Mesenchymal stem cells from umbilical cord blood: parameters for isolation, characterization and adipogenic differentiation

Tatiana Taís Sibov · P. Severino · L. C. Marti · L. F. Pavon ·
D. M. Oliveira · P. R. Tobo · A. H. Campos · A. T. Paes ·
E. Amaro Jr. · L. F. Gamarra · C. A. Moreira-Filho

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Abstract Isolation of mesenchymal stem cells (MSCs) from umbilical cord blood (UCB) from full-term deliveries is a laborious, time-consuming process that results in a low yield of cells. In this study we identified parameters that can be helpful for a successful isolation of UCB-MSCs. According to our findings, chances for a well succeeded isolation of these cells are higher when MSCs were isolated from UCB collected from normal full-term pregnancies that did not last over 37 weeks. Besides the duration of pregnancy, blood volume and storage period of the UCB should also be considered for a successful

isolation of these cells. Here, we found that the ideal blood volume collected should be above 80 mL and the period of storage should not exceed 6 h. We characterized UCB-MSCs by morphologic, immunophenotypic, protein/gene expression and by adipogenic differentiation potential. Isolated UCB-MSCs showed fibroblast-like morphology and the capacity of differentiating into adipocyte-like cells. Looking for markers of the undifferentiated status of UCB-MSCs, we analyzed the UCB-MSCs' protein expression profile along different time periods of the differentiation process into adipocyte-like cells. Our results

T. T. Sibov (✉) · L. F. Pavon · E. Amaro Jr. ·
L. F. Gamarra
Instituto do Cérebro, Instituto Israelita de Ensino e
Pesquisa Albert Einstein-IEPAAE, Hospital Israelita
Albert Einstein, Avenida Albert Einstein, no 627/701,
Morumbi, São Paulo, SP CEP: 05651-901, Brazil
e-mail: tatianats@einstein.br

L. F. Pavon
e-mail: lorenep@einstein.br

E. Amaro Jr.
e-mail: amarojr@einstein.br

L. F. Gamarra
e-mail: lgamarra@einstein.br

P. Severino · L. C. Marti · P. R. Tobo ·
A. H. Campos · A. T. Paes
Centro de Pesquisa Experimental,
Instituto Israelita de Ensino e Pesquisa
Albert Einstein-IEPAAE, São Paulo, SP, Brazil
e-mail: psever@einstein.br

L. C. Marti
e-mail: lmarti@einstein.br

P. R. Tobo
e-mail: patriciatobo@natura.net

A. H. Campos
e-mail: holthausen@einstein.br

A. T. Paes
e-mail: angelatp@einstein.br

D. M. Oliveira
Universidade de Brasília, Brasília, DF, Brazil
e-mail: dmoliveira@unb.br

C. A. Moreira-Filho
Departamento de Pediatria, Faculdade de Medicina da
Universidade de São Paulo-USP, São Paulo, SP, Brazil
e-mail: carlos.moreira@icr.usp.br

showed that there is a decrease in the expression of the markers CD73, CD90, and CD105 that correlates to the degree of differentiation of UCB-MSCs. We suggest that CD90 can be used as a mark to follow the differentiation commitment degree of MSCs. Microarray results showed an up-regulation of genes related to the adipogenesis process and to redox metabolism in the adipocyte-like differentiated MSCs. Our study provides information on a group of parameters that may help with successful isolation and consequently with characterization of the differentiated/undifferentiated status of UCB-MSCs, which will be useful to monitor the differentiation commitment of UCB-MSC and further facilitate the application of those cells in stem-cell therapy.

Keywords Mesenchymal stem cells · Umbilical cord blood · Cell isolation · Cell characterization and adipogenesis

Introduction

Mesenchymal stem cells (MSCs) are multipotent cells capable of differentiating into adipocyte-, osteoblast-, and chondrocyte-like cells. These cells can be isolated from different tissues such as bone marrow (BM), umbilical cord (UC), adipose tissue, dental pulp, and umbilical cord blood (UCB). UCB is an interesting source of these cells because the collection process is painless and non-invasive, it causes no harm to the mother or infant, and it is a material usually discarded (Bieback et al. 2004; Erices et al. 2000; Gang et al. 2004; Lee et al. 2004).

