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Oct-4, Rex-1, and Gata-4 Expression in Human MSC Increase the Differentiation Efficiency But Not hTERT Expression

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Abstract Micro-environment seems to exert an important influence on human mesenchymal stem cell (MSC) differentiation and proliferative capacity in bone marrow as well as in culture ex vivo. Oct-4, Rex-1, and TERT genes are well-known for the maintenance of pluripotentiality differentiation and the proliferative capacity of embryonic stem cells. Some previous data report expression of these embryonic factors in selected clones from bone marrow adult stem cells. Our goal was to study expression of Oct-4, Rex-1, and TERT in primary cultured human MSC according to the serum concentration. In addition, we have studied the expression of Gata-4 since this factor plays a key role in organogenesis. We hypothesized that low serum concentration with appropriate growth factors may induce an undifferentiated status with a re-expression of embryonic factors and extend differentiation capacity. Thus, using a defined culture medium, we report on the increased expression of Oct-4, Rex-1, and Gata-4 in human MSC. We have correlated this expression to an increase in differentiation efficiency towards osteogenic and adipogenic phenotypes. Our data suggest that the culture medium used permits the emergence of adult stem cells with a high differentiation capacity and expression of embryonic factors. These cells may have important implications for cell therapy.

Key words: human mesenchymal stem cell; Oct-4; Rex-1; Gata-4; TERT; define medium; differentiation

The factors involved in the differentiation process of mesenchymal stem cells (MSC) have not been completely elucidated. Among them, the micro-environment (ME) or "niche" seems to play a critical role. The ME controls the undifferentiated status of the MSC as well as the multipotentiality in different environments including bone marrow, or the extensive ex vivo culture.

Various studies have identified factors normally restricted to embryonic stem cells (ESC) and which may be expressed in adult stem cells under specific conditions. Reyes et al. [2001] have isolated Multipotent Adult Progenitor Cells (MAPC) described as precursors of MSC. The MAPC express Oct-4 and Rex-1 transcription factors and several common surface antigens with MSC. MAPC share important characteristics with embryonic stem cells, in particular large telomeres after long-term culture [Reyes et al., 2001] and probably pluripotency [Jiang et al., 2002a]. Pochampally et al. [2004] have described the selection of MSC precursors using serum deprivation. These cells express Oct-4, telomerase reverse transcriptase (TERT) and maintain large telomeres.

Oct-4 [Boiani et al., 2002] and Rex-1 [Ben-Shushan et al., 1998] are transcription factors characteristic of pluripotentiality. They have been characterized as embryonic and germinal restricted factors [Baddoo et al., 2003]. The molecular events underlying the separation of distinct cell lineages are still not well understood. However, it is known that the transcription factor Oct-4 is expressed throughout oogenesis and becomes restricted to the inner cell mass at the blastocyst stage and is later confined to developing germ cells [Scholer et al., 1990; Palmieri et al., 1994]. Niwa et al. [2000] have demonstrated a relationship between Oct-4 and Rex-1 expression. Paradoxically, an over- or under-expression of

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Oct-4 leads to a down-regulation of Rex-1 expression. These transcription factors regulate the totipotency of the ESC. Their down-regulation triggers trophectoderm differentiation and their up-regulation triggers primitive endoderm and mesoderm differentiation [Niwa et al., 2000]. Among other embryonic mesendodermic transcription factors, some factors from the GATA family play a critical role in mesendoderm and endodermal differentiation. These zinc finger proteins are composed of two conserved zinc fingers and their adjacent basic domains bind to (A/T)GATA(A/G) sequences. Only six GATA factors have been isolated from vertebrates. They are subdivided in two classes, GATA 1–3 and 4–6 [Technau and Scholz, 2003]. GATA factors 4–6 are controlled by signaling pathways that act in the early specification of endoderm and mesoderm [Shoichet et al., 2000; LaVoie, 2003; Yoshida-Koide et al., 2004].

In contrast to embryonic stem cells and MSC precursors, human and murine primary MSC are negative for TERT expression [B anchi et al., 2003] and have a limited number of divisions before senescence. The unlimited propagation of ES cells is related to the expression and activity of telomerase [Kim et al., 1994]. Some studies have reported the establishment of human MSC with an infinite life span which maintain their differentiation potency and their physiological growth rate by the enforced expression of TERT [Okamoto et al., 2002; Mihara et al., 2003].

