TECHNOLOGY REPORT

Efficient Method to Generate Single-Copy Transgenic Mice by Site-Specific Integration in Embryonic Stem Cells

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Summary: Transgenic and gene-targeted mutant mice provide powerful tools for analysis of the cellular processes involved in early development and in the pathogenesis of many diseases. Here we describe a transgene integration strategy mediated by site-specific recombination that allows establishment of multiple embryonic stem (ES) cell lines carrying tetracycline-inducible genes targeted to a specific locus to assure predictable temporal and spatial expression in ES cells and mice. Using homologous recombination we inserted an frt homing site into which tetracycline-inducible transgenes can be integrated efficiently in the presence of FLPe recombinase. This strategy and the vectors described here are generally applicable to any locus in ES cells and should allow for the rapid production of mice with transgenes efficiently targeted to a defined site. genesis 44:23–28, 2006. **© 2006 Wiley-Liss, Inc.**

Key words: transgenic; embryonic stem cells; inducible gene expression; tetracycline; site specific recombination; FLPe recombinase

Inducible control of gene expression has many applications in the study of gene function and genetic regulatory mechanisms. The ability to vary expression levels provides a powerful tool to study the biological role particularly of genes whose constitutive expression might be detrimental or lethal to the cells. Temporal, spatial, and titratable control of gene expression becomes very important in the study of genes involved in development and in disease progression.

The tetracycline-inducible system has been used effectively in vitro and in vivo to regulate gene expression. The system consists of two components, one encoding the tetracycline controllable transactivator (rtTA) and the other consisting of the tetracycline operator minimal promoter (tetOP) driving the gene of interest. These components need to be sequentially targeted into cells. Random integration of these elements into the genome is problematic because position effect variegation can profoundly affect expression and the insertion event itself may disrupt endogenous genes (Costantini *et al.*, 1989; Palmiter and Brinster, 1986; Soriano *et al.*, 1987). In addition, foreign DNA usually integrates as a linear array, resulting in multiple copies giving variable levels of gene dosage that may subsequently be silenced by epigenetic mechanisms. To circumvent these problems we developed a system that uses homologous recombination combined with an flp-in strategy in ES cells to efficiently target inducible transgenes to a specific locus. This strategy is similar to previously described methods that use Cre recombinase and lox sites to target transgenes to specific cassette-acceptor loci (Masui *et al.*, 2005; Soukharev *et al.*, 1999; Wutz *et al.*, 2002). We modified this approach using an Flpe recombinase and an frt homing site and further extended the study by showing that the flp-in transgenes function in vivo. Combining this targeting strategy with tetraploid embryo complementation allowed us to accelerate the production of mice carrying inducible genes whose expression can be analyzed without laborious and time-consuming screening of transgenic offspring and/or subsequent breeding of mice to obtain experimental animals carrying the necessary alleles.

RESULTS

Modification of the ColA1 Locus for Frt-Mediated Targeting

Our strategy for frt-mediated targeting is based on a single loxP-based strategy described by Wutz *et al.* (2002) and a system developed by Invitrogen (La Jolla, CA) (Fig. 1B). This strategy consists of two steps. In the first step an frt-flanked neomycin resistance gene and a hygromycin resistance gene that lacks a promoter and an ATG initiation codon that has an FRT site embedded in the 5′ coding region are introduced into targeting arms that will be used to mediate homologous recombination at a specific

