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Z/EG, a Double Reporter Mouse Line That Expresses Enhanced Green Fluorescent Protein Upon Cre-Mediated Excision

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Summary: The Cre/*loxP* system has become an important tool in designing postintegrational switch mechanisms for transgenes in mice. The power and spectrum of application of this system depends on transgenic mouse lines that provide Cre recombinase activity with a defined cell type-, tissue-, or developmental stage-specificity. We have developed a novel mouse line that acts as a Cre reporter. The mice, designated Z/EG (*lacZ/EGFP*), express *lacZ* throughout embryonic development and adult stages. Cre excision, however, removes the *lacZ* gene, which activates expression of the second reporter, enhanced green fluorescent protein. We have found that the double-reporter Z/EG line is able to indicate the occurrence of Cre excision from early embryonic to adult lineages. The advantage of the Z/EG line is that Cre-mediated excision can be monitored in live samples and that live cells with Cre-mediated excision can be isolated using a single-step FACS. It will be a valuable reagent for the increasing number of investigators taking advantage of the powerful tools provided by the Cre/*loxP* site-specific recombinase system. *genesis* 28: 147–155, 2000. © 2000 Wiley-Liss, Inc.

Key words: Cre; *loxP*; transgenic mice; conditional gene expression; *lacZ* enhanced green fluorescent protein

INTRODUCTION

The Cre recombinase of bacteriophage P1 has become a powerful tool in making alterations to the mouse genome. The *loxP* recognition sites for Cre recombinase are first placed in a strategic manner into the mouse genome. The Cre enzyme is then introduced to recombine the DNA between the *loxP* sites and create specific types of genome alterations, including deletions, insertions, inversions, and translocations (LePage *et al.*, 2000; Matsusaka *et al.*, 2000; Ramirez-Solis *et al.*, 1995; Sauer, 1993; Sauer and Henderson, 1988; Zheng *et al.*, 2000). No recombination occurs between the *loxP* sites until the Cre enzyme is introduced, thus the alterations are

referred to as conditional alterations. The Cre recombinase can be expressed in a transgenic mouse in a tissue- or temporal-specific manner, depending on the promoter used to regulate its expression (Lakso *et al.*, 1992; Orban *et al.*, 1992). Consequently, the genome alterations can be made in a tissue-specific or stage-specific manner.

The growing popularity of this system in mouse transgenesis has resulted in the creation of a number of Cre transgenic lines (Nagy, URL). This battery of transgenic mice express Cre under tissue-specific regulation, either by linking Cre to the promoter in a transgene construct or by “knocking in” the Cre coding sequence into a particular endogenous gene. The specificity of Cre excision activated by these transgenic lines must be tested to validate their use for desired target tissues and stages. Several reporter transgenic mouse lines have been developed for this purpose (Akagi *et al.*, 1997; Kawamoto *et al.*, 2000; Sakai *et al.*, 1995; Soriano, 1999; Tsien *et al.*, 1996). These reporter strains are used by crossing the Cre transgenic mice to the reporter mice and analyzing double-transgenic offspring for expression of the given reporter transgene. Most of the lines utilize the activation of a *lacZ* reporter gene upon Cre excision. A problem with these lines has been that there is no reporter gene expression until Cre excision. Therefore, in any tissues without reporter gene expression, Cre activity may be present but the reporter gene will not be expressed to reflect that activity. We have developed a reporter line that, instead, expresses *lacZ* in a widespread manner before Cre excision. Cre excision then excises the *lacZ* reporter and replaces it with human placental alkaline phosphatase (hPLAP) gene expression.

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This double-reporter strain, designated Z/AP, has been used to characterize several Cre transgenic mouse lines (Guo *et al.*, 2000a,b; Hebert and McConnell, 2000; Lobe *et al.*, 1999; Lomeli *et al.*, 2000).

A novel reporter gene has been developed for use in mice, the enhanced green fluorescent protein (EGFP), derived from the jellyfish, *Aequorea victoria* (Chalfie *et al.*, 1994). The EGFP reporter has several advantages over conventional enzymatic reporters, such as the β galactosidase encoded by *lacZ* and the hPLAP proteins. EGFP can be visualized in live animals and cells, requires no treatment of the tissues to be observed, and cells expressing EGFP can be separated using fluorescence-activated cell sorting (FACS).

We have developed a Cre reporter mouse that takes advantage of the properties of the EGFP reporter. This Cre reporter line incorporates the *lacZ* reporter as the first marker, expressed prior to Cre excision, and the

EGFP reporter as the second marker, expressed after Cre excision.

RESULTS

Z/EG Expression Vector

We previously designed a Cre reporter transgene, Z/AP, that provides *lacZ* expression prior to Cre excision and hPLAP gene expression after Cre excision (Lobe *et al.*, 1999). The same double-reporter system was used here, but the second reporter (hPLAP) was replaced with the enhanced green fluorescence protein (EGFP) reporter gene. This vector was designed to provide *lacZ* expression before Cre excision and EGFP expression after Cre excision and is referred to as Z/EG (*lacZ*/EGFP; Fig. 1).

