Study of telomerase activity, proliferation and differentiation characteristics in umbilical cord blood mesenchymal stem cells

Karimi, T.[1]; Eslaminejad, M. B.[2*]; Aminlari, M.[1]; Shahverdi, A.[3] and Bahmanpour, S.[4]

[1]Department of Biochemistry, School of Veterinary Medicine, Shiraz University, Shiraz, Iran; [2]Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran; [3]Department of Embryology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran; [4]Department of Anatomical Sciences, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran

[*]**Correspondence:** M. B. Eslaminejad, Department of Stem Cells and Developmental Biology, Cell Science Research Center, Royan Institute for Stem Cell Biology and Technology, Academic Center for Education, Culture and Research (ACECR), Tehran, Iran. E-mail: eslami@royaninstitute.org

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Summary

In recent years, considerable advances have been made in the field of regenerative medicine. Unlike embryonic stem cells, which pose the problems of ethical concerns and cause severe immunological reactions as well as neoplasma formation after transplantation, umbilical cord blood is a primitive source of mesenchymal stem cells that covers the benefits of both embryonic and adult stem cells. It has been determined that the proliferation capacity of cells is critically linked to the maintenance of the length of telomeres by telomerase activity. Since there is no information accessible regarding the pattern of telomerase activity in UCB-MSCs through several passages, the aim of this study was the evaluation of telomerase activity in UCB-MSCs, as a predisposing factor for cell immortalization. No telomerase activity was detected in UCB-MSCs from several passages applying telomerase rapid amplification protocol (TRAP). Since there is a direct correlation between the activation of telomerase expression and neoplasma formation in adult somatic cells, UCB can be assumed as an excellent source of MSCs for therapeutic application with a high level of safety. According to the histological results, RT-PCR and biochemical assays, MSCs derived from UCB showed high differentiation capacity to bone and cartilage. UCB-MSCs showed very low level of differentiation potential to adipocytes. Our results showed that UCB-MSCs maintain their self-renewal and differentiation potential through several passages. Since a large number of metabolically active cells must be available in cell therapy, high proliferation capacity through several passages is a great advantage for large scale expansion of UCB-MSCs.

Key words: Umbilical cord blood mesenchymal stem cells, Isolation, Telomerase activity, Senescence, Proliferation potential

Introduction

Combination of two unique properties, self-renewal and differentiation potential into multiple cell lineages in stem cells made them the major focus of clinicians and basic scientists. As a source of mesenchymal stem cells (MSCs), umbilical cord blood (UCB) provides significant clinical benefits over the other adult stem cell resources. First, umbilical cord blood is regarded as clinical waste in the delivery room; the collection of cord blood units is an easy and non-invasive method for mothers and infants. Moreover, UCB is a very young source of MSCs and the immaturity of UCB-MSCs is associated with their lower immunogenicity in comparison with the other sources of MSCs. Even in allogenic transplantation UCB-MSCs have the ability to modulate immune

responses, without any subsequent risk of immunorejection (Malgieri *et al*., 2010). Adult stem cells including MSCs cease their proliferation after long-term culture or through several passages which is determined as senescence (Terai *et al*., 2005). Since a large number of metabolically active cells must be accessible for cell therapy, senescence is a major problem that must be solved. It has been determined that the proliferation capacity of human cells is critically linked to the maintenance of the length of telomeres which are repetitive DNA sequences (thousands of TTAGGG repeats) located at the end of chromosomes (Counter *et al*., 1992). Telomeres protect the end of chromosomes from the exonuclease activity, end-to-end fusion and from the loss of coding sequences due to the end replication problem. Maintaining stable telomere length is associated with the activation of telomerase, which is a ribonucleoprotein. Immortal cells such as tumor cells and embryonic stem cells are able to maintain telomere function by activation of telomerase (Chan and Blackburn, 2004). In general, normal somatic cells exhibit no telomerase activity, but low to moderate level of telomerase activity has been detected in some adult stem cells from skin (Harle-Bachor and Boukamp, 1996), gut (Kolquist *et al*., 1998), and from the hematopoietic system (Chiu *et al*., 1996). However, no information is available regarding the pattern of telomerase activity in UCB-MSCs through several passages. According to the results of previous studies, UCB-MSCs can be a potentially practical source of cells for cell therapy and regenerative medicine, but biological properties of UCB-MSCs including their proliferation, differentiation capacity and factors related to their safety must be studied prior to clinical trials. Here, we tried to isolate MSCs from UCB by a simple and efficient method and determine their characterization including proliferation and differentiation capabilities. Since there is a close correlation between high level of proliferation capacity, telomerase expression and the risk of neoplasma formation after transplantation, an attempt was made to evaluate the amount of telomerase activity through several passages (P3-P9) by telomerase repeat amplification protocol (TRAP) to determine the safety of UCB-MSCs for therapeutic applications.

