Chapter 5

Transduction of Murine Hematopoietic Stem Cells with Tetracycline-regulated Lentiviral Vectors

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Abstract

Tetracycline-regulated integrating vectors allow pharmacologically controlled genetic modification of murine and human hematopoietic stem cells (HSCs). This approach combines the stable transgene insertion into a host genome with the opportunity for time- and dose-controlled reversible transgene expression in HSCs. Here, we describe the step-by-step protocol for transduction of murine stem-cell enriched populations of bone marrow cells, such as lineage negative cells (Lin^-), with a lentiviral vector expressing the enhanced green fluorescent protein (EGFP) under the control of the tetracycline-regulated promoter. This chapter explains how to establish in vitro and in vivo systems to study transgene dose-dependent mechanisms affecting cell fate decisions of genetically modified hematopoietic cells.

Key words Tetracycline-regulated lentiviral vectors, Murine hematopoietic stem cells, Transduction, Gene transfer, Dose-dependent transgene expression

1 Introduction

Tetracycline-regulated retroviral vectors allow genetic modification of hematopoietic stem cells (HSCs) via drug-controlled transgene overexpression and are widely used to study determinants of normal and malignant hematopoiesis such as self-renewal, proliferation/survival, and impaired differentiation [1–3]. Furthermore, recently developed tetracycline-regulated RNAi technologies enable the study of loss-of-function phenotypes and thus characterization of genes encoding putative drug targets in hematopoietic disorders [3]. Tetracycline-regulated vectors based on lentiviral (LV) backbone integrate into the genome of target cells independently of cell division [4, 5] and thus allow further optimization of existing transduction protocols [6, 7]. Accumulating studies show that

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tetracycline-regulated lentiviral vectors provide efficient dose- and time-controlled reversible transgene expression to investigate immediate [8, 9] and long-term effects of transgene upregulation and downregulation in murine HSCs [5, 10, 11] using in vitro and in vivo experimental systems. Importantly, long-term murine bone marrow (BM) transplantation (BMT) experiments demonstrated stable and reversible transgene expression in serial recipients with development of benign clonal selection, both in the presence and absence of doxycycline (DOX) induction [11]. Here, when lentiviral self-inactivating vectors were used to express enhanced green fluorescent protein (EGFP) under the control of the tetracycline-regulated promoter, the majority of tetracycline-regulated vector integration sites were identified in introns and exons of transcription units and in non-coding/repeat regions of the genome, as previously described for constitutively expressing lentiviral vectors [11–13]. However, there is no guarantee that insertional mutagenesis will be avoided in the case of increased vector copy numbers (VCN), prolonged animal observation time and particularly when fluorescent markers are co-expressed with potent proto-oncogenes or genes involved in signaling cascades [6, 11, 14, 15]. Nevertheless, the opportunities to control vector and doxycycline dose, to monitor background activity of tetracycline-regulated promoters (TRPs) and to characterize the vector insertional profile [11, 16] allow establishment of promising in vitro and in vivo systems to study the dose-dependent role of transgene overexpression in mechanisms triggering fate decisions of genetically modified HSCs.

In this chapter, we provide the detailed transduction protocol of lineage negative (Lin^-) bone marrow cells from Rosa26rtTA-nls-Neo2 (Rosa26rtTA) mice expressing the reverse tetracycline-inducible transactivator (rtTA-M2) under the control of the ubiquitously active Rosa26 locus [8, 11, 17]. For transduction, we used a lentiviral self-inactivating vector expressing EGFP [11] under the control of the T11 tetracycline-regulated promoter [2], which is an improved version of the TRP originally described by Gossen and Bujard [18]. We present a detailed description of all steps, including pre-stimulation of the HSC-enriched fraction (Lin^-) of Rosa26rtTA BM, transduction of (Lin^-) Rosa26rtTA cells by tetracycline-regulated lentiviral vectors with different multiplicities of infection (MOIs), doxycycline dose-dependent induction of transgene overexpression and determination of gene transfer/expression levels. Emphasis is given to the important characteristics of tetracycline-regulated system, such as inducibility and background activity of TRPs in the absence of doxycycline. We describe the specific details of culturing transduced cells for in vitro expansion or transplantation into lethally irradiated mouse recipients for long-term in vivo murine transplantation studies (Fig. 1).

