Published in final edited form as:
Nat Protoc. ; 7(2): 374–393. doi:10.1038/nprot.2011.446.

A pipeline for the generation of shRNA transgenic mice

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Abstract

RNA interference (RNAi) is an extremely effective tool for studying gene function in almost all metazoan and eukaryotic model systems. RNAi in mice, through the expression of short hairpin RNA s (shRNA s), offers something not easily achieved with traditional genetic approaches—inducible and reversible gene silencing. However, technical variability associated with the production of shRNA transgenic strains has so far limited their widespread use. Here we describe a pipeline for the generation of miR30-based shRNA transgenic mice that enables efficient and consistent targeting of doxycycline-regulated, fluorescence-linked shRNA s to the *Col1a1* locus. Notably, the protocol details crucial steps in the design and testing of miR30-based shRNA s to maximize the potential for developing effective transgenic strains. In all, this 14-week procedure provides a fast and cost-effective way for any laboratory to investigate gene function *in vivo* in the mouse.

INTRODUCTION

RNAi in mice

RNAi is a conserved cellular mechanism that directs targeted suppression of transcripts through small RNA species. Understanding the basic principles of this endogenous process has allowed RNAi to be adopted in various forms as a tool for suppressing gene expression in almost all metazoan and eukaryotic model systems. Early iterations of RNAi used transfected small interfering RNAs (siRNAs) or vector-based stem-loop shRNAs driven by RNA polymerase III (PolIII) promoters. Later, it was shown that endogenously expressed

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Note: Supplementary information is available via the HTML version of this article.

AUTHOR CONTRIBUTIONS L.E.D. designed and performed experiments, analyzed data and wrote the paper. P.K.P. and J.Z. designed and performed experiments and analyzed data. C.F., K.M., C.M. and Y.P. designed and performed experiments. R.A.D. and G.J.H. designed experiments. S.W.L. designed experiments and wrote the paper.

COMPETING FINANCIAL INTERESTS The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.natureprotocols.com/.

small RNAs known as microRNAs (miRNAs) could provide a scaffold in which to embed a synthetic sequence targeting a gene of interest[1]. This not only enabled more efficient engagement of the RNAi processing machinery and enhanced gene silencing, but also it enabled the expression of these miRNA-based shRNAs (shRNAmirs) from RNA PolII promoters[2,3], including tetracycline (tet) response elements (TREs), which are widely used to reversibly control the expression of protein coding cDNAs. In this form, RNAi delivers a key advantage not offered by traditional genetic approaches—inducible and reversible gene silencing.

Since the first application of RNAi in mammalian cells, a large amount of work has focused on the development of transgenic RNAi mouse strains, to enable gene silencing *in vivo*. These efforts have ranged from pronuclear transgenesis to lentiviral transduction, and, more recently, directed targeting to defined loci (Supplementary Table 1). In most cases, such strategies have highlighted the enormous potential of transgenic RNAi, but the inherent cost and clone-to-clone variability in the generation of RNAi animals by these methods has limited the widespread use of the approach. Indeed, we and others have spent many years designing and evaluating different methods for producing regulated RNAi in mice, but consistently met the same barrier—laborious and time-consuming screening of embryonic stem (ES) cell clones or founder animals to identify a single effective strain. We recently showed that by combining fluorescence-linked shRNA technology, *Col1a1*-based recombinase-mediated gene targeting and tetraploid complementation, the development of RNAi transgenic animals can not only be efficient, but also a straightforward approach for almost any laboratory[4].

The basics of *Col1a1*-targeted shRNA transgenic mice

With the right tools, the process of shRNA transgenic mouse production (outlined in Fig. 1) can be extremely fast and effective. Success relies on two things: a potent shRNA trigger and a reliable system for introducing and expressing these shRNAs. This protocol details the design and testing of miR30-based shRNAs and recombinase-mediated cassette exchange (RMCE) for efficient and reproducible ES cell transgenesis.

In theory, any siRNA or shRNA sequence can be adapted to the miR30 expression cassette and be used to generate a transgenic mouse. However, our experience has shown that ‘validated’ siRNAs or PolIII-driven shRNAs do not always translate well to the miR30 context. Moreover, few, if any, vector-based shRNAs (including shRNAmirs) are validated for gene silencing when expressed as single copies. Unlike most transfection or virus-based shRNA applications that often rely on high levels of plasmid integration, *Col1a1*-targeted shRNA transgenic mice harbor only a single copy of the shRNAmir cassette. Expression at the single-copy level is beneficial as it avoids the complications of shRNA toxicity and/or saturation apparent in other settings[5–7]; however, it demands the use of extremely potent shRNAmirs to achieve effective gene silencing. To identify these shRNAmirs, we use either a large-scale ‘Sensor assay’[8] or a two-step *in silico* filter to triage the thousands of potential shRNA sequences for a given gene (see Experimental design). We then carefully test 10–15 candidates for gene silencing (protein knockdown) when expressed from a single genomic copy. We exclusively use shRNAs embedded within the human miR30 scaffold, and both the retroviral and *Col1a1*-targeting vectors described here are developed to enable simple and standardized XhoI/EcoRI shuttling of shRNAs between all available miR30 backbones. In the targeting vector, the shRNAmir is placed in the 3′ untranslated region of a fluorescent reporter (commonly GFP or RFP) controlled by a tet-responsive element (TRE), providing fluorescence-linked, inducible and reversible gene silencing in cells that express a tet-transactivator. Notably, the TRE promoter provides equal or better levels of shRNA expression than all constitutive promoters we have tested (CMV, MSCV retroviral LTR, EF1-α).