Materials and Methods

Isolation of MSCs from UCB

Human UCB samples (n=10) were harvested from full-term C-section deliveries with the informed consent of mothers. Anti-coagulated cord blood samples were diluted 1:1 with 2mM ethylenediaminetetraacetic acid (EDTA)-phosphate-buffer saline (PBS) (Merck, Germany). Diluted samples were loaded on Lymphodex solution (Inno-Train, Diagnostik, Germany). The mononuclear cell (MNC) fraction was separated by density gradient centrifugation at 430 xg for 30 min at room temperature. MNC fraction was collected and washed twice with PBS and seeded at a density of 1×10^8 cells per cm^2 into T75 cell culture flasks. Isolated cells were cultured in complete alpha-modified Eagle's minimal essential medium (α-MEM, Sigma, Germany) supplemented with 10% fetal bovine serum (FBS, Gibco, Germany), 4 mM L-glutamine (Gibco, UK), 100 IU/ml penicillin (Gibco, Germany) and 100 mg/ml streptomycin (Gibco, Germany). Cultures were maintained at 37°C in a humidified atmosphere containing 5% carbon dioxide. Media were exchanged twice weekly. When MSC colonies formed and approximately 70-80% confluence was achieved, the cells were detached with 0.05% trypsin-0.03% EDTA (Sigma, USA) and sub-cultivated at a density of $1 \times 10^6/cm^2$ (Terai *et al*., 2005; Kern *et al*., 2006; Wan *et al*., 2006).

Immunophenotyping of UCB-MSCs

For further characterization of isolated cells, surface antigen phenotyping was performed on UCB derived fibroblastoid-like adherent cells at passages 4. Mononuclear UCB cells were analysed for expression of hematopoietic and mesenchymal markers by flow cytometry (Kern *et al*., 2006; Wan *et al*., 2006). The following cell-surface epitopes were marked with anti-human antibodies:

CD90-Fluorescein isothiocyanate (FITC), CD105 Phycoerythrin (PE), CD73-PE, CD44-PE, CD146-PE, CD 34-PE, CD45-FITC, CD-11b-PE, CD31-FITC, CD33-FITC. Monoclonal antibodies were purchased from BD Pharmingen (Becton Dickinson, San Diego, USA).

Mouse isotype antibodies served as control, 1×10^4 labeled cells were acquired for each antibody labeling. After washing of cells by cold PBS, they were incubated with 1 µg/ml of each antibody for 30 min at room temperature (Terai *et al*., 2005; Kern *et al*., 2006). Cells were fixed with 4% paraformaldehyde and analysed using BD FACS Calibur (USA) flow cytometer.

Differentiation capacity of UCB-MSCs

To promote bone differentiation, UCB-MSCs at passage 4 were seeded at a density of $10^3/cm^2$, in 6 well plates and cultured in α-MEM basal medium containing 10% FBS. As soon as 80% confluence was reached, bone differentiation of cells was induced by feeding them for 2-3 weeks with osteo-inductive medium consisting of DMEM as a basal medium and supplemented with 10 nM dexamethasone (Sigma, USA), 10 mM B-glycerol phosphate (Sigma, USA), 50 µg/ml ascorbate 2-phosphate (Sigma, USA) and 10% FBS (Eslaminejad and Eftekhari, 2007). After 15 days of cell culture under osteogenic condition, Alkaline phosphatase (ALP) activity was assayed applying ALP assay kit (Bio Vision, USA). Enzyme activity was determined colorimetrically using p-nitrophenyl phosphate (PNPP) as substrate which turns yellow (λmax = 405 nm) when dephosphorylated by ALP (Heino *et al*., 2004). At day 21, mineralized nodules in osteogenic cultures were fixed with 95% ethanol for 10 min, then treated for 2 min with 0.1% alizarian red stain (ARS) (Sigma, USA) dissolved in Tris-HCl buffer at pH = 8.3 to stain the calcium salts and observed under light microscope (Wan *et al*., 2006).

To induce adipogenic differentiation, confluent cells at passage 4 were cultured in adipogenic medium composed of DMEM supplemented with 100 nM dexamethasone and 50 mg/ml indomethacin (Sigma, USA) (Eslaminejad *et al*., 2009). Then the cultures were incubated for 21 days. To confirm adipogenic differentiation oil red staining was done; cells were fixed with 4% formalin at room temperature, washed by 70% ethanol and stained, using oil red solution (Sigma, USA) in 99% isopropanol for 15 min.