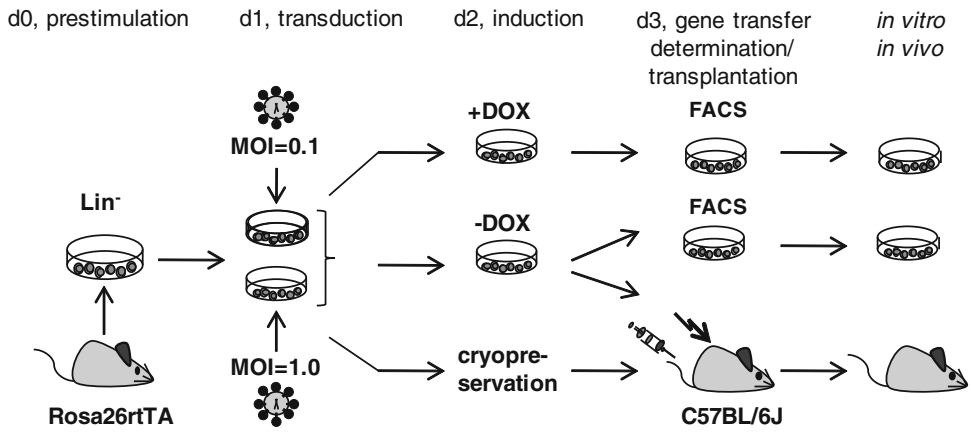

Fig. 1 Flow sheet of experimental procedures. *DOX* doxycycline, *MOI* multiplicity of infection, *d0–3* day 0–3

2 Materials

Prepare all solutions using ultrapure water (Biochrom, Berlin, Germany) under sterile conditions. Follow the appropriate S2 biosafety regulations when working with Vesicular Stomatitis Virus glycoprotein (VSVg)-pseudotyped LV vector particles.

2.1 Pre-stimulation of Purified Lineage Negative Rosa26rtTA Bone Marrow Cells

1. StemSpan medium (STEMCELL Technologies SARL, Cologne, Germany). For long-term storage, keep aliquots at −20 °C. Before use, thaw and store at 4 °C.
2. Cytokines (Peprotech, Hamburg, Germany): recombinant murine stem cell factor (mSCF), recombinant murine interleukin 3 (mIL-3), recombinant human FMS-like tyrosine kinase 3 ligand (hFlt-3), recombinant human interleukin 11 (hIL-11). Store aliquots at −20 °C.
3. 200 mM L-glutamin. For long-term storage, keep aliquots at −20 °C.
4. 10,000 U/mL penicillin/10 mg/mL streptomycin. Aliquots should be kept at −20 °C for long-term storage.
5. 20 μg/μL meropenem HEXAL (HEXAL AG, Holzkirchen, Germany). Keep aliquots at −20 °C.
6. Culturing medium: serum-free StemSpan medium with 2% penicillin/streptomycin, 1% L-glutamine, 20 μg/mL meropenem HEXAL, 50 ng/mL mSCF, 20 ng/mL mIL-3, 50 ng/mL hFlt-3, 50 ng/mL hIL-11 (*see* **Note 1**).
7. Türk's solution (Merck KGaA, Darmstadt, Germany).
8. APC-conjugated streptavidin antibody (eBioscience, San Diego, CA, USA).
9. Phosphate-buffered saline (PBS) (DPBS Dulbecco's Phosphate Buffered Salt Solution).

10. Fetal bovine serum (FBS) Standard Quality, heat inactivated for 30 min at 56 °C. For long-term storage, keep aliquots at −20 °C. Before use, thaw and store at 4 °C.
11. Staining buffer: 4% FBS in PBS.
12. FACS-buffer: 4% FBS, 2 mM EDTA in PBS.
13. Cell culture plastic ware: 48-well tissue plates for suspension cell culture, 50 mL tubes.
14. Cell culture incubator set at 37 °C, 5% CO_2, 100% humidity.
15. Neubauer counting chamber and cover glasses.
16. Automatic counter CASY-TT instrument (Roche Diagnostics, Mannheim, Germany).
17. Suitable Eppendorf centrifuge, e.g., Heraeus Fresco 17 Centrifuge (Thermo Fisher Scientific, Waltham, MA, USA).
18. FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).
19. 100 µg/mL propidium iodide (PI) (100×).
20. Freezing medium: 10% DMSO in FBS.