Introduction of Z/EG Into ES Cells and Mice

The Z/EG vector was introduced into R1 embryonic stem (ES) cells (Nagy *et al.*, 1993), and ES cell clones that carried the Z/EG transgene were selected through neomycin resistance, conferred by the β -geo fusion gene. We have found that strong, uniform expression of the *lacZ* reporter in the ES cell clones correlates well with widespread expression in corresponding embryos or animals derived from the ES cells (Lobe *et al.*, 1999). Thus we further screened the neomycin-resistant ES cell clones for a high level of overall *lacZ* expression. Of the 144 neomycin-resistant clones, 29 of the ES cell clones exhibited intense *lacZ* expression in more than 90% of the cells. These clones were further screened by Southern blot to determine which had a single transgene copy. We wished to use only ES cell clones with a single copy of the transgene to avoid recombination between multiple *loxP* sites, which might lead to loss of reporter gene expression or chromosome instability and loss (Lewandoski and Martin, 1997). Eight of the 29 ES cell clones had a single copy of the transgene. As a final test, an expression construct for Cre recombinase, which also carried a puromycin-resistance gene, was introduced

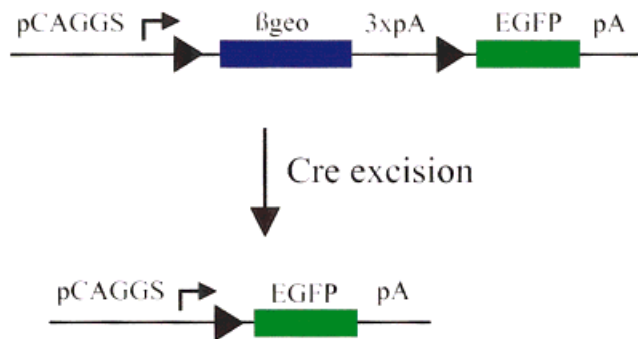


FIG. 1. Z/EG expression construct. The Z/EG transgene consists of the strong pCAGGS promoter, directing expression of a *loxP*-flanked β geo (*lacZ*/neomycin-resistance) fusion gene and three SV40 polyadenylation sequences. Following that is the coding sequence of the enhanced green fluorescent protein and a rabbit β globin polyadenylation sequence. In this configuration, β geo is expressed before Cre excision and EGFP is expressed after Cre excision.

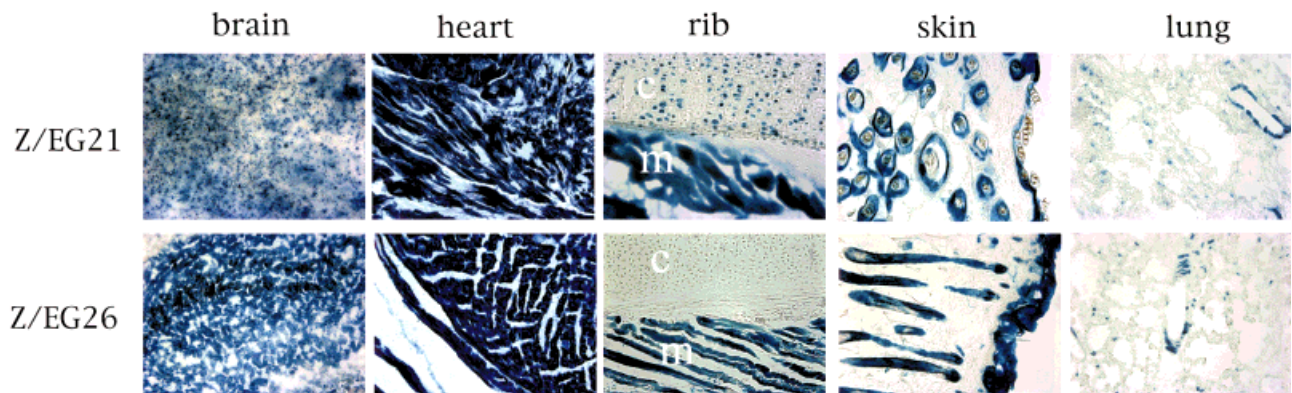


FIG. 2. *lacZ* expression in tissues from the Z/EG21 and Z/EG26 transgenic lines. Tissue sections, as indicated, from hemizygous Z/EG21 and Z/EG26 mice were stained for *lacZ* expression. c, chondrocytes; m, muscle.

into these eight ES cell lines. After selection for puromycin resistance, cells were monitored for EGFP expression. Of the eight ES cell lines tested this way, six of the lines had a strong EGFP signal.

Three of the Z/EG ES lines (Z/EG5, Z/EG21, and Z/EG26) were used to generate chimeric mice by ES cell (<-) embryo aggregation (Nagy, 1997). The chimeric mice were bred to CD1 mice to produce hemizygous transgenic offspring. We initially assessed *lacZ* expression in the transgenic mice from each of the three lines to determine which had the strongest and most extensive transgene expression. Tissues from two- to three-week-old mice were assayed in whole-mount stains and in slide sections. Two of the lines, Z/EG21 and Z/EG26, expressed *lacZ* in all tissues tested except liver, and Z/EG26 lacked *lacZ* expression in chondrocytes (Fig. 2). Both Z/EG21 and Z/EG26 also had low expression in the lung. The Z/EG5 mice had low overall *lacZ* expression and were not characterized further.

EGFP Expression After Cre Excision

To test whether the Z/EG mice would correctly switch from *lacZ* to EGFP expression when Cre recombinase was introduced, the Z/EG mice were crossed to a general Cre deleter mouse line (pCX-NLS-Cre; Nagy, 2000). Single (Z/EG) and double (Z/EG;pCX-NLS-Cre) transgenic offspring were examined for *lacZ* and EGFP expression at various stages of embryonic development and postnatally.