To investigate the chondrogenic differentiation potential of isolated cells, 2×10^5 cells in passage 4 were cultured in the tip of a 15-ml canonical tube and allowed to form micromasses applying chondrogenic medium. Chondrogenic medium involves DMEM basal medium supplemented with 10 ng/ml transforming growth factor-β3 (TGF-β3, Sigma, Germany), 10 ng/ml bone morphogenic protein-6 (BMP-6, Sigma, Germany), 50 mg/ml insulin transferrin selenium + premix (Sigma, Germany), 1.25 mg/ml bovine serum albumin (Sigma, Germany) and 1% fetal bovine serum (Kern *et al*., 2006; Eslaminejad *et al*., 2009). After 21 days cartilage micro masses were fixed and embedded in paraffin wax, and serial 5 µm sections were achieved by microtome. Histological sections were fixed and then stained using toluidine blue (Sigma, Germany) for 30 sec at room temperature.

Population doubling time

To determine population-doubling time (PDT), cells from passages 3-9 were plated at 10^3 cells/cm^2 in basal α-MEM, supplemented with 10% FBS and incubated at humidified atmosphere and 5% CO_2. Cells were detached and counted with hemocytometer on day 5. The population doubling time was calculated using the following equation:

$$PDT = \frac{CT}{\log \frac{N}{N0} \times 3.31}$$

Where,
N: The harvesting cell number
N0: The initial cell number
CT: The culture time (Eslaminejad *et al*., 2009)

RT-PCR and primer sequences

Differentiated cells were washed with PBS twice and collected with TRIZOL (Sigma, USA). Total cellular RNA was extracted using Qiagen kit (USA). Any DNA contamination was removed by DNase

Table 1: Nucleotide sequences of primers used for RT-PCR detection of specific genes of bone and cartilage

Gene name	Primer sequences	Product size (bp)
GAPDH (house keeping)	F: 5′ CTG AGC AGA CCG GTG TCA CAT C3′ R: 5′ GAG GAC TTT GGG AAC GAC TGA G3′	166
Collagen type II (Col II)	F: 5′ TCT ACC CCA ATC CAG CAA AC 3′ R: 5′ GCG TAG GAA GGT CAT CTG GA 3′	170
Aggrecan (AGC)	F: 5′ CTG GAC AAG TGC TAT GCC G3′ R: 5′ GAA GGA ACC GCT GAA ATG C3′	191
Sox9	F: 5′ CCC TTC AAC CTC CCA CAC TAC 3′ R: 5′ GCT GTG TGT AGA 3′	232
Osteonectin (ON)	F: 5′CGA GCT GGA TGA GAA CAA CA 3′ R: 5′AAG TGG CAG GAA GAG TCG AA 3′	126
Osteocalcin (OC)	F: 5′ GGC AGC GAG GTA GTG AAG AG 3′ R: 5′ CAG CAG AGC GAC ACC CTA GAC3′	196
Osteopontin (OPN)	F: 5′ TGA GGT GAT GTC CTC GTC TG 3′ R: 5′ GCC GAG GTG ATA GTG TGG TT 3′	100
Runx-2	F: 5′ ATG ACA CTG CCA CCT CTG A 3′ R: 5′ ATG AAA TGC TTG GGA ACT GC3′	118

treatment. Reverse transcriptase polymerase chain reaction was performed and first strand cDNA was synthesized using Revert aid minus first strand cDNA synthesis kit, (Fermentase, USA), according to the manufacturer's protocol. Primer sequences used for RT-PCR to identify the expression of specific genes of bone and cartilage are listed in Table 1. RT-PCR was performed for 35 cycles at 95°C for 10 sec, 60°C for 20 sec, and 72°C for 1 min. The RT-PCR products were analysed on 2.5% agarose gel and stained with ethidiumbromide. Expression of GAPDH gene as a house keeping gene was evaluated (Gaur *et al*., 2005; Titorencu *et al*., 2007).

Telomerase activity

Telomerase activity in each sample was detected through different passages (3-9) by telomeric repeat amplification protocol, using TRAPeze kit (Telo TAGGG Telomeras PCR ELISA, Roche, Germany) according to the manufacturer's protocol. 2 × 10^5 cells were applied per single reaction. Telomerase activity was expressed as a percentage of relative telomerase activity (RTA) when compared to the positive control (Zimmermann *et al*., 2003). P19 immortal cell line was applied as the positive control.

