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# Isolation and differentiation of mesenchymal stem-like cells from human umbilical cord vein endothelium and subendothelium

**A. Pivoriūnas[1,*], E. Bernotienė[1],**

**A. Ungurytė[1], S. Valiūnienė[1],**

**G. Drąsutienė[2], A. Venalis[1]**

*[1] Department of Experimental Research, Institute of Experimental and Clinical Medicine, Vilnius University, Žygimantų 9, LT-01102 Vilnius, Lithuania*

*[2] Clinic of Obstetrics and Ginaecology, Vilnius University, Antakalnio 57, LT-10207 Vilnius Lithuania*

Mesenchymal stem cells (MSC) are multipotent stem cells found in several adult tissues. These cells are capable to differentiate into cells of connective tissue lineages, including bone, fat and cartilage. MSC have generated a great deal of interest because of their potential use in regenerative medicine and tissue engineering. Currently, the most common source of MSC is bone marrow. However, the use of bone marrow-derived cells present some problems, including the necessity of harvesting bone marrow from donors and donor's age restriction. Therefore, the search for alternative MSC sources is of significant importance.

We report herein a successful isolation of MSC-like cell population from human umbilical vein endothelium and subendothelium. These cells have a characteristic MSC morphology and can be rapidly expanded *in vitro*. Specific staining with Sudan III and silver nitrate (von Kossa) confirmed the adipogenic and osteogenic differentiation of MSC-like cells cultured under specific conditions.

Importantly, long-term storage in liquid nitrogen did not affect the proliferation and differentiation capacities of MSC-like cells.

These findings suggest that MSC derived from human umbilical cord endothelium and subendothelium is an efficient and easily obtainable source for studies of adult stem cell biology and a potentially useful tool for future cell and gene therapy protocols.

**Key words:** mesenchymal stem cells, umbilical cord vein, adipocytes, osteocytes

## INTRODUCTION

MSC are characterized by their ability under appropriate stimuli differentiate into lineages of mesenchymal tissues including bone, cartilage, muscle and fat [1]. In addition, MSC have the potential to differentiate into other types of tissue forming cells such as hepatic [2], cardiac [3] and neural [4]. Initially MSC were characterized in bone marrow. The isolation method was based on the ability of the bone marrow-derived fibroblast-like cells to adhere on the plastic substrate of the cell culture plate [5]. The ease of isolation and the high expansion potential *in vitro* make MSC attractive as a model system for studies of cell differentiation and potentially useful for cell and gene therapy.

To date, the most common source of MSC is bone marrow. However, the number of bone marrow derived MSC significantly decreases with age [6]; in addition, aspiration of bone marrow from donor is an invasive and painful procedure. This has led many reseachers to search for alternative sources of MSC. Recently, several reports have demonstrated a successful isolation of MSC from subcutaneous adipose tissue [7], umbilical cord blood endothelial and subendothelial layers [8, 9], Warrton's jelly [10] and chorionic villi of human placenta [11]. Umbilical cord as a source of MSC is particulary attractive for two reasons. First, umbilical cords in delivery rooms are still regarded as a medical waste and therefore are abundantly available for scientific needs. The second and most important consideration is that the proliferation and differentiation potential of MSC decreases with donor's age [6]. In this respect, umbilical cord is an excellent alternative source for MSC.

However, controversy exists about the umbilical cord blood as a source of MSC. A recent report has demonstrates that only 23% of the cord blood samples analyzed generated MSC-like cells; furthermore, the proliferative capacity of isolated MSC varied greatly [12]. Much less information is available on the isolation of MSC from endothelial and subendothelial layers of the umbilical cord vein. To our knowledge, only a few attempts have been successful [9, 13]. Furthermore, so far very little

* Corresponding author. E-mail: augustas.pivoriunas@ekmi.vu.lt

information in literature is available about the proliferative and differentiation potential of those cells during long-term cultivation *in vitro*. We address these issues in the current study.