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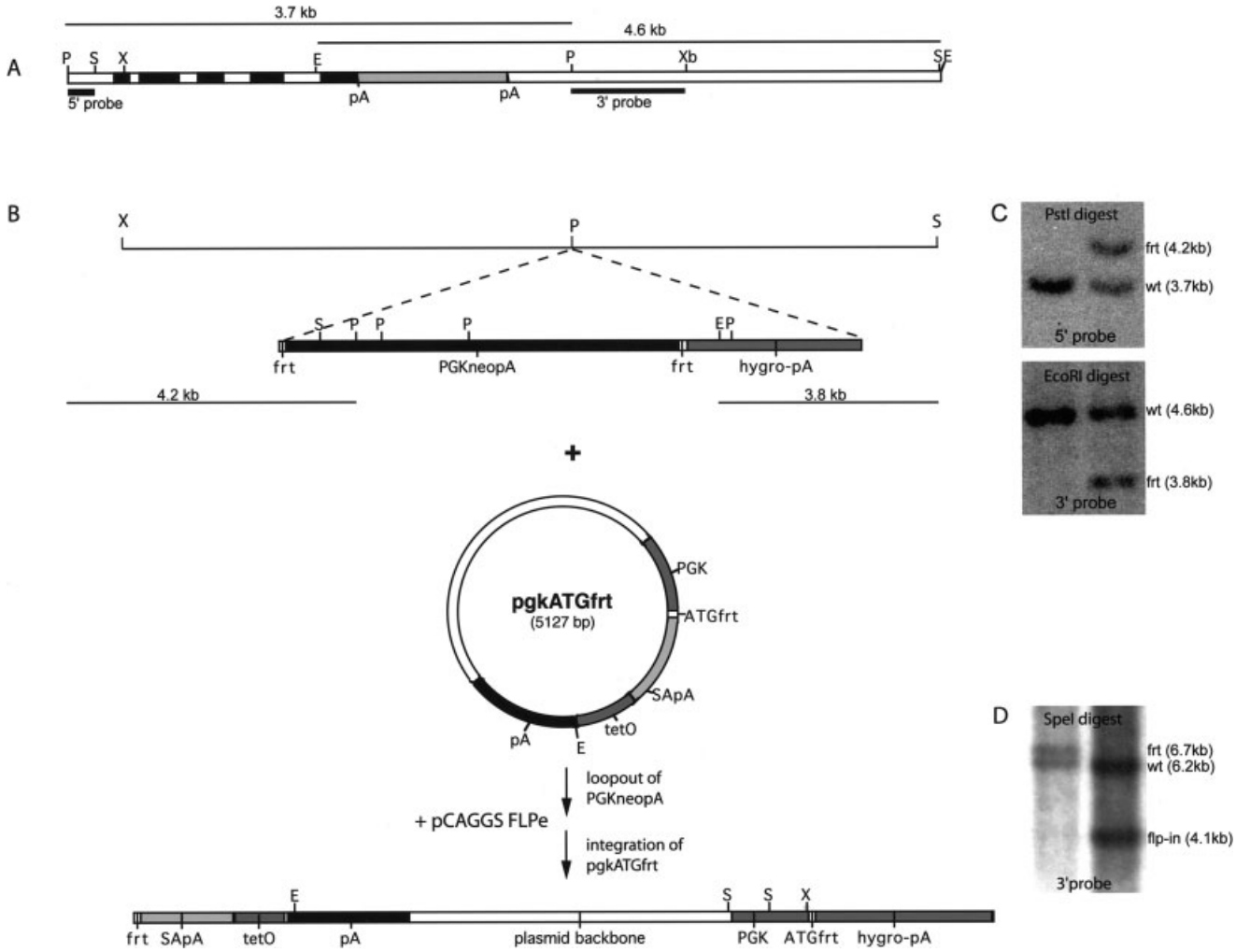

FIG. 1. Flp-in system. **A:** Schematic representation of the ColA1 locus and downstream region. Exons are shown as black boxes and 3′ UTR as a gray box. pA, polyadenylation signal. P, PstI; S, SpeI; X, XhoI; E, EcoRI; Xb, XbaI. **B:** Strategy for FLPe-mediated recombination. Homologous recombination was used to place an frt-hygro-pA "homing" cassette downstream of the ColA1 locus. A gene of interest is then targeted to the modified locus by coelectroporation of the pgkATGfrt vector carrying the gene and an Flpe transient expression vector. Inter- and intrachromosomal recombination at the frt sites results in loss of the PGKneopA cassette and insertion of the gene of interest and pgkATG cassette to restore and confer hygromycin resistance. **C:** Representative results of Southern analysis of G418-resistant clones using 5′ external and 3′ internal probes. **D:** Representative results of Southern analysis of hygromycin-resistant flp-in clones using a 3′ internal probe.

site in the genome. In the second step the modified locus is targeted using a plasmid that contains the PGK promoter and an ATG initiation codon, an frt site, and a tetracycline-inducible minimal CMV promoter driving the gene of interest. Coelectroporation of this plasmid with a plasmid expressing the FLPe recombinase followed by hygromycin treatment will select for those cells in which recombination has occurred between frt sites such that the entire flp-in plasmid is inserted with the PGK promoter and the ATG initiation codon upstream and into frame with the hygromycin resistance gene.