Statistical analysis

Data of PDT are presented as mean ± standard deviation. One-way ANOVA was used to analyse the PDT data. Differences were considered significant at $P<0.01$. Minitab 16 software was used for the statistical tests.

Results

According to the culturing condition applied in this research, gradually over a 2-week period, two different kinds of colonies appeared; 1) typical MSC colonies containing spindle-shaped cells (Fig. 1A) 2) large number of round colonies consisting of a highly heterogeneous population of round cells, very large multinucleated cells and triangular cells (Fig. 1B). Over 3-4 weeks colonies of fibroblastoid like cells expanded and their number increased while other heterogeneous cell populations gradually detached. UCB-MSCs achieved 80-90% confluence after approximately 30-35 days culturing under normal condition in P0, but they achieved 80%-90% confluence after 14 days in passage 1 and after 5-7 days in passage 2 or more (Figs. 2A-H).

Immunophenotypic characteristics of fibroblastoid like cells derived from UCB

Results of flow cytometry indicated that adherent cells derived from UCB were strongly positive for MSC markers including CD105, CD73, CD44, CD90 as well as

CD146. Very low level of expression was detected for hematopoietic markers (including CD34, CD45, CD33 and CD11b) and CD31which is an endothelial marker (Fig. 3).

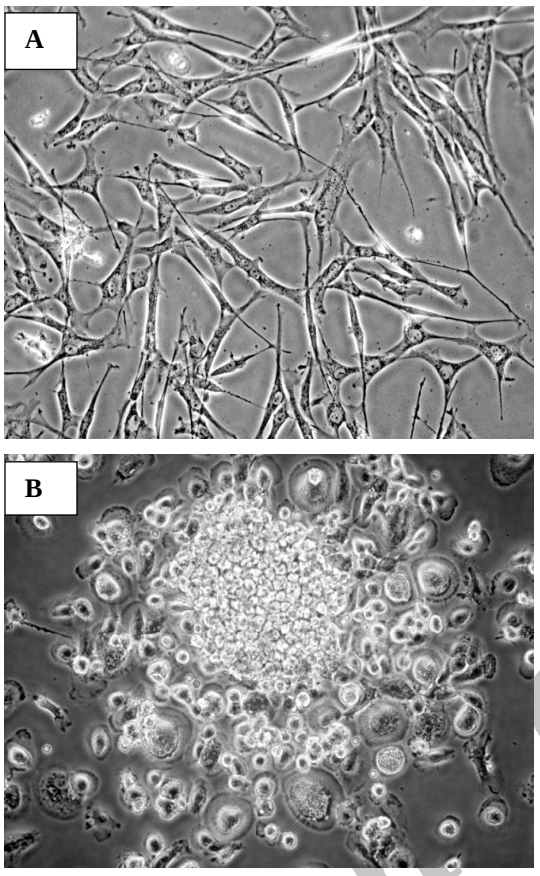

Fig. 1: Primary culture of MSC. A) MSC colonies containing fibroblastoid like cells, P0/Day 14. B) Round colonies containing a heterogeneous population of cells P0/Day 7

Differentiation capacity of UCB-MSCs

Active bone differentiation of UCB-MSC was detected after 2-3 weeks of culturing in osteo-inductive medium by evaluation of ALP activity and alizarian red staining (Fig. 4A). Nodule-like structures were observed in some areas. In most cell culture areas nodule like structures were not detected but instead mineral deposition had an even distribution in all of the UCB-MSCs, when they remained in osteo-inductive condition for 3 weeks (Fig. 4B). Bone differentiation potential of UCB-MSCs was further confirmed by evaluation of ALP activity; according to our results, high level of ALP activity was detected after 2 weeks culturing in osteo-inductive condition. Results of RT-PCR indicated high expression levels of osteocalcin, osteonectin, osteopontin and Runx-2 (Fig. 5).

Toluidine blue staining of cartilage micromasses demonstrated complete differentiation of UCB-MSCs to cartilage (Fig. 4). Results of RT-PCR indicated high expression levels of collagen typeII, aggrecan and Sox-9 (Fig. 5).

Oil red staining was done to demonstrate the accumulation of neutral lipid vacuoles. According to our results, UCB-MSCs showed a very low level of differentiation potential to adipocytes after 21 days adipogenic-induction condition.

Growth characteristics of UCB-MSCs

According to our data mean population, doubling time of isolated fibroblastoid like cells in P3 was 60.595 ± 0.516 h and remained approximately constant over the following passages until P9. There were no significant differences between PDT of isolated MSCs from UCB through passages 3-9 (Table 2). According to our cultivation condition, UCB-MSCs have the potential of tolerating several passages without any changes in their morphological characteristics until P15 (Figs. 2A-H).