The second phase of the protocol takes advantage of a targeting system developed in Rudolf Jaenisch's laboratory that allows FlpE-mediated integration of a transgenic cassette at single copy to a defined locus—downstream of the *Col1a1* gene[9,10]. Originally designed for regulated expression of cDNAs, transgenic targeting to this defined locus eliminates variation in copy number and expression pattern, often associated with transgenic mouse production. After cloning and testing, potent miR30- based shRNAs are subcloned into the ColA-TRE-GFP-miR30 (cTGM)-targeting construct that is integrated at single copy into KH2 ES cells by Flp-driven RMCE. The KH2 ES cells (developed by Jaenisch's laboratory) contain both the Flp-RMCE recipient locus downstream of the *Col1a1* gene and a second-generation reverse tettransactivator (rtTA-M2) expressed from the *Rosa26* promoter, allowing immediate validation of regulated GFP-shRNAmir expression and gene silencing. Finally, targeted KH2 ES cells can be used to generate transgenic mice by either blastocyst injection or tetraploid embryo complementation, which produces wholly ES cell–derived animals and avoids the need for F1 screening.

Alternative approaches

In the past 10 years, we and others have evaluated a variety of different approaches for generating effective shRNA transgenic animals (Supplementary Table 1). Early efforts from a number of groups reported effective gene knockdown using targeted integration of stem-loop shRNAs expressed by modified PolIII promoters. This approach has since been largely replaced by shRNAmirs, as these synthetic miRNAs show less toxicity than stem-loop–based approaches[5,7] and offer the flexibility to directly link reporter genes, such as GFP, to shRNAmir transcript expression[3,4,11]. In terms of shRNA delivery, the most basic options—viral transduction and pronuclear transgenesis—require minimal vector construction and little or no ES cell manipulation, but, in our experience, they require extensive screening of many founder lines to identify those with efficient knockdown[12]. Moreover, the user has little control of the number of integrated transgenes or the sites of integration. Such issues can be overcome through targeting by homologous recombination, and this approach has been used effectively to derive ES cells and mice carrying a single shRNA at a defined locus[11]. However, as with simple transgenesis, homologous recombination is a relatively inefficient process and usually requires extensive screening to identify functional integrations. We have found that by far the most efficient strategy to reliably introduce shRNA cassettes at single copy is RMCE downstream of *Col1a1*. Although the application of RMCE is not restricted to the *Col1a1* locus, we have recently shown that *Col1a1*-targeted shRNAs can be robustly expressed in almost all tissues in the adult mouse[4]. Thus, in combination with strong ubiquitous or tissue-specific tTA/rtTA strains, this system can provide spatial, temporal and reversible silencing of any gene in the mouse.

Recently, Vidigal *et al.*[13] reported the use of a RMCE strategy for targeting shRNAmirs to the *Rosa26* locus in ES cells and showed that doxycycline (dox)-controlled silencing of well-known developmental transcription factors could generate both hypomorphic and loss-of-function phenotypes during embryogenesis. In essence, both this and the approach described here operate on the same principles and both can be effective; however, there are two key differences to consider: (i) Vidigal *et al.*[13] use an RMCE approach whereby the tTA or rtTA is expressed from the *Rosa26* promoter and is physically linked to the shRNAmir cassette. This genetic linkage is convenient for mouse breeding, but at the same time restricts the flexibility of shRNAmir expression, in that it cannot be combined with other tissue-specific tTA or rtTA transactivator strains. (ii) Both systems incorporate GFP as a fluorescent reporter; however, the configuration of the GFP-shRNAmir cassette reported by Vidigal and colleagues appears to moderately reduce shRNA potency, whereas we and others have noted that the inclusion of GFP as a 'spacer' increases gene silencing[3,4]. This difference in shRNA potency is likely due to the placement of the shRNAmir within an

intronic region of GFP, which we have also noted to reduce shRNA efficacy (J. Pelletier, unpublished data).

In all, we believe that the *Col1a1* system described here offers greater flexibility in breeding and fluorescence-linked shRNA expression; however, these technologies are not mutually exclusive and could be effectively combined to investigate complex genetic questions in the mouse.

Applications of the system

The use of RMCE to target tet-regulated cDNAs to the *Col1a1* locus in KH2 has been well described by Jaenisch's laboratory[9]. We have also recently adapted the platform to allow inducible expression of endogenous miRNAs, and in theory the approach could be used to express any RNA transcript, including long noncoding RNAs. It should be noted that changing the miRNA backbone downstream of GFP can affect fluorescence levels, perhaps due to altered processing of the nascent transcript (Y.P., unpublished data). Regulated shRNAmirs can also be effectively combined with existing genetic models to interrogate more complex biological questions, as we recently showed using a *Kras*-driven model of lung adenocarcinoma[4]. Although such multi-allelic models can be achieved through traditional mouse breeding, we showed that rederiving ES cells containing disease-relevant alleles in addition to the untargeted (empty) *Col1a1* homing cassette substantially reduces the cost and increases the speed of generating flexible models of disease.

