Appl Biochem Biotechnol (2015) 177:1040–1054
DOI 10.1007/s12010-015-1795-8

Characterization and Differentiation of Stem Cells Isolated from Human Newborn Foreskin Tissue

Özge Sezin Somuncu[1] · Pakize Neslihan Taşlı[1] · Hatice Burcu Şişli[1] · Salih Somuncu[2] · Fikrettin Şahin[1]

Received: 27 February 2015 / Accepted: 2 August 2015 /
Published online: 25 August 2015

Abstract Circumcision is described as a cultural, medical, and religious process which states surgical removal of the foreskin either partly or fully. Cells isolated from the circumcised tissues are referred as foreskin cells. They have been thought as feeder cell lines for embryonic stem cells. Their fibroblastic properties were also utilized for several experiments. The waste tissues that remain after the circumcision thought to have stem cell properties. Therefore, there have been very few attempts to expose their stem cell properties without turning them into induced pluripotent stem cells. Although stem cell isolation from prepuce and their mesenchymal multilineage differentiation potential have been presented many times in the literature, the current study explored hematopoietical phenotype of newborn foreskin stem cells for the first time. According to the results, human newborn foreskin stem cells (hnFSSCs) were identified by their capability to turn into all three germ layer cell types under in vitro conditions. In addition, these cells have exhibited a stable phenotype and have remained as a monolayer in vitro. hnFSSCs suggested to carry different treatment potentials for bone damages, cartilage problems, nerve damages, lesion formations, and other diseases that are derive from mesodermal, endodermal, and ectodermal origins. Owing to the location of the tissue in the body and differentiation capabilities of hnFSSCs, these cells can be considered as easily obtainable and utilizable even better than the other stem cell sources. In addition, hnFSSCs offers a great potential for tissue engineering approaches due to exhibiting embryonic stem cell-like characteristics, not having any ethical issues, and teratoma induction as in embryonic stem cell applications.

Electronic supplementary material The online version of this article (doi:10.1007/s12010-015-1795-8) contains supplementary material, which is available to authorized users.

✉ Fikrettin Şahin
fsahin@yeditepe.edu.tr

[1] Department of Genetics and Bioengineering, Faculty of Engineering and Architecture, Yeditepe University, 26 Ağustos Campus, Kayisdagi Cad., Kayisdagi, 34755 Istanbul, Turkey

[2] Department of Pediatric Surgery, School of Medicine, Bahçeşehir University, Istanbul, Turkey

Keywords Neurogenesis · Osteogenesis · Adipogenesis · Chondrogenesis · Human newborn foreskin stem cell

Introduction

A major part of recent attention has focused on the probability that adult human stem cells are a practical therapeutic option to embryonic stem cells. This curiosity has improved highly regarding to the recent studies, indicating that different adult stem cells show efficient varied differentiation potentials [15, 32].

Circumcision, performed due to cultural, medical, or religious aspects, is defined as the removal of foreskin tissue either partly or fully. The surgery is very easily carried out when applied by professionals with sterile settings and suitable pain management. Male circumcision implemented through the newborn period carries significantly lower complication ratios than when applied in subsequent periods in life [6, 21].

Throughout the third month of intraurine life, a twist of skin evolves at the bottom of the glans penis. Skin enlarges distally and transforms into the foreskin. Under the presence of androgens, cells begin to keratinize, disperse, and locate among the prepuce. This is mainly unfinished at birth and continues during childhood. These cells may carry significant treatment potentials for several diseases [30].

In this current study, we aimed to investigate the stem cell properties of these cells. Therefore, there have been other studies involving the human foreskin cells. One of them has covered the generation of induced pluripotent stem cells (iPS cells). As known, embryonic stem (ES) cells, originated from inner cells of blastocytes carry the capability to grow for an indefinite period while maintaining their pluripotent state. These features have directed the prospects that human ES cell may be beneficial to identify disease mechanisms, to monitor efficient and harmless drugs and to treat patients of different diseases and problems like spinal cord injury and diabetes. Therefore, human induced pluripotent (iPS) cells were defined as similar to ES cells as in proliferation, morphology, gene expression, surface antigen, and telomerase activity profiles. In addition, iPS cells can also turn into cell lineages of three germ layers in vitro and in teratoma structures. These outcomes indicated that iPS cells can be produced from human fibroblasts. Recently, fibroblasts from neonatal foreskin tissues were obtained for the generation of iPS cells. Studies have demonstrated the creation of iPS cells from human foreskin fibroblasts with the same factors as human ES cells. These factors have identified as Oct3/4, Sox2, Klf4, and c-Myc [25, 33].

A different study was performed for describing the properties of human foreskin as feeder cell lines to human ES cells. Researchers have shown that human ES cells protected their stem cell features such as immortality, pluripotency, and proliferation capacity after enhanced culture of 70 passages which was approximately 250 doublings. The main benefit of foreskin feeder cells has defined as their potential to be cultured for at least 42 passages, thus facilitating correct examination for unknown agents and genetic alteration like antibiotic resistance covered in the long-lasting preparation of new feeder lineages [1, 12].

Foreskin cells have also been utilized for tissue engineering as well. Different cell types and tissue forms have been suggested as initiating material for the process. Allogeneic skin grafts suitable for therapeutic applications have mainly developed via cell culture and foreskin cells of young subjects. The dermal keratinocytes and fibroblasts have been operated in products like Dermagraft, Apligraft, and TransCyte. Clinical outcomes have showed that these products

were not enough for wound healing fully but were used for wound surface while expecting for mesh grafting [11, 24].

