CHAPTER ONE

Adipocyte-Specific Transgenic and Knockout Models

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Abstract

Adipose tissue plays a major role in metabolic homeostasis, which it coordinates through a number of local and systemic effectors. The burgeoning epidemic of metabolic disease, especially obesity and type 2 diabetes, has focused attention on the adipocyte. In this chapter, we review strategies for genetic overexpression and knockout of specific genes in adipose tissue. We also discuss these strategies in the context of different types of adipocytes, including brown, beige, and white fat cells.

1. INTRODUCTION

1.1. Adipose tissue: Brown, white, and beige

Adipose tissues play key roles in energy balance, glucose and lipid homeostasis, in addition to a variety of other functions ranging from immunity to hemostasis to angiogenesis (Rosen & Spiegelman, 2006). Because adipose tissue affects so many physiological processes, and is in turn affected by

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the systemic environment, there has been great interest in establishing systems for determining the molecules and pathways that operate within the adipocyte itself. One cannot assume, for example, that an animal (or person) exhibiting a disorder of adiposity, such as obesity or resistance to weight gain, has a primary defect in the adipocyte.

Traditionally, adipocytes have been divided into two types: white and brown. White adipocytes make up the bulk of mammalian adipose tissue; these cells store lipid and expand during overnutrition. Brown adipocytes, conversely, are rich in mitochondria and burn energy through activity of uncoupling protein-1 (UCP-1), which dissipates the mitochondrial proton gradient with allowing ATP synthesis, thus generating heat (Seale, Kajimura, & Spiegelman, 2009). Recently, brown adipocytes have been further divided into two groups: "classic" or interscapular brown fat, which are derived from $Pax7^{+}/Myf5^{+}$ precursor cells, and so-called "beige" or "brite" adipocytes, which are induced within white adipose depots in response to cold, sympathetic stimulation, and other stimuli, and which derive from $Pax7^{-}/Myf5^{-}$ cells (Wu, Cohen, & Spiegelman, 2013). The expression profiles of white, brown and beige adipocytes overlap extensively, which have made the identification of transgenic drivers that target one cell type over another extremely difficult (discussed in more detail below).

An additional consideration is the existence of multiple white adipose depots. These are traditionally broken down into "visceral" and "subcutaneous" fat pads, although each group is made up of many smaller depots, each of which may have its own biological properties. The reader is referred to recent reviews on adipose depot heterogeneity for more details (Lee, Wu, & Fried, 2013). Importantly for the purposes of this discussion, however, there are no molecular markers which absolutely distinguish between white depots, and thus one cannot make a true "subcutaneous fat-only" or "visceral fat-only" transgenic or knockout mouse.

1.2. Cellular heterogeneity of adipose tissue

Like most organs, adipose tissue is comprised of several different cell types, including mature adipocytes, preadipocytes, fibroblasts, endothelial cells, and a wide variety of immune cells. These cells interact with one another in very significant ways with profound consequences for local and systemic metabolic function. Many of these cell types express specific markers that can be used to evaluate their contributions to overall metabolic function (e.g., *Tie2* for endothelial cells and *Lyzs* for myeloid-derived cells), but there

are no markers as yet that can be used to specifically study the populations of these cells that reside within the adipose depot. Thus, while one can knock out a gene in all endothelial cells and then study the effects on an isolated fat pad, one cannot knock out a gene *only* in intra-adipose endothelial cells. For the purposes of this chapter, we focus our attention on mature adipocytes, which comprise approximately 50% of the cellular content of a fat pad.

