression of insulin and expression of three transcription factors involved in beta-cell differentiation, *FOXA2*, *PAX4,* and *ISL1*. In the second experiment, we lowered the MOI ratio (40:1), and hMSCs were concomitantly cultured either in islet-conditioned medium or in control medium. Insulin expression and only one transcription factor expression, *PAX4*, could be detected in hMSCs cultured with islet-conditioned medium. In the third experiment, a MOI ratio of 20:1 was used either in the presence or in the absence of pancreatic human islets. Infected hMSCs cocultured with human islets expressed insulin gene at a very low level, but no expression of transcription factors was detected. In the fourth experiment, coinfection of hMSCs with Adm*HLXB9* (MOI 50:1) and Adm*FOXA2* (MOI 50:1) was performed with or without islets. In the presence of human islets, insulin as well as *NEUROD1* and *ISL1* expressions were detected.
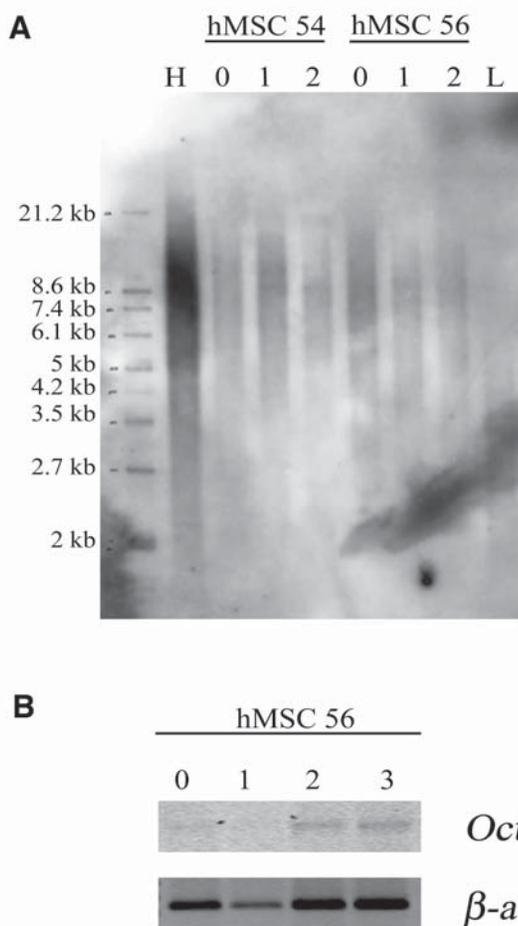

Figure 3. Endogenous expression of **(A)** key pancreatic developmental transcription factors and **(B)** pancreatic genes and epithelial or surface MSC markers in hMSCs. Reverse transcription–polymerase chain reaction (RT-PCR) amplifications of RNAs extracted from hMSCs harvested at different passages (0, 1, 2, and 3) were analyzed by agarose gel electrophoresis. RNA isolated from human pancreatic islets or fetal pancreas was used as a positive control. For *NEUROG3* RT-PCR analysis, human fetal pancreas was used because adult pancreatic islets do not express this gene. Abbreviation: hMSC, human mesenchymal stem cell.

