Chapter 29

Efficient Transduction of Hematopoietic Stem Cells and Its Potential for Gene Correction of Hematopoietic Diseases

Dolly Thomas and Gustavo Mostoslavsky

Abstract

The ability to efficiently transduce hematopoietic stem cells (HSC) represents a powerful methodology by which to study the role of specific genes on HSC function, as well as to broaden the potential of gene therapy for hematopoietic related diseases. While retroviruses have been used extensively to transduce a variety of cell types, HIV-derived lentiviruses prove superior for transduction of quiescent HSC due to their ability to infect non-dividing cells. Quality of lentiviral supernatants and starting cells are vital to obtain reproducible consistent results, and therefore, here we describe the production of concentrated lentiviral preparations, the purification of HSC from total mouse bone marrow, and their transduction to obtain long-term HSC engraftment with persistent gene transfer and expression of the desired transgene.

Key words Lentiviral transduction, Gene Therapy, Ultracentrifugation, Hematopoietic stem cells, Hoechst 33342 staining, Side population, Artemis immunodeficiency

1 Introduction

For more than two decades, simple retroviruses were the most commonly used vector for gene transfer into mammalian cells [1–3]. However, the use of these vectors was limited mostly due to their inefficient capacity to induce gene transfer into non-dividing cells [4, 5]. The development of HIV-1 based lentiviral vectors bypassed this obstacle, allowing for efficient transduction of a wide range of mammalian cells including their ability to integrate into the genome of non-proliferating cells [6–8]. This became integral especially in regards to gene transfer into quiescent HSC populations, first demonstrated by Uchida et al. in 1998 [9]. Since then lentiviral gene transfer into purified HSC populations has served as the basis for gaining insights into basic HSC biology as well as developing potential therapies for certain human hematological diseases [10–13]. One such example are our previous studies using

Francesca Storici (ed.), *Gene Correction: Methods and Protocols*, Methods in Molecular Biology, vol. 1114,
DOI 10.1007/978-1-62703-761-7_29,

Artemis-deficient mice [14], which present with symptoms of human RS-SCID (Radiosensitive Severe Combined Immuno deficiency disorder), including severe lymphocyte deficiency. Lentiviral mediated overexpression of the human Artemis gene within transplanted Artemis-deficient HSC resulted in complete rescue of depleted B and T cell populations upon bone marrow transplantation [14].

While the use of gene therapy in humans has been progressing cautiously, due to past concerns regarding viral integration, and the development of secondary insertional mutagenesis [15, 16], it is clear that the use of more sophisticated viral vectors, including self-inactivating lentiviral-based vectors such as those mentioned above [10–14] would be beneficial for a safer more promising gene therapy approach to human disease. The rationale behind the success of gene therapy in the hematopoietic system lies in the use of viral vectors capable of effectively transducing quiescent HSC while at the same time limiting the risk of insertional mutagenesis. The viability and long-term engraftment of transplanted cells is dependent upon maintenance of cellular integrity during transduction protocols. Herein resides a main obstacle with clear practical implications that has hampered progress in this field (i.e., finding the right balance between achieving high efficiency of transduction while maintaining the multipotential capacity of the transduced HSC). We here present a detailed protocol that allows first the purification of a highly homogenous HSC population, followed by the efficient lentiviral transduction of these purified HSC that preserve their robust multipotent activities, in vitro and in vivo. This methodology provides a basis for an optimized approach to use gene therapy in the clinical arena.

