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Technical note

Lineage-specific transgene expression in hematopoietic cells using a Cre-regulated retroviral vector

Vivian M. Turner [a], Sandra Gardam [a,1], Robert Brink [a,b,*]

[a] Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst NSW 2010, Australia
[b] St. Vincent's Clinical School, University of New South Wales, Darlinghurst NSW 2010, Australia

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ABSTRACT

Transduction of bone marrow stem cells with retroviral expression vectors represents a cheaper and more rapid alternative to conventional transgenesis for studies of in vivo gene function. However, achieving tissue-specific expression of genes inserted into retroviral vectors is notoriously difficult. We have developed a single tri-cistronic retroviral vector (MG^{f}I4) that facilitates Cre-dependent, lineage-specific gene expression within hematopoietic cells. Bone marrow stem cells transduced with MG^{f}I4 co-express a loxP-flanked (floxed) eGFP cDNA together with truncated human CD4 (hCD4Δ). Open reading frames (ORFs) cloned between these two cDNAs are not constitutively translated but are activated upon Cre-mediated removal of the eGFP cDNA. Mice reconstituted with transduced bone marrow stem cells obtained from *Cd19*-Cre, *Cr2*-Cre or *Lck*-Cre, donors were shown to specifically express an ORF insert in the appropriate lymphocyte subsets. Cells that had activated ORF expression were identifiable by transition from a GFP^{+}, hCD4^{+} to a GFP^{-}, hCD4^{+} phenotype. The use of this novel vector in conjunction with the wide range of well-characterized Cre-transgenic lines will be a versatile tool for exploring gene function within the immune system. In particular, this approach will provide a convenient way to test the functional significance of naturally occurring genetic mutations linked to human disease.

1. Introduction

Transgenic mice remain one of the principal tools for investigating gene function in vivo. However, generation of mice transgenic for a particular DNA construct typically involves production and analysis of multiple independent lines to avoid transgene integration site and copy number effects. These effects include poor expression as well as loss of tissue specificity in transgenic promoters designed to direct expression to a particular cell type. One strategy developed to circumvent this problem is the use of gene targeting to introduce single copy transgenes into the ROSA26 locus together with a loxP-flanked (floxed) transcriptional silencer (Jager et al., 2004). Crossing of such lines with transgenic or gene "knock-in" mice in which Cre recombinase is expressed in a specific and well-characterized pattern can then be performed to achieve activation of transgene expression in specific cell types (Jager et al., 2004; Sasaki et al., 2006). Whilst there is great flexibility in this approach by virtue of the many Cre-transgenic lines that exist, producing a separate gene-targeted mouse for every gene construct of interest is prohibitively expensive and time consuming for most laboratories.

To retain the flexibility of Cre-regulated gene expression but circumvent the need for either conventional transgenesis or gene targeting, we set out to develop a retroviral vector that could be used to transduce hematopietic stem cells and

Abbreviations: BM, bone marrow;eGFP, enhanced green fluorescent protein;GOI, gene of interest;HEL, hen–egg lysozyme;LTR, long terminal repeat;ORF, open reading frame.

* Corresponding author. Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst NSW 2010, Australia. Tel./fax: +61 2 9295 8454.
E-mail address: r.brink@garvan.org.au (R. Brink).
[1] Present address: Department of Biomedical Molecular Research, VIB - Ghent University, Technologiepark 927, 9052 Gent - Zwijnaarde, Belgium.

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express a "gene of interest" (GOI) in a Cre-dependent fashion in reconstituted mice. Since production and packaging of retroviral particles requires transcription of the full retroviral genome, it was not possible to use a floxed transcriptional silencer in this scenario. Instead, we have produced a tricistronic vector that directs Cre-dependent expression in vivo by regulating translation rather than transcription of cloned open reading frames (ORFs) encoding GOIs.

2. Materials and methods

2.1. Mice

Cd19-Cre (Rickert et al., 1997), *Cr2*-Cre (Kraus et al., 2004) and *Lck*-Cre (Orban et al., 1992) transgenic mice have been previously described. C57BL/6 and B6.SJL-Ptprc^a (C57BL/6 CD45.1 congenic) mice were purchased from Animal Resources Centre, Perth. All animal experiments were carried out under protocols approved by the Garvan/St. Vincent's Animal Ethics Committee.

2.2. Retroviral constructs

pMG^{f}I4 (Fig. 1A) was assembled from pMIG (Van Parijs et al., 1999) using standard recombinant DNA techniques (sequence available on request). Retroviruses were produced in the Phoenix cell line (Swift et al., 2001). Cells cultured in 10 cm plates were transfected with 10 μg of pMG^{f}I4 and 5 μg of pCL-Eco (Naviaux et al., 1996) using a FuGENE 6 transfection reagent. At 48 h and 72 h after transfection, viral supernatants were collected and filtered through 0.45 μM filters. Purification was performed with 100 kDa Amicon tubes (Millipore) and retrovirus was snap frozen in liquid nitrogen for storage at −80 °C.

2.3. Bone marrow chimeras

Single cell suspensions of bone marrow were cultured in RPMI with 10% FCS, 10 μg/ml IL-3, 10 μg/ml IL-6, 50 μg/ml mSCF, 50 U/ml penicillin and 50 μg/ml streptomycin for 40 h at 10^7 per well in 6 well plates (BD Biosciences). Cells were then transduced with retrovirus by centrifugation at 780×*g* for 90 min at 30 °C. Hexadimethrine bromide (Sigma) was used at 4 μg/ml with the retrovirus. 24 h later, transduced cells were purified via autoMACS using hCD4 microbeads (Miltenyi). C57BL/6 mice, given two doses of 425 cGy whole body irradiation the day before (X-RAD 320 Biological Irradiator, PXI), were reconstituted with 1.5×10^6 CD45.1 congenic C57BL/6 BM cells plus 4×10^5 transduced BM cells.

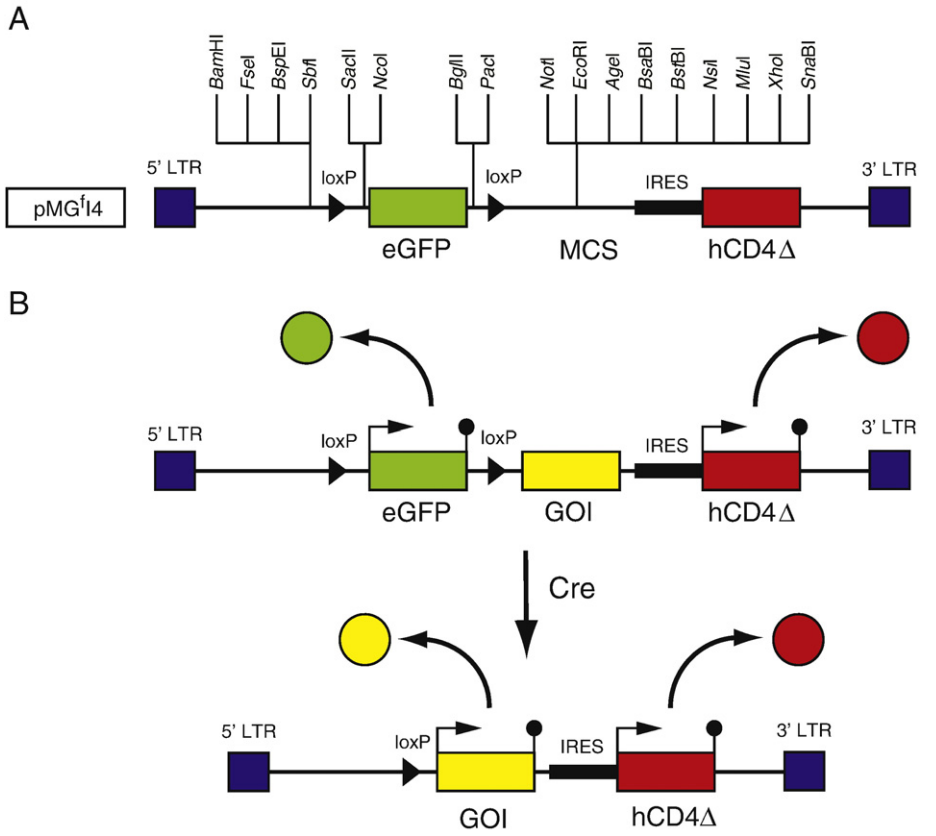

Fig. 1. Schematic representation of pMGfI4 vector (A) and summary of it's activity (B). The 5′ LTR controls transcription of all three ORFs: the eGFP, flanked by loxP sites; a GOI cloned into the MCS; and truncated hCD4Δ preceded by an IRES. The baseline configuration (in the absence of Cre recombinase) results in the translation of eGFP and hCD4Δ, however the GOI is not efficiently translated due to a lack of an IRES. In the presence of Cre, the eGFP cassette is removed and the GOI and hCD4Δ are expressed. Round coloured balls indicate protein expression.

2.4. Flow cytometry

Flow Cytometric analysis was performed on the FacsCantoII flow cytometer (BD Biosciences) with FacsDiva software (BD Biosciences) and analysis performed with FlowJo (TreeStar). Single cell suspensions were prepared from spleens and bone marrow. Samples were subjected to erythrocyte depletion in red blood cell lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM Na_2EDTA, pH 7.4). The following antibodies were purchased from BD Biosciences: anti-CD16/32 (2.4G2), anti-B220-Pacific Blue (RA3 6B3), and anti-CD3-PerCP (145-2C11). Anti-hCD4-APC (S3.5) was purchased from Invitrogen. mHEL was detected using HyHEL9-biotin plus streptavidin-PE.

3. Results and discussion

As a starting point for the development of a Cre-regulated expression vector, the pMSCV-IRES-GFP (pMIG) retroviral vector (Van Parijs et al., 1999) was used. The major alterations to pMIG were: i) replacement of the 3′ GFP cDNA with one encoding a truncated form of human CD4 lacking the cytoplasmic signaling domain (hCD4Δ), and ii) insertion of a floxed eGFP cDNA, without an associated polyadenylation signal, upstream of the multiple cloning site (MCS) (Fig. 1A). The new vector was named pMSCV-eGFP^{flox}-IRES-hCD4Δ or pMG^{f}I4.

The principle behind pMG^{f}I4 (and the resulting MG^{f}I4 retroviral particles) is that the GOI inserted into the MCS is transcribed as a component of tri-cistronic RNA molecule but is not translated. This is predicted by virtue of the presence of a strong Kozak translation initiation signal (Kozak, 1989) associated with the upstream eGFP cassette and the absence of an IRES associated with the GOI (Fig. 1B). Thus, in its initial conformation, MG^{f}I4 should only encode protein synthesis from the 5′ eGFP cDNA and the 3′ IRES-linked hCD4Δ cDNA (i.e. transduced cells = eGFP$^+$, GOI$^-$, hCD4$^+$). Following Cre-mediated removal of the eGFP cassette, however, the GOI possesses the most 5′ Kozak sequence and should be strongly translated (Kozak, 1989) together with the IRES-associated hCD4Δ cDNA from the resulting bicistronic RNA (i.e. transduced cells = eGFP$^-$, GOI$^+$, hCD4$^+$) (Fig. 1B).

To test the effectiveness of MG^{f}I4 at achieving the predicted Cre-regulated induction of GOI expression in vivo, a cDNA encoding a membrane-bound version of hen–egg lysozyme (mHEL) (Hartley et al., 1991) was cloned into the MCS. MG^{f}I4 vector carrying the mHEL insert (1.4 kb) was packaged into retroviral particles with high efficiency, as were other vectors carrying inserts of up to 2.5 kb (data not shown). From known retroviral packaging limits (9–10 kb of RNA), it should be possible to produce retroviral particles from MG^{f}I4 containing inserts of up to 4–5 kb. Bone marrow (BM) cells from wild-type (WT) mice or from transgenic mice encoding Cre recombinase expression in either the B-cell (*Cd19*-Cre, *Cr2*-Cre) or the T-cell (*Lck*-Cre) lineage (Rickert et al., 1997; Kraus et al., 2004; Orban et al., 1992) were used for transduction with MG^{f}I4-mHEL. Transduced BM cells were used to reconstitute lethally irradiated C57BL/6 mice and spleen cells and BM from these chimeras analysed by flow cytometry following 8–10 weeks of reconstitution.