Figure 2. Characteristics of undifferentiated human mesenchymal stem cells (hMSCs). **(A):** Cells from two donors (hMSC 54 and hMSC 56) were expanded and harvested at each passage (0, 1, and 2) and telomere lengths were evaluated. **(B):** Reverse transcription (RT)–polymerase chain reaction for *OCT4* and *ACTB* (used as RNA quality and RT efficiency control). RNA obtained at passages 0, 1, 2, and 3 for hMSC 56 was analyzed.
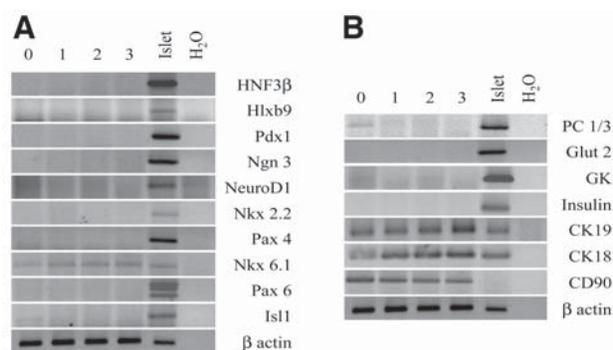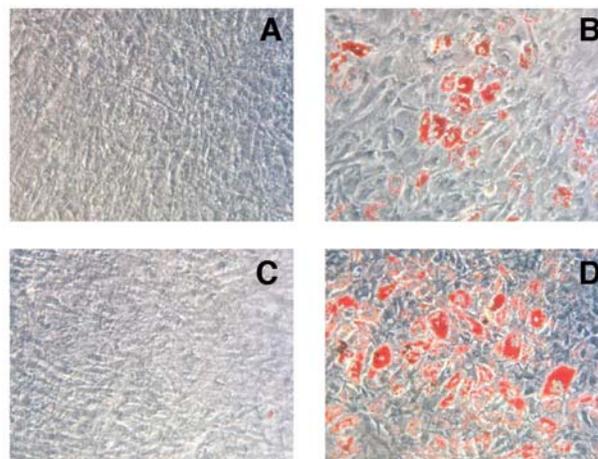

Figure 4. In vitro differentiation of human mesenchymal stem cells into adipocytes. Oil Red-O staining of the lipid vesicles performed 3 weeks after adipogenic stimulation demonstrates an ongoing adipogenesis (magnification, ×20). **(A):** Control cells, **(B)** control cells cultured in adipogenic medium, **(C)** infected cells, and **(D)** infected cells cultured in adipogenic medium.

DISCUSSION

Several studies have demonstrated that ES cells can be induced to differentiate into pancreatic beta cells [6–8]. Assady et al. [6] show that pluripotent undifferentiated human ES cells spontaneously differentiate in vitro into cells with characteristics of insulin-producing cells. Mouse ES cells have also been shown to normalize blood glucose when transplanted into streptozotocin-induced diabetic mice [8]. Similar results have been obtained by Castaing et al. [7] by grafting human pancreatic tissue in beta cell–deficient severe combined immunodeficiency mice, but use of embryonic tissues raises ethical issues. The present study demonstrates that, alternatively, hMSCs can be shifted toward an endocrine phenotype with the gene expression of insulin and some transcription factors implicated in the beta-cell differentiation.

We characterized hMSC phenotype and pluripotency more extensively. Previous reports demonstrated that hMSCs are relatively easy to expand [31, 32]. In this study, we examined the extent to which the cells could be expanded in culture by repeated passaging. Despite some tiny strain-dependent variations, exponential growth is maintained over four passages and phenotype is conserved. Moreover, we showed that hMSCs have long telomeres, approximately 10.2 kbp on average, compared with the

high telomere length control. This length remains unchanged during passaging, but hMSCs do not possess telomerase activity. Telomerase activity and long telomere have been associated with immortality in tumors and ES cells. Conflicting results are found in the literature; Pittenger et al. [15] described a telomerase activity in marrow MSCs even at passage 12, whereas no activity was detected in two other reports [33, 34]. Our study provides some information regarding the telomere length in hMSCs. Our results are in agreement with those of Parsch et al. [35], who describe a length of 11.4 ± 2.5 kbp [35]. Other studies show different telomere lengths, ranging from 6.6 to 15 kbp, depending on origin (bone marrow or placental cord blood) and culture conditions (serum-deprived or MAPC-selective medium) [22, 23, 36, 37]. Expression of *OCT4* and *REX1*, characteristically expressed in embryonic cells, was observed in MAPCs [23] and MIAMI cells [24]. In our study, we also found *OCT4* expression in hMSCs as reported in others works [38]. Thus, because hMSCs can be selected and expanded easily while maintaining their multipotential capability, they may be a potential source of cells for diabetes mellitus therapy.