The migration potentials of human foreskin cells have been examined by other studies. Fibronectin can be defined as a glycoprotein with a high density, which is found in an insoluble type at the fibroblast surfaces. Results have specified that the existence of fibronectin inhibits the migrating capability of human foreskin cells through matrix. These data also have given information about the cell-cell interactions and cell-matrix interactions of human foreskin cells [8, 22].

There have been trials to investigate foreskin cells as stem cell reservoirs. The present study showed the isolation of stem cells from human newborn foreskin tissue, characterization of their stem cell properties, and differentiation potentials for the first time. Results indicated that stem cells were located among prepuce and carry enormous potentials for differentiating into ectoderm-, mesoderm-, and endoderm-originated cell lineages. These outcomes suggested that obtaining the foreskin tissue via circumcision from newborn and storing them for future disease progression possibilities can be used for treatment procedures.

Method

Isolation and Culture Conditions of Human Foreskin Stem Cells

Human foreskin stem cells were collected from the newborn foreskin (prepuce) tissue in Yeditepe University/Turkey Biotechnology Laboratories. The collected tissues were harvested and minced. The foreskin tissue was plated in 6-well plates (BIOFIL, TCP, Switzerland) and grown to confluency in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % (*v*/*v*) fetal bovine serum (FBS) and 1 % (*v*/*v*) PSA (10,000 units/mL penicillin, 10,000 μg/mL streptomycin, 25 μg/mL amphotericin B) (Invitrogen, Gibco, UK). The harvested cells started to appear after 3–4 days and covered the surface after 8 days. Thereafter, the cells were trypsinized using 0.25 % (*v*/*v*) trypsin/EDTA (Invitrogen, Gibco, UK). A medium was added to detached cells to inhibit the activity of trypsin. After the cells were centrifuged at 300×*g* for 5 min at room temperature, the pellet was dissolved in fresh medium and seeded on a T-75 flask (Zelkultur Flaschen, Switzerland). The cells were maintained at 37 °C and 5 % CO_2 in a humidified incubator. Cells from passages 3~4 were used in all experiments.

Characterization of Human Foreskin Stem Cells

Human newborn foreskin stem cells (hnFSSCs) were characterized according to the protocol described previously by our group [27]. Cells were trypsinized and incubated with the primary antibodies which were prepared in PBS. For characterization, primary antibodies against CD14 (ab82434), CD31 (ab27333), CD34 (ab18227), CD44 (ab58754), CD45 (ab134202), CD73 (ab157335), CD90 (ab95700), CD105 (ab53321), Integrin beta 1 (ab27314) (Abcam, UK), and CD29 (Zymed, San Francisco, CA, USA) were used. The cells were incubated with fluorescein-iso-thio-cynate (FITC) or phyco-erythrin (PE)-conjugated antibodies at 4 °C for 1 h. The flow cytometry analysis of the cells was performed by using Becton Dickinson FACS Calibur flow cytometry system (Becton Dickinson, San Jose, CA, USA).

Differentiation Process

hnFSSCs were induced to differentiate osteogenic, neurogenic, adipogenic, chondrogenic, and epithelial cell types. The cells were seeded on a 6-well plate for RNA isolation and 48-well plate (BIOFIL, TCP, Switzerland) for immunocytochemistry at a density of 150×10^3 cells/well and 10×10^3 cells/well, respectively, for each differentiation. Differentiation media contents are shown in Table 1.

Alkaline Phosphatase Activity Assay

Alkaline phosphatase (ALP) activity was determined to demonstrate osteogenic differentiation. After the process of osteogenic differentiation, medium was removed and cells were treated with 0.2 % (*w*/*v*) Triton X-100 (BioBasic Inc., Canada) for lysis. Cells were collected from plates and mixed by vortex for 20 min at room temperature. Thereafter, 10 μL of cell lysate was added to each well of a 96-well plate followed by the addition of 90 μL of ALP solution (BioAssay Systems, USA). After the incubation for 15 min, the absorbance at 405 nm was measured by an ELISA plate reader [26].

Alizarin Red Staining

Alizarin red was conducted to detect calcium deposits. Cells were seeded on 24-well plates at a concentration of 20,000 cells/well. At the end of osteogenic differentiation, the medium from cells was removed and the cells were washed three times with PBS. Then, the cells were incubated with 100 % methanol for 20 min for fixation. After the addition of Alizarin red, cells were incubated for 15 min. After washing the cells three times with dH_2O, the cells were observed under a light microscope (Zeiss Primo Vert,Germany) [10].

Oil Red O Staining

Oil red oil solution was prepared by dissolving 0.5 g oil red oil (Sigma, USA) in 100 mL isopropanol. The cells were then fixed with 2 % (*w*/*v*) paraformaldehyde for 30 min followed by washing three times with PBS. Incubation with oil red oil solution diluted 6:4 in PBS for 1 h was performed for staining. Cells were washed with PBS and observed under a light microscope [9].

Alcian Blue Staining

One gram Alcian blue dye (Sigma, USA) was dissolved in 100 mL of 3 % (*w*/*v*) acetic acid to prepare an Alcian blue staining solution. The cells were fixed with 2 % paraformaldehyde for 30 min and incubated with Alcian blue staining solution for 30 min. After the incubation, the cells were washed three times with PBS and the samples were observed under a light microscope [28].