The aim of this work has been to demonstrate that the culture conditions of adult human MSC may modify the expression of embryonic genes associated with their potential for differentiation. A culture medium with a low serum concentration seems to play a major role in restoring the multipotentiality of human MSC [Reyes et al., 2001; Hu et al., 2003]. Our data shows an increase in Oct-4, Rex-1, and Gata-4 by MSC and their differentiation potency.

MATERIALS AND METHODS

Mesenchymal Stem Cell Isolation and Expansion

Bone marrow aspirates were collected on ACD-heparin from healthy adult volunteers after informed consent. Nucleated cells were directly plated at 50,000 cells per cm^2 on plastic culture dishes. Expansion medium consisted of αMEM basal medium (Sigma Aldrich, Saint Quentin Falavier, France) supplemented with 10% FCS (Invitrogen), 2 mM glutamine and penicillin/streptomycin (Roche Diagnostic, Meylan, France) noted αMEM medium. Two to three days later non-adherent cells were discarded and primary culture was performed for 21 days. The complete medium was changed twice a week. After reaching subconfluence, cells were lifted with 0.25% trypsin and 1 mM EDTA, suspended in fresh medium, plated at 1,000 cells/cm^2 and incubated for 14 days at 37°C in 5% CO_2.

Induction Phase

Two different conditions of culture were tested (see Fig. 1) for their capacity to facilitate cell differentiation. After trypsination, cells were plated either on plastic in αMEM medium or on plastic coated with human fibronectin (Sigma Aldrich) in a modified MCDB201 medium. This modified MCDB201 medium was composed of: MCDB201/DMEM (60/40), 2% FCS, 1× ITS + 1 (insulin-transferrine

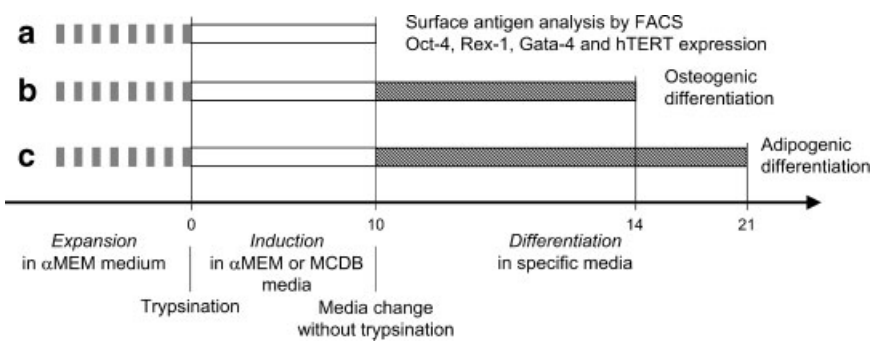

Fig. 1. Design of the study. Cells were cultured in modified MCDB201 medium or αMEM medium for 10 days. After this induction phase, RT-PCR and quantitative PCR analysis were performed (**lane a**) as well as differentiation to osteoblastic phenotype (**lane b**) and adipocyte phenotype (**lane c**).

selenium containing linoleic acid and BSA), 10^{-9} M dexamethasone, 10 ng/ml PDGF-BB, 10 ng/ml EGF and 0.2 mM ascorbate-2-P (All from Sigma Aldrich).

Fluorescence Activated Cell Sorting (FACS) Analysis

Cells were washed once with culture medium, once with PBS, resuspended in PBS-BSA 1% and stained with coupled fluorochrome antibodies as described by the manufacturer. Analysis was performed with a FACSCalibur (Beckton Dickinson, Pont De Claix, France). CD90-PE, CD73-PE, CD45-Cy, CD34-Cy, CD44-FITC, CD49b-PE were purchased from Beckton Dickinson; CD105-PE and GlyA-FITC from Santa Cruz (Santa Cruz, CA); CD133-PE from Miltenyi Biotech (Paris, France).

RNA Extraction and RT-PCR

Total RNA was extracted from each sample with RNA plus (Qbiogen, Illkirch, France), according to the recommendations of the manufacturer. The harvested RNA was reverse transcribed using a M-MLV reverse transcriptase kit (Roche Diagnostic). The cDNA was amplified with specific primers as shown in Table I in a reaction mixture containing "FastTaq" Taq polymerase (Roche Diagnostic). PCR was performed in a DNA thermal Cycler (T3 thermocylcer, Biometra, Goettingen, Germany). The amplified samples were visualized on 2% agarose gels stained with ethidium bromide and photographed under UV light using a GelDoc (BioRad, Marnes-la-Coquette, France) system.