1.3. Developmental stages of adipocytes

Adipocytes develop from precursor cells in a process that can be split into two stages: lineage commitment and terminal differentiation. Because most of the heavily studied cellular models of adipogenesis represent already committed preadipocytes, we know much more about terminal differentiation. There has been a recent flurry of work that has defined pericyte-like cells that represent much earlier stages in adipogenesis, and this has spurred the identification of transcription factors that both promote and repress lineage commitment. Furthermore, the study of multipotent bone marrow stromal cells has also enabled the elucidation of pathways involved in the "bone-fat switch." However, from the perspective of this chapter, it is unclear how to use this information to direct transgenic gene expression at specific developmental stages. For example, *Pdgfra* is expressed in early adipose progenitor cells, but it is also associated with vascular cells not destined to become adipocytes (Lee, Petkova, Mottillo, & Granneman, 2012). Other markers of early adipose precursor cells such as CD24 are also expressed in other stem cells (Elghetany & Patel, 2002). This is also true for markers of committed preadipocytes, such as Pref-1, which is "specific" for preadipocytes versus adipocytes, but which is expressed in several other tissues during embryonic development, including pancreatic β-cells (Carlsson et al., 1997).

2. LOSS-OF-FUNCTION STRATEGIES IN ADIPOCYTES *IN VIVO*

2.1. Overall considerations

In addition to the Cre-loxP-dependent strategies described below, other methods have been employed that should be mentioned, although they will not be described in detail here. First, one can study animals that are global knockouts of the gene of interest. The caveat here of course is that an observed phenotype in adipose tissue might be due to an indirect effect of the gene in another cell type. This can be mitigated somewhat by studying primary adipocytes from such animals (Chiang et al., 2009; Oh et al., 2005;

Shaughnessy, Smith, Kodukula, Storch, & Fried, 2000), or by harvesting preadipocytes, or embryonic fibroblasts for differentiation and subsequent experimentation *ex vivo* (Eguchi et al., 2011; Fisher et al., 2012). Another approach has been to use antisense oligonucleotides or adenoviral-mediated knock-down (Inoue et al., 2008; Jarver et al., 2012; Levine, Jensen, Eberhardt & O'Brien, 1998; Rondinone et al., 2002; Samuel et al., 2006; Yu et al., 2008). When applied via intraperitoneal injection, one can get knock-down in visceral fat pads, but the effect is not seen in all depots nor is it exclusive to fat; liver may be the dominant tissue affected in these studies. Adenovirus can also be directly injected into the fat pad, but again the effect may be seen systemically, it may affect multiple cell types within the pad, and reproducibility between injections can be a major issue. Finally, one can knock-down genes in cultured preadipocytes (e.g., 3T3-F442A cells), implant those cells under the skin of a nude mouse, and then study the fat pad that develops there (Kang et al., 2012; Mandrup, Loftus, MacDougald, Kuhajda, & Lane, 1997). This can be a convenient technique to study gene expression or whether a gene affects adipogenesis, but the amount of fat that grows is typically insufficient to affect the metabolism of the recipient animal.

2.2. Knocking out genes in all fat depots

Pan-adipose gene knockout can be theoretically achieved using any fat-selective marker to drive Cre recombinase. The first and thus most widely used model is the aP2-Cre mouse; aP2 is a lipid-binding protein that is highly expressed in adipocytes, and is encoded by the *Fabp4* gene. Spiegelman's group identified several upstream *cis*-regulatory elements that drive *Fabp4* expression in fat (Graves, Tontonoz, Platt, Ross, & Spiegelman, 1992; Graves, Tontonoz, & Spiegelman, 1992; Ross et al., 1990), and this enabled three different groups to generate mice expressing Cre driven by a 5.4 kb piece of the *Fabp4* flanking sequence (Abel et al., 2001; Barlow et al., 1997; He et al., 2003). Two of these lines (aP2-Cre^{Salk} and aP2-Cre^{BI}) have been used with great frequency to achieve adipose-specific gene deletion. There are general concerns about specificity when using aP2-Cre lines, as aP2 is also expressed in activated macrophages (Fu, Luo, Lopes-Virella, & Garvey, 2002; Makowski et al., 2001). This issue is of more than theoretical concern, as macrophage infiltration into adipose tissue, especially under conditions of overnutrition, has significant effects on local and systemic metabolism (Chawla, Nguyen, & Goh, 2011; Osborn & Olefsky, 2012).

