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Lentiviral vector system for coordinated constitutive and drug controlled tetracycline-regulated gene co-expression

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ABSTRACT

Constitutive co-expression of cooperating transgenes using retroviral integrating vectors is frequently used for genetic modification of different cell types to establish therapeutic or cancer models. However, such approaches are unable to dissect the influence of dose, order and reversibility of transgene expression on the fate of newly developed therapeutic/malignant phenotypes. We present a modular lentiviral vector system, which provides expression of constitutive and inducible components. To demonstrate its functionality, we constitutively expressed the well-described transcription factor *Meis1* followed by inducible co-expression of collaborating partner *Hoxa9* under the control of tetracycline responsive promoters in murine fibroblasts and primary hematopoietic progenitor cells (HPCs). Fluorescent markers to track transgene co-expression revealed tightly controlled, efficiently inducible and reversible but cell type dependent gene transfer over time. We demonstrated dose-dependent blockade of myeloid differentiation when both *Meis1*|*Hoxa9* were concomitantly overexpressed in primary HPCs *in vitro*, but the absence of the transformed phenotype in non-induced samples or when *Hoxa9* expression was down-regulated. This system combines the advantages of lentiviral gene transfer and the opportunity for drug-controlled co-expression of multiple transgenes to dissect, among others, gene networks governing complex cell behavior, such as proto-oncogene dose-dependent leukemogenic pathways or collaborating mechanisms of genes enhancing competitive fitness of hematopoietic cells.

1. Introduction

Expression of selected gene(s) of interest (GOI) using retroviral (RV) and lentiviral (LV) vectors is a common tool to modify different cell types in a wide variety of biomedical research areas, including gene therapy, stem cell transplantation and reprogramming studies [1–5]. Currently, when co-expression of more than one GOI is required to affect the cell fate, most models utilize constitutive co-expression of several collaborative GOIs. Thus, to convert differentiated somatic cells into induced pluripotent stem cells (iPSC), multicistronic all-in-one SIN LV (RV) vectors expressing selected reprogramming factors via 2A self-cleavage sites are commonly used [3,6]. However, parameters such as dose-control and reversibility of resulting transformed phenotypes are still difficult to properly control using such systems.

Monocistronic tetracycline-regulated LV vectors [7–10], which are widely used in murine bone marrow transplantation (BMT) studies, provide an optimal combination of drug-dependent dose and time controlled expression of selected GOI after establishment of steady-state hematopoiesis [9,10]. Long-term murine BMT studies demonstrated stable and reversible transgene expression in serial recipients and relatively safe integration profiles [10,11], suggesting tetracycline-regulated LV vector system as a valuable tool for modification of hematopoietic stem cells (HSCs) to address the dose-dependent role of selected GOI in promotion of HSC self-renewal or (pre)malignant transformation [12].

Although very efficient, neither approach described above is designed to address important features like the role of sequential order of GOIs expression in cell fate decision or larger gene networks. However, accumulating data indicate the importance of this

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issue. Several mouse models have suggested that combined effects of enhanced proliferation (Class I abnormalities) and differentiation block (Class II) result in acute myelogenous leukemia (AML) [13–16]. In t(8; 21) human AML, it was recently shown that HSCs transform into leukemia stem cells (LSCs) via definitively-ordered acquisition of Class II (*AML1/ETO*) and Class I (*c-KIT* mutant) abnormalities [16].

It is possible to achieve definitively-ordered co-expression of several selected GOIs through co-transduction approaches, when target cells are concomitantly transduced with vectors expressing GOI1 and GOI2 [17–19]. However, in an effort to obtain sufficient amounts of cells co-expressing GOIs, in particular in cell types that are difficult to transduce (including primary HSCs), increasing vector doses can lead to a lack of balanced expression (1:1 ratio per cell) of both selected GOIs and increased side effects due to insertional mutagenesis [19–21].

We present an approach, which allows expression of more than one selected GOI with the opportunity to control dose, time and order of GOIs expression. Our newly developed lentiviral vector system combines a constitutive expression of GOI1 and tetracycline-regulated co-expression of a second collaborating GOI2. To test the functionality of our system, we selected the transcription factors *Meis1* and *Hoxa9*, as they are well-described collaborative partners in murine AML development [17,18]. Here, first the *Meis1* gene was expressed under the control of different phosphoglycerate kinase (PGK) promoters and then the *Hoxa9* gene was co-expressed under the control of tetracycline responsive promoters (TRPs) [6,22–24]. The system was validated using a murine fibroblast cell line that expresses rtTA2 and functionally tested in primary murine hematopoietic Rosa26rtTA [10,12] progenitor cells (HPCs) *in vitro*. The inhibition of myeloid differentiation and enhanced proliferation activity of transduced HPCs was dependent on the doxycycline dose and related transcriptional level of *Hoxa9* expression. This system demonstrated tight control characterized by lack of immature cells in the absence of doxycycline induction. Efficient reversibility of *Hoxa9* expression upon removal of doxycycline resulted in loss of proliferation advantages and out-selection of immature cells. Similarly to previously described constitutively expressing lentiviral vectors, the majority of vector integration sites (VIS) were localized in introns/exons of transcription units and in non-coding/repeat regions of the genome [10,11].