Self-inactivating lentiviral vectors are packaged via transfection of HEK-293T (293T) cells with the lentiviral backbone in conjunction with four helper constructs that provide in trans expression of enzymatic and structural viral proteins. Transfected 293T cells allow for the packaging and release of lentiviral particles, which are then collected and concentrated by ultracentrifugation to obtain viral titers that range between 5×10^8 and 5×10^9 viral particles per milliliter. Accurate titering of obtained viral particles is key to ensure proper MOI (multiplicity of infection = number of infectious particles per target cell). For HSC purification, we use and describe here Hoechst 33342 staining of total bone marrow, named "SP method" first described by Goodell et al. in 1996 [17]. Hoechst 33342 allows for a highly specific staining pattern based on the unique ability of HSC to exclude the Hoechst dye due to the actions of the ABCG2 transporter, highly expressed by HSC [18]. Lastly, in order to further optimize levels of gene transfer, we include minimal prestimulation with low levels of SCF and TPO during viral transduction [13], both important to preserve HSC function as well as inducing HSC to become activated from a G0 to G1 state [19].

2 Materials

2.1 Lentiviral Preparation

1. TransIT® Transfection Reagent (Mirus Bio LLC).
2. Transfection Media: Dulbecco's Modified Eagle Medium, 10 % Fetal Bovine Serum, 100 μg/ml Primocin (InVivoGen).
3. Helper Plasmids (HDM-Tat1b, pRC1-Rev1b, HDM-Hgpm2, HDM-Vsv-G) (Originally developed by the Harvard Gene Therapy Initiative).
4. SW-28 Beckman Coulter Rotor.
5. Ultra-Clear Centrifuge Tubes (Beckman Coulter).
6. XL-100K Optima UltraCentrifuge (Beckman Coulter).
7. 15 cm tissue culture treated plates.
8. 150 ml Bottle Top Filter.
9. 5 ml Polypropylene tubes.
10. 293T cells.

2.2 Hematopoietic Stem Cell Purification

1. Purification Media: Hanks Buffered Saline Solution, 2 % Fetal Bovine Serum, 1 % HEPES, 1 % Penicillin/Streptomycin. Store at 4 °C.
2. Wash Media: Phosphate Buffered Saline (1X), 2 % Fetal Bovine Serum. Store at 4 °C.
3. Hoechst 33342, Trihydrochloride, Trihydrate (100 mg) (Invitrogen). Resuspend in water to a concentration of 10 mg/ml. Aliquot and store at −20 °C.
4. Ficoll-Paque™ PLUS (GE Healthcare).
5. Beckman Coulter Z series Z2 Cell counter.
6. BD FACSAria cell sorter or Beckman–Coulter MoFlo cell sorter. Multiple laser excitation is required. A 488-nm laser was used for propidium iodide excitation. Hoechst was excited using a 350-nm emission UV laser and its signal was collected with a 405/30 filter (Hoechst blue) and a 670/40 filter (Hoechst Red).
7. FACS Media: Phosphate Buffered Saline (1X), 2 % Fetal Bovine Serum, 1 μg/ml Propidium Iodide. Prepare fresh and keep on ice.

2.3 Transduction

1. HSC Transduction Media: StemPro-34 (Invitrogen) (supplemented with L-glutamine and Penicillin/Streptomycin), StemPro-34 Nutrient Supplement (Invitrogen), 10 ng/ml mouse SCF (R&D Systems), 100 ng/ml human TPO (R&D Systems), 5 μg/ml Polybrene (Hexadimethrine Bromide) (Sigma). Prepare fresh and keep at 4 °C.
2. 96-well round bottom plates.
3. Concentrated lentiviral preparations (from Subheading 3.1).

3 Methods

3.1 Transfection of 293T Cells for Viral Packaging/ Concentration of Viral Supernatants

Carry out all steps in this section in a tissue culture hood using proper aseptic tissue culture practices unless otherwise noted.

