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# MORPHOLOGY OF *IN VITRO* EXPANDED HUMAN MUSCLE – DERIVED STEM CELLS

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**Background**: Skeletal muscle contains populations of multipotent adult stem cells also referred to as muscle-derived stem cells.

**Aim**: The main goal of this study was to isolate and culture human adult stem cells from skeletal muscle and characterize them.

**Methods**: Muscle-derived stem cells were isolated from biopsy specimens of femoral muscle. The cells were cultured in Dulbecco's modified Eagle's minimal essential medium supplemented with 10% fetal calf serum and gentamycin. When they reached confluence, they were sub-passaged up to the third passage. Cells from the last passage were prepared for TEM analysis. Production of α-actin and desmin was confirmed by histochemistry. Moreover, the phenotypic characterization was performed.

**Results**: Primary isolated muscle-derived stem cells had a fibroblast-like shape. During subsequent passages they maintained this morphology. TEM analysis showed typical ultrastructural morphology of mesenchymal stem cells. They had large pale nuclei with a large amount of euchromatine. Nuclei were irregular with noticeable nucleoli. Dilated cisterns of rough endoplasmic reticulum were present in cytoplasm. In certain parts of the cytoplasm there were aggregates of granules of glycogen. The products of cells were actively secreted into the extracellular matrix. They expressed α-actin and desmin. The results of phenotypic characterization showed that almost all analyzed cells were CD13, CD34, CD56 positive and CD45 negative. Moreover, they did not express anti-human fibroblast surface protein.

**Conclusions**: Muscle-derived stem cells exhibited typical characteristics typical for mesenchymal stem cells. After analysis of their differentiation potential they could be used in tissue engineering and regenerative medicine.

## INTRODUCTION

Stem cells are characterised as undifferentiated cells which have been derived from embryonic, foetal and adult organisms[1-3]. These cells are unique in their potential to generate various types of tissues under proper conditions *in vitro* and *in vivo*[4]. Embryonic and foetal stem cells are considered pluripotent but their utilization is restricted by ethical considerations[5]. For this reason, multipotent adult stem cells are promising for tissue engineering and regenerative medicine.

Over the past few years, adult stem cells have been derived from various types of tissues including bone marrow, umbilical cord blood, adipose tissue, skin, periosteum, dental pulp, etc.[6-11]. Adult stem cells are adherent and have a fibroblast-like morphology when cultured *in vitro*. These cells are heterogeneous and express a variety of surface markers including CD29, CD44, CD90, CD105, STRO-1 and Sca-1. Moreover, they are negative for haematopoietic markers CD34, CD45 and for HLA Class II (ref.[12, 13]).

Skeletal muscle contains populations of myogenic cells (satellite cells) which are capable of differentiation into myoblasts and a population of multipotent stem cells also referred as multipotent muscle-derived stem cells[14, 15]. Satellite cells express myogenic markers MyoD, Myf5, desmin and PAX-1, and haematopoietic marker CD34 (ref.[16, 17]), while muscle-derived stem cells are predominantly positive for Sca-1, CD13, CD34 and CD56 (ref.[18, 19]) and negative for CD45 (ref.[20]). Muscle-derived stem cells are usually isolated by the serial plating technique which is based on the different propensity of cells to adhere to cultivation substrate and lead into the purification of myogenic cells[21].

The aim of this study was to isolate and culture *in vitro* human muscle-derived stem cells and do a morphological and phenotypical analysis of them.

