Methodology article

Cre reporter strains produced by targeted insertion of *EYFP* and *ECFP* into the *ROSA26* locus

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Abstract

Background: Several Cre reporter strains of mice have been described, in which a *lacZ* gene is turned on in cells expressing Cre recombinase, as well as their daughter cells, following Cre-mediated excision of a *loxP*-flanked transcriptional "stop" sequence. These mice are useful for cell lineage tracing experiments as well as for monitoring the expression of Cre transgenes. The green fluorescent protein (GFP) and variants such as EYFP and ECFP offer an advantage over *lacZ* as a reporter, in that they can be easily visualized without recourse to the vital substrates required to visualize β-gal in living tissue.

Results: In view of the general utility of targeting the ubiquitously expressed ROSA26 locus, we constructed a generic ROSA26 targeting vector. We then generated two reporter lines of mice by inserting *EYFP* or *ECFP* cDNAs into the *ROSA26* locus, preceded by a *loxP*-flanked stop sequence. These strains were tested by crossing them with transgenic strains expressing Cre in a ubiquitous (*β-actin-Cre*) or a cell-specific (*Isl1-Cre* and *En1-Cre*) pattern. The resulting EYFP or ECFP expression patterns indicated that the reporter strains function as faithful monitors of Cre activity.

Conclusions: In contrast to existing *lacZ* reporter lines, where *lacZ* expression cannot easily be detected in living tissue, the EYFP and ECFP reporter strains are useful for monitoring the expression of Cre and tracing the lineage of these cells and their descendants in cultured embryos or organs. The non-overlapping emission spectra of EYFP and ECFP make them ideal for double labeling studies in living tissues.

Background

The Cre-*loxP* site specific recombination system [1] is widely used for production of tissue-specific and conditional knockout alleles in mice [2,3]. Recently, a Cre-dependent *lacZ* reporter strain (R26R) was produced by targeted insertion of a lacZ gene, preceded by a loxP-flanked (floxed) strong transcriptional termination sequence (tpA), into the ubiquitously expressed ROSA26 locus [4, 5]. The *R26R* allele terminates transcription prematurely, but when the mice are crossed with Cre-ex-

pressing transgenic mice, the Cre-mediated excision of the floxed termination sequence leads to constitutive *lacZ* expression. Thus, these doubly transgenic animals express *lacZ* only in the cells that have expressed Cre, as well as in all of their daughter cells. Similar Cre reporter strains have been produced using different promoters to express *lacZ* [6–9], Another variation on this theme has been the development of the Z/AP reporter strain, which switches from lacZ to alkaline phosphatase expression upon exposure to Cre recombinase activity [10]. All of these strains are useful for monitoring the expression of Cre recombinase, as well as for cell lineage tagging experiments [9, 11–14].

Here, we describe the production of two similar Cre reporter alleles that express enhanced yellow or cyan fluorescent protein (EYFP or ECFP), two color variants of the green fluorescent protein (GFP). GFP and its variants are autofluorescent proteins that can be visualized in living cells, and are therefore particularly useful for monitoring gene expression in whole embryos, animals or cultured cells and organs [15]. EYFP and ECFP were chosen because their emission spectra overlap minimally, so they can be distinguished when used simultaneously, whereas the emission spectra of EYFP and ECFP overlap to a greater extent with that of EGFP [16, 17] http://www.clontech.com/gfp/pdf/LivingColors.pdf.

During the course of this work, three new Cre reporter strains that conditionally express EGFP were reported. Two transgenic strains [18, 19] use the β-actin promoter/CMV enhancer to express EGFP following Cre-mediated excision of a stop sequence, and in one of these, lacZ is expressed prior to the Cre-mediated excision event. In the third strain, which is similar in principle to the YFP and CFP alleles reported here, EGFP was inserted at the *ROSA26* locus [20]. The availability of different Cre reporter strains will be valuable, not only because of the advantages of different reporter proteins, but also because the efficiency of Cre-mediated excision may be dependent on the target locus.