We report herein a successful establishment of MSC-like cell culture from the endothelial and subendothelial compartments of the umbilical cord vein. Our results demonstrate that the obtained MSC-like cells could be rapidly expanded in culture. These cells are able to differentiate into at least two lineages of mesenchymal tissues (adipogenic and osteogenic). Importantly, MSC-like cells retained their proliferative and differentiation potential after cryopreservation.

Thus, MSC-like cells derived from the endothelial and subendothelial layers of the umbilical cord vein is a promising experimental model and a useful tool for future cell and gene therapy applications.

## MATERIALS AND METHODS

### Materials

Dullbecco's modified Eagle's medium with low glucose (DMEM), fetal calf serum, penicillin/streptomycin, L-glutamine, 0.25% trypsin/1mM EDTA were obtained from Biological Industries (Kibbutz Beit Haemek, Israel). The basal medium for human mesenchymal stem cells (Mesencult™), osteogenic and adipogenic stimulatory supplements, dexamethasone, ascorbic acid, β-glycerophosphate, collagenase (type IV) were obtained from StemCell Technologies (Vancouver, Canada). Dimethylsulfoxide (DMSO) was from Wak-Chemie Medical GMBH (Steinbach, Germany). Silver nitrate, sodium thiosulfate and Sudan III were obtained from Sigma Chemical. (St. Louis, MO). Cell culture plastic was from Becton Dickinson (Franklin Lakes, NJ). Cell culture photographs were taken with a Moticam 2000 digital imaging camera from Motic (Hong Kong, China).

### Isolation and culture of MSC-like cells

Umbilical cords were obtained from term deliveries according a protocol approved by the Lithuanian Bioethics Committee. Umbilical cords were collected and processed within 3–5 hours after normal delivery. The umbilical vein was catheterized and washed five times with PBS and its distal end was clamped. The vein was then filled with 0.1% collagenase in DMEM medium with 20% FCS and antibiotics and placed in a 37 °C thermostat for 20 min. The collagenase solution was then drained, and the suspension of endhotelial and subendhotelial cells was collected by washing several times with PBS. The cells were centrifuged for 5 min at 500 g and resuspended in DMEM supplemented with 20% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1 mM sodium pyrophosphate. After counting the cells were plated into 25 $cm^2$ cell culture flasks at the approximate concentration of $2 \times 10^4/cm^2$. The cultures were maintained at 37 °C in a humidified atmosphere containing 5% $CO_2$. After two days of culture, the medium was changed and nonadherent cells were removed, then the medium was routinely changed twice a week. When cells with fibroblastoid-like morphology became dominant in the culture (approximately two weeks later), the cells were washed with PBS, harvested with 0.25% trypsin / 1 mM EDTA solution, resuspended in culture medium and plated onto 175 $cm^2$ cell culture flasks for expansion (dilution 1:4).

Part of the subconfluent cell culture (passage 2) was cryopreserved according to the following protocol. The cells were washed with PBS and harvested with 0.25 % trypsin/1mM EDTA solution for 7 min at 37 °C. The trypsinized cells were mixed with complete medium to inactivate the trypsin collected by centrifugation for 5 min at 400 g, resuspended in complete medium, counted and gently suspended at a concentration of $1.25 \times 10^6$/ml in medium containing 10% DMSO and 25% of FCS. Aliquots of 2 ml each were slowly frozen at -70 °C and stored in liquid nitrogen for 2 months. The frozen stocks of cells were thawed at 37 °C, diluted with complete medium (20% FCS), and recovered by centrifugation to remove DMSO. The cells were resuspended in complete medium and plated at a concentration of about 2 x $10^4/cm^2$. One day later nonandherent cells were removed, adherent cells were harvested with 0.25% trypsin/1mM EDTA and replated for expansion.

### Adipogenic induction

MSC-like cells were seeded at a density $5 \times 10^3/cm^2$ in DMEM (low glucose) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1mM sodium pyrophosphate in 35 mm plastic dishes until the cultures reached subconfluence. Then the cultures were treated with adipogenic-induction medium prepared according to manufacturer's instructions (StemCell Technologies) for two weeks. The adipogenic medium was changed twice a week.