Immunocytochemistry

Immunocytochemistry analyses were completed according to the previously described protocol [27]. At the end of the differentiation procedures of hnFSSCs, the cells were incubated with

Table 1 Differentiation medium contents for each differentiation processes of human newborn foreskin stem cells

Differentiation list	Differentiation medium content	Days
Osteogenic differentiation	Dulbecco's modified Eagle's medium–low glucose (DMEM-LG; Life Technologies, Gaithersburg, MD, USA) supplemented with 10 % fetal bovine serum (FBS; Sigma, St. Louis, MO, USA) and 1 % antibiotic antimycotic solution (Life Technologies), dexamethasone (0.1 μmol/L), ascorbic acid (0.05 mmol/L), and glycerophosphate (10 mmol/L)	21
Chondrogenic differentiation	DMEM-LG (Life Technologies, Gaithersburg, MD, USA) supplemented with 10 % FBS (Sigma, St. Louis, MO, USA) and 1 % antibiotic antimycotic solution (Life Technologies), insulin (6.25 μg/mL), transferrin (6.25 μg/mL), linolenic acid (5.33 μg/mL), bovine serum albumin (1.25 mg/mL), pyruvate (1 mmol/L), ascorbate-2-phosphate (0.17 mmol/L), dexamethasone (0.1 mol/L)	7
Adipogenic differentiation	DMEM-LG (Life Technologies, Gaithersburg, MD, USA) supplemented with 10 % FBS (Sigma, St. Louis, MO, USA) and 1 % antibiotic antimycotic solution (Life Technologies), 1-methyl-3 isobutylxanthine (0.5 mmol/L), dexamethasone (1 μmol/L), insulin (10 μg/mL), and indomethacin (100 μmol/L)	14
Neurogenic differentiation	DMEM-LG (Life Technologies, Gaithersburg, MD, USA) supplemented with 10 % FBS (Sigma, St. Louis, MO, USA) and 1 % antibiotic antimycotic solution (Life Technologies), 0.5 mM isobutyl methyl xanthin (IBMX; Sigma Chemical Co), 1 mM dibutyryl-cAMP (dbcAMP; Sigma Chemical Co), and 10 mM retinoic acid (Sigma Chemical Co)	10
Epithelial differentiation	DMEM-LG (Life Technologies, Gaithersburg, MD, USA) supplemented with 10 % FBS (Sigma, St. Louis, MO, USA) and 1 % antibiotic antimycotic solution (Life Technologies),10 ng/mL kerstinocyte growth factor (KGF), 25 ng/mL epidermal growth factor (EGF), 10 ng/mL hepatocyte growth factor (HGF), 60 ng insulin-like growth factor 2 (IGF 2)	21

2 % (*w*/*v*) paraformaldehyde for 30 min at 4 °C for fixation and permeabilized with 0.1 % (*v*/*v*) Triton X-100 for 5 min and prepared in PBS. The cells were incubated with 2 % (*v*/*v*) goat serum (Sigma, USA) for 20 min at 4 °C for preventing nonspecific binding of primary antibodies. The cells were again washed three times with PBS. The cells were incubated overnight with primary antibodies at 4 °C.

For osteogenic differentiation, collagen type 1 (COL1A; ab96723, Abcam, UK) and osteocalcin (OCN; sc-30044, Santa Cruz Biotechnology, TX, USA) were used. For neurogenic differentiation, enolase (sc-59536, Santa Cruz Biotechnology, TX, USA), nestin (sc-20978, Santa Cruz Biotechnology, TX, USA), neurofilament (sc-25652, NF; Santa Cruz Biotechnology, TX, USA), and tyrosine hydroxylase (TH; sc-53456, Santa Cruz Biotechnology, TX, USA) were used. For adipogenic differentiation, adipocyte protein 2 (aP2; sc-635, Santa Cruz Biotechnology, TX, USA) and PPAR-γ (ab19481, Abcam, UK) were used. For chondrogenic differentiation, collagen type 2 (COL2A; (ab3092, Abcam, UK) was used.

The cells were washed three times with PBS to remove the excess antibody after incubating with primary antibodies. Thereafter, the cells were treated with secondary antibodies (goat antirabbit IgG Alea Fluor 488, goat antimouse IgG Alea Fluor 488) and incubated for 1 h at 4 °C followed by rinsing three times with PBS. 4′,6-Diamidino-2-phenylindole (DAPI) (AppliChem, Germany) was used to stain the nuclei of the cells by incubating for 20 min at

4 °C. The cells were then rinsed three times with PBS and observed under fluorescence microscope (Nicon Eclipse TE200).

Quantitative Real-Time PCR

Total RNA isolation from differentiated cells was performed using High Pure RNA Isolation Kit (Roche, Germany) according to the manufacturer's instructions. Complementary DNA (cDNA) synthesis from isolated RNA samples was done using High Fidelity cDNA Synthesis Kit (Roche, Germany). Real-time PCR was performed using Maxima SYBR Green/ROX (Fermentas, USA) for the determination of expression levels of marker genes after differentiations [29]. cDNAs of the differentiated cells were used as template and were mixed with primers and Maxima SYBR Green/ROX qPCR Master Mix (2X). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as the house-keeping gene for the normalization of the data. The results of real-time PCR data were normalized to the mRNA level of GAPDH. Primer sequences for marker genes are shown in Table 2**.**

Statistical Analysis

All data are shown as means ± standard errors (SD). Graphics were drawn using GraphPad Prism 5 software (GraphPad Prism, USA). The statistical analysis of the results were performed by one-way ANOVA followed by multiple-comparison Turkey's test using GraphPad Prism 5 software. Statistical significance was determined at $P < 0.05$.

