Video Article

Protocol for MicroRNA Transfer into Adult Bone Marrow-derived Hematopoietic Stem Cells to Enable Cell Engineering Combined with Magnetic Targeting

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Abstract

While CD133$^+$ hematopoietic stem cells (SCs) have been proven to provide high potential in the field of regenerative medicine, their low retention rates after injection into injured tissues as well as the observed massive cell death rates lead to very restricted therapeutic effects. To overcome these limitations, we sought to establish a non-viral based protocol for suitable cell engineering prior to their administration. The modification of human CD133$^+$ expressing SCs using microRNA (miR) loaded magnetic polyplexes was addressed with respect to uptake efficiency and safety as well as the targeting potential of the cells. Relying on our protocol, we can achieve high miR uptake rates of 80–90% while the CD133$^+$ stem cell properties remain unaffected. Moreover, these modified cells offer the option of magnetic targeting. We describe here a safe and highly efficient procedure for the modification of CD133$^+$ SCs. We expect this approach to provide a standard technology for optimization of therapeutic stem cell effects and for monitoring of the administered cell product via magnetic resonance imaging (MRI).

Video Link

The video component of this article can be found at https://www.jove.com/video/57474/

Introduction

CD133$^+$ SCs represent a heterogeneous stem and progenitor cell population with promising potential for regenerative medicine. Their hematopoietic, endothelial, and myogenic differentiation potential[1,2,3] enables the CD133$^+$ cells, *e.g.*, to contribute to neovascularization processes through differentiation into newly forming vessels and activation of pro-angiogenic signaling by paracrine mechanisms[4,5,6,7].

Despite their high potential demonstrated in more than 30 approved clinical trials (ClinicalTrails.gov), their therapeutic outcome is still under controversial discussion[4]. Indeed, a clinical application of SCs is hampered by low retention in the organ of interest and massive initial cell death[5,8,9]. Additional engineering of CD133$^+$ SCs prior transplantation could help overcome these challenges.

One prerequisite for an efficient cell therapy would be the reduction of the massive initial cell death to enhance the engraftment of therapeutic relevant cells[10]. Current studies demonstrated an immense cell loss of 90–99% in highly perfused organs such as the brain and heart during the first 1–2 h, independent of the transplanted cell type or application route[11,12,13,14,15,16,17,18,19,20,21]. SC labeling using magnetic nanoparticles (MNPs) enables an innovative non-invasive strategy to target cells to the site of interest[22,23,24,25,26] and simultaneously allows cell monitoring using MRI[27] and magnetic particle imaging (MPI). The most efficient *in vivo* studies applying magnetized cell targeting used cell retention after local administration in preference to cell guidance after intravenous injection[23,24,28]. Therefore, our group designed a delivery system consisting of superparamagnetic iron oxide nanoparticles[29]. With this technique, CD133$^+$ SCs and human umbilical vein endothelial cells (HUVECs) could efficiently be targeted, as demonstrated by *in vitro* attempts[30,31].

Another hurdle for SC therapies is the hostile inflammatory environment of the affected tissue after transplantation, which contributes to the initial cell death[32]. In addition to several pre-conditioning studies, the application of therapeutic relevant miRs was tested[33]; it has been successfully demonstrated that anti-apoptotic miRs inhibit apoptosis *in vitro* and enhance cell engraftment *in vivo*[33]. These small molecules, composed of 20–25 nucleotides, play a crucial role as posttranscriptional modulators of messenger RNAs (mRNAs), and thus affect stem cell fate and behavior[34]. Moreover, the exogenous introduction of miRs avoid the undesired stable integration into the host genome[34].

Current attempts for efficient introduction of nucleic acids (NAs) into primary SCs are mostly based on recombinant viruses[8,35]. Despite the high transfection efficiency, recombinant virus manipulation presents a major obstacle for a bench-to-bedside translation, *e.g.*, uncontrollable gene expression, pathogenicity, immunogenicity, and insertional mutagenesis[35,36]. Therefore, non-viral delivery systems such as polymer-based

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constructs are critical to develop. Among those, polyethylenimine (PEI) represents a valid delivery vehicle offering benefits for miRs such as NA condensation to protect from degradation, cellular uptake, and intracellular release through endosomal escape[37,38]. Furthermore, miR-PEI complexes demonstrated a high biocompatibility in clinical trials[39]. Therefore, our delivery system consists of a biotinylated branched 25 kDa PEI bound to a streptavidin-coated MNP-core[30,31,40].