Limitations of the system

Robust tTA/rtTA expression—In this system, inducible production of shRNAs requires robust expression of two components from two different genomic loci. These are the TRE-driven shRNAmir downstream of *Col1a1* and a tTA or rtTA. Our initial testing of existing 'ubiquitous' rtTA strains, CMV-rtTA and *Rosa26*-rtTA, showed limited GFP-shRNAmir expression in a variety of adult tissues such as the liver, lung, kidney, pancreas and muscle[4]. We subsequently showed that GFP-shRNAmirs can be efficiently expressed in these tissues using a more sensitive and widely expressed rtTA strain, CAGs-rtTA3 (ref. 4); however, despite these marked improvements, dox-induced GFP-shRNAmir expression is not universal *in vivo* (Supplementary Table 2). The results suggest that TREdriven expression at the *Col1a1* locus is achievable in most tissues, but that adequate tTA/rtTA expression is a key rate-limiting step for potent gene silencing. Thus, although many existing tTA/rtTA strains have been validated using inducible cDNA transgenes, each should be independently validated for its ability to achieve the levels of activation necessary for effective dox-regulated RNAi. In addition, we have recently reported that shRNAs that target the essential gene *Rpa3* can be silenced *in vivo*[14], especially in tissues with a high proliferative rate such as the bone marrow and intestine. The mechanism underlying this silencing remains unclear, but could result from suppression of either the shRNA or rtTA expression. It should also be noted that there are some tissues (e.g., the spleen) in which we consistently see mosaic induction of GFP regardless of the rtTA strain used. Although the reason for this phenomenon is not known, it is a topic of active investigation in our group. Supplementary Table 2 summarizes the expression of GFP and shRNA silencing efficiency in different organs *in vivo* using two alternate tTAs—*Rosa26*-rtTA and CAGs-rtTA3. Notably, in this system, both cell-type specificity and shRNAinduction using different tTA/rtTA strains can be assessed using GFP as a surrogate, quantitative readout.

Dox bioavailability—In addition to robust expression of tTA or rtTA, a further requirement for regulated activation (rtTA) or inactivation (tTA) of the TRE promoter is the bioavailability of dox *in vivo* (a tetracycline analog). As mentioned above, strains such as CAGs-rtTA3 promote widespread dox-dependent TRE expression, suggesting that dox

accessibility is not a major issue in most tissues. However, in at least one tissue—the brain—restricted availability to dox substantially affects shRNAinduction. Whereas dox or minocycline administered through the diet results in little to no induction of GFP-shRNAmir expression in neurons, direct injection of dox into the ventricle of the brain or dox treatment of cultured brain slices promotes strong GFP induction in many neuronal cell types (B. Burbach and A. Zador, unpublished observations). This effect is likely to be a consequence of the blood-brain barrier and specific to neuronal cell types, but it highlights the necessity of dox bioavailability to each organ to maintain shRNAmir regulation.

Depletion is not deletion—shRNA-mediated silencing is different from genetic deletion. Although we and others have shown that RNAi *in vivo* can approximate null phenotypes[4,12,13,15], for some genes (particularly for short transcripts) it is conceivable that there will be no shRNAs that provide 'near-null knockdown'. In circumstances where complete gene loss is required, conventional conditional knockouts represent a viable alternative, although in some cases transgenic shRNAs represent a more flexible option for studying gene function. Moreover, the intentional use of less potent shRNAs to produce hypomorphic states will—as has proven informative in lower organisms—enrich studies of gene function and in some instances better model the partial loss of gene function seen in many human conditions. For example, we recently showed that depletion of adenomatous polyposis coli (APC) in T cells using *Rosa26*-rtTA leads to T-cell acute lymphoblastic leukemia (T-ALL)[4]; however, increasing the dosage of rtTA (using *Rosa26*-rtTA$^{+/+}$ or CAGs-rtTA3), and thus APC knockdown, leads to rapid thymic involution and no T-ALL (L.E.D., unpublished data), similarly to what has been reported using conditional mutant APC alleles[16,17]. In this example, shRNA-mediated depletion of APC more accurately models the situation in human disease, where reduced APC expression (not loss) is linked to T-ALL[18]. Ultimately, we believe that shRNA transgenic mice and traditional genetic alleles will provide a complementary approach to comprehensively study gene function *in vivo*.