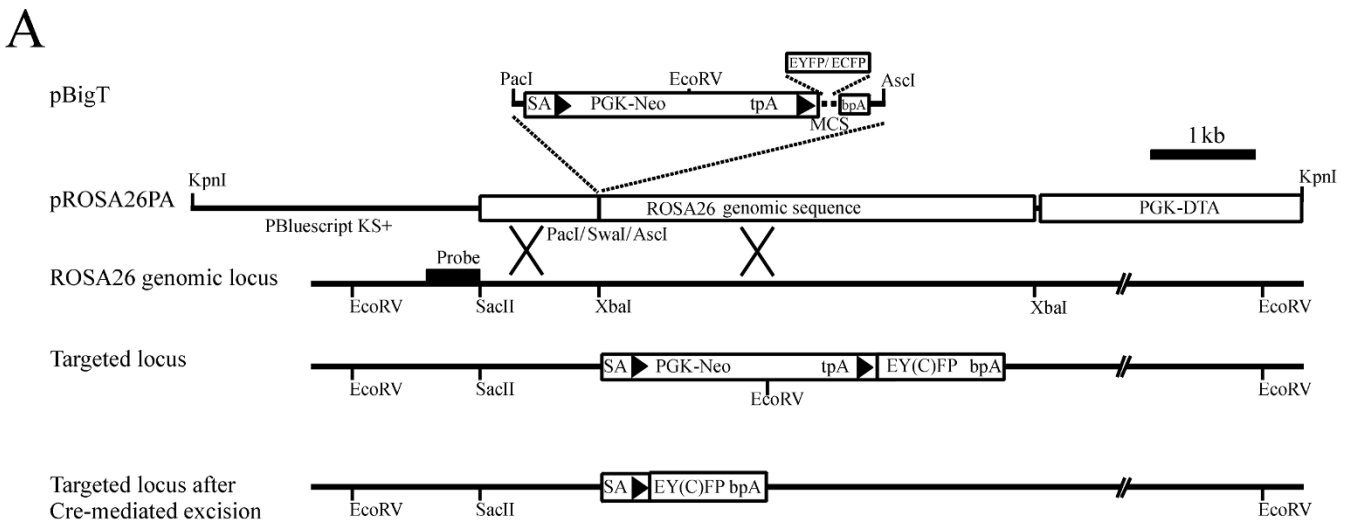
Results and Discussion

Considering the utility of the *ROSA26* locus for expressing various sequences in a ubiquitous manner [4, 5, 21], we generated a generic targeting vector (pBigT), into which any sequence of interest can easily be inserted downstream of a floxed neo-tpA cassette, and then subsequently cloned into a plasmid with the *ROSA26* genomic flanking arms. *ECFP* or *EYFP* was inserted into pBigT, and the resulting sequence was inserted into a vector containing *ROSA26* genomic sequences to permit homologous recombination (Fig 1A). The targeting vector was electroporated into ES cells, and 27 G418-resistant colonies were analyzed for each construct. Three R26R-EYFP and two R26R-ECFP colonies carried the targeted allele, as determined by Southern blot hybridization (Figure 1B). The targeted ES cells, originally derived from strain 129X1/SvJ [22], were injected into C57BL/6J blastocysts, and the resulting chimeric mice were bred to C57BL/6J females to pass the mutation through the germ line. Thus, the resulting mice were a mixture of strains 129X1/SvJ and C57BL/6J.

No background expression of EYFP or ECFP could be detected in the R26R-EYFP or R26R-ECFP mice, as expected (data not shown), due to the strong transcriptional stop sequence inserted between the promoter and the coding sequences. However, when the reporter mice were crossed to β-actin-Cre transgenic mice [23], which express Cre constitutively in most or all cells including those in the early embryo, the resulting doubly transgenic embryos displayed apparently ubiquitous expression of EYFP or ECFP (Figure 2), indicating efficient excision of the floxed stop sequence. The expression of EYFP vs. ECFP could be clearly distinguished using appropriate filter sets (Fig 2). This ubiquitous expression of EYFP or ECFP was heritably transmitted to the following generations (data not shown), resulting in ubiquitously expressing lines of mice that will be useful for lineage tracing in chimeric mice or embryos, analogous to the original ROSA26 lacZ promoter trap strain [21].