In this manuscript, we present a comprehensive protocol describing (i) the manual isolation of $CD133^+$ SC from human bone marrow (BM) donation with a detailed characterization of the SC product and (ii) an efficient and gentle transfection strategy of a magnetically non-viral polymer-based delivery system for genetic engineering of $CD133^+$ SCs using miRs. $CD133^+$ SCs are isolated and magnetically enriched from human sternal BM aspirates using a surface antibody-based magnetic-activated cell sorting (MACS) system. Afterwards, the cell viability as well as the cell purity are analyzed using flow cytometry. Subsequently, miR/PEI/MNP complexes are prepared and $CD133^+$ SCs are transfected. 18 h after transfection, the uptake efficiency and the impact of transfection on SC marker expression and cell viability are analyzed. Moreover, evaluation of the intracellular distribution of the transfection complex compounds is performed using four-color labeling and structured illumination microscopy (SIM).

Protocol

Sternal human BM for cell isolation was obtained from informed donors, who gave their written consent to use their samples for research according to the Declaration of Helsinki. The ethical committee of the University of Rostock has approved the presented study (reg. No. A 2010 23, prolonged in 2015).

1. Cell Preparation

NOTE: Use heparin sodium (250 IU/mL BM) to prevent coagulation for BM examination.

1. **$CD133^+$ SC isolation**
    1. Preparation of required solutions
        1. Prepare phosphate buffered saline (PBS)/ ethylenediaminetetraacetic acid (EDTA) stock solution (2 mM) by mixing 996 mL of 1x PBS with 4 mL of EDTA (0.5 M). Vigorously mix the solution and filtrate using a 0.45 µm filter. Store at room temperature (RT).
        2. Prepare the MACS buffer by mixing 995 mL of 2 mM PBS/EDTA with 5 g of bovine serum albumin (BSA). Vigorously mix the solution and filtrate using a 0.45 µm filter. Store at 4 °C.
        3. Prepare collagenase B stock solution (40 mg/mL) by diluting 500 mg of collagenase B (from *Clostridium histolyticum*) in 12.5 mL of PBS. Vigorously mix the solution, make aliquots of 350 µL, and store at -20 °C.
        4. Prepare deoxyribonuclease (DNAse) I stock solution (10 mg/mL) by diluting 100 mg of DNase I (from bovine pancreas) in 10 mL of PBS. Vigorously mix the solution, make aliquots of 350 µL, and store at -20 °C.

    2. Enzymatic digestion of human BM
        1. Pre-warm the human lymphocyte medium to RT and thaw enzymes (1 aliquot each of collagenase B and DNAse per 20 mL BM).
        2. Collect the BM from syringes into a 50 mL conical tube and mix gently. Discard any existing clots.
           NOTE: Transfer 200 µL of undiluted BM into a 1.5 mL tube for subsequent flow cytometric analysis. Store at 4 °C until use.
        3. Transfer 10 mL BM into a new 50 mL conical tube, add 6 mL of PBS/EDTA (2 mM), 20 mL of human lymphocyte medium, 175 µL of Collagenase B (40 mg/mL), and 175 µL of DNAse I (10 mg/mL). Gently mix the solution and incubate for 30 min at RT on a shaker. Repeat for the remaining BM sample.

    3. Density gradient centrifugation
        1. Prime a 50 mL density gradient centrifugation tube by applying 15 mL of human lymphocyte separating medium into a 50 mL tube. Centrifuge at 1,000 x *g* for 30 s.
        2. Carefully layer 35 mL of diluted BM on top of the density gradient centrifugation tube filter and centrifuge at 445 x *g* for 35 min at RT.
           NOTE: Centrifuge settings are low acceleration (3) and no brake (1).
        3. Carefully remove the tube from the centrifuge without shaking.Carefully discard ~20 mL from the upper clear solution without touching the cloudy layer directly on top of the filter.
        4. Carefully transfer the cloudy layer (which is directly on top of the filter and contains the mononuclear cells (MNCs)) into a new 50 mL conical tube and fill up with PBS/EDTA to a final volume of 50 mL.
           NOTE: Combine all MNCs from one BM patient sample into one new tube.
        5. Count the MNCs by transferring 10 µL of the 50 mL cell suspension into a 1.5 mL tube. Add 10 µL of 3% acetic acid with methylene blue. Gently mix and apply 10 µL into a counting chamber. Calculate the number of MNCs.
        6. Centrifuge the MNC suspension at 300 x *g* for 10 min. Discard the supernatant.
           NOTE: During centrifugation, cool down the centrifuge from RT to 4 °C.

    4. Magnetic selection of $CD133^+$ SCs using human CD133 antibody-linked superparamagnetic iron dextran particles
       NOTE: During this step, work on ice. Store solutions until use at 4 °C. Store MACS permanent magnet, separation columns, and pre-separation filter at 4 °C.
        1. Choose the column size. For <1.2 x 10^8 MNCs, use MS columns, and for >1.2 x 10^8 MNCs, use LS columns (see **Table of Materials**).
        2. To prepare of magnetic selection for 1 x 10^8 total cell number, carefully resuspend MNCs in 300 µL MACS buffer (4 °C). Add 100 µL FcR blocking reagent (4 °C) and 100 µL CD133 antibody-linked superparamagnetic iron dextran particles (4 °C).
        3. Gently mix the cell suspension and incubate for 30 min at 4 °C. Gently shake the cell suspension during incubation (2–3x).
        4. Add 2 mL of MACS buffer (4 °C) per 1 x 10^8 total MNCs. Gently mix the cell suspension and centrifuge at 300 x *g* for 10 min at 4 °C.