Our study presents a promising tool for dose controlled and time coordinated genetic modifications of different cell types via ectopic expression of cooperating GOIs, allowing development of new physiological or pathophysiological models. Modifications of HSCs can help to identify the collaborating mechanisms of genes enhancing competitive fitness of healthy hematopoietic cells, aberrant pathways leading to leukemogenesis, as well as events common to or distinct for both processes.

2. Materials and methods

2.1. Cloning of constructs

The coding cDNA for murine *Hoxa9* (GenBank: BC055059.1) with a HIS epitope tag at the 5'-end was cloned into a lentiviral self-inactivating (LV SIN) vector [25] under the control of the tetP [23] or T11 [8] TRPs. The coding cDNA for murine *Meis1* (NM_001193271.1) (kind gift from M. Heuser, Hannover Medical School) was cloned into the same vector under the control of human (hPGK) (GenBank: M11958) or murine (GenBank: M18735) phosphoglycerate kinase (mPGK) promoters. To allow tracking of *Hoxa9* expression, we introduced the red fluorescent protein dTomato via an internal ribosomal entry sequence (IRES) derived from encephalomyocarditis virus downstream of the reading frame. To track *Meis1*, Enhanced Green Fluorescent Protein (EGFP) was linked by 2A self-cleavage sites [6] resulting in four constructs pRRL.PPT.tetP(T11).Hoxa9.IRES.dTomato/mPGK(hPGK).Meis1.p2A.EGFP.pre. Cloning details are available on request.

2.2. Cell lines and vector production

Human embryonic kidney 293T and modified murine fibroblasts (SC1), which express rtTA2 (SC1/rtTA2 cell line) [10,12], were cultured in Dulbecco's modified eagle's medium (DMEM, high glucose; Biochrom, Berlin, Germany) as previously described [26,27]. Vector production was performed as described before [12,26,27]. In short, 293T cells were transfected with 10 μg of lentiviral vector, 10 μg of pcDNA3.GP.4xCTE (gag/pol), 5 μg of pRSV-Rev and 1.5–2 μg of pMD.G (VSVg) or a murine ecotropic envelope plasmid using the calcium phosphate method. Virus supernatant was subjected to ultracentrifugation using the Ultracentrifuge Optima LE-80 K (Beckman Coulter, Brea, CA, USA) at 10,000 rpm (13,238 × g) 16–24 h (ecotropic) or at 25,000 rpm (82,740 × g) for 2 h (VSVg) at 4 °C. Titration was performed on the SC1/rtTA2 cell line. For SC1/rtTA2 transduction, cells were seeded the day before on to 24- or 12- well plates in the absence or presence of doxycycline hyclate (Sigma–Aldrich, St. Louis, MO, USA) at final concentrations of 0, 0.01, 0.1, and 1.0 μg/ml. The following day, DMEM medium was replaced and supplemented with 4 μg/ml protamine sulfate (Sigma, Seelze, Germany) and 0, 0.01, 0.1, or 1.0 μg/ml doxycycline (DOX) before addition of viral supernatants. Spinoculation was performed at 2000 rpm (700 × g) (Multifuge 3S-R, Heraeus, Berlin, Germany) at 32 °C for 1 h.

2.3. Mice

C57BL/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France) and housed in the pathogen-free animal facility of Hannover Medical School. Rosa26rtTA-nls-Neo mice were bred with C57BL/6J (CD45.2) mice and genotyped as described [12,28]. Experiments were approved by the local ethical committee and performed according to their guidelines.

2.4. Isolation, transduction and cultivation of primary cells

BM cells from Rosa26rtTA-nls-Neo mice were harvested from femurs and tibias and lineage negative (Lin^-) cells were purified using lineage specific antibodies (Lineage Cell Depletion Kit, Miltenyi Biotech, Bergisch Gladbach, Germany). (Lin^-) cells were prestimulated 12–24 h in StemSpan medium (Stem Cell Technologies, Vancouver, Canada) as described before [12] with the following cytokine (Peprotech, Hamburg, Germany) cocktail: 36S = 6 ng/ml murine interleukin 3 (mIL-3); 10 ng/ml murine interleukin 6 (mIL-6); 20 ng/ml murine stem cell factor (mSCF) [29]. (Lin^-) cells were transduced overnight (one infection cycle) with lentiviral vectors on tissue culture plates coated with Retronectin (TaKaRa, Otsu, Japan) [12]. Doxycycline hyclate (Sigma–Aldrich, St. Louis, MO, USA) was used at final concentrations of 0.1 or 1.0 μg/ml. Cell number and viability were determined using a CASY-TT instrument (Roche Diagnostics GmbH, Mannheim, Germany).

2.5. Flow cytometry and cell sorting

dTomato or EGFP expression data were acquired on a FACSCalibur flow cytometer (Becton Dickinson, Heidelberg, Germany). Dead cells were excluded by propidium iodide staining. Raw data were analyzed with FlowJo software (Tree Star, Ashland, OR, USA).