1. Prepare a 15 cm tissue culture treated plate of 293T cells to a confluence of 85–90 % (*see* **Note 1**).
2. Prepare the transfection mix by first pipetting 2 ml of DMEM into a 5 ml polypropylene tube. While vortexing, add 112.5 μL of TransIT® transfection reagent to the DMEM drop by drop. Try to avoid the TransIT® hitting the sides of the tube. For each 15 cm plate of 293T cells, make one tube of transfection mix. Allow the transfection mix to incubate for 10 min at room temperature.
3. During this incubation prepare the DNA mix, containing your lentiviral vector and the four helper plasmids. In a 1.5 ml eppendorf tube add 30 μg of lentiviral vector DNA. Mix with 1.5 μg of HDM-Tat1b, 1.5 μg of pRC1-Rev1b, 1.5 μg of HDM-Hgpm2, and 3 μg of HDM-Vsv-G helper plasmids. Pipette gently to mix (*see* **Note 2**).
4. Add the DNA mix to the transfection mix drop by drop while vortexing. Avoid hitting the sides of the tube. Incubate the mix for 15 min at room temperature.
5. During this incubation, change the media of your 293T to 13 ml of new transfection media (*see* **Note 3**).
6. Add the Transfection/DNA mix to the cells GENTLY drop by drop. Gently push the plate front to back and then left to right several times to evenly distribute the mix to all the cells of the plate to ensure homogeneous efficient transfection.
7. Incubate the plate for 48 h in a 37 °C, 5 % CO_2 incubator. Do not change media during this time.
8. To collect your viral supernatant, pipette up the media and transfer through a 150 ml bottle top filter into a sterile glass bottle (using vacuum). Keep the bottle at 4 °C for further collections. Replenish media with 15 ml of fresh transfection media. Repeat viral collection to a total of five times. Pool all collections together and place filtered unconcentrated virus at 4 °C until ready for concentration (*see* **Note 4**).
9. To concentrate, make sure ultracentrifuge has been set to 4 °C and allow the inner chamber to cool. Once cooled, place an ultracentrifuge ultraclear tube into swinging bucket and weigh on a scale. Pipette viral supernatants into ultracentrifuge tubes. The weight of virus plus ultracentrifuge tube plus swinging bucket must be equivalent in order to keep the ultracentrifuge balanced during centrifugation (*see* **Note 5**).

10. Once all samples have been loaded into the rotor, carefully place the rotor into the ultracentrifuge. Spin viral supernatants for 90 min at 36,000 × *g* at 4 °C.
11. Following centrifugation, carefully remove the ultracentrifuge tubes. Use an empty beaker for waste. In one swift motion, dump out the supernatant from the tube. Hold the tube facing down, until the last two drops fall from the edge of the tube. Turn the tube upright and immediately wrap paraffin over the top of the tube (*see* **Note 6**).
12. Place the tubes on ice for 2–3 h, then prepare 10 μL aliquots and store at −80 °C (*see* **Note 7**).
13. Before using concentrated virus for transduction experiments it is necessary to titer all viruses by FACS or Southern blot (*see* **Note 8**).

3.2 Purification of Hematopoietic Stem Cells by Hoechst 33342 Staining

1. Sacrifice mice according to IACUC approved protocols for your institution.
2. Spray bottom half of mouse with 70 % ethanol to sterilize and wet fur. Harvest femurs and tibias and place them into 10 ml of cold wash buffer (*see* **Note 9**). Keep on ice until all bones have been collected.
3. To harvest cells, pour all bones plus wash buffer into a mortar. Crush bones using a pestle to release the cells into solution (*see* **Note 10**).
4. Pipette the wash buffer plus cells up and down to break up any clumps and pass through a 70 μm cell strainer placed on top of a 50 ml centrifuge tube. Wash the mortar twice with 10 ml of cold wash buffer and pass all wash buffers through the filter.
5. Centrifuge harvested cells at 244 × *g* for 6 min at 4 °C. Pour out the supernatant and resuspend in 10 ml of Purification media (*see* **Note 11**).
6. Using a Coulter counter, count the number of total bone marrow cells. Set the parameters for size to include cells between 4 and 10 μm. Calculate the total number of cells in your sample (*see* **Note 12**).
7. For Hoechst staining, resuspend cells to a concentration of 4.5×10^6 cells/ml in a glass bottle and stain with 8.8 μg/ml of Hoechst 33342 for 90 min in a 37 °C water bath. Every 30 min swirl the bottle gently to avoid settling of the cells.
8. After staining, transfer all cells to 50 ml centrifuge tubes and spin down at 244 × *g* for 6 min at 4 °C. Pour out the supernatant and resuspend the cells in 5 ml of Purification media.
9. Pipette 5 ml of room temperature Ficoll-Paque Plus into a 15 ml centrifuge tube. Tilt the tube slightly and carefully (very slowly)