Intracellular accumulation of lipids was visualized using Sudan III staining. Briefly, cells were fixed for 10 min with 1% formalin in PBS and stained with Sudan III for 2 h. Control cultures without differentiation stimuli were stained in the same manner.

### Osteogenic induction

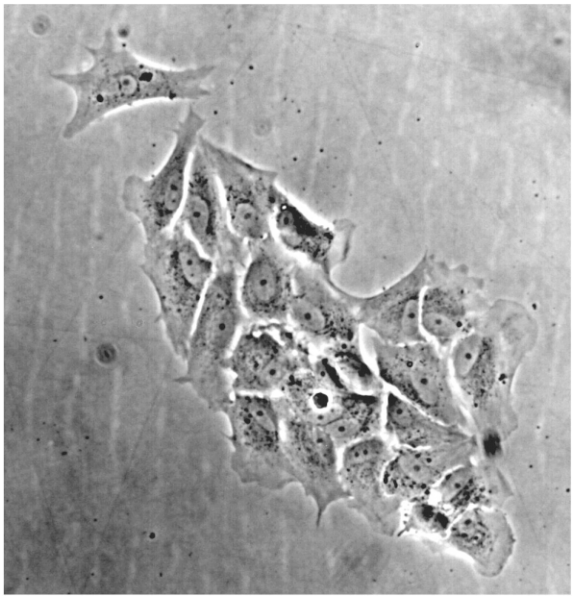
MSC-like cells were seeded at a density $5 \times 10^3/cm^2$ in DMEM (low glucose) supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine and 1mM sodium pyrophosphate in 35-mm diameter plastic dishes until the cultures reached subconfluence. Then the cultures were treated with osteogenic-induction medium prepared according to manufacturer's instructions (StemCell Technologies), plus dexamethasone (final concentration $10^{-8}$ M) and ascorbic acid (final concentration 50 μg/ml). When the formation of cell multilayers became evident, β-glycerophosphate was added into the medium (final concentration $10^{-6}$ M). The cell cultures were treated with osteogenic-induction medium for two weeks; the medium was changed twice a week.

Osteogenic differentiation was assessed by staining for the identification of hydroxyapatite crystals (von Kossa). The cells were fixed for 10 min with 1% formalin in PBS and stained with 1% silver nitrate for 30 min, followed by 5% sodium thiosulfate for 5 min. Control cultures without differentiation stimuli were stained in the same manner.

## RESULTS AND DISCUSSION

Initially, after 48 h of culture, two types of adherent cells were observed. Clusters of small flattened cells morfologically similar to the endothelial cells were dominant (Fig. 1A). A few spindle-shaped cells with typical fibroblastoid morphology (initially identified as MSC-like cells) were also observed. Subsequently, MSC-like cells formed colonies and expanded. At the end of the second week of the cell culture MSC-like cells became predominant (Fig. 1B). After the first passage the MSC-like cell cultures became homogeneous and demonstrated a high proliferation potential (Fig. 2A). Typically, MSC-like cells doubled on an average rate of about twice per 24 hours. The high replicative potential remained unchanged until the 6th passage when the cells almost completely lost their proliferation potential and morphological changes became apparent. MSC-like cells transformed from the spindle-shaped morphology to a larger, flatter phenotype (Fig. 2B). By contrast, several studies have demonstrated that MSC derived from different sources retained a high proliferation potential over 20 cell passages [14]. Cell culture density has been shown to be a critical factor influencing the proliferation potential. When the cells reached high confluence levels they lost their replicative