Evidence has emerged that MSCs may be used for supporting new clinical concepts in cellular therapy. However, MSC isolation from UCB from full-term deliveries is laborious, time-consuming, and results in a low yield of cells (Bieback et al. 2004; Secco et al. 2008). Some crucial steps in the isolation process of MSCs from UCB are known to be the time between collection and isolation, the net volume of blood, and the mononuclear cell (MNC) count (Bieback et al. 2004). Other parameters that might relate to a successful isolation of these cells have not been described.

The establishment of a reproducible adult stem cell culture system, like MSCs, is important to elucidate the role of molecular markers associated with the undifferentiated state of these cells and with the

adipogenic differentiation process (Ng et al. 2008; Rim et al. 2005; Schilling et al. 2007).

Global gene expression analysis has been used as a valuable tool for a better understanding of adipocyte metabolism and for identification of MSCs' undifferentiated state. Using this approach, many markers of the undifferentiated state of MSCs and of the MSCs' differentiation process in adipocyte-like cells have been identified. Nevertheless, no specific markers for the undifferentiated state have been described (Gregoire 2001; Grimaldi 2001; Linhart et al. 2001; MacDougald and Mandrup 2002; Ng et al. 2008; Rosen and Spiegelman 2000; Urs et al. 2004).

In this study, we describe some important parameters to be considered for a successful isolation of MSCs from UCB. We characterize MSCs by their morphology, immunophenotypic characteristics, cytochemical and ultrastructural properties, and protein/gene expression profile during the adipocyte differentiation process. We have identified transcripts which showed to be selectively expressed in undifferentiated MSCs, as well transcripts expressed in a differentiated progeny, the adipocyte-like cell.

Materials and methods

Harvest of human umbilical cord blood samples

A total of 118 UCB units were collected after informed consent was obtained in accordance with the Ethics Committee of the Albert Einstein Research Institute (São Paulo, Brazil). Samples were collected by venous puncture of the umbilical vein at the time of the full-term delivery and conserved in 100 mM of EDTA anticoagulant at 22 °C and processed within 6 h after the collection. Pregnancy duration, volume collected, and period of storage (collection period up to processing) were recorded for each sample.

Isolation and culture of human UCB-derived MSCs

Mononuclear cells (MNCs) were separated by means of Ficoll-Hypaque gradient (density 1.077 g/mL; GE). MNCs were seeded into 25-cm² flasks at a density of 1×10^7 – 10^8 cells/cm² containing Dulbecco's modified Eagle's Medium–Low Glucose (DMEM-LG, GIBCO Invitrogen) supplemented with 1% L-Glutamine

200 mM, 1% Antibiotic—Antimycotic 10,000 U/mL sodium penicillin, 1% 10,000 µg/mL streptomycin sulfate, 25 µg/mL amphotericin B (GIBCO/Invitrogen Corporation), and 10% Fetal Bovine Serum (GIBCO/Invitrogen Corporation). UCB-MSC cultures were maintained at 37 °C in 5% CO₂ and non-adherent cells were removed after 48 h. Medium was changed every other day. The experiments were performed at the fourth passage of the cultures with approximately 80–90% confluence. UCB-MSC lineages were established in culture up to the fourth passage.

Immunophenotyping of UCB-MSCs by flow cytometry

We analyzed cell-surface expression using a pre-defined set of protein markers. We used monoclonal antibodies commercially available, following the manufacturer's instructions. Briefly, UCB-MSC samples at passage 4 were harvested by treatment with 0.25% Tryple Express (GIBCO-Invitrogen, Carlsbad, CA, USA), washed with PBS (pH = 7.4), stained with the selected monoclonal antibodies, and incubated in the dark for 30 min at 4 °C. Cells were then washed and fixed with 1% paraformaldehyde. The following human antibodies were used: CD14-FITC, CD29-PE, CD31-PE, CD34-PE, CD44-PE, CD73-PE, CD90-APC, CD106-FITC, CD166-PE (BD Pharmingen, San Diego, CA, USA), CD45-PerCP-Cy5, HLA-DR-PerCP-Cy5 (Biosciences, San Jose, CA, USA), and CD105-PE (clone: 8E11; Chemicon, Temecula, CA, USA).

