

## ARTICLE

# Lentiviral vectors containing mouse *Csf1r* control elements direct macrophage-restricted expression in multiple species of birds and mammals

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The development of macrophages requires signaling through the lineage-restricted receptor *Csf1r*. Macrophage-restricted expression of transgenic reporters based upon *Csf1r* requires the highly conserved *Fms*-intronic regulatory element (FIRE). We have created a lentiviral construct containing mouse FIRE and promoter. The lentivirus is capable of directing macrophage-restricted reporter gene expression in mouse, rat, human, pig, cow, sheep, and even chicken. Rat bone marrow cells transduced with the lentivirus were capable of differentiating into macrophages expressing the reporter gene *in vitro*. Macrophage-restricted expression may be desirable for immunization or immune response modulation, and for gene therapy for lysosomal storage diseases and some immunodeficiencies. The small size of the *Csf1r* transcription control elements will allow the insertion of large “cargo” for applications in gene therapy and vaccine delivery.

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## INTRODUCTION

Haemopoietic stem cells (HSC) in the bone marrow (BM) give rise to monocytes in circulation which can then enter tissues to become resident macrophages. Mammalian macrophages are numerous in all tissues, and apart from their role in the innate immune response are required for many aspects of development and homeostasis (reviewed in ref. 1). Macrophages are also involved in the pathogenesis of inflammatory diseases such as atherosclerosis<sup>2</sup> and rheumatoid arthritis<sup>3</sup> and in the initiation, progression, and metastasis of tumors.<sup>4</sup> The active recruitment of monocyte-macrophages in all forms of inflammation makes them an attractive candidate for therapeutic gene delivery. Gene-targeted macrophages could therefore be an effective method to focus protein expression to the site of diseased tissues and nearby blood vessels. Macrophages can cross the blood–brain barrier and therefore could deliver proteins to the brain in the treatment of neurological disorders.<sup>5</sup> Macrophage lineage cells function as antigen-presenting cells (APC) for the activation of T lymphocytes.<sup>6</sup> Restricted expression in vaccine vectors could provide increased immunogenicity by avoiding expression in non-APC, where the outcome may be tolerogenic.

A feature of macrophages is the high expression of lysosomal enzymes.<sup>7</sup> Lineage-restricted gene therapy has been considered in the treatment of lysosomal storage diseases and other phagocyte defects. Lysosomal storage diseases are a large group of metabolic disorders caused by a deficiency in lysosomal enzymes.<sup>8</sup> In affected patients, these defects are most obvious in macrophages. Phagocyte function is specifically compromised in chronic granulomatous disease where defects in genes coding for components of the NADPH oxidase enzyme system result in impaired macrophage

function.<sup>9</sup> A recent study by Brendel *et al.*<sup>10</sup> used a novel myeloid-specific mIR223 promoter for chronic granulomatous disease therapy in mice. While expression of p47(PHOX) and gp91(PHOX) was targeted to granulocytes and macrophages, expression was also detected in other cells such as HSC and lymphocytes.

Colony-stimulating factor 1 receptor (*Csf1r*) is a member of the type III protein tyrosine kinase receptor family whose expression in adults is restricted to cells of the macrophage lineage.<sup>11</sup> We have previously identified the elements of the *Csf1r* locus required for lineage-restricted expression in the MacGreen reporter mice.<sup>12</sup> The construct contained a 7.2kb region comprising the promoter and first intron, which includes a critical conserved enhancer termed the *Fms*-intronic regulatory element (FIRE). A construct of this size is clearly not practical for gene therapy. Here, we have extracted sequences of the mouse *Csf1r* promoter and FIRE to create various lentiviral vectors. These vectors drive expression of several different reporter genes in monocytes and macrophages in a variety of species, including human, rat, pig, cow, sheep, and chicken. Aside from the practical applications for delivery to macrophages, the results indicate that the high sequence conservation of FIRE is reflected in conserved functional activity from birds to mammals.

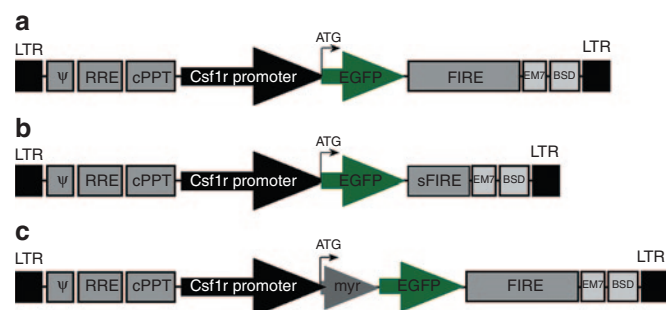
## RESULTS

The *Csf1r* promoter and FIRE drives EGFP and mCherry expression in RAW264.7 cells

