Arch Biol Sci. 2017;69(4):715-721 https://doi.org/10.2298/ABS170313018S

# Generation of human induced pluripotent stem cells from adipose-derived stromal/stem cells isolated from a 75-year-old patient

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**Received**: March 13, 2017; **Revised**: May 10, 2017; **Accepted**: May 17, 2017; **Published online**: June 6; 2017

**Abstract:** Human adipose-derived stromal/stem cells (hASCs) have been considered a valuable cell source for generating induced pluripotent stem cells (iPSCs). Adipose tissue is easy to obtain. Moreover, the isolated cells rapidly proliferate to reach the required number of cells. Some authors have already shown that iPSCs can be successfully obtained using adult hASCs. Nevertheless, little is known about the generation of iPSCs using hASCs isolated from the tissues of patients over the age of 70. In this study, we examined the generation of iPSCs from hASCs isolated from a 75-year-old man. We transduced hASCs with human transcription factors OCT4, SOX2, c-MYC and KLF4 and observed the formation of human embryonic stem cell (hESC)-like colonies. The efficiency of the reprogramming process was 0.08% at day 18 post-infection. Reprogrammed cells expressed pluripotent state-specific transcription factors OCT4, SOX2, NANOG and KLF4, and were able to differentiate into three germ layers *in vitro*.

**Key words:** adipose cells; stem cells; reprogramming; transcription factor

## INTRODUCTION

The discovery that somatic cells can be induced into pluripotent cells provides an unique tool for studying disease pathogenesis, for toxicological testing and for the identification of new therapeutic targets [1,2]. In principle, human induced pluripotent stem cells (iPSCs) can be obtained from various somatic cells and are capable of forming any cell type in the body. Different types of reprogrammed parental cells are not equivalent in their ability to convert into iPSCs.

The majority of iPSC studies use dermal fibroblasts from adult skin biopsies as a parental cell source. However, the taking of punch biopsies is an invasive procedure and requires more than two weeks to obtain sufficient amounts of cells for reprogramming. Moreover, the reprogramming efficiency of adult human fibroblasts using OCT4, SOX2, c-MYC and KLF4 ("Yamanaka" factors) is about 0.01% [3]. In comparison, the keratinocytes from a 4-year-old child and 28-35-year-old adult have been reprogrammed to a pluripotent state at least 100-fold more efficiently and 2-fold faster when compared to the reprogramming of the human fibroblasts [4]. The main disadvantage of using keratinocytes for this purpose is that they accumulate more mutations than cells inside the body due to higher UV exposure.

The accessibility of parental cells for reprogramming is a critical consideration within the field of iPSCs. In humans, some cell types, such as neural stem cells, are not available in large quantities and this makes them unsuitable for reprogramming in a clinical setting [5]. Conversely, the adipose-residing cells are considered as an excellent autologous cell type for cell-based therapies. The usage of adipose cells for iPSC generation has several advantages over other cell types. First, adipose tissue can be easily obtained by surgical resection and liposuction using relatively safer and less invasive medical procedures. Second, human adipose-derived stromal/stem cells (hASCs) are multipotent cells that can be isolated in very large quantities [6,7]. Finally, hASCs can be derived from patients of different ages and can be grown easily and rapidly in culture [8,9].

Sun et al. [8] have reported the efficient generation of iPSCs using hASCs from 40- to 65-year-old patients by transduction with OCT4, SOX2, KLF4 and c-MYC. Recently, Kang et al. [10] described the reprogramming of stem cells and stromal cells derived from the adipose tissues of 27-53 year-old male or female patients. However, there are no reports on the generation of iPSCs using adipose-derived cells from patients aged over 70 years.

In this study, we focused on the investigation of reprogramming capability of hASCs derived from a 75-year-old man. Reprogramming of hASCs was accompanied by changes in cell morphology, induction of endogenous pluripotency genes and the ability to differentiate into the three germ layers *in vitro*. Our results indicate that ASCs from elderly patients can be successfully reprogrammed into adult, individual-specific iPSCs.