Detection of Telomerase Components hTERT

Telomerase components hTERT were detected by quantitative, one-step RT-PCR with the LightCycler instrument (Roche Diagnostic) using the *T*elo*TAGGG* hTERT Quantification Kit (Roche Diagnostic) according to the manufacturer's instructions. In a separate one-step RT-PCR, mRNA encoding for porphobilinogen deaminase (PBGD) was processed for use as a housekeeping gene. The reaction product serves both as a control for RT-PCR performance and as a reference for relative quantification.

Differentiation

After cell maintenance for 10 days in αMEM or in modified MCDB201 medium, cells were cultured in specific differentiation media (Fig. 1).

To induce adipocyte differentiation, human MSC were cultured in MCDB201/DMEM (60/40) with 2× ITS + 1 (containing linoleic acid and BSA), 10% horse serum (Sigma Aldrich) 10% foetal calf serum, 10^{-7} M dexamethasone and 60 μM indomethacine and maintained for 21 days with medium exchanges every 5 days. For oil-red staining, cells were fixed with cold methanol (−20°C) for 2 min and rinsed with propan-2-ol 50%. Cells were stained by oil-Red-O at 0.2% in propan-2-ol for10 min. After photography, coloration was solved by propan-2-ol 100% and quantify at 500 nm [Ramirez-Zacarias et al., 1992; Sekiya et al., 2002, 2004].

To induce osteoblast differentiation, human MSC were cultured in DMEM low glucose (1 g/L) with 10% FCS, 10 mM β-glycerophosphate,

TABLE I. Sequence of Primer, Size of Product, Number of PCR Cycle and Annealing Temperature

Gene		Primers	PCR cycle	Annealing temperature (°C)
RPL27	5′	GAACATTGATGATGGCACCTC	26	55
	3′	GGGGATATCCACAGAGTACC		
CD90	5′	TCGCTCTCCTGCTAACAGTCTTG	30	58
	3′	GCCCTCACACTTGACCAGTTTG		
CD73	5′	TCGGCTCTTCACCAAGGTTCAG	30	58
	3′	CCCACAACTTCATCACCAACAGG		
CD105	5′	CATTGTGGCATCCTTCGTGG	30	58
	3′	AAACTTGTCACCCCTGTCCTCTG		
Oct-4	5′	CGACCATCTGCCGCTTTGAG	36	58
	3′	CCCCCTGTCCCCCATTCCTA		
Rex-1	5′	GCGTACGCAAATTAAAGTCCAGA	36	58
	3′	CAGCATCCTAAACAGCTCGCAGAAT		
Gata-4	5′	GATGCCTTTACACGCTGATG	34	60
	3′	TGGGTTAAGTGCCCCTGTAG		

Oct-4 and Rex-1 primers were defined by Henderson et al. [2002].

10^{-7} M dexamethasone, and 0.2 mM ascorbic acid with medium changes twice a week. After 14 days, human MSC were fixed with methanol. Minerealization was assayed using 2% alizarine red for 2 min. Alkaline phosphatase activity was detected by 5-bromo-4-chloro-3-indoly phosphate and nitroblue tretrazolium for 5–10 min and then washed with 100 mM Tris HCl pH 9.5 100 mM NaCl and 10 mM $MgCl_2$ buffer [Stanford et al., 1995; Sakaguchi et al., 2004; Abdallah et al., 2006].