To view early-stage embryos, a Z/EG female was mated with a pCX-NLS-Cre male, sacrificed at 2.5 days postcoitum (dpc), and the embryos were flushed from the oviducts. When viewed under the GFP light, one of 11 eight-cell-stage embryos and morulae exhibited a strong green signal and two embryos had a faint green signal (Fig. 3a). Although these embryos were not genotyped by any molecular means, this observation suggested that in double-transgenic (Z/EG;pCX-NLS-Cre) embryos, Cre excision of the *lacZ* gene activates the EGFP reporter, which becomes visible already at the early morulae stage. The difference in fluorescence intensity between the embryos may correspond to the onset of Cre excision, which would occur at the two- to four-cell stage.

Embryos from the same cross were observed at postimplantation stages, E9.5 and E12.5. Embryos were also genotyped by Southern blot using the yolk sac for DNA. At both E9.5 and E12.5, the green fluorescent signal from the double-transgenic embryos was sufficiently strong to be seen through the uterine wall. Double-transgenic embryos displayed strong, overall EGFP fluorescence (Fig. 3b,c). In addition, the yolk sac and embryonic portion of the placenta were fluorescent green (Fig. 3c). We quantitated the expression in the embryos by making single-cell suspensions of individual double-transgenic E9.5 and E12.5 embryos and counting the GFP-positive cells out of the total number of cells. We also analyzed the same samples by flow cytometry. Both methods showed that 44% of the Z/EG26 embryo

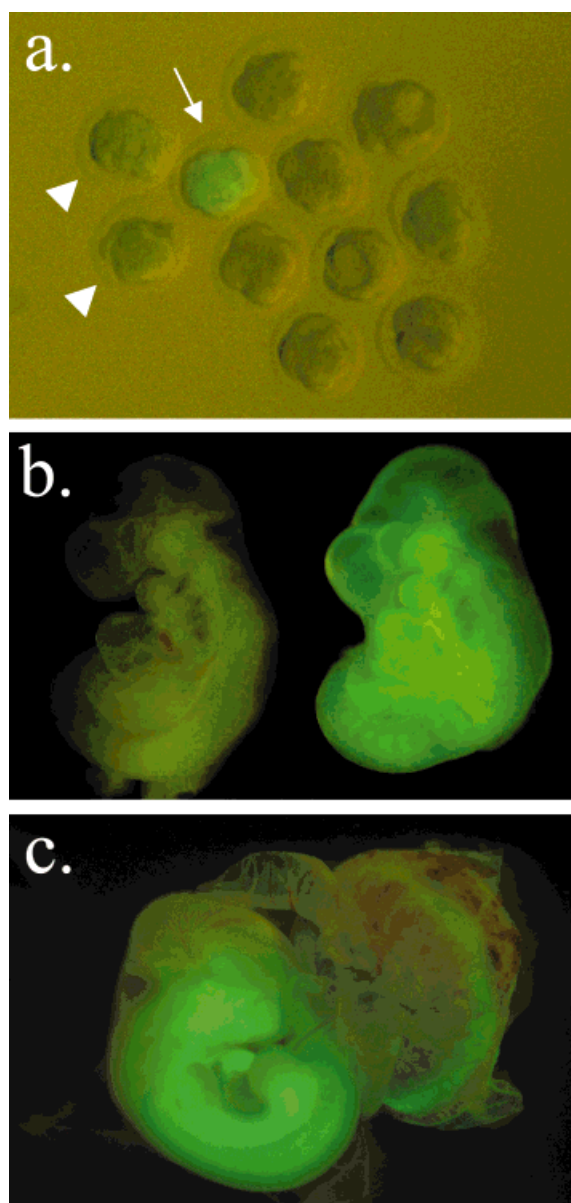


FIG. 3. EGFP expression in embryos after Cre excision. Z/EG females were crossed with the general deleter mouse line pCX-NLS-Cre. Embryos were dissected at (a) E2.5, (b) E9.5, and (c) E12.5. (a) The strong EGFP-positive embryo is marked with an arrow and the two weak embryos are marked with arrowheads. (b) The embryo on the left is a single-transgenic Z/EG embryo, negative for EGFP expression, while the embryo on the right is a double-positive Z/EG;pCX-NLS-Cre embryo that is expressing EGFP. (c) A double-transgenic Z/EG;pCX-NLS-Cre embryo that is EGFP-positive, including the embryonic region of the placenta.

cells were positive for green fluorescence (SD = 6.7%, n = 10), and 70% of the Z/EG21 embryo cells were EGFP-positive (SD = 8.8%, n = 5).

We also examined newborn mice from Z/EG x pCX-NLS-Cre matings for reporter gene expression. The double-transgenic pups could be easily identified, since they were strong fluorescent green. Fluorescence was most

intense in striated muscle and the central nervous system, showing through the less-fluorescent skin and bone (Fig. 4a). The strong EGFP signal persisted in the animals until they were at least three months of age, the age we have tested them so far. When the double-transgenic pups were dissected, we found that all organs expressed the EGFP reporter gene (Fig. 4b-i). Sections of the tissues revealed that most cells were positive for GFP expression (Fig. 5), except that the Z/EG26 mice had very little EGFP expression in chondrocytes. Thus, although the mice from both lines had restricted *lacZ* expression in liver and lung, they had strong levels of EGFP expression in those tissues after Cre excision.

To measure EGFP-positive cells in the thymus and spleen, single-cell suspensions were made of the tissues and used for flow cytometric analysis (Fig. 6). For the EGFP26 mice, 84% of the thymus cells were positive and 56% of the spleen cells were positive. The EGFP21 mice had a higher proportion of EGFP-positive thymus and spleen cells (95% and 81%, respectively). For the Z/EG26 mouse line, we also analyzed the cells by double labeling. For the thymus, cells were sorted according to CD4- and CD8-positive cells, representing the T-cell population, and for spleen, CD19 antibody was used to identify predominantly B cells. Approximately 79% of the CD4-positive and 73% of the CD8-positive T cells expressed GFP, and 33% of the CD19 B cells expressed GFP.