Other issues related to the adipose specificity of available aP2-Cre lines are discussed below.

Other adipose-specific Cre lines have also been developed. Most notably, two lines of mice that express Cre driven by control elements from the adiponectin (encoded by *Adipoq*) locus were generated by the Scherer lab (Adipoq-Cre^{S}) (Wang, Deng, Wang, Sun, & Scherer, 2010) and the Rosen lab (Adipoq-Cre^{R}) (Eguchi et al., 2011). The former is a traditional promoter-driven construct using 5.4 kb of the upstream *Adipoq* flanking sequence. Adipoq-Cre^{R}, in contrast, is a BAC transgenic, with Cre inserted into the translational start site of the *Adipoq* gene in the context of >150 kb of flanking sequence. This means that the majority of the *Adipoq* regulatory elements are present in the Adipoq-Cre^{R} mouse; a potential caveat with this animal is that there are passenger genes on the large BAC that could also affect the resulting phenotype. Yet another mouse was generated by the Lazar lab using a 33 kb fragment of the resistin (*Retn*) gene (Mullican et al., 2013). Resistin was chosen as a driver because its expression was believed to be restricted to white adipose tissue (Steppan et al., 2001), although the Retn-Cre mouse does not adhere to this expectation (Mullican et al., 2013).

2.2.1 Temporal control of adipose-specific recombination

The transgenes described above are all expressed preferentially in mature adipocytes, but the timing of their expression is not well characterized *in vivo*. Studies of adipogenesis *in vitro* suggest that aP2 is induced by Day 2 of differentiation, while adiponectin appears to be turned on around Day 4. We have no idea, however, how this translates into the biology of a living fat pad, in which cells turn over at a low but constant rate (Arner & Spalding, 2010; Spalding et al., 2008; Tchoukalova et al., 2012). One concern is that as these transgenes express at some point during differentiation, the gene knockout effect may alter the subsequent development of the cells. This means that an observed phenotype would represent both developmental and physiological consequences of gene loss, which can be difficult to deconvolute. To circumvent this, one can consider adding a layer of temporal control, most commonly achieved by fusing the Cre transgene to a mutated form of the estrogen receptor that responds to tamoxifen in preference to native estrogens (Danielian, Muccino, Rowitch, Michael, & McMahon, 1998). Such a mouse has been developed by the Chambon/Metzger labs, using the 5.4 kb aP2 promoter to generate a

tamoxifen-inducible aP2-CreERT2 line (Imai, Jiang, Chambon, & Metzger, 2001; Imai et al., 2004).

2.2.2 Tissue specificity

Despite the theoretical concerns about macrophage expression of aP2 that could confound metabolic phenotypes, early studies with both aP2-Cre^{BI} and aP2-Cre^{Salk} lines suggested that both were fairly adipose-specific. However, later studies reported widespread expression during early embryogenesis (Urs, Harrington, Liaw, & Small, 2006), as well as expression in several other tissues including adult lymphatic tissue (Ferrell, Kimak, Lawrence, & Finegold, 2008) and the peripheral and central nervous system (Martens, 2010). This suggests that Cre expression may have become increasingly "leaky" over time in these animals.