Experimental design

shRNA design and cloning—Identification of shRNA sequences that induce potent gene silencing at single copy is one of the most crucial steps in developing an effective shRNA strain. Unfortunately, RNAi triggers that elicit efficient knockdown at single copy are relatively rare (~3% of all possible sequences)[8], thereby underscoring the importance of shRNA design and thorough testing. Currently, the most effective way to confidently identify the best possible shRNAmirs for a given gene is to functionally test every possible sequence using a multiplexed Sensor-based assay that we recently described[8]. Ultimately, this technology will lead to the identification of validated shRNAs targeting all genes, but until then we recommend that each shRNAmir be individually tested for silencing efficacy prior to engineering ES cells. For the identification of new shRNAs, it is possible to use the high-throughput Sensor assay; however, this large-scale approach may not be suitable for every project. As an alternative, we recommend using a two-step *in silico* filter that increases the identification of potent RNAi triggers seven- to eightfold (~3% to ~25%)[8]. To maximize the likelihood of recovering at least two shRNAs that induce effective silencing, we recommend cloning and testing 10–15 high-confidence candidates against each gene of interest. To first narrow the large pool of potential target sequences from each transcript, we use an online siRNA prediction tool: 'Designer of Small Interfering RNAs—DSIR' (http://biodev.extra.cea.fr/DSIR/DSIR.html)[19]. Sequences identified by DSIR can then be cross-checked against the transcript database to exclude sequences that show high similarity or exactly seed matches to 'off-target' genes. Finally, using the 21-mer 'Guide strand' output from DSIR, we apply a series of seven Sensor exclusion criteria recently defined in our lab by using a large-scale nonbiased approach for defining effective shRNA sequences (Fig. 2a)[8]. The top DSIR-scoring 21-mer guide strand predictions that pass the Sensor criteria are

then embedded within PCR or linker cloning templates as detailed in Figure 2b. To generate the appropriate shRNAmir template, the nucleotide immediately 5′ to the 21-mer sense strand is adjusted according to the nucleotide 5′ to the 21-mer target site in the mRNA transcript (Fig. 2b); if the 5′ nucleotide in the mRNAis an A or U, the first base of the 22-mer sense strand becomes a C, and if the 5′ nucleotide in the mRNAis a C or G, the first base of the 22-mer sense strand becomes an A. This adjustment creates a mismatch at the base of the stem-loop that mimics the structure of endogenous miR30 (ref. 20), thereby enabling more efficient processing by the RNAi machinery.

Once they are embedded within a template (Table 1), individual shRNAs can be cloned by two alternate methods (Fig. 2). Although each produces an identical final product in a similar time frame, PCR-based cloning is far more cost efficient and easily scaled, and therefore the preferred method for most laboratories. In rare cases and for unknown reasons, we do not recover specific shRNAs by using the PCR-based approach. As an alternative, 'difficult' shRNA sequences can often be cloned by annealing complementary 110-bp oligos that generate 'sticky' XhoI/EcoRI cloning sites for ligation into miR30-based vectors. We have not observed any positive correlation between those shRNAs that do not amplify by PCR and the level of gene silencing ultimately achieved. In our hands, 97- and 110-bp 'Ultramers' from Integrated DNA Technologies provide the lowest rate of mutations per oligo and are the best option for both cloning approaches.

shRNA testing—Our laboratories (as well as the laboratory of Stephen Elledge) have developed a large number of shRNAmir viral vectors that can be used for testing shRNAmir efficacy[2,3,21,22]. The choice of viral vector and cell type for testing each shRNAis largely dependent on the target gene, although we recommend that the vector confer resistance to an antibiotic (puromycin, hygromycin and so on) for selection of transduced cells. For routine testing, we use the retroviral vector pLMP (Fig. 3)[2], which provides robust constitutive expression of the shRNAmir and a fluorescent and antibiotic selection marker. However, in cases where depletion of the target protein is predicted to have negative effects on cell proliferation or viability, the use of constitutive vectors often leads to premature silencing of the retroviral promoter and an underestimation of gene knockdown. To avoid this, we recommend using a dox-inducible shRNA expression vector such as pTGMP (Fig. 3)[4] or TRMPV-hygro[21] in an rtTA-expressing cell line to allow control of the timing of gene silencing. It should be noted that although TRE-driven shRNAs are still prone to silencing, selection of the transduced cells before shRNA expression provides a window for evaluating knockdown.

To accurately validate knockdown, there are two key requirements: (i) target cells that show expression of the target protein and can be transduced by retro- or lentivirus under conditions that lead to a single genomic integration of the provirus and (ii) an assay that allows direct measurement of the target protein (not RNA) or its activity (for example, by western blotting, ELISA or kinase assay). We discourage the use of quantitative PCR to evaluate RNAi- mediated gene silencing as, in our experience, it does not always accurately reflect the reduction in cellular protein (Supplementary Fig. 1). For testing, we routinely use NIH3T3 cells because they are easily transduced with retrovirus and grow well in cell culture, although any mouse cells can be used. In settings in which NIH3T3 cells do not express the target protein and no viable cell alternative can be found, we generate a Flag-tagged cDNA expression construct (containing the 3′ untranslated region of the endogenous gene) in a hygromycin-selectable retrovirus and produce a stable NIH3T3 cell line expressing this exogenous gene. The stable cell line can then be subsequently retransduced with pLMP shRNAs (see Steps 13–24) and knockdown measured by western blotting using an anti-Flag antibody. We discourage the use of transient co-transfection of shRNAmir and

cDNA expression vectors as a means of testing knockdown efficacy, as this generally results in an overestimation of gene silencing.