Table 2: Population doubling time of UCB-MSCs after several passages (P3-P9)

Passages	PDT ± SD
P3	60. 595 ± 0.516
P5	60. 435 ± 0.260
P7	60. 985 ± 0.978
P9	60. 665 ± 0.646

Telomerase activity

Telomerase activity was measured through passages 3, 5, 7 and 9 by telomerase repeat amplification protocol (TRAP). Results of the present study indicated that UCB-MSCs were negative for telomerase activity after several subcultivations. Telomerase activity was expressed as the percentage of relative telomerase activity (RTA) of MSCs when compared to the positive control. P19 immortal cell line was used as the positive control and illustrated a high level of telomerase activity.

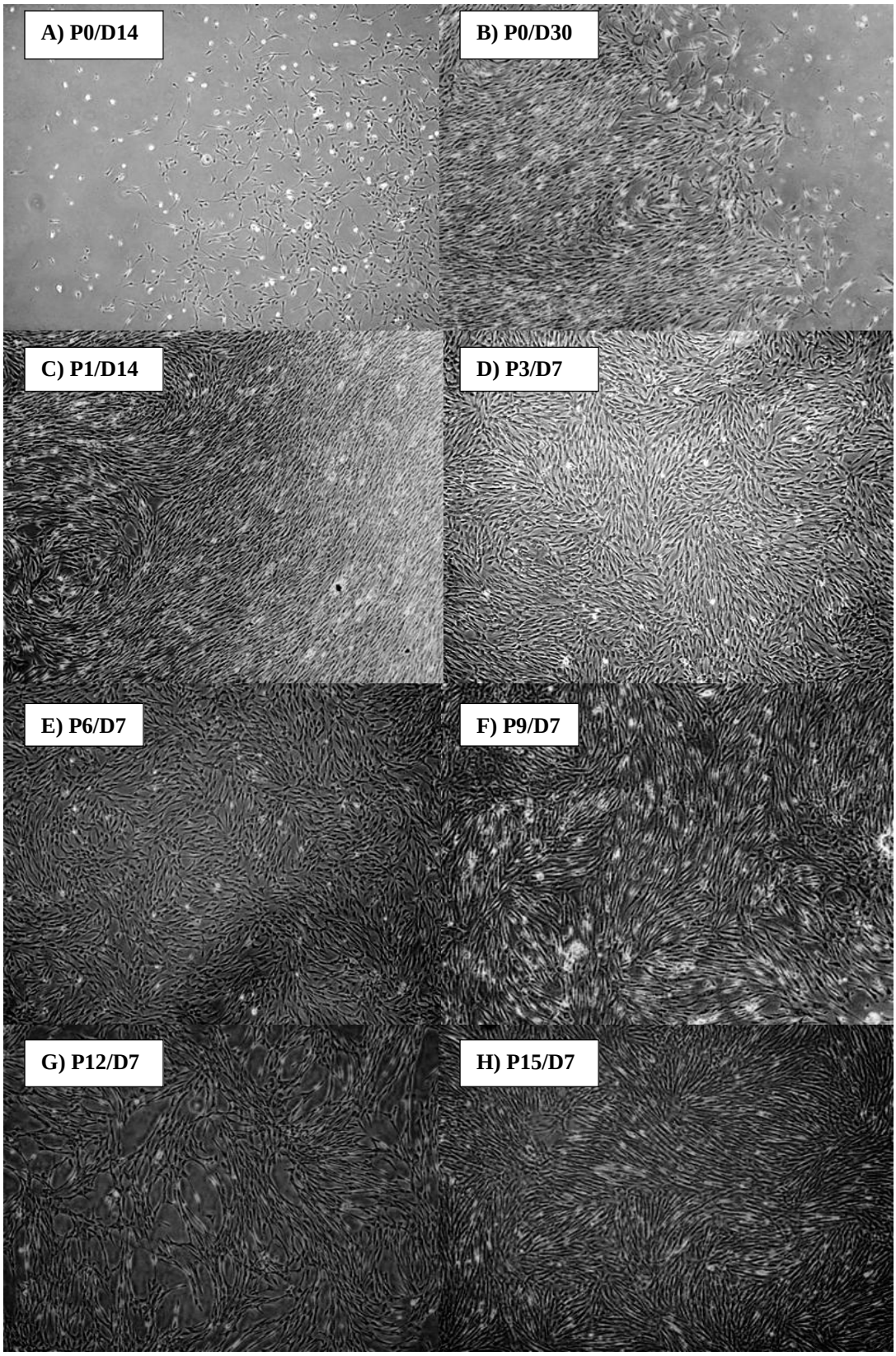

Fig. 2: Morphology of umbilical cord blood mesenchymal stem cells (UCB-MSCs) from different passages. A) The culture of UCB-MSCs appeared at day 14, B) 30 after initial plating, C) The expanded UCB-MSC culture at passage 1/day 14, D) passage 3/day 7, E) passage 6/day 7, F) passage 9/day 7, G) passage 12/day 7, and H) passage 15 /day 7