Regardless of the genotype of BM used, 10–60% of splenic B cells or T cells were identifiable as transduced by virtue of their expression of hCD4 (Fig. 2A). In recipients reconstituted with WT BM, transduced cells showed proportional expression of hCD4 and eGFP (Fig. 2A, i–ii). However, recipients of BM cells from each of the three Cre-transgenic lines showed the predicted loss of eGFP expression in the majority of transduced lymphocytes of the appropriate lineage. Thus hCD4$^+$ B cells but not T cells derived from *Cd19*-Cre (Fig. 2A, iii) or *Cr2*-Cre (Fig. 2A, iv) BM showed extensive loss of eGFP expression, whilst the converse was true for transduced lymphocytes derived from *Lck*-Cre BM (Fig. 2A, v). Importantly, simultaneous analysis of cell surface levels of mHEL on transduced cells showed that its expression was undetectable in the absence of Cre but was efficiently and specifically activated by Cre-mediated removal of eGFP (Fig. 2A, ii–v).

To determine whether activation of the expression of GOIs cloned into MG^{f}I4 could also be temporally regulated, the activation (loss of eGFP expression) of the construct was examined in BM cells from mice reconstituted with transgenic cells that expressed Cre either early (*Cd19*-Cre) or late (*Cr2*-Cre) during B cell development (Rickert et al., 1997; Kraus et al., 2004). Analysis of transduced B-lineage cells in the BM (hCD4$^+$, B220$^+$) showed that the most immature (CD21$^-$) of these showed little or no inactivation of eGFP expression in either WT or *Cr2*-Cre-transgenic BM but that *Cd19*-Cre-transgenic BM cells already showed extensive eGFP deletion (Fig. 2B) and expression of mHEL (data not shown) at this stage of development. At the more mature recirculating B cell stage (CD21$^+$), most *Cd19*-Cre and *Cr2*-Cre-transduced cells had lost eGFP expression whilst WT B cells remained eGFP$^+$ (Fig. 2B).

These data show that transduction of Cre-transgenic BM stem cells with retroviral particles derived from pMG^{f}I4 provides a robust and efficient system to manipulate in vivo gene expression in hematopoietic cells. Due to the large number of well-characterised transgenic lines that express Cre in different hematopoietic lineages, this vector should prove extremely versatile. Indeed, in theory it could be employed in vivo using any type of transplantable stem cell. Although the efficiency of deletion may vary in different Cre-transgenic lines, a great advantage of this system is that transduced cells that have activated expression of the GOI can be readily identified due to their loss of eGFP. In addition, differences in expression due to integration site variations (e.g. compare T cells in Fig. 2, ii–iv) can be readily identified by hCD4 expression and thus considered when comparing phenotypes associated with different transduced populations.

In addition to achieving lineage-specific expression, this vector can also be combined with existing conditional (floxed) gene knockout mice to carry out in vivo structure–function studies. Thus expression of MG^{f}I4-encoded mutant proteins can be activated in specific cell types in conjunction with the Cre-mediated inactivation of the endogenous wild-type alleles. This has the potential to allow testing of a variety of gene mutants in physiological settings and should prove particularly useful for testing the functional significance of naturally occurring mutations in human genes associated with heritable diseases.

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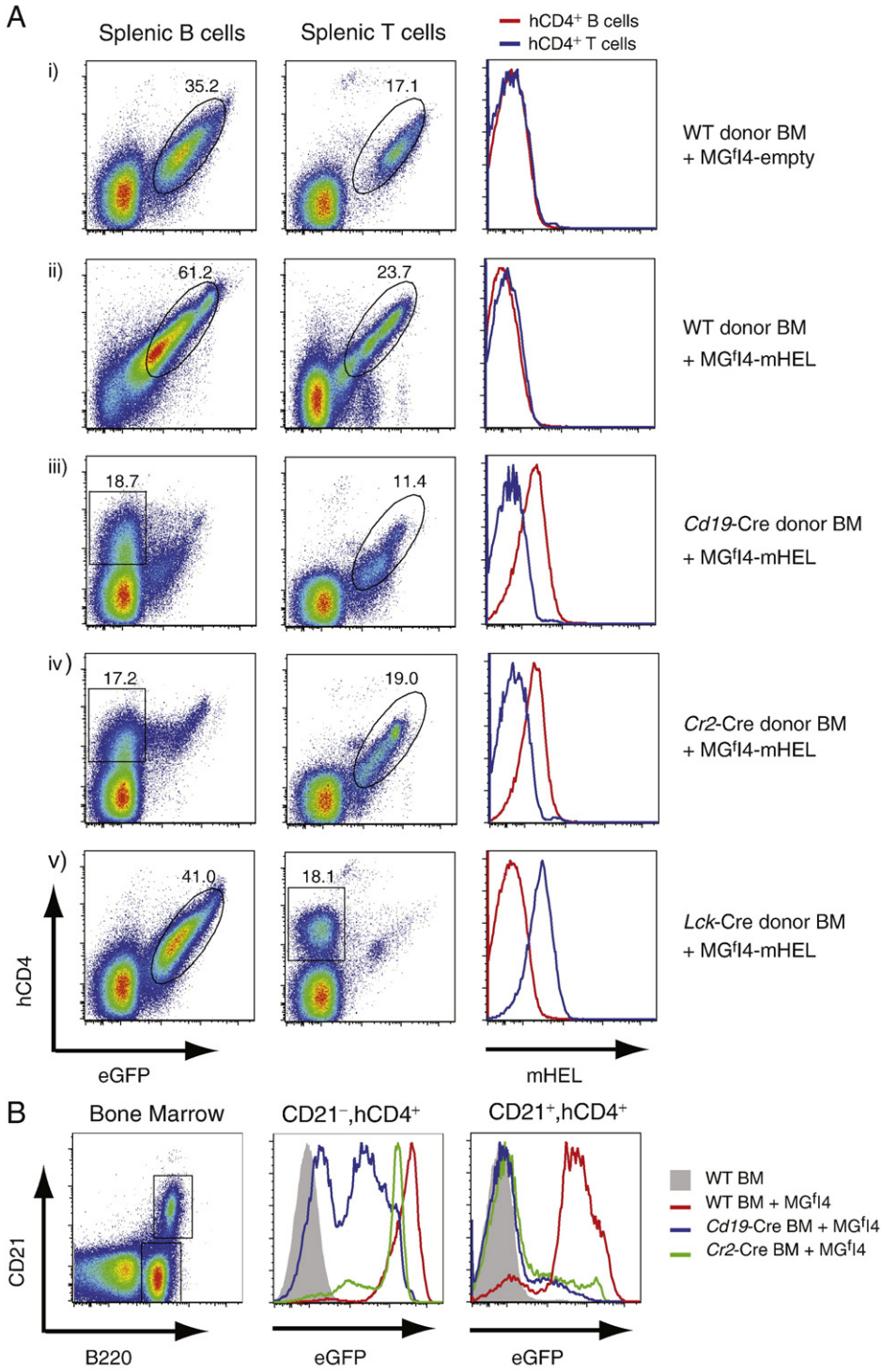

Fig. 2. In vivo validation of the pMGfl4 vector. (A) FACS analysis of hCD4$^+$ (transduced) splenic B (CD45.1$^-$, B220$^+$) and T (CD45.1$^-$, CD3$^+$) cells derived from WT, *Cd19*-Cre, *Cr2*-Cre and *Lck*-Cre donor BM transduced with the indicated viruses. Loss of eGFP expression and gain of mHEL expression occurs in Cre-dependent manner. (B) eGFP expression on immature (CD45.1$^-$, hCD4$^+$, B220$^+$, CD21$^-$) and mature (CD45.1$^-$, hCD4$^+$, B220$^+$, CD21$^+$) BM cells derived from transduced WT, *Cd19*-Cre and *Cr2*-Cre BM. The equivalent populations from unmanipulated C57BL/6 BM are indicated in the left hand panel and were included as negative controls for eGFP fluorescence (grey histograms).

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