To test this strategy in ES cells, we targeted the ColA1 locus that encodes the type I collagen protein (Fig. 1A). This locus was chosen because it has been shown to support high transgene expression even in cell types that do not normally express the type I collagen gene (McCreath *et al.*, 2000). We chose to position the frt target site in a region that lies ~500 basepairs (bp) downstream of the 3′ untranslated region (Fig. 1B). This left the coding portion and 3′ UTR transcriptional control region of the ColA1 gene intact. V6.5 ES cells were electroporated with this homologous targeting construct and stable G418 clones were derived. The targeting efficiency as determined by Southern blot analysis was 80% (data not shown). One of these clones, C10, was chosen for subsequent targetings.

Generation of R26-M2rtTA Allele

A gene trap vector was used to generate the tetracycline-inducible M2rtTA transactivator driven from the

endogenous Rosa26 promoter. Homologous recombination-based knock-in at this locus is highly efficient and results in ubiquitous expression of transgenes (Zambrowicz *et al.*, 1997). The construct used here is similar to the targeting construct described by Wutz *et al.* (2002) except that nls-rtTA has been replaced by the M2rtTA transactivator. This modified transactivator has been used in vitro in eukaryotic cells and has been shown to function at a 10-fold lower doxycycline concentration than rtTA, is more stable, and has very low background activity in the absence of doxycycline (Urlinger *et al.*, 2000). C10 ES cells were electroporated with the vector and puromycin-resistant clones were screened by Southern blot analysis. Approximately 20% of the clones were correctly targeted (data not shown). Of these clones KH2 was chosen for subsequent targeting of cDNAs by FLPe-mediated recombination.

FLPe-Mediated Recombination of Tet-Inducible EGFP Downstream of ColA1 Locus

To establish an ES cell line that expressed a cDNA under the control of the tetracycline-inducible promoter, we constructed an flp-in vector carrying the gene encoding EGFP. In addition, this flp-in vector contains a splice acceptor (SA) and a polyadenylation sequence upstream of the minimal tetracycline-responsive promoter that should reduce tetracycline-independent transcription by blocking readthrough from potential upstream promoters. This vector and a vector that expresses the FLPe recombinase from the highly expressed CAGGS promoter (Buchholz *et al.*, 1998) were coelectroporated into KH2 ES cells. Integration of this vector into the frt site linked to an ATG initiation codon and a Pgk1 promoter restores the hyg^r gene. Hygromycin-resistant colonies were selected and screened by Southern blot analysis (Fig. 1D). Very few hygromycin colonies survived selection, but 80% of the clones showed correct targeting to the frt site upstream of the hygromycin cassette.

To confirm that EGFP was tetracycline-inducible, ES cells were cultured in the presence of increasing amounts of doxycycline for 2 days and then analyzed for intensity of GFP fluorescence by FACS. As can be seen in Figure 2, the percentage of cells expressing high levels of GFP increased with increasing concentrations of doxycycline. Without doxycycline all cells expressed a very low level of GFP that was detected by FACS analysis but not by fluorescence microscopy (data not shown). These data indicate tight and titratable regulation of this reporter gene in ES cells.

In Vivo Expression of Tetracycline-Inducible EGFP

In order to test the tetracycline-inducible system in vivo, mice were derived by tetraploid embryo complementation (Eggan *et al.*, 2002) from ES cells carrying both the R26-M2rtTA allele and the flp-in tetO-EGFP allele. The production of "ES mice" from ES cells that have been subjected to multiple rounds of in vitro selection has been shown to be highly efficient. Since mice produced by this method are derived entirely from the ES cells, they can be used directly without further breeding. Mice were treated with doxycycline administered in drinking water for 5 days and tissues were harvested and analyzed by immunocytochemistry. As seen in Figure 3 and summarized in Table 1, the highest EGFP expression was found in the thymus, lymph nodes, spleen, pancreatic islets, the crypt cells and villi of the intestine, and epidermal layer of the skin. All cells of the liver expressed EGFP, but at a relatively low level. Patchy expression of EGFP was observed in the lung, heart, and kidney, only the peritubular cells of the testis showed expression, and no expression was seen in the brain or skeletal muscle. The lack of expression in the brain and testis is likely due to the inability of doxycycline to cross the blood–brain and blood–testis barriers. Transgenic mice that were not exposed to doxycycline showed no EGFP expression (data not shown).