## MATERIALS AND METHODS

### Cell culture

Primary hASCs were isolated from an adipose tissue sample of a 75-year-old man donor. Informed consent was obtained from the donor (Project 4/2006 NSF).

The tissue was washed with phosphate buffered saline (PBS), and digested with type II collagenase (Sigma-Aldrich) at 37°C for 2 h. Primary cultures of mouse embryonic fibroblasts (MEFs, feeder cells) were prepared from 13-14-day old embryos from imprinting control region (ICR) mice after digestion with 0.05%/0.02% trypsin/EDTA (Sigma-Aldrich) at 37°C for 20 min. The cells were then washed with PBS and cultured in Dulbecco's modified Eagle's medium (DMEM) low glucose (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Sigma-Aldrich) and penicillin/streptomycin/amphotericin B (PAN-Biotech). The culture medium was changed 48 h after cell plating to remove debris and nonadherent cells. After 2 passages, 90% confluent MEFs were mitotically inactivated by 5 mg/mL mitomycin C (Sigma-Aldrich) at 37°C for 1h.

Embryonic stem cells from the BG01V line (ATCC® Number SCRC-2002™), as well as the reprogrammed hASCs, were maintained on amitotic MEFs in embryonic stem cell (ESC) media containing DMEM/F12 (Sigma-Aldrich), 15% FCS, 5% knockout serum replacement (KOSR, Gibco, Invitrogen), 1% MEM non-essential amino acids (Gibco, Invitrogen), 2.0 mM L-alanyl-L-glutamine (Gibco, Invitrogen), 0.1 mM β-mercaptoethanol (Sigma-Aldrich), 4 ng/ml hFGF-2 (Genaxxon) and penicillin/streptomycin/amphotericin B. Cells cultured without a feeder layer were maintained in DMEM/F12 supplemented with 15% KOSR, 10% MEFs-conditioned medium, 1% MEM non-essential amino acids, 2 mM L-alanyl-L-glutamine, 0.1 mM β-mercaptoethanol, 4 ng/ml hFGF-2 and penicillin/streptomycin/amphotericin B. The cultures were passaged mechanically. The pluripotent cells were cultured on mitomycin-C-inactivated MEFs. All cultures were carried out in a humidified atmosphere at 37°C in 5% $CO_2$.

### Preparation of MEF-conditioned medium

MEFs at 80% confluence were cultured in DMEM/F12, supplemented with 15% KOSR, 1% MEM non-essential amino acids, 2.0 mM L-alanyl-L-glutamine, 0.1 mM β-mercaptoethanol, 4 ng/ml hFGF-2 and penicillin/streptomycin/amphotericin B. Conditioned medium were collected after 48-h incubation.

### Flow cytometry

Flow cytometry was used for phenotypic characterization of hASCs. The cells were trypsinized, washed, and a single cell suspension was stained with fluorochrome-conjugated anti-human CD29 PE (BD Biosciences, Clone MAR4), CD73 PE (BD Biosciences, Clone AD2), CD90 FITC (BD biosciences, Clone 5E10), and CD45 CD45 FITC (BD Biosciences, Clone HI30). Data were collected by FACSCalibur cell analyzer (BD Biosciences) and analyzed with Cell Quest software (BD Biosciences).

### Cellular reprogramming

Retrovirus vectors (pMXs-based) expressing one of the reprogramming factors OCT4, SOX2, c-MYC orKLF4 were used. To produce high-titer viruses, each of the vectors was cotransfected into 4 million 293Tcells together with packaging vectors (pUMVC and pCMV-VSV-G) using Lipofectamine 2000 (Invitrogen). Virus

