Multimodal Lentiviral Vectors for Pharmacologically Controlled Switching Between Constitutive Single Gene Expression and Tetracycline-Regulated Multiple Gene Collaboration

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Multimodal lentiviral vectors (LVs) allow switching between constitutive and tetracycline-regulated gene co-expressions in genetically modified cells. Transduction of murine primary hematopoietic progenitor cells (HPCs) with multimodal LVs in the absence of doxycycline ensures the constitutive expression of gene of interest 1 (GOI1) only. In the presence of doxycycline, induced tetracycline-regulated expression of a second GOI (GOI2) allows evaluation of the collaboration between two genes. Drug removal retains constitutive expression, which allows the contribution of an individual gene into created networks to be studied. Doxycycline-dependent switching can be tracked via fluorescent markers coupled to constitutive and tetracycline-regulated GOIs. This article describes transduction of murine primary HPCs with different doses of multimodal LVs, distinct cytokine conditions, and their influence on the number and viability of cells co-expressing both collaborating GOIs upon doxycycline induction. A 2-week protocol is provided for multimodal LV production, titer determination, and evaluation of tetracycline responsive promoter background activity in a murine fibroblast cell line. The power of this model to assess the dose/time/order-controlled contribution of single and multiple genes into hematopoietic networks opens new routes in reprogramming, stem cell, and leukemia biology.

Keywords: lentiviral vectors, constitutive and tetracycline-regulated, genetic modification of HSCs and HPCs, fate tracking

INTRODUCTION

LENTIVIRAL VECTORS (LVs) ALLOW efficient expression of selected transgenes in different cell types and are valuable tools to establish therapeutic and cancer models.^{1–6} Many research articles and protocols provide comprehensive information of how to design conventional lentiviral systems for constitutive or drug-controlled gene expression in hematopoietic stem cells (HSCs).^{7–14} Lentiviral systems that simultaneously co-express several genes of interest (GOIs) can be generated in case expression of more than one transgene is required to affect cell fate decisions. For example, to reprogram differentiated somatic cells into induced pluripotent stem cells (iPSC), constitutive LVs

successfully co-express several reprogramming factors via 2A self-cleavage sites.⁵ However, parameters such as the transgene expression level and reversibility of generated phenotype are difficult to control properly with such vectors. Conventional tetracycline-regulated systems help to enable dose- and time-controlled reversible transgene expression *in vitro* and *in vivo*.^{13–17} However, they are also limited by the general capacity of conventional LVs to overexpress or downregulate all delivered transcripts simultaneously. Thus, although multiple gene transfer via LVs is efficient, neither constitutive nor inducible conventional LVs allow sufficient regulation of single genes to study impact on gene network signaling/activity

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and therefore do not permit the investigation of the role of an individual gene in cell fate decision.

In addition, neither conventional constitutive nor inducible LVs are designed to address the role of sequential order of transgene expression in cellular processes such as transformation or reprogramming. For experimental strategies that use concomitant co-transduction of target cells with two different vectors expressing GOI1 and GOI2, it is difficult to achieve a 1:1 transduction ratio of two different GOIs. Although lentiviral self-inactivating (SIN) vectors are considered to be less genotoxic than gammaretroviral vectors (GVs),^{13,14,18,19} increased vector doses to obtain maximal numbers of cells co-expressing both GOIs can lead to increased side effects due to insertional mutagenesis.^{20,21} Such approaches are even more mutagenic for cell types that are difficult to transduce, that is, HSCs and hematopoietic progenitor cells (HPCs).

Recently, a multimodal lentiviral SIN vector system was developed, which combines both constitutive and inducible cassettes in the same backbone (Fig. 1a) to coordinate sequential order, dose, and temporal control of expression for multiple transgenes.²² Using this system, the well-described transcription factors (TFs) *Meis1* and *Hoxa9* were co-expressed (Fig. 1a), as they are well-known collaborative partners in murine acute myeloid leukemia development.^{22–24} In the absence of doxycycline (DOX), *Meis1* (GOI1) was constitutively expressed via the murine phosphoglycerate kinase (mPGK) promoter (Fig. 1a).²² DOX administration induced co-expression of collaboration partner *Hoxa9* (GOI2) by the tetracycline responsive promoter (TRP) T11, which is an improved version of the TRP initially introduced by Gossen and Bujard.^{14,22,25,26} To track *Meis1*, enhanced green fluorescent protein (EGFP) was linked by 2A self-cleavage sites.^{5,22} To monitor *Hoxa9* expression, the red fluorescent protein dTomato was introduced via an internal ribosomal entry sequence (IRES) derived from encephalomyocarditis virus downstream of the reading frame (Fig. 1a).²² EGFP and dTomato fluorescent markers to track transgene co-expression identified optimal vector design with a fixed 1:1 ratio of transduction for both constitutive and conditionally expressed genes.²² Functional *in vitro* tests of this multimodal vector system in primary murine HPCs, expressing the reverse tetracycline-inducible transactivator (rtTA) M2 under the control of the ubiquitously active Rosa26 locus (Rosa26rtTA), demonstrated a dose-dependent blockade of myeloid differentiation, when first *Meis1* and second *Hoxa9* were consistently over-

expressed.^{22,27,28} However, the absence of the transformed phenotype was obtained in non-induced samples or when *Hoxa9* expression was downregulated.²² Efficient reversibility of the transformed phenotype after DOX removal was associated with a significantly decreased level of *Hoxa9* transcript expression (>150-fold).²²

The current work provides a detailed protocol to establish this recently developed multimodal lentiviral SIN vector system to co-express collaborative GOIs *Meis1* and *Hoxa9* coupled with fluorescent markers (Fig. 1a).²² The functionality of the system in murine primary HPCs *in vitro* is demonstrated in two different cytokine cocktails, which are both described to support myeloid differentiation of transduced HPCs (Fig. 1b).^{16,22} In addition, two different multiplicities of infection (MOIs) are used to investigate vector dose effects. Characteristics of collaborative gene co-expression such as percentage and number of viable transgene positive cells are tracked over time to investigate any diversity due to different cytokine composition or transduction level. It is demonstrated that time points characterized by maximal numbers of *Meis1*_EGFP⁺/*Hoxa9*_dTomato⁺ double-positive cells are cytokine specific (Fig. 2). Since the source and amount of primary hematopoietic cells are often limited, identification of optimal time points to conduct further experimental procedures (*e.g.*, morphology determination, immunophenotyping, sorting, etc.) could significantly improve future studies.

Transduction with LVs is cell type-specific, and genetic modification of primary HPCs is less efficient than of established cell lines, especially when LVs express TFs, which could affect cell metabolic pathways.^{13,22} Thus, for titer determination and validation of generated multimodal LVs, it is recommended to utilize cell lines (*e.g.*, the murine fibroblast cell line SC1), which are generally easier to transduce than primary cells.

One possible limitation of this multimodal LV system is the relatively large sizes of both constitutive and tetracycline-regulated components incorporated into an all-in-one vector. Thus, the LVs presented here do not contain the reverse tetracycline-inducible transactivator and therefore require target cells with integrated rtTA for *in vitro* validation, such as the murine fibroblast cell line SC1/rtTA2 that harbors the *rtTA*-M2.^{13,14,16,17,22,27} To establish functional tests *in vitro* and *in vivo*, as the source of murine HSCs and HPCs, the Rosa26rtTA-nls-Neo2 mouse strain is suggested.^{13,14,16,17,22,28}

The protocol is started with the calcium phosphate-based experimental procedure to produce multimodal LVs with constitutive and inducible

Figure 1. Multimodal lentiviral vectors (LVs) combining constitutive and tetracycline-regulated expression cassettes. **(a)** Vector design. Constitutive module: gene of Interest 1 (GOI1) or murine *Meis1* under the control of murine phosphoglycerate kinase (mPGK) promoter. To track *Meis1*, enhanced green fluorescent protein (EGFP) was linked to the *Meis1* sequence by 2A self-cleavage sites. Inducible module: GOI2 or murine *Hoxa9* under the control of the T11 tetracycline-regulated promoter (TRP). To track *Hoxa9* expression, red fluorescent protein dTomato was introduced via an internal ribosomal entry site (IRES) sequence derived from encephalomyocarditis virus downstream of the *Hoxa9* reading frame. p2A, porcine teschovirus. **(b)** Experimental design to transduce murine primary Rosa26rtTA lineage negative cells (Lin⁻) with vesicular stomatitis virus glycoprotein (VSVg) pseudotyped multimodal LVs using different cytokine conditions (S3F11 and 36S) and multiplicities of infection (MOI). Transduction in the doxycycline (DOX) absence (0 μg/mL) allows constitutive expression of only the *Meis1*_EGFP. DOX induction (1 μg/mL) allows collaborative *Hoxa9*_dTomato co-expression. Selected contour plots to demonstrate percentages of EGFP⁺ and dTomato⁺ cells in (Lin⁻) Rosa26rtTA cells transduced with multimodal LVs with MOI=10 on days 2 and 21 post transduction (PT; corresponds to days 1 and 20 after DOX induction). NT, not transduced.

components (Figs. 1a and 3a). Second, multimodal LV titration is described via transduction of murine fibroblasts SC1/rtTA2 and the fluorescence-activated cell sorting (FACS)-based procedure to estimate the percentages of cells expressing *Meis1*_EGFP in the absence of DOX and *Meis1*_EGFP/*Hoxa9*_dTomato in the presence of DOX (Figs. 3b and

4).^{13,16,22,27} It is described that TRPs in LVs in the absence of DOX can exhibit some background activity characterized by basal transgene expression (so-called leakiness).^{13,25} In addition, a linear relationship between transduction rate and basal TRP activity in the absence of DOX has been described.¹³ How to determine if the tetracycline-regulated

Figure 2. Tracking of transduced murine primary hematopoietic cells via double-positive fluorescent markers. **(a)** Tracking EGFP⁺/dTomato⁺ cells over time indicated the maximal percentage of transgene positive cells at day 9 after DOX induction for S3F11 cytokine conditions and at day 20 for 36S cytokine conditions (corresponds to day 10 and 21 post transduction). **(b)** Tracking of viable EGFP⁺/dTomato⁺ cells over time indicated the maximal number at day 9 after DOX induction for S3F11 cytokine conditions and at day 20 for 36S cytokine conditions.

component of multimodal LVs exhibits leakiness is explained (Fig. 4b). Finally, it is demonstrated how to transduce lineage negative (Lin⁻) Rosa26rtTA bone marrow cells with multimodal LVs using different experimental conditions (Fig. 1b). How to monitor the co-expression of Meis1_EGFP and Hoxa9_dTomato *in vitro* and how to determine the optimal conditions to track and study the fate of transduced cells over time is described (Figs. 1b and 2).^{13,17,22}

Thus, these protocols help to establish the new multimodal constitutive/inducible LV system as a promising alternative to conventional lentiviral constitutive or tetracycline-regulated vectors. The multimodal vectors will help to develop new models and approaches for genetic modification of HSCs to study the biology of normal and malignant hematopoiesis in order to examine the role of collaborat-

ing and single genes in cooperative gene signaling networks that control cell fate decision processes. The vector is an advanced tool for delivery and controlled expression of multiple transgenes including fluorescent markers, which are highly useful for clonal tracking in stem cell, leukemia, and reprogramming studies.

1. MATERIALS

1.1. Reagents

All reagents and their abbreviations are listed in Table 1.

1.2. Equipment

1. Biological safety cabinet with sterile conditions (follow the appropriate S2 biosafety

Figure 3. Production and titration of multimodal LVs with constitutive and tetracycline-regulated co-expression of collaborative GOIs. **(a)** Experimental schema to produce multimodal LVs using the calcium-phosphate method. VSVg, envelope plasmid; Rev and Gag/Pol, packaging plasmids; GOI2/GOI1, transfer plasmid. **(b)** Experimental procedure to titrate multimodal LVs using murine fibroblast cell line SC1/rtTA2. FACS, fluorescence-activated cell sorting; d, day.

Figure 4. Transduction of murine fibroblast cell line with multimodal LVs containing constitutive and tetracycline-regulated cassettes. **(a)** Titer estimation of multimodal VSVg pseudotyped LV particles via transduction of murine fibroblast cell line SC1/rtTA2 with different volumes of LV supernatant (without compensation settings). EGFP gene transfer (percentage of EGFP⁺ cells) and EGFP expression level (mean fluorescence intensity [MFI]) were estimated in the absence and presence of doxycycline. **(b)** EGFP and dTomato gene transfer in SC1/rtTA2 cells transduced with different volumes of multimodal VSVg pseudotyped LVs (with compensation settings). Background activity of T11 TRP (percentage of dTomato⁺ cells) was evaluated in the absence of DOX. EGFP gene transfer was evaluated in the absence and presence of DOX. EGFP and dTomato co-expression (percentage of dTomato⁺/EGFP⁺ cells) was estimated in the presence of DOX. NT, not transduced; TU/mL, transducing units per milliliter.

regulations when working with Vesicular Stomatitis Virus glycoprotein [VSVg]-pseudotyped LV particles).

2. Cell culture incubator set at 37°C, 5% CO₂, 100% humidity.
3. Cooling centrifuge Heraeus Multifuge 3 S-R (Thermo Fisher Scientific, Waltham, MA) with tissue culture plate holders.

4. FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany).
5. FlowJo software (Tree Star, Ashland, OR) or similar flow cytometry analysis program.
6. Vortex mixer.
7. Water bath.
8. Eppendorf centrifuge (e.g., Heraeus Fresco 17 Centrifuge; Thermo Fisher Scientific).

Table 1. Reagents

<i>Reagents</i>	<i>Supplier</i>	<i>Specific handling</i>	<i>Storage conditions</i>
Fetal bovine serum Brazil One	PAN Biotech, Aidenbach, Germany Cat. No. P30-3309	Heat inactivated for 30 min at 56°C, prepare 50 mL aliquots	–20°C long term (according to manufacture expiry date), 4°C short term (up to 2 months)
Water Ultra pure	Biochrom GmbH, Berlin, Germany Cat. No. L0020		Room temperature
Dulbecco's modified Eagle's medium with glutamine	Biochrom GmbH Cat. No. FG 0435		4°C
Sodium pyruvate, 100 mM	PAN Biotech Cat. No. P04-43100		4°C
10,000 IU/mL penicillin and 10 mg/mL streptomycin	PAN Biotech Cat. No. P06-07100	10 mL aliquots recommended	–20°C long term (according to manufacture expiry date), 4°C short term (up to 2 months)
L-glutamine	Biochrom GmbH Cat. No. K 0282	5 mL aliquots recommended	–20°C long term (according to manufacture expiry date), 4°C short term (up to 2 months)
HEPES-buffer, 1 M	PAN Biotech Cat. No. P05-01100		4°C
Calcium chloride dihydrate (CaCl ₂ × 2H ₂ O)	Sigma-Aldrich, St. Louis, MO Cat. No. C3306		Room temperature
Sodium chloride (NaCl)	Sigma-Aldrich Cat. No. S5886		Room temperature
Disodium hydrogen phosphate (Na ₂ HPO ₄)	Sigma-Aldrich Cat. No. S5136		Room temperature
Hydrochloric acid (HCl), 37%	VWR Chemicals, Radnor, PA Cat. No. 20252.335		Room temperature
Sodium hydroxide (NaOH)	Carl Roth GmbH + Co. KG, Karlsruhe, Germany Cat. No. 9356.1		Room temperature
EDTA, 0.5 M, pH 8.0, Ultra pure	Thermo Fisher Scientific, Waltham, MA Cat. No. 15575020		4°C
Dulbecco's Phosphate Buffered Saline	PAN Biotech Cat. No. P04-36500		Room temperature
Propidium iodide, 1 mg/mL	Sigma-Aldrich Cat. No. P4864		4°C
Chloroquine diphosphate salt	Sigma-Aldrich Cat. No. C6628		Room temperature
Protamine sulfate	Sigma-Aldrich Cat. No. P3369		4°C
Dimethyl sulfoxide	Merck KGaA, Darmstadt, Germany Cat. No. 1.02950.0500		Room temperature
Doxycycline hyclate	Sigma-Aldrich Cat. No. D9891	Light sensitive	4°C
Meropenem HEXAL	HEXAL AG, Holzkirchen, Germany PZN 10553214		–20°C
StemSpan SFEM Medium	STEMCELL Technologies SARL, Cologne, Germany Cat. No. 09650	5 and 10 mL aliquots recommended	–20°C long term (according to manufacture expiry date), 4°C short term (up to 3 weeks)
Murine stem cell factor	Peprotech, Hamburg, Germany Cat. No. 250-03		–20°C
Murine interleukin 3	Peprotech Cat. No. 213-13		–20°C
Human FMS-like tyrosine kinase 3 ligand	Peprotech Cat. No. 300-19		–20°C
Human interleukin 11	Peprotech Cat. No. 200-11		–20°C
Human interleukin 6	Peprotech Cat. No. 200-06		–20°C
Retronectin 1 mg/mL	TaKaRa, Saint-Germain-en-Laye, France Cat. No. T100B		–20°C
Hank's balanced salt solution (10 ×)	Gibco by Life Technologies, Darmstadt, Germany Cat. No. 14185-045		4°C
Bovine serum albumin	PAN Biotech Cat. No. P06-139350		4°C
Trypsin 0.5%/EDTA 0.2% in phosphate-buffered saline (10 ×)	PAN Biotech Cat. No. P10-024100	10 ml aliquots recommended	–20°C long term (according to manufacture expiry date), 4°C short term (up to 2 months)
Murine embryonic kidney 293T cell line	ATCC		Liquid nitrogen
Murine fibroblast SC1 cell line	ATCC		Liquid nitrogen

9. Automatic cell counter CASY-TT instrument (Roche Diagnostics, Mannheim, Germany).
10. Refrigerator (4°C).
11. Freezer (−20°C, −80°C, liquid nitrogen).
12. Ultracentrifuge Optima LE-80K with rotor SW 32Ti (Beckman Coulter, Brea, CA).

1.3. Supplies

1. Cell-culture plasticware: 48- and 24-well tissue plates and 10 cm dishes for adherent and suspension cell culture, 1.5 mL Eppendorf, 15 mL and 50 mL Falcon tubes or equivalent.
2. Neubauer counting chamber and cover glasses.
3. Millex-GP Syringe Filter Unit, 0.22 μ m, polyethersulfone, 33 mm (Merck KGaA, Darmstadt, Germany).
4. Millex-GV Syringe Filter Unit, 0.22 μ m, PVDF, 33 mm (Merck KGaA).
5. Falcon™ Cell Strainer, 70 μ m Nylon (Thermo Fisher Scientific).
6. Syringes, 10 mL or 20 mL.
7. Bottle Top Filter 250 mL, Filtropur BT25 0.2 (0.22 μ m; Sarstedt AG & Co., Nümbrecht, Germany).

1.4. Reagent and buffer preparation

Prepare all solutions under sterile conditions using biological safety cabinet.

1. Normal Dulbecco's modified Eagle's medium (DMEM): based on DMEM with glutamine. Contains 10% fetal bovine serum (FBS; volume/volume), 1 mM sodium pyruvate, 2% penicillin/streptomycin, 2 mM L-glutamine. Store at 4°C up to 2 months.
2. Transfection (TF) medium: the same as normal DMEM but in addition supplemented with 20 mM HEPES buffer. Store at 4°C up to 2 months.
3. Calcium chloride solution (2.5 M). Dissolve 36.7525 g $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ in water at a final volume of 100 mL; sterilize the final solution by filtration with 0.22 μ m Millex-GP Syringe Filter, make aliquots and store them at −20°C.
4. 2×HEPES-buffered saline (2×HeBS; 50 mM HEPES, 280 mM NaCl, and 1.5 mM Na_2HPO_4). Dissolve 16.3632 g NaCl, 0.21294 g Na_2HPO_4 , and 50 mL 1 M HEPES-buffer in 500 mL water. Adjust pH to 7.05 with HCl/NaOH and add water to a final volume of 1,000 mL. Sterilize by filtration with 0.22 μ m bottle top filter and store aliquots at −20°C.

5. Chloroquine diphosphate salt (25 mM, 1,000×). Dissolve 0.645 g in 50 mL water. Sterilize by filtration with 0.22 μ m Millex-GP Syringe Filter, aliquot, and store at −20°C.
6. FACS buffer: 4% FBS, 2 mM EDTA in phosphate-buffered saline (PBS). Store at 4°C.
7. Propidium iodide (PI; 100 μ g/mL, 100×). Dilute stock solution (1 mg/mL, 1,000×) 10 times with water. Store at 4°C.
8. Protamine sulfate (4 mg/mL, 1,000×). Prepare 4 mg/mL solution in water. Sterilize by filtration with 0.22 μ m Millex-GP Syringe Filter, aliquot, and store at −20°C.
9. Transduction (TD) medium: the same as normal DMEM but in addition supplemented with 4 μ g/mL protamine sulfate (1×). Prepare fresh before use.
10. Freezing medium: 10% dimethyl sulfoxide in FBS. Prepare fresh.
11. DOX (1 mg/mL, 1,000×). Prepare 1.0 mg/mL solution in water: dissolve 0.1 g doxycycline hyclate in 100 mL water. Sterilize by filtration with 0.22 μ m Millex-GP syringe filter. Keep 1 mL aliquots in Eppendorf tubes protected from light at −20°C for long-term storage (up to 2 years) and at 4°C for short-term storage (up to 4 months).
12. Trypsin (1×): dilute stock solution 10 times with PBS. Store at 4°C.
13. Meropenem HEXAL (20 μ g/ μ L, 1,000×). Dissolve 1,000 mg Meropenem HEXAL in 50 mL water under sterile conditions, aliquot, and store at −20°C.
14. S3F11 culture medium: serum-free StemSpan medium with 2% penicillin/streptomycin, 1% L-glutamine, 20 μ g/mL meropenem HEXAL, 50 ng/mL mSCF, 20 ng/mL mIL-3, 50 ng/mL hFlt-3L, and 50 ng/mL hIL-11. Take 10 mL serum-free StemSpan and add 200 μ L penicillin/streptomycin, 100 μ L L-glutamine, 10 μ L meropenem HEXAL (1,000×), 5 μ L mSCF (100 ng/ μ L), 10 μ L mIL-3 (20 ng/ μ L), 5 μ L hFlt3L (100 ng/ μ L), and 5 μ L hIL-11 (100 ng/ μ L).¹⁶ Store at 4°C.
15. 36S culture medium: serum-free StemSpan medium with 2% penicillin/streptomycin, 1% L-glutamine, 20 μ g/mL meropenem HEXAL, 6 ng/mL mIL-3, 10 ng/mL hIL-6, and 20 ng/mL mSCF. Take 10 mL serum-free StemSpan and add 200 μ L penicillin/streptomycin, 100 μ L L-glutamine, 10 μ L meropenem HEXAL (1,000×), 3 μ L mIL-3 (20 ng/ μ L),

1 μ L hIL-6 (100 ng/ μ L), and 2 μ L mSCF (100 ng/ μ L).²² Store at 4°C.

16. Retronectin (RN; 48 μ g/mL). Mix 2.5 mL RN (1 mg/mL) with 2.5 mL water. Dilute with 47 mL PBS. Sterilize by filtration with 0.22 μ m Millex-GV Syringe Filter and store at -20°C.

Note: Do not confuse with 0.22 μ m Millex-GP syringe Filter.

17. 1 $\times$ Hank's balanced salt solution (HBSS): add 5 mL 10 $\times$ HBSS to 45 mL water. Store at 4°C.
18. RN-blocking solution: 2% bovine serum albumin (BSA) in PBS. Weigh 1 g BSA and transfer to 50 mL tube. Dissolve with 50 mL PBS. Sterilize by filtration with 0.22 μ m Millex-GP syringe filter. Store at 4°C.
19. RN-washing solution: 2.5% (volume/volume) 1 M HEPES in 1 $\times$ HBSS. Dilute 1.25 mL of 1 M HEPES with 48.75 mL 1 $\times$ HBSS. Store at 4°C.

1.5. Cells and vectors

1. Human embryonic kidney 293T cell line.
2. Murine SC1 fibroblasts expressing rtTA2 (SC1/rtTA2 cell line).^{13,16,22,27}
3. Lentiviral vector transfer plasmid. The coding cDNA for murine *Hoxa9* (GenBank: BC055059.1) was cloned under the control of the T11 TRP into a third-generation lentiviral self-inactivating vector containing the post-transcriptional regulatory element of woodchuck hepatitis virus (wPRE).^{2,22,25,29,30} The coding cDNA for murine *Meis1* (NM_001193271.1) was cloned into the same vector under the control of mPGK (GenBank: M18735) promoter.²² To allow tracking of *Hoxa9* expression, dTomato was introduced via an IRES derived from encephalomyocarditis virus downstream of the reading frame.²² To track *Meis1*, EGFP was linked by 2A self-cleavage sites resulting in vector pRRL.PPT.T11.*Hoxa9*.IRES.d*Tomato*/mPGK.*Meis1*.p2A.*EGFP*.pre (T11_ *Hoxa9*_mPGK_ *Meis1*; Fig. 1a).²²
4. Packaging Gag/Pol plasmid pcDNA3.GP.4xCTE.^{2,8}
5. Packaging pRSV-Rev plasmid.^{2,31}
6. Envelope plasmids pMD.G (VSVg) or pEnv(eco)-IRES-puro (ecotropic).^{1,8,32-34}

Note: All plasmids including encoding the multimodal lentiviral vector are available on

request according to university policy and after a standard material transfer agreement.

7. Lineage negative (Lin⁻) bone marrow cells from Rosa26rtTA-nls-Neo2 mice.^{13,14,16,17,22,28}

2. EXPERIMENTAL PROCEDURE

2.1. Production of multimodal LVs with constitutive and tetracycline-regulated components

1. Day 0. Ensure that all plasmids are available with the optimal concentration (1 μ g/mL). Design of the multimodal LV to enable constitutive expression of GOI1 (*Meis1*) followed by inducible expression of GOI2 (*Hoxa9*) is provided in Fig. 1a. In the absence of DOX, the EGFP fluorescent marker allows detection of *Meis1* positive cells only (when there is no TRP background activity). Upon DOX application, the dTomato fluorescent marker allows detection of *Hoxa9* positive cells. Ideally (e.g., absence of any selection pressure due to proliferative advantages or disadvantages conferred onto transduced cells), all single-positive *Meis1*/EGFP⁺ cells should become double-positive *Meis1*/EGFP⁺/*Hoxa9*/dTomato⁺ upon administration of optimal DOX concentration.
2. Day 1. Seed 5 $\times$ 10⁶ 293T cells per 10 cm dish in 10 mL normal DMEM.
3. Day 2 (9–11 am). Prepare plasmid DNA mix in 1.5 mL Eppendorf tube (Fig. 3a):
T11_ *Hoxa9*_mPGK_ *Meis1* (LV): 10 μ g
pcDNA3. GP.4xCTE (gag/pol): 10 μ g
pRSV-Rev: 5 μ g
pMD. G (VSVg) or pEnv(eco)-IRES-puro (envelope): 2 μ g
4. Dilute plasmid DNA mix in water to a total volume of 450 μ L and add 50 μ L 2.5 M calcium chloride solution.
5. Add 500 μ L 2 $\times$ HeBS to a 15 mL conical tube. Add the plasmid DNA/calcium chloride mixture to the 2 $\times$ HeBS while bubbling (e.g., by forcing air through a 2 mL pipet with the tip placed under the surface of the mixture) and incubate the resulting plasmid DNA/calcium phosphate mixture at room temperature (RT) for 20 min.
6. Remove the old medium from the 293T cells and add 10 mL TF medium supplemented with 25 μ M chloroquine diphosphate salt (1 $\times$). Add the plasmid DNA/calcium phosphate mixture by pipetting dropwise onto the cells.

Note: Chloroquine diphosphate salt is expected to increase transfection efficiency.

7. Incubate cells for 6–8 h in an incubator at 37°C and 5% CO₂, and replace the used medium with 10 mL fresh TF medium (5–8 pm).
 8. Day 3 (2 pm). Collect the LV particle supernatant (SNT) approximately 24 h post transfection: from one 10 cm dish, remove 10 mL LV SNT carefully with a syringe and filter through a 0.22 µm Millex-GP Syringe filter into a 15 mL or 50 mL conical Falcon tube. After each harvest, replace media with 10 mL TF medium.
- Note:* The LV particle SNT can be harvested every 12 h, beginning at 24 h, up to 72 h after transfection. SNT can be placed at –80°C or kept up to 36 h at 4°C. Aliquot a small amount in a 1.5 mL Eppendorf tube for subsequent titration analysis.
9. Day 4. Collect SNT at approximately 36 h (*e.g.*, 9 am) and 48 h (*e.g.*, 6 pm).
 10. Day 5. Collect SNT at approximately 60 h (*e.g.*, 9 am) and optionally 72 h (*e.g.*, 6 pm; Fig. 3a).
 11. Perform ultracentrifugation (optionally) to concentrate LV particle SNT. Concentration of ecotropic- or VSVg- pseudotyped LV particles recommended via ultracentrifugation at 13,238 *g* for 16–24 h or at 82,740 *g* for 2 h at 4°C, correspondingly. If subsequent experiments to transduce murine hematopoietic cells are planned, dissolve the concentrated LV particles in pure serum-free StemSpan medium. For example, concentrated LV particles derived from 200 mL unconcentrated SNT can be dissolved in 2 mL pure StemSpan medium. Store LV SNT aliquots (*e.g.*, 200 µL and one 50 µL aliquot for titration) at –80°C.

2.2. Titration of multimodal LVs with constitutive and tetracycline-regulated components via transduction of a murine fibroblast cell line in the absence and presence of DOX

1. Day 1. Seed 2 × 10⁴ SC1/rtTA2 cells in 1 mL normal DMEM per well onto 24-well plates in the absence and presence (1 µg/mL) of DOX (Fig. 3b).

Note: For example, when one time point aliquot of SNT will be analyzed, consider both ±DOX conditions and three different amounts of SNT, and thus seed a minimum of six wells (in case of triplicates, 18 wells).

Note: Transduction in the absence of DOX allows constitutive expression of only the *Meis1* gene coupled with the *EGFP* fluorescent marker. Transduction in the presence of DOX allows *Meis1_EGFP//Hoxa9_dTomato* co-expression.

2. Day 2. Remove the old medium and add fresh TD medium supplemented with 0 and 1 µg/mL DOX. Add 0.1, 1, and 10 µL LV particle supernatant to both 0 and 1.0 µg/mL DOX-treated cells (Fig. 3b).

Note: Take LV SNT from –80°C and store it on ice to thaw.

3. Centrifuge 24-well plate at 800 *g* and 32°C for 60 min. Place the plate into a cell culture incubator and incubate until the next day (12–24 h).
4. Day 3. Remove the old TD medium and add fresh normal DMEM supplemented with 0 and 1 µg/mL DOX, correspondingly.

Note: Follow the appropriate biosafety regulations when working with VSVg pseudotyped LV particles (*e.g.*, in case of transduction with VSVg pseudotyped LV particles, wash transduced cells twice with PBS before adding the fresh normal medium in order to remove vector particles).

5. Day 5. Remove the old medium. Wash each well twice with 1 mL PBS. Add 400 µL 1 × trypsin. Incubate for 5 min at RT and resuspend cells by pipetting.
6. Take 100 µL cells and add 400 µL FACS buffer without PI for FACS analysis (without compensation settings). Acquire EGFP-positive cells in samples treated with or without DOX and determine the percentage of EGFP⁺ cells with FlowJo software or equivalent (Fig. 4a). Use the following formula to determine the LV titer as infectious particles or transducing units per milliliter (TU/mL):

$$\frac{\% \text{ of positive cells}}{100} \times \text{cell number (day of infection)} \times 2$$

$$\frac{\text{volume of virus SNT (1 µL = 0.001 mL)}}{\text{volume of virus SNT (1 µL = 0.001 mL)}}$$

Note: Percentage of transgene positive cells for titer estimation should not exceed 30% to avoid multiple vector integration and related LV titer underestimation.³⁵ Here, LV titer estimation based on SC1/rtTA2 transduction with 1 µL SNT resulted in 25% of EGFP⁺ cells and already reflects underestimation features

when compared to the 4% of EGFP⁺ cells obtained with 0.1 μ L SNT (Fig. 4a).

7. Add PI (100 $\times$) to cells in FACS buffer to achieve a final concentration of 1 μ g/mL (1 $\times$), mix by vortex mixer pulse, and acquire EGFP⁺, dTomato⁺, and double-positive EGFP⁺/dTomato⁺ cells from samples cultured with or without DOX on a FACSCalibur flow cytometer (with compensation settings; Fig. 4b).
8. Analyze with FlowJo or equivalent software.
9. Estimate background activity (leakiness) of T11 TRP (% of dTomato⁺ cells) in SC1/rtTA2 cells transduced with multimodal LVs in the absence of DOX (with compensation settings; Fig. 4b). Percentages of dTomato⁺ cells are expected to be <1 in the absence of DOX (Fig. 4b).
10. Estimate percentages of dTomato⁺, EGFP⁺, and dTomato⁺/EGFP⁺ cells in SC1/rtTA2 cells transduced with multimodal LVs in the presence of DOX (with compensation settings; Fig. 4b).
11. Percentages of EGFP⁺ cells in the absence and presence of DOX for the same SNT volume (e.g., 1.0 μ L) without and with compensation settings are expected to be similar (Fig. 4). However, the mean fluorescence intensity of EGFP in the presence of DOX could be increased in comparison with samples cultivated without DOX (positive regulatory loop; Fig. 4a).²²

2.3. Transduction of murine primary hematopoietic progenitor cells with multimodal LVs in the absence of DOX allows constitutive gene expression only

1. Day 0. Pre-stimulate purified (Lin⁻) Rosa26rtTA bone marrow cells in two different cytokine myeloid conditions: S3F11 and 36S.^{16,22} For this purpose, dilute the (Lin⁻) Rosa26rtTA cells to a density of 0.5 $\times$ 10⁶/0.5 mL in the corresponding culturing medium in the absence of DOX and plate in independent wells of a 48- or 24-well suspension culture plate. Cultivate for 12–24 h in a cell culture incubator (Fig. 1b).

Note: Take into consideration that if Lin⁻ cells were cryopreserved, the number of viable cells on the next day could be about half of that estimated before freezing.

Note: Transduction in the absence of DOX allows constitutive expression of only the *Meis1* gene coupled with the *EGFP*.

2. Day 1. Prepare RN pre-coated plates. For example, to transduce 1 $\times$ 10⁵ (Lin⁻) Rosa26rtTA cells with one vector, two different MOIs (MOI=2 and MOI=10), and two myeloid cytokine conditions (S3F11 and 36S), pre-coat a total of four wells of a 48-well (1.1 cm²) suspension culture plate (Fig. 1b). Add 228 μ L of 48 μ g/mL RN solution per well (final concentration 10 μ g/cm²). Incubate for 2 h at RT to let the RN adhere to the plate. Before transduction, remove the RN solution and block with 500 μ L RN-blocking solution for at least 30 min at RT. Discard the blocking solution and add 700 μ L RN-washing solution.

Note: Consider if your experiment requires biological triplicates.

Note: To pre-coat one well in a 24-well plate (2.0 cm²) with final RN concentration of 10 μ g/cm², add 420 μ L of 48 μ g/mL RN stock to the well, as described above.³⁶

Note: RN pre-coating could be started on day 0. In this case, plates could be incubated with 48 μ g/mL RN stock solution for 2 h at RT at day 0, and then kept overnight at 4°C. Alternatively, add 48 μ g/mL RN stock solution and incubate the plates overnight at 4°C.

3. Calculate the amount of LV particles and StemSpan medium required for cell transduction in one well of a 48-well plate (the well bottom should be optimally covered: both pure StemSpan medium or corresponding S3F11 and 36S culture medium could be used to adjust the optimal final volume). For example, to transduce 1 $\times$ 10⁵ of (Lin⁻) Rosa26rtTA cells with a VSVg pseudotyped vector T11_ *Hoxa9*_mPGK_ *Meis1* with a titer 1 $\times$ 10⁷ TU/mL and MOI=2, 40 μ L SNT should be supplemented with 360 μ L StemSpan medium (optimal final volume per well of 48-well plate is 400 μ L). To transduce the cells with MOI=10, 200 μ L SNT should be supplemented with 200 μ L StemSpan medium. Prepare corresponding SNT/StemSpan master mix when more than one well will be transduced, and keep it on ice.

Note: It is estimated that only half of the LV particles will bind to the RN pre-coated plate.¹⁷

4. Remove the washing solution from RN pre-coated wells.
5. Add prepared SNT/StemSpan mixture to RN pre-coated wells.
6. Centrifuge the plate at 800 *g* and 32°C for 60 min to promote the binding of LV vector particles with RN.
7. During the centrifugation step, carefully re-suspend and count the (Lin⁻) Rosa26rtTA cells pre-stimulated in S3F11 and 36S culture medium. The recommended density of (Lin⁻) Rosa26rtTA cells for transduction in one well of 48-well plate is 1×10^5 cells/400 μ L. If needed, add corresponding culture medium to achieve the recommended density.
8. Remove the supernatants from the wells after the centrifugation step (it is assumed that the LV particles were bound to RN on the well bottom). Add prepared (Lin⁻) Rosa26rtTA cells in S3F11 and 36S culture conditions to the respective LV-coated wells with MOI=2 and MOI=10 (Fig. 1b).
9. Incubate the cells in a cell culture incubator until the next day (12–24 h).

2.4. DOX induction allows collaborative tetracycline-regulated gene co-expression in murine primary hematopoietic progenitor cells transduced with multimodal LVs

1. Day 1 post transduction (PT; Fig. 1b). For each well, re-suspend the cells transduced in the absence of DOX. Divide the volume (400 μ L for a 48-well plate) into two aliquots (200 μ L) and transfer into two wells of a new 48-well suspension culture plate.

Note: Transduced (Lin⁻) Rosa26rtTA donor cells that were not treated with DOX could be cryopreserved at day 1 PT and transplanted later into lethally irradiated recipient mice (C57BL/6J).^{14,17} DOX could be delivered to mice with drinking water or dry food either 1 week before transplantation or 8 weeks after, according to experimental plans.^{13,14} If transplantation of freshly transduced (Lin⁻) Rosa26rtTA donor cells is planned, it is recommended to do it on day 2 PT, considering that 1 day is required for DOX induction and determination of transduction efficiency.¹⁷

2. Supplement the first culture aliquot with an additional equal volume of fresh culture medium containing no DOX and the second

culture aliquot with medium containing double of the final DOX concentration. For instance, the first 200 μ L aliquot of S3F11 MOI=2 could be supplemented with 200 μ L of pure S3F11 culture medium (0 μ g/mL DOX), and the second aliquot with 200 μ L of S3F11 culture medium containing 2 μ g/mL DOX (final DOX concentration 1 μ g/mL). This generates culture conditions containing 0 μ g/mL DOX for only Meis1_EGFP constitutive expression and 1 μ g/mL DOX conditions for Meis1_EGFP//Hoxa9_dTomato co-expression (Fig. 1).

3. Incubate the cells in a cell culture incubator until the next day (12–24 h).

2.5. Tracking of transduced murine primary hematopoietic cells via single- and double-positive fluorescent markers

1. Day 2 PT (day 1 after DOX induction). Carefully re-suspend cells in each well.
2. Take 10 μ L aliquot to count the number of living cells.
3. Take 40 μ L aliquot for FACS analysis ($\sim 3 \times 10^4$ – 1×10^5 cells). Add 300 μ L of FACS buffer and 3 μ L of 100 $\times$ PI solution, mix via vortex. Keep on ice.
4. Acquire EGFP⁺ cells (0 μ g/mL DOX) and EGFP⁺/dTomato⁺ (1 μ g/mL DOX) cells using compensation settings on a FACSCalibur flow cytometer and analyze the data with FlowJo or equivalent software.
5. Determine the background activity (% of dTomato⁺ cells) of the tetracycline-regulated promoter in the absence of DOX for S3F11 and 36S cytokine conditions for MOI=2 and MOI=10 (Fig. 1b).
6. Compare percent of double positive EGFP⁺/dTomato⁺ cells for all culture conditions tested (*e.g.*, S3F11 and 36S cytokine conditions) and transduction conditions (*e.g.*, MOI=2 and MOI=10; Fig. 1b).
7. Keep cells in culture to continue the planned FACS analysis and live cell counts (*e.g.*, on days 5, 9, 13, 16, and 20 after DOX induction; Fig. 2). To keep the optimal cell density ($\leq 1 \times 10^6$ /mL), split the cells into larger tissue culture plates. To feed cells, add fresh S3F11 or 36S culture medium (no DOX or final 1 μ g/mL DOX concentration) two to three times per week. For both feeding and

splitting, include half fresh and half conditioned medium.

Note: Transduced material could be also cryopreserved at any selected time point.

8. On day 9 (S3F11) or 20 (S3F11 and 36S cytokine conditions) after DOX induction (corresponds to 10 and 21 days PT), sort for single-positive EGFP⁺ (no DOX) or double-positive EGFP⁺/dTomato⁺ cells (1 μ g/mL DOX).²² Consider the type of analysis to be performed at this point: for example, cytopspin analysis to determine cell morphology, extract DNA for vector copy or insertional analysis, extract RNA for transcriptional studies, or continue further *in vitro* culture to identify clones with enhanced competitive fitness.^{16,22,37}

Note: Sorting procedure helps to enrich the population with double-positive Meis1_EGFP⁺/Hoxa9_dTomato⁺ cells to investigate the cell morphology at relatively early time points. The sorted single-positive Meis1_EGFP⁺ expressing cells serve as a control.

Note: Sorting procedure is important to enrich the population with Meis1_EGFP⁺/Hoxa9_dTomato⁺ cells to investigate the collaborative abilities of co-expressed GOIs (*e.g.*, to establish further limiting dilution assay, to select immortalized clones, to extract RNA for gene expression microarray analysis, etc.). If an “immortalization” assay is to be performed, the number of primary transduced HPCs plated per 96-well could be reduced (to 1–10 per well) but the screening capacity will be increased and result in a higher number of potential clones that arise from the 96-well plate. The reduced cell number in the “immortalization” assay will also help to minimize the background level provided by non-transduced cells, which might proliferate in S3F11 and 36S cytokine conditions for up to 3 months when the cell density is optimal (>10 cells per well).

3. TROUBLESHOOTING

1. 293T cells detach easily from the plate bottom when the cell density is too high. To prevent this, seed the recommended number of the cells on day 1 and carefully replace TF medium via placing the pipet against the culture plate wall (steps 2.1.2., 2.1.6–2.1.9.).
2. VSVg pseudotyped particles could be toxic for 293T cells. To minimize the toxicity

during vector production, the amount of VSVg envelope vector could be decreased to 1–1.5 μ g. Alternatively, the transfection time for 293T cells could be shortened to 6 h (steps 2.1.3. and 2.1.7.), or another less toxic envelope vector (*e.g.*, ecotropic) could be considered for vector production (step 2.1.3.).^{8,22}

3. In order to minimize the possible toxicity of VSVg (or other envelope) pseudotyped vector particles for target cells, transduction time could be shortened from 24 to 12 h. In addition, transduced cells could be washed with PBS or StemSpan medium once or twice (steps 2.2.4. or 2.3.9.–2.4.1.).
4. In case the vector titer is too low but no toxicity is observed in 293T cells, the transfection time could be prolonged up to 10 h and TF medium volume decreased to 8 mL (steps 2.1.7.–2.1.10.). Alternatively, LV vector particles could be concentrated by ultracentrifugation (step 2.1.11.).
5. Percentage of dTomato⁺ SC1/rtTA2 cells in the DOX absence and presence determined via flow cytometry analysis using “no compensation settings” could be different from those determined with “compensation settings.” This can occur due to the physical overlap between the emission spectra of EGFP and dTomato, which results in significant optical background by EGFP into dTomato measurements. Thus, using proper compensation setups to estimate the percentages of dTomato⁺ cells in the absence (background activity of T11 TRP) and presence (gene transfer) of DOX is recommended. For titer evaluation, determination of the percentage of EGFP⁺ SC1/rtTA2 cells in the absence and presence of DOX using “no compensation settings” (steps 2.2.6.–2.2.11.) is recommended.
6. On day 2 post transduction of Lin[−] Rosa26rtTA cells with multimodal LVs (Fig. 1b), percentages of single-positive EGFP⁺ (no DOX treatment) and double-positive EGFP⁺/dTomato⁺ cells (day 1 after DOX induction) could both be lower than expected for the applied MOI (steps 2.5.1.–2.5.4.).

Here, MOI calculations based on titer estimation resulted from LV transduction of a murine fibroblast cell line. Genetic modification of primary hematopoietic cells is less efficient than of established cell lines, especially when GOIs that potentially affect cell

metabolism are expressed in addition to the inert fluorescent markers.

7. It is possible that remarkable fluctuation in the percentages of EGFP⁺ and EGFP⁺/dTomato⁺ cells will occur over time, depending on GOI nature, order of expression, cytokine cocktail, and other experimental conditions (Fig. 2). In the experiments, the increased detected percentages of EGFP⁺ and EGFP⁺/dTomato⁺ cells at later time points reflected the resulting selective advantages. The culture conditions can exert great selection pressure, as the maximum level of Meis1_EGFP⁺/Hoxa9_dTomato⁺ cells was observed on day 9 after DOX induction for cells cultured under S3F11 cytokine conditions, but much later (day 20) when the 36S cytokine conditions were used (Fig. 2).²²

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AUTHOR DISCLOSURE

The authors confirm that there are no conflicts of interest associated with this publication.