As *Col1a1*-targeted shRNAmir transgenic mice generated by this method carry only a single copy of the shRNAmir cassette, it is essential to test the efficacy of each shRNA under these conditions. Although each cell type used for testing will differ in viral transduction, in immortalized fibroblasts (e.g., NIH3T3s, mouse and chicken embryonic fibroblasts), transduction rates between 5% and 20% are optimal for generating cell populations that carry a single viral integration[8]; subsequent antibiotic selection ensures the expansion of only shRNA-expressing cells (Supplementary Fig. 1a). Transduction rates above 30% are often indicative of multiple viral integrations per cell and can lead to an overestimation of the potency of individual shRNAs, as shown in Supplementary Figure 1b. In our experience, shRNAs that show effective knockdown using pLMP at single copy show equivalent or better silencing when they are linked to GFP and expressed from the TRE promoter in the targeted ES cells.

Off-target effects: two strains are better than one—Off-target effects, in the context of RNAi, refer to both sequence-dependent and sequence-independent consequences of siRNA or shRNA expression that are not caused by depletion of the target protein. We recently showed that expression of single-copy, transgenic miR30-based shRNAs in cultured cells does not affect processing of endogenous miRNAs, thereby suggesting that, in this system, sequence-independent off-target effects are not a major source of artifact[4]. However, we still strongly recommend the use of a control transgenic strain such as those targeting Firefly or *Renilla* luciferase[4] to control for potential nonspecific effects of expression of an shRNAmir. Unfortunately, there is currently no way to predict the degree of sequence-dependent off-target effects induced by each individual shRNAmir. As such, the use of appropriate controls is crucial for the generation of reliable experimental data (for a review, see ref. 23). The expression of RNAi-resistant cDNAs to 'rescue' shRNA-induced phenotypes offers one alternative; however, these experiments are technically much more challenging in transgenic mice compared with *in vitro* cell culture, and they create other potential artifacts associated with protein overexpression. Accordingly, we recommend the generation of two independent strains of mice carrying unique shRNAs targeting the same gene, particularly in cases where there is no existing knockout strain to compare and validate null phenotypes. The characterization of two independent strains also offers the opportunity to identify dose-dependent effects of gene silencing if the shRNAs used differ in their level of depletion.

Genotyping shRNA transgenic mice—All transgenic mice produced by *Col1a1* targeting contain common, transgenic-specific sequences that allow the use of a generic genotyping PCR to recognize any integrated transgene at this locus. This PCR is essential for distinguishing between *Col1a1*-transgene heterozygous and homozygous mice. To distinguish different shRNAmir transgenic lines by a simple PCR-based approach, we take advantage of the 22-bp shRNA guide sequence unique to every strain and design a forward PCR primer that spans the loop and guide strand of the shRNA cassette and a reverse primer in the common polyadenylation sequence (RBG-R1 and RBG-R2; Fig. 4a and Table 2). This PCR approach has worked effectively in every case we have tested and it allows the identification of compound transgenic mice that carry both an experimental shRNAmir and a control shRNAmir (for example, Luc.1309 or Ren.713). We routinely generate compound transgenic breeders carrying both an experimental and control shRNAmir because it ensures the segregation of the shRNAmirs in the F1 generation and thus provides age-matched littermate control animals in each experiment (Fig. 4b).

MATERIALS

REAGENTS

- EcoRI (20,000 U ml^{-1}; NEB, cat. no. R0101)
- XhoI (20,000 U ml^{-1}; NEB, cat. no. R0146)
- AgeI (5,000 U ml −1; NEB, cat. no. R0552)
- NcoI (10,000 U ml^{-1}; NEB, cat. no. R0193)
- T4 DNA ligase (2,000,000 U ml^{-1}; NEB, cat. no. M0202)
- Calf intestinal phosphatase (CIP, 10,000 U ml^{-1}; NEB, cat. no. M0290)
- Platinum Pfx DNA polymerase (2.5 U μl^{-1}; Invitrogen, cat. no. 11708-021)

  ▲ CRITICAL Of the DNA polymerases we have tested, only Pfx platinum provides a consistently high yield of the desired product.
- PCR nucleotide mix (Roche, cat. no. 11-581-295-001)
- Ampicillin (Sigma, cat. no. A0166)
- Luria broth (LB; Sigma, cat. no. L3522)
- Competent bacteria (XL10-GOLD; Stratagene, cat. no. 200314)
- QIAquick PCR purification kit (Qiagen, cat. no. 28104)
- QIAquick gel extraction kit (Qiagen, cat. no. 28704)
- KH2 ES cells (Open Biosystems, cat. no. MES4304)
- Drug-resistant 4 (DR4) Mouse embryonic fibroblasts (MEFs) (Open Biosystems, cat. no. MES3948)
- Plat-E ecotropic packaging cells (Cell Biolabs, cat. no. RV-101)
- NIH 3T3 cells (ATCC, cat. no. CRL-1658)
- pCAGGs-FlpE (Open Biosystems, cat. no. MES4488)
- cTGM[4]
- pLMP[2]
- *Col1a1* 3′ probe (Addgene, cat. no. 20731)
- Gelatin (0.1% (wt/vol) in water; Millipore, cat. no. ES-006-B)
- Dulbecco's phosphate-buffered saline (Gibco, cat. no. 14190)
- M15 medium (see REAGENT SETUP)
- Knockout MEM (Gibco, cat. no. 10829)
- ESGRO (LIF; Millipore, cat. no. ESG1106)
- β-Mercaptoethanol (β-ME; Sigma, cat. no. M7154) ! CAUTION Handle concentrated β-ME in a fume cupboard.
- Penicillin-streptomycin-glutamine (Gibco, cat. no. 10378)
- Fetal bovine serum (ES-Cult; Stem Cell Tech, cat. no. 06952)
- 3T3 medium (see REAGENT SETUP)

