Video Article

Isolation, Characterization and MicroRNA-based Genetic Modification of Human Dental Follicle Stem Cells

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Abstract

To date, several stem cell types at different developmental stages are in the focus for the treatment of degenerative diseases. Yet, certain aspects, such as initial massive cell death and low therapeutic effects, impaired their broad clinical translation. Genetic engineering of stem cells prior to transplantation emerged as a promising method to optimize therapeutic stem cell effects. However, safe and efficient gene delivery systems are still lacking. Therefore, the development of suitable methods may provide an approach to resolve current challenges in stem cell-based therapies.

The present protocol describes the extraction and characterization of human dental follicle stem cells (hDFSCs) as well as their non-viral genetic modification. The postnatal dental follicle unveiled as a promising and easily accessible source for harvesting adult multipotent stem cells possessing high proliferation potential. The described isolation procedure presents a simple and reliable method to harvest hDFSCs from impacted wisdom teeth. Also this protocol comprises methods to define stem cell characteristics of isolated cells. For genetic engineering of hDFSCs, an optimized cationic lipid-based transfection strategy is presented enabling highly efficient microRNA introduction without causing cytotoxic effects. MicroRNAs are suitable candidates for transient cell manipulation, as these small translational regulators control the fate and behavior of stem cells without the hazard of stable genome integration. Thus, this protocol represents a safe and efficient procedure for engineering of hDFSCs that may become important for optimizing their therapeutic efficacy.

Video Link

The video component of this article can be found at https://www.jove.com/video/58089/

Introduction

The human dental follicle is a loose ectomesenchymally-derived connective tissue surrounding the developing tooth[1,2]. Beside its function to coordinate osteoclastogenesis and osteogenesis for the tooth eruption process, this tissue harbors stem and progenitor cells especially for the development of the periodontium[3,4,5]. Therefore, the dental follicle is considered as an alternative source to harvest human adult stem cells[6,7].

Several studies demonstrated that human dental follicle stem cells (hDFSCs) are capable of differentiating into the periodontal lineage including osteoblasts, ligament fibroblasts and cementoblasts[8,9,10]. Furthermore, these cells were shown to match all characteristics of mesenchymal stromal cells (MSCs) including self-renewing capacity, plastic adherence, expression of specific surface markers (e.g., CD73, CD90, CD105) as well as osteogenic, adipogenic and chondrogenic differentiation potential[11,12,13]. Other studies also revealed a neural differentiation potential of hDFSCs[2,14,15,16,17,18].

Due to their promising properties and easy access, hDFSCs became recently relevant for tissue engineering[19,20,21]. The first studies concentrated on the potential of DFSCs to regenerate bone, periodontal and tooth roots[19,22,23,24,25,26,27,28,29,30]. Since the knowledge of the neurogenic capability of hDFSCs, their application as potential treatment for neurodegenerative diseases has been investigated[31,32,33]. HDFSCs have also gained importance with respect to the the regeneration of other tissues (*e.g.*, corneal epithelium)[34,35]. The therapeutic potential of hDFSC is not only based on their direct differentiation potential but also on their paracrine activity. Recently, hDFSCs have been shown to secrete a wealth of bioactive factors, such as matrix metalloproteinases (MMPs), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF) and hepatocyte growth factor (HGF), which play a crucial role for angiogenesis, immunomodulation, extra cellular matrix remodeling and reparative processes[36].

However, broad clinical translation of stem cell therapy is still impaired by several challenges, such as massive initial cell death and low beneficial stem cell effects[37,38]. Genetic engineering provides a promising strategy to address these challenges and therefore can greatly

enhance the therapeutic efficacy of stem cells[38,39,40]. For transient cell manipulation, microRNAs (miRs) are suitable candidates, as these small translational regulators control the fate and behavior of stem cells without the hazard of stable genome integration[41,42,43]. To date, several beneficial miRs have been identified promoting stem cell proliferation, survival, homing, paracrine activity as well as their differentiation into several lineages[44]. For instance, miR-133a engineered MSCs showed an increased survival and engraftment in infarcted rat hearts resulting in an improved cardiac function when compared to unmodified MSCs[45]. Likewise, miR-146a overexpressing MSCs were shown to secrete higher amounts of VEGF which in turn led to an enhanced therapeutic efficiency in ischemic tissue[46].