Results

Cell Culture of Human Foreskin Stem Cells

Mesenchymal stem cells were isolated regarding the standard procedures for the isolation of monolayer cells from human newborn foreskin tissue. Light microscopy from cells in passage 0 indicated a fibroblast-homologous, spindle-formed morphology (Fig. 1a). In passage 3, the spindle-shaped cells began to show a widened, horizontal morphology (Fig. 1b).

Flow Cytometry Analysis of Human Foreskin Stem Cells

Flow cytometry analysis demonstrated that hnFSSCs expressed mesenchymal markers as CD29, CD44, CD73, and CD90. They were also identified as positive for the hematopoietic markers like CD14, CD34, and CD45. hnFSSCs was found negative for CD31 which was identified as an endothelial surface marker [20] (Fig. 2).

Differentiation of Human Foreskin Stem Cells into Adipogenic Cells in vitro

The potential of human foreskin stem cell differentiation into adipocytes, chondrocytes, osteocytes, epithelial, and neural cells is examined in this present study. After 14 days of culture with an adipogenic-stimulating medium, oil red O-positive lipid groups are found in hnFSSC cultures with adipogenic differentiation medium. Negative control cultures that only included DMEM-LG, supplemented with 10 % FBS and 1 % antibiotic antimycotic solution

Table 2 List of primers used in quantitative RT-PCR

Marker	Sequences (5′-3′)	Ref.
OCN	GTGCAGAGTCCAGCAAAGGT	[27]
	TCAGCCAACTCGTCACAGTC	
ALP	GACATCGCCTACCAGCTCAT	
	TCACGTTGTTCCTGTTCAGC	
COL1A	CCACGCATGAGCGGACCCTAA	
	ATTGGTGGGATGTCTTCGTCTTGG	
ON	ATGAGGGCCTGGATCTTCTT	[9]
	CTGCTTCTCAGTCAGAAGGT	
BGN	CAGCCCGCCAACTAGTCA	[23]
	GGCCAGCAGAGACACGAG	
Nestin	GGAGTCCTGGATTTCCTTCC	
	GCCCTGACCACTCCAGTTTA	
CD133	GCCAGCCTCAGACAGAAAAC	
	CCAAGCCTTAGGAGCATCTG	
NF	GTGACCAAGCCCGACCTTT	
	ATTCCTCAGCGTTCTGCATGT	
Adiponectin	TATCCCCAACATGCCCATTCG	[2]
	TGGTAGGCAAAGTAGTACAGCC	
PPAR-γ	CCTATTGACCCAGAAAGCGATT	[2]
	CATTACGGAGAGATCCACGGA	
LPL	ACAAGAGAGAACCAGACTCCAA	[2]
	AGGGTAGTTAAACTCCTCCTCC	
αP2	AACCTTAGATGGGGGTGTCCT	[2]
	TCGTGGAAGTGACGCCTTTC	
Sox9	GAACGCACATCAAGACGGAG	
	TCTCGTTGATTTCGCTGCTC	
Aggrecan	ACTGCTGCAGACCAGGAGGT	
	TCCTCGGGGGTGACGATGCT	
COL2A	GTGTGGAAGCCGGAGCCCTG	[28]
	GGTCCTGGTTGCCCACTGGC	
CY 18	AAATCCGGGAGCACTTGGAG	
	CAATCTGCAGAACGATGCGG	
CY 19	AGAGCTGGCCTACCTGAAGA	
	ACCAGGCTTCAGCATCCTTC	
GAPDH	TGGTATCGTGGAAGGACTCA	[16]
	GCAGGGATGATGTTCTGGA	

All the real-time PCR experiments were performed using iCycler RT-PCR detection system (Bio-Rad, Hercules, CA, USA)

Abbreviations: ALP alkaline phosphatase, *CD133* cluster of differentiation 133, *NF* neuroflament, *PPAR-γ* peroxisome proliferator-activated receptor gamma, *LPL* lipoprotein lipase, *αP2* adipocyte protein 2, *COL2A* collagen type 2, *BGN* byglican, *OCN* osteocalcin, *COL1A* collagen type 1, *Sox9* SRY-box-containing gene 9, *CY 18* cytoglobin 18, *CY 19* cytoglobin 19, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase.