Our recent study suggested that the function of FIRE, *in situ*, is position and orientation dependent,<sup>13</sup> but it has previously been reported to have conventional enhancer activity.<sup>14</sup> In order to drive expression of enhanced green fluorescent protein (EGFP) specifically in monocytes

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and macrophages, the *Csf1r* promoter (including start codon) was fused to the coding sequence of *EGFP* and cloned upstream of FIRE in a lentiviral vector (Figure 1). mCherry constructs (data not shown) were also created as well as one in which expression of EGFP or mCherry was localized to membranes using a myristoylated motif (Figure 1c). The specificity of the lentivirus was initially tested by transduction of the murine cell lines EL4 (T lymphocyte) and RAW264.7 (monocyte/macrophage) with *Csf1r*:EGFP-FIRE. After 10 days of selection in blasticidin, only RAW264.7 cells expressed EGFP when viewed by confocal microscopy (Figure 2a). Cells transduced with *Csf1r*:myr:EGFP-FIRE displayed EGFP expression at slightly lower levels via flow cytometry (Figure 2b). However, in these cells, as anticipated, the EGFP was only localized to the cell membrane and also to membranes of intracellular vesicles (Figure 2c). Lentiviral transduction of RAW264.7 cells with



**Figure 1** *Csf1r*:EGFP lentiviral constructs. Schematic of murine *Csf1r*:EGFP constructs. All constructs contained a blasticidin-resistance gene (BSD) driven by the EM7/SV40 promoter. (a) *Csf1r*:EGFP-FIRE (b) *Csf1r*:EGFP-sFIRE (c) *Csf1r*:myr:EGFP-FIRE. cPPT, central polypurine tract; FIRE, Fms-Intronic Regulatory Element;  $\psi$ , HIV-1 packaging signal; LTR, long terminal repeat; RRE, rev response elements; sFIRE, short FIRE; myr, myristoylated motif.

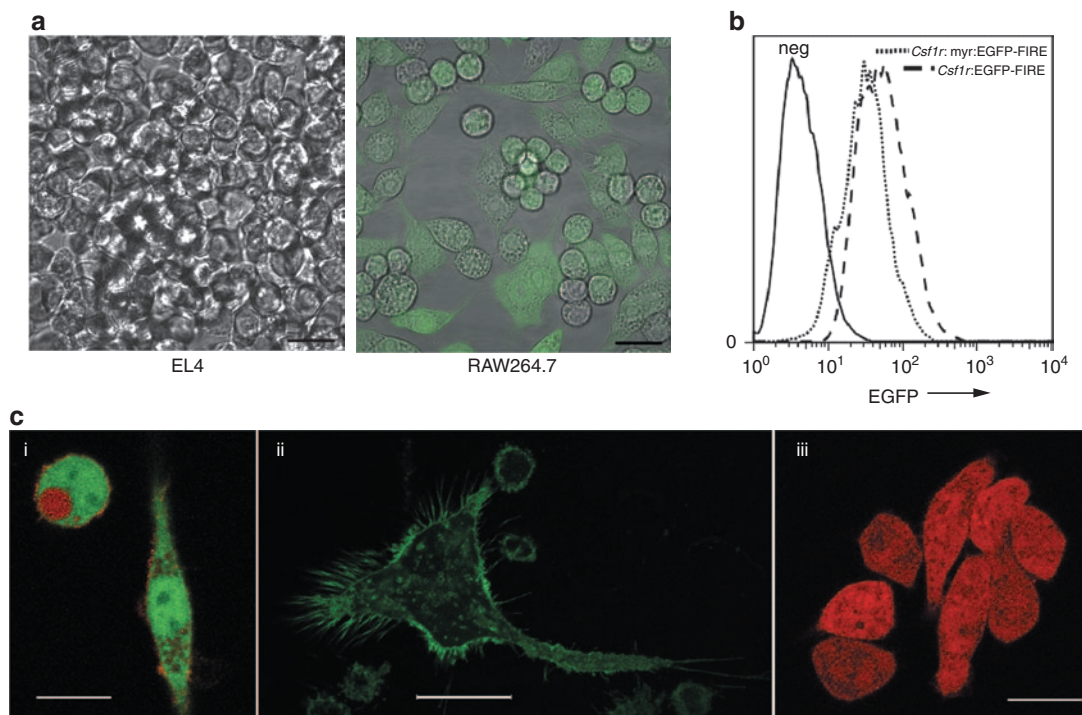
the alternative vector containing mCherry also resulted in reporter expression as detected by confocal microscopy (Figure 2c). In summary, with all reporters, the expression was not only restricted to macrophages, but was sufficient to enable ready visualization.