A



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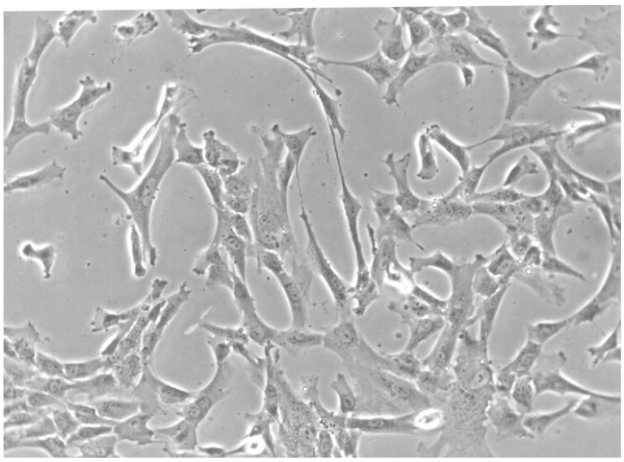
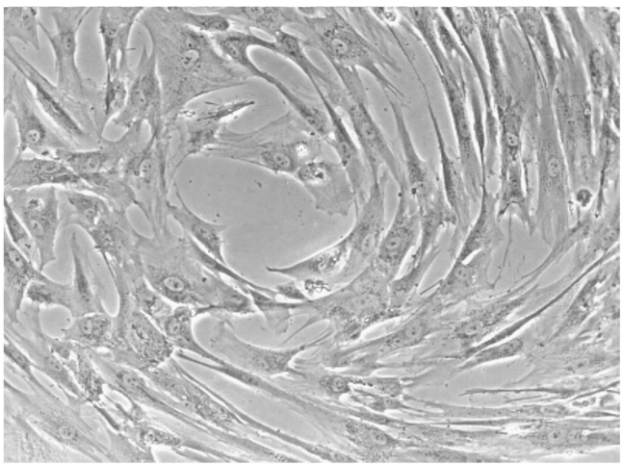


**Fig. 1.** Appearance of primary cell culture derived from human umbilical vein endhotelial and subendothelial layers
(A) Compact cluster of small endothelial cells after 7 days of culturing. (B) Expanding MSC-like cells in primary culture, after 15 days of culturing. Phase contrast, original magnification × 200 (A), × 100 (B).

A



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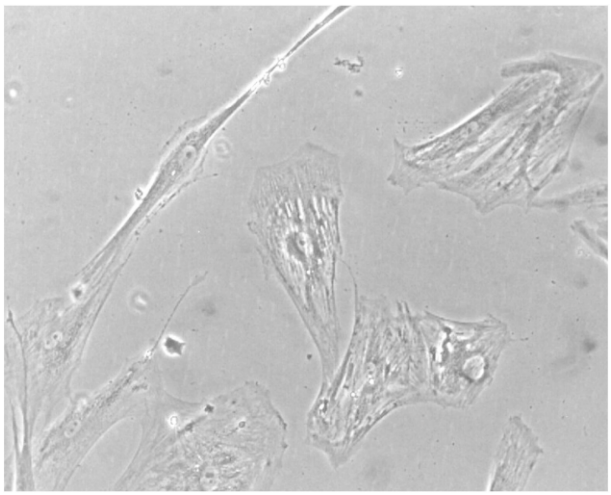

**Fig. 2.** Morphological changes of MSC-like cells during long-term cultivation *in vitro*
Appearance of MSC-like cells from second (A) and sixth (B) passage cultures. Phase contrast, original magnification x 100.

potential [13, 15]. In our study, MSC-like cells were usually replated at subconfluence (70–80%), therefore we think that some other factors could influence the loss of proliferation potential after the 6th passage. Interestingly, the MSC-like cells from the same donor, which were recovered after cryopreservation, also entered stationary phase at the 6th passage.

Further studies on MSC-like cells revealed their ability to differentiate into adipocytic and osteocytic cells. To investigate the adipogenic and osteogenic potential, we used 2 and 5 passage cells. MSC-like cells recovered after cryopreservation (3 passage) also have been used in differentiation experiments.

When induced to differentiate under adipogenic conditions, the spindle shape of MSC-like cells flattened and broadened; by the end of the second week most cells contained numerous lipid droplets visualized by Sudan III staining (Fig. 3).