2.2 Transduction of Lineage Negative Rosa26rtTA Bone Marrow Cells with Tetracycline-regulated Lentiviral Vectors

1. 48 µg/mL retronectin (RN) (TaKaRa, Saint-Germain-en-Laye, France), store at −20 °C.
2. 10× Hank's balanced salt solution (HBSS). To prepare 1× HBSS: add 5 mL 10× HBSS to 45 mL of water.
3. Bovine serum albumin (BSA).
4. 1 M Hepes buffer.
5. RN-blocking solution: 2% BSA in PBS. Weigh 1 g of BSA and transfer to 50 mL tube. Dissolve with 50 mL PBS. Sterilize by filtration with 0.22 µm MILLEX®-GP (Merck KGaA). Store at 4 °C.
6. RN-washing solution: 2.5% (volume/volume) 1 M Hepes in 1× HBSS. Dilute 1.25 mL of 1 M Hepes with 48.75 mL of 1× HBSS. Store at 4 °C.
7. Cooling centrifuge Heraeus Multifuge 3 S-R (Thermo Fisher Scientific) with tissue culture plate holders.
8. Cell-free tetracycline-regulated lentiviral vector supernatant (SNT) with known titer. Store aliquots at −80 °C. Ecotropic- or VSVg-pseudotyped LV vector particles are recommended. For example, concentrated ecotropic-pseudotyped (*see* **Note 2**) LV vector pRRL.PPT.T11.EGFP.pre [11] SNT with titer of 3.7×10^7 tu/mL (transducing units per milliliter) (*see* **Note 3**).

2.3 Doxycycline Induction of Transgene Expression

Doxycycline hyclate (Sigma-Aldrich, St. Louis, MO, USA), 1.0 mg/mL stock solution: dissolve 0.1 g of Doxycycline hyclate in 100 mL of water. Sterilize by filtration with 0.22 µm

MILLEX®-GP (Merck KGaA). For long-term storage, keep 1 mL aliquots at −20 °C. The working aliquot can be stored at 4 °C (*see* **Note 4**).

2.4 Determination of Gene Transfer/ Expression Levels

1. 100 µg/mL propidium iodide (100×).
2. FACSCalibur flow cytometer.
3. FlowJo software (Tree Star, Ashland, OR, USA) or similar flow cytometry analysis program.

3 Methods

Here, we describe the step-by-step routine transduction protocol of lineage negative Rosa26rtTA bone marrow cells. Modification of this HSC-enriched fraction of murine bone marrow cells with tetracycline-regulated lentiviral vectors can be accomplished in four main steps: (Subheading 3.1) pre-stimulation of purified lineage negative Rosa26rtTA bone marrow cells; (Subheading 3.2) transduction of lineage negative Rosa26rtTA bone marrow cells with tetracycline-regulated lentiviral vectors; (Subheading 3.3) doxycycline induction of transgene expression; (Subheading 3.4) determination of gene transfer/expression levels.

3.1 Pre-stimulation of Purified Lineage Negative Rosa26rtTA Bone Marrow Cells

Work under sterile conditions (day 0) (Fig. 1).

1. Resuspend purified (Lin^-) Rosa26rtTA BM cells (*see* **Note 5**) in 1 mL of culturing medium. Keep on ice.
2. Determine the number of vital purified (Lin^-) Rosa26rtTA BM cells by diluting 1:100 in Türk's solution and counting in a Neubauer counting chamber. Alternatively, an automatic counter can be used.
3. Determine the purity of (Lin^-) Rosa26rtTA bone marrow cells. To accomplish this, stain 10^5 lineage negative and lineage positive cells with 0.1 µg of APC-conjugated streptavidin antibody for 30 min at 4 °C in 0.1 mL of staining buffer. After staining add 1 mL of PBS, centrifuge for 2 min at 400 × *g* in a Heraeus Fresco 17 Centrifuge and carefully discard the supernatant. Repeat the washing procedure and resuspend the pellet in 0.3 mL of FACS buffer. Add 3 µL of 100× PI solution to stain dead cells, mix by vortex. Acquire the amount of APC-positive cells on a FACSCalibur flow cytometer: a purity ≥85 % of APC negative cells in (Lin^-) fraction is recommended.
4. Dilute the (Lin^-) Rosa26rtTA cells at a density of 0.5×10^6/0.5 mL in culturing medium and plate in independent wells of a 48- or 24-well suspension culture plate (*see* **Note 6**). One well corresponds to one biological replicate.
5. Cultivate for 12–24 h in a cell culture incubator (Fig. 1).