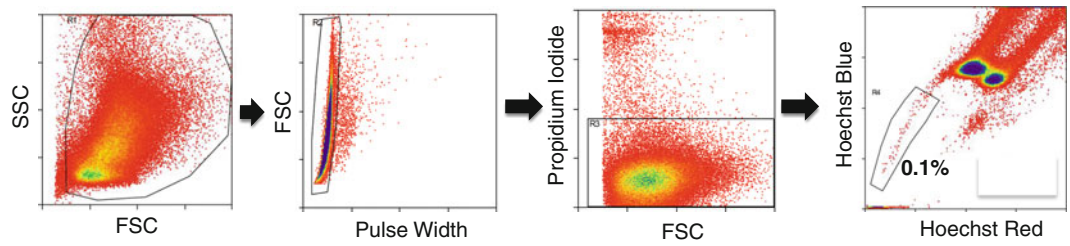

Fig. 1 Gating strategy for sorting HSC contained within the SP fraction of total bone marrow. Total bone marrow samples stained with Hoechst 33342 were depleted of red blood cells using a Ficoll density gradient. Remaining cells were analyzed using a MoFlo cell sorter (BD). Doublets and dead cells were excluded and the remaining Hoechst profile was analyzed using a UV laser. Proper gating of SP cells is critical to ensure that the final sorted population contains pure HSC. Understained samples or improper gating could result in the inclusion of progenitors and mature blood cell types within the purified side population

layer the cells atop the Ficoll layer. Spin the cells at 805 × *g* for 20 min at 20 °C.

10. Carefully pipette out the buffy coat and transfer to a clean 15 ml centrifuge tube. Wash cells thoroughly with wash buffer by pipetting up and down several times. Spin at 244 × *g* for 6 min at 4 °C.
11. Resuspend cells in 1.5–2 ml of FACS media. Filter cells through a 40 μm cell strainer directly into a 5 ml FACS polypropylene tube. Keep cells on ice to avoid Hoechst efflux.
12. Analyze and sort cells using a Beckman Coulter MoFlo cell sorter, BD FACSAria cell sorter or other (Fig. 1) (*see* **Note 13**). Sort cells directly to wells of a 96-well round bottom plate containing 50 μL of HSC Transduction Media.

3.3 Lentiviral Transduction of Purified Hematopoietic Stem Cells

1. To each well containing HSC from Subheading 3.2 (Fig. 2), carefully add the volume of virus that corresponds to 200–300 MOI and mix gently by pipetting slowly to prevent bubbles (*see* **Notes 14** and **15**).
2. Place cells at 37 °C, 5 % CO_2 overnight (*see* **Note 16**).

4 Notes

1. When transfecting 293T for viral production, it is imperative that the cells are at the proper confluence. Improper confluence may affect transfection efficiency ultimately leading to inefficient viral production. Although instructions from manufacturers of transfection reagents recommend to transfect cells at relatively low confluence, for viral production we strongly recommend to perform transfection when cells are 85–90 % confluent.