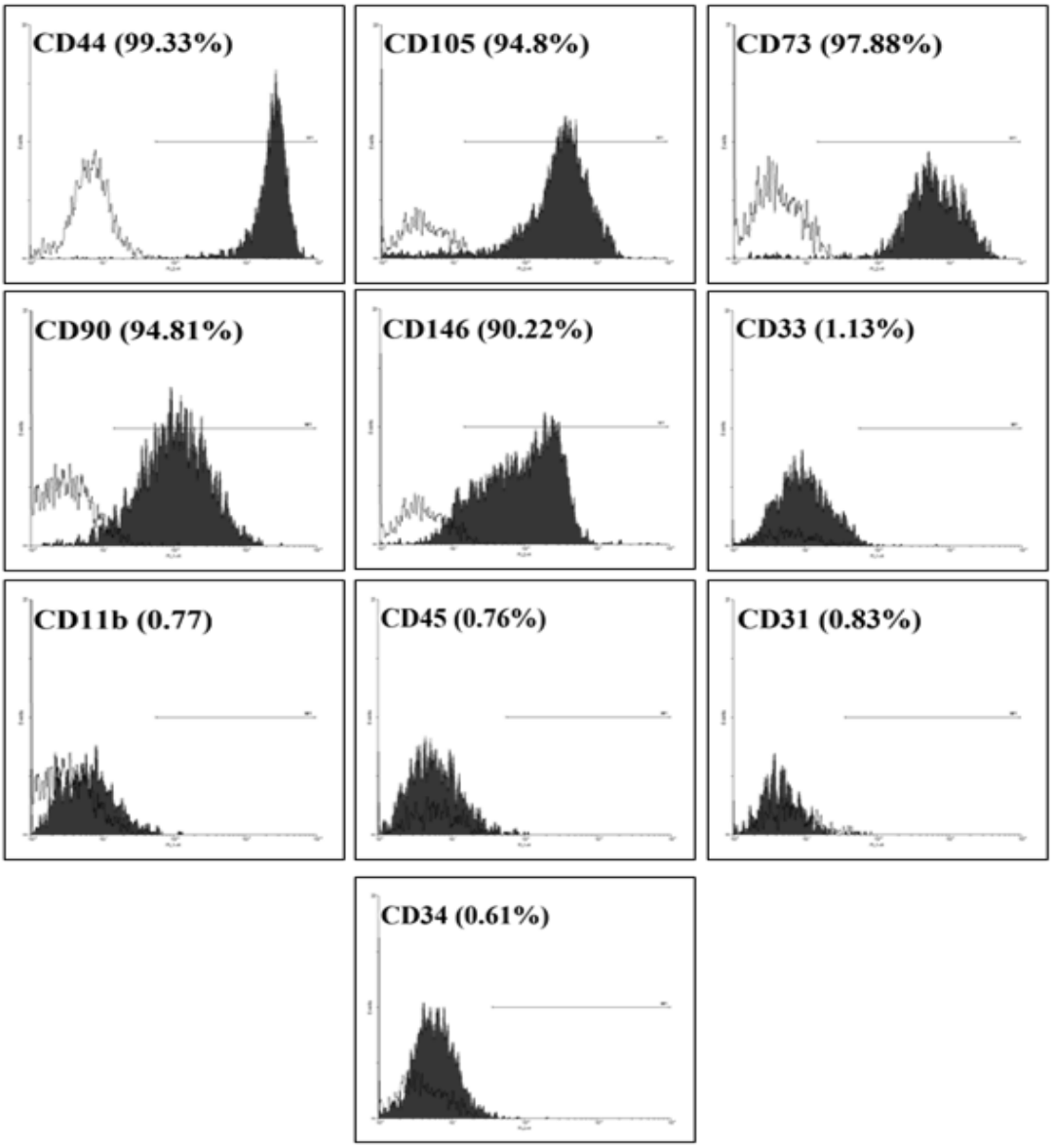

Fig. 3: Immunophentypical characteristics of spindle shaped cells derived from UCB