hMSCs easily differentiate into lineages of mesenchymal tissues, including bone, cartilage, fat, tendon, muscle, and mar-
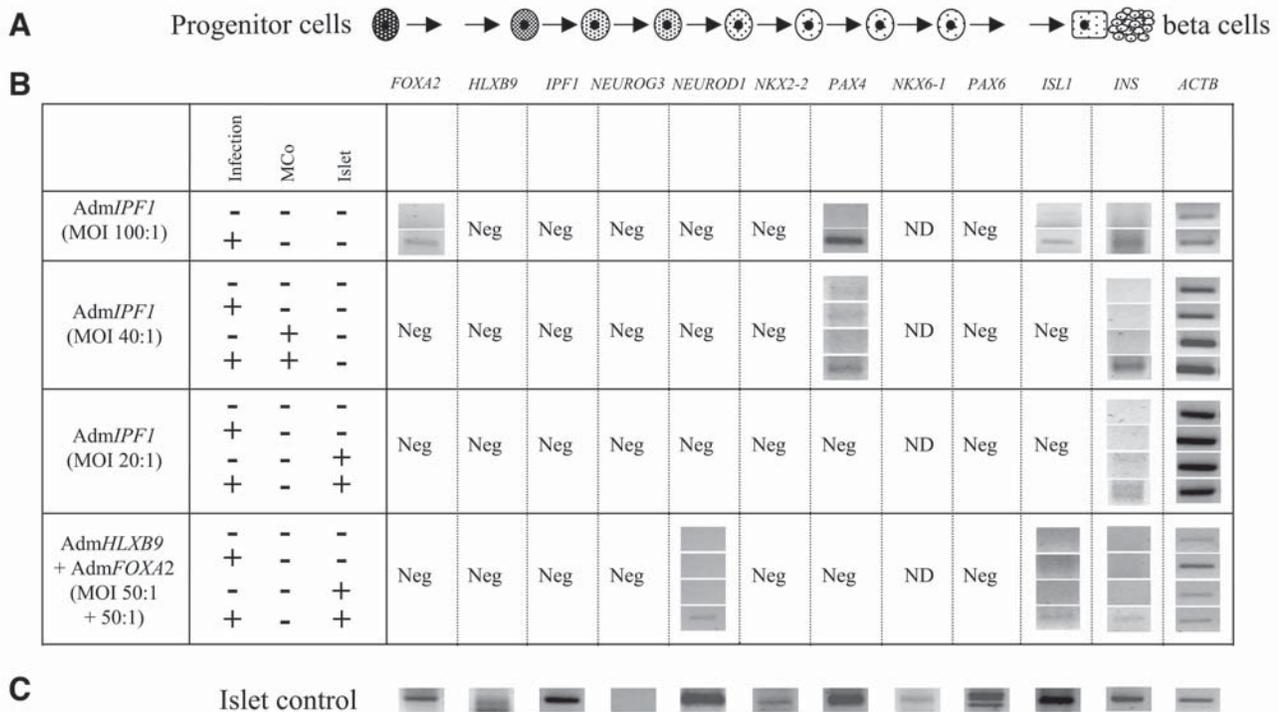

Figure 5. Effect of adenovirus-mediated ectopic expression of pancreatic transcription factors (Adm*IPF1*, Adm*HLXB9*, and Adm*FOXA2*) and islet environment on insulin and key transcription factor expression in hMSCs. **(A):** Model of the cascade of transcription factors controlling beta-cell differentiation as described by Schwitzgebel et al. [25]. **(B, C):** RT-PCR analysis of infected hMSCs cultured with or without **(B)** islet environment and **(C)** control human islet. *ACTB* PCR amplification was used as RNA quality and RT efficiency control. Abbreviations: hMSC, human mesenchymal stem cell; MCo, islet-conditioned medium; MOI, multiplicity of infection; ND, nondetermined; Neg, nonexpressed; RT-PCR, reverse transcription–polymerase chain reaction.