5. Set up the MACS magnet holder and attach the MACS permanent magnet. Install the MACS column and apply the pre-separation filter on top.
6. Equilibrate the first MACS MS/LS column and pre-separation filter with 0.5 mL (MS) or 3 mL (LS) of MACS buffer (4 °C).
   NOTE: Never allow the MACS columns to dry out after equilibration.
7. Discard the supernatant and resuspend the obtained pellet in 500 µL of MACS buffer (4 °C) per 1 x 10^8 total cell amount. Apply the cell suspension into the pre-separation filter.
8. Wash the MACS column and pre-separation filter three times using, for each wash, 0.5 mL (MS) or 3 mL (LS) of MACS buffer (4 °C).
   NOTE: During the third washing-step, install a second MACS column into the MACS permanent magnet and equilibrate the second MACS MS/LS column with 0.5 mL (MS) or 3 mL (LS) MACS buffer (4 °C).
9. Discard the pre-separation filter.
10. Elute the cell fraction directly onto the second MACS column: add 1 mL (MS) or 5 mL (LS) MACS buffer (4 °C) onto the first MACS column and remove the MACS column from the MACS permanent magnet. Immediately transfer the first MACS column above the second MACS column and push the cell suspension through the MACS column using the supplied plunger.
11. Wash the MACS column three times, using for each wash 0.5 mL (MS) or 3 mL (LS) MACS buffer (4 °C).
12. Elute the cell fraction from the column by adding 1 mL (MS) or 5 mL (LS) MACS buffer (4 °C) onto the second MACS column and removing the MACS column from the MACS permanent magnet. Immediately transfer the second MACS column above a 1.5 mL tube (MS) or 15 mL conical tube (LS) and push the cell suspension through the MACS column using the supplied plunger.
13. Centrifuge at 300 x *g* for 10 min at 4 °C.Carefully discard the supernatant and resuspend the obtained pellet in 100 µL of MACS buffer (4 °C).
14. Count the $CD133^+$ cells: transfer 6 µL of the cell suspension into a 1.5 mL tube. Add 6 µL trypan blue solution (0.4%). Gently mix and apply 10 µL into a counting chamber. Calculate the number of $CD133^+$ cells.
15. Store the $CD133^+$ cells on ice until seeding.

2. **Characterization of freshly isolated $CD133^+$ SCs**
   NOTE: The following work should be carried out in a room protected from bright light (dim lighting only). Keep tubes, buffers, and antibodies on ice unless stated otherwise.
   1. Preparation of the SCs for flow cytometric analysis
      1. Use two samples (each 10 µL) of BM aliquots and one sample of freshly isolated $CD133^+$ SCs (minimum, 1 x 10^4 cells) for analysis. Transfer the cells into a 1.5 mL tube. Add 10 µL of FcR blocking reagent and fill up with MACS buffer (4 °C) to a volume of 33 µL.
      2. Add the following antibodies onto the inner side of the respective tube (see **Table 1**): anti-CD34-fluorescein isothiocyanate (FITC) (clone: AC136), anti-CD133/2-phycoerythrin (PE) (clone: 293C3), isotype control mouse IgG 2b-PE, anti-CD45-allophycocyanin (APC)-H7 (clone: 2D1), and 7-aminoactinomycin (AAD). After all antibodies have been added, shake down the antibodies side of the tube. Gently mix the solution (final volume 50 µL) and incubate for 10 min at 4 °C.
      3. Add 1 mL of 1x red blood cell (RBC) lysis buffer, gently mix the suspension, and incubate on ice 10 min. Centrifuge the suspension at 300 x *g* for 10 min at 4 °C.
      4. Discard the supernatant and resuspend the obtained pellet in 100 µL PBS (4 °C). Store on ice until flow cytometric analysis.
   2. Flow cytometric analysis of $CD133^+$ SCs
      1. To examine the cell viability and purity of $CD133^+$ SC, use an adapted International Society of Hematotherapy and Graft Engineering (ISHAGE) flow cytometry gating strategy[41] (see the representative depiction in **Figure 1**). Use the following order:
         Step 1: selection of cell population (**Figure 1A**)
         Step 2: selection of $CD45^+$ cells (**Figure 1B**)
         Step 3: selection of viable $CD45^+$ cells (**Figure 1C**)
         Step 4: selection of viable $CD45^+/CD34^+$ cells (**Figure 1D**)
         Step 5: selection of viable $CD45^+/CD34^+/CD133^+$ cells (**Figure 1E**)
         Run flow cytometer (see **Table of Materials**) according to the manufacturer's instructions.
      2. Calculate the cell viability and purity using the following equations:

$$Viability\ [\%] = \frac{viable\ CD45^+\ cells}{CD45^+\ cells} \times 100$$ **Equation 1**

$$Purity\ [\%] = \frac{viable\ CD45^+/CD34^+/CD133^+ cells}{viable\ CD45^+ cells} \times 100$$ **Equation 2**