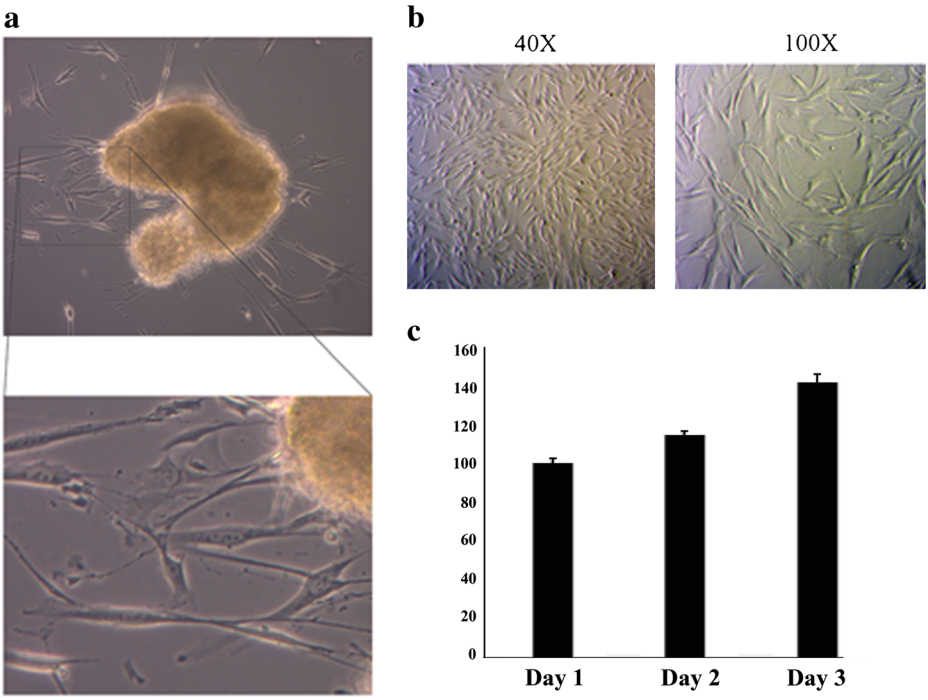


Fig. 1 Isolated human newborn foreskin stem cells (hnFSSCs). **a** Basal photomicrographic representation of hnFSSCs at day 3 of isolation, **b** hnFSSCs morphology at passage 3, and **c** cell viability at passage 3

did not verify lipid clusters (Fig. 3a). Immunocytochemistry experiment results for adipogenic differentiation were presented for two adipocyte-specific antibodies. Positive control cultures demonstrated fluorescently positive outcomes for both adiponectin and PPAR-γ antibodies; whereas, the negative control cultures demonstrated no immunopositive outcomes. Quantitative real-time polymerase chain reaction (qPCR) trials were also performed for four adipogenic differentiation indicator genes such as LPL, aP2, adiponectin, and PPAR-γ (Fig. 3b). Genes encoding transcription factors involved in mesenchymal differentiation into adipocyte were found to be expressed. Positive control LPL, aP2, and adiponectin gene expressions were situated two times more upregulated than the negative control LPL gene expressions; whereas, the positive control PPAR-γ expression was identified to be approximately three times more overexpressed than the negative control (Fig. 3c). These experiments pointed out that hnFSSCs turned into adipocytes in vitro successfully.

Differentiation of Human Foreskin Stem Cells into Chondrogenic Cells in vitro

Seven days of culture with a chondrogenic-stimulating medium were established. Alcian blue-stained regions were detected, representing the existence of acidic proteoglycans, suggestive for cartilaginous tissue. Negative control cultures that only included DMEM-LG, supplemented with 10 % FBS and 1 % antibiotic antimycotic solution showed very few acidic proteoglycans (Fig. 3d). Immunocytochemistry experiment results for chondrogenic differentiation were displayed for two chondrocyte-specific antibodies. Positive control cultures signified fluorescently highly positive results for Col2A antibody; whereas, the negative control cultures

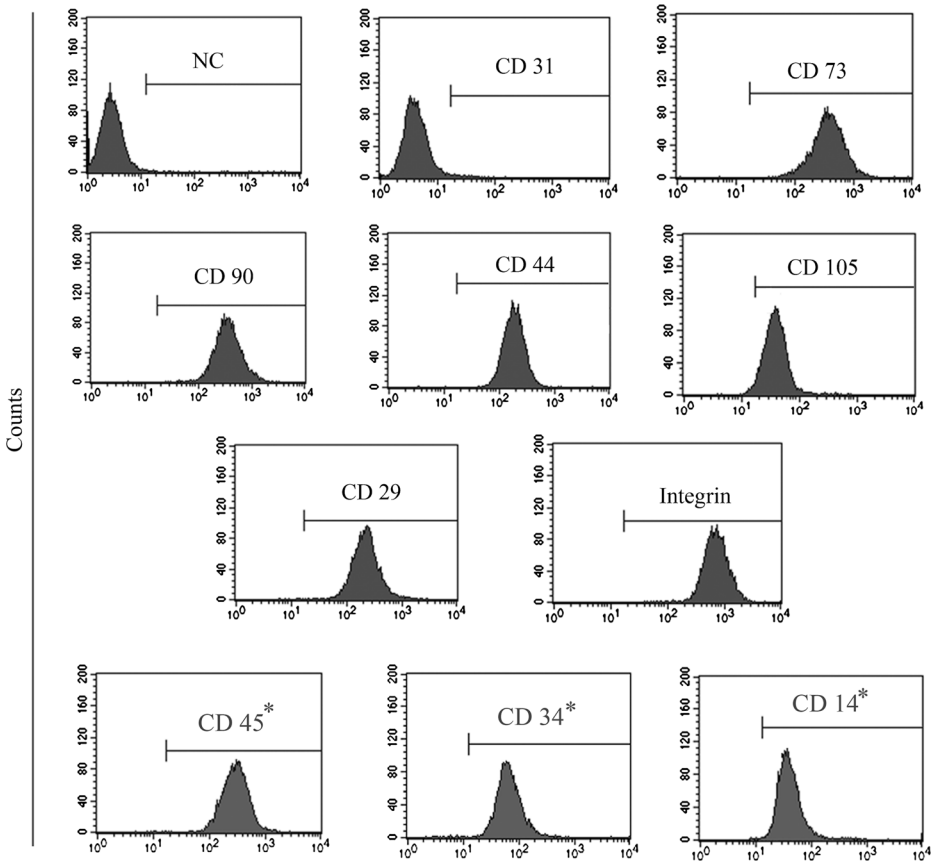


Fig. 2 Mesenchymal stem cell characterization of human newborn foreskin stem cells at passage 3. Major population of cells maintained the characteristics of stem cells. Flow cytometry analysis data shows the positive mesenchymal stem cell surface markers CD29, CD44, CD73, and CD90 and the negative endothelial stem cell surface marker CD31. Data also indicates the positive hematopoietic stem cell surface markers CD45, CD34, and CD14. *NC* negative control

pointed less immune-positive outcomes (Fig. 3e). Quantitative real-time trials were also implemented for chondrogenic differentiation indicator genes such as Col2A, Sox2, and ACAN. Genes covered in mesenchymal differentiation into chondrocyte were detected to be expressed. Positive control Col2A, Sox2, and ACAN gene expressions illustrated to be two times more upregulated than the negative control gene expressions (Fig. 3f). Chondrogenic differentiation of hnFSSCs was completed effectively by these tests.