Tissue-Specific Cre Excision

To test the utility of the Z/EG mice as a Cre reporter line, they were crossed with the tissue-specific Cre line, K5-Cre (Tarutani *et al.*, 1997). The K5-Cre mice express Cre recombinase under the regulation of the Keratin-5 promoter in the basal layer of the skin epidermis and in some regions of the gut.

In the newborn litters, double-transgenic (Z/EG;K5-Cre) offspring could be identified by their green fluorescence (Fig. 7). However, the Z/EG;K5-Cre double-transgenic mice had a uniform green fluorescence and lacked the intense signal from muscle and central nervous system seen in the double-transgenic mice for which pCX-NLS-Cre was used. Dissection of the Z/EG;K5-Cre mice revealed that the skin and gut were positive for GFP expression, as expected (Fig. 7b-d), whereas other tissues were negative. In serial sections of skin, the epidermis and hair follicles were positive for EGFP (Fig. 7h) but negative for *lacZ* expression (Fig. 7e; compare with Fig. 2), indicating that Cre activity in the epidermal cells resulted in excision of *lacZ* and consequent expression of the EGFP reporter. On the other hand, the dermis and adjacent adipocytes and muscle remained positive for *lacZ* expression and did not express EGFP (Fig. 7e), demonstrating a lack of Cre expression in those cells.

Within the gut of Z/EG;K5-Cre double-transgenic mice, Cre excision could readily be seen in the esophagus and fundus of the stomach (Fig. 7c). Visualization of EGFP and *lacZ* expression in serial tissue sections showed the squamous epithelial cell lining of the esophagus and fundus were fluorescent green (Fig. 7i,j),

whereas the outer muscle layers remained *lacZ*-positive (Fig. 7f,g). This confirmed that the Cre-expressing epithelial cells had undergone excision of the *loxP*-flanked *lacZ* sequence, resulting in EGFP expression. This result indicates the Z/EG mice serve as a sensitive reporter for Cre excision when crossed to tissue-specific Cre transgenic lines.

DISCUSSION

Cre recombinase is widely used in strategies for mouse transgenesis and genome alteration (reviewed in Nagy, 2000). The power of the Cre/*loxP* system depends on the availability of well-characterized Cre reporter mice to determine the expression pattern of Cre transgenic lines. Careful analysis of Cre activity in the Cre transgenic mouse lines is important because a low level of excision early in development may produce only a few cells with Cre excision, but all the descendants of those cells will carry the Cre-induced genomic alteration. In addition, Cre recombinase transgenes expressed under the regulation of a tissue-specific promoter will have different levels of expression and therefore efficiency of Cre activity, dependent on the transgene integration site.

The Z/EG mice provide a sensitive assay for Cre-mediated excision when used with either a general Cre deleter line or a tissue-specific Cre line. The advantages of using EGFP as the reporter for Cre excision is that it can be monitored in live mice and cells and that it requires no treatment of the tissues for visualization. The expression of EGFP after Cre excision is strong enough in Z/EG mice to be seen even if a small population of cells undergoes Cre excision, as with a tissue-specific promoter. This can be useful in cell-lineage tracing (Chai *et al.*, 2000; Jiang *et al.*, 2000). The use of EGFP as the Cre reporter also allows the use of flow cytometric analysis for quantitation and FACS sorting for separation of cells that have undergone Cre excision. The use of the double-reporter, *lacZ* to EGFP, system affords an important visualization of animals and tissues that have not undergone Cre excision.

Our strategy to establish the Z/EG transgenic mice by introducing the transgene into ES cells and selecting ES cell clones for desired features works efficiently to identify clones that provide widespread expression *in vivo*. The screen for ES cell clones that express *lacZ* in a uniform and strong level provided two out of three mouse lines with broad transgene expression at a high level in the mouse. A similar strategy of prescreening for desired expression patterns in ES cells has been successfully applied to other transgenes with tissue-specific (Ding *et al.*, submitted) or inducible expression (Guo and Lobe, unpublished).

We have observed that *lacZ* is not expressed at high levels in some tissues. This was noted previously for the Z/AP mice, where hPLAP was expressed in chondrocytes and adipocytes after Cre excision, even though *lacZ* expression was absent in those tissues prior to Cre excision (Lobe *et al.*, 1999). In the Z/EG mice, we ob-