Two groups have recently performed a systemic evaluation of several adipose-specific Cre lines by crossing them to reporter mice (R26R-lacZ) in which Cre-mediated recombination can be visualized and quantified by lacZ staining (Lee, Russell, et al., 2013; Mullican et al., 2013; Soriano, 1999). All Cre lines were able to induce recombination in adipose tissue when assessed by X-gal staining or expression of Cre mRNA or protein. However, aP2-Cre^{BI}, aP2-Cre^{Salk}, aP2-CreERT2, and Retn-Cre lines exhibited significant recombination in other tissues including brain and skeletal muscle (see Table 1.1). Such non-adipose recombination can cause unexpected lethality; animals in which *Dicer* or *Hdac3* were knocked out using aP2-Cre^{Salk} die shortly after birth while knockout animals generated with Retn-Cre and/or Adipoq-Cre^{S} survive without gross abnormalities. Of note, aP2-Cre^{BI} mice showed recombination in the developing spermatogonia of ~2% of the seminiferous tubules, which suggest occasional clonal germ-line recombination during spermatogenesis. Indeed, Cre-mediated excision of a target gene was observed in tail DNA when insulin receptor or *Tfam^{flox}* mice were crossed to aP2-Cre^{BI} (Lee, Russell, et al., 2013). Such germ-line recombination (also called the "delta" effect) can potentially cause lethality (Dubois, Hofmann, Kaloulis, Bishop, & Trumpp, 2006) and may result in whole body heterozygous knockout in the next generation if these litters are not excluded from breeding pairs. Unlike aP2-Cre mice, non-adipose tissue recombination was not observed with Adipoq-Cre lines (Eguchi et al., 2011; Lee, Russell, et al., 2013; Mullican et al., 2013; Wang et al., 2010). It is worth mentioning, however, that adiponectin is also expressed in osteoblasts (Erwin Wagner, personal communication), and this

Table 1.1 Comparison of available adipose-specific care lines

	aP2-Cre^{Salk}	aP2-Cre^{BI}	Retn-Cre	Adipo-Cre^{S}	Adipo-Cre^{R}	aP2-CreERT2	UCP1-Cre	UCP1-CreER
Developed by	Evans RM	Kahn BB	Lazar MA	Scherer P	Rosen ED	Chambon/ Metzger Laboratory	Benito M	Wolfrum C
Driver	5.4 kb piece of *aP2/Fabp4* promoter	5.4 kb piece of *aP2/Fabp4* promoter	BAC transgenic containing 23 kb up- and 10 kb downstream of *Retn*	5.4 kb piece of adiponectin promoter/5′-end-coding region	BAC transgenic containing ~150 kb flanking sequence of *Adipoq*	5.4 kb piece of *aP2/Fabp-4* promoter	8.4 kb piece of promoter and 4.3 kb coding region of *Ucp1*	BAC transgenic containing up and downstream of *Ucp1*
Tissue expression	iBAT WAT Liver Skeletal muscle Brain	iBAT WAT Lung Liver Heart Testis Skeletal muscle Brain Clonal germ-line recombination (~2%) Early embryonic development	iBAT WAT Brain	iBAT WAT Osteoblasts (not tested but expected)	iBAT WAT Osteoblasts	iBAT WAT Lung Heart Skeletal muscle Salivary gland	iBAT Beige/brite fat (not tested but expected)	iBAT Beige/brite fat

needs to be considered when interpreting a metabolic phenotype (Karsenty & Ferron, 2012).

2.2.3 Efficiency of recombination

Differences in recombination efficiency might be due to copy number variation and/or positional effects caused by the location of the Cre transgene. This may explain lower recombination efficacy of aP2-CreERT2 compared to aP2-Cre mice, which were both generated with the same 5.4 kb fragment of *Fabp4* promoter (Lee, Russell, et al., 2013). Adipoq-Cre^{R} appears to be more efficient at promoting recombination in fat than either aP2-Cre line, despite a lower level of Cre expression (Lee, Russell, et al., 2013). This might be because Adipo-Cre^{R} directs Cre expression to a more complete population of adipocytes, but this is unclear.

Recombination efficiency can be influenced by the genomic location and distance between the loxP sites of the target locus (Feil, Valtcheva, & Feil, 2009). Adipose-specific Cre lines showed great variation depending on targeted alleles and depots, which is well summarized by Lee, Russell, et al. (2013). It has been shown that knockout efficiency with the use of aP2-Cre^{BI} greatly vary in the adipose tissue depending on the floxed alleles. Multiple genes including *Pparg*, *Hif1a*, *Hif1b*, *Slc2a4*, and *IR* were efficiently ablated but genes like *Tfam* and *Ptp1b* were poorly recombined with the aP2-Cre^{BI}. Similarly, allele-specific differences in recombination efficiency have been shown with aP2-CreERT2. Ablation of *Rxra* and *Pparg* was efficient in this model, while other target alleles such as *Insr* and *Dicer* were poorly recombined. Adipoq-Cre^{R} has shown efficient targeting for multiple alleles including *Tfam*, *Ptp1b*, and *Dicer*, but may prove to be less efficient for other alleles.

