Heparan sulfate proteoglycans in B cell maturation and myeloma plasma cell survival

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Instant conditional transgenesis in the mouse hematopoietic compartment

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Instant conditional transgenesis in the mouse
Abstract

Adoptive transfer of retrovirally transduced stem cells has recently been described for instant transgenesis in the hematopoietic compartment of mice. This method circumvents the need to manipulate the germline. However, cell type specific gene expression in this 'retrogenic' mouse model has remained tedious. Here we report a single retroviral vector-based method to rapidly generate conditional retrogenic mice. For this purpose, mutated LoxP-flanked DNA segments are transduced into hematopoietic stem cells isolated from Cre-recombinase transgenic mice, which are subsequently transferred into immunodeficient mice. In this way gene expression can be restricted to hematopoietic cell lineages of choice in the acquired immune system.
Introduction

To investigate the biology of a wide range of immune cells in vivo, it is common practice to generate conditional transgenic mice. However, the classical approach to produce transgenic mice is labor intensive, time consuming and therefore expensive. The recent development of the retrogenic mouse model allows for the rapid generation of transgenic mice (Holst et al., 2006; Nakagawa et al., 2006). In this system, a gene of interest is introduced into hematopoietic stem cells (HSCs) by retroviral infection. The infected cells are subsequently injected into immunodeficient mice, which eventually will lead to the development and differentiation of a donor-derived, transgenic, immune system. Although instant in vivo transgenesis is a powerful approach to rapidly study gene function during immune cell development, the generation of cell-specific gene expression remains inefficient and complex in this system: for gene expression in a differentiated hematopoietic cell type of interest, suitable promoter sequences, either with or without proximal enhancers, must be designed (Marodon et al., 2003; Zhang et al., 2005), cloned in a retroviral vector and tested. We have created a method, which obviates these additional steps and significantly simplifies the procedure to obtain conditional retrogenic mice. Our system of conditional retrogenic gene expression is based on the inversion of mutated loxP-flanked DNA segments by Cre recombinase (Nagy, 2000; Zhang and Lutz, 2002). The flanked gene of interest is introduced in HSCs derived from Cre recombinase transgenic strains. Thus, the cell-specific expression of the introduced gene is determined by the promoter driving Cre recombinase and not by cell type specific promoters cloned in the retroviral vector. Hence, a single cloning vector can be used to express the transgene in a broad spectrum of hematopoietic cell types, both spatially and temporarily.

Materials and methods

Materials

Mouse stem cell virus cloning vector (pMSCVpuro) was obtained from Clontech Labs. Inc. (Mountain View, CA, USA). The anti-mouse CD45R/B220 (clone RA3-6B2) and anti-mouse CD3ε (clone 145-2C11) monoclonal antibodies (both APC-conjugated) were from Pharmingen BD (San Jose, CA, USA). Recombinant Murine IL-3 and IL-6 were derived from PeproTech Inc. (Rock Hill, NJ, USA). Recombinant Rat Stem Cell Factor (SCF) was obtained from Amgen (Thousand Oaks, CA, USA). Phosphate Buffered Saline (PBS) was from Fresenius Kabi (the Netherlands). Ficoll-Paque™ PLUS was obtained from Amersham Biosciences (AB, Uppsala, Sweden). Ecotropic Phoenix cells were derived from the lab of Gary Nolan (http://www.stanford.edu/group/nolan/retroviral_systems/phx.html). The Adeno-cre virus (Ad5CMVCre) was obtained from Gene Transfer Vector Core (University of Iowa, USA). NIH3T3 mouse fibroblast were obtained from the American Type Culture Collection (ATCC Rockville, MD, USA). DNA modifying enzymes, Iscove’s modified Dulbecco’s modified Eagle’s medium (IMDM) cell culture media and its components Penicilline/Streptomycine/
Glutamine (Pen/Strep/Glu) were purchased from Invitrogen (Carlsbad, CA, USA). Fetal Bovine Serum (FBS) was produced by HyClone (Logan, UT, USA). Polybrene and primers were from Sigma-Aldrich.com. The FuGENE transfection reagent was from Roche (Roche Diagnostics, Roche Applied Science, Indianapolis, IN, USA). Fluorescent cell culture pictures were generated with a camera coupled fluorescent microscope (Olympus, Japan).