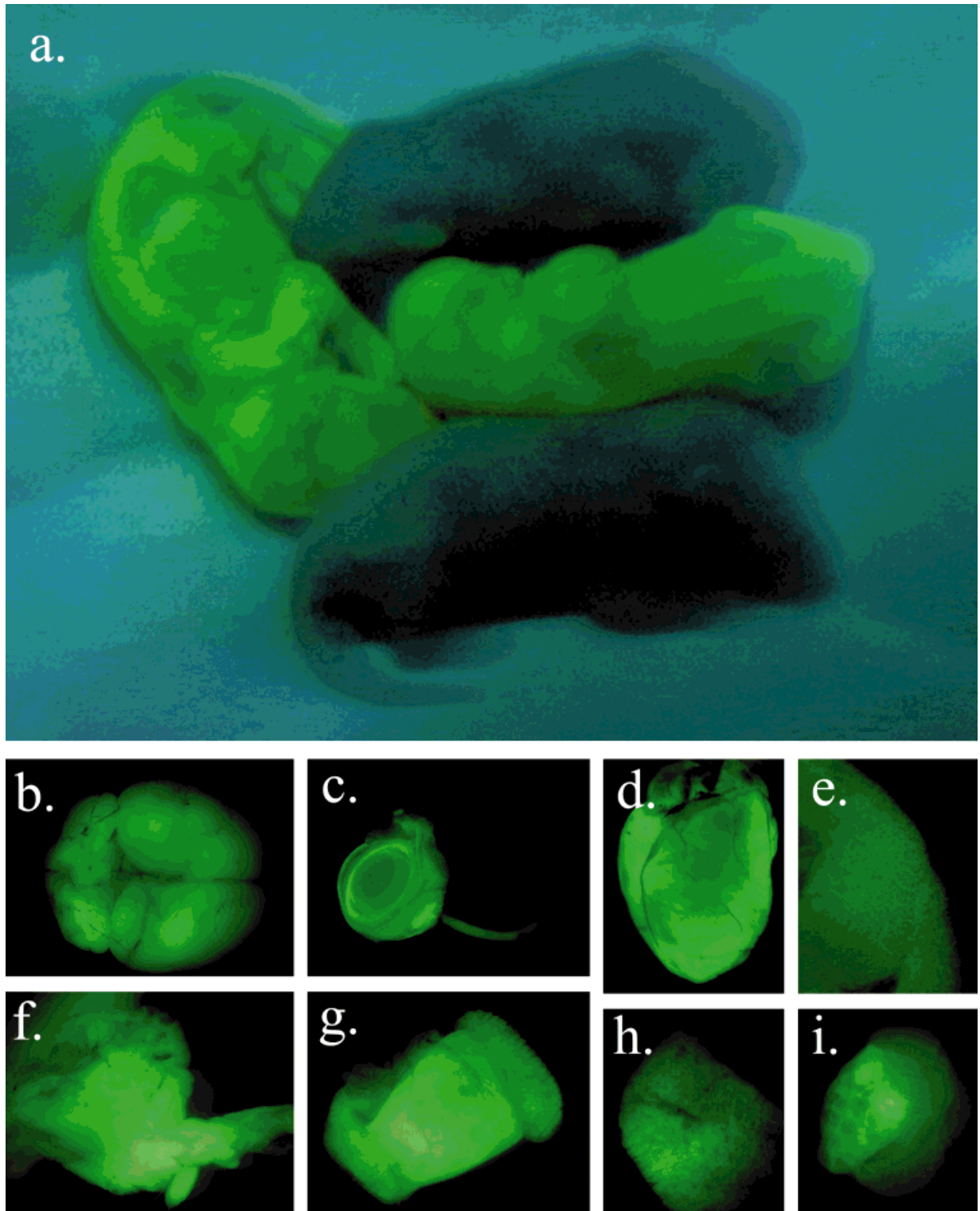


FIG. 4. Newborn pups and organs from a Z/EG x pCX-NLS-Cre cross. **(a)** Newborn double-transgenic pups can be readily visualized by green fluorescence against their single-transgenic littermates. Expression in whole organs was visualized for **(b)** brain, **(c)** eye, **(d)** heart, **(e)** lung, **(f)** pancreas, **(g)** intestine, **(h)** liver, and **(i)** ovary.

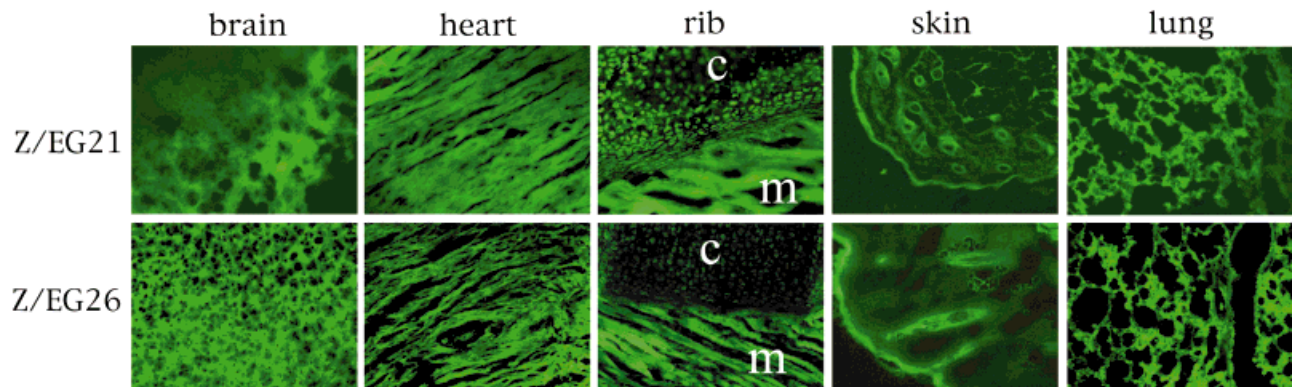


FIG. 5. EGFP expression in tissues from the Z/EG21 and Z/EG26 transgenic lines. Tissue sections from double-transgenic Z/EG21;pCX-NLS-Cre and Z/EG26;pCX-NLS-Cre mice were visualized for green fluorescence. c, chondrocytes; m, muscle.