supernatants were harvested 48 h later and used for first infection that was performed at day 0. For second infection, fresh media was added to infected 293T cells and collected after another 48 h. The supernatants containing OCT4, SOX2, c-MYC and KLF4 virus particles were pooled and concentrated 600-fold using Amicon Ultra concentrators (Millipore) with a cut-off 50000 nominal molecular weight limit (NMWL). Virus concentrates were diluted in culture media at a ratio 1:50 and polybrene (Sigma-Aldrich) and a final concentration of 8 μg/μl was added. The mixture was used for infection of $3x10^5$ hASCs/50 $cm^2$ and the cells were incubated overnight. Next, the wells were supplemented with 50% v/v culture media (DMEM low glucose with 10% fetal calf serum and penicillin/streptomycin/amphotericin B). On day 7, the reprogrammed cells were transferred to amitotic MEFs in ESC media.

**Calculation of reprogramming efficiency**

The reprogramming efficiency was determined as the number of ESC-like colonies formed per number of infected seeded cells on day 18. The colonies with non-ESC-like morphology were not included in the calculation.

**Positive selection of reprogrammed cells**

A method for the selection of human iPSCs based on the expression of transgenous OCT4 and surface pluripotent marker TRA-1-60 was used [11]. Briefly, 8-11 days after formation of the first colonies (on the $25^{th}$ day after first infection), the puromycin-resistant transgenous OCT4-expressing cells were selected by culturing in ESC media with 1 mg/mL puromycin for 5 days. The medium was changed every second day. Next, the cells were passaged and expanded for another 7 days. Then, $5x10^5$ OCT4-positive cells were sorted by a magnetic column according to expression of TRA-1-60 on the $37^{th}$ day after first infection. The positive OCT4, TRA-1-60 fraction was resuspended in ESC media and transferred onto amitotic MEFs.

**Evaluation of alkaline phosphatase activity**

OCT4 and TRA-1-60 positive hASCs cells were cultured on MEFs for a week and alkaline phosphatase activity was evaluated 18 and 65 days after first infection. The cells were washed with buffer (0.1 M Tris, 0.1 M NaCl, 0.005 M $MgCl_2$, pH 9.5) and incubated with 0.15 mg 5-Br-4-Cl-3-indolyl phosphate and 0.3 mg nitro blue tetrazolium diluted in washing buffer for 20 min at room temperature (RT). After washing with PBS, images were taken using a Leica DMI3000B microscope (Leica Microsystems, Germany).

**Quantitative real-time polymerase chain reaction**

Total RNA was isolated using Tri Reagent (Sigma-Aldrich), followed by cDNA synthesis using Affinity Script QPCR cDNA Synthesis Kit (Stratagene) according to the manufacturer's recommendation. Quantitative PCR was performed using a Go Taq qPCR master Mix Kit (Promega) and primers to endogenous transcripts as in [12]: OCT4 (forward primer 5′-GCACTGTACTCCTCGGTCCCTTTCCC-3′; reverse primer 5′-CTTCCCTCCAACCAGTTGCCCCAAAC-3′); NANOG (forward primer 5′-AAAGAATCTTCACCTATGCC-3′; reverse primer 5′-GAAGGAAGAGGAGAGACAGT-3′) and reference gene β-actin (forward primer 5′TGACGGGGTCACCCACACACTGTGCCCATCTA-3′, reverse primer 5′ CTAGAAGCATTTGCGGACGATGGAGGG-3′).

For each sample, the threshold cycle (Ct) for the internal control (β-actin) amplification was subtracted from the threshold cycle of the corresponding transcription factor amplification (Ct, TF) to yield ΔCt. The Pfaffl method was used to calculate the ratio of relative gene expression related to β-actin (internal control), and represented in the bar graphs [13]. All values were expressed as means±SD. Statistical analysis was performed by Student's t test. A probability value of $p<0.05$ was considered statistically significant.

**Embryoid body-based *in vitro* test of pluripotency**

The colonies of reprogrammed cells were scraped and gravity sedimented for 10 min at 37°C. The cells were then seeded on a nonadherent surface, precoated with 0.8 mg/$cm^2$ polyHEMA (Sigma-Aldrich). The differentiation medium (80% DMEM/F12, 20% FCS, 1% MEM non-essential amino acids, 2 mM L-alanyl-L-glutamine,

0.1 mM β-mercaptoethanol and penicillin/streptomycin/amphotericin B) was changed every second day.