This manuscript presents a detailed protocol for the selective extraction and genetic engineering of hDFSCs. For this purpose, we described the harvesting and enzymatic digestion of human dental follicles as well as the subsequent isolation of hDFSCs. In order to characterize isolated cells, important instructions for the verification of MSC properties have been included in accordance with the guidelines of the International Society for Cellular Therapy[13]. In addition, we provide a detailed description for the generation of miR-modified hDFSCs by applying a cationic lipid-based transfection strategy and the evaluation of transfection efficiency and cytotoxicity.

Protocol

HDFSCs are isolated from the dental follicles of extracted wisdom teeth provided by the Department of Oral and Maxillofacial Plastic Surgery of the Rostock University Medical Center. Informed consent and written approval was obtained from all patients. This study was authorized by the local ethics committee of the University of Rostock (Permission No. A 2017-0158).

1. Isolation of hDFSCs

NOTE: To prevent bacterial contamination, wisdom teeth should not be erupted before extraction

1. **Preparation of required solutions**
    1. Prepare phosphate-buffered saline (PBS)/Penicillin-Streptomycin-Glutamine (P-S-G) solution: mix 495 mL of PBS with 5 mL of P-S-G solution. Store aliquots of 50 mL at 4 °C.
    2. Prepare hDFSC culture medium: mix 445 mL of basal medium with 5 mL of antibiotic agent and 50 mL of fetal bovine serum (FBS). Store at 4 °C.
    3. Prepare Collagenase type I stock solution (30 mg/mL): dilute 300 mg of Collagenase type I in 10 mL of basal medium supplemented with 1% antibiotic agent. Vigorously mix the solution. Filter the solution using a 0.2 µm filter. Store aliquots of 500 µL of Collagenase type I stock solution at -20 °C.
    4. Prepare Dispase II stock solution (40 mg/mL): dilute 40 mg of Dispase II in 10 mL of basal medium supplemented with 1% antibiotic agent. Vigorously mix the solution. Filter the solution using a 0.2 µm filter. Store aliquots of 500 µL of Dispase II stock solution at -20 °C.

2. **Surgical procedure**
    1. Inject a sufficient amount (maximum: 2 mL) of local anesthetic.
    2. Create and raise a three-sided mucoperiosteal flap. Raising a lingual flap is not required.
    3. Protect the lingual aspect with a periosteal elevator.
    4. Gently mill buccal and distal bone with a round hard metal burr.
    5. Loosen the tissue of the follicle with a periosteal elevator. Carefully remove the complete tooth (germ) and follicle by pulling out the crown (and follicle) with posterior (third-molar) forceps.
    6. Debride and irrigate the socket thoroughly with NaCl solution.
    7. Replace the mucoperiosteal flap with vicryl sutures (see **Table of Materials**).
    8. Remove the tooth follicle from the oral cavity and place them into an aliquot of 50 mL of PBS/P-S-G solution.
    NOTE: The sample can be stored at 4 °C until further processing.