The specificity of the R26R-EYFP and ECFP lines was tested by crossing them with strains of mice that express Cre in a tissue-specific fashion. Isl1 is a LIM homeodomain transcription factor expressed in motor neurons and dorsal root and cranial sensory ganglia [24]. Isl1-Cre mice were produced by targeting *Cre* into the *Isl1* locus (see Materials and Methods). Heterozygous Isl1-Cre mice were viable and fertile, while homozygous mice died at E9-E10.5, as has been previously described with mice in which the exon encoding the second LIM domain of Isl1 had been deleted [25]. Figure 3A shows an experiment in which the R26R-YFP mice were crossed with the Isl1-Cre mice, resulting in expression of EYFP in motor neurons and dorsal root ganglia in the doubly transgenic offspring. Thus R26R-YFP mice faithfully report the cell-specific patterns of Isl1/Cre in subsets of neurons. As a control, the Isl-1/Cre mice were also mated to the original *R26R lacZ* allele [4], resulting in a similar pattern of expression of *lacZ*.

Engrailed-1 (En-1) is a homeodomain protein expressed specifically at the mid-hindbrain junction of the early somite stage embryo, as well as in a number of sites later in embryogenesis. Figure 4 shows an experiment in which R26R-YFP mice were crossed with a strain of mice in which Cre was knocked into the *En-1* locus [26], resulting in the expression of YFP specifically at the mid-



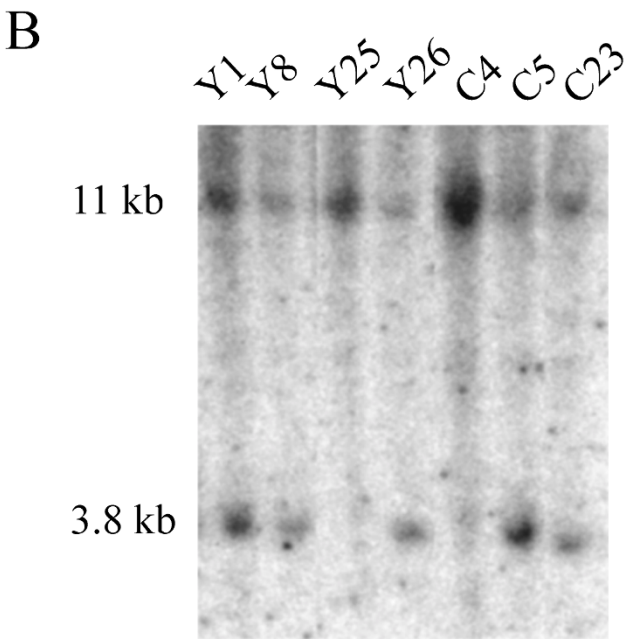


Figure 1
Targeting of the *ROSA26* locus. A, top to bottom: pBigT, a plasmid containing a loxP-flanked cassette with a *PGK-neo* selectable marker and a tpA transcriptional stop sequence, into which the *EYFP* or *ECFP* was cloned; pROSA26PA, containing genomic *ROSA26* sequences for homologous recombination, and a diphtheria toxin gene (*PGK-DTA*) for negative selection in ES cells; the wild type *ROSA26* locus, with the location of the probe indicated; the structure of the targeted locus; and the structure of the targeted locus after Cre-mediated excision of the *loxP*-flanked (*PGK-neo*, tpA) cassette. *LoxP* sites are indicated by solid arrowheads. B, Southern blot of DNA from seven ES cell lines, digested with EcoRV and hybridized with the probe indicated in A. The 11 kb band is the wild type band and the 3.8 kb band represents the targeted allele. Lines Y25 and C4 are wild type, while the remainder are heterozygous for the targeted allele.

hindbrain junction in an E8.5 embryo. For comparison, we also crossed the En-1/Cre mice with the original *R26R lacZ* allele [4], resulting in lacZ expression in the same region at E8.5 (Fig 4C).

The expression of ECFP proved more difficult to detect in fixed and sectioned tissue (data not shown), although it was clearly detectable in unfixed embryonic tissue (Fig 2C). This is not surprising, given the higher quantum yield and extinction coefficient of EYFP as compared to

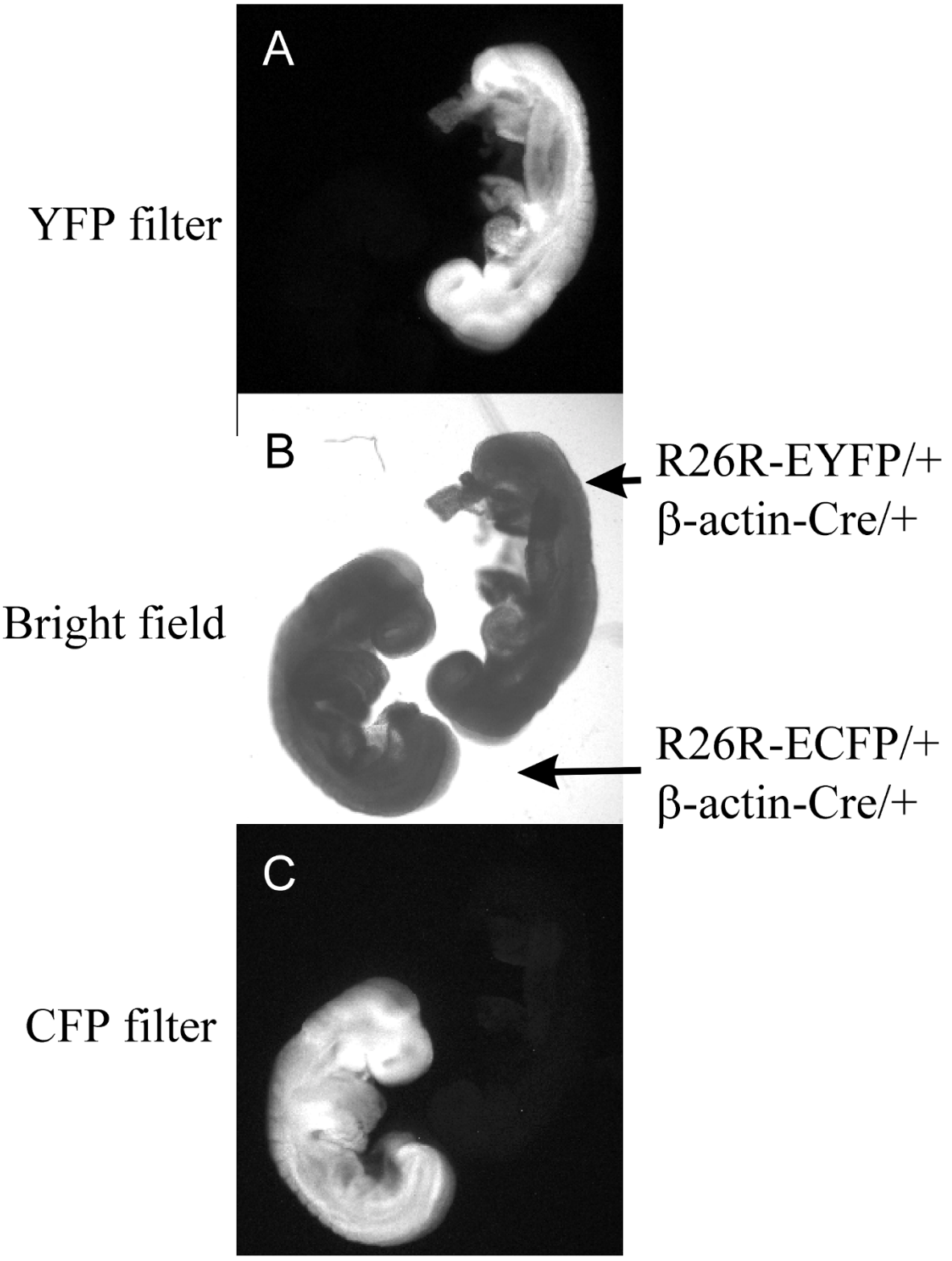


Figure 2
Ubiquitous expression of EYFP or ECFP in R26R E8.5 embryos carrying a *β-actin-Cre* transgene. The two embryos, one carrying *R26R-EYFP* (right) and one carrying *R26R-ECFP* (left), were both heterozygous for the *β-actin-Cre* transgene. They are visualized with a YFP filter set (A), with bright field illumination (B), or with a CFP filter set (C).

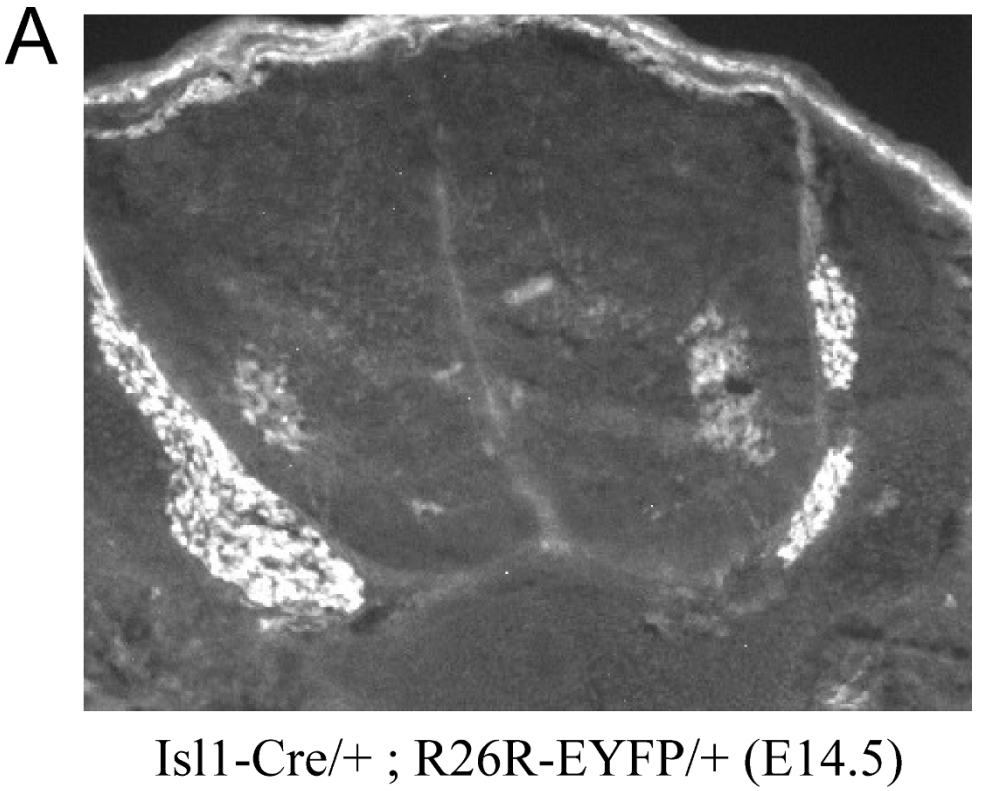


Figure 3
Specific expression of EYFP in R26R-EYFP mice carrying *Isl1-Cre*. A, transverse section of an E14.5 R26R-EYFP/+; *Isl1-Cre*/+ embryo, revealing expression of EYFP in the motor neurons and dorsal root ganglia. The apparent expression in surface ectoderm is an artifact, as it was also seen in non-transgenic embryos (data not shown). Panel B, transverse section of E12.5 *R26R-lacZ; Isl1-Cre* embryo, showing a comparable pattern of β-gal staining.

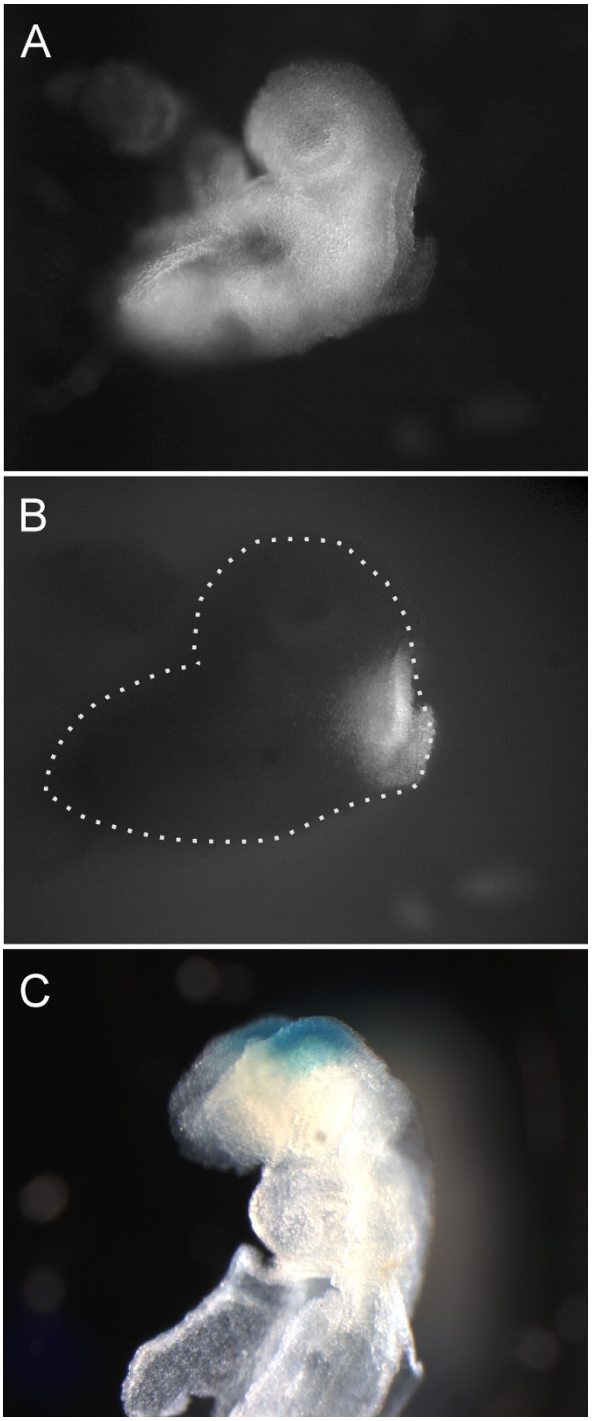


Figure 4
Specific expression of EYFP at the mid-hindbrain junction in a R26R-EYFP E8.5 embryo carrying *En1*^{Cki}, an *Engrailed-1 Cre* knock-in allele. A, dark field illumination, showing anterior portion of embryo. B, YFP expression in the same embryo. The outline of the embryo is indicated by the dotted line. C, E8.5 embryo from a cross between *En1*^{Cki} and the *R26R lacZ* allele [4], resulting in *lacZ* expression in the same mid-hindbrain region.

ECFP, both of which result in higher fluorescence intensity.

Conclusions

We have constructed two reporter lines of mice that express EYFP or ECFP only in cells expressing the Cre recombinase, and their daughter cells, by targeting these cDNAs into the ubiquitously expressed ROSA26 locus, preceded by a *loxP* flanked "stop" sequence. Crosses with a general Cre expressing strain (βactin-Cre) and two tissue-specific Cre strains (Isl1-Cre and En1-Cre) showed that the reporter strains function as expected, based on their similarity to the well characterized R26R-lacZ strains. In contrast to *lacZ* expression, which cannot be easily detected in living tissue, the EYFP and ECFP reporter strains (together with the GFP reporter strains currently available) will be very useful for monitoring the expression of Cre in living tissues, or tracing the lineage of these cells and their descendants, in cultured embryos or organs. Furthermore, by using modified forms of Cre whose recombinase activity is inducible, one can use these reporter mice to perform detailed analysis on the lineage of cells at different time points during development.