REFERENCES

1. Naldini L, Blomer U, Gallay P, et al. *In vivo* gene delivery and stable transduction of post mitotic cells by a lentiviral vector. *Science* 1996;272:263–267.
2. Dull T, Zufferey R, Kelly M, et al. A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998;72:8463–8471.
3. Vigna E, Naldini L. Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med* 2000;2:308–316.
4. Schambach A, Bohne J, Chandra S, et al. Equal potency of gammaretroviral and lentiviral SIN vectors for expression of O6-methylguanine-DNA methyltransferase in hematopoietic cells. *Mol Ther* 2006;13:391–400.
5. Warlich E, Kuehle J, Cantz T, et al. Lentiviral vector design and imaging approaches to visualize the early stages of cellular reprogramming. *Mol Ther* 2011;19:782–789.
6. Naldini L. *Ex vivo* gene transfer and correction for cell-based therapies. *Nat Rev Genet* 2011;12:301–315.
7. Vigna E, Cavalieri S, Ailles L, et al. Robust and efficient regulation of transgene expression *in vivo* by improved tetracycline-dependent lentiviral vectors. *Mol Ther* 2002;5:252–261.
8. Schambach A, Galla M, Modlich U, et al. Lentiviral vectors pseudotyped with murine ecotropic envelope: increased biosafety and convenience in pre-clinical research. *Exp Hematol* 2006;34:588–592.
9. Kutner RH, Zhang XY, Reiser J. Production, concentration and titration of pseudotyped HIV-1-based lentiviral vectors. *Nat Protoc* 2009;4:495–505.
10. Laurenti E, Barde I, Verp S, et al. Inducible gene and shRNA expression in resident hematopoietic stem cells *in vivo*. *Stem Cells* 2010;28:1390–1398.
11. Zuber J, McJunkin K, Fellmann C, et al. Toolkit for evaluating genes required for proliferation and survival using tetracycline-regulated RNAi. *Nat Biotechnol* 2011;29:79–83.
12. Jiang W, Hua R, Wei M, et al. An optimized method for high-titer lentivirus preparations without ultracentrifugation. *Sci Rep* 2015;5:13875.
13. Kustikova OS, Stahlhut M, Ha T-C, et al. Dose response and clonal variability of lentiviral tetracycline-regulated vectors in murine hematopoietic cells. *Exp Hematol* 2014;42:505–515.e7.
14. Stahlhut M, Ha T, Morgan M, et al. Comparison of tetracycline-regulated promoters in lentiviral-based vectors in murine transplantation studies. *Curr Gene Ther* 2016;16:242–248.
15. Konrad TA, Karger A, Hackl H, et al. Inducible expression of EVI1 in human myeloid cells causes phenotypes consistent with its role in myelodysplastic syndromes. *J Leukoc Biol* 2009;86:813–822.
16. Kustikova OS, Schwarzer A, Stahlhut M, et al. Activation of Evi1 inhibits cell cycle progression and differentiation of hematopoietic progenitor cells. *Leukemia* 2013;27:1127–1138.
17. Stahlhut M, Schambach A, Kustikova OS. Transduction of murine hematopoietic stem cells with tetracycline-regulated lentiviral vectors. *Methods Mol Biol* 2016;1448:65–76.
18. Beard BC, Dickerson D, Beebe K, et al. Comparison of HIV-derived lentiviral and MLV-based gammaretroviral vector integration sites in primate repopulating cells. *Mol Ther* 2007;15:1356–1365.
19. Kustikova OS, Schiedlmeier B, Brugman MH, et al. Cell-intrinsic and vector-related properties cooperate to determine the incidence and consequences of insertional mutagenesis. *Mol Ther* 2009;17:1537–1547.
20. Montini E, Cesana D, Schmidt M, et al. 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* 2009;119:964–975.
21. Heckl D, Schwarzer A, Haemmerle R, et al. Lentiviral vector induced insertional haploinsufficiency of Ebf1 causes murine leukemia. *Mol Ther* 2012;20:1187–1195.
22. Stahlhut M, Schwarzer A, Eder M, et al. Lentiviral vector system for coordinated constitutive and drug controlled tetracycline-regulated gene co-expression. *Biomaterials* 2015;63:189–201.
23. Kroon E, Krosl J, Thorsteinsdottir U, et al. Hoxa9 transforms primary bone marrow cells through specific collaboration with Meis1a but not Pbx1b. *EMBO J* 1998;17:3714–3725.
24. Thorsteinsdottir U, Kroon E, Jerome L, et al. Defining roles for *HOX* and *MEIS1* genes in induction of acute myeloid leukemia. *Mol Cell Biol* 2001;21:224–234.
25. Heinz N, Schambach A, Galla M, et al. Retroviral and transposon-based tet-regulated all-in-one vectors with reduced background expression and improved dynamic range. *Hum Gene Ther* 2011;22:166–176.
26. Gossen M, Bujard H. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* 1992;89:5547–5551.
27. Urlinger S, Baron U, Thellmann M, et al. Exploring the sequence space for tetracycline-dependent transcriptional activators: novel mutations yield expanded range and sensitivity. *Proc Natl Acad Sci U S A* 2000;97:7963–7968.
28. Magnusson M, Brun ACM, Miyake N, et al. HOXA10 is a critical regulator for hematopoietic stem cells and erythroid/megakaryocyte development. *Blood* 2007;109:3687–3696.

29. Zufferey R, Donello JE, Trono D, et al. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J Virol* 1999;73:2886–2892.
30. Schambach A, Mueller D, Galla M, et al. Overcoming promoter competition in packaging cells improves production of self-inactivating retroviral vectors. *Gene Ther* 2006;13:1524–1533.
31. Schambach A, Swaney WS, van der Loo JC. Design and production of retro- and lentiviral vectors for gene expression in hematopoietic cells. *Methods Mol Biol* 2009;506:191–205.
32. Yang Y, Vanin EF, Whitt MA, et al. Inducible, high-level production of infectious murine leukemia retroviral vector particles pseudotyped with vesicular stomatitis virus G envelope protein. *Hum Gene Ther* 1995;6:1203–1213.
33. Kafri T, van Praag H, Ouyang L, et al. A packaging cell line for lentivirus vectors. *J Virol* 1999;73:576–584.
34. Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000;7:1063–1066.
35. Kustikova OS, Wahlers A, Kuhlcke K, et al. Dose finding with retroviral vectors: correlation of retroviral vector copy numbers in single cells with gene transfer efficiency in a cell population. *Blood* 2003;102:3934–3937.
36. Modlich U, Schambach A, Li Z, et al. Murine hematopoietic stem cell transduction using retroviral vectors. *Methods Mol Biol* 2009;506:23–31.
37. Kustikova OS, Modlich U, Fehse B. Retroviral insertion site analysis in dominant haematopoietic clones. *Methods Mol Biol* 2009;506:373–390.

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