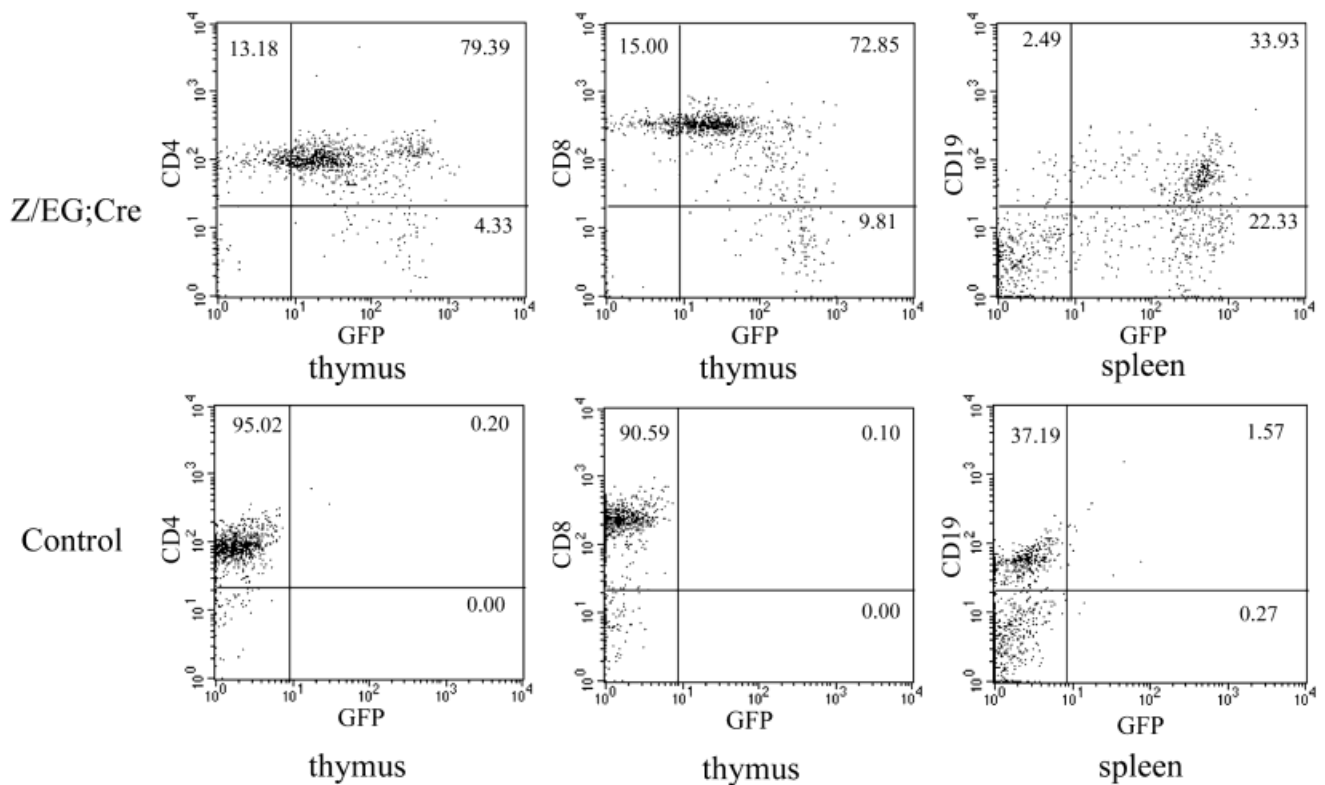


FIG. 6. Measurement of EGFP expression by flow cytometry. Flow-cytometric analysis of thymus and spleen from Z/EG;pCX-NLS-Cre mice or a nontransgenic littermate, as indicated. Cells were double-sorted for EGFP expression and either CD4 or CD8 antibody to mark T cells of the thymus, and CD19 to mark B cells in the spleen. The percentage of cells in each quadrant is listed.

served this phenomenon for the liver, where neither the Z/EG21 nor Z/EG26 mice expressed *lacZ* prior to Cre excision but both expressed EGFP after Cre excision. Likewise, *lacZ* expression in the lung is limited for both mouse lines, whereas EGFP expression is not. The basis for the difference in reporter-gene expression may be that the *lacZ* coding sequence is of bacterial origin, whereas hPLAP and EGFP are of eukaryotic origin and may be more efficiently expressed in murine cells.

Mouse reporter lines for Cre activity have been made previously. Each line utilizes different combinations of reporter genes, allowing flexibility in their use with a given Cre/*loxP* experiment. The majority of the lines utilize activation of *lacZ* expression as a readout of Cre activity (Akagi *et al.*, 1997; Soriano, 1999; Tsien *et al.*, 1996). Another pair of Cre reporter lines incorporates the chloramphenicol acetyl transferase (CAT) gene as the reporter prior to Cre excision and switches to the