**Mice**
Cre-recombinase transgenic- and C57BL/6 wild type mice (Harlan) were kept under standard housing conditions, whereas recipient \( \text{Rag-2}^{-/-}\gamma_c^{-/-} \) mice (Colucci et al., 1999) were kept under sterile conditions in individual ventilated cages. All mice were treated according to regulations concerning laboratory animal experiments in the Netherlands.

**Retroviral particle production**
Virus was produced using the ecotropic Retroviral System of Gary Nolan’s laboratory according to their recommendations (http://www.stanford.edu/group/nolan/retroviral_systems/retsys.html). Usually 5 µg of \( \text{pRetroSwitch-EGFP}^{inv} \) was used to transfect Phoenix cells in a 10 cm culture dish, using the FuGENE transfection reagent according to manufacturers recommendations. Two days after transfection, virus was harvested and stored at -80 °C until needed.

**Transduction, adoptive transfer and FACS analysis**
The transduction of HSCs has been described previously in detail (Nakagawa et al., 2006). Briefly, fetal livers, were removed from E14.5 \( \text{CD19cre}^{+/-} \) or \( \text{CD4cre}^{+/-} \) embryos. HSCs from fetal livers were enriched through discontinuous gradient centrifugation using Ficoll and subsequently cultured for 48 h in IMDM, supplemented with Pen/Strep/Glu and 10% FBS, in the presence of 10 ng ml\(^{-1}\) IL-3, IL-6 (PeproTech Inc., Rock Hill, NJ, USA) and SCF (Amgen, Thousand Oaks, CA, USA), 5 µg ml\(^{-1}\) polybrene (Sigma-Aldrich.com) and virus supernatant. HSCs were spin-infected once at 600 g for 60 min at room temperature prior to a 48 h incubation. Before and after transduction, the HSC population (Lin\(^{-}\)/c-Kit\(^{+}\)/Sca-1\(^{+}\)) was 0.1% of the total population of fetal liver cells (data not shown). Cells were harvested and resuspended at a density of \( 2 \times 10^7 - 4 \times 10^7 \) cells ml\(^{-1}\) PBS. Non-irradiated neonates (\( \text{Rag-2}^{-/-}\gamma_c^{-/-} \)) were injected with 50 µl of cell suspension intra liver. Four weeks post-adoptive transfer, mice were sacrificed, and splenic cells were measured by FACS (FACSCantoll, BD, USA), supported by the Cell Quest Pro or FACS Diva software. FACS data were analyzed with FlowJo (version 7.2.1).

**Results and discussion**

**Construction of \( \text{pRetroSwitch-EGFP}^{inv} \)**
First, we designed a vector, \( \text{pRetroSwitch-EGFP}^{inv} \) (Figure 1A,B), to express an enhanced green fluorescent protein (EGFP) reporter, specifically in either B or
T cells in vivo. For this purpose, we modified a mouse stem cell virus vector to contain a multiple cloning site flanked by the mutant LoxP sites Lox66 and Lox71 in an opposite head-to-head orientation (pRetroSwitch) (Figure 1A). Cre-mediated recombination of this mutant LoxP pair will result in unidirectional genetic inversion as it produces an inactive double mutant (Lox72), thereby locking any insert in the reverse orientation (Figure 1B) (Albert et al., 1995; Zhang and Lutz, 2002). Subsequently, an IRES-EGFP reporter was cloned in the anti-sense orientation in between the mutant LoxP pair. Consequently, Cre recombinase activity will revert this insert to its sense orientation, resulting in expression of EGFP (and any other gene of interest) (Figure 1B).