3. **Enzymatic digestion of the tooth follicle**
    1. Pre-warm hDFSC culture medium and PBS/P-S-G solution to room temperature (RT).
    2. Thaw one aliquot of each Collagenase type I and Dispase II stock solutions. Prepare a digestion solution of 3 mg/mL Collagenase type I and 4 mg/mL Dispase II by adding 500 µL of each Collagenase type I and Dispase II stock solution to 4 mL of basal medium containing 1% antibiotic agent.
    3. Place extracted follicle in a sterile Petri dish and add 10 mL of PBS/P-S-G solution to wash the extracted tissue. Repeat this washing step twice.
    4. Mince the extracted follicle to pieces of about 1 mm x 1 mm with a sterile scalpel within the Petri dish containing 10 mL of PBS/P-S-G solution.
    5. Transfer minced tissue and PBS/P-S-G solution from the Petri dish into a 50 mL conical centrifuge tube. Wash the Petri dish with 10 mL of PBS/P-S-G solution and transfer the solution into the same tube.
    6. Centrifuge the conical centrifuge tube for 10 min at 353 x *g* at RT and discard the supernatant.
    7. Add 5 mL of digestion solution to the pelleted tissue. Gently mix the solution and the tissue. Incubate the mixture at 37 °C and 5% CO_2 for 2 h in a shaking incubator.
    8. Centrifuge digested cell/tissue suspension at 353 x *g* for 10 min at RT. Discard the supernatant and re-suspend the obtained pellet in 6 mL of hDFSC culture medium.
    9. Seed cell suspension in a 25 cm^2 cell culture flask and incubate cells at 37 °C, 5% CO_2 and 20% O_2.
    NOTE: If tissue is not completely digested, transfer the remaining tissue to the cell culture flask as well.
    10. Change medium carefully 24 h after cell seeding. Afterwards change medium every three days until confluency.
    NOTE: HDFSCs should be plastic-adherent 24 h after cell seeding and can be separated from non-adherent cells and blood components by simply changing the medium.

4. **Cell harvesting**
    1. Pre-warm hDFSC culture medium, PBS/P-S-G solution and Trypsin/Ethylenediaminetetraacetic acid (EDTA) solution to RT.
    2. Discard the supernatant from culture flask and wash confluent cells with 5 mL of PBS/P-S-G solution.
    3. Add 1 mL of Trypsin/EDTA solution to the culture flask and incubate for 3 min at 37 °C. Stop the digestion process by adding 3 mL of hDFSC culture medium to the culture flask.
    4. Transfer cell suspension into a 15 mL conical centrifuge tube and centrifuge the suspension for 10 min at 353 x *g* at RT. Discard the supernatant and re-suspend the pellet in an appropriate amount of hDFSC culture medium.
    5. Count cells: gently mix 10 µL of cell suspension with 10 µL of Trypan blue solution. Apply 10 µL into a counting chamber and calculate the hDFSC cell amount.

2. Characterization of hDFSCs

1. **Immunophenotyping**
    1. Preparation of cells for flow cytometric analysis
        1. Preparation of required solutions
            1. Prepare PBS/EDTA (2 mM): mix 996 mL of PBS with 4 mL of EDTA (0.5 M).
            2. Prepare staining buffer: mix 995 mL of PBS/EDTA (2 mM) with 5 g of BSA. Filter the solution using a 0.22 µm filter unit and store at 4 °C until usage.
            3. Prepare paraformaldehyde (PFA) stock solution (4%): dilute 4 g PFA in 100 mL of PBS and heat the solution to 80 °C. Mix the solution and adjust the pH value to 7.3. Aliquot obtained PFA solution in respective amounts (1.5 mL) and store at -20 °C until usage.
            CAUTION: Because PFA fumes are toxic, prepare the PFA solution in a ventilated fume hood.
        2. After cell harvesting (1.4), transfer 14 samples of 5 x 10^4 cells into 1.5 mL microcentrifuge tubes. Centrifuge the suspensions at 300 x *g* for 10 min at 4 °C. Discard the supernatant.
        NOTE: Perform the following work in a rather shaded room. Keep cells and reagents on ice unless stated otherwise.
        3. Re-suspend cells in certain amounts of staining buffer (4 °C) and add FcR blocking reagent (4 °C) as indicated in **Table 1**.
        4. Add the following antibodies onto the inner side of the respective sample as indicated in **Table 1**: Allophycocyanin (APC) mouse anti-human CD29; APC mouse IgG1 κ isotype control; Peridinin-chlorophyll protein (PerCP)-Cyanine5.5 mouse anti-human CD44; PerCP-Cyanine5.5 mouse IgG2b κ isotype control; V500 mouse anti-human CD45; V500 mouse IgG1 κ isotype control; Phycoerythrin (PE) mouse anti-human CD73; PE mouse IgG1 κ isotype control; PerCP-Cyanine5.5 mouse anti-human CD90; PerCP-Cyanine5.5 mouse IgG1 κ isotype control; mouse anti-human CD105: Alexa Fluor (AF)488; mouse IgG1 negative control: AF488; PE-Cyanine7 mouse anti-human CD117; PE-Cyanine7 mouse IgG1, κ isotype control. After antibodies have been added to each sample, spin down antibodies and mix gently. Incubate the solutions for 10 min at 4 °C.
        5. Add 1 mL of PBS (4 °C) and centrifuge the samples at 300 x g for 10 min at 4 °C. Discard the supernatant.
        6. Re-suspend cells in 100 µL of PBS (4 °C) and add 33 µL of PFA (4%). Mix the solutions and store on ice or at 4 °C until flow cytometric analysis.
    2. Flow cytometric measurement of cells
    NOTE: Perform the following work in a rather shaded room. Keep cells on ice until measurement.
        1. To examine the expression of surface antigens, transfer samples into tubes suitable for flow cytometric measurements.
        2. Measure at least 2 x 10^4 events using a flow cytometer. Analyze as depicted in **Figure 2**.