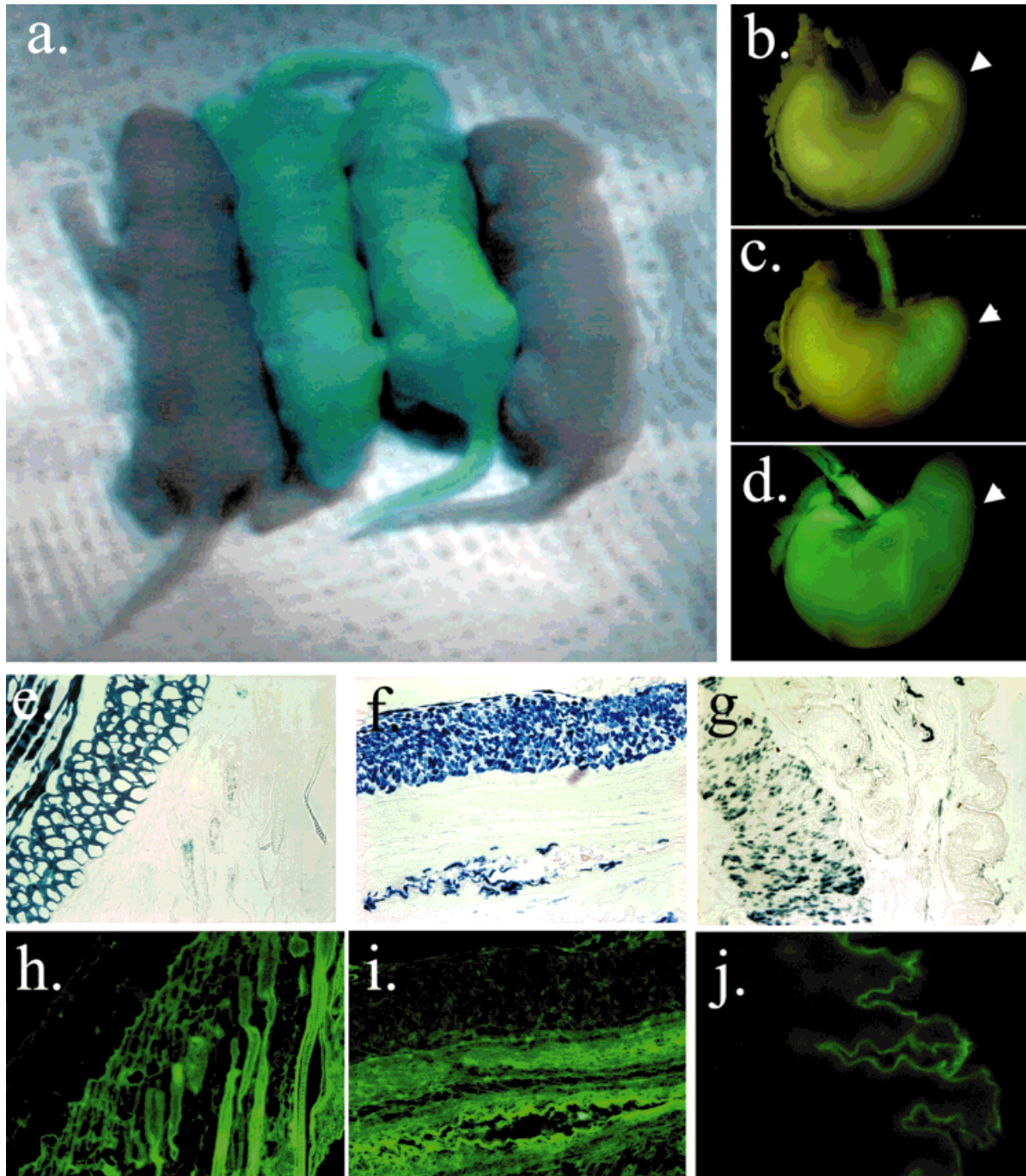


FIG. 7. EGFP and *lacZ* expression in Z/EG x K5-Cre offspring. **(a)** Double-transgenic Z/EG;K5-Cre mice can be readily detected by green fluorescence in the skin. **(b–d)** Stomach and esophagus from a **(b)** Z/EG mouse, showing no EGFP expression, **(c)** Z/EG;K5-Cre mouse, showing Cre expression in the lining of the esophagus and fundus of the stomach, and **(d)** Z/EG;pCX-NLS-Cre mouse, with EGFP expression throughout the esophagus and stomach. The fundus region is marked with an arrowhead. **(e–j)** Reporter gene expression in tissue sections showing *lacZ* **(e–g)** and EGFP **(h–j)** expression in tissue sections of skin **(e,h)**, esophagus **(f,i)**, and fundus region of the stomach **(g,j)**.

lacZ reporter (Sakai *et al.*, 1995) or the EGFP reporter (Kawamoto *et al.*, 2000) upon Cre excision. The Z/AP mouse reporter line expresses *lacZ* initially but switches to hPLAP expression in cells that undergo Cre excision

of the *lacZ* reporter (Lobe *et al.*, 1999). The Z/EG line described here also utilizes the binary reporter system of the Z/AP mice but with EGFP as the second reporter indicating Cre activity. In many cases, GFP expression

will be valuable for analysis in embryos, live mice, or using FACS. In other experiments, *lacZ* or hPLAP will be more useful, such as when analyzing Cre excision at the cell level in tissue sections. Furthermore, each reporter line may have a different sensitivity to Cre excision. This variation probably reflects differences in accessibility of integration sites and transgene *loxP* sites and suggests it is worth testing Cre lines against more than one reporter line (Hebert and McConnell, 2000).

The Z/EG mouse line is novel in that it provides a double-reporter system for Cre excision utilizing *lacZ* and EGFP. It will complement the use of the Cre/*loxP* system in the mouse, where it has become a critical component for genome alterations.

METHODS

DNA Constructs

The Z/EG vector was based on the pCCALL construct (Lobe *et al.*, 1999). pCCALL has a chicken β actin promoter with upstream CMV (cytomegalo virus) enhancer elements to direct strong, widespread expression (Lobe *et al.*, 1999; Niwa *et al.*, 1991). The promoter is followed by a *loxP*-flanked β geo (*lacZ* and neomycin-resistance) fusion gene and three polyadenylation sites. Thus, prior to Cre excision, the *lacZ* reporter and the neomycin-resistance gene are expressed. A rabbit β -globin pA signal is 3' to the second *loxP*.