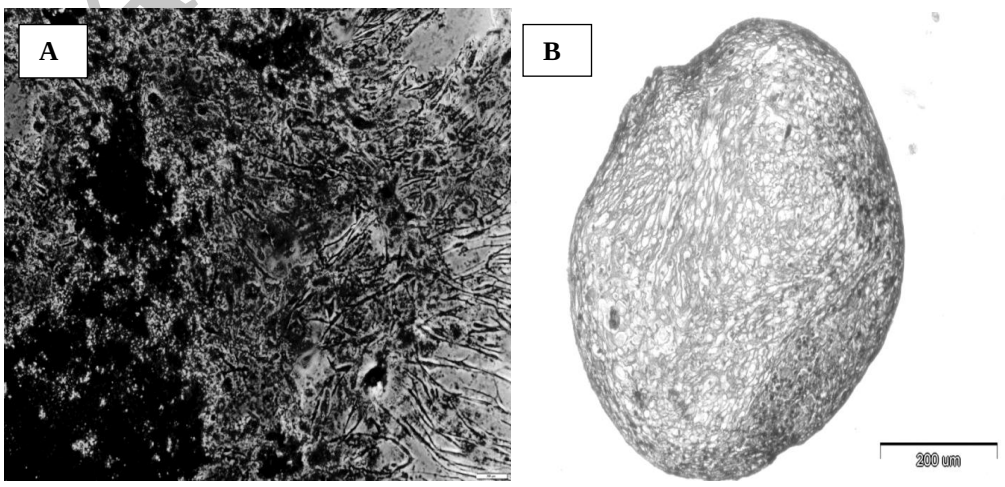

Fig. 4: A) Alizarian red staining for determination of osteogenesis. B) Toluiden blue staining for determination of chondrogenic differentiation

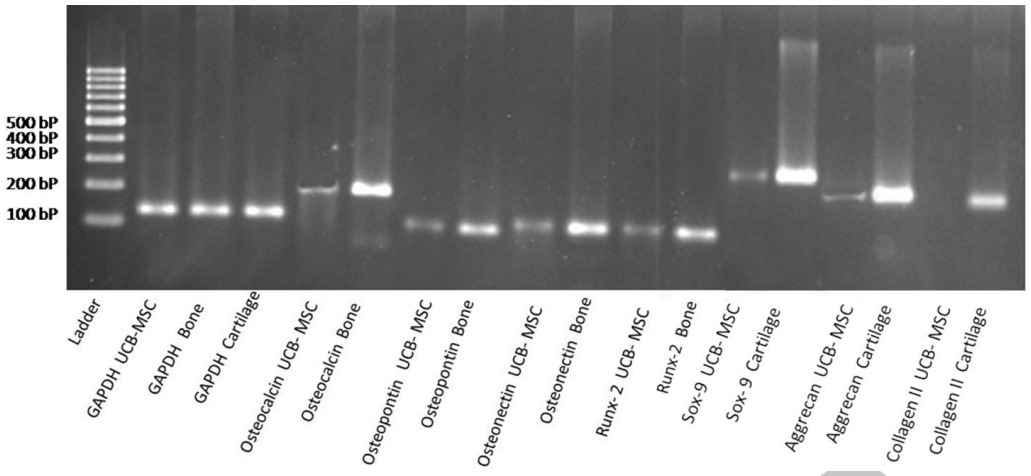

Fig. 5: Results of RT-PCR to determine the expression of GAPDH (house-keeping gene), specific genes of bone and cartilage in UCB-MSCs before and after 21 days cultivation in osteo- and chondro-inductive conditions

Discussion

In this study an attempt was made to determine UCB-MSCs characteristics from different aspects including, telomerase activity, proliferation and differentiation potential. According to our data no telomerase activity was detected at different passages (P3, P5, P7 and P9) of UCB-MSCs. Since there is a close correlation between telomerase activity and tumorogenesis, no telomerase activity in UCB-MSCs can be assumed as a benefit for their therapeutic application. Regarding telomerase activity, our data are in agreement with most of the previous investigations in MSCs (Terai *et al*., 2005; Zimmermann *et al*., 2003; Machado *et al*., 2009; Zhang *et al*., 2011). On the other hand, our results are in contrast to the findings of Pittenger *et al*. (1999) who detected high telomerase activity in BM-MSCs. However in this study fibroblasts as a typical negative control illustrated a high level of telomerase activity, too (Pittenger *et al*., 1999).