Differentiation of Human Foreskin Stem Cells into Osteogenic Cells in vitro

Osteogenic differentiation of hnFSSCs was also investigated. Twenty-one days of culture with an osteogenic-inducing medium were assigned. Alizarin red-stained regions were detected, on behalf of the existence of matrix mineralization. Negative control cultures certified no matrix mineralizations (Fig. 3g). ALP activity tests were also applied for proving the osteogenic differentiation. Related studies showed that ALP upsurges if there is active bone formation [17]. ALP activity was found to be doubled in differentiated foreskin cell (DF) cultures when

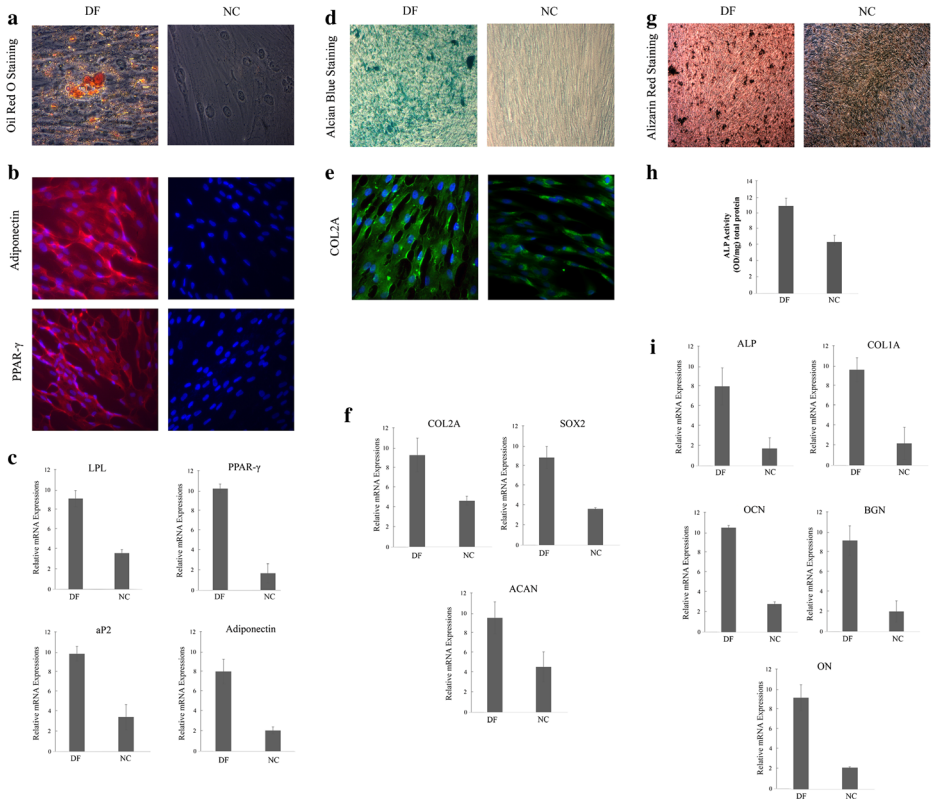


Fig. 3 Mesoderm-derived adipo-, chondro-, and osteogenic differentiation of hnFSSCs. **a–c** Adipogenic differentiation (oil red O staining, immunocytochemistry, and qPCR). **d–f** Chondrogenic differentiation (Alcian blue staining, immunocytochemistry, qPCR). **g–i** Osteogenic differentiation (Alizarin red staining, ALP activity, qPCR). *NC* negative control (growth medium-applied group), *DF* differentiated foreskin cells, *ALP* alkaline phosphatase, *CD133* cluster of differentiation 133, *NF* neuroflament, *PPAR-γ* peroxisome proliferator-activated receptor gamma, *LPL* lipoprotein lipase, *αP2* adipocyte protein 2, *COL2A* collagen type 2, *BGN* byglican, *OCN* osteocalcin, *COL1A* collagen type 1, *Sox9* SRY-box-containing gene 9, *CY 18* cytoglobin 18, *CY 19* cytoglobin 19, *GAPDH* glyceraldehyde 3-phosphate dehydrogenase, *qPCR* quantitative real-time polymerase chain reaction; $^{*}p < 0.05$ versus NC

compared with the NC cultures. According to this information, osteogenic differentiation of hnFSSCs was detected to have been positively developed (Fig. 3h). Quantitative real-time trials were also employed for osteogenic differentiation pointer genes such as ALP, Col1A, OCN, BGN, and ON. These genes involved in mesenchymal differentiation into osteocyte were detected to be expressed. Positive control ALP, Col1A, OCN, BGN, and ON gene expressions were explained to be three times more upregulated than the negative control gene expressions (Fig. 3i). These results suggested that osteogenic differentiation of hnFSSCs has finalized significantly.