The data acquisition was done using a FACSARIA flow cytometer (BD Biosciences, San Jose, CA, USA), and data were analyzed using FACSDIVA software (BD Biosciences, San Jose, CA, USA) or Flow Jo Software (TreeStar, Ashland, OR, USA).

In vitro differentiation assays

To evaluate UCB-MSCs' differentiation ability, cells were subjected to adipogenic differentiation in vitro, according to established protocols (Gang et al. 2004; Jiang et al. 2002). Briefly, cells at passage 4 were seeded in 12-well plates with 5×10^4 cells/well. When the 80% confluence was achieved; the regular medium was changed by the induction medium, which was refreshed every 72 h for 21 days. The adipogenic medium contained 5 mg/mL insulin (Sigma), 50 mmol/L indomethacin (Sigma), 1 mmol/L dexamethasone (Sigma),

and 0.5 mmol/L isobutyl-1-methyl xanthine (Sigma) in regular medium.

Ultrastructural characterization by transmission electron microscopy (TEM)

After 3 weeks, UCB-MSC samples differentiated into adipocyte-like cells and their respective controls at fourth passage were also ultrastructurally characterized. The pellets of these cells were fixed directly in 0.5% glutaraldehyde fixative solution, following standard procedures to TEM. The meshes were analyzed and photographed under a transmission electron microscope PHILIPS CM100.

Flow cytometry analysis of the MSC immunophenotypic marker set

The markers CD29, CD44, CD73, CD90, and CD105 were also evaluated by Flow Cytometry (FACS) in different phases of the cellular differentiation process (24 h and 7 days), in 4 UCB-MSC samples differentiated in adipocyte-like cells, regarding the respective control. These assays were performed to evaluate the protein expression of these cells during the adipogenic differentiation process. The data acquisition was also done using the FACSARIA flow cytometry (BD Biosciences, San Jose, CA, USA) and data analyses were performed using FACSDIVA software (BD Biosciences, San Jose, CA, USA) or Flow Jo Software (TreeStar, Ashland, OR, USA).

Experimental design of the microarray study

For DNA microarrays two biological replicates for each differentiated UCB-MSC samples (A7-1; A7-2) and three technical replicates for each sample control (M1; M2) were used, using the MSCs obtained from two UCB samples at passage 4 (Table 1). All these samples were hybridized to Human Gene 1.0 st chips (Affymetrix). Undifferentiated MSCs (M1; M2) kept in culture for 7 days were compared to their adipocyte-like differentiated progeny (A7-1; A7-2).

RNA extraction, DNA microarray assay, and analysis

For RNA extraction of 4 UCB-MSC samples and their progeny differentiated in adipocyte-like cells at fourth

Table 1 Samples used in this study

Sample ID	No of treatment days	Lineage
M1	–	Undifferentiated MSCs (sample control)
M2	–	Undifferentiated MSCs (sample control)
A7-1	7 days	Adipogenic
A7-2	7 days	Adipogenic

M undifferentiated MSCs, *A7* progeny differentiated in adipocyte-like cells in 7 treatment days, *1* and *2* different UCB samples

passage, we used RNeasy® Mini Kit (Uniscience, Qiagen, Alabama, USA), following the manufacturer's instructions. DNase treatment was also performed (Uniscience, Qiagen, Alabama, USA) and total RNA quantified by spectrophotometer and its integrity assessed by agarose gels.

Experimental procedures for microarray assays were performed according to the GeneChip Whole Transcript Sense Target Labeling Assay (Affymetrix), following the manufacturer's instructions.

Due to the small number of replicates, differentially expressed genes were identified according to the HTself method (Vencio and Koide 2005), which takes advantage of self-self experiments to define an intensity-dependent fold-change cut-off.

Differentially expressed genes were functionally clustered using DAVID (Database for Annotation, Visualization, and Integrated Discovery; <http://niaid.abcc.ncifcrf.gov/home.jsp>) using the parameter KEGG Pathways (Kyoto Encyclopedia of Genes and Genomes) for pathway mapping and functional annotation of gene sets. Since we used whole-genome KEGG terms as background for this analysis, as provided by DAVID, a cut-off at *p* value <0.1 was used for selecting significantly enriched pathways in a gene set.