According to our data, UCB-MSCs underwent 24 population doublings during 60 days and population doubling time remained constant through several passages (P3-P9). Since for cell therapy a large number of cells must be available, there exists a need for MSCs to be expanded prior to each transplantation, highly ex-*vivo* expansion potential is a great advantage for UCB-MSCs. Results of Machado *et al*. (2009) indicated that telomerase or telomere state is a critical factor for proliferation of MSCs. Although UCB-MSCs did not express telomerase, UCB cells as the most primitive source of adult stem cells, have very long telomeres, which cause a delay in their senescence, providing a high proliferation capacity above the other adult MSC sources (Tondreau *et al*., 2005). Indeed it has been determined that stem cells from diverse tissues exhibit changes in their self-renewal capacity during aging (He *et al*., 2009). However, as a part of neonatal bodies, UCB cell tolerated the lowest level of changes in their stemness and self-renewal characteristics (Can and Karahuseyinoglu, 2007; He *et al*., 2009).

Here we isolated a phenotypically homogenous population of MSCs from UCB applying their adherence characteristics. Fibroblast like cells isolated from UCB expressed a set of surface markers including CD105 (SH2/endoglin), CD 73 (SH3), matrix receptor CD44 (hyaluronate receptor), CD90 (Thy-1) and CD 146. All of which were reported as MSC-specific surface antigens by previous studies (Lee *et al*., 2004; Tondreau *et al*., 2005; Kern *et al*., 2006; Wan *et al*., 2006, Zhang *et al*., 2011).

Very low expression levels were detected for typical hematopoietic markers including CD34, CD45 and for CD33 which is expressed by myeloid precursors and CD11b (Mac-1) that is expressed on dendritic cells or for CD31, which is a marker of endothelial cells.

Our data are in agreement with the preliminary studies on BM and UCB (Bieback *et al*., 2004; Terai *et al*., 2005; Tondreau *et al*., 2005; Kern *et al*., 2006) and it can be concluded that we achieved a homogenous population of MSCs from UCB after four passages.

Bone differentiation capacity of UCB-MSCs was detected after 3 weeks culturing in osteo-inductive medium by alizarian red staining and these results were confirmed by the high level of alkaline phosphatase activity after 2 weeks of culturing in osteo-inductive condition. Results of RT-PCR indicated high expression levels of osteocalcin, osteonectin, osteopontin and Runx-2. Osteocalcin, osteonectin and osteopontin are specific gene markers of bone and Runx-2 is a transcription factor involved in the osteogenesis process (Gaur *et al*., 2005). Toluidine blue staining of cartilage micromasses demonstrated complete differentiation of UCB-MSCs to cartilage. High expression levels of specific gene markers of cartilage including Collagen type II, Aggrecan and Sox-9 were detected by RT-PCR. Our data are in agreement with the results of previous studies and indicated UCB-MSCs differentiate into bone and cartilage in appropriate inductive condition (Bieback *et al*., 2004; Lee *et al*., 2004; Tondreau *et al*., 2005; Kern *et al*., 2006; Zhang *et al*., 2011). However, results of oil red staining for determination of adipogenic differentiation potential of UCB-MSC showed a very low level of differentiation to adipocytes after 3 weeks of adipogenic induction. Our data confirmed the results of Kern *et al*. (2006) that showed UCB-MSCs did not differentiate into adipocytes after 3 weeks maintenance in adipogenic induction medium, in contrast to BM-MSCs that differentiated into adipocytes easily. Results of Bieback *et al*. (2004) indicated that MSCs derived from UCB cannot differentiate into adipocytes under standard differentiation condition but adipogenic differentiation could be induced at least after 5 weeks continuous adipogenic induction. The reduced sensitivity of UCB-MSCs for differentiation to adipocytes in comparison with BM-MSCs may be related to the inherent differences between the two cell sources. According to previous studies adipocytes, as characteristics of adult human bone marrow, are absent in fetal bone marrow and the potential of MSCs to become adipocytes was gained with aging (Gimble *et al*., 1996; Moerman *et al*., 2004).

According to our results, UCB-MSCs sustain their expansion capabilities after several subcultivations. Since in cell therapy a large number of cells must be available, high proliferation capacity through several passages is a great benefit for application of UCB-MSCs in cell therapy. Results of telomerase amplification protocol illustrated that UCB-MSCs are telomerase negative. Since there is a direct correlation between activation of telomerase expression and teratoma formation in adult somatic cells, telomerase negative UCB-MSCs can be regarded as an excellent source of MSCs for clinical application with a high level of safety.

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