3. **Cell culture of freshly isolated $CD133^+$ SCs**
   1. Aliquot 100x recombinant human cytokine supplement in 100 µL aliquots and store at -20 °C. Thaw one 100x aliquot before the medium preparation.
   2. Prepare the $CD133^+$ SC culture medium, serum-free hematopoietic cell expansion medium supplemented with recombinant human cytokine supplement (final 1x) and 1% penicillin/streptomycin.
   3. Seed 5 x 10^4 freshly isolated $CD133^+$ SCs for transfection in a 24-well plate with 500 µL culture medium. Incubate the cells at 37 °C, 5% CO_2 and 20% O_2.
      NOTE: Seed 5 x 10^4 freshly isolated $CD133^+$ SCs as a control for flow cytometric analysis.

2. Preparation of Transfection Complexes

NOTE: During this step, keep the entire work space ribonuclease (RNAse)-free using RNAse decontamination solution and use only RNAse-free consumable material.

1. **Preparation of miR**
   NOTE: The following work should be carried out in a room protected from bright light (dim lighting only).
   NOTE: For the miR-specimen: use Cyanine 3 dye labeled precursor miR and unlabeled precursor miR.
   1. To prepare the miR stock solution (50 µM), dilute the desired amount of miR in the appropriate volume of nuclease-free water (*e.g.*, dilute 5 nmol of miR in 100 µL water). Gently mix the solution, aliquot the miR stock (5 µL), and store below -20 °C.

2. **Preparation of PEI**
   1. Prepare the PEI following standard protocols and note the PEI stock concentration[31,42] .

3. **Preparation of MNP**
   1. Prepare the MNPs following standard protocols and note the MNP stock concentration[31,42].

4. **Preparation of miR/PEI complexes**
   1. Prepare 5% glucose solution by diluting D-(+)-glucose in nuclease-free water. Gently mix the solution, aliquot the stock (1.5 mL), and store at 4 °C.
      NOTE: The following work should be carried out in a room protected from bright light (dim lighting only).
   2. Use 20 pmol miR per well of a 24-well plate[30]. Dilute miR in the 5% glucose solution to a final concentration of 0.25 pmol miR/µL.
   3. Calculate the amount of PEI required based on the miR amount (20 pmol, step 2.3.1) and optimal nitrogen (of PEI) to phosphate (of miR) (N/P) ratio based on the PEI stock concentration (step 2.2.1; here, a ratio of 7.5 was used)[30]. Dilute the PEI in an equal amount of 5% glucose solution based on the equation:

      $$N/P\ ratio = \frac{[\mathrm{PEI}] \times \mathrm{Volume\ of\ PEI}}{pmol\ miR \times 0.12}$$

      which rearranges to

      $$Volume\ of\ PEI = \frac{\frac{\mathrm{N}}{\mathrm{P}} \times \mathrm{pmol\ miR} \times 0.12}{[\mathrm{PEI}]}$$ **Equation 3**

      where PEI volume is in µL and [PEI] is in mM, and N/P (optimized for this protocol) is 0.75. 0.12 is a conversion factor specific to miR.
   4. Add the pre-diluted PEI into the pre-diluted miR and vortex for 30 s.
   5. Incubate the miR/PEI complex for 30 min at RT.

5. **Preparation of the miR/PEI/MNP complexes**
   NOTE: The following work should be carried out in a room protected from bright light (dim lighting only).
   1. While incubating the miR/PEI complexes, prepare the MNPs by sonicating the MNPs for 20 min at 35 kHz in the sonicating water bath at RT to ensure the particles are in suspension and not aggregating.
   2. Calculate the number of required MNPs (3 µg iron/mL) based on the MNP stock solution (step 2.3.1).
   3. Add the sonicated MNPs into the miR/PEI complexes and vortex for 30 s. Incubate the miR/PEI/MNP complexes for 30 min at RT.