The pCCALL plasmid contained two potential cryptic ATG translation start sites in the cloning site between the 3' *loxP* sequence and the rabbit β -globin pA signal. To optimize expression of the second reporter, we re-constructed this area of the plasmid to create pCCALL2. The 238-bp Sall-BglIII fragment, containing the 3' *loxP* site and the two ATGs, was replaced with a 72-bp sequence containing a 3' *loxP* site and a multiple cloning site (BglIII, KpnI, XhoI, NotI). To produce the ZEG expression vector, the XhoI-NotI fragment of pEGFP-1 (Clontech) was inserted into the XhoI-NotI sites of pCCALL2. Therefore when the *loxP*-flanked β geo/polyadenylation sequence is removed by Cre excision, the promoter is placed adjacent to the EGFP reporter gene.

ES Cell Clones

The R1 ES cell line (Nagy *et al.*, 1993) was maintained essentially as described (Wurst and Joyner, 1993). The cells were grown in Dulbecco's Modified Eagle's Medium (DMEM)/high glucose (Gibco) supplemented with 15% fetal calf serum (Hyclone), 1 mM sodium pyruvate (Gibco), 1 mM nonessential amino acids (Gibco), 2 mM L-glutamine, 10^{-6} M β -mercaptoethanol, and 10 ng/ml of leukemia inhibitory factor (LIF; Mereau *et al.*, 1993). Except as noted below, cells were grown on feeder plates of mitomycin C-treated embryonic fibroblast cells.

To create clones of ES cells expressing Z/EG, approximately 6×10^6 cells were electroporated with 10 μ g of Z/EG DNA (digested with ScaI and StuI to remove the plasmid backbone) at 220 V and 500 μ F in a Biorad Gene Pulser. Transfected ES cells were selected for 7–8 days in

medium containing 150 μ g/ml G418 (Gibco), whereafter 144 surviving colonies were picked and transferred to 96-well plates in triplicate. One of the 96-well plates contained mitomycin C-treated mouse embryonic feeder cells, while the other two plates were treated with 0.1% gelatin. The ES clones growing on feeders were frozen at -70°C for subsequent aggregation with embryos, while one set of ES clones growing on the gelatinized plate was stained for *lacZ* expression (Lobe *et al.*, 1999) and the other set was used to extract DNA for genotyping by Southern analysis. In 29 of the ES cell clones, more than 90% of the cells stained intensely for *lacZ* activity. For these clones, DNA was digested with EcoRV, gel electrophoresed, transferred to a Hybond-N membrane (Amersham), and hybridized with a ^{32}P -labelled 464-bp BglIII-HindIII fragment, including the rabbit β globin polyadenylation sequence of the Z/AP vector. Eight of the clones with the high level of *lacZ* expression were found to carry one copy of the Z/EG transgene.

Transgenic Mice

Three of the clones with a single copy of the Z/EG transgene that expressed *lacZ* at a high level were thawed and aggregated with eight-cell stage CD1 mouse embryos (2.5 dpc), then transferred to pseudo-pregnant recipients to produce chimeric mice (Nagy, 1997). Male chimeras were mated with CD1 females to identify germline transmitters. Pups were genotyped by staining ear clips for *lacZ* expression (Lobe *et al.*, 1999). To detect excision of floxed β geo and expression of EGFP, germline-transmitting chimeric males and hemizygous F1 offspring were crossed with mice expressing pCX-NLS-Cre (Nagy, 2000). Pups were analyzed for the Cre transgene by Southern analysis, using BglIII for DNA digest and the Cre coding sequence as a ^{32}P -labelled probe.

Whole-Mount and Sectioned Tissue Preparation

Embryos and organs were dissected into ice-cold PBS and visualized for EGFP fluorescence as described below. For tissue sections, samples were embedded directly into OCT (Tissue Tek, Sakura), frozen at -70°C and cryosectioned at 7 μ m onto polylysine-coated slides. After drying 1–4 h at room temperature, slides with sections were stored at -20°C .

For EGFP, the slides were mounted directly with Cytoseal mounting solution (Fischer). For *lacZ* staining, slides were fixed for 5 min in 0.2% glutaraldehyde and washed three times for 5 min in *lacZ* wash buffer (2 mM MgCl_2 , 0.01% sodium deoxycholate, 0.02% Nonidet-P40 in 100 mM sodium phosphate pH 7.3 or PBS). They were then stained in *lacZ* stain solution (0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in *lacZ* wash buffer) for 4–6 h at 37°C , protected from light. When the staining was complete, slides were rinsed in PBS before dehydration through a graded ethanol series and mounting coverslips.

Visualization of GFP

EGFP was viewed in live animals using a GFP-5 light source from BLS-Ltd (www.BLS-Ltd.com, Hungary). For embryos and whole-mount organs, green fluorescence was observed on a Leica Wild M3C stereo microscope with an MAA-02 Universal light source from BLS-Ltd. Tissue sections were viewed using a Leitz DMRB microscope equipped with epifluorescence lighting, using the FITC filter set. Animals were photographed using a Nikon E2 digital camera, and all microscope samples were photographed using a CoolSnap digital camera.

Flow cytometry

Tissues were minced with a scalpel, then broken into a single-cell suspension by passage through a 30 μ M nitex nylon mesh (B/SH Thompson, cat HCS-30). The cells were analyzed on a Becton Dickinson FACScan Flow Cytometer, using a 480 nm laser for excitation, 530 nm emission for GFP, and 570 nm emission for the R-Phycoerythrin-conjugated antibodies CD4, CD8, CD13, and CD19.

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