## MATERIAL AND METHODS

### *Isolation and cell culture*

Human muscle cells were obtained under sterile conditions from a biopsy specimen (sized 2 × 5 mm) of femoral muscle (male individual, 3-month-old). The sampling was indicated for a genetic examination and was performed in accordance with The Helsinki Declaration. The muscle was carefully rinsed with sterile phosphate buffered saline (PBS, Oxoid, UK) supplemented with gentamycin in final concentration of 200 μg.ml$^{-1}$ (Lek, Slovenia). Then cut into small pieces and digested with 0.1% collagenase type I (Pan Biotech, Germany) for 60 min. at 37 °C. The obtained suspension was centrifuged at 1000 rpm for 10 min. The resultant supernatant was aspirated and sediment was resuspended in 10 ml of 0.25 % trypsin–EDTA solution (PAA, Austria) for 30 min at 37 °C. The final suspension was filtered through 70 μm pore-size cell strainer (BD Falcon, US) and centrifuged at 1000 rpm for 5 min. The supernatant was carefully removed and sediment was resuspended in Dulbecco's modified Eagle's minimal essential medium (DMEM, Pan Biotech, Germany) containing 10% foetal calf serum (FCS, PAA, Austria) and gentamycin in a final concentration of 80 μg.ml$^{-1}$. The suspension was plated on an uncoated Petri dish (Ø 40 mm, Swiss) assigned as PP1 and paced in $CO_2$ incubator (37 °C, 5 % of $CO_2$ in air) for 120 min. After 2 h, unattached cells floating in medium were collected and plated on other Petri dish assigned as PP2. The procedure was repeated after 24 h incubation and the last Petri dish was designated PP3. Cells from PP3 were cultured for 21 days to obtain a sufficient number of cells, and the culture medium was refreshed every 48 h. When the cells reached confluence, they were trypsinized and subpassaged up to the 3$^{rd}$ passage. Cells from last passage were prepared for histological and histochemical analysis according to standard protocols. The phenotypic characterization was done by flow cytometry.

### *Phenotypical analysis*

Bone marrow and adipose tissue derived MSCs from the third passage were analyzed by direct and indirect immunofluorescence, according to protocols specific for each antibody. In each case, 10 000 events were acquired and analyzed by a Coulter Epics ALTRA flow cytometer. The following anti-bodies were used for cell staining: anti-CD34-FITC; anti-CD56-FITC; anti-human fibroblast surface protein (Sigma Aldrich, USA); anti-CD-13-PECy5; anti-CD45-PE-Cy5 (Dako Glostrup, Denmark); with a secondary FITC-conjugated donkey anti-mouse IgG antibody (Chemicon, USA).

### Morphological analysis

The morphology of the *in vitro* expanded cells was repeatedly examined under inverted microscope Zeiss Axiovert 100 during cultivation.

Cells intended for immunohistochemical analysis were fixed with cold methanol for 1 min. After that they were prepared for immunoflurescence staining against α–actin and desmin (Dako Glostrup, Denmark) according to standard protocol.

Cells set aside for transmission electron microscopy observation were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, Germany) for 4 h. After fixation, samples were