Differentiation of Human Foreskin Stem Cells into Epithelial Cells in vitro

In this current study, differentiation of hnFSSCs into human epithelial cells was also examined. Quantitative real-time trials engaged for epithelial differentiation pointer genes CY 18 and CY

19. Genes contained within mesenchymal differentiation into epithelial cells were detected. Positive control CY 18 and CY 19 gene expressions were illustrated to be about two times more upregulated than the negative control gene expressions (Fig. 4). These results suggested that epithelial differentiation of hnFSSCs may be achieved in vitro.

Differentiation of Human Foreskin Stem Cells into Neurogenic Cells in vitro

Immunocytochemistry experiment results for neurogenic differentiation were exhibited for two neurogenesis-specific antibodies. Positive control cultures signified fluorescently positive results for NF, MAP2, TH, enolase, and nestin antibodies (Fig. 5a). Quantitative real-time trials were also implemented for neurogenic differentiation indicator genes such as NF, nestin, and CD133. Genes enclosed in mesenchymal differentiation into neurons and glial cells were detected. Positive control NF, nestin, and CD133 gene expressions were exemplified to be three times more upregulated than the negative control gene expressions (Fig. 5b). Neurogenic differentiation results of hnFSSCs were accomplished efficiently by these tests.

Discussion

Since circumcision takes a major role in preventing several sexual diseases and is being applied by different cultures, the waste tissue from the surgical operation can be maintained and can be used for treatment models. Recent studies have shown that newborn prepuce tissue development lasts for a while even after birth [7]. According to the hypothesis that pluripotent stem cells persevere even after birth in numerous organs and that when stimulated they proliferate and differentiate in response to native signals delivered by the organ they are employed to [31], we aimed to examine the cells of newborn prepuce. In this current study, human foreskin stem cells managed to differentiate into all three germ layer cell lines. In our perspective, hnFSSCs utilization may be considered as a more beneficial therapy than other adult stem cell lines and human embryonic stem (hES) cells. Regarding ethical cases and the chances of teratoma development, human embryonic stem cell utilization carries limitations. Attainability of the hES cells can be listed as one of them. In contrast, the new stem cell source

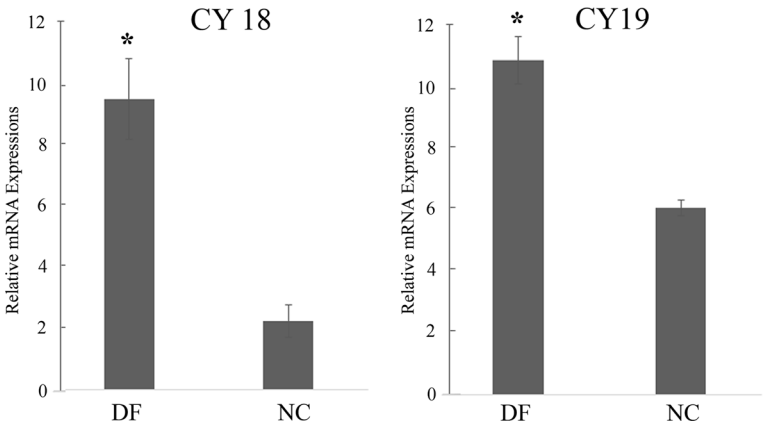
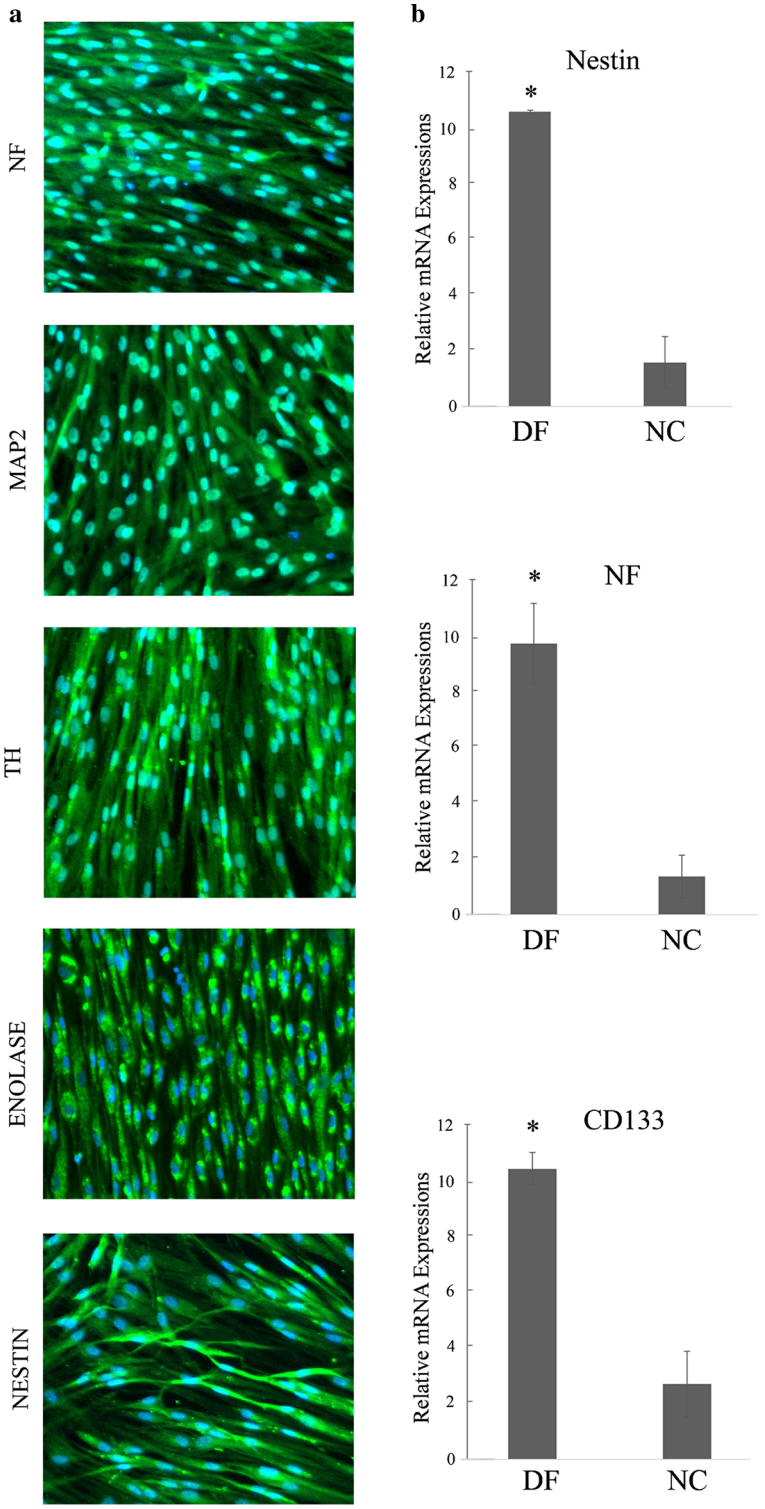