2. **Multipotent differentiation potential of hDFSCs**
    1. Use a commercially available Human Mesenchymal Stem Cell Functional Identification Kit to confirm adipogenic, osteogenic and chondrogenic differentiation potential of hDFSCs. Apply donkey anti-goat AF488 secondary antibody for staining of fatty acid binding protein 4 (FABP4) and aggrecan as well as donkey anti-mouse AF488 secondary antibody for staining of osteocalcin. For staining of nuclei, use mounting medium with 4',6-Diamidino-2-phenylindole (DAPI).
    NOTE: Prepare samples without primary antibody as negative controls for microscopic analyses.
    2. Analyse expression of proteins by laser scanning confocal microscopy. Microscopy settings: 40x objective with oil immersion; excitation laser 488 nm (for AF488) and 405 nm (for DAPI).

3. Transfection of hDFSCs

1. After cell harvesting (1.4), seed hDFSCs on a 24-well cell culture plate 24 h prior to transfection.
NOTE: Starting cell density is approximately 4 x 10^4 cells per well. Cells should reach ~80% confluence on day of transfection.
Note: Seed one additional cell sample as control for flow cytometric analysis.
2. Preparation of transfection complexes
NOTE: To prevent contamination from RNases, clean the workspace directly before preparation of transfection complexes using RNase decontamination solution. Use only RNase-free material and solutions.
NOTE: Perform the following work in a rather shaded room.
    1. Prepare miR stock solution (50 µM): re-suspend Cy3-labelled precursor miR (5 nmol) in 100 µL nuclease-free water. Aliquot obtained miR stock solution in respective amounts (5 µL) and store in the dark at -20 °C until usage.
    2. Dilute 40 pmol of miR (0.8 µL of the 50 µM miR stock solution) in 66.7 µL of reduced serum medium. Mix the solution gently
    3. Dilute 0.67 µL of cationic lipid-based transfection reagent in 66.7 µL of reduced serum medium. Mix the solution gently and incubate for 5 min at RT.

4. After incubation, add the pre-diluted transfection reagent to the pre-diluted miR. Mix the solution gently and incubate for 15 min at RT.

3. Add the prepared transfection complexes dropwise directly to the culture medium on cells. Mix gently by rocking the 24-well cell culture plate back and forth.
4. Incubate the cells at 37 °C, 5% CO_2, and 20% O_2 for 24 h.

4. Analysis of Transfection

NOTE: Perform the following work in a rather shaded room.