Validation of microarray assays by quantitative RT-PCR

The cDNA was synthesized in a reaction containing 1 µg of total RNA of 4 UCB-MSC samples and their progeny differentiated in adipocyte-like cells at passage 4 using SuperScript™III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA), under conditions recommended by the manufacturer.

We worked with the key transcription factors Peroxisome Proliferator-Activated Receptor γ (PPAR

γ), LPL (Lipoprotein Lipase), and FABP4 (Fatty Acid Binding-Protein 4) for validation of microarrays results. PCR primers were designed using Primer Express® Software v2.0 (Applied Biosystems, USA). The Kit QuantiTect SYBR Green PCR (Uniscience, Qiagen, Alabama, USA) was used to quantify gene expression by qRT-PCR, under conditions recommended by the manufacturer. GAPDH mRNA was used for data normalization. The sequences of primers used were Peroxisome Proliferator-Activated Receptor gamma (NM_005037) Forward: CCCCTATTCCATGCTGTTATG; Reverse: CTTCCATTACGGAGAGATCCA, Lipoprotein Lipase (NM_000237) Forward: CCACCTCATTCCTCCGAGTAGCA; Reverse: CAGCCAGTCCACCACAATGACA, Fatty Acid Binding Protein 4 (NP_001433) Forward: CGTGGAAGTGACGCCTTTCATG; Reverse: ACTGGGCCAGGAATTTGACGAA, GAPDH (NM_002046.3) Forward: GGAGAAGGCTGGGGCTCAT; Reverse: GTCCTTCACGATACCAAAGTT.

Statistical analysis

Quantitative data were expressed as mean $\pm$ standard deviations (SD). Student's *t* test and Chi-square test were used to identify parameters related to successful isolation of MSCs from UCB. Gene expression results were analyzed by one-way repeated measures ANOVA. Results with *p* < 0.05 were considered statistically significant. The statistics software used was SAS, version 9.0.

Results

Isolation and expansion of UCB-MSCs

After the processing of UCB samples, the MNC fraction from these samples was seeded. After 20 days, we observed fibroblastic adherent cells in 11 UCB samples, representing an isolation success rate of approximately 10%.

Our results showed that pregnancy of up to 37 weeks (*p* < 0.001; Fig. 1a), collected blood volume above 80 mL (*p* = 0.039; Fig. 1b) were relevant parameters for the successful isolation of these cells. We only could isolate UCB-MSCs from samples that were processed less than 6 h after the collection.

Fig. 1 Distribution and variance curve graphic (*box-plot*). **a** Correlation between pregnancy and successful and unsuccessful isolation of UCB-MSCs; **b** Correlation between collected volume and unsuccessful isolation of UCB-MSCs. Student *t* test and Chi-square test were used to identify parameters related to successful isolation of MSCs from UCB

These isolated adherent cells presented fibroblast appearance (Fig. 2a). These isolated adherent cell samples were expanded in culture up to passage 13 (data not shown), because after this passage the cells reach senescence, and the characterization assays were performed in 11 samples of isolated cells.

Immunophenotypic profile of isolated adherent cells

Adherent cells isolated from 11 samples of UCB at passage four were analyzed by FACS and gated for granularity, size, and surface markers. These gated cells were analyzed for the expression of cell membrane protein markers and were negative for the expression of hematopoietic markers such as CD14, CD31, CD34, CD45, CD106, and HLA-DR and positive for CD29, CD44, CD73, CD90, CD105, and CD166.

In vitro differentiation assay, cytochemical, and transmission electron microscopy analysis of UCB-MSCs

Another important characteristic observed in the isolated adherent cells that helped us identify them as UCB-MSCs was the adipogenic differentiation potential. We assessed the differentiation capacity of 11 isolated adherent cell lines using medium containing adipogenic lineage-specific induction factors.

The process of differentiation of all established cellular lineages in the fourth passage into adipocyte-like cells was confirmed after 21 days and can be demonstrated by the Oil Red cytochemical test (Fig. 2d).