Figure 1. The construction and validation of the pRetroSwitch expression vector. (A) The modified multiple cloning site (MCS) was cloned into the pMSCV expression vector, hereafter known as pRetroSwitch. An oligonucleotide was made with the restriction sites BamHI and NotI which are flanked by the mutated LoxP sites (Lox71 and Lox66) in an opposite orientation. A gene of interest can be cloned into the BamHI site. Dashed arrows represent the DNA primers which contain appropriate restriction sites to facilitate cloning into pMSCV, following PCR. The solid arrows represent the direction of the core 8 bp sequence in the LoxP sites, and the sequences in bold with stars are the mutations within the LoxP sites. (B) Schematic representation of pRetroSwitch-EGFP before and after Cre recombinase activity. In the presence of Cre recombinase activity the IRES-EGFP cassette is switched into the sense orientation leading to EGFP expression. The double mutated Lox72 site is no longer recognized by Cre recombinase. Stars represent the mutations in the LoxP sites. EGFP activity in living cells transduced for 48 h. (C) NIH3T3 cells after transduction with Adeno-cre virus or with (D) pRetroSwitch-EGFPinv. Scale bar, 50 μm. (E) NIH3T3 fibroblasts after co-transduction with pRetroSwitch-EGFPinv and Adeno-cre. Scale bar, 50 μm. (F) B220 MACS-sorted B cells isolated from a CD19cre adult mouse, transduced with pRetroSwitch-EGFPinv. Scale bar, 25 μm. Histogram plots (C–F) show percentages EGFP positive cells. White arrows point to EGFP expressing B cells. All pictures (C–F) were taken with normal (phase/contrast) and fluorescent light simultaneously.
Figure 2. (A) FACS analysis of a total spleen cell population isolated from an adult Rag-2^{-/-} recipient mice after reconstitution with CD19cre-/pRetroSwitch-EGFPinv-transduced hematopoietic stem cells. FSC/SSC plot of total spleen cell population with lymphocyte gate (upper left panel). Lymphocytes were stained for CD45R (B220), the left and right gate represent the non-B cell (CD45Rlow/-) and B cell (CD45Rhigh) populations (upper right panel). Non-B cell and B cell populations were analyzed for EGFP expression. EGFP expression was detected only in B cells (lower left and lower right panels, respectively). (B) FACS analysis of a total spleen cell population from an adult Rag-2^{-/-} recipient mouse, after reconstitution with CD4cre+/- pRetroSwitch-EGFPinv-transduced hematopoietic stem cells. FSC/SSC plot of total spleen cell population with lymphocyte gate (upper left panel). Lymphocytes were stained for CD3, the left and right gate represent the non-T cell (CD3-) and T cell (CD3+) populations, respectively (upper right panel). Non-T cell and T cell populations were analyzed for EGFP expression. EGFP expression was detected only in T cells (lower left and lower right panels, respectively). Figures are representative of at least 3 recipient mice per genotype.

Conditional EGFP expression in vitro

First, we designed a vector, pRetroSwitch-EGFPinv (Figure 1A,B), to express an enhanced green fluorescent protein (EGFP) reporter, specifically in either B or T cells in vivo. For this purpose, we modified a mouse stem cell virus vector to contain a multiple cloning site flanked by the mutant LoxP sites Lox66 and Lox71 in an opposite head-to-head orientation (pRetroSwitch) (Figure 1A). Cre-mediated recombination of this mutant loxP pair will result in unidirectional genetic inversion as it produces an inactive double mutant (Lox72), thereby locking any insert in the reverse orientation (Figure 1B) (Albert et al., 1995; Zhang and Lutz, 2002). Subsequently, an IRES-EGFP reporter was cloned in the anti-sense orientation in between the mutant LoxP pair. Consequently, Cre recombinase activity will revert this insert to its sense orientation, resulting in expression of EGFP (and any other gene of interest) (Figure 1B).