row [15]. They have also been shown to differentiate into cells of ectodermal origin, such as neurons, although with a much lower efficiency compared with mesoderm derivatives [20, 21]. We thus hypothesize that hMSCs could be induced to differentiate into endoderm derivatives. We therefore searched for markers of endocrine beta cells. We report high expression levels of cytokeratin 18 and 19. Cytokeratin proteins are the intermediate filaments of the cytoskeleton in epithelial cells. In the human pancreas, *KRT7* (*CK7*) and *KRT19* (*CK19*) have been identified in ductal epithelium, whereas *KRT8* and *KRT18* are expressed in acini, ducts, and islets [39]. In MAPCs, *KRT18* (*CK18*) and *KRT19* (*CK19*) were also detected at low levels [40]. Using RT-PCR analysis, we observed *NKX6-1* mRNA in hMSC expression in all conditions. *NKX6-1* is expressed in the developing and mature pancreas as well as in the central nervous system [41]. In the developing mouse pancreas, NKX6-1 protein could be detected as early as embryonic day 10.5 (E10.5) in most epithelial cells. In the mature pancreas, expression of *NKX6-1* is restricted to beta cells. Its mRNA is expressed in beta- and alpha-cell lines, but no protein can be detected in the alpha-cell line, suggesting that post-transcriptional regulation contributes to the restriction of *NKX6-1* to the beta cells [42]. *NKX6-1* as *NeuroD1* and *ISL1*, two other transcription factors involved in beta-cell differentiation, was expressed in MIAMI cells [24]. *NEUROD1* was also detected in rat bone marrow stem cells [43]. Among all hMSCs tested, we did not detect any *NEUROD1* expression. In contrast, we occasionally observed *PCSK1* (PC1/3 convertase) expression. *PCSK1* belongs to a family of cellular endoproteolytic processing enzymes. These convertases are mostly found in neural and endocrine cells and are involved in proinsulin processing [44].

Data from the literature report that hMSCs could be transduced with high efficiency using retroviral vectors and maintained their multipotentiality [45–47]. In our study, adenoviral infection using a high MOI ratio (100:1) did not alter the adipogenic differentiation ability of hMSCs and did not make them tumorigenic. These results proved that adenoviral infection had no negative effects on hMSC properties and could be used to deliver specific genes of the beta-endocrine pathway. hMSCs were infected with adenoviral vectors coding for three of the transcription factors of the beta pathway (*FOXA2*, *HLXB9,* and *IPF1*) and cultured in the presence of pancreatic islets or islet-conditioned medium. Insulin expression was analyzed. During development, *HLXB9* is transiently expressed in regions of endoderm that give rise to the respiratory and digestive tubes as well as the pancreatic anlage [48, 49]. Dorsally, *HLXB9* is required for specifying the gut epithelium to a pancreatic fate, whereas ventrally it regulates endocrine cell differentiation. *FOXA2* is a member of the forkhead/winged helix transcription factor family, which is essential for all endodermal lineage development. It plays an essential role in the cell type–specific transcription of the *IPF1* gene in the pancreas [26, 50]. The homeodomain *IPF1* transcrip-