These cells showed marked morphological changes compared to their undifferentiated control (Fig. 2c), i.e., the adherent cells differentiated into adipocyte-like cells showing to be oval, with peripheral basophilic nuclei due to the presence of many lipid droplets, shown in red, as observed by the Oil Red test (Fig. 2d). The electron micrograph of these cells confirmed the presence of many lipid droplets (Figs. 2e, f). No lipid droplets were observed in the negative control (Fig. 2b), i.e. adherent cells presenting fibroblast appearance (Fig. 2a).

Immunophenotypic analysis during the UCB-MSC cellular differentiation process

During the UCB-MSC differentiation process in adipocyte-like cells we assessed MSC immunophenotypic marker set by FACS, such as CD29, CD44, CD73, CD90, and CD105 in two different phases of the cellular differentiation process (24 h and 7 days).

The results, obtained with 4 UCB-MSC samples and their respective controls, showed a significant decrease in the expression of this immunophenotypic marker set in the adipocyte-like cells in the seventh day of treatment, when compared with the 24-hour period. However, CD90 presented the best profile for a MSC undifferentiation marker, because it presented the greatest decrease in expression levels after the 7 days of treatment (Fig. 3).

Gene expression of UCB-MSCs

Using global gene expression analysis, we identified differentially expressed genes in undifferentiated

Fig. 2 **a** Morphology of adherent cells in primary culture of UCB-MSCs. **b** TEM of undifferentiated UCB-MSCs. **c** Cytochemical analysis; Control sample (undifferentiated MSCs), 400x. **d** Cytochemical analysis; Adipogenesis was detected by the formation of intracytoplasmic lipid droplets stained with oil

red; arrow = lipid droplets; 400x. **e, f** TEM of adipocytes-like differentiated UCB-MSCs. **b, e, f** Bar: 1 μ m. *n* nucleus; *c* cytoplasm; *nu* nucleolus; *li* lipid droplets; *mi* mitochondria; *rer* rough endoplasmic reticulum

UCB-MSCs, as compared to progeny differentiated in adipocyte-like cells. From this analysis, we identified a total of 1,628 genes differentially expressed between the two studied groups.

Functional classification of these genes highlighted, as expect, alterations in pathways related to fatty acid metabolism. For instance, we observed in the adipocyte-like cells the up-regulation of the well-known genes PPAR-Gama, LPL, and FABP4, involved in the PPAR-Gama signaling pathway, which is a pathway with an essential role in adipogenesis (Table 2).

Moreover, we identified that a series of genes related to redox metabolism were also up-regulated in the adipocyte-like cells (ACSL3, ACAT2, ALDH2, ACSL1, ADHFE1, CPT1A, ACADVL, ALDH3A2, ADH1A, ADH1B, and ADH1C). These genes are thought to have important functions in adipocyte metabolism, thus possibly representing good markers for the initial phase of adipogenesis (Table 2).

In the undifferentiated UCB-MSCs, we observed the up-regulation of 11 genes related to TGF- β signaling pathway, 19 genes to axonal guidance, and

Fig. 3 FACS analysis of the immunophenotypic markers set (CD29, CD44, CD73, CD90 and CD105) in adipocyte-like differentiated UCB-MSCs. ADIP 24H: treated UCB-MSCs in adipogenic medium during 24 h; ADIP 7D: treated UCB-MSCs in adipogenic medium during 7 days. Gene expression results were analyzed by one-way repeated measures ANOVA, $p < 0.05$ was considered statistically significant

Table 2 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways significantly changed between undifferentiated and adipocyte-like differentiated UCB-MSCs

KEGG pathways	<i>p</i> value	Differentially expressed genes
hsa01040:Polyunsaturated fatty acid biosynthesis	1.53E−06	FADS2, SCD, GPSN2, ELOVL5, FADS1, PECCR, FASN, PTPLB, HADHA
hsa00071:Fatty acid metabolism	3.03E−04	ACSL3, HADHB, ACAT2, ALDH2, ACSL1, ADHFE1, HADHA, CPT1A, ACADVL, ALDH3A2, ADH1A, ADH1B, ADH1C
hsa00100:Biosynthesis of steroids	4.28E−04	IDI1, DHCR24, NQO1, FDFT1, DHCR7, HMGCR, CYP51A1, SC5DL
hsa00061:Fatty acid biosynthesis	0.003995	ACACA, FASN, THEDC1, ACACB
hsa03320:PPAR signaling pathway	0.001255	ACSL3, ME1, UBA52, FADS2, SCD, AQP7, ACSL1, DBI, FABP4, PPARG, ANGPTL4, LPL, CPT1A
hsa00620: Pyruvate metabolism	0.003461	ACAT2, ALDH2, ACACA, ME1, ME2, ACACB, MDH2, LDHB, ALDH3A2
hsa00640:Propanoate metabolism	0.003915	ACAT2, ALDH2, ACACA, MLYCD, HADHA, ACACB, LDHB, ALDH3A2
hsa00480:Glutathione metabolism	0.008607	GCLM, GSR, GPX3, TXNDC12, G6PD, GPX4, IDH1, IDH2
hsa00020:Citrate cycle (TCA cycle)	0.008643	SDHA, IDH3A, AC01, MDH2, IDH1, ACQ2, IDH2

Genes described in the table are up-regulated in the adipocyte-like differentiated UCB-MSCs when compared with undifferentiated UCB-MSCs

18 to tight junction pathways. The genes involved in these pathways seem to be mostly related to cellular migration, proliferation, and differentiation, cellular cycle progression, and apoptosis control (Table 3).

Validation of microarray data using quantitative RT-PCR

Results of microarray assays were validated by qRT-PCR through the expression evaluation of some selected genes. Four UCB-MSC samples and their differentiated progeny in adipocyte-like cells were analyzed on the

basis of gene-expression differences. We compared expression levels at different time points of the differentiation process (1 h and 7 days of treatment) and compared differentiated versus control samples.

We validated the expression of three genes that were down-regulated in the differentiated UCB-MSCs. These genes were CD73, CD90, and CD105. High levels of expression were observed after 1 h of treatment. After 7 days of treatment, we observed a significant decrease of the relative expression of these genes, mainly of CD90, corroborating data of protein expression (Fig. 4).

Table 3 Kyoto Encyclopedia of Genes and Genomes (KEGG) significantly changed between undifferentiated and adipocyte-like differentiated UCB-MSCs

KEGG pathways	<i>p</i> value	Differentially expressed genes
hsa04510:Focal adhesion	1.31E−11	PDGFA, BIRC3, PRKCA, ITGA6, ITGA11, MYL9, TNC, PDGFC, COL1A2, COL2A1, LAMB2, MET, ITGA2, FLNB, VEGFC, COL3A1, VEGF, ITGA3, SHC3, CAV2, BIRC2, VTN, ITGB1, COL5A1, ITGB3, FN 1, THBS2, ITGA5, PXN, MRCL3, MRLC2, LOC442204, COL5A2, VASP, CDC42, COL1A1, ITGA7, CCND1, THBS3, MYLK
hsa04512:ECM-receptor interaction	1.98E−09	COL3A1, FNDC1, ITGA3, ITGA6, ITGA11, VTN, ITGB1, COL5A1, ITGB3, FN 1, CD47, TNC, THBS2, ITGA5, COL1A2, COL2A1, COL5A2, COL1A1, LAMB2, ITGA7, ITGA2, AGRN, THBS3
hsa04810:Regulation of actin cytoskeleton	9.38E−08	PDGFA, TMSL3, LIMK1, FGF5, ARPC2, ITGA6, ITGA11, ARPC1B, CFL2, MYL9, TMSB4X, FGF2, ITGA2, PFN2, BDKRB2, F2R, BDKRB1, ACTA1, ITGA3, ITGB1, ITGB3, FN1, IGGAP1, CFL1, ITGA5, ACTA2, ACTG2, PXN, MRCL3, MRLC2, LGC442204, CDC42, PFN1, ITGA7, CD14, MYLK
hsa04514:Cell adhesion molecules (CAMs)	0.006043	PTPRF, CDH2, CD276, JAM2, ALCAM, ITGA6, ITGB1, PVR, PVRL2, ICAM1, NEGRI, PDCD1LG2, CLDN1, CLDN11, NFASC, VCAM1
hsa04530:Tight junction	0.038232	AMOTL1, PRKCA, JAM2, RAB3B, THY-1, EPB41L2, MYL9, SPTAN1, MRCL3, MRLC2, LOC442204, CLDN1, CLDN11, CDC42, GNAI2, CTTN, NT5E
hsa04360:Axon guidance	4.57E−04	SEMA3C, LIMK1, SEMA5A, SEMA7A, NTN4, PPP3CC, ROB01, EPHA2, ITGB1, CFL2, SEMA3A, CFL1, NRP1, EFNA2, CDC42, GNAI2, RASA1, MET, EPHB2
hsa04350:TGF-beta signaling pathway	0.03219	THBS2, RPS6KB1, TGFB1, TFDP1, INHBA, SMAD3, DCN, LTBP1, TGFB2, THBS3, SMURF2, ENG