There are also depot-dependent differences in recombination efficiency seen with these models. For example, aP2-Cre^{BI} and Adipoq-Cre mice show greater recombination efficiency in BAT than in WAT, aP2-CreERT2 shows modest recombination in subcutaneous WAT with poor recombination in the BAT and perigonadal fat, and Retn-Cre shows high recombination in WAT and a mosaic pattern of recombination in BAT (Lee, Russell, et al., 2013; Mullican et al., 2013). This is in accordance with previous observations from mouse models using these Cre lines. For example, crossing *Hif1b*^{flox}, *Shox2*^{flox}, or *Pparg*^{flox} mice to aP2-Cre mice resulted in more efficient ablation of target genes in BAT than in WAT. When *Shox2* ^{flox} mice were crossed with aP2-Cre^{BI} mice, *Shox2* mRNA was reduced by 48%, 58%, and 81% in perigonadal, subcutaneous, and

brown fat, respectively. When *Tfam*^{flox} mice were crossed with aP2-Cre, a 54% reduction of *Tfam* mRNA was observed in isolated subcutaneous adipocytes, but no difference in Tfam expression in the isolated perigonadal adipocytes, indicating a major depot-specific difference in recombination.

Further, an age-dependent increase in recombination was observed in the fat-specific knockout of *Insr* (FIRKO) mice (Bluher et al., 2002). This might be more apparent than real, as there is a relative reduction in the number of preadipocytes as an animal ages (Alt et al., 2012). Another possibility is that age causes epigenetic modifications to the *Insr* locus that might affect recombination efficiency (Serrano et al., 2005).

Finally, one consideration that is often overlooked is that it is difficult to segregate Cre and floxed alleles when they are present on the same chromosome. This does not affect recombination efficiency at the locus *per se*, but it does make it more difficult to generate large cohorts of floxed, Cre only, and knockout mice.

2.3. Knocking out genes in brown and beige fat

While the field awaits a WAT-specific Cre line, there are options for those who wish to knock out genes in brown fat only. A mouse line that drives Cre from the *Ucp1* promoter was developed by the Benito group in 2001 (Guerra et al., 2001). This line has been used to drive BAT-specific recombination but has not been tested to see if it also expresses in beige cells. Furthermore, there have been concerns about both recombination efficiency and specificity of this line. Additional Ucp1-Cre lines are currently under development. Interestingly, at the time of this writing, a new Ucp1-CreER line has been published which should allow temporal control of recombination in brown and beige fat (Rosenwald, Perdikari, Rulicke, & Wolfrum, 2013).

At present, there is no Cre line that allows for beige-specific recombination without also knocking genes out in interscapular BAT. However, Wu and Spiegelman have identified markers that are selective for beige cells, such as *Slc27a1*, *CD40*, *CD137*, and *TMEM26* (Wu et al., 2012), making such a tool at least theoretically possible.

3. GAIN-OF-FUNCTION STRATEGIES IN ADIPOCYTES *IN VIVO*

In general, strategies to overexpress genes in adipose tissue mirror those used to knock them out, in that both paradigms involve finding an

adipose-specific marker to drive expression of a transgene, be it Cre recombinase or another gene of interest. Accordingly, most adipose-specific transgenic studies have used the 5.4 kb aP2 promoter sequence, as it is the best known. As mentioned above, caveats about non-adipose expression, particularly in macrophages, must be considered. Furthermore, the usual issues applying to any transgenic model can confound the results obtained with these models, such as variation in transgene copy number and position effects. Because of this, it has been generally recommended that more than one transgenic line be studied, to be sure that the observed phenotype is truly due to overexpression of the gene of interest.