6. **Preparation of the transfection complexes for four-color labeling using SIM**
   NOTE: Use unlabeled precursor miR.
   1. Label the miR with Cyanine 5 dye using a Cyanine 5 dye miR labeling kit (see **Table of Materials**) following the manufacturer's instructions. Measure the final miR concentration using the listed spectrophotometer (see **Table of Materials**) in accordance to the pre-setting provided by the manufacturer (nucleic acid, RNA-40). Aliquot the miR (5 µL) and store at -80 °C protected from light.
   2. Label the PEI with a bright green fluorescence dye using an appropriate protein labeling kit (see **Table of Materials**) following the manufacturer's instructions. Define the final PEI concentration using the Ninhydrin assay (step 2.2.1). Aliquot the PEI (in the volume calculated based on **Equation 3**) and store at 4 °C protected from light.
   3. Label the MNPs with rhodamine dye conjugated to biotin using a ratio of 1:1,000 w/w, dye to MNP. Mix the rhodamine dye and MNPs concurrently with the preparation of the miR/PEI complex formation and incubate the solution for 30 min in the dark. Directly use labeled MNPs.

3. Transfection of $CD133^+$ SCs

NOTE: During this step, keep the entire work space ribonuclease (RNAse)-free using RNAse decontamination solution and use only RNAse-free consumable material
NOTE: The following work should be carried out in a room protected from bright light (dim lighting only).

1. Add the prepared miRNA/PEI/MNP complex dropwise directly into the appropriate well containing the freshly isolated $CD133^+$ SCs in culture medium.
2. Mix gently by rocking the plate back and forth. Incubate the cells for 18 h at 37 °C, 5% CO_2 and 20% O_2.

4. Analysis of the miR Transfection

1. **Examination of the miR uptake efficiency and the cytotoxicity of transfection complexes**

NOTE: For the miR-specimen: use Cyanine 3 dye labeled precursor miR for the uptake evaluation and non-transfected CD133$^+$ SCs for the flow cytometry control gates.
NOTE: The following work should be carried out in a room protected from bright light (dim lighting only).
NOTE: Keep the tubes, buffers, and antibodies on ice unless stated otherwise.

1. Prepare the paraformaldehyde stock solution (PFA, 4%) by diluting 4 g PFA in 100 mL PBS and heating the solution to 80 °C. Vigorously mix the solution and adjust the pH value to 7.3. Aliquot the PFA stock (1.5 mL) and store at -20 °C.
2. 18 h after transfection, collect each well in a separate 1.5 mL tube. Wash each well once with 500 µL of PBS and transfer this cell suspension to the same tube.
3. Centrifuge at 300 x g for 10 min at 4 °C.Discard the supernatant and resuspend the obtained pellet in 100 µL of 4 °C MACS buffer.
4. Add 0.5 µL of an amine reactive dye to distinguish between live and dead cells, gently mix the suspension, and incubate for 10 min at 4 °C. Add 1 mL PBS (4 °C) and centrifuge at 300 x *g* for 10 min at 4 °C.
5. Discard the supernatant, resuspend the obtained pellet in 100 µL PBS, add 33 µL PFA (4%), and store on ice or at 4 °C until flow cytometric analysis.
6. Arrange the gating strategy according to the representative depiction in **Figure 2**. Run the samples on the flow cytometer (see **Table of Materials**) according to the manufacturer's instructions.

2. **Characterization of the miR-transfected CD133$^+$ SCs**
NOTE: Used unlabeled precursor miR as well as non-transfected CD133$^+$ SCs for flow cytometry control gates.
   1. Collect each well in a separate 1.5 mL tube and centrifuge at 300 x *g* for 10 min at 4 °C.
   2. Use the gating strategy as described in step 1.2.

3. **Examination of the intracellular distribution of transfection complexes by SIM**
   1. Prepare the complexes and transfect the cells as described in Section 2 and Section 3.
   2. 18 h after the transfection, collect each well in a separate 1.5 mL tube. Wash each well once with 500 µL of PBS and transfer the wash into the well's respective tube. Centrifuge at 300 x *g* for 10 min at 4 °C.
   3. Discard the supernatant and resuspend the obtained pellet in 1 mL of 2% fetal bovine serum (FBS) in PBS, gently mix the suspension, and centrifuge at 300 x *g* for 10 min at 4 °C.
   4. Discard the supernatant, resuspend the obtained pellet in 100 µL of PBS, and add 33 µL PFA (4%) for 20 min.
   5. Transfer the cell suspension into a new 24-culture plate containing sterile glass coverslips and centrifuge at 300 x *g* for 10 min at 4 °C.
   6. Discard the supernatant and add 500 µL of PBS. Gently shake the plate and discard PBS.
   7. Place the prepared coverslips on the microscopic slides using aqueous mounting medium to preserve fluorescence and dry them at RT for at least 1 h.
   8. Acquire images using a 100X oil immersion objective. SIM settings: 405, 488, 561, and 633 nm laser lines for excitation and z-stacks with a 16-bit depth at 3 angles, 3 phases, with averaging 4, grids per laser line: 23 µm – 405, 34 µm – 488, 42 µm – 561, 51 µm – 633.

Representative Results

The presented protocol describes a manual isolation and magnetic enrichment of human BM-derived CD133$^+$ SCs with a subsequent virus independent cell engineering strategy, as a non-invasive technology for *in vitro* cell manipulation and *in vivo* monitoring tool.