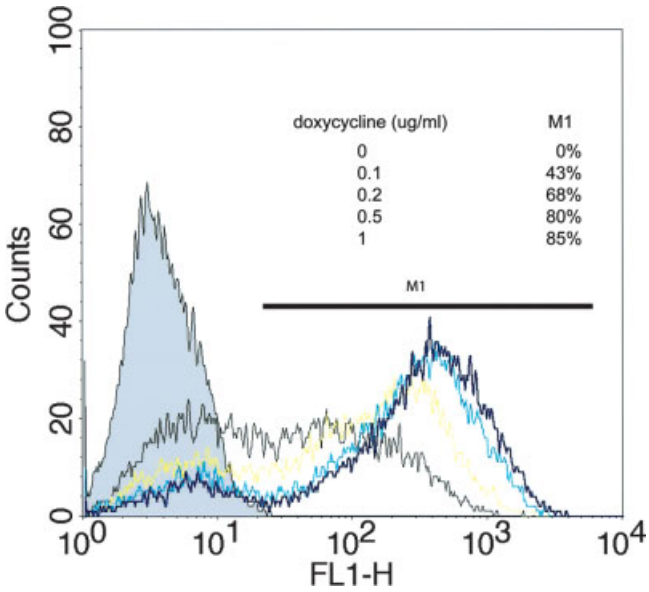

FIG. 2. FACS analysis of EGFP expression. ES cells carrying the R26-M2rtTA transactivator and tetO-EGFP alleles were grown in the absence or presence of increasing concentrations of doxycycline for 48 h and analyzed for intensity of EGFP fluorescence. Doxycycline concentrations (μg/ml): gray-filled, no drug; black line, 0.1; yellow line, 0.2; light blue line, 0.5; dark blue line, 1.0. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

DISCUSSION

We have shown that FLPe recombinase-mediated targeting is a very efficient method for introducing transgenes into a defined site in the genome. We initially chose the ColA1 locus as the frt homing site for integration of doxycycline-inducible genes and have shown that we get good doxycycline responsive expression both in ES cells and in mice. In a separate study, we used this same strategy to evaluate the oncogenic potential of Oct4, a POU-