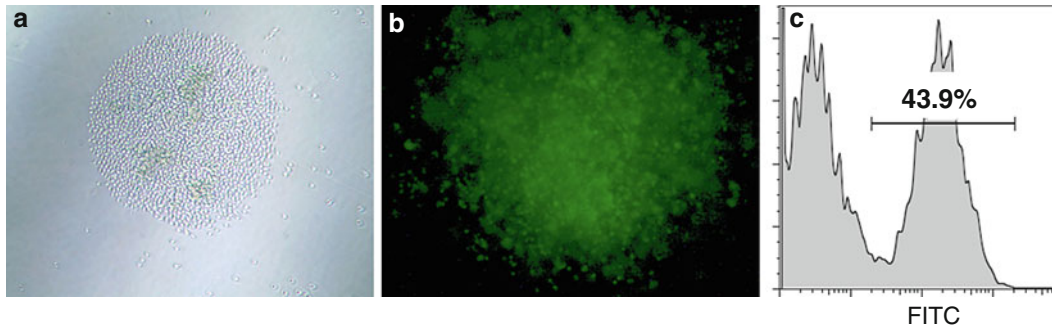

Fig. 2 Lentiviral transduction of purified HSC. (**a**) Purified HSC were sorted directly to a 96-well round bottom plate for lentiviral transduction. (**b**) Transduced HSC were cultured in methylcellulose media for 7 days to allow for colony formation. Fluorescence microscopy image shows a FITC positive colony arising from a successfully transduced HSC. (**c**) Resulting methylcellulose cultures can be analyzed by FACS to determine the percentage of cells that are positive for the fluorescent reporter in order to track efficiency of transduction

2. All plasmid DNA used for viral production should be of high quality and purity (normally the DNA obtained from a Midiprep or Maxiprep purification kit works well).
3. 293T cells can be easily detached from tissue culture treated surfaces, and therefore take extreme care when changing media in between viral collections. Slowly pipette media to the side wall of the plate in order to prevent loss of cells.
4. We recommend collecting viral supernatants a total of five times. To make collections easier, we suggest collecting virus twice on days 1 and 2 (starting 48 h after transfection) once in the morning and again in the evening (8–10 h apart). On the third day of collection, collect supernatant once in the morning, and proceed with concentration (**step 9**). If necessary, unconcentrated viral particles can be stored at 4 °C for up to 4–5 days without losing any viral activity.
5. To limit chances of contaminating viral supernatants carry out this step next to a gas flame. To make balancing of samples easier, use a glass beaker. Zero the beaker and place the bucket plus ultracentrifuge tube inside the beaker. Then slowly add your viral supernatant to the tube.
6. After centrifugation, you may or may not see a small loose white pellet. This is normal. Continue with aliquoting and titering your virus.
7. This 2–3 h incubation allows for any virus to come down off the sides of the centrifuge tubes and also helps viral particles to come into solution. When aliquoting, we recommend making 10 μL working aliquots, and one to two tubes of larger volumes of virus that can be frozen and thawed to aliquot later on. We recommend not to freeze–thaw viral aliquots more than twice.