#### Primary rat macrophages transduced with *Csf1r*:EGFP-FIRE lentivirus express EGFP

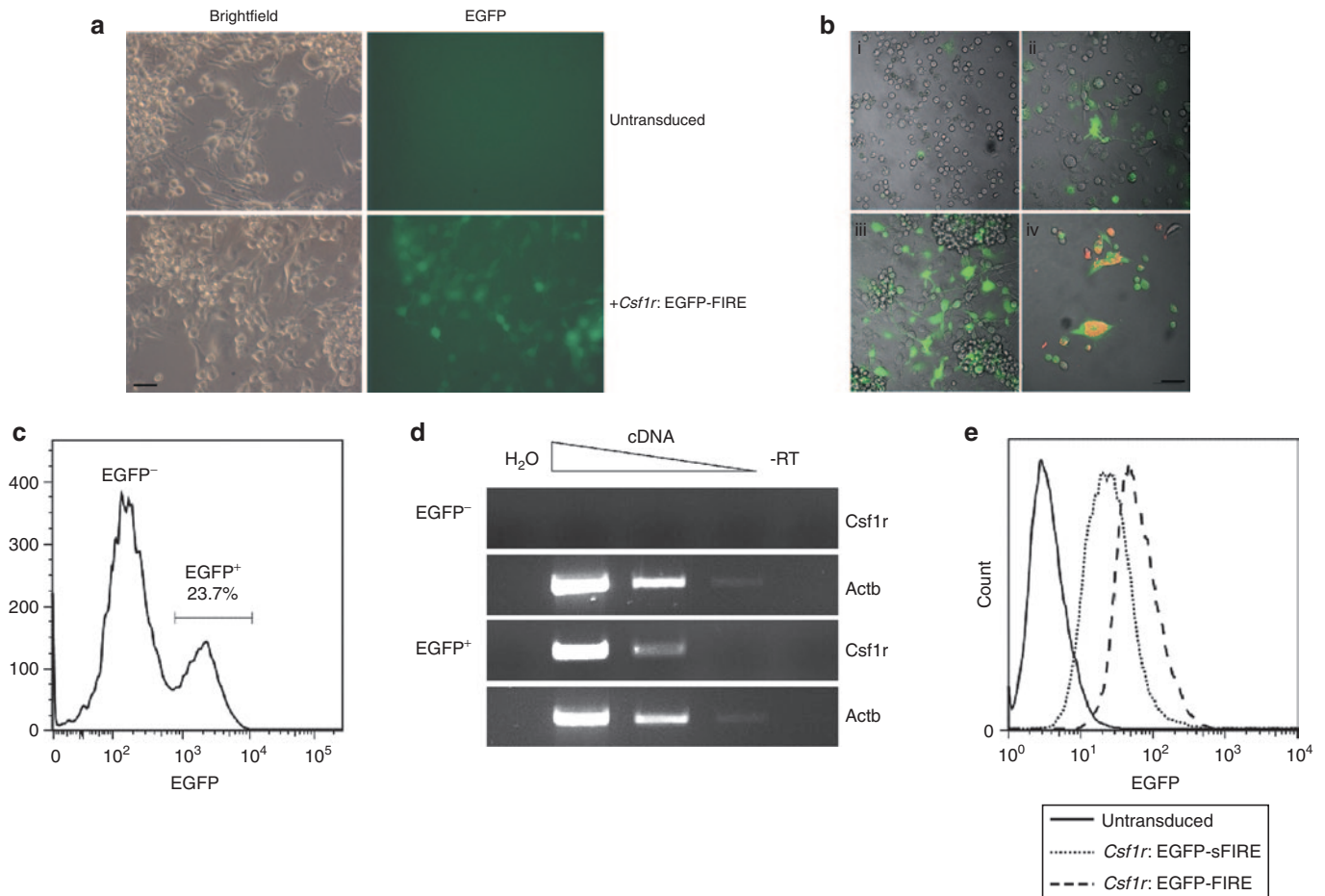
We currently have *Csf1r*:EGFP-positive transgenic mice, which have been widely used.<sup>12</sup> A similar reporter in rat would have great utility, but rat transgenesis is less straightforward and lentiviral delivery would provide an alternative route. The *Csf1r*:EGFP-FIRE lentivirus was also active in primary cells and in the rat. Rat BM-derived macrophages were transduced with the lentivirus and the majority of transduced cells were EGFP<sup>+</sup> as determined by fluorescent microscopy (Figure 3a). We therefore wished to determine whether the virus, once integrated into progenitors, would maintain expression during differentiation. Rat BM cells were cultured for 2 days in IL-3, IL-6, and stem cell factor (SCF) to support myeloid progenitors and then transduced with *Csf1r*:EGFP-FIRE. Two days following transduction, growth factors were replaced with rhCSF1 to allow macrophage development. Prior to addition of rhCSF1, few cells were EGFP<sup>+</sup> (Figure 3b, i), but as the cells differentiated into macrophages, the number of EGFP<sup>+</sup> increased. After 7 days, the large majority of cells were EGFP<sup>+</sup> (Figure 3b, iii) and were confirmed as functional macrophages by their ability to phagocytose fluorescently labeled zymosan particles (Figure 3b, iv).