### Immunofluorescence staining of pluripotency-associated proteins

To detect pluripotency-specific or germ layer-specific marker expression, the reprogrammed cells were cultured on confluent amitotic MEF feeder cells on coverslips for 7-9 days until defined colonies were obtained. The embryoid bodies (EBs) were seeded onto gelatin-coated coverslips 30 days after formation and cultured for 5 more days to allow attachment and monolayer spreading of EB cells. The coverslips were collected, washed with PBS and the cells fixed with 4% paraformaldehyde for 20 min at RT. Next, the cells were blocked in PBS with 1% bovine serum albumin and permeabilized with 0.1% Triton X 100 for 1h.

Immunofluorescence staining was performed using primary antibodies against pluripotency-specific markers: SOX2 ((R&D Systems, MAB2018, 1:50OCT4A (R&D Systems, MAB17591, 1:50), NANOG (R&D Systems, AF1997, 1:20), KLF4 (R&D Systems, AF3640, 1:20), and germ layer-specific markers: VIMENTIN (eBioscience, 14-9897-82, 1:500; NESTIN (R&D Systems, MAB1259, 1:100), α-ACTININ (Abcam, ab78505, 1:500), DESMIN (R&D Systems, AF3844, 1:20), AFP (Sigma-Aldrich, A8452, 1:500), and GATA4 (Abcam, ab61767, 1:500). The cells were incubated with primary antibodies overnight. They were then washed and incubated for 1 h at RT with secondary antibodies conjugated with Alexa Fluor 488 (Invitrogen, A21202, 1:1000) or Alexa Fluor 594 (Invitrogen, A21203, 1:1000). Cell nuclei were stained with l μg/ml Hoechst 33258 (Sigma-Aldrich) for 5 min at RT. Images were taken using a confocal microscope Leica TCS SPE (Leica Microsystems).

## RESULTS

### Reprogramming of hASCs

hASCs were isolated from a 75-year-old man. When the hASCs were cultured in DMEM with 10% FCS, they grew attached to the culture surface and exhibited spindle-shaped fibroblast-like morphology (Fig. 1A). At passage four, 95% of the cells were positive for mesenchymal surface markers CD29, CD73 and CD90 and negative for leukocyte common antigen CD45 (Fig. 1B).

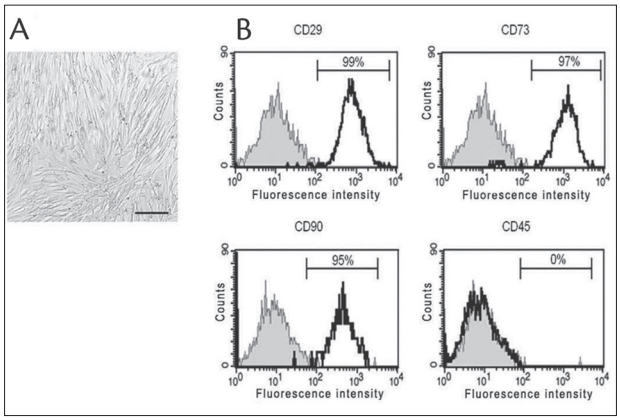


**Fig. 1**. Characteristics of hASCs. **A** – monolayer of ASCs (adherent fibroblast-like cells). Scale bar 200 μm. **B** – flow cytometry analysis showed that more than 95% of hASCs are positive for the mesenchymal surface markers CD29, CD73 and CD90, and all cells are negative for leukocyte common antigen CD45.