1. **Cell harvesting after transfection**
   NOTE: Collect all cell solutions of one sample in the same 15 mL conical centrifuge tube.
   1. 24 h after transfection, collect the supernatant of samples in respective centrifuge tubes.
   2. Wash cells with 1 mL of PBS, transfer PBS into the respective centrifuge tube and add 500 µL Trypsin/EDTA (RT) to cells. Incubate the solutions for 3 min at 37 °C.
   3. Stop trypsinization by adding 1 mL of culture medium (RT) to the cells and transfer the solution into the respective centrifuge tube. Centrifuge cells at 300 x *g* for 10 min at 4 °C.
      NOTE: From this time, keep cells and reagents on ice unless stated otherwise.

2. **Preparation of cells for flow cytometric analysis**
   1. Discard the supernatant. Re-suspend cells in 100 µL of staining buffer (4 °C) and transfer cell solution to a 1.5 mL microcentrifuge tube.
   2. Add 0.5 µL of amine reactive dye to the samples in order to distinguish between live and dead cells. Gently mix the solution and incubate for 10 min at 4 °C.
   3. Add 1 mL of PBS (4 °C) to cells and centrifuge at 300 x *g* for 10 min at 4 °C. Discard the supernatant.
   4. Re-suspend cells in 100 µL of PBS (4 °C) and add 33 µL of PFA (4%). Mix the solutions and store on ice or at 4 °C until flow cytometric analysis.

3. **Flow cytometric measurement of cells**
   NOTE: Perform the following work in a rather shaded room. Keep cells on ice until measurement.
   1. Transfer samples into tubes suitable for flow cytometric measurements.
   2. Examine cell viability and miR uptake efficiency using a flow cytometer. Measure at least 2 x 10^4 events. Use the gating strategy depicted in **Figure 4**.
      NOTE: Use untransfected cells as negative control to arrange the gating for Cy3 positive cells and to calculate cell death caused by transfection.

Representative Results

Here, we present a detailed isolation instruction to harvest hDFSCs from human dental follicle tissue. Due to the easy access of the dental follicle during routine surgery, it is a promising source for the extraction of adult stem cells.

The isolated hDFSCs showed all characteristics described for the definition of MSCs[13]. In fact, cells were plastic-adherent under described culture conditions and displayed a fibroblast-like morphology (**Figure 1**). Flow cytometric analyses revealed that hDFSCs expressed a panel of certain surface antigens, including CD29, CD44, CD73, CD90 and CD105, while CD45 and CD117 were absent (**Figure 2**). Moreover, the adipogenic, osteogenic and chondrogenic differentiation potential of cells under specific in vitro culture conditions was confirmed by immunostaining of fatty acid binding protein 4 (FABP4), osteocalcin and aggrecan (**Figure 3**).

The described cationic lipid-based transfection strategy enabled efficient transient genetic modification of hDFSCs with a miR-uptake in ~100% of viable cells 24 h post-transfection (**Figure 4B**). Moreover, transfected (**Figure 4A**) and untransfected (**Figure 4C**) samples showed comparable amounts of dead cells proving gentle cell processing conditions.

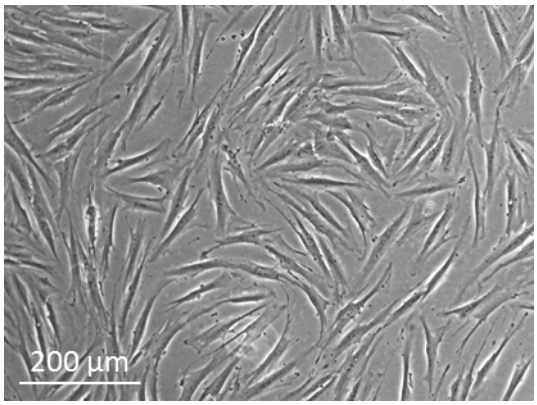

Figure 1: Representative light microscope picture of hDFSCs. Cells show a fibroblast-like morphology under standard culture conditions.

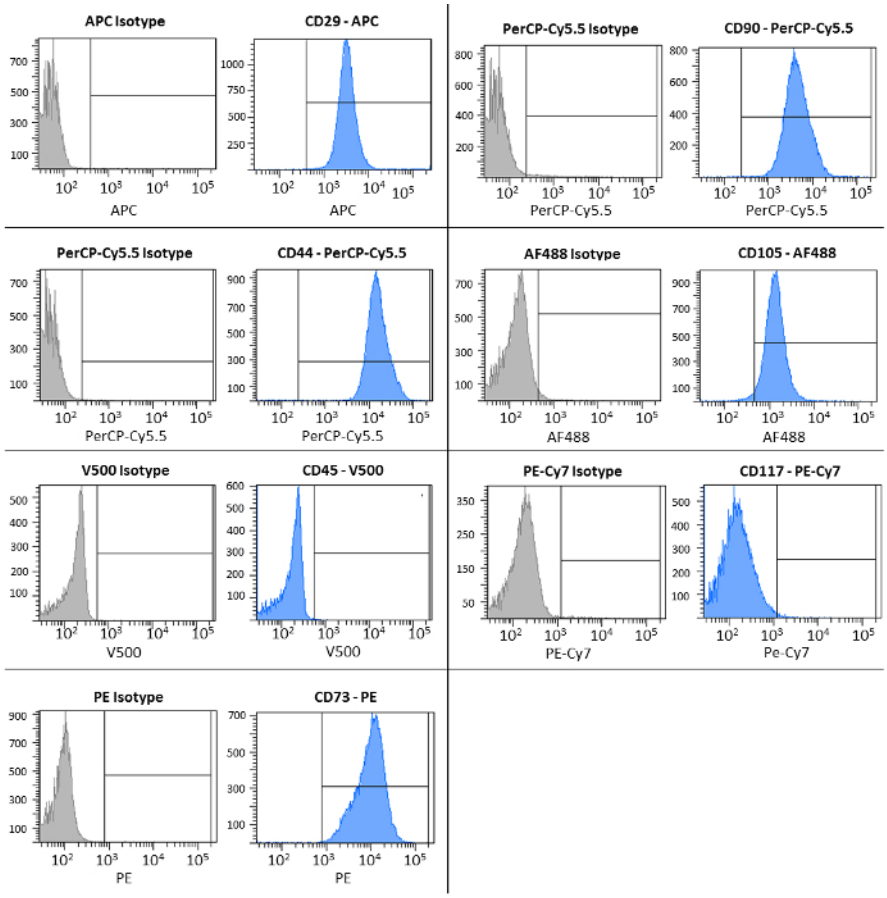

Figure 2: Representative flow cytometric immunophenotyping of hDFSCs. Flow cytometric analysis of cells after staining for specific cell surface markers (blue). Corresponding isotype controls were used as negative controls (grey). Please click here to view a larger version of this figure.