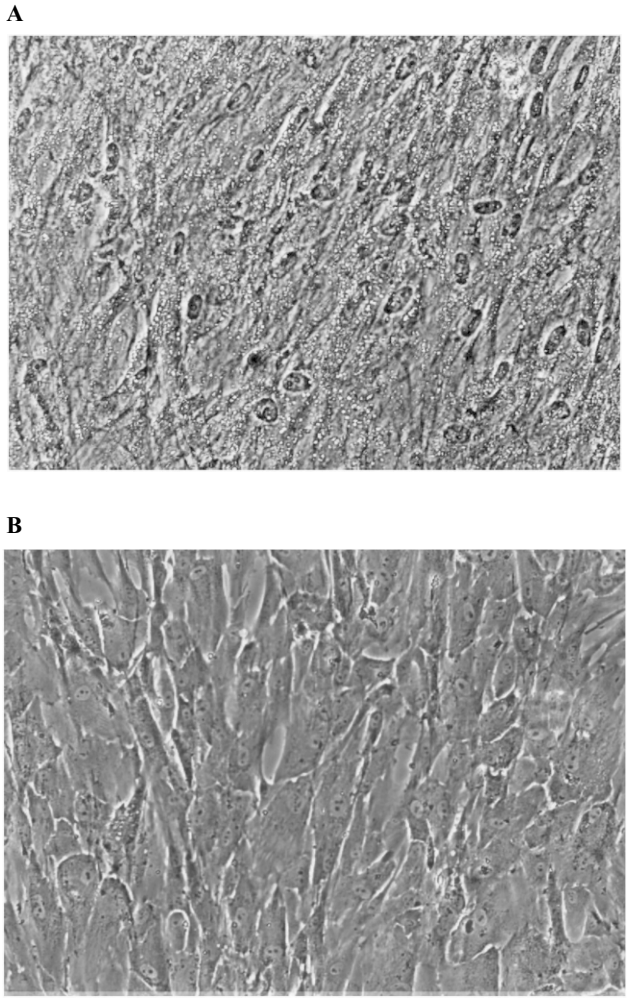


**Fig. 3.** Adipogenic differentiation of MSC-like cells
(A) Adipogenic differentiation was evaluated by culturing cells in adipogenic induction and maintenance medium for 2 weeks and evidenced by formation of lipid vacuoles using Sudan III staining. (B) Control cultures without differentiation stimuli were stained in the same manner. Original magnification ×100 (A), ×200 (B).

The MSC-like cells cultured under osteogenic conditions started to proliferate faster and formed multilayers within the first week. After two weeks, staining with silver nitrate (von Kossa) revealed a characteristic pattern of a mineralized matrix (Fig. 4). Importantly, MSC-like cells from different passages and cells recovered after cryopreservation demonstrated a similar, if not identical, differentiation potential.

In conclusion, we report herein successful isolation of MSC-like cell population from human umbilical vein endothelium and subendothelium. These cells have a characteristic MSC morphology and are able to differentiate into at least adipocytes and osteocytes. MSC-like cells can be rapidly expanded in vitro and stored for long-term periods in liquid nitrogen without losing

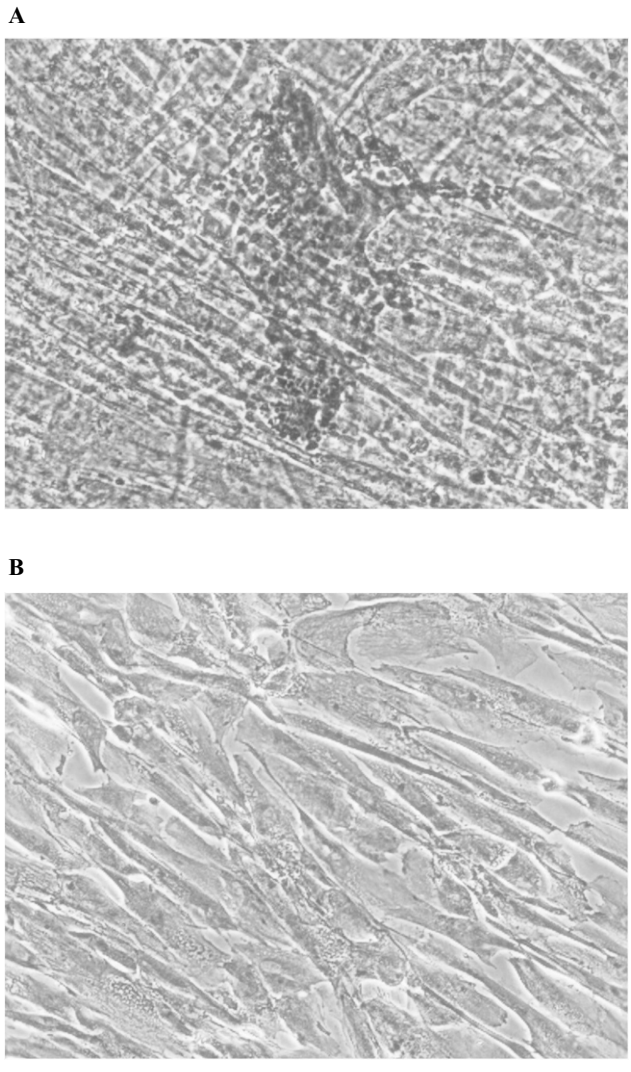


**Fig. 4.** Osteogenic differentiation of MSC-like cells
(A) For osteogenic differentiation, cells were cultured for up to 2 weeks in osteogenic induction and maintenance medium. Staining with silver nitrate (von Kossa) revealed characteristic elements of mineralized matrix. (B) Control cultures stained with silver nitrate (von Kossa). Original magnification ×100 (A), ×200 (B).

capacity to proliferate and differentiate. Although their futher characterization is needed, results presented in this study suggest that MSC-like cells derived from umbilical cord endothelium and subendothelium have the potential to be a useful source for stem cell research and future cell and gene therapy applications.

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**A. Pivoriūnas, E. Bernotienė, A. Ungurytė, S. Valiūnienė, G. Drąsutienė, A. Venalis**

**MEZENCHIMINIŲ KAMIENINIŲ LĄSTELIŲ IŠSKYRIMAS IR DIFERENCIAVIMAS IŠ ŽMOGAUS VIRKŠTELĖS VENOS ENDOTELINIO IR SUBENDOTELINIO SLUOKSNIŲ**

Santrauka

Mezenchiminės kamieninės ląstelės (MKL) aptinkamos daugelyje suaugusio organizmo audinių. Sukūrus specialias aplinkos sąlygas, šias ląsteles galima diferencijuoti įvairių jungiamojo audinių tipų kryptimis (kaulinio, riebalinio, kremzlinio). Kadangi ne visada pavyksta panaudoti kaulų čiulpus MKL išskirti, pastaruoju metu ypač aktyviai ieškoma technologinių sprendimų, leidžiančių išskirti MKL iš alternatyvių šaltinių.

Šiame darbe aprašomas MKL išskyrimas iš žmogaus virkštelės venos endotelinio ir subendotelinio sluoksnių. Ląstelės pasižymėjo tipiška, MKL būdinga morfologija ir dideliu proliferaciniu aktyvumu *in vitro*. Specifiniai dažymo metodai patvirtino, kad sukūrus specialias kultivavimo sąlygas, šios ląstelės diferencijuoja adipogenine ir osteogenine kryptimis. Ilgalaikis saugojimas skystame azote nepaveikė ląstelių proliferacinio ir diferenciacinio potencialo.

Gauti rezultatai rodo, kad MKL, išskirtos iš žmogaus virkštelės venos endotelinio ir subendotelinio sluoksnių, gali būti sėkmingai naudojamos kaip eksperimentinis modelis tyrinėjant kamieninių ląstelių biologiją bei kuriant naujus genų ir ląstelių terapijos protokolus.