These concerns can be largely circumvented using a simple knock-in strategy for transgenic expression involving the ROSA26 locus, identified in 1991 by Friedrich and Soriano (1991). The ubiquitous expression of ROSA26 in embryonic and adult tissues, together with the high frequency of gene-targeting events observed at this locus in murine ES cells has enabled a large number of knock-in lines to be generated. The strategy used by our lab and others is to introduce the transgene of interest into the ROSA26 locus downstream of a "STOP" cassette consisting of multiple polyadenylation signals flanked by loxP sites (Soriano, 1999). Upon temporal and cell-type-specific induction of Cre, transcription of the transgene from the ROSA26 promoter (or from an exogenous promoter inserted into the ROSA26 locus) is induced as a result of the deletion of the loxP-flanked STOP cassette. To trace expression of the transgene *in vivo*, it can be useful to introduce a downstream or upstream reporter gene (usually GFP or lacZ) separated from the transgene by a viral 2A peptide (Fig. 1.1A and B). The 2A peptide cleaves autocatalytically (Szymczak et al., 2004) and allows efficient expression of both transgene and reporter after translation. An internal ribosomal entry site (IRES) can be used instead of the 2A peptide, but the large size and difference in expression levels between genes before and after the IRES can make this a less desirable strategy. The Cre/loxP system controls transgene expression in a time- and cell-type-specific fashion. However, once induced, the transgene can no longer be silenced. To overcome this limitation, the tetracycline (Tet)-controlled system can be superimposed to generate inducible ROSA26 transgenes (Beard, Hochedlinger, Plath, Wutz, & Jaenisch, 2006).

Because this strategy is a gene-targeting approach in embryonic stem cells, it involves homologous recombination and selection of positive clones, which adds to the burden of work early in transgenic generation. However, incorporation directly into the ROSA26 locus eliminates copy number and position effects, which means that there is no longer a need to generate