Conditional EGFP expression in vivo

Having confirmed Cre-recombinase-induced switching, we pursued the generation of retrogene mice specifically expressing the EGFP reporter in B cells. For this purpose, HSCs were isolated from CD19cre-/- transgenic embryos (E14.5), transduced with pRetroSwitch-EGFPinv, and subsequently injected into alymphoid neonates (Colucci et al., 1999). Four weeks after injection, the reconstituted mice were sacrificed. Splenic cells were isolated and analyzed by FACS. A gate was set for lymphocytes (empirical observation) in the total splenic cell population (Figure 2A, upper left panel), which was
stained with the B cell marker CD45R (aka. B220, Fig. 2A, upper right panel). EGFP expression was determined for both the CD45R<sup>low</sup> (non-B cells) and CD45R<sup>high</sup> (B cells) population (Figure 2A, lower left and lower right panel, respectively). As expected, B cells carried the EGFP signal, whereas all other splenic cells, including those outside the lymphocyte gate (data not shown), lacked the EGFP signal. In a comparable, but separate experiment, we transduced HSCs isolated from CD4cre<sup>−/−</sup> fetal livers with pRetroSwitch-EGFP<sup.rgb</sup> to obtain EGFP expression specifically in T cells. FACS analysis shows that EGFP expression was observed in CD3 positive cells only (Figure 2B).

**Concluding remarks**

We present a system which enables the instant specific expression of a transgene in vivo in a broad range of hematopoietic cell lineages. Using a single vector backbone in combination with HSCs derived from Cre transgenic mouse strains, this system permits the expression of a gene of interest in B (CD19cre) or T lymphocytes (CD4cre), but also in myeloid progenitor-derived lineages including monocytes, mature macrophages, and granulocytes (LysMcre) or in megakaryocytes and platelets (Pf4cre). Additional Cre transgenic mouse strains commonly used in immunology and hematology will allow for instant expression of the transgene in diverse developmental and differential stages of B and T cells. Thus, the choice of cell type for transgene expression is only dogged by the availability of Cre transgenic mice (Table 1).

**Table 1. Available Cre transgenic mouse strains used in immunology**

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<th>Cre strain</th>
<th>Cre activity in cell population</th>
<th>Original publication</th>
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In our system, it is not necessary to design and clone promoters and/or enhancers to obtain conditional gene expression, which significantly reduces the cloning effort. In addition, the insert size is restricted to the transgene’s open reading frame only, thus improving packaging of viral particles, which is size dependent. Although ectopic recombination is a major concern when applying Cre-Lox systems (Schmidt-Supprian et al., 2007) and cannot be ruled out in the present model, it will be exclusively limited to the hematological compartment, making data interpretation less complex (Schmidt-Supprian et al., 2007). Furthermore, the relatively low costs and simple logistics of our system allows for ample controls, including mice carrying
the Cre transgene alone or mice carrying only IRES-reporters, even in littermates (Schmidt-Supprian et al., 2007). Obviously, other than in the previously described retrogenic system (Holst et al., 2006; Nakagawa et al., 2006), the nature of our system prevents premature phenotype development as reporter activity becomes only apparent after Cre expression, which can easily be monitored even prior to reconstitution. Hence, transgene-induced side effects such as interference with HSC homing/migration to the bone marrow niche, or untimely differentiation, proliferation, or induction of apoptosis are excluded. The development of sophisticated transgenic mouse models is a major asset to the study of hematological diseases. We suggest that our conditional retrogenic model can be applied to test a set of open reading frames with, for example, oncogenic potential in a relatively short period of time, in contrast to the efforts involved to generate classical conditional mice. This is especially relevant when dealing with complex genes, such as CD44, which sprouts lots of different open reading frames (Koopman et al., 1993), either described or predicted in silico, all of which could play a role in tumorigenesis. Compared to the classical transgenic mouse models (Jonkers et al., 2002), Cre-Lox models are considered to more closely resemble human oncogenesis, as they allow the study of gene expression in specific cell populations. Concerning the hematological compartment, we argue that our conditional retrogenic mouse system even more faithfully recapitulates the pathobiology of sporadic tumor formation in humans, since only a relatively small proportion of cells within a specific population is modified. At least, this novel mouse model could provide exciting new opportunities to gain insight into the pathogenesis of hematological malignancies, viz. leukemia, malignant lymphoma, and multiple myeloma.

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Authorship

Contribution: T.C. and R.M.R. equally designed, initiated and completed the work, A.U. provided material, technical assistance and advice, M.S. and S.T.P. supervised the project. All authors contributed to the writing of the manuscript. Conflict-of-interest disclosure: The authors declare no competing financial interests.
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