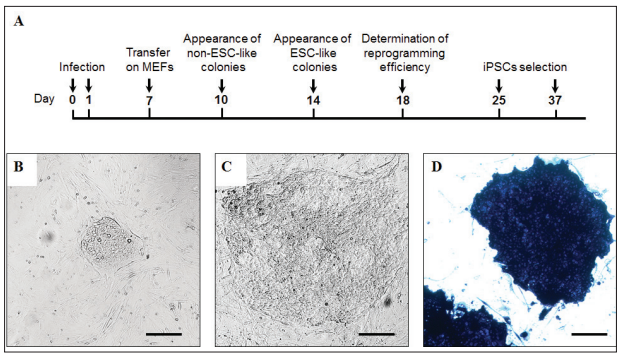


**Fig. 2.** Reprograming of hASCs. **A** – scheme of hASC reprograming. **B** – early colony formed on MEFs on day 10. **C** – flat and compact ESC-like colony of reprogrammed hASCs with clear-cut round edges growing on MEFs. **D** – example of AP positive colonies (blue). Scale bar 200μm.

A schematic diagram of the iPSC generation protocol is shown in Fig. 2A. The cells were infected with a mix of lentiviruses carrying one of the four genes (OCT4, SOX2, c-MYC and KLF4) produced in 293T cells [11]. On day 10, we detected the first non-ESC-like colonies (Fig. 2B) that had a morphology similar to the "early colonies" or "background colonies" described previously [8,14,15]. Four days later, the treated hASCs were evaluated for the appearance of tightly-packed colonies, resembling to hESCs (Fig. 2C). The colonies were composed of small, fast-grow-

ing cells and were positive for alkaline phosphatase (AP) activity (Fig. 2D) on day 18. Forty-one ESC-like colonies, representing 0.08% mean reprogramming efficiency, were obtained.

In our previous study, we reported a method for the selection of human iPSCs based on the expression of transgenous OCT4 (the OCT4 vector has a puromycin resistance gene) and the surface pluripotent marker TRA-1-60 [11]. Here, we used the same protocol to pick out reprogrammed cells. We selected $1x10^5$ cells positive for OCT4- and TRA-1-60-reprogrammed hASCs and expanded them on MEFs. hASC-derived ESC-like colonies were morphologically indistinguishable from hESCs. We manually picked six individual colonies. A further three iPSCs clones, iP-hASC-1, iP-hASC-2 and iP-hASC-3, were successfully established from this reprogramming experiment and characterized with regard to pluripotency-associated properties.

## Characterization of human iPSCs

Two human iPSC clones (iP-hASC-1, iP-hASC-2) were continuously cultured for approximately 3 months and characterized to demonstrate their fully reprogrammed character. The expression of pluripotency-specific markers OCT4, SOX2, KLF4 and NANOG, was examined in clones iP-hASC-1 and iP-hASC-2 (Fig. 3A). The analyzed clones were positive for these markers. Although ASC were not negative for the tested markers, the observed expression was definitely much lower than in the iP-hASC clones. The expression intensities for the four markers in the iP-hASC clones were comparable with those detected in the embryonic stem cell line BG01V.

Reprogramming of ASCs was induced by virus delivery of transgenous *oct4*, *sox2*, *c-myc* and *klf4*, which activated the transcription of corresponding endogens. The activation of endogenous *oct4* and *nanog* transcription is a key step in the reprogramming of somatic cells into induced pluripotent stem cells. A statistically significant increase in mRNA expression levels of endogenous *oct4* and *nanog* in reprogrammed hASCs compared to parental cells was determined. In reprogrammed cells, the endogenous *oct4* were from 6- to 15-fold higher than in parental cells. As regards endogenous *oct4*, its expression was increased from 3- to