tion factor is expressed in pluripotent precursor cells in the dorsal and ventral pancreatic bud. In the adult, *IPF1* is mainly expressed within insulin-secreting pancreatic islet beta cells and cells of the duodenal epithelium [51]. *IPF1* is the only transcription factor specific of the endocrine pathway, whereas the two other factors, *HLXB9* and *FOXA2*, are implicated in hepatic development. Expression of human *FOXA2*, *NEUROD1*, *PAX4*, and *ISL1* genes was consistently detected after infection of hMSCs with adenovirus vectors expressing mouse *HLXB9*, *FOXA2*, and *IPF1*. These four transcription factors regulate cell differentiation processes within the developing pancreas [52, 53]. *FOXA2* was detected after Adm*IPF1* infection using a high MOI ratio. This result is surprising in that *FOXA2* is a transcriptional activator of *IPF1* in beta cells [50]. In the same culture conditions, we also observed *PAX4* and *ISL1* expression. Mice deficient for *PAX4* fail to develop beta and delta cells within the pancreas [54], whereas *ISL1* is required for all endocrine islet cells [55]. When the Adm*IPF1* viral dose was reduced to 40:1 and cells were cultured in islet-conditioned medium, only *PAX4* expression was detected. At an MOI ratio of 20:1, whatever the cell culture conditions, we did not observe any transcription factor expression. It seems that when low MOIs (20:1 and 40:1) were used, a pancreatic environment (islets or conditioned medium) was required to lead to insulin expression. Ferber et al. [11] demonstrated that adenovirus-mediated gene transfer of *IPF1* to the mouse liver activated expression of the endogenous genes for mouse insulin 1 and 2 and prohormone convertase 1/3. Coexpression of *FOXA2* and *HLXB9*, via dual infection of hMSCs with Adm*FOXA2* and Adm*HLXB9*, was performed in coculture experiments with the pancreatic islets. Again, insulin was expressed concomitantly with *NEUROD1* transcription factor. *NEUROD1*, a basic helix-loop-helix transcription factor, is expressed in the developing endocrine pancreas, the small intestine, and the nervous system. Homozygous *NEUROD1*-null mice have a striking reduction in the number of insulin-producing beta cells and fail to develop mature islets [56]. Only Heremans et al. [57] have studied the effects of ectopic expression of *NEUROG3* and *NEUROD1*, using adenoviral vectors, in human ductal cells, showing that *PAX4*, *NKX2-2,* and *PAX6* as well as insulin and somatostatin were expressed.

Whether hMSCs were driven to activate silent genes or whether our manipulations enhanced the expression of already active promoters is speculative. It can be hypothesized that pluripotent stem cells reside in the adult bone marrow in a standby state but their differentiation potential is restricted by their natural environment. Whatever mechanism is involved, the next step is the identification, selection, and amplification of hMSCs expressing insulin gene to determine if all of the cells express insulin at a low level or a restricted population expresses it at a high level. Apart from MSCs, bone marrow also contains a hematopoietic stem cell population that can engraft in epithelial tissues and differentiate into epithelial cells of the liver, lung, gastroin-

testinal tract, and skin [17]. Our cell population was negative for CD45, and we favor the hypothesis that cells undergoing insulin gene expression were mesenchymal and not hematopoietic stem cells. Recently, a new population of nontransformed pluripotent human cells isolated from bone marrow was described [24]. When these cells were treated with factors known to promote the expression of a beta-like cell phenotype, expression of insulin and glucagon genes was detected by RT-PCR. Elsewhere, in vivo experiments showed that transplantation of adult bone marrow–derived cells expressing c-kit reduce hyperglycemia in mice with streptozotocin-induced pancreatic damage [58]. Moreover, bone marrow cells that selectively express the enhanced green fluorescent protein (EGFP) if the insulin gene is actively transcribed were transplanted into lethally irradiated recipient mice and gave rise to EGFP-positive insulin-producing cells in pancreatic islets [59]. However, other studies suggest that stem cells may act through differentiation into endothelial cells that may affect the islet regeneration process [60]. In conclusion, our results demonstrate that human bone marrow stem cells are able to express insulin gene by a mechanism involving both transcription factors involved in beta-cell differentiation and appropriate microenvironment.

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Human Bone Marrow Mesenchymal Stem Cells Can Express Insulin and Key Transcription Factors of the Endocrine Pancreas Developmental Pathway upon Genetic and/or Microenvironmental Manipulation In Vitro

Christine Moriscot, Florence de Fraipont, Marie-Jeanne Richard, Mélanie Marchand, Pierre Savatier, Domenico Bosco, Marie Favrot and Pierre-Yves Benhamou

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