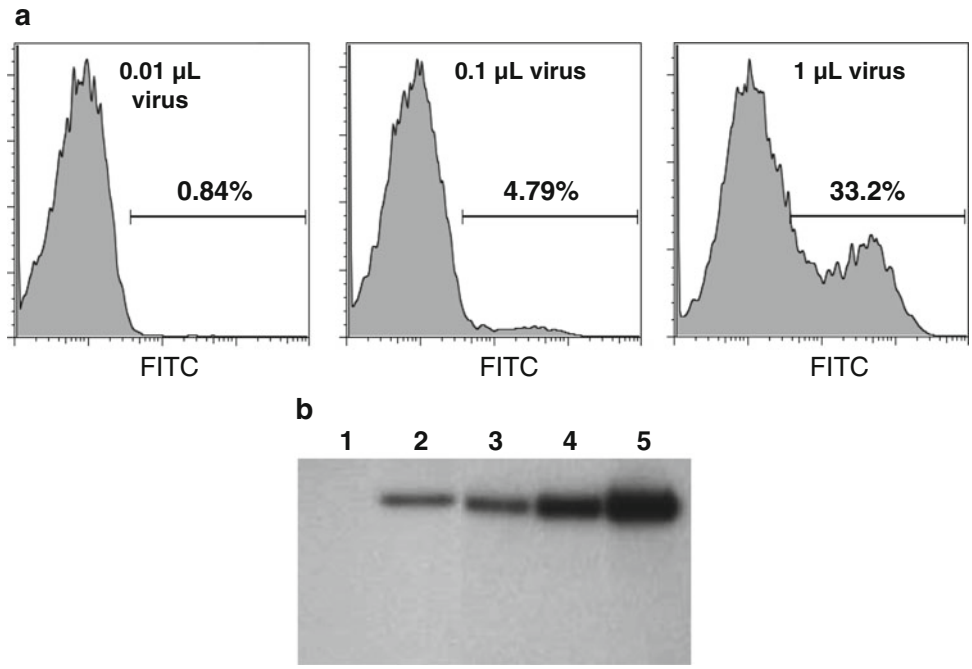

Fig. 3 Titering of concentrated lentivirus by flow cytometry and southern blot. (**a**) Lentiviruses containing a GFP fluorescent reporter titered by FACS. HEK293 cells were transduced with increasing volumes of concentrated lentiviral supernatants and cultured for 3 days prior to analysis by FACS. Titer of infectious particles per ml is calculated based on percentage of positive cells from the total of transduced cells. (**b**) Titering of lentiviral preparation by Southern blot. *Lane 1* represents uninfected HEK293 control, *Lane 2*: Copy number control (1 copy), *Lanes 3*, *4*, and *5*: HEK293 transduced with 1 µL, 5 µL, or 10 µL of concentrated virus, respectively

8. To titer viruses by FACS, transduce HEK293 cells (6-well plate) (~90 % confluent), with 0.01, 0.1, and 1 µL of concentrated virus. For Southern blot, transduce HEK293 with 1, 5, and 10 µL of concentrated virus. Transduction is performed in 1 ml of 10 % DMEM media containing 5 µg/ml Polybrene. Add the appropriate volume of virus. Swirl the plate gently to distribute virus. The next day, change media and leave cells for 2 more days before analyzing cells by FACS, or for gDNA extraction (Fig. 3).
9. When harvesting long bones, be sure to remove excess muscle and tissues from the bone so that the extracted bones are as clean as possible. Improper dissection will result in contamination from other cell types and improper filtering of samples in **step 4** of the purification process.
10. When crushing harvested long bones, the cells from the marrow will be released into the wash solution and will start to turn pink or light red in color. Continue to crush until the bones appear white in color. You will see small red clumps in the solution, which is why we recommend pipetting up and down thoroughly in the following step.

11. Resuspend cells in 10 ml of purification media/for every mouse used for bone marrow harvest. Cell counts are critical for Hoechst staining, and therefore we recommend resuspending total bone marrow cells in an appropriate volume to be sure that the coulter counter used in the following step gives an accurate count. For example, for cells from four mice, resuspend in 40 ml of purification media.
12. We normally obtain approximately 1×10^8 total bone marrow cells per mouse; however, this number will vary depending upon the age and health of your mice. Below is a sample calculation, which will help you determine the appropriate volume of purification media and Hoechst 33342 required to obtain optimal staining.

    For 1×10^7 cells/ml in 10 ml (total of 1×10^8 total cells):

    1×10^8 total cells/4.5×10^6 cells/ml for staining = 22.2 ml of purification media.

    For staining: Take 10 ml of your cells + 12.2 ml of purification media + 22.2 μl of Hoechst 33342 (8.8 mg/ml 1,000X stock).
13. HSC contained within the side population account for only 0.05–0.1 % of the total bone marrow.
14. If your viral aliquots do not appear clean or you suspect the presence of debris (which can interfere with transduction efficiencies), do a quick spin before adding the virus to your cells.
15. Alternatively additional spinfection of cells with virus for 2 h at $800 \times g$ at 37 °C, may increase efficiency of transduction. For spinfection, spin cells in 100 μl of HSC transduction media and add double the volume of virus as used in 50 μl. Then leave cells overnight as in **step 2**. The increased volume of media maintains cell viability during centrifugation.
16. The next day transduced HSC are ready to be used for in vitro assays such as methylcellulose colony forming unit assays or for in vivo transplant experiments. For lentiviruses containing a fluorescent reporter, allow for up to 3 days to observe reporter expression.