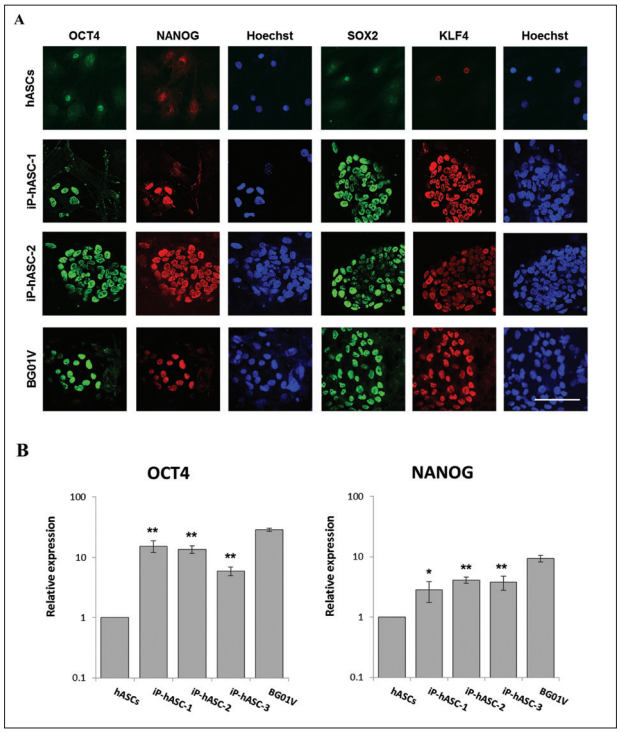


**Fig. 3.** Expression of pluripotent state-specific markers. **A** – immunofluorescent staining shows expression of pluripotent markers, OCT4, SOX2, KLF4 and NANOG in clones of reprogrammed hASC and parental cells. Human embryonal stem cells from the BG01V line served as a positive control. The nuclei of cells were stained with Hoechst (blue). Immunofluorescent staining shows expression of tested pluripotent markers in iPSC clones. Single hASCs display low nuclear expression of tested markers. The scale bar is 100 μm. **B** – quantitative-PCR analysis of gene expression levels of endogenous OCT4 and NANOG within reprogrammed hASCs relative to those in hASCs. The data are shown as means±SD (n=3). Statistically significant differences between parental and reprogrammed cells are indicated (* – P<0.05; ** – P<0.01; *** – P<0.001).

4-fold. These genes were found to be from 2- to 3-fold lower in reprogrammed cells than in hESCs (Fig. 3B).

Finally, the ability to differentiate into nearly all tissue types as a hallmark of pluripotent stem cells was analyzed employing embryoid body (EB)-based differentiation *in vitro*. The reprogrammed cells were cultured in differentiation medium and by preventing cell adhesion to the bottom of the culture dishes. Under these conditions, the cells formed spherical aggregates (EBs) shown in Fig. 4A. For evaluation of the ability for *in vitro* differentiation of reprogrammed cells, we investigated the expression of nestin (neural stem cells, pancreatic islet progenitors), vimentin (glial cells, pancreatic precursor cells), α-actinin (muscle

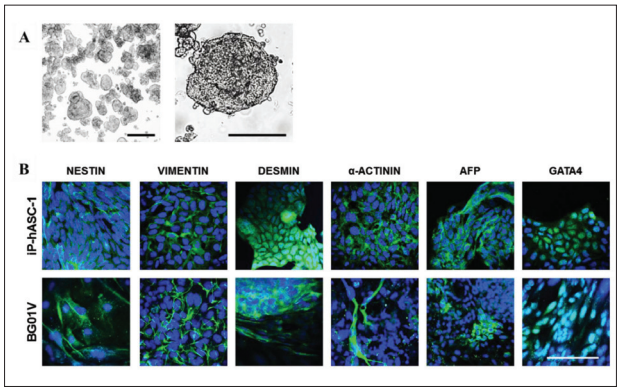


**Fig. 4.** *In vitro* differentiation of iP-hASC-1. **A** – Image of EBs generated in suspension in culture 20 days after formation. **B** – immunofluorescent staining showing the expression of differentiation markers, nestin, vimentin, α-actinin, desmin, AFP and GATA4 on day 35 after EB formation. EBs of BG01V cells served as a positive control. The nuclei of cells are stained with Hoechst (blue). The scale bar is 100 μm.

cells), desmin (muscle cells), α-fetoprotein (AFP) (visceral endodermal cells) and GATA4 (primitive endodermal cells) in 35-day-old EBs. Vimentin and nestin were expressed in the periphery of the adherent EBs, suggesting their expression in migrating cells. Muscle markers α-actinin and desmin and endodermal markers GATA4 and AFP were found inside the EBs as well as at the periphery (Fig. 4B). the EBs of BG01V cells showed similar expression of examined differentiation markers.