#### *Csf1r*:EGFP-FIRE drives EGFP expression exclusively in cells expressing *Csf1r*

To confirm the specificity of *Csf1r*:EGFP-FIRE, rat BM cells were transduced with the lentivirus after 2 days in culture and reverse



**Figure 2** Murine *Csf1r* elements in a lentivirus drive reporter gene expression in macrophages but not T lymphocytes. (a) Representative confocal microscopy images of EL4 and RAW264.7 cells transduced ( $n = 3$ ) with *Csf1r*:EGFP-FIRE. (b) FACS analysis of RAW264.7 cells transduced with *Csf1r*:EGFP-FIRE or a membrane-bound EGFP (*Csf1r*:myr:EGFP-FIRE). (c) RAW264.7 cells transduced with (i) *Csf1r*:myr:mCherry-FIRE and *Csf1r*:EGFP-FIRE, (ii) *Csf1r*:EGFP-FIRE, (iii) and *Csf1r*:mCherry-FIRE. Bar = 20  $\mu$ m or 10  $\mu$ m in C i and iii.



**Figure 3** *Csf1r*:EGFP-FIRE can transduce primary rat macrophages and EGFP expression is restricted to haemopoietic cells expressing *Csf1r*. **(a)** Rat bone marrow (BM) cells were differentiated into macrophages with rhCSF1 then transduced with *Csf1r*:EGFP-FIRE. Bar = 50  $\mu$ m. **(b)** Rat BM cells were cultured in interleukin (IL)-6, IL-3, and SCF and transduced with *Csf1r*:EGFP-FIRE, then differentiated into macrophages with rhCSF1 and images taken via confocal microscopy on days (i) 0, (ii) 3, and (iii) 7 of differentiation. (iv) A phagocytosis assay was performed on transduced macrophages. Bar = 50  $\mu$ m. **(c)** Rat BM cells were cultured in IL-6, FLT3-L, TPO, and SCF and transduced with *Csf1r*:EGFP-FIRE. After 3 weeks in selection with blasticidin, cells were FACS sorted for EGFP<sup>-</sup> and EGFP<sup>+</sup> populations. **(d)** mRNA was prepared from sorted EGFP<sup>-</sup> and EGFP<sup>+</sup> populations and used in RT-PCR to analyze *Csf1r* expression. Each image is representative of two experiments. **(e)** Murine RAW264.7 cells were transduced with  $1 \times 10^5$  viral titer units per  $3 \times 10^5$  cells and FACS analysis performed for EGFP expression following blasticidin selection. Histogram is representative of three independent experiments. RT-PCR, reverse transcriptase PCR; SCF, stem cell factor; TPO, thrombopoietin.

transcriptase PCR performed for *Csf1r* expression in sorted GFP<sup>-</sup> and GFP<sup>+</sup> populations following selection in blasticidin. Cells were cultured in the presence of IL-6, FLT3-L, thrombopoietin, and SCF to support the growth of progenitors and provide a mixture of haemopoietic lineages for analysis. Three weeks following transduction, approximately one quarter of the cells expressed EGFP (Figure 3c) and only these cells expressed *Csf1r* as determined by reverse transcriptase PCR (Figure 3d). These results confirm that *Csf1r*:EGFP-FIRE drives EGFP expression only in haemopoietic cell types expressing *Csf1r*.

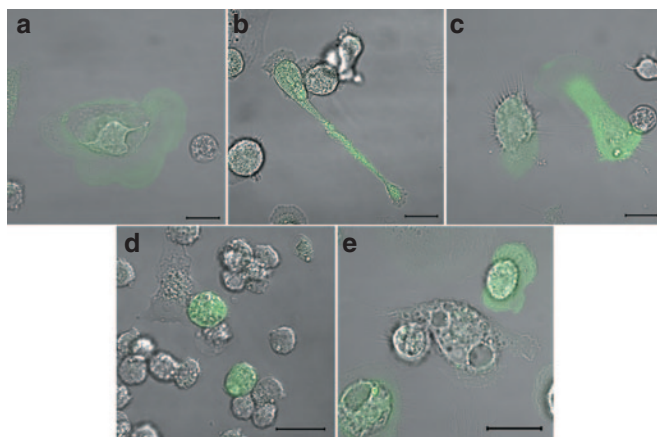
A lentivirus containing a smaller region of intronic DNA results in EGFP expression but at lower levels in RAW264.7 cells. The plasmid encoding *Csf1r*:EGFP-FIRE consists of 6.5 kb sequence between the two lentiviral long terminal repeats (LTR). To increase the potential "cargo" capacity available for expression of genes of interest, we created a smaller vector (*Csf1r*:EGFP-sFIRE), which reduced the region surrounding FIRE by around 1.2 kb, so that there was only 5.3 kb between LTRs. The truncated construct retained EGFP expression, albeit at a slightly lower level compared to *Csf1r*:EGFP-FIRE (Figure 3e).