Fig. 4 Endoderm-derived epithelial differentiation of hnFSSCs. Quantitative real-time PCR of differentiated samples. *DF* differentiated foreskin cells, *NC* negative control (growth medium-applied group). *$p < 0.05$ versus NC


a
NF
MAP2
TH
ENOLASE
NESTIN
b
Nestin
Relative mRNA Expressions
12
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◀ **Fig. 5** Ectoderm-derived neurogenic differentiation of hnFSSCs**. a** Immunocytochemistry imsges of differentiated cells**, b** quantitative real-time PCR of differentiated samples. *DF* differentiated foreskin cells, *NC* negative control (growth medium-applied group); *$p < 0.05$ versus NC

human foreskin tissue was easily accessible. Therewith, hnFSSCs showed no differentiation into cancer cells. On the other hand, human adult stem cells display restrictions in differentiation capabilities. These disadvantages of the other stem cell reservoirs make hnFSSCs more substantial.

Optimization experiments of our study indicated that newborn prepuce carries cells with stem cell (especially hematopoietic characteristic) features, but with the age factor, differentiation potentials of the cells decrease (Electronic Supplementary Material). This may be related to several reasons such as varied gene expressions.

Throughout the isolation process, we have not used any chemicals, because of the risk of harming the cells. Conversely, other studies that carry stem cell isolations mostly have shown that chemicals need to be used [19]. Our colorimetric cell viability assay data indicated that cell viability increased throughout the three consecutive days (data not shown). This situation suggested that human foreskin stem cells were healthy and proliferating as expected. With respect to the differentiation outcomes, human foreskin stem cells carried a huge potential of turning into endodermal, ectodermal, and mesodermal cell lineages. Normally, mesenchymal stem cells can be described by their capability to differentiate into osteoblasts, chondrocytes, and adipocytes [4, 5]. Therefore, as a new mesenchymal stem cell source, we demonstrated that hnFSSCs can also differentiate into neurogenic and epithelial cells. These headways suggested that hnFSSCs can also be used for the diseases involving neuronal loss or neuronal degeneration such as Parkinson's or Alzheimer's disease [3]. At the meantime, hnFSSCs may also be a possible treatment source for skin burns with respect to their epithelial differentiation capability in vitro. Bone marrow stem cells have been shown as a model for treating the skin burns before [18], but our experiments displayed that foreskin can be defined as much of a strong resource as the bone marrow.

Another important point of this recent study was the difference of hnFSSCs surface markers from the other mesenchymal cell lineages. Normally, mesenchymal stem cells express no hematopoietic stem cell surface markers [13, 14]. However, our flow cytometry experiment indicated an opposite outcome even when repeated for three times. Cells also were separated by fluorescent-activated cell sorting, so that only hnFSSCs were used for further experiments (data not shown).

As future prospects, hnFSSCs may be considered as an easily-accessible source for mesenchymal stem cells and may be used for all three germ line diseases. Since these cells differentiated into adipocytes, chondrocytes, osteocytes, neurons, and epithelial cells, we have proved that human foreskin tissue holds a potential which can compete with cord blood or other mesenchymal stem cell sources such as bone marrow. Other than these advantages, the human foreskin tissue can be obtained easily by circumcision operation. Generally, the waste circumcised tissues are mostly supposed to be thrown away. Therefore, with this study of ours, these tissues were suggested to be maintained in licensed facilities for future utilizations.

As stated, hematopoietic stem cell surface markers were also expressed by hnFSSCs. This data indicated that hnFSSCs may be a potential treatment source for different cancer types of the blood such as leukemia and multiple myeloma. hnFSSCs can also be used for other blood diseases and immunological deficiencies. Regarding the fact that these cells actually express both mesenchymal and hematopoietic stem cell surface markers, they can be utilized for numerous diseases.

In summary, hnFSSCs can be thought as dynamic and precious stem cell sources for a great number of diseases if it is isolated from a newborn prepuce tissue. According to the situation, this present study suggests that circumcision right after birth, specifically in the first month, can be very beneficial for future complications.

Acknowledgments We would like to thank Burçin Keskin for her help. The authors deny any conflicts of interest.

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