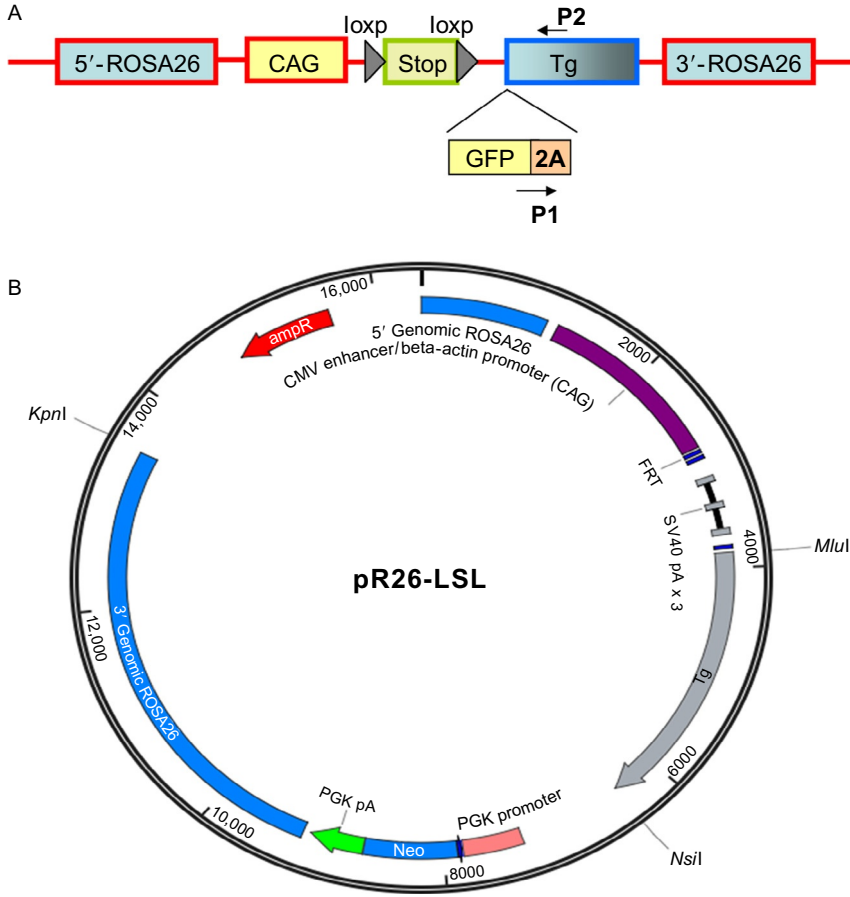

Figure 1.1 Targeting strategy to insert a transgene into the ROSA26 locus. (A) Schematic depicting an example of a targeting construct. ROSA26 targeting arms surround a CAG promoter, a Stop cassette flanked by loxP sites, and a transgene of interest which may be attached to a reporter by a self-cleaving 2A peptide, (B) another view of pR26-LSL showing the entire plasmid.

multiple transgenic lines. Most importantly, the transgene of interest can be expressed in any cell type for which an appropriate Cre line is available, without the need for creating a new construct and founder line.

3.1. Methods

1. Amplify your transgene from cDNA and clone into pR26-LSL using *Mlu*I and *Nsi*I.
2. Linearize the targeting cassette with *Kpn*I (ensure that there is no *Kpn*I site in your transgene) and purify the DNA using gel electrophoresis or

Elutip-D. The Elutip-D method yields the purest DNA for microinjection, whereas the gel purification method is a quick and easy and yields adequately clean DNA for microinjection.

3. Measure the DNA concentration using a spectrophotometer.
4. Microinjection of DNA per the specifications of your local facility. ES cells from 129Sv mice are most commonly used.
5. ES cell selection with neomycin.
6. Identification of correctly targeted ES cell clones. Collect DNA from each neo+ clone using digestion buffer (10 m*M* Tris–HCl, pH 7.6–8.0; 25 m*M* EDTA; 100 m*M* NaCl; 0.5% SDS; 0.25 mg/ml Proteinase K) and probe for the correct insertion of the transgene into the ROSA26 locus by Southern blot or long-range PCR. We currently employ long-range PCR to screen ES cells, using the following primers to screen the 5′ insertion site: 5′-GCCAAGTGGGCAGTTTACCG-3′ (outside of the 5′-arm) and 5′-TAGGTAGGGGATCGGGACTCT-3′ (in the CAG). For the 3′ insertion site, the primers are: 5′-GCCAGCTCATTCCTCCCACTC-3′ and 5′-GGCATGGCAATG TTCAAGCAG-3′ (outside of 3′-arm).
   - **6.1.** Expand ES cell till confluent in 24-well plate
   - **6.2.** Aspirate medium; Add 400 μl of digestion buffer
   - **6.3.** Incubate 60 °C for >3 h
   - **6.4.** Transfer the solution into 1.5 ml tubes
   - **6.5.** Add equal volume of phenol/chloroform, vortex well
   - **6.6.** Extract and precipitate with 100% EtOH
   - **6.7.** Wash twice with 70% EtOH
   - **6.8.** Resuspend in 30–40 μl of TE depending on the size of pellet
   - **6.9.** Measure the concentration of DNA
   - **6.10.** Store the samples at −20 °C or do PCR
   - **6.11.** PCR program:

95 °C	4 min
95 °C	45 s
61 °C	30 s
72 °C	7 min
Go to step 2	34 cycles
72 °C	10 min
4 °C	

7. Generation of chimeric mice.
8. Identification of germ-line transmission. Tail biopsies (5 mm) are collected from 2-week-old mice. Genomic DNA is extracted and tested for the presence of ROSA26 transgene DNA with transgene-specific PCR. We design one pair of primers unique to our gene as shown in Fig. 1.1A. The other pair of primers is: forward 5′-GGCATTAAAGC AGCGTATCC-3′ and reverse 5′-CTGTTCCTGTACGGCATGG-3′ as wild-type.

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