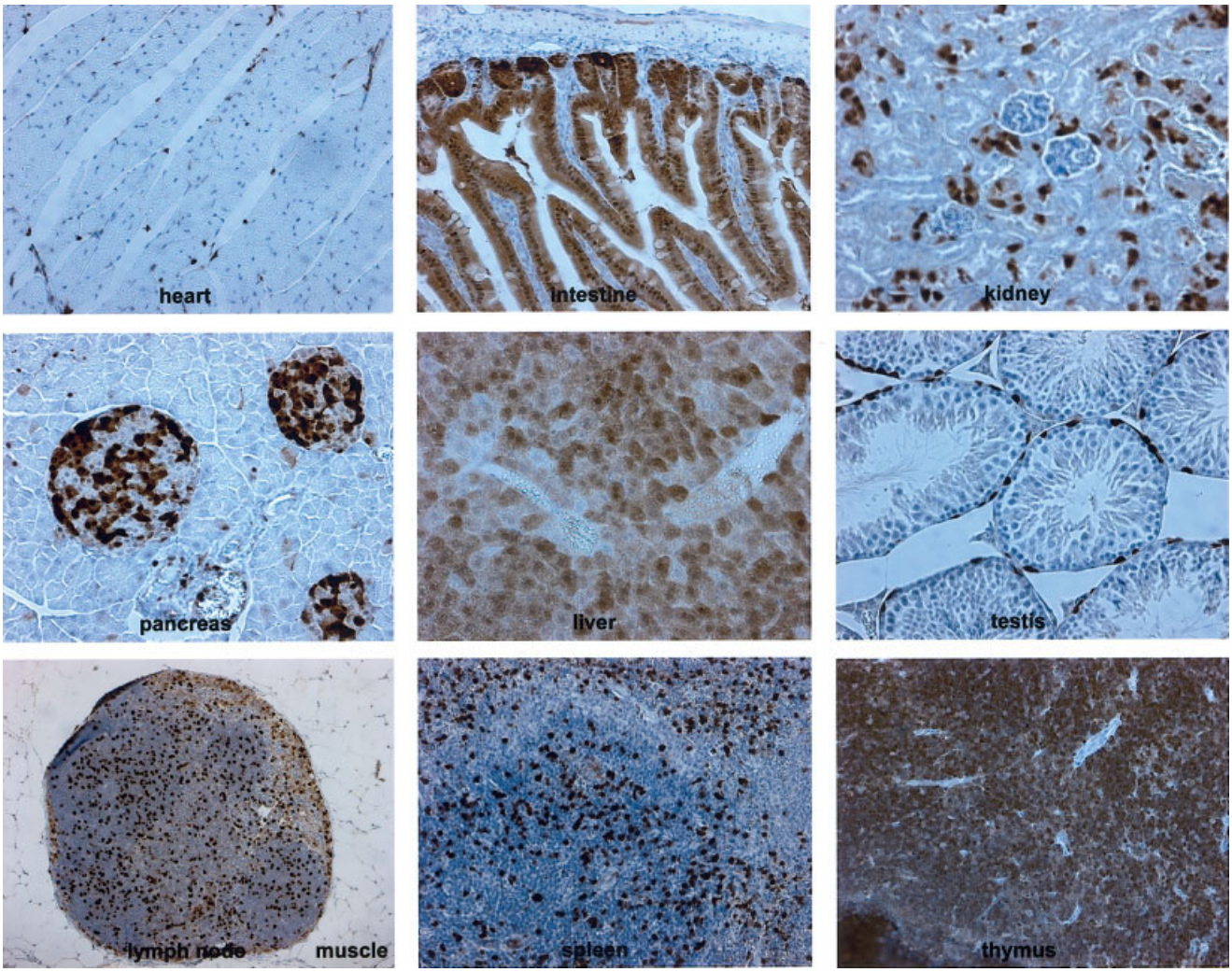

FIG. 3. Induction of EGFP expression in vivo. Mice were treated for 5 days with doxycycline (2 mg/ml) administered in drinking water. Paraffin sections of representative tissues were immunostained with anti-EGFP antibody (brown signal) and counterstained with hematoxylin.

domain transcription factor that is normally expressed in pluripotent cells, and observed Oct4 expression patterns similar to the EGFP expression reported in this study (Hochedlinger *et al.*, 2005). This inducible expression of EGFP is similar to the expression of EGFP driven from the ROSA26 promoter (Mao *et al.*, 2001) in all tissues and cell types examined except for the lack of expression in skeletal muscle, brain, and testis. As discussed above, this lack of expression in the brain and testis is likely due to the inability of doxycycline to cross the blood–brain and blood–testis barriers.

Recombinase-mediated targeting can be applied to any locus in the genome and we are currently building homologous recombination targeting vectors to introduce frt sites into different loci in the genome. One caveat to this approach is the possibility that expression of certain neighboring genes could be affected or the transgenes could be silenced due to the fact that the plasmid backbone and the PGK hygromycin cassette will also be integrated along with the transgene. There are, however, several advantages to generating a collection of ES cell lines using this strategy. First, once the frt sites have been targeted using homologous recombination, transgenes can be introduced without having to build additional targeting vectors with homologous arms. Second, the same flp-in vector can be used to introduce a given cDNA into several selected sites in the genome. We anticipate that transgene expression will vary, especially in vivo from tissue to tissue, depending on the integration site. Third, a series of cDNAs can be introduced into the same integration site to evaluate gene function without the variation in level and pattern of gene expression due to position effects. Fourth, flp-in vectors that express cDNAs driven off of tissue-specific promoters can be constructed and these can be easily and efficiently integrated into different loci to evaluate their patterns of gene expression. The flexibility and ease of this strategy combined with tetraploid embryo complemen-

Table 1
In Vivo Expression of Inducible EGFP

Tissue	Cell type: EGFP Expression
Spleen	B-cells: ++
	T-cells: ++
Thymus	All cells: +++
Lymph node	B-cells: +++
	T-cells: +++
Liver	Hepatocytes: +++
	Endothelial cells: +
	Bile ducts: −
Kidney	Tubular cells (cortex): +
	Collecting ducts (medulla): +
	Interstitial cells (medulla): −
Heart	Myocytes: −
	Interstitial fibroblasts: +
	Endothelial cells: +
	Pericardial cells: +
Intestine	Crypt cells: +++
	Villi: +++
	Mesenchymal cells: −
Pancreas	Islet cells: +++
	Acinar cells: +
Brain	No expression
Testis	Peritubular cells: +
	All other cells: −
Lung	Bronchial epithelial cells: +
	Alveolar cells: +
	Interstitial fibroblasts: +
	Endothelial cells: −
Skin	Epithelial (epidermis): +++
	Fibroblast (dermis): ++
	Adipocytes: +
Skeletal muscle	No expression

+ <10% of the cells score GFP positive.
++ 10–50% of the cells score GFP positive.
+++ >50% of the cells score GFP positive.

tation will provide a powerful tool for generating and evaluating mice carrying multiple transgenes.