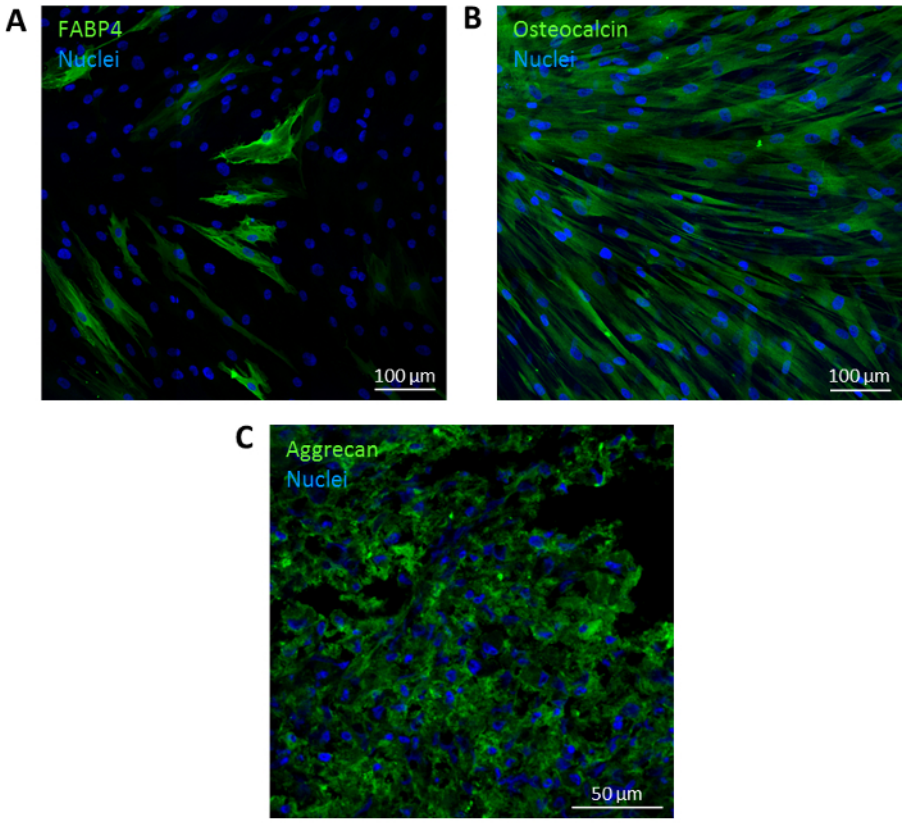

Figure 3: Representative verification of the adipogenic, osteogenic and chondrogenic differentiation potential of hDFSCs. After differentiation, adipocytes, osteocytes and chondrocytes were identified by immunostaining of (**A**) fatty acid binding protein 4 (FABP4) (green), (**B**) osteocalcin (green) and (**C**) aggrecan (green). Nuclei were stained with DAPI (blue). Please click here to view a larger version of this figure.