Genes described in the table are up-regulated in the undifferentiated UCB-MSCs when compared with adipocyte-like differentiated UCB-MSCs

Fig. 4 Gene validation of CD73, CD90 and CD105 in adipocytes-like differentiated UCB-MSCs by qRT-PCR. ADIP-1H: treated UCB-MSCs in adipogenic medium during 1 h; ADIP-7D: treated UCB-MSCs in adipogenic medium during 7 days. Gene expression results were analyzed by one-way repeated measures ANOVA, $p < 0.05$ was considered statistically significant

The opposite situation was found for *Peroxisome Proliferator-Activated Receptor γ* (*PPAR γ*), *LPL* (*Lipoprotein Lipase*), and *FABP4* (*Fatty Acid Binding-Protein 4*), which were up-regulated in differentiated UCB-MSCs after 7 days of treatment (Fig. 5).

Discussion

The successful isolation of MSCs from UCB is a matter of great interest due to the non-invasive nature of collection and its broad availability. However,

Fig. 5 Gene validation of PPAR-gama, LPL and FABP4 in adipocytes-like differentiated UCB-MSCs by qRT-PCR. ADIP-1H: treated UCB-MSCs in adipogenic medium during 1 h; ADIP-7D: treated UCB-MSCs in adipogenic medium during 7 days. Gene expression results were analyzed by one-way repeated measures ANOVA, $p < 0.05$ was considered statistically significant

difficulties in the isolation and differentiation of these cells have been reported (Bieback et al. 2004; Gang et al. 2004; Romanov et al. 2003; Wexler et al. 2003).

In this study, the success rate of the isolation of UCB-MSCs was approximately 10% ($n = 11/118$), a rate that is the same described by Secco and coworkers (Secco et al. 2008) and it is slighter lower than the rate (20–35%) described by other authors (Bieback et al. 2004; Chang et al. 2006; Kang et al. 2006; Park et al. 2006). The difficulty in isolating UCB-MSCs has been explained by two main factors: low amount of MSCs in UCB and the presence of clot and hemolysis. In order to overcome these difficulties, we studied some potentially critical parameters. The samples of UCB_MSCs processed in this work were initially collected for a public UCB Banks and further rejected. The more frequent reason for the rejection of the samples by the UCB cord Bank was low volume of the samples (a volume lower than 50 mL). The samples also were collected from pregnancies that have duration of at least 35 weeks to 40 weeks of gestation. According to our findings, chances for an optimal isolation are higher when UCB from normal full-term pregnancies that did not last over 37 weeks was used as the source for MSCs. MSCs are present in the human fetus blood circulation during the fist trimester of gestation (Campagnoli et al. 2001). High number of MSCs can be observed in the fetus blood until 12 weeks of gestation when the number of MSCs starts to decline and almost disappear from fetal blood (Campagnoli et al. 2001; Gotherstrom et al. 2004).

Besides the duration of pregnancy, blood volume and storage period should also be considered for the successful isolation of these cells. In our study we found that the ideal blood volume collected should be above 80 mL and the period of storage should not exceed 6 h. Kang and coworkers (2006) also reported that they could not isolate UCB-MSCs from samples that were collected and stored for more than 6 h. We found that when blood samples were collected in volumes lower than 80 mL no cells could be isolated. This finding led us to evaluate some parameters important for the successful isolation of MSCs from UCB. However, here we have not tested the effects of FBS concentration and media supplements on isolation success rate, culture and maintenance of UCB-MSCs.