References

1. Kohn DB, Sadelain M, Dunbar C et al (2003) American Society of Gene Therapy (ASGT) ad hoc subcommittee on retroviral-mediated gene transfer to hematopoietic stem cells. Mol Ther 8(2):180–187
2. Maetzig T, Galla M, Baum C et al (2011) Gammaretroviral vectors: biology, technology and application. Viruses 3(6):677–713
3. Somia N, Verma IM (2000) Gene therapy: trials and tribulations. Nat Rev Genet 1(2): 91–99
4. Peters SO, Kittler EL, Ramshaw HS et al (1996) Ex vivo expansion of murine marrow cells with interleukin-3 (IL-3), IL-6, IL-11, and stem cell factor leads to impaired engraftment in irradiated hosts. Blood 87(1):30–37
5. Yonemura Y, Ku H, Hirayama F et al (1996) Interleukin 3 or interleukin 1 abrogates the reconstituting ability of hematopoietic stem cells. Proc Natl Acad Sci U S A 93(9):4040–4044
6. Blomer U, Naldini L, Kafri T et al (1997) Highly efficient and sustained gene transfer in

adult neurons with a lentivirus vector. J Virol 71(9):6641–6649

7. Kafri T, Blomer U, Peterson DA et al (1997) Sustained expression of genes delivered directly into liver and muscle by lentiviral vectors. Nat Genet 17(3):314–317
8. Naldini L, Blomer U, Gallay P et al (1996) In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science 272(5259):263–267
9. Uchida N, Sutton RE, Friera AM et al (1998) HIV, but not murine leukemia virus, vectors mediate high efficiency gene transfer into freshly isolated G0/G1 human hematopoietic stem cells. Proc Natl Acad Sci U S A 95(20):11939–11944
10. Heckl D, Wicke DC, Brugman MH et al (2011) Lentiviral gene transfer regenerates hematopoietic stem cells in a mouse model for Mpl-deficient aplastic anemia. Blood 117(14): 3737–3747
11. Cavazzana-Calvo M, Payen E, Negre O et al (2010) Transfusion independence and HMGA2 activation after gene therapy of human beta-thalassaemia. Nature 467(7313):318–322
12. Sakuma T, Barry MA, Ikeda Y (2012) Lentiviral vectors: basic to translational. Biochem J 443(3):603–618
13. Mostoslavsky G, Kotton DN, Fabian AJ et al (2005) Efficiency of transduction of highly purified murine hematopoietic stem cells by lentiviral and oncoretroviral vectors under conditions of minimal in vitro manipulation. Mol Ther 11(6):932–940
14. Mostoslavsky G, Fabian AJ, Rooney S et al (2006) Complete correction of murine Artemis immunodeficiency by lentiviral vector-mediated gene transfer. Proc Natl Acad Sci U S A 103(44):16406–16411
15. Baum C, Dullmann J, Li Z et al (2003) Side effects of retroviral gene transfer into hematopoietic stem cells. Blood 101(6):2099–2114
16. Hacein-Bey-Abina S, von Kalle C, Schmidt M et al (2003) A serious adverse event after successful gene therapy for X-linked severe combined immunodeficiency. N Engl J Med 348(3):255–256
17. Goodell MA, Brose K, Paradis G et al (1996) Isolation and functional properties of murine hematopoietic stem cells that are replicating in vivo. J Exp Med 183(4):1797–1806
18. Zhou S, Schuetz JD, Bunting KD et al (2001) The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7(9):1028–1034
19. Ema H, Takano H, Sudo K et al (2000) In vitro self-renewal division of hematopoietic stem cells. J Exp Med 192(9):1281–1288