## DISCUSSION

At present, it is possible to reprogram cells derived from patients over 60 years to obtain more youthful features, resetting telomere lengths and gene expression profiles similar to those observed in hESCs [16,17]. Several groups have succeeded in the establishment of iPSCs from healthy elderly donors or diseased aged persons. Ohmine et al. [18] generated iPSC lines from epidermal keratinocytes derived from elderly type 2 diabetes patients of different age, using the four Yamanaka factors. Although reprogramming efficiency is not high – 2 to 10 expandable clones per $10^5$ (~0.002-0.01%), iPSC lines could be derived and these lines expressed a range of pluripotency markers and exhibited the ability to differentiate into the three germ layers. Also, Sun et al. [8] reported the generation of iPSCs from patients' hASCs (40- to 65-year-old) by transduction with OCT4, SOX2, KLF4 and c-MYC, with an efficiency of 0.01-0.03%. Nevertheless, there are no reports on the reprogramming of adipose-derived iPSCs from patients aged over 70 years.

The present work contributes to the development of knowledge for the reprogramming of hASCs. Here we report the successful establishment of iPSCs from hASCs of a 75-year-old man. hASC-iPSCs with hESC-like morphology were generated with an efficiency of 0.08%, on day 18. However, the efficiency is a higher than reported for iPSCs generated from the fibroblasts of a 74-year-old donor, with a mean reprogramming efficiency of 0.06% [16]. We suggest that using hASCs as parental cells might resolve the low efficiency of transduction. Because adipose tissue often accumulates in the human body, hASCs might represent an abundant cell source for reprogramming.

The reprogrammed cells were characterized by alkaline phosphatase expression which, along with expression of normal pluripotent stem cell markers OCT4, NANOG, KLF4 and SOX2, indicated undifferentiated cells with the potential to self-renew. Moreover, all iPSC clones activated the transcription of the endogenous homeodomain transcription factors, OCT4 and NANOG, which have been identified as important to both early embryo development and pluripotency maintenance in hESCs [19,20]. We found a significant increase in mRNA levels for endogenous OCT4 and NANOG at more than 3 months post-infection, providing proof of a successful reprogramming procedure.

The differentiation potential of the *in vitro*-formed iPSCs was investigated using the protocol for differentiation by subjecting the cells to EB formation. EBs were formed by a spontaneous aggregation of cells [20,21]. This structure facilitates multicellular interactions comprising ectodermal, mesodermal, and endodermal tissues and leading to cell differentiation during early mammalian embryogenesis [18,19]. In the tested clones, we demonstrated the expression of the molecular markers nestin, vimentin, α-actinin, desmin, AFP and GATA4. Together, these results indicate that reprogrammed elderly hASCs have pluripotent properties, since only pluripotent cells are able to differentiate towards the three embryonic germ tissues (ectoderm, mesoderm and endoderm).

In conclusion, herein we report the efficient generation of iPSCs from hASCs obtained from an elderly patient. iPSCs generated from patients of different age should provide a valuable experimental model for investigating the cellular reprogramming process as well as practical alternatives for the generation of patient-specific cells.

**Acknowledgments:** This work was partly supported by the grants ReProForceFP-7- REGPOT-2009-1 and №BG-051PO001-3.3.06-0059.

**Authors' contribution:** ES, the main author, provided the design and implementation of the experiment, data collection, data analysis and manuscript compilation. TO contributed to the analysis of data. MM coordinated the research.

**Conflict of interest disclosure:** The authors declare no conflict of interest in the study.

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