- DMEM (Gibco, cat. no. 11995-065)
- Newborn calf serum (Invitrogen, cat. no. 16010-159)
- Trypsin-EDTA (0.25% (wt/vol); Gibco, cat. no. 25200)
- DMSO (Sigma, cat. no. D2650)
- Hygromycin B (Roche, cat. no. 10-843-555-001)
- Puromycin-dihydrochloride (Sigma, cat. no. P8833)
- Hexadimethnine bromide (polybrene; Sigma, cat. no. H9268)
- Doxycycline hyclate (Sigma, cat. no. D9891)
- Oligonucleotides (4 nmol; Integrated DNA Technologies)
- Agarose
- Calcium chloride
- Potassium acetate
- Magnesium acetate
- HEPES-KOH
- Ethanol
- Isopropanol
- Blood/tissue harvest kit (Qiagen)
- PrimeIt II labeling kit (Stratagene)

EQUIPMENT

- Cell culture plates (10 cm; BD Falcon)
- Cell culture plates (6 cm; BD Falcon)
- Cell culture plates (6 well; BD Falcon)
- Cell culture plates (24 well; BD Falcon)
- Cell culture plates (96 well; BD Falcon)
- Syringe (20 ml; BD Falcon)
- Syringe filters, 40 μm
- PCR tubes per plates, 0.2 ml
- PCR hood (DNA clean)/DNA clean area
- PCR thermal cycler with ramp/increment mode
- Cell culture incubator (37 °C; CO_2 regulated)
- Bacterial incubator, 37 °C
- Shaking bacterial incubator, 37 °C
- Shaking bacterial incubator, 32 °C
- Water bath, 37 °C
- Water bath, 42 °C

- Water bath, 70 °C
- Electroporator with capacitance extender (Gene Pulser II; BioRad)
- γ-Irradiator
- Flow cytometer
- Cell culture laminar flow hood
- Pipettes
- Spectrophotometer

REAGENT SETUP

Oligonucleotides for PCR cloning (97-mer oligos; MW = ~30,000 Da, 4 nmol = ~120 μg) Resuspend 4 nmol of oligonucleotides in 120 μl of H_2O to make a 1 mg ml^{-1} stock. Serially dilute the stocks in ddH_2O to a final concentration of 0.02 ng μl^{-1}. Resuspended oligos can be stored at −20 °C indefinitely.

Oligonucleotides for linker cloning (110-mer oligos; MW = ~34,000 Da, 4 nmol = ~135 μg) Resuspend 4 nmol of oligonucleotides in 135 μl of ddH_2O to make a 1 mg ml^{-1} stock (if the oligo amount is limiting, concentrations down to 0.25 mg ml^{-1} will also work—complementary oligonucleotides must be combined at equal molar concentrations). Resuspended oligos can be stored at −20 °C indefinitely.

Annealing buffer, 5× For 1 ml of 5× annealing buffer, combine 500 μl 1 M potassium acetate, 300 μl 0.5 M HEPES-KOH (pH 7.4), 20 μl 0.5 M magnesium acetate and 180 μl of ddH_2O. The buffer can be stored at room temperature (25 °C) indefinitely.

M15 culture medium To 500 ml of Knockout MEM, add 90 ml of ES-Cult serum, 6 ml of penicillin-streptomycin-glutamine, 6 ml of 100× β-ME (to make the 100× stock, add 37 μl of β-ME to 50 ml of PBS) and 60 μl of ESGRO (LIF). Store for a maximum of 1–2 months at 4 °C.

M15 1× freezing medium For 100 ml of M15 freezing medium, combine 50 ml of M15 medium, 40 ml of ES-Cult serum and 10 ml of DMSO. Store for a maximum of 2–3 months at 4 °C.

Feeder cells medium To 500 ml of Knockout MEM, add 60 ml of ES-Cult serum, 6 ml of penicillin-streptomycin-glutamine and 6 ml of 100× β-ME. Store for a maximum of 2–3 months at 4 °C.

3T3 medium To 500 ml of DMEM, add 50 ml of calf serum and 5 ml of penicillin-streptomycin-glutamine. The medium should be used or discarded within 2–3 months at 4 °C.

PROCEDURE

Design ● TIMING **1 h**

1 Define the region of mRNA sequence to be targeted—usually the minimal common transcript region from the current NCBI database (http://www.ncbi.nlm.nih.gov/gene) —and run shRNA predictions at http://biodev.cea.fr/DSIR/DSIR.html. Check the predicted 21-mer guide strands for compliance with Sensor criteria (Fig. 2). Exclude sequences that do not comply.

Optionally, for large-scale testing across multiple genes, validate 60–70 predictions per gene by using the Sensor assay[8].