3.2 Transduction of Lineage Negative Rosa26rtTA Bone Marrow Cells with Tetracycline-regulated Lentiviral Vectors

1. Define the size and number of wells for RN pre-coating. Considerations for this step include the cell number to be transduced, multiplicity of infection and number of replicates (*see* **Notes 7** and **8**). For example, to transduce 1×10^5 (Lin^-) Rosa26rtTA cells with one vector but two different multiplicities of infection (MOI 0.1 and 1) in three biological replicates, pre-coat a total of six wells of a 48-well (1.1 cm^2) suspension culture plate (*see* **Note 9**).
2. For RN pre-coating of 48-well plate, add 228 µL of 48 µg/mL RN solution per well (final concentration 10 µg/cm^2) (*see* **Note 10**). Incubate for 2 h at room temperature (RT) to allow the RN to adhere to the plate (*see* **Note 11**). Before transduction, remove the RN solution and block with 500 µL of RN-blocking solution for at least 30 min at RT. Discard the blocking solution and add 700 µL of RN-washing solution.
3. Thaw LV vector SNT (*see* **Notes 2** and **12**), and store it on ice. In an Eppendorf tube, supplement the calculated amount of SNT to achieve the selected MOI with ice-cold pure StemSpan medium. For example, to obtain an MOI 1.0 in order to transduce 1×10^5 of (Lin^-) Rosa26rtTA cells with the ecotropic vector pRRL.PPT.T11.EGFP.pre [11], supplement 100 µL of SNT (titer 2×10^6 tu/mL) with pure StemSpan medium to a final volume of 400 µL (for 48-well plate) (*see* **Note 13**). We recommend preparing a master mix for several biological replicates. Mix carefully.
4. Remove the washing solution from RN pre-coated wells.
5. Add prepared supernatant to RN pre-coated well. For instance, add SNT with a final volume of 400 µL to RN pre-coated well of 48-well tissue culture plate.
6. Centrifuge the plate at 2000 rpm ($800 \times g$) and 32 °C for 60 min in a Heraeus Multifuge to facilitate the binding of LV vector particles with RN (*see* **Notes 14** and **15**).
7. During the centrifugation step, carefully resuspend and count the pre-stimulated (Lin^-) Rosa26rtTa cells. Calculate if any additional culture medium is needed to achieve the required density and amount of (Lin^-) Rosa26rtTA cells for transduction. Have culture medium and cell suspension prepared in the sterile hood.
8. After the centrifugation step to bind the LV vector particles to the RN, carefully remove the supernatants from wells (LV vector particles are expected to be bound to RN on the bottom of the well). It is important to work fast and not allow the wells to dry. Add prepared cells to LV vector-coated wells. For instance, add 1×10^5 of (Lin^-) Rosa26rtTA cells in 400 µL of culture medium to one LV vector-coated well of a 48-well tissue culture plate.
9. Incubate the cells in a cell culture incubator (day 1) (Fig. 1).

3.3 Doxycycline Induction of Transgene Expression

1. On day 2 (Fig. 1), carefully resuspend the transduced cells in each well (*see* **Notes 16** and **17**). Divide the volume of each well (400 µL for 48-well plate) into two aliquots (200 µL) and transfer into two wells of a new 48-well suspension culture plate.
2. Supplement the one culture aliquot with an additional equal volume of fresh culturing medium containing no DOX and the second culture aliquot with medium containing double the final DOX concentration. For example for 48-well plate, the first aliquot (200 µL) could be supplemented with 200 µL of 0 µg/mL DOX culturing medium ("no DOX" control), and the second aliquot with 200 µL of 2.0 µg/mL DOX culturing medium (final DOX concentration 1.0 µg/mL).
3. It is recommended to include intermediate DOX concentrations corresponding to final 0.01 and 0.1 µg/mL of DOX (*see* **Note 18**).
4. Identical DOX concentrations including the "no DOX" control are recommended to be applied when different MOIs of lentiviral tetracycline-regulated vector are used to transduce (Lin^-) Rosa26rtTA cells (*see* **Note 18**).
5. Incubate the cells in a cell culture incubator (day 2) (Fig. 1).

3.4 Determination of Gene Transfer/Expression Levels

1. On day 3 (Fig. 1), carefully resuspend cells in each well.
2. Take an aliquot to count the number of living cells.
3. Take an aliquot for FACS analysis: 3×10^4 cells are sufficient (usually ~40 µL). Add 300 µL of FACS buffer and 3 µL of 100× PI solution, mix by vortex. Keep on ice.
4. Acquire EGFP-positive cells on a FACSCalibur flow cytometer and analyze with FlowJo or equivalent software (Fig. 2).
5. Determine the background activity of the tetracycline-regulated promoter in the absence of DOX (Figs. 2 and 3) (*see* **Note 19**).
6. Determine gene transfer (percent of $EGFP^+$ cells) and expression (MFI of EGFP) levels after 24 h of DOX induction (Fig. 3).
7. Further in vitro expansion to determine gene transfer/expression levels in transduced (Lin^-) Rosa26rtTA cells on days 5 and 10 after DOX induction is recommended (*see* **Note 20**). On days 8–10, material for DNA preparation and vector copy number identification could be taken (*see* **Note 19**).
8. Transplantation of transduced (Lin^-) Rosa26rtTA donor cells, which were not treated with DOX, into lethally irradiated recipient mice (C57BL/6J) is recommended on day 3 (Fig. 1). Alternatively, the material cryopreserved on day 2 could be transplanted (Fig. 1, *see* **Notes 16** and **17**).

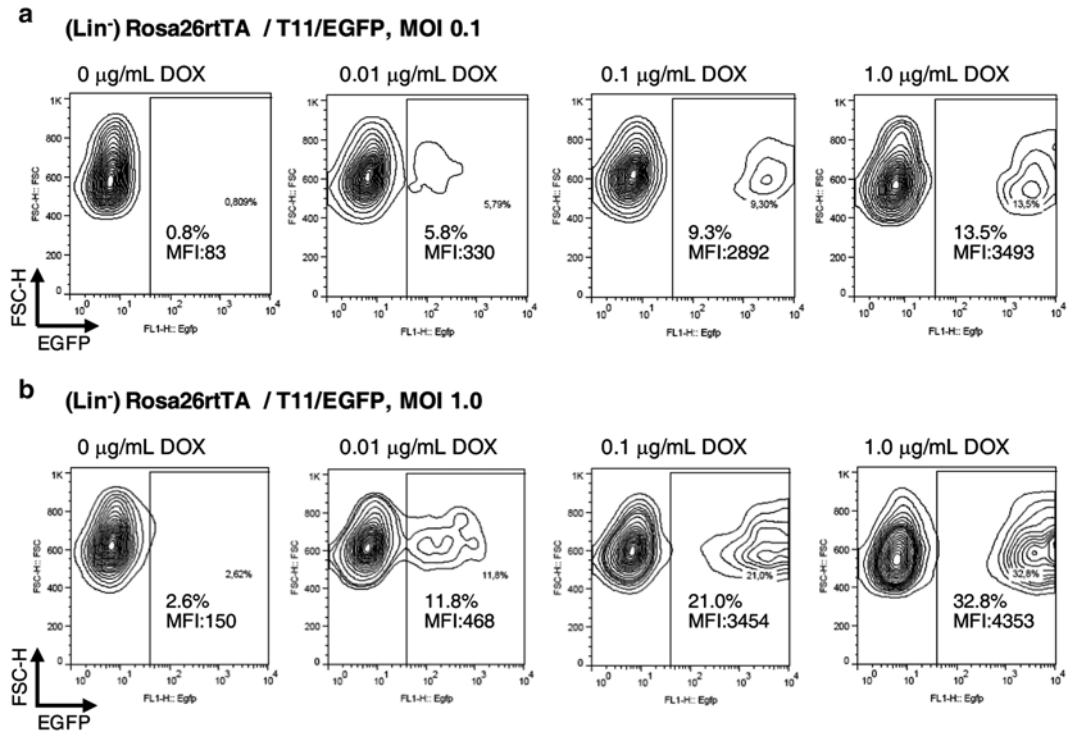

Fig. 2 Estimation of gene transfer/expression levels and background activity of the lentiviral T11/EGFP vector in transduced (Lin⁻) Rosa26rtTA cells. (**a**, **b**) T11 background activity (at 0 µg/mL of DOX) and transduction rate (percentage of EGFP⁺ cells; MFI of EGFP), when different concentrations of DOX (0.01, 0.1, 1.0 µg/mL) were applied for 24 h to induce EGFP expression in (Lin⁻) Rosa26rtTA cells transduced with the T11/EGFP vector using MOI = 0.1 (**a**) and MOI = 1.0 (**b**). Selected contour plots from three biological replicates are presented. *MFI* mean fluorescence intensity of EGFP, *DOX* doxycycline, *MOI* multiplicity of infection

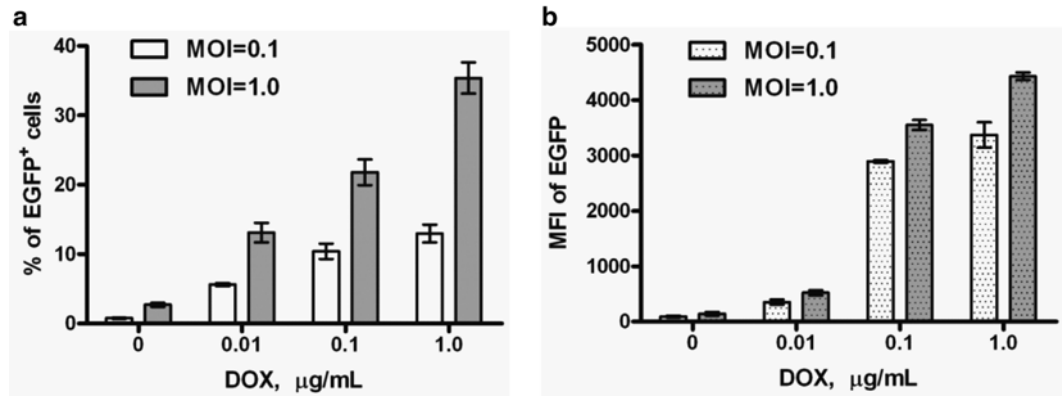

Fig. 3 T11 promoter inducibility in (Lin⁻) Rosa26rtTA cells transduced with lentiviral T11/EGFP vector. Gene transfer (percentage of EGFP⁺ cells) (**a**) and gene expression (MFI of EGFP) (**b**) levels, when different concentrations of DOX (0, 0.01, 0.1, 1.0 µg/mL) were applied for 24 h to induce EGFP expression in (Lin⁻) Rosa26rtTA cells transduced with the T11/EGFP vector using MOI = 0.1 and MOI = 1.0. Data are summarized from experiments performed in biological replicates and presented as mean ± SD, $n = 3$. *MFI* mean fluorescence intensity of EGFP, *DOX* doxycycline, *MOI* multiplicity of infection

4 Notes

1. In addition to the presently described cytokine composition of the culturing medium, others can also be used [8, 11, 19–21].
2. We recommend concentration of ecotropic- or VSVg-pseudotyped LV vector particles [22, 23] via ultracentrifugation at 10,000 rpm (13,238 × *g*) 16–24 h or at 25,000 rpm (82,740 × *g*) for 2 h at 4 °C, correspondingly (Ultracentrifuge Optima LE-80K, Beckman Coulter, Brea, CA, USA). Dissolve the concentrated LV vector particles in pure serum-free StemSpan medium to avoid exposure of HSCs to serum components resulting in unwanted differentiation. Store LV vector aliquots at −80 °C.
3. To estimate the titer of tetracycline-regulated lentiviral vectors, we use modified murine fibroblasts (SC1), which express rtTA2 (SC1/rtTA2) [8, 11, 24].
4. Doxycycline is light sensitive. For short-term usage, keep 1 mg/mL stock solution aliquots at 4 °C in Eppendorf tubes made from dark plastic or use aluminum foil to cover the Eppendorf tube and protect the DOX from light. For long-term storage, keep 1 mg/mL stock solution at −20 °C.
5. On average, we harvest ~4×10^7 BM cells from one mouse (age ≥8 weeks) when total BM is flushed out from femurs, tibiae and pelvis. For preparation of (Lin^-) Rosa26rtTA BM cells, we use the Lineage Cell Depletion kit (Miltenyi Biotec, Bergisch Gladbach, Germany) and magnetic cell separation (MACS) technology. The expected amount of (Lin^-) cells is ~1 % of the total BM cells [6], but may vary depending on the purity of isolated (Lin^-) cells. Purity of (Lin^-) cells ≥85 % is recommended depending on experimental purpose.
6. (Lin^-) cell aliquots can be cryopreserved immediately after MACS purification in freezing medium at −80 °C and later transferred to liquid nitrogen. After thawing, it is important to count viable cell numbers: in our experience, the number of viable cells is usually about half of that estimated before freezing.
7. Tissue culture plate format and corresponding amounts of reagents depend on the amount of cells to be transduced [6]. When ≤2×10^4 cells are transduced (for example LSK: Lin^- $Sca1^+$ $cKit^+$) with tetracycline-regulated lentiviral vectors, the use of a 96-well plate is recommended.
8. For further in vitro experiments (Fig. 1), we recommend plating freshly prepared or thawed (Lin^-) cells in triplicates (three biological replicates) [11].

9. We recommend using empty wells to separate experimental wells for mock untransduced cells and/or cells transduced with different vectors. When possible, different tissue culture plates could be used.
10. Amounts of RN depend on tissue culture plate format [6]. For instance, to achieve a final RN concentration of 10 μg/cm^2 to pre-coat one well in a 24-well plate (2.0 cm^2), add 420 μL of 48 μg/mL RN stock to the well [6].
11. RN pre-coating could be started at day 0 (Fig. 1). If transduction is performed on the next day (day 1) RN pre-coated plates could be prepared by incubation for 2 h at RT at day 0, and then kept overnight at 4 °C.
12. If the titer is high but experimental MOI and amount of cells are low, dilute the calculated amount of SNT with ice-cold pure StemSpan medium in an Eppendorf tube to achieve the lower titer. For example, to transduce 1×10^5 of (Lin$^-$) Rosa26rtTA cells with an ecotropic vector pRRL.PPT.T11.EGFP.pre [11] with a titer 3.7×10^7 tu/mL and an MOI = 1.0, we recommend diluting the SNT stock to a titer of 2×10^6 tu/mL.
13. We suppose that only half of the LV vector particles will bind to the RN pre-coated plate.
14. LV vector particles preloading (via spinoculation) can be performed at 4 °C, but the efficiency of (Lin$^-$) transduction was observed to be increased at 32 °C.
15. LV vector particles preloading procedure can be repeated when the titer of the supernatant is low, but high MOIs are needed.
16. If further in vivo murine BMT experiments will be performed, we recommend culturing the majority of the transduced cells without DOX induction until day 3, when the transduced cells will be transplanted (Fig. 1). Alternatively, transduced material could be cryopreserved already at day 2 (Fig. 1). In both cases, we recommend using an aliquot of transduced cells for DOX treatment and transduction rate evaluation and/or for further in vitro expansion.
17. Cell differentiation during the culture period should be taken into consideration when calculating the amount of cells needed for BMT experiments [6].
18. For biological replicates within independent experiments [11], we recommend use of ecotropic- or VSVg-pseudotyped LV vector supernatants from the same preparation batch in order to minimize the "batch to batch" variability.
19. It was shown that transduction of (Lin$^-$) Rosa26rtTA cells with increasing MOIs of tetracycline-regulated lentiviral vectors resulted in increased vector copy number and a linear increase

of background activity of tetP TRP in the absence of DOX [11, 18]. The level of "leakiness" could be decreased by using improved TRPs, for instance such as T11 [2, 11] (Figs. 2 and 3).

20. For further in vitro expansion, we give the cells fresh culturing medium (no DOX or final DOX concentration) 2–3 times per week. It could be necessary to split the cells into larger tissue culture plates to maintain the recommended cell density. For both feeding and splitting we include half fresh and half conditioned medium [6].

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References

1. Kenny PA, Enver T, Ashworth A (2002) Retroviral vectors for establishing tetracycline-regulated gene expression in an otherwise recalcitrant cell line. BMC Mol Biol 3:13
2. Heinz N, Schambach A, Galla M et al (2011) Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. Hum Gene Ther 22:166–176
3. Zuber J, McJunkin K, Fellmann C et al (2011) Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. Nat Biotechnol 29:79–83
4. Vigna E, Naldini L (2000) Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. J Gene Med 2:308–316
5. Vigna E, Cavalieri S, Ailles L et al (2002) Robust and efficient regulation of transgene expression in vivo by improved tetracycline-dependent lentiviral vectors. Mol Ther 5:252–261
6. Modlich U, Schambach A, Li Z, Schiedlmeier B (2009) Murine hematopoietic stem cell transduction using retroviral vectors. In: Baum C (ed) Genetic modification of hematopoietic stem cells. Humana Press, New York, pp 23–31
7. Loew R (2009) The use of retroviral vectors for tet-regulated gene expression in cell populations. In: Baum C (ed) Genetic modification of hematopoietic stem cells. Humana Press, New York, pp 221–242
8. Kustikova OS, Schwarzer A, Stahlhut M et al (2013) Activation of Evi1 inhibits cell cycle progression and differentiation of hematopoietic progenitor cells. Leukemia 27:1127–1138
9. Konrad TA, Karger A, Hackl H et al (2009) Inducible expression of EVI1 in human myeloid cells causes phenotypes consistent with its role in myelodysplastic syndromes. J Leukoc Biol 86:813–822
10. Laurenti E, Barde I, Verp S et al (2010) Inducible gene and shRNA expression in resident hematopoietic stem cells in vivo. Stem Cells 28:1390–1398
11. Kustikova OS, Stahlhut M, Ha T-C et al (2014) Dose response and clonal variability of lentiviral tetracycline-regulated vectors in murine hematopoietic cells. Exp Hematol 42:505–515.e7
12. Beard BC, Dickerson D, Beebe K et al (2007) Comparison of HIV-derived lentiviral and MLV-based gammaretroviral vector integration sites in primate repopulating cells. Mol Ther 15:1356–1365

13. Kustikova OS, Schiedlmeier B, Brugman MH et al (2009) Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. Mol Ther 17:1537–1547
14. Heckl D, Schwarzer A, Haemmerle R et al (2012) Lentiviral vector induced insertional haploinsufficiency of Ebf1 causes murine leukemia. Mol Ther 20:1187–1195
15. Montini E, Cesana D, Schmidt M et al (2009) The genotoxic potential of retroviral vectors is strongly modulated by vector design and integration site selection in a mouse model of HSC gene therapy. J Clin Invest 119:964–975
16. Kustikova OS, Modlich U, Fehse B (2009) Genetic modification of hematopoietic stem cells. In: Baum C (ed) Genetic modification of hematopoietic stem cells. Humana Press, New York, pp 373–390
17. Magnusson M, Brun ACM, Miyake N et al (2007) HOXA10 is a critical regulator for hematopoietic stem cells and erythroid/megakaryocyte development. Blood 109:3687–3696
18. Gossen M, Bujard H (1992) Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. Proc Natl Acad Sci U S A 89:5547–5551
19. Schnabel CA, Jacobs Y, Cleary ML (2000) HoxA9-mediated immortalization of myeloid progenitors requires functional interactions with TALE cofactors Pbx and Meis. Oncogene 19:608–616
20. Zhang CC, Lodish HF (2005) Murine hematopoietic stem cells change their surface phenotype during ex vivo expansion. Blood 105:4314–4320
21. Li Z, Schwieger M, Lange C et al (2003) Predictable and efficient retroviral gene transfer into murine bone marrow repopulating cells using a defined vector dose. Exp Hematol 31:1206–1214
22. Schambach A, Bohne J, Baum C et al (2006) Woodchuck hepatitis virus post-transcriptional regulatory element deleted from X protein and promoter sequences enhances retroviral vector titer and expression. Gene Ther 13:641–645
23. Schambach A, Swaney WP, van der Loo JC (2009) Design and production of retro- and lentiviral vectors for gene expression in hematopoietic cells. In: Baum C (ed) Genetic modification of hematopoietic stem cells. Humana Press, New York, pp 191–205
24. Urlinger S, Baron U, Thellmann M et al (2000) Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. Proc Natl Acad Sci U S A 97:7963–7968