Murine *Csf1r*:EGFP-FIRE drives expression of EGFP in macrophages from multiple species

The FIRE element is highly conserved from birds to humans. We therefore examined whether murine *Csf1r*:EGFP-FIRE could direct the expression of EGFP in macrophages of other species. Figure 4 clearly shows EGFP expression in BM-derived macrophages from pig (Figure 4a), monocyte-derived macrophages from cow (Figure 4b) and human (Figure 4c) as well as a chicken macrophage cell line (HD11, Figure 4d), and sheep alveolar macrophages (Figure 4e).

## DISCUSSION

Differentiation of BM progenitors into macrophages requires macrophage colony-stimulating factor (Csf1), which signals via its receptor Csf1r.<sup>15,16</sup> Expression of *Csf1r* mRNA in mice is restricted to cells of the myeloid lineage. The only other expression occurs in placental trophoblasts during development, where it is driven by a distinct promoter.<sup>17</sup> MacGreen mice were created previously by placing EGFP expression under the control of the *Csf1r* proximal promoter and FIRE located in the first intron.<sup>12</sup> These mice have consistent expression of EGFP in the same locations as the endogenous gene and



**Figure 4** Murine *Csf1r*:EGFP-FIRE transduced primary macrophages from multiple species express EGFP. The following cells were differentiated into macrophages with rhCSF1 and then transduced with the lentivirus: (a) pig bone marrow–derived macrophages (BMDM), (b) cow monocyte-derived macrophages (MDM), (c) human MDM. A chicken macrophage cell line, HD11 (d) and sheep alveolar macrophages (e) were also transduced. These cells were not selected in blasticidin. Bar = 20  $\mu$ m. Transductions were performed at least twice for each cell type.

provide a valuable tool for those interested in macrophage biology. Lentiviral gene delivery to embryos results in higher percentages of transgenic animals when compared to standard pronuclear injection techniques.<sup>18</sup> The region of *Csf1r* used to create the MacGreen mice (7.2 kb) was considered too large to incorporate into a lentiviral vector. Including EGFP, the sequence between the two LTRs would be 10.5 kb. HIV-based lentiviral vectors generally contain a maximum sequence of 9 kb between the two LTR as this is the size of the native HIV genome.<sup>19</sup> Insertion of larger sequences is possible but typically results in lower titers of virus.<sup>20</sup> To enable inclusion of cargo, we created a vector in which a 0.5 kb region of the murine *Csf1r* promoter was placed 5' to the EGFP coding sequence with a distal portion of the *Csf1r* second intron containing FIRE (1.8 kb) placed 3' to the EGFP stop codon. We also created a smaller vector, in which the region surrounding FIRE was further reduced. This decreased expression somewhat, possibly due to the exclusion of a second known DNase hypersensitive site upstream of FIRE.<sup>14</sup> Both vectors enabled expression of the EGFP reporter. We also tested a membrane-targeted reporter. Myristoylation is a protein modification and one of the mechanisms involved when a protein associates with a membrane.<sup>21</sup> When this motif was fused to EGFP in the construct *Csf1r:myr*:EGFP-FIRE and used to transduce cells, it resulted in EGFP that was not only localized to the cell membrane but also to membranes of intracellular vesicles. This would therefore be useful in colocalisation studies as pathogens are known to assemble within endosomes<sup>22</sup> and phagosomes<sup>23</sup> and would also aid the visualization of pseudopodia during infection.

Rat BM cells were capable of differentiation into EGFP<sup>+</sup> macrophages following transduction with the *Csf1r*:EGFP-FIRE lentivirus highlighting both the potential use of this vector in gene therapy and its lineage-restricted expression. Although there remains a formal need to confirm that the vector would be induced during human macrophage differentiation from progenitors, the FIRE element is highly conserved and also enters open chromatin during human macrophage differentiation.<sup>24</sup> Current gene therapy strategies are based on *ex vivo* transduction of autologous HSC with viral vectors containing therapeutic genes. Recent studies in humans have highlighted the benefit of HSC gene therapy in metachromatic leukodystrophy, a neurodegenerative lysosomal storage disease. Metachromatic leukodystrophy patients are deficient in arylsulfatase

A causing build-up of the enzyme's substrate in various cell types including microglia and macrophages of the peripheral nervous system.<sup>25</sup> Biffi *et al.*<sup>26</sup> reported promising results in a phase 1/2 clinical trial involving three patients where HSC were transduced with a lentivirus overexpressing arylsulfatase A from a ubiquitous promoter. Gene delivery driven by such promoters could have undesirable effects in nontarget cell types, and current lentivirus-based technologies aim to deliver proteins only to target cells. As macrophages have the potential to deliver therapeutic proteins to many target tissues, they make an attractive target cell for gene therapy. Several macrophage promoters such as CD68 and the scavenger receptor-A have been studied. Their limitations are reviewed in ref. 27. He *et al.*<sup>28</sup> generated macrophage-specific synthetic promoters by random ligation of myeloid/macrophage cis elements and reported that these promoters were 100-fold more effective at delivering green fluorescent protein (GFP) to macrophage cell lines than the native *Csf1r* promoter. However, their *Csf1r* constructs did not include the intronic region containing FIRE, which is essential for EGFP expression in the MacGreen mice, so the baseline for comparison was very low.<sup>12</sup> The promoter of CD68 has been suggested as the best currently available macrophage-specific promoter,<sup>29</sup> but CD68 reporter mice do not express GFP in the majority of cells expressing F4/80,<sup>30</sup> which is a well-documented marker of macrophages.<sup>31</sup> For myeloid-restricted gene expression, the *Csf1r* promoter/enhancer appears superior in terms of efficacy, specificity, and reproducibility. The production of transgenic sheep using the *Csf1r*-based lentivirus is currently underway, and initial analysis of peripheral blood mononuclear cell in these animals shows EGFP expression is restricted to CD14<sup>+</sup> monocytes (unpublished data). We have shown that macrophages from chicken, cow, human, and pig as well as sheep alveolar macrophages can also be transduced with the vector, and express EGFP, so the mouse sequence provides a generic vehicle that could be tested in both veterinary, preclinical, and human clinical applications.

This remarkable cross-species reactivity highlights the widespread implications that this vector could have in current gene therapy technologies. If the *Csf1r*:EGFP-FIRE lentivirus is to be used as a gene therapy vehicle, it would be advantageous to further decrease the size of the vector in order for sequences larger than EGFP to be inserted. The smaller vector we created apparently reduced expression and the titer of the two lentiviruses was similar, indicating the larger vector was not inhibiting virus production (data not shown). We may be able to optimize expression further by creating a hybrid or synthetic combination of FIRE (which is only around 300 bp) and the upstream element. What is clearly the case is that the enhancer can be located conveniently downstream of the gene of interest or reporter gene, so further optimization will be straightforward. The safety concerns of using lentiviral vectors in gene therapy technologies are well documented. There is the risk of insertional mutagenesis following viral transduction and current gene therapy research focuses on the use of integrase-deficient packaging plasmids.<sup>32</sup> If *Csf1r*:EGFP-FIRE were to be used in clinical settings, it would be quite simple to mutate (D64V) the viral integrase, which decreases integration up to 10<sup>4</sup>-fold.<sup>33</sup>

Aside from gene therapy, lentiviral vectors have also been considered as vaccines<sup>34</sup> to drive efficient antigen expression and presentation in APC both *in vitro* and *in vivo*, leading to the activation of cellular immunity and humoral immune responses. The injection of a lentivirus containing ovalbumin driven by a ubiquitous promoter protected mice from the development of OVA-expressing tumor cells.<sup>35</sup> However, in some cases, ubiquitous expression of a potential antigen in non-APC could potentially drive peripheral tolerance, rather than effective immunization (*e.g.*, ref. 36). The *Csf1r* lentivirus addresses this question by

**Table 1** Oligonucleotides used in cloning

Oligonucleotide	Sequence/s
Mouse Csf1r promoter	5' TTACCAGTTGGTCCCAGAGG
Csf1r:EGFP splice overlap	5' CACGCTATCCCCTGGAGACTATGGTGAGCAAGGGCG; 3' AACAGCTCCTCGCCCTTGCTCACCATAGTCTCCAGGGGATAGCGTGAGCC
EGFP	3' GCGGCCGCTTTACTTGT
FIRE	5' GAGTGTGTGAGTGGCTGCAT; 3' AGACCATCCATCCTCCTCCT
sFIRE	5' GGGTCAGCAAACAGGACAGT; 3' TCCCCTGCTCCAGTACAGAC
Csf1r:myrEGFP splice overlap	5' CACGCTATCCCCTGGAGACTATGGGCTGCATCAAGAGCAAG; 3' GCGCTTGCTCTTGATGCAGCCCATAGTCTCCAGGGGATAGCGTGAGCC
myr:EGFP splice overlap	5' CGACGACGAGGCACCGGTGCGCCACCATGGTGAGCAAGGGC; 3' GCCCTTGCTCACCATGGTGGCGACCGGTGCCTCGTCGTCG
mCherry	3' TTGAATTGCTCCTCGAGGCCG
Csf1r:mCherry splice overlap	5' GGCTCACGCTATCCCCTGGAGACTATGGTGAGCAAGGGCGAGGAGGATAA; 3' TTATCTCCTCGCCCTTGCTCACCATAGTCTCCAGGGGATAGCGTGAGCC

FIRE, Fms-intronic regulatory element.

**Table 2** Cell culture media

Cells	Media	Polybrene ( $\mu\text{g/ml}$ )	Blasticidin ( $\mu\text{g/ml}$ )
RAW264.7	DMEM, 10% FCS	8	5
HEK293T,D-17	DMEM, 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, nonessential amino acids	8 (D-17)	15 (D-17)
EL4	DMEM, 10% horse serum	8	10
HD11	RPMI, 10% FCS, 2% chicken serum	8	7.5
Rat BM, rat BMDM	DMEM, 10% FCS, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin	5	8 (Rat BM)

BMDM, bone marrow-derived macrophages; DMEM, Dulbecco's modified Eagle medium; RPMI, Roswell Park Memorial Institute medium.

driving expression in professional APC. Classical Steinman–Cohn dendritic cells as well as macrophages express Csf1r-EGFP in the MacGreen mouse.<sup>37</sup> As Csf1r:EGFP-sFIRE is only 5.3 kb between LTRs, there is the possibility to insert larger antigens for which previous vaccination approaches have proved difficult. One such example is the blood-stage of malaria. There is currently no vaccine and previous attempts at production have been hampered by the size of the antigens.<sup>38</sup>

In summary, we have created a lentiviral vector that drives expression of genes of interest in macrophage lineage cells of species from birds through livestock to humans.

## MATERIALS AND METHODS

### Constructs

Lentiviral vector plasmid pLenti6-R4R2-V5DEST (Invitrogen, Paisley, UK) was modified by removal of the attR4-attR2 region with EcoRV and self-ligation. The resultant plasmid was cut with ClaI/HpaI and a polymerase chain reaction (PCR) product encoding central polypurine tract inserted. Two genomic DNA regions containing the FIRE were amplified by PCR and cloned into the XbaI site. The sizes of the genomic DNA containing FIRE were 1,798 bp and 537 bp, respectively. The murine Csf1r promoter (mm10, chr18:61,105,457-61,105,950) was amplified via splice overlap PCR so that the ATG of Csf1r initiates translation of EGFP. The construct used to produce MacGreen mice was used as template DNA.<sup>12</sup> The Csf1r promoter:EGFP fragment was cloned into the EcoRV site upstream of FIRE. A construct was also created where mCherry replaced EGFP. mCherry or EGFP was also fused to a myristoylated motif (myr) to localize expression to membranes. The vector containing

myr:mCherry<sup>39</sup> was a generous gift from Mary E. Dickinson (Baylor College of Medicine, Houston, TX). A list of oligonucleotides used in the construction of these vectors can be found in Table 1.

### Cell culture

All cells were maintained at 37 °C in a humidified 5% CO<sub>2</sub> incubator and cultured as per Table 2. All media contained 1 mmol/l GlutaMAX (Life Technologies, Paisley, UK) except for rat BM, which contained 2 mmol/l. If cells were selected with blasticidin following lentiviral transduction, the concentration is listed in Table 2.

### Lentivirus production and titration

High-titer lentivirus was prepared by transfection of HEK293T cells with a Csf1r construct as well as psPax2 (encoding gag/pol/rev proteins) and pLP/vesicular stomatitis virus G (for the expression of vesicular stomatitis virus G glycoprotein). Prior to transfection, HEK293T cells were plated at 10<sup>5</sup> cells/cm<sup>2</sup> and cotransfection of the three plasmids was performed with Fugene HD (Promega, Southampton, UK) according to manufacturer's instructions. The following day medium was replaced with serum-free culture media. Supernatant was harvested 48 hours post-transfection and filtered through 0.45  $\mu\text{m}$  filters prior to concentration in Centricon Plus-70 columns (Millipore, Watford, UK). Once the supernatant was concentrated to 10 ml, it was then ultracentrifuged for 2 hours at 69K xg at 4 °C. Viral pellets were resuspended in 100 mmol/l NaCl, 20 mmol/l Tris pH 7.3, 10 mg/ml sucrose, and 10 mg/ml mannitol and stored at –80 °C until use. The viral titer (typically between 8.5 × 10<sup>7</sup> and 2 × 10<sup>8</sup> transducing units/ml) was assayed by end-point dilution on the D-17 cell line. For some experiments, unconcentrated lentivirus (10<sup>5</sup> transducing units/ml) was used.

### Lentiviral transduction

Cells were cultured in 24-well plates or Lab-Tek II 4-well chamber slides and on the morning of transduction, placed in a minimal volume of media containing polybrene and lentivirus (as specified in more detailed protocols below and in Table 2). Cells were incubated at 37 °C for 3–4 hours before the wells were topped up with 1 ml fresh media.

### Lentiviral transduction of cell lines

EL4 and RAW264.7 cells were transduced with  $9.2 \times 10^4$  transducing units of lentivirus (Figure 2a). Unconcentrated virus was used to transduce RAW264.7 cells in Figure 2c.

### FACS analysis of RAW264.7 cells

RAW264.7 cells were harvested and placed in PBS containing 2% fetal calf serum (FCS) and 1  $\mu$ g/ml propidium iodide (Sigma, Dorset, UK) to exclude dead cells. EGFP expression was analyzed on a FACSCalibur (BD, Oxford, UK).

### Microscopy

Cells were imaged via confocal microscopy using either an eC1 (Nikon, Kingston Upon Thames, UK) or LSM710 (Zeiss, Cambridge, UK). Standard light microscopes were also used. For detection of EGFP expression in macrophages from multiple species, EGFP gain was initially set using untransduced cells and the same EGFP gain used for all samples.

### Lentiviral transduction of rat BM-derived macrophages

All animal care and experimentation was conducted in accordance with guidelines of Roslin Institute and the University of Edinburgh and under Home Office Project License PPL 60/4259. Cryopreserved BM cells from a male Wistar rat were thawed and cultured on bacteriological plates as per Table 2 in media containing  $10^4$  U/ml (100 ng/ml) recombinant human CSF1 (rhCSF1, a gift from Chiron, Emeryville, CA). On day 8,  $1.9 \times 10^5$  cells/well were plated in a 24-well plate and transduced the following day with *Csf1r*:EGFP-FIRE with  $4 \times 10^4$  transducing units of lentivirus.

### Lentiviral transduction of rat BM

BM cells from an 8-week-old male Dark Agouti rat were isolated from femurs by flushing the bones with cell culture media. Red blood cells were removed using lysis buffer (BioLegend, London, UK) according to instructions. Cells were cultured at a concentration of  $1 \times 10^6$  cells/ml in media (Table 2) containing 10 ng/ml rat IL-3b (PeproTech, London, UK), 100 ng/ml murine stem cell factor, and 50 ng/ml murine IL-6 (Sigma). The following day cells were concentrated back to  $1 \times 10^6$  cells/ml via centrifugation. On day 2, cells were transduced with  $1.2 \times 10^5$  transducing units of *Csf1r*:EGFP-FIRE lentivirus. Media was replaced the following day and on day 4, rhCSF1 ( $10^4$  U/ml) was added for macrophage differentiation. In experiments where BM was FACS sorted for EGFP expression, cells were harvested as described above and cultured in 15% FCS, penicillin, streptomycin, 50  $\mu$ mol/l 2-Mercaptoethanol, 2 mmol/l GlutaMAX, 50 ng/ml murine IL-6, 120 ng/ml murine FLT3-L, 50 ng/ml rat thrombopoietin, and 100 ng/ml murine SCF (Sigma). Cells were concentrated to  $1 \times 10^6$  cells/ml and transduced on day 2 as described above. On day 3, the media was replaced and on day 4 Blastidin (8  $\mu$ g/ml) was added to select for transduced cells. Cells were cultured for 3 weeks before EGFP positive and negative cells were sorted on a FACSria III (BD). SYTOX Blue (Invitrogen) was used to exclude dead cells. Following cell sorting, cells were centrifuged and RNA isolated using an RNeasy Mini kit (QIAGEN, Venlo, Netherlands) according to instructions.

### Phagocytosis assay

Phagocytosis assays were performed as described on [www.macrophages.com](http://www.macrophages.com). Zymosan A BioParticles labeled with Alexa Fluor 594 (Invitrogen) were used.

### cDNA synthesis and PCR

DNA was removed from RNA with Ambion DNA-free and cDNA prepared using SuperScript III reverse transcriptase and random hexamers (Invitrogen). Rat *Actb* and *Csf1r* were amplified from EGFP<sup>-</sup> and EGFP<sup>+</sup>

cells with Invitrogen Taq polymerase using the following oligonucleotides. *Actb* 5' caacctcttcgagctctc, *Actb* 3' ctctcagctgtggtggtgaa, *Csf1r* 5' AGGAAGCTGTGCTGCCCTGT, and *Csf1r* 3' CTCCCCCGAATCCTCACCA.

### Isolation of primary macrophages from multiple species

Pig BM-derived macrophages, cow, and human monocyte-derived macrophages were derived according to protocols described in ref. 40–42 respectively. Sheep alveolar macrophages were isolated as per ref. 43 and cultured in Roswell Park Memorial Institute medium containing 20% sheep serum containing  $10^4$  U/ml (100 ng/ml) rhCSF1.

### Lentiviral transduction of macrophages from multiple species

All cells were transduced with  $2 \times 10^5$  transducing units of lentivirus in media containing 8  $\mu$ g/ml polybrene except for human and pig monocyte-derived macrophages, which were transduced with unconcentrated lentivirus.

### CONFLICT OF INTEREST

The authors declared no conflict of interest.

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