2 Design miR30 97-mer PCR templates for PCR cloning or 110-bp oligonucleotides for linker cloning according to the instructions in Figure 2.

shRNA cloning

3 Prepare PCR templates or linker oligonucleotides as described in REAGENT SETUP.

4 Clone shRNAs by either PCR (option A) or linker cloning (option B). PCR is generally preferable (see INTRODUCTION).

A. PCR cloning of shRNAs ● TIMING **8 h**

i. Prepare the PCR master mix using Pfx Platinum polymerase in a 'DNA clean' area such as a PCR hood.

PCR reaction		
Component	**Amount per well (µl)**	**Final**
H_2O	33.5	
PCR buffer, 10×	5	1×
$MgSO_4$ (50 mM)	1	1 mM
DNTP (2.5 mM each)	5	0.25 mM each
5′miR-Xho (10 µM)	2	0.4 µM
3′miR-Eco (10 µM)	2	0.4 µM
Pfx platinum DNA polymerase	0.5	1.25 U

▲ CRITICAL STEP Do not prepare PCR reagents in an area of the laboratory that is in frequent contact with miR30-based plasmids. The same primers are used for PCR cloning into each miR30 vector, and as such the PCR is very sensitive to contamination.

ii. Add 1 µl of template oligonucleotide (0.02 ng μl^{-1}) and cycle the PCR as detailed below. Include one well that does not contain template as a negative control.

Cycle number	Denature	Anneal	Extend
1	94 °C for 4:00		
2–33	94 °C for 0:15	54 °C for 0:30	68 °C for 0:25
34			68 °C for 5:00

iii. Run 5 µl of PCR product on a 2.5% (wt/vol) agarose gel to check amplification. The expected band is 131 bp. Occasionally, this PCR produces an additional higher band, although this does not markedly interfere with subsequent cloning.

? TROUBLESHOOTING

iv. Isolate the PCR product by using a Qiagen PCR purification column (per the manufacturer's instructions) and elute the DNA in 30 µl of Tris (pH 8.0) (EB buffer; part of the Qiagen kit) after a 10-min incubation on the column. At this point, you can check the presence of DNA by sensitive spectrophotometry.

PAUSE POINT PCR product can be stored at 4 °C.

v. Digest the purified PCR product for 3–4 h at 37 °C.

Restriction digest		
Component	**Amount per well (µl)**	**Final**
Purified PCR product	30	
EcoRI buffer, 10×	5	1×
BSA (10 mg ml^{-1}) 100×	0.5	0.1 mg ml^{-1}
XhoI (20,000 U ml^{-1})	1	20 U
EcoRI (20,000 U ml^{-1})	1	20 U
DNase-free H_2O	12.5	

vi. Run the digested sample on a 2% (wt/vol) agarose gel and excise the 110-bp digested PCR product.

vii. Purify the digested fragment using the Qiagen gel extraction kit. Melt the gel in 6 volumes of Buffer QG (part of the Qiagen kit) and add 1 volume of isopropanol to help the precipitation of the small fragment. Incubate at room temperature for 5–10 min before loading onto the column. Elute DNA in 30 µl of Qiagen EB buffer after a 10-min incubation on the column.

B. Linker cloning of shRNAs ● TIMING 3 h

i. Order each 110-bp oligonucleotide with 5′-end phosphorylation. This phosphate addition ensures that the annealed linker will ligate with the dephosphorylated vector backbone.

ii. Resuspend the phosphorylated 110-bp oligonucleotides for cloning at 1 µg $µl^{-1}$. If the amount of oligo is limited, they can be resuspended at a lower concentration (down to 0.25 µg $µl^{-1}$), but each pair to be annealed must be present at the same molar ratio.

iii. Combine the complementary oligonucleotides in 0.2-ml PCR tubes as outlined below.

Component	**Amount per well (µl)**	**Final**
DNase-free H_2O	20	
Annealing buffer (REAGENT SETUP), 5×	10	1×

Component	Amount per well (μl)	Final
Sense oligo	10	10 μg
Antisense oligo	10	10 μg

iv. Run the following PCR program to denature and slowly cool the reaction to anneal.

Cycle number	Temperature	Time
1	95 °C	5 min
1	80 °C	10 min
Ramp (50 cycles)	80–55 °C	−0.5 °C/2.5 min

■ PAUSE POINT Annealed oligos can be stored at 4 °C for 6 months.

v. Purify the annealed linker by using the Qiagen PCR purification kit according to the manufacturer's instructions. Determine the DNA concentration by spectrophotometry.

XhoI/EcoRI subcloning of shRNAs ● TIMING 1–2 d

5 Digest the recipient miR30-based vector (usually pLMP) with XhoI/EcoRI for 4 h at 37 °C.

Component	Amount per tube (μl)	Final
EcoRI buffer, 10×	5	1×
BSA (10 mg ml^{-1}), 100×	0.5	0.1 mg ml^{-1}
pLMP (1 μg $μl^{-1}$)	5	5 μg
EcoRI (20,000 U ml^{-1})	1.5	30 U
XhoI (20,000 U ml^{-1})	1.5	30 U
DNase-free H_2O	36.5	

6 Heat-inactivate the enzymes at 70 °C for 15 min.

7 Add 10 U (1 μl) of CIP and incubate for 1 h at 37 °C.

8 Purify the vector backbone with a Qiagen PCR purification kit. It is not necessary to gel-purify the vector backbone; excessive exposure to DNA-damaging UV light and impurities remaining from the agarose gel extraction may decrease subsequent cloning efficiency.