Successfully isolated UCB-MSCs were characterized by morphologic, immunophenotypic, and protein/gene expression analyses, and by multi-lineage differentiation potential. Isolated UCB-MSCs showed fibroblast-like morphology and showed the capacity of differentiating into adipocyte-like cells when cultured under defined conditions, as previously reported (Gang et al. 2004).

Despite the therapeutic potential of UCB-MSCs, markers for the unmistak characterization of MSCs are still needed. Cell surface antigens such as CD29, CD44, CD73, CD90, and CD105 may assist with the recognition of undifferentiated MSCs. However they are not specific for undifferentiated cells, other types

of cells also express these markers and differentiated MSCs may express low levels of them (Gang et al. 2004). Clinical application of UCB-MSCs requires the identification of suitable markers that would indicate the differentiation status of these cells under specific conditions. Thus, in this study we also characterized the UCB-MSC protein expression profile during the differentiation process into adipocyte-like cells. Using FACS we observed a decrease in the expression of markers CD73, CD90, and CD105 during the UCB-MSC differentiation process. Among these markers we observed that the UCB-MSC undifferentiated state was better characterized by the CD90 expression profile, because the decrease in its expression after 7 days of treatment was more noteworthy than the decrease observed for CD73 and CD105. These results were in agreement with the qRT-PCR assays.

Our data corroborate some recent reports that show that the expression of CD73, CD90, and CD105 on the cell surface of bone marrow mesenchymal stem cells (BM-MSCs) was down-regulated during the differentiation process into mesodermic lineages (Delorme et al. 2008; Jin et al. 2009; Yang et al. 2006). Therefore, CD73, CD90, and CD105 might be useful cell surface markers to determine the undifferentiation status of MSCs.

CD105, which is an integral membrane glycoprotein known as endoglin, is a receptor for TGF- β 1 and - β 3 (Duff et al. 2003; Jin et al. 2009) which modulates TGF- β signaling by interacting with related molecules, such as TGF- β 1, - β 3, BMP-2, -7, and activin A. It is speculated that these members of the TGF- β superfamily are mediators of cell proliferation and differentiation (Jin et al. 2009). Previous studies reported that members of the TGF- β family control the differentiation of MSCs (Roelen and Dijke 2003). However, whether CD105 plays a functional role during the process of stem-cell differentiation has not yet been clarified. In our microarray results we identified up-regulation of some genes related to TGF- β signaling pathway. A recent report showed that TGF- β signaling is an important event in MSC proliferation and also an inhibitor of adipogenesis via Smad-3 signaling. These findings were supported by the observation that blocking of Smad-3-mediated TGF- β signaling resulted in enhanced adipogenic differentiation (Ng et al. 2008).

Microarray results showed the up-regulation of some known genes related to the adipogenesis process

in the MSCs differentiated into adipocyte-like cells, in agreement with the expected results for the differentiation process. These were *LPL*, *PPAR gamma*, *FABP4*, *RASD1*, *RAP2A*, *AKR1C*, *ADFP*, *APO*, *GPX3*, *GLUL*, *CRYAB*, *KNC*, *IGF2*, *PTPN*, and *INSR*. In our study, we also identified up-regulation of genes such as *ACSL3*, *ACAT2*, *ACACA*, *FASN*, and *ACADVL* that may be directly related to redox metabolism—and consequently to metabolic diseases like obesity in differentiated cells.

Some studies have shown the correlation between plasmatic quantities of antioxidants and the increase of systemic markers of oxidative stress associated with obesity and metabolic syndromes (Furukawa et al. 2004; Reitman et al. 2002). This finding should be investigated further as it may open new avenues into the search for molecular therapeutic targets for obesity treatment, as well as other metabolic syndromes.

Our study provides information on some critical parameters that may help with the successful isolation and characterization of the UCB-MSC, which might be used for supporting clinical approaches in cellular therapy.

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Conflict of interest The authors declare no conflict of interest.

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