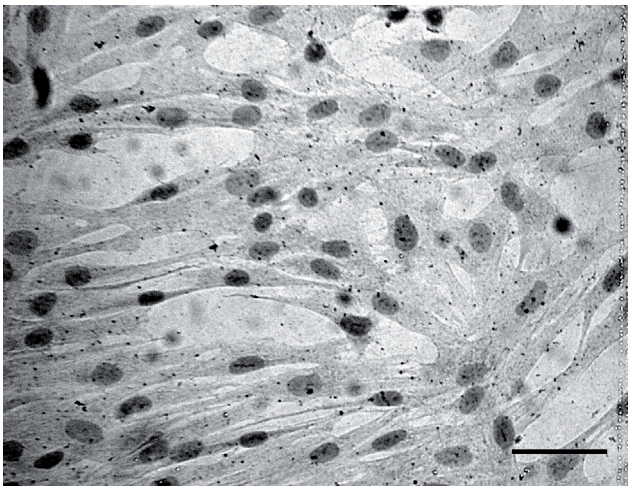

**Fig. 1.** Cell cultures of human muscle-derived stem cells. Scalebar = 100 μm.

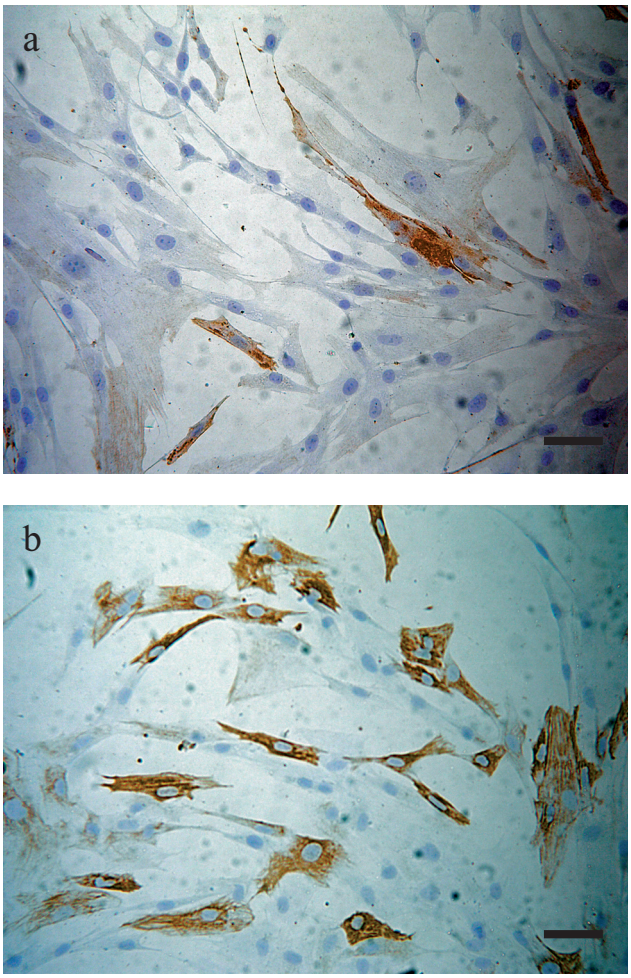


**Fig. 2.** Immunohistochemical staining for α–actin (a) and desmin (b). Scalebar = 100μm.

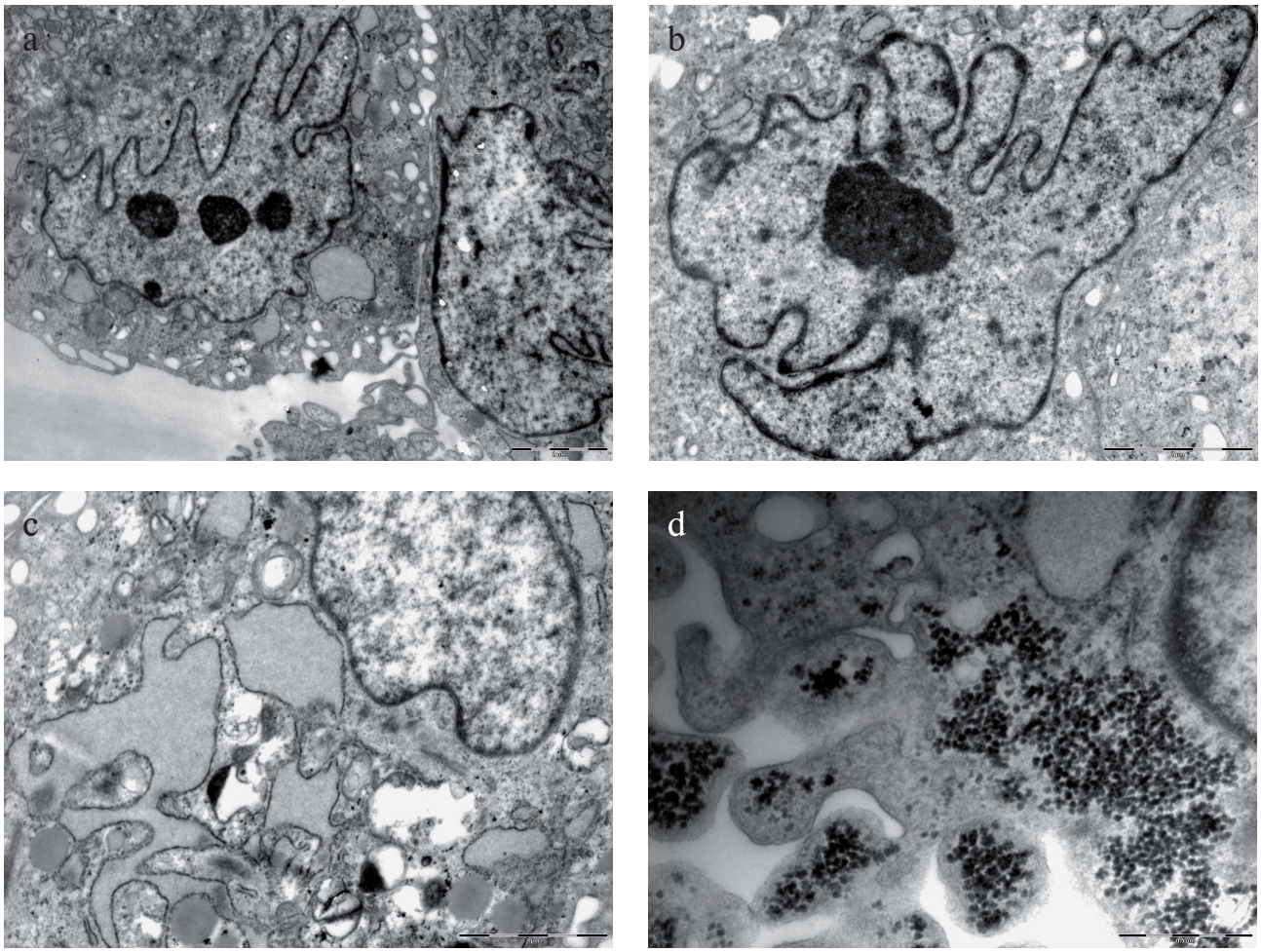


**Fig. 3.** TEM analysis of human muscle-derived stem cells

rinsed in PBS and postfixed in 2% osmium tetraoxide (Serva, Germany) for 2 h, then rinsed in distilled water and dehydrated in a graduated series of ethanol. Subsequently, the samples were embedded in EPON and cut into semi-thin sections. Ultra-thin sections were mounted on 200 mesh copper grids, then double stained using uranyl acetate and lead citrate (Serva, Germany) and examined using a Philips Morgagni transmission electron microscope.

## RESULTS AND DISCUSSON

In the present study we have performed morphological and phenotypical characterization of human muscle-derived stem cells. These cells were considered to be multipotent and so they are interesting for cell therapy[16, 19, 20]. We used the generally accepted serial plating technique to obtain a population of stem cells[21]. Primary isolated muscle–derived stem cells had a fibroblast–like morphology (Fig. 1). During subsequent passages they maintained this morphology. Phenotypical analysis showed that almost all of the analyzed cells were CD13, CD34 and CD56 positive and, CD45 negative. This is in accord with other studies[17, 22, 23]. They did not express anti–human fibroblast surface protein. The immunohistochemical analysis showed expression of α–actin and desmin (Fig. 2), which indicates their mesenchymal origin as well as their myogenic capacity[24]. TEM analysis showed typical ultrastructural morphology of mesenchymal stem cells (Fig. 3). They had large pale nuclei with a large amount of euchromatine. Nuclei were irregular with noticeable nucleoli. Dilated cisterns of rough endoplasmic reticulum were present in the cytoplasm. In certain parts of the cytoplasm there were aggregates of granules of glycogen. The products of cells were actively secreted into the extracellular matrix. In summary, muscle–derived stem cells exhibit characteristics typical for mesenchymal stem cells. After other analysis they could be used in tissue engineering and regenerative medicine.

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