This three-step isolation technology permits a separation of MNCs from the pre-digested sternal BM through density gradient centrifugation. Afterwards, a CD133$^+$ cell fraction can be magnetically enriched using the appropriate isolation technology. This allows an enrichment of CD133$^+$ SCs with a viability and purity higher than 80%, which can be determined by flow cytometry (**Figure 1**). Hereinafter, only SC products which match the requirements were used for further experiments.

The transfection method described here allows a highly effective introduction of miR in these freshly isolated human CD133$^+$ SCs with an uptake of approximately 80% of viable cells (**Figure 2**)[30]. In addition, there are no significant cytotoxic effects evident 18 h after transfection compared to control cells (**Figure 2**)[30]. Moreover, a sufficient delivery and usual distribution of the transfection complex compounds (miR, PEI, and MNPs) can be observed within the cytoplasm of cells using SIM (**Figure 3**).

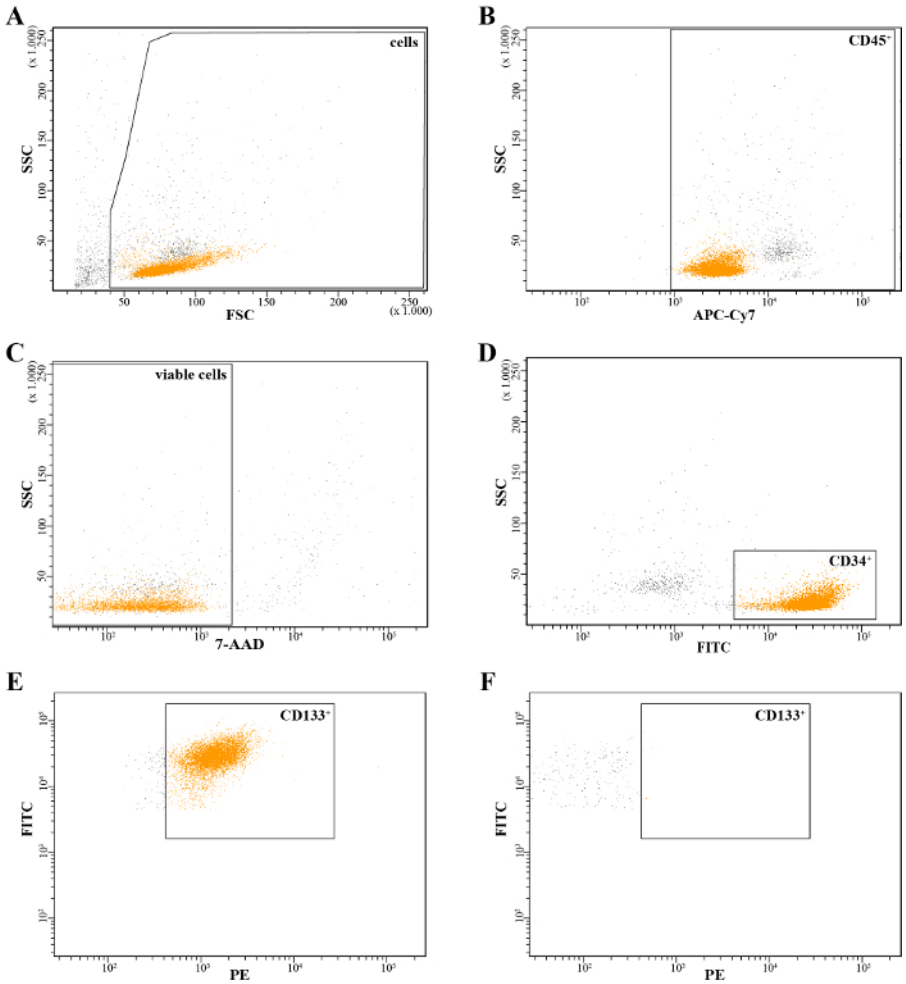

Figure 1: Representative Boolean gating strategy for CD133$^+$ SC characterization. (**A-E**) Depiction of a 5-Step selection strategy for a strict evaluation of cell viability and purity of isolated CD133$^+$ SC as well as (**F**) CD133 isotype control using the appropriate untreated BM sample. (**A**) Excluding debris from intact cell population in a forward/side scatter (FSC/SSC) dot plot, followed by successive selection of (**B**) CD45$^+$ cell population, (**C**) viable CD45$^+$ cell population, (**D**) viable double positive CD45$^+$/CD34$^+$ cell population, and (**E**) viable triple positive CD45$^+$/CD34$^+$/CD133$^+$ cell population. Orange: CD45$^+$/CD34$^+$/CD133$^+$ cells highlighted within each selection step. Legend: APC-Cy7: anti-CD45, FITC: anti-CD34, FSC: Forward Scatter Channel, PE: anti-CD133, SSC: Side Scatter Channel, 7-AAD: 7-aminoactinomycin. Please click here to view a larger version of this figure.