RESULTS

We first checked that human Mesenchymal Stem Cells (human MSC), selected and expanded in αMEM medium, were not contaminated with hematopoietic cells. Indeed, CD45 positive cells represent 5% ± in the primo culture, 1% ± of cells above second sub-culture and were not detectable after this sub-culture. Human MSC cultured in αMEM medium did not express other hematopoietic markers such as CD34 and CD133 and expressed CD90 (Thy-1), CD73, CD105 (endoglin), CD44 and CD49b (Fig. 2a) in agreement with the expression pattern of surface antigens previously described for human MSC. The proportion of positive cells for these markers was $97.6 \pm 2.3\%$ CD90, $97.7 \pm 2.2\%$, CD73 and $97.5 \pm 1.9\%$ CD105 (Fig. 2a). This expression was maintained during the expansion phase in each sub-culture and we checked that cells have osteogenic, adipogenic, and chondrogenic differentiation capability (data not shown). The surface antigens on the human MSC expanded 10 days in modified MCDB201 medium were analyzed by flow cytometry and compared with cells expanded in αMEM. No differences were observed in the level of CD90, CD73, and CD105 antigens (Fig. 2b). CD49b expression was not modified by culture on plastic coated with 10 μg/ml human fibronectin (Fig. 3a). A previous study observed a modification of CD44 antigen expression in relation to the percentage of FCS in culture [Lodie et al., 2002]. To test whether our culture conditions could interfere with CD44 expression, we performed an additional experiment using modified MCDB201 medium supplemented or not with 2 or 10% FCS

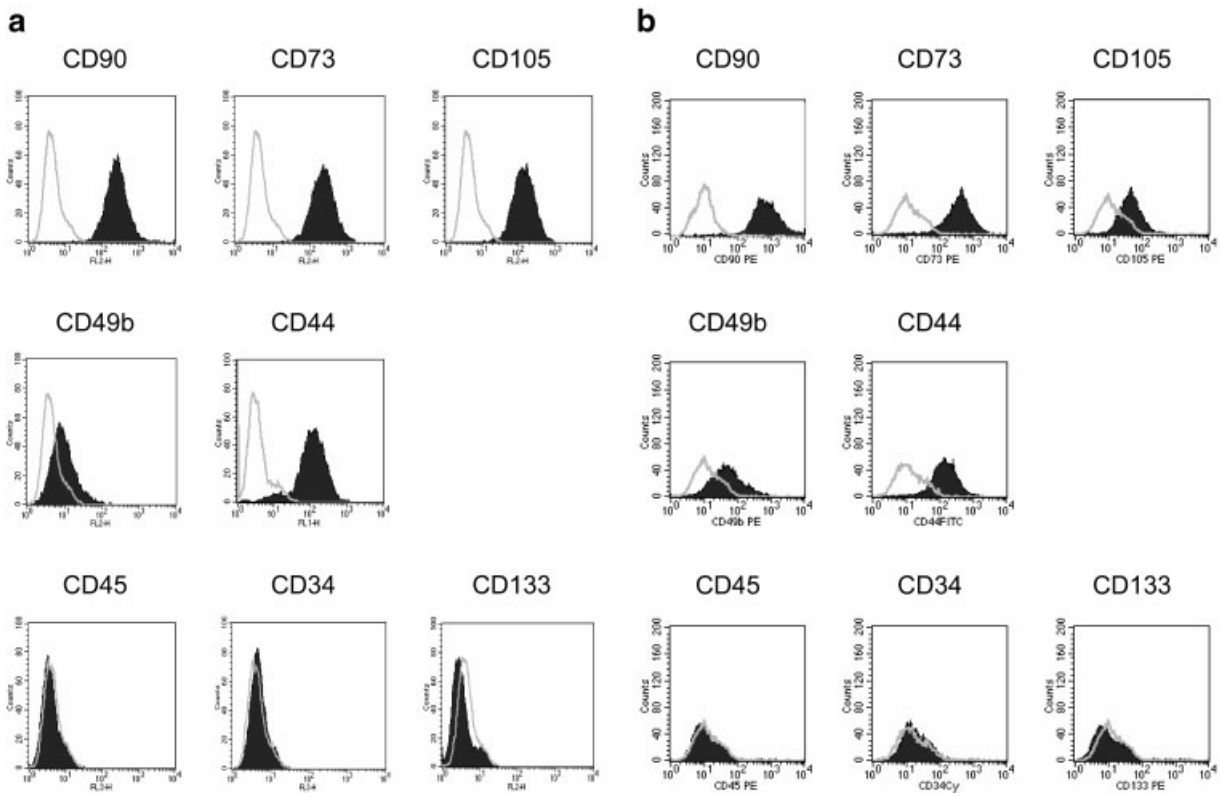

Fig. 2. Phenotype of human mesenchymal stem cell (human MSC) in culture in (**a**) αMEM medium and (**b**) modified MCDB201 The black shaded curve represents antigen specific recognition and the unshaded curve the negative isotypic control. These blots are representative of three different experiments.