9 Ligate 4 ng of digested 110-bp PCR product or annealed linker with 100 ng of XhoI/EcoRI-digested pLMP (molar ratio ~3:1) overnight at 15 °C (most efficient) or at room temperature (25 °C) for 1 h.

▲ CRITICAL STEP Do not increase the insert/vector molar ratio over 5:1, as this will result in shRNA concatemer insertions.

Ligation		
Component	**Amount per tube**	**Final**
Ligation buffer, 10×	1 µl	1×
XhoI/EcoRI digested vector (e.g., pLMP)	100 ng	100 ng
XhoI/EcoRI digested PCR product/linker (4 ng µl^{-1})	1 µl	4 ng
NEB DNA ligase (2,000,000 U ml^{-1})	0.5 µl	1,000 U
DNase-free H_2O	Up to 10 µl	

Include a vector-only control ligation (no PCR product/linker) to assess the background colony number after transformation.

10 Combine each ligation reaction with competent XL10-GOLD bacteria in a 1:20 (ligation/bacteria) ratio on ice (e.g., 5 µl of ligation mixture with 100 µl of bacteria). Mix by gently agitating the tube. Do not pipette up and down.

11 Incubate the tubes on ice for 10–20 min and transform bacteria as required (heat shock or electroporation), depending on how the competent cells were prepared. For individual shRNA cloning and subcloning, the ligation efficiency is generally high and it is not necessary to use commercial ultracompetent bacteria. We routinely use homemade chemically competent XL10-GOLD bacteria prepared using calcium chloride[24] and then transform the reactions by heat shocking at 42 °C for 45 s. We have also successfully used Top10, DH5-α, JM110 and STBL3 bacterial strains for expanding miR30-based plasmids. If you are using commercial competent bacteria, follow the manufacturer's instructions for the transformation of ligation reactions.

12 Streak out on LB agar + ampicillin (100 µg ml^{-1}) plates and grow overnight at 37 °C.

13 If the number of colonies on each ligation plate is at least four- to fivefold greater than that on the control plate, pick three or four colonies per shRNAinto 4 ml of LB + ampicillin (100 µg ml^{-1}) and culture them overnight at 37 °C with shaking. Miniprep using the Qiagen (or similar) DNA miniprep kit and screen clones by sequencing them with the miR30seq primer. If the number of colonies on the control plate is equivalent to that of the ligation plates, repeat the ligation and/or cloning procedure.

? TROUBLESHOOTING

Testing knockdown: virus production and collection ● TIMING **3 d**

14 Plate Plat-E cells at 2 × 10^6 cells per 6-cm plate.

15 When cells are 90% confluent (usually 6–8 h later), transfect 5 µg of each miR30 vector by calcium phosphate precipitation[25] or by using a lipid-based transfection reagent such as FuGENE (Promega) or Lipofectamine (Invitrogen).

▲ CRITICAL STEP Virus from a control shRNA such as Luc.1309 (ref. 26) or Ren. 713 (ref. 21) should also be produced.

16 At 18 h after transfection, rinse the cells twice with 4 ml of room-temperature PBS and replace it with 2.5 ml of viral collection medium (DMEM with 10% (vol/vol) calf serum; used to culture the target cells).

17 Collect viral supernatant 36, 48 and 54 h after transfection. After the final viral collection, pool supernatants and remove debris using a 0.45-µm syringe filter.

■ PAUSE POINT Viral supernatant can be stored at 4 °C for up to 2 weeks and indefinitely at −80 °C. Titer will rapidly reduce over time at 4 °C.

Target cell transduction and selection ● TIMING 8–10 d

18 For each shRNA to be tested (including the control shRNA), plate 75,000 NIH 3T3 cells per well in three wells of a six-well plate, 24 h before transduction. Plate one additional well to use as a nontransduced control for flow cytometry and antibiotic selection.

19 To 2 ml of cell culture medium, add 2 µl of polybrene (4 mg ml^{-1}) and 200 µl, 40 µl or 10 µl of viral supernatant to generate working viral dilutions of ~1/10, 1/50 and 1/200; optimal virus dilution will depend on both viral titer and target cell type.

20 Incubate the cells with virus for 24 h at 37 °C.

21 Remove the virus-containing medium and recover cells in 2 ml of normal culture medium for 24 h.

22 Trypsinize cells and analyze each transduced population by fluorescence flow cytometry to determine the percentage of transduced cells (transduced cells express GFP).

23 For each shRNA, choose a transduced population that contains 5–20% GFP-positive cells (approximating a low multiplicity of infection (MOI)) and replate all cells in one well of a six-well plate.

24 Add puromycin to a final concentration of 2 µg ml^{-1} and select for 3–5 d, or until 24 h after all nontransduced control cells are dead. Do not let cells become 100% confluent; passage as necessary. Confirm selection of the retrovirus by flow cytometry; expect >99% GFