A Multifunctional Reporter Mouse Line for Cre- and FLP-Dependent Lineage Analysis

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Summary: The Cre/lox and FLP/FRT recombination systems have been used extensively for both conditional knockout and cell lineage analysis in mice. Here we report a new multifunctional Cre/FLP dual reporter allele (R26N2G) that exhibits strong and apparently ubiquitous marker expression in embryos and adults. The reporter construct, which is driven by the CAG promoter, was knocked into the ROSA26 locus providing an open chromatin domain for consistent expression and avoiding site-of-integration effects often observed with transgenic reporters. R26N2G directs Cre-dependent nuclear-localized β-galactosidase (β-gal) expression, and can be converted into a Cre-dependent EGFP reporter (R26N2G) by germline excision of the FRT-flanked nlslacZ cassette. Alternatively, germline excision of the floxed PGKNEO primary stop cassette results in transcription of the marker gene. After the excision event, which is irreversible, expression of the marker gene is independent of the continued presence of the SSR. Thus, any cell type of interest can be followed throughout its lifetime, providing that the promoter driving expression of the marker gene is constitutively active.

We have developed a multifunctional Cre/lox-FLP/FRT reporter mouse line (R26N2G) that uniquely combines a number of desirable features for lineage analyses. The structure of R26N2G is shown in Figure 1. The main body of the targeting vector consists of a ubiquitous promoter, a floxed PGKNEO primary stop cassette, an FRT-flanked nlslacZ marker gene that also serves as a secondary stop cassette, and EGFP. The reporter construct was knocked into the ROSA26 locus, which is transcriptionally active at all developmental stages and in almost all adult tissues (Friedrich and Soriano, 1991; Zambrowicz et al., 1997), thereby avoiding potential silencing effects of local chromatin structure that are often observed with randomly integrated transgenic constructs (Bonnerot et al., 1990; Novak et al., 2000). Unlike most ROSA26-based reporters, in which marker gene expression is driven by the endogenous ROSA26 promoter (Awatramani et al., 2001; Soriano, 1999; Stoller et al., 2008), we utilized the CAG promoter, which is a composite regulatory sequence consisting of the CMV-IE enhancer and the modified chicken β-actin promoter (Niwa et al., 1991). The CAG promoter provides strong marker gene expression in all cell types examined both in embryos and adult mice. As shown in Figure 1, Cre-
mediated excision of the PGKNEO cassette results in nlslacZ expression. β-gal includes a nuclear localization signal for greater sensitivity and resolution in some experimental settings. R26NZG can be easily modified by FLP-dependent germline excision of the nlslacZ cassette to produce a Cre-dependent EGFP reporter line (R26NG). The EGFP signal from the recombined R26NG is robust and can be used readily for live cell imaging and sorting, as well as for histological analyses. Germline excision of the PGKNEO cassette converts R26NZG to a FLP-dependent EGFP reporter (R26ZG), which expresses nlslacZ in the absence of FLP. By the simultaneous use of appropriate Cre and FLP deleters, R26ZG also functions as a dual reporter that expresses nlslacZ after Cre-mediated recombination and EGFP only after both Cre- and FLP-mediated recombination. The transcriptionally active genes are colored blue (nlslacZ), green (EGFP), or red (PGKNEO) in (b).

FIG. 1. (a) Schematic representation of the R26NZG targeting strategy. A reporter cassette composed of the CAG promoter, a floxed (orange triangles) PGKNEO-4pA cassette, an nlslacZ-3pA cassette flanked by FRT sites (purple double triangles), and EGFP-pA was knocked into the ROSA26 locus. 5′ and 3′ homology arms are shown as bold lines. B, BamHI; X, XbaI. (b) Schematic diagram of Cre- and FLP-dependent recombination and derivative reporter alleles. R26NG is a Cre-dependent nlslacZ reporter that can be converted to a Cre-dependent EGFP reporter, R26NG, by FLP-dependent excision of the nlslacZ cassette. Germ line excision of the PGKNEO cassette by Cre converts R26NG into a FLP-dependent EGFP reporter, R26ZG, which expresses nlslacZ in the absence of FLP. By the simultaneous use of appropriate Cre and FLP deleters, R26ZG also functions as a dual reporter that expresses nlslacZ after Cre-mediated recombination and EGFP only after both Cre- and FLP-mediated recombination. The transcriptionally active genes are colored blue (nlslacZ), green (EGFP), or red (PGKNEO) in (b).

Double heterozygous F1 embryos generated by crossing Hprt1Cre+ male and R26NZG/+ female mice exhibited intense and ubiquitous X-gal staining at the whole-mount level (Fig. 2a). There was no leaky EGFP expression observed in the double heterozygotes (data not shown), and R26NZG/+ embryos lacking the Cre allele did not show any X-gal staining (Fig. 2a, data not shown). The ubiquity of reporter expression was further assessed in the R26NG line. To generate R26NG, R26ZG/+,

and R26FLPe/FLPe (a ROSA26-based FLP allele; Farley et al., 2000) mice were crossed. The excision of the nlslacZ cassette was incomplete (data not shown), probably reflecting the lower recombination activity of FLPe relative to Cre (Raymond and Soriano, 2007; Schaft et al., 2001). The resulting mosaic F1 offspring were crossed with R26FLPe/+ mice to establish the R26NG line. Approximately 50% of F1 embryos generated by crossing R26NG/+ male and Hprt1Cre+ female mice showed widespread and robust EGFP signal (Fig. 2b). As maternal Cre activity is provided in the egg in this mating scheme (Tang et al., 2002), excision of the floxed PGKNEO cassette is independent of inheritance of the Hprt1Cre allele. Cryosections of E13.5 embryos showed EGFP expression in all tissues except for primitive erythrocytes in the heart chambers and blood vessels (Fig. 2c,d). At adult stages, intense EGFP fluorescence was observed in all tissues/organs examined, including the adrenal gland, bone, brain (representative regions of forebrain, midbrain, and hindbrain examined), cartilage, heart, intestine, kidney, liver, lung, ovary, pancreas (exocrine and endocrine), skeletal muscle, skin, spleen, stomach, testis, thymus, thyroid, and all bone marrow-derived cells except red blood cells (Fig. 2e–w, data not shown).
EGFP signal was easily visualized in paraformaldehyde-fixed tissues without the need for enhancement with immunohistochemistry. Representative sections through each organ did not reveal any EGFP-negative cell populations, although more extensive analyses are required to prove truly ubiquitous expression.

FACS analysis of total bone marrow was performed to quantify EGFP signal intensity of the $R26^{NG}$ allele relative to the commonly used reporters, $R26R$-EYFP (Srinivas et al., 2001) and Z/EG (Novak et al., 2000). Following lysis of red blood cells, apparently all bone marrow-derived cells from $Hprt^{Cre/\pm}$-$R26^{NG/\pm}$ mice were EGFP+ (Fig. 2w). The recombined $R26^{NG}$ allele generated approximately nine-fold and two-fold greater signal intensity than the recombined $R26R$-EYFP and Z/EG alleles, respectively (Fig. 2w). In addition, $R26^{NG}$-derived cells showed a narrower distribution of signal intensities compared with Z/EG, indicating greater uniformity of
EGFP expression levels among different cell types despite the use of similar regulatory elements in these reporter lines. Both $Hprt^{Cre/+}; R26^{NGI/+}$ and $Hprt^{Cre/+}; R26^{NGI/+}$ mice are viable and fertile, suggesting no significant deleterious effect of high level, ubiquitous, reporter gene expression.

In some experimental settings, nuclear-localized β-gal is preferable to cytoplasmic β-gal, as it can provide greater detection sensitivity and resolution, and can readily be distinguished from background staining due to endogenous enzymatic activities or to “bluing” of the tissue during X-gal staining. We confirmed the nuclear-localized β-gal signal in isolated skeletal muscle fibers, which are syncytia containing many nuclei. Adult extensor digitorum longus (EDL) muscle fibers from the hind leg of adult $Hprt^{Cre/+}; R26^{NGI/+}$ mice showed intense nuclear X-gal staining (Fig. 3a,b). The recombined $R26^{NGI}$ allele also is expressed in muscle stem cells (satellite cells), as shown by X-gal staining combined with immunohistochemistry for CD34, a marker specific to satellite cells in single muscle fiber preparations (Fig. 3c,d; Beauchamp et al., 2000). Because of the close juxtaposition of the satellite cell and the muscle fiber, nuclear localized β-gal greatly facilitates the discrimination of satellite cell and fiber staining. Indeed, we were unable to score satellite cells for X-gal staining when the R26R line, which generates cytoplasmic β-gal, was used (Fig. 3e,f). We also assessed expression of EGFP in myofibers and satellite cells of $Hprt^{Cre/+}; R26^{NGI/+}$ adult mice using confocal microscopy. As expected, muscle fibers and Pax7+ satellite cells were intensely EGFP+ (Fig. 3g,h,i). As Pax7 labels the vast majority of satellite cells (Scale et al., 2000), we conclude that the $R26^{NGi}$ and $R26^{NG}$ can be used to label essentially all satellite cells.

To test the efficiency of tissue-specific excision of the floxed PGKneo cassette, we crossed $R26^{NGI/+}$ or $R26^{NGI/+}$ reporter mice with $MyoD^{Cre/+}$ mice, in which the iCre gene (Shimshek et al., 2002) was knocked into the MyoD locus (unpublished data). $MyoD$ is a skeletal muscle-specific transcription factor expressed in all developing skeletal musculature, including somite myotomes, limb buds, and head and neck musculature (Chen et al., 2005; Sassoon et al., 1989). Intense and specific X-gal signal and green fluorescence were detected in all myogenic regions of $MyoD^{Cre/+}; R26^{NGI/+}$ and $MyoD^{Cre/+}; R26^{NGI/+}$ embryos, respectively (Fig. 4a–e).

As proof-of-concept for the use of $R26^{NGi}$ as a dual Cre/lox-FLP/FRT reporter, we crossed $MyoD^{Cre/+}; R26^{NGI/+}$ and $R26^{FLP/E}$ mice and analyzed β-gal and EGFP expression in F1 embryos. $MyoD^{Cre/+}; R26^{NGI/+}$ embryos expressed β-gal exclusively in myogenic domains as demonstrated earlier (Fig. 4g) and did not express EGFP (Fig. 4f). However, embryos that inherited both the $MyoD^{Cre/+}$ and $R26^{FLP/E}$ delets ($MyoD^{Cre/+}; R26^{FLP/E/NGi}$) exhibited green fluorescence in myogenic domains (Fig. 4h), the intensity of which varied between embryos (data not shown). Variation in fluorescence reflected embryo-to-embryo differences in the efficiency of FLP-mediated excision of the nslacZ cassette, as EGFP+ embryos also stained with X-gal in myogenic domains with intensities inversely proportional to the EGFP signals (Fig. 4i). As predicted from the design of $R26^{NGi}$, we observed fewer blue dots of X-gal signal in $MyoD^{Cre/+}; R26^{FLP/E/NGi}$ embryos than in $MyoD^{Cre/+}; R26^{NGi/+}$ littermates (Fig. 4j,k). We also confirmed mutually exclusive expression of β-gal and EGFP in individual cells of the developing limb musculature before myogenic cell fusion at E11.5 (Fig. 4l–o).
The ability to interrogate cell lineage relationships based on the historical or ongoing expression of two independent genes provides a powerful tool that will refine SSR-based lineage analyses. Awatramani et al. described another ROSA26 dual reporter line, which expresses alkaline phosphatase from the endogenous ROSA26 promoter after FLP-dependent stop cassette excision and EGFP only after both FLP- and Cre-dependent recombination (Awatramani et al., 2003). A current limitation of the dual reporter technology is the limited number of available FLP deleter lines and the lower efficiency of FLP-mediated recombination compared with Cre-mediated recombination (Raymond and Soriano, 2007; Schaft et al., 2001). Recently, however, a codon-optimized FLP gene (FLPo) was developed that achieves similar recombination efficiency as Cre (Raymond and Soriano, 2007). We anticipate that the improvements in FLP-mediated recombination efficiencies and the availability of dual Cre/lox-FLP/FRT reporters such as R26NZG, which exhibits intense and apparently ubiqui-

FIG. 4. Muscle-specific recombination of the R26NZG and R26NG reporters. (a) X-gal-stained MyoD<sup>Crei</sup>+/R26NZG<sup>/+</sup> embryo at E11.5 showing labeling in all myogenic regions of the head, trunk and limb buds. Staining accurately reflects the pattern of MyoD expression by in situ hybridization (data not shown). (b) The intense EGFP signal in MyoD<sup>Crei</sup>+/R26NZG<sup>/+</sup> embryos matches the X-gal signal in (a). (c) X-gal-stained forelimb from an E18.5 MyoD<sup>Crei</sup>/R26NZG<sup>/+</sup> fetus. (d,e) Paraffin sections of the X-gal-stained forelimb shown in (c). R, radius; U, ulna; H, humerus. (f-k) EGFP fluorescence and X-gal staining of E13.5 F1 embryos generated by crossing MyoD<sup>Crei</sup>/R26FLPe<sup>/+</sup> male × R26<sup>NZG</sup><sup>/+</sup> female mice. (f,g) A MyoD<sup>Crei</sup>+/R26NZG<sup>/+</sup> embryo showing intense X-gal signal in myogenic domains after 75 min of X-gal staining (g), but no EGFP signal (f). (h,i) A MyoD<sup>Crei</sup>+/R26<sup>FLPe/NZG</sup> embryo showing a robust EGFP signal (h) and relatively weak X-gal staining (i) compared with its MyoD<sup>Crei</sup>/R26NZG<sup>/+</sup> litter mate (g). Persistent X-gal staining is due to incomplete excision of the nlslacZ cassette by FLP. (j,k) Magnified views of the head muscle region of embryos stained overnight in X-gal (dotted boxes in g and i). The EGFP+, MyoD<sup>Crei</sup>+/R26<sup>FLPe/NZG</sup> embryo had a fewer number of blue "dots" than its MyoD<sup>Crei</sup>/R26NZG<sup>/+</sup> litter mate. (l-o) A horizontal section of the forelimb shown in (f). (l) Fluorescent whole mount image of a MyoD<sup>Crei</sup>/R26<sup>FLPe/NZG</sup> forelimb at E11.5. Fluorescence and bright-field images were superimposed. (m–o) A horizontal section of the forelimb shown in (l). (m) Merged EGFP and DAPI (red) images. (n) X-gal staining with Fast Red counter staining. (o) Merged image of EGFP fluorescence and X-gal staining, which was colorized red for better visualization of the mutually exclusive EGFP and X-gal signals. Arrowheads in (m–o) show representative X-gal positive cells.
tous marker gene expression, will encourage the development of new FLP deleter lines to investigate cell lineage relationships in development and disease.

METHODS

Targeting Vector

The R26\(^{NZG}\) knockin vector (see Fig. 1), CLNFZG, contains a CAG promoter derived from pCAGGS (Niwa et al., 1991), a floxed PGKNEO primary stop cassette containing three polyadenylation sequences (pA) from the bovine growth hormone (bGH) gene (Liu et al., 2003) and one pA from PGK (derived from PGKneotPa-lox2; gift from Dr. Philippe Soriano), an FRT-flanked nlslacZ cassette containing an SV40 nuclear-localization signal from pCS2-NLS (Rupp et al., 1994) and three SV40 pA sequences excised from pDnLacZ3pA (Yamamoto et al., 2007), and EGFP-bGHpA derived from pCMS-EGFP (Clontech, Mountain View, CA). To generate the final targeting vector, pR26-CLNFZG, CLNFZG sequences were introduced into the XbaI site of ROSA26 genomic DNA that was retrieved by standard recombineering techniques from a C57BL/6 BAC clone. The resulting vector contains 5' and 3' homology arms of 2.3 kb and 5.7 kb, respectively, which targets the construct to the XbaI site of intron 1 at the ROSA26 locus. The detailed cloning strategy and complete sequence of plasmids are available on request.

Generation of R26\(^{NZG}\), R26\(^{NG}\), and R26\(^{ZG}\) Reporter Knockin Mice

ES cell electroporation and production of chimera was performed by the University of Connecticut Gene Targeting and Transgenic Facility (GTTF). The pR26-CLNFZG targeting vector was linearized with SfiI and electroporated into 129S6/C57BL/6 hybrid ES cells (D1: established by GTTF). Southern blot hybridization on BamHI-digested genomic DNA was used to screen for homologous recombination on the 5' end using a 0.67 kb probe outside of the 5' homology arm. The probe was liberated by EcoRV digestion of the PCR product generated with the following primers: 5'-TTCCCTCTCAATATGCTGACACAAA-3' and 5'-GCCAGAGAAAGGCTCTCCTCA-3'. The targeted and wild-type alleles produced products of 11.5 kb and 5.8 kb, respectively. Nested PCR was used to assay for correct targeting on the 5' end. The targeted allele generated a 5.8 kb diagnostic fragment with the following primers: 1st PCR, 5'-GGGAA GACAATAGCAGGCAATGCGTGA-3' and 5'-GATGGCCAATTTCACTGTGAAGAC-3'; and 2nd PCR, 5'-TTCCCTCTCAATATGCTGACACAAA-3' and 5'-TTCCCTCTCAATATGCTGACACAAA-3'.

Chimeric mice were produced from two targeted ES cell clones by aggregation with CD1 embryos. Germ line transmission of the targeted allele was assessed by LacZ PCR with primers (5'-GGGATCCCGAGAATTCGAAGTTCC-3' and 5'-GGGTCTCAACAGCTGACTG-3') that generate a 333-bp product. R26\(^{ZG}\) was generated by a cross between R26\(^{NZG}/+\) and HpR1\(^{Cre}/+\) mice. Removal of the PGKNEO cassette was verified by the presence of a 193-bp PCR product using primers that flank PGKNEO (5'-ACTGGGACACAGAATTCGAAGTTCC-3' and 5'-GTCTCACTGGAACGCTGAC-3'). R26\(^{NG}\) was generated by a cross between R26\(^{NZG}/+\) and R26\(^{FLPe}/FLPe\) mice. As R26\(^{FLPe}\)-driven excision of the nlslacZ cassette was incomplete, the resulting mosaic F1 offspring were crossed with R26\(^{FLPe}/+\) mice to establish the R26\(^{NG}\) line. Removal of the nlslacZ cassette was assessed by PCR with a forward PGK pA cassette primer (5'-GATGGCAAGTTCCGTTTGACTG-3') and a reverse EGFP primer (5'-GGCGCTAATGGCTGAACTTTGCTGACCC-3') that amplifies a 264-bp product. Lines were maintained by breeding to FVB mice.

Histology and X-Gal Histochemistry

For whole-mount detection of EGFP, embryos and adult organs were fixed in 2% paraformaldehyde (PFA) in PBS (pH 7.4) for at least 2 hr at 4°C, and rinsed in three changes of PBS over 30 min. The whole-mount samples were photographed using epifluorescence illumination. X-gal staining of whole mount embryos was performed as described previously (Yamamoto et al., 2007). For sequential detection of EGFP and β-gal, EGFP signal was photographed before X-gal staining. Detection of EGFP and β-gal in embryonic and adult organ cryosections were performed as described previously (Yamamoto et al., 2007), with minor modifications. Briefly, samples were fixed in 2% PFA/PBS for at least 3 hr, rinsed with PBS and cryoprotected in 30% sucrose/PBS for at least 1 hr at 4°C. After embedding in O.C.T. (Sakura Finetek, Torrance, CA), 10 μm or 12 μm cryosections were collected using a tape-transfer system (CryoJlane; Instrumented, St. Louis, MO). Standard methods were used for paraffin histology of X-gal stained samples. Images were captured using a Hamamatsu C5810, Retiga EXi, Retiga 2000R or Spot RT3 video camera, and a Leica MZFLIII stereomicroscope (whole mounts) or Nikon E600 microscope (sections) with either bright-field or epifluorescence illumination.

FACS Analysis

Total bone marrow was isolated from recombined, heterozygous R26\(^{NG}\), R26R-EYFP, and Z/EG adult mice with minor modifications (Watt et al., 1980). Briefly, bone marrow plugs were isolated from dissected femurs using 0.2 μm-filtered 10% PBS in PBS. Erythrocyte lysis was performed by pelleting the cells and replacing the solution with 900 μl of distilled water for 10 seconds. The solution was made isotonic by addition of 100 μl of 10X PBS. Cells were pelleted and suspended in 10% FBS/PBS. Cytometric analysis was performed on 20,000 events for each bone marrow preparation with a BD FACSAria II flow cytometer utilizing FACSQ流6.1 software. The FITC filter set was applied using a 488-nm coherent sapphire solid state laser for excitation; a 505 long-pass dichroic and a 530/50 band-pass filter for emission. A primary gate based on physical parameters (forward and side light scatters) was set to exclude small de-
Single Muscle Fiber Isolation

Muscle fibers were isolated from the EDL muscle of adult mice as previously described (Shefer et al., 2004). Fibers were fixed in 2% (for X-gal staining) or 4% (for EGFP observation) PFA/PBS for 20 min at 4°C, washed three times for 20 min each in PBS, and processed for immunohistochemistry.

Immunohistochemistry

For Pax7 immunohistochemistry, muscle fibers were blocked in 2% BSA/5% goat serum/0.1% Triton X-100/ PBS at room temperature for 45 min, followed by incubation in a 1:10 dilution of hybridoma supernatant of mouse anti-chicken Pax7 monoclonal antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) for 2 h in 2% BSA/5% goat serum/PBS at room temperature. Fibers were washed three times for 5 min each in PBS and incubated with a 1:500 dilution of Alexa Fluor 555-conjugated goat anti-mouse antibody (Invitrogen, Carlsbad, CA) in 2% BSA/5% goat serum/PBS at room temperature. After washing three times for 5 min each, fibers were stained with DAPI and mounted on slides using aqueous mounting medium (Biomeda, Foster City, CA). For CD34 immunohistochemistry, fibers were blocked in 5% goat serum and 0.1% Triton X-100 in PBS (pH 7.4) overnight at 4°C, followed by incubation with a 1:400 dilution of rat anti-mouse CD34 monoclonal antibody (RAM34, eBioscience, San Diego, CA) for 3 h in fresh blocking solution at room temperature. After washing in PBS, fibers were incubated with 6 μg/ml biotinylated goat anti-rat IgG (BD Biosciences, San Jose, CA) in blocking buffer for 1 h. After washing in PBS, fibers were incubated with Alexa Fluor 488 or 555-conjugated streptavidin for 1 h in the blocking solution. Fibers were washed, stained with DAPI and coverslipped as earlier. CD34 signal detected by Alexa Fluor 488 was colorized red in Photoshop.

Confocal Microscopy

Stained fibers were observed with a TCS SP2 Leica Laser Scanning Spectral Confocal Microscope. This microscope is equipped with an Argon laser (488 nm excitation) for EGFP, and a Helium-Neon laser (543 nm excitation) for Alexa Fluor 555. Samples were visualized with a 40X oil objective and captured and analyzed with Leica Confocal Software version 2.61. Each satellite cell was first identified using a standard UV epifluorescence to detect Pax7 staining, and DAPI staining was verified before scanning. A Z-stack was collected for each satellite cell to assess EGFP fluorescence throughout the cell, which aided in scoring both positive and negative cells. To avoid emission bleed-through, images were taken in sequential scan mode and laser intensity, gain and offset on PMTs were adjusted using single-stained specimens.

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