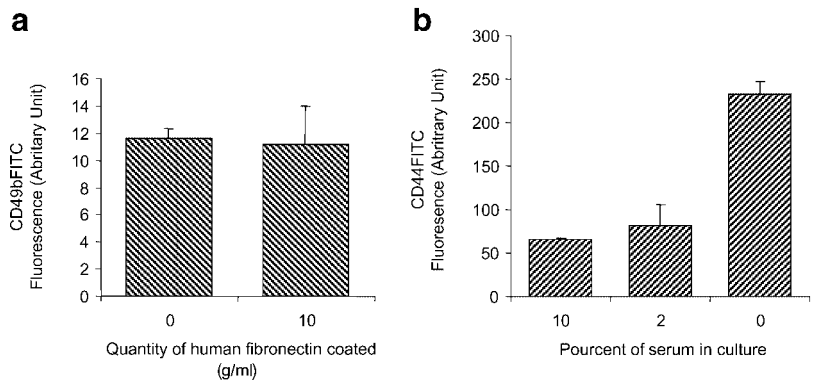

Fig. 3. Quantification. of matrix receptor CD49b (**panel a**) and CD44 (**panel b**) level of expression by flow cytometry. Plots show isotype control versus specific antibody staining profile.

(Fig. 3b). Cells cultured in serum free medium (0% FCS) for 10 days expressed a high level of CD44 (level 233 ± 13 in arbitrary unit of fluorescence). During culture with 2 or 10% of FCS, CD44 expression diminished to 65 ± 1 arbitrary unit (Fig. 3b).

Cells cultured in modified MCDB medium show a more rapid growth than cells cultured in αMEM medium until the 6th passage analyzed (Fig. 4). To further determine the extent of differentiation of cells cultured for 10 days in modified MCDB201 or in αMEM medium, the expression of Oct-4 and Rex-1 was studied. In MSC cultured in αMEM medium, no expression of Rex-1 and very low levels of Oct-4 were detected (Fig. 5 column a) whereas Rex-1 and Oct-4 were expressed in human MSC after the induction phase obtained by expansion in modified MCDB201 medium (Fig. 5 column b).

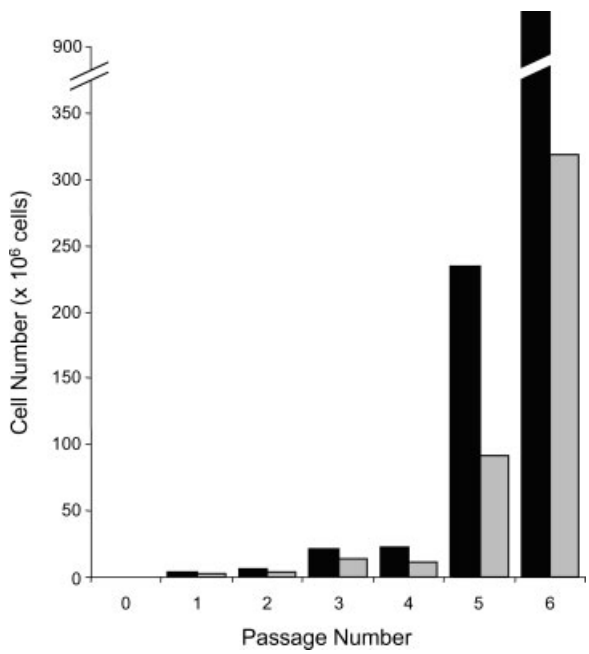

Fig. 4. Cell growth capability of human mesenchymal stem cells in αMEM medium (gray bar) and modified MCDB201 (black bar). After each passage, cells were plated at 1,000 cells per cm^2 and the culture medium changed every 3 days. This experiment is representative of three independent cultures.

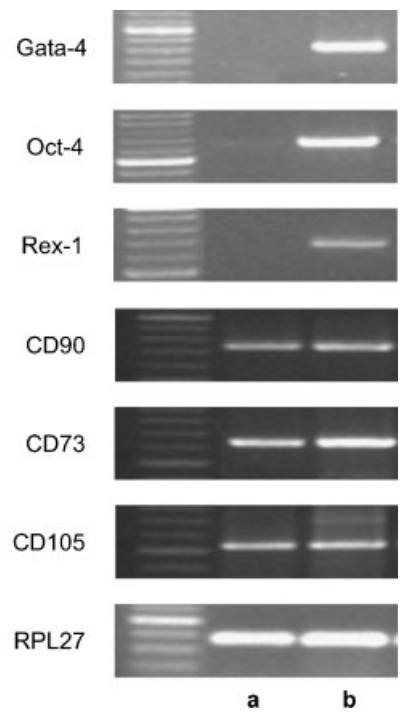

Fig. 5. Expression of transcription factors by RT-PCR. Reverse transcription was performed using 0.5 μg of total RNA and oligo dT nucleotides. Primers and PCR conditions are described in Table I. RPL27 is a ubiquitous protein; CD90, CD73, and CD105 genes were positive controls of MSC. We carried out a 10-day culture in αMEM (column **a**) or in modified MCDB201 (column **b**) media before RNA extraction. This figure is representative of three independent experiments.

In addition, we observed an increase in Gata-4 gene expression during the same induction phase. The phenotype of cells was confirmed by measuring expression of CD90, CD73, and CD105 mRNA in the same experiments. According to the FACS analysis, no difference was observed between expression of RPL27 and CD90, CD73 and CD105 in the two media.

Using a real time PCR detection kit, we compared the expression of the hTERT gene in cells maintained in αMEM versus cells induced by culture in the modified MCDB201 medium. Human MSC maintained in αMEM medium, do not express the hTERT gene (Fig. 6). Similarly, no hTERT mRNA was observed in cells cultured in modified MCDB201 medium.

As shown in Figure 7, we observed a greater deposition of mineral in cells pre-treated in modified MCDB201 medium (panel B) than cells maintained in αMEM medium (panel A). The same observation can be made concerning the activity of alkaline phosphatase. These levels are higher in modified MCDB201 medium pre-treated cells (panel D) compared with αMEM medium (panel C).

Adipogenesis as shown in Figure 7 panel E and F is significantly higher in modified MCDB201 medium pre-treated cells. These observations were confirmed by measuring fat droplets stained with fresh Oil Red-O (*t*-test, $P < 0.0025$).

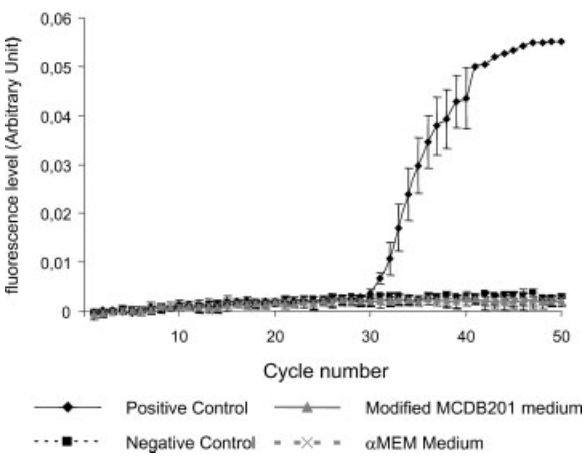

Fig. 6. Detection of hTERT mRNA by realtime PCR. A one-step detection kit and a LightCycler system were used in this study. The plain black line illustrates the positive control provided in the kit, the dotted black line the negative control, dotted gray line shows αMEM medium and the plain gray line shows the modified MCDB201 medium. As supplied by the manufacturer, we used the PBGD housekeeping gene as a control of RT-PCR reaction. This graph is representative of three independent experiments.