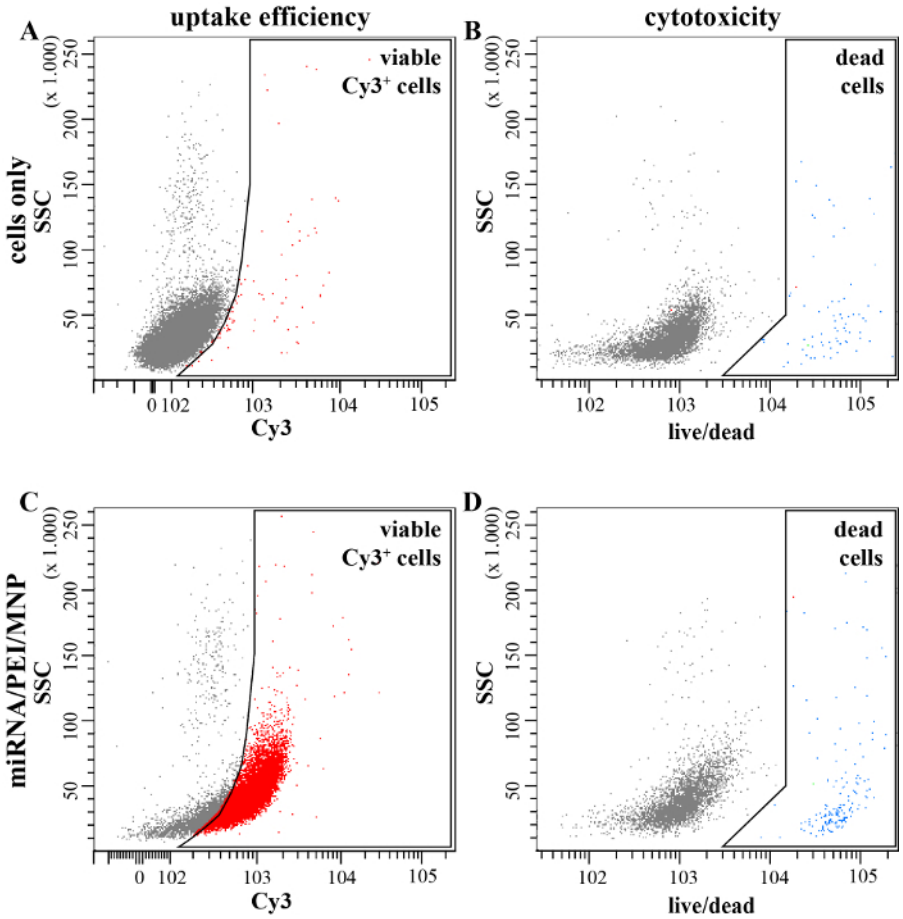

Figure 2: Representative gating strategy for the evaluation of miR uptake efficiency and cytotoxicity of the transfection complex. Gating strategies of (**A**, **B**) transfected CD133$^+$ SCs as well as (**C**, **D**) non-transfected CD133$^+$ SCs for the analyses of (**A**, **C**) Cyanine3 dye labeled precursor-miRNA uptake efficiency (red: viable Cy3$^+$ cells) and (**B**, **D**) cytotoxic effects of the transfection procedure marked by an amine reactive dye to distinguish between live and dead cells (blue: dead cells). Legend: SSC: Side Scatter Channel, cells only: non-transfected CD133$^+$ SCs, miRNA/PEI/MNP: CD133$^+$ SCs transfected with Cy3-labeled miR Please click here to view a larger version of this figure.

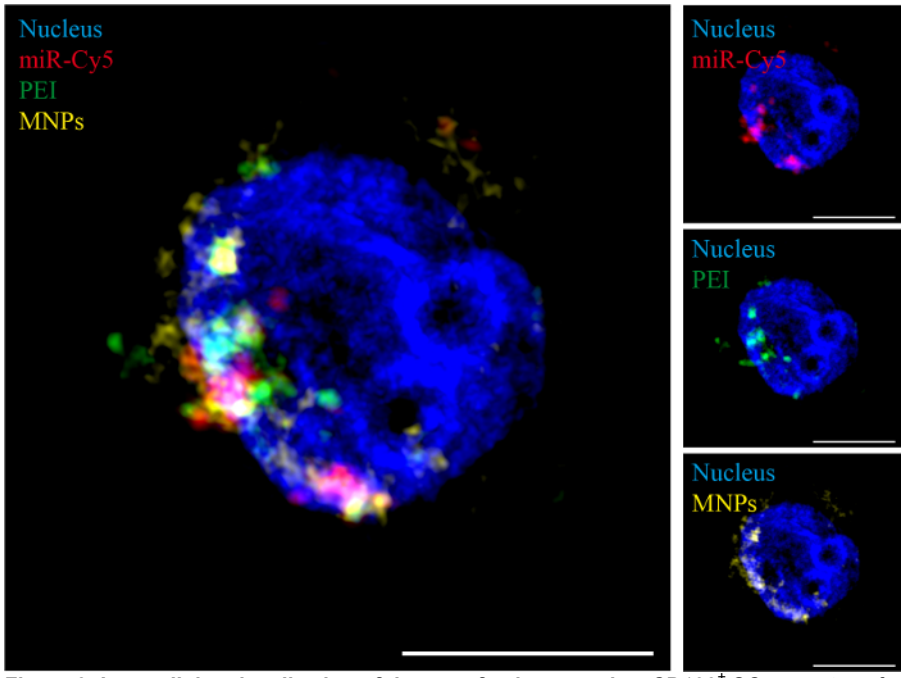

Figure 3: Intracellular visualization of the transfection complex. CD133$^+$ SCs were transfected with three-color labeled miR/PEI/MNP complex (miR: 20 pmol, PEI: N/P ratio 7.5, MNP: 3 µg/mL). Representative visualization was performed 18 h after transfection using structured illumination microscopy. The miR was labeled with Cyanine5 dye (red), PEI with a bright green fluorescence dye (green), and MNPs with rhodamine dye conjugated to biotin (yellow), and the nucleus was counterstained with DAPI (blue). Scale bar = 5 µm. Please click here to view a larger version of this figure.

sample			FCR blocking reagent [µL]	MACS buffer [µL]	antibodies [µL]					total [µL]
number	specimen	cells [µL]			CD133-PE isotype	CD133-PE	CD34-FITC	CD45-APC-Cy7	ADD	
1	BM	10	10	12.5	5	-	5	2.5	5	50
2	BM	10	10	12.5	-	5	5	2.5	5	50
3	CD133$^+$	($1x10^4$)	10	12.5	-	5	5	2.5	5	50

Table 1: Pipetting layout for characterization of CD133$^+$ SCs.

Discussion

In recent years, CD133$^+$ SCs have emerged as a promising cell population for SC-based therapies as evidenced by several phase I, II, and III clinical trials[43,44,45,46,47,48,49,50,51,52,53,54]. Here, we present a detailed protocol for the standardized manual purification and flow cytometric characterization of these cells from human sternal BM. The described MACS-based isolation procedure represents a gentle, fast, and efficient strategy to obtain a highly pure and viable hematopoietic SC fraction. The good compatibility combined with the rapid degradation of co-injected MACS MicroBeads used for cell sorting has been demonstrated previously by our group[55,56].

This isolation procedure was developed using human sternal BM as a source material, and therefore, the protocol currently enables purification of hematopoietic SCs intended for use in non-clinical research. If CD133$^+$ SCs are isolated from other tissues (*e.g.,* BM obtained from iliac crest) several steps of the protocol such as enzymatic tissue digestion and density centrifugation may require adaption. For clinical cell application, our group further established a GMP-conformant on-site manufacturing procedure using the an automatic system in order to satisfy additional demands on behalf of clinical needs[57].

Engineering of SCs prior to their application is a novel strategy to overcome certain obstacles in SC therapy, including low cell retention and massive cell death. The current protocol comprises instructions for the production of a viral-free multifunctional transfection system based on branched PEI bound to MNPs and its efficient introduction into the CD133$^+$ SCs[30]. Application of this system enables genetic cell modification, magnetically controlled cell guidance, and non-invasive cell tracing via MRI or MPI[30,31,40,42,58,59,60].

Importantly, the described transfection conditions have been optimized for transient genetic modification of CD133$^+$ BM-derived SCs[30]. In previous works, this transfection system has been also successfully applied for the delivery of plasmid DNA and miR to other cell types[31,40,60]. These experiments have demonstrated that transfection efficiency as well as cytotoxicity are cell type-dependent. Therefore, the optimal compositions of NAs, PEI, and MNPs need to be defined for each cell type.

Due to its high efficiency, PEI is one of the most commonly used polymers for non-viral genetic cell engineering[61]. However, the clinical application[61,62] of PEI is very limited to date because of its general toxicity[61,63]. Interestingly, our group recently demonstrated that MNPs reduce

PEI toxicity when included in the transfection system[31,60]. At the same time, the possible toxicity of iron oxide based nanoparticles caused by the production of reactive oxygen species (ROS) is dose-dependent and several types of superparamagnetic nanoparticles are approved for MRI[64,65]. However, attention must be paid to the toxicity of the system *in vitro* and *in vivo*.

Overall, the protocol is quite complex, containing many critical steps that need to be very carefully addressed. For this purpose, we have highlighted these points in the protocol section with notes. In particular, we wish to point out the importance of a completely RNAse-free environment and avoiding any light exposure when handling the applied fluorescent dyes.

Disclosures

The authors declare no conflict of interest.

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