MATERIALS AND METHODS

Targeting Constructs and Screening of ES Cells

A 6kb XhoI/SpeI fragment cloned from an RPCI-22 mouse BAC library was used to generate the arms of the ColA1/frtneomycin/hygromycin targeting vector. An XhoI/SalI fragment containing the frt/hygromycin/SV40pA from pcDNA5/FRT (Invitrogen) was cloned into the SalI site of pFNtpa (Possemato *et al.*, 2002) to generate a plasmid that contains a cassette consisting of an frt-flanked neomycin selectable marker and a promoter-less, ATG-less hygromycin resistance gene. A NotI/SalI fragment containing this cassette was blunt-ended and cloned into the blunt-ended PstI site of the ColA1 targeting arms. The targeting vector was linearized with XhoI, introduced into V6.5 ES cells by electroporation, and the cells were selected with 350 μg/ml G418. DNAs from picked clones were digested with PstI and analyzed for targeting using a ColA1 5′ external 400-bp PstI/XhoI probe. To check for correct targeting at the 3′ end, DNAs from picked clones were digested with EcoRI (one external and one internal site) and analyzed using an internal 840-bp PstI/XbaI ColA1 3′probe. A correctly targeted ES cell line (C10) was subsequently electroporated with an R26 targeting construct that allows expression of the M2rtTA tetracycline-responsive transactivator under the control of the R26 promoter. DNAs from picked clones were digested with EcoRV and analyzed using an R26 5′ external 380-bp EcoRV/SalI probe. This probe was polymerase chain reaction (PCR)-amplified from genomic DNA using the primer pair 5′aggcgcccgatagaataaat and 5′ctttacacaccattgcaccg. A correctly targeted ES cell line, KH2, was used for subsequent experiments.

Tet-Inducible Flp-in Expression Vector, Flp-in and Screening

The pBS31 flp-in vector consists of the following four cassettes: the pgk promoter followed by an ATG and an frt site, the CMV minimal promoter containing tetracycline-responsive operator binding sequences derived from pTETOP (Wutz and Jaenisch, 2000), a splice acceptor double polyA cassette derived from the ROSA26 targeting vector (Soriano, 1999), the rabbit beta globin polyadenylation signal sequence, and a unique EcoRI site for cloning genes. The sequence of this plasmid will be provided upon request. Approximately 1.5×10^7 KH2 ES cells were electroporated with 50 μg of flp-in vector and 25 μg of pCAGGS-FLPe-puro (Buchholz *et al.*, 1998) at 500 V and 25 μF using two pulses in a Gene PulserII (Bio-Rad, Hercules, CA). Hygromycin (Roche, Nutley, NJ) selection (140 μg/ml) was started after 24 h. DNAs from picked clones were digested with SpeI and analyzed for correct targeting using the ColA1 3′ probe.

Induction of Transgene Expression

ES cells were cultured on primary embryonic fibroblasts in the absence or presence of doxycycline ranging in concentrations from 0.1–1 μg/ml for 48 h. Doxycycline (2 mg/ml, supplemented with sucrose at 10 mg/ml) was administered to mice in drinking water for 5 days.

FACS Analysis

Cells were trypsinized, resuspended in medium, and analyzed for EGFP fluorescence on a FACS Calibur (Becton Dickinson, San Jose, CA).

Histological Analysis

Tissue samples were fixed in 10% buffered formalin for 24 h and embedded in paraffin. Immunostaining of 5-μm sections was performed using an avidin-biotin immunoperoxidase assay. Anti-EGFP antibody (Molecular Probes, Eugene, OR; A11122) was diluted 1:1,500 and incubated overnight at 4°C and then with biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for 2 h at room temperature followed by avidin-coupled peroxidase (Vector Laboratories) for 30 min. Diamobenzidine was used as a chromogen and hematoxylin as a counterstain. Transgenic mice that were not

treated with doxycycline were used as a negative control.

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