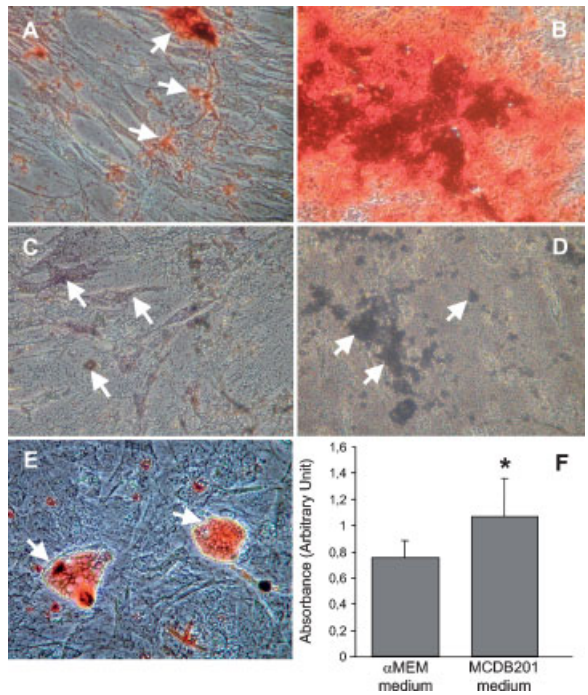

Fig. 7. Differentiation into osteoblastic and adipogenic phenotype: influence of culture medium. (1) Mineral deposition (**panels A and B**) and phosphatase alkaline (PA) activity (**panels C and D**) were observed either in modified MCDB201 (panels B and D) or αMEM (panels A and C). The mineral deposition is observed with red alizarin coloration phospho-calcic crystal. The PA activity is measured using BCIP/NBT substrate transformation. This is representative of three independent experiments. (2) Adipocytic vesicles are colored by Oil Red O (white arrow in **panel E**). A quantification by solubilization of coloration in 2 ml propan-2-ol and measurement of absorbance at 500 nm demonstrated a significative increase of differentiation in modified MCDB201 medium (*t*-test, $P < 0.0025$, represented by a asterisk in **panel F**). These results are a mean of three representative independent experiments.

DISCUSSION

In this study, using adult MSC, we demonstrate that their culture in modified MCDB lead to (1) the transcription of embryonic factors involved in totipotency (e.g., Oct-4 and Rex-1) and organogenesis (e.g., Gata-4) but not TERT; and (2) a facilitation of the osteogenic and adipogenic differentiation potential. These physiologic modifications were not correlated with modification of classical membrane antigens expressed by MSC in culture.

The MSC phenotype needs to be clearly defined and checked to avoid any contamination

by precursor cells from other sources. Previous studies have defined the phenotype of MSC as negative for CD45 antigen and progenitor specific markers such as CD133 and CD34, and positive for CD44, CD73, CD90, CD105, and low for CD49b [Reyes et al., 2001; Lodie et al., 2002; Jiang et al., 2002b; Baddoo et al., 2003]. MSC used for this study express CD44, CD73, CD90, CD105, and were low for CD49b and negative for CD45, CD34, and CD133 as expected. Various precursor cells within bone marrow, negative for CD45, may also be found as endothelial precursor cells (EPC). EPC are $CD45^-$ $CD133^+$ and become $CD133^-$ $CD34^+$ during differentiation to mature endothelial cells [Quirici et al., 2001; Salven et al., 2003]. In bone marrow, these $CD133^+$ cells are the common precursors to endothelial and hematopoietic lineages [Quirici et al., 2001]. Conversely, MSC are negative for CD133 [Vogel et al., 2003; Oswald et al., 2004]. This MSC phenotype is stable in the two types of medium used in this study. Reyes et al. [2001] observed with MAPC that the increase of FCS concentration induces an increase in CD44 and a decrease in CD49b expression in relation with a loss of differentiation potentiality and of proliferation potential beyond 30 cell doublings [Jiang et al., 2002a,b]. Thus, we analyzed the expression level of these two antigens under our culture conditions. In contrast with MAPC, we demonstrate that the level of CD49b expression was not modified by culture of MSC on fibronectin. In human MSC, CD49b associates non-covalently with CD29 [Baddoo et al., 2003; Meirelles Lda and Nardi, 2003; Zimmermann et al., 2003] and form the integrin $\alpha 2\beta 1$, a receptor for collagen and laminin [Miyake et al., 1994] and not fibronectin. This could explain the absence of the influence of fibronectin on CD49b expression. The second matrix receptor analyzed was CD44, a member of the hyaluronan-binding proteins, with structural similarities to selectins. Differences may be related to the concentration of FCS [Lodie et al., 2002]. However, between 10 and 2% (inverse!) of FCS, no significant modification was showed but we could demonstrate an important increase in the absence of FCS.

Our work demonstrating the expression of Oct-4, Rex-1, and Gata-4 is likely to be due to the culture conditions used. The expression of these embryonic factors has rarely been reported in adult cells [Moriscot et al., 2005]. Data using embryonic stem cells in murine models demonstrated the importance of Oct-4 expression for generating the three primordial tissues (endoderm, ectoderm, and mesoderm) [Niwa et al., 2000; Niwa, 2001]. This transcription factor was expressed only in cells which can differentiate in these three embryonic layers or in an adult organism but are restricted to the germinal cell line. Regulation of this factor seems to be critical because its up or down-regulation reduces the totipotency of embryonic cells [Niwa et al., 2000; Niwa, 2001], represses target genes such as Rex-1 and induces differentiation [Niwa et al., 2000]. In our study, we both induced expression of Oct-4 and Rex-1 by culturing human MSCs with low serum concentration in a modified MCDB201 medium.

The presence of TERT expression in adult stem cells is subject to debate. The expression of TERT in cells is associated with immortal cells [Kim et al., 1994]. Several studies have demonstrated that MSC do not express TERT catalytic subunits. However, one study has demonstrated TERT expression during a limited time but failed to identify the activity of this enzyme [Bianchi et al., 2003]. Although regarded as tissue-specific stem cells, human MSCs have a relative low proliferative ability with a limited life span. The average number of population doublings is reported to be approximately 38 at which time cells finally cease to divide, being broad and flattened [Bruder et al., 1997]. Verfaillie's group has demonstrated that MAPC expressed Oct4 and have extended growth capacity due to presence of large telomeres without any evidence of hTERT expression [Reyes et al., 2001; Jiang et al., 2002a]. Prockop's group has identified in the primary cultures of hMSCs a subpopulation (named RS) which has a greater potential of proliferation and differentiation [Pochampally et al., 2004]. This population can be isolated by serum deprivation. They have demonstrated that their RS cells express Oct-4 and have an extended growth capacity due to higher TERT expression and larger telomeres than hMSCs. In our culture conditions, with PDGF and EGF growth factors, we have demonstrated that the total population of hMSCs can express embryonic transcription factors such as Oct-4 and Rex-1 and display a greater growth potential but not express hTERT. In addition, we have previously demonstrated that MSCs showed large telomeres [Moriscot et al., 2005].

We did not observe Gata-4 expression in MSC cultured in αMEM medium as previously described [Lodie et al., 2002] but we were able to demonstrate an increase in the expression of Gata-4 transcription factor in a modified MCDB201 medium. This factor plays an important role in the mechanism of differentiation into meso- and endodermal tissues [Shoichet et al., 2000; Yoshida-Koide et al., 2004] in organogenesis of most vertebrates. The expression of this transcription factor into MSC was observed after treatment by the 5-azacytidine drug [Hakuno et al., 2002; Xu et al., 2004] and after hepatocyte growth factor treatment of adult stem cells [Schwartz et al., 2002; Forte et al., 2006]. Gata-4 expression may play a key role in the differentiation of these stem cells due to its role in the development of various foetal [Arceci et al., 1993; Technau and Scholz, 2003] and adult tissues [Gillio-Meina et al., 2003] such as heart, intestinal epithelium, primitive endoderm, and gonads.

The presence of Gata-4 in conjunction with the expression of Oct-4 and Rex-1 may be related to the pluripotentiality of these cells and their capacity to differentiate to primitive endoderm and mesoderm. We analyzed the differentiation potential of osteoblastic and adipocytic phenotypes. Although induction was observed in the two differentiation pathways in most MSCs maintained in αMEM containing 10% FCS, we never observed differentiation in 100% of cells. When cells were cultured in modified MCDB201 medium, their capacities of differentiation were increased both for osteoblastogenesis and adipogenesis. We hypothesized that culture in a modified MCDB201 medium, with specific growth factors such as PDFG BB or EGF helps to maintain cells in an undifferentiated state as demonstrated by the re-expression of embryonic markers. Reyes et al. [2001] suggest that FCS reduce MAPC differentiation capacity. Similarly, in our work, we demonstrate a more efficient and complete differentiation with low FCS concentration. On the other hand, it was suggested that Oct-4 could be a co-activator of an osteoblastic gene like osteopontin (OPN) in embryonic stem cells from a potential binding site within the cis-regulatory sequences (named Palindromic Oct Regulatory Element or PORE) [Botquin et al., 1998; Pesce and Scholer, 2001]. We may hypothesize that Oct-4 is a co-activator of some genes involved in the expression of differentiation programs which may increase the outcome of differentiated phenotypes.

CONCLUSION

Our data suggest that modification of culture conditions (serum concentration) as well as cell density may contribute to the emergence of stem cells with a greater potentiality. These cells are characterized by the expression of Oct-4 and Rex-1 totipotency factors and by the expression of Gata-4 organogenesis transcription factor. This may have implications for reconstructive surgery using adult stem cells.

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