Materials and Methods

Sources of Plasmids

The components of the *ROSA26* targeting vectors were a gift from Philippe Soriano. The adenovirus splice acceptor (SA) and bovine growth hormone polyadenylation sequence (bpA) were from plasmid pSAβgeo [21]. The *loxP* flanked neo cassette, and trimer of the SV40 polyadenylation sequence (tpA), were from plasmid PGKneotpAlox2 [4]. The *ROSA26* genomic sequence and the diphtheria toxin (DTA) expression cassette were from plasmid pROSA26-1 [4], and the template for the external probe used to genotype ES cells was from plasmid pROSA26-5' [4].

The plasmids pEYFP-N1 and pECFP containing cDNA for EYFP and ECFP were purchased from Clontech Laboratories Inc.

Targeting Constructs

The plasmid pBigT consists of the adenovirus splice acceptor sequence followed by a *loxP* site, neo expression cassette, strong transcriptional stop sequence (triple SV40 polyadenylation sequence), another *loxP* site in the same orientation as the first, a multiple cloning site (MCS), and the bovine growth hormone polyadenylation sequence. A PacI site was included 5' to the SA, and an AscI site 3' to the bpA. These two enzymes are rare eight base pair cutters and result in sticky ends upon digestion and can be used to excise the entire construct, for insertion into the plasmid with the *ROSA26* genomic arms.

The XbaI site used for insertion into the *ROSA26* genomic locus [4] was replaced by a linker (PacI, SwaI, AscI), so that it could be digested with PacI and AscI, and receive the bigT sequence.

To make pBigT, plasmid pSAβgeo was digested with ClaI and XbaI to remove the *βgeo* and bpA sequence but leave behind the SA, the ends were Klenow filled and the plasmid self-ligated. The resulting plasmid was digested with SacI and PstI to remove the 5' MCS, which was replaced by a PacI linker. The resulting plasmid was called pPacSA.

Next, pSAβgeo was digested with SacI to remove the SA and *βgeo* sequences, but leave behind the bpA, and self-ligated. The resulting plasmid was digested with XbaI and ApaI to remove most of the 3' MCS (except for the terminal KpnI site), which was replaced with an AscI linker. The resulting plasmid was called pbpAAsc.

The *loxP-neo*-tpA-*loxP* was excised from plasmid PGKneotpAlox2 by cutting with NotI, Klenow filling, and then cutting with KpnI. This was inserted downstream of the SA in pPacSA digested with SalI, Klenow filled, and then cut with KpnI. A MCS containing the restriction sites for XhoI, ApaI, SacII, NotI, SacI, EcoRV, and KpnI was synthesized and inserted downstream of the second *loxP* site by digesting the DNA with XhoI and KpnI and ligating in the MCS, which had been synthesized to have compatible cohesive ends. This plasmid was called pSAleotpA. The bpA sequence along with the 3' AscI site was excised from pbpAAsc by digesting it with SacI and KpnI, and inserted downstream of the MCS in pSAleotpA by digesting it with SacI and KpnI. The resulting plasmid was called pBigT, and its MCS contains sites for the restriction enzymes NheI, SalI, AccI, XhoI, ApaI, SacII, NotI, SacI and BclI.

To make the *ROSA26* genomic sequence compatible with the pBigT plasmid, pROSA26-1 was digested with XbaI, Klenow filled and a linker (PacI, SwaI, AscI) inserted. This plasmid was called pROSA26PA.

To make the R26R-YFP targeting construct, the *EYFP* cDNA was excised from pEYFP-N1 with ApaI and NotI and inserted into BigT digested with ApaI and NotI. BigT was then digested with PacI and AscI to release the entire floxed *neo*-tpA and *EYFP* assembly, and inserted into pROSA26PA digested with PacI and AscI. This plasmid was subsequently linearized with KpnI and used for electroporation.

To make the R26R-CFP targeting construct pECFP was digested with AgeI, Klenow filled then digested with NotI to excise the *ECFP* cDNA. This was inserted into BigT digested with XhoI, Klenow filled, and then digested with NotI. BigT was then digested with PacI and AscI to release the entire floxed neo-tpA and CFP assembly, and inserted into pROSA26PA digested with PacI and AscI. This plasmid was subsequently linearized with KpnI and used for electroporation.

An appreciable amount of recombination was observed while trying to grow up the final targeting vector, resulting in an aberrant plasmid of smaller molecular weight. Therefore, the mixture of theses two plasmids was digested with KpnI which linearized both plasmids, and the correct targeting vector was gel purified. It was subsequently confirmed to be the correct targeting vector by diagnostic PCR, restriction digests and sequencing (data not shown).

Targeting of the ROSA26 locus in ES cells

JM-1 ES cells [22] were expanded by culturing on feeder cells in medium supplemented with LIF. Approximately 15×10^6 cells were electroporated with 10 μg of each targeting vector and grown without feeders under selection in 300 μg/ml G418 for seven days. 96 colonies were picked and 27 screened, for electroporations with each of the two constructs R26R-YFP and R26R-CFP. Genomic Southern blot hybridization was performed on DNA from ES cells digested with EcoRV. The 5' probe used detects a 11 kb wild type band and a 3.8 kb targeted band, due to the presence of an extra EcoRV site in the targeted allele.

Mice carrying the targeted allele were genotyped by the PCR as described [4].

Construction of the Isl1-Cre mouse strain.

Isl1 genomic DNA had been previously isolated from a mouse 129/Sv genomic library (Stratagene) as described by Pfaff et al. [25]. A PacI site had been introduced into an EcoRI site in the exon encoding the second LIM domain of Isl1. A cassette coating IRES Cre SV40 pA and pgk-neomycin was cloned into this PacI site to create a targeting construct with flanking 5' and 3' genomic DNA arms of 5 kb and 2 kb, respectively. ES cells were targeted and screened as described in Pfaff et al.[25].

Mouse strains

En-1/Cre mice (En1*Cki* allele) were a gift of Dr. Alexandra Joyner and Dr. Wolfgang Wurst [26]. β-actin/Cre mice were a gift of Dr. Gail Martin [23]. R26R-lacZ reporter mice were a gift of Dr. Philippe Soriano [4].

Detecting EYFP and ECFP expression.

For whole mount photographs (Figures 2 and 4), unfixed embryos were photographed using a Nikon epifluorescence microscope fitted with Chroma filter sets for ECFP

(cyan GFP Ex436/20 Dm455 Bar480/40) and EYFP (yellow GFP Ex500/20 Dm515 Bar535/30). Digital images were acquired using a Spot camera.

For histological sections (Fig 3), embryos were fixed overnight in 4% paraformaldehyde at 4°C, washed 2x for 10 min. in PBS, then equilibrated in the following solutions until the embryos settled at the bottom (approx. 30 min): PBS, 5% sucrose in PBS, 10% sucrose in PBS, and 15% sucrose in PBS. They were then equilibrated in a 1:1 mixture of OCT (Tissue-Tek, Mile, Inc.) and 15% sucrose in PBS for >1 hour, and embedded in OCT over dry ice. Sections were cut at 8 - 12 µM, blow-dried for 30 min. at low heat, then stored at -80°C with desiccant in an air tight bag. Before being photographed, the slides were brought to room temperature, washed 3x in PBS, mounted in Vectashield (Vector Laboratories), covered with a cover glass and sealed with clear nail polish. Sections were photographed as described above.

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