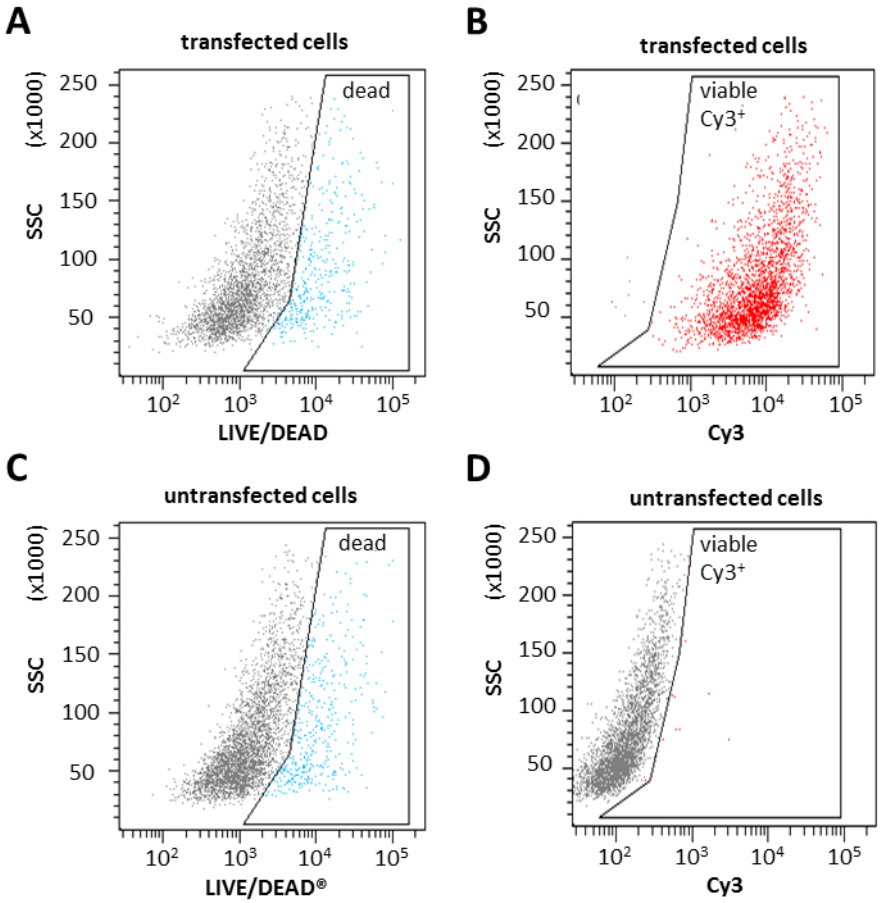

Figure 4: Representative gating strategy for analysis of transfection. Schematic representation of gating strategy used for the quantification of (**A**) cytotoxicity (blue: dead cells) and (**B**) Cy3-labeled miR uptake efficiency (red: $Cy3^+$ cells) 24 h post-transfection. Untransfected cells (**C**,**D**) were used as control.

	MACS	**FcR**	**Antibodies [µL]**							**Isotype controls [µL]**						
Sample	**buffer**	**blocking**	CD29	CD44	CD45	CD73	CD90	CD105	CD117	APC	PerCP-Cy5.5	V500	PE	PerCP-Cy5.5	AF488	PE-Cy7
	[µl]	**reagent [µl]**	APC	PerCP-Cy5.5	V500	PE	PerCP-Cy5.5	AF488	PE-Cy7	IgG1	IgG2b	IgG1	IgG1	IgG1	IgG1	IgG1
1	30	10	10													
2	37.5	10		2.5												
3	37.5	10			2.5											
4	30	10				10										
5	35	10					5									
6	35	10						5								
7	37.5	10							2.5							
8	30	10								10						
9	30	10									10					
10	37.5	10										2.5				
11	30	10											10			
12	40	10												10		
13	30	10													5	
14	37.5	10														2.5

Table 1: Pipetting layout for immunophenotyping of hDFSCs.

Discussion

Adult stem cells are currently in focus for the treatment of several degenerative diseases. In particular, bone marrow (BM)-derived stem cells, including hematopoietic stem cells (HSCs) and MSCs, are under intensive clinical investigation[47]. However, BM harvesting is an invasive procedure causing pain at the site of donation and may lead to adverse events[48]. Recently, the postnatal dental tissue has emerged as a novel and easily accessible source for stem cells. These dental stem cells were shown to meet all MSC characteristics and showed higher proliferation capacity as BM-derived stem cells[49]. Here, we presented a detailed protocol for the extraction, characterization and engineering of hDFSCs.

The described isolation procedure has been developed on human dental follicles of impacted wisdom teeth, as this tissue is commonly extracted and disposed of as medical waste[19]. Nevertheless, other dental tissues, including dental pulp[50,51], periodontal ligament[52], exfoliated deciduous teeth[53], and root apical papilla[54], can be utilized for the extraction of dental stem cells.

Genetic engineering of stem cells by inserting miRs is a novel strategy to overcome certain difficulties in stem cell-based therapies, such as low stem cell survival[43,55,56,57]. This protocol presented crucial instructions for the efficient introduction of synthetic miR into hDFSCs using a commercially available cationic lipid-based transfection reagent. The application of cationic liposomal formulations for the delivery of therapeutic reagents, such as drugs and nucleic acids, has been investigated in numerous clinical trials[58,59]. However, cationic liposomes are potentially cytotoxic in a dose-dependent manner by causing e.g., damage to the integrity of the cell membrane or alterations in gene expression[59,60,61]. Therefore, particular attention must be paid to toxic effects on cells induced by transfection.

Notably, indicated transfection conditions have been optimized for miR-mediated genetic modification of hDFSCs in respect of efficiency and cytotoxicity. Nevertheless, other studies demonstrated the successful application of this transfection reagent for the delivery of additional nucleic acids, including plasmid DNA, mRNA and siRNA, into different cell types[62,63,64,65,66,67,68]. Results of these studies revealed that ideal delivery conditions varied significantly and have to be defined for each cell type.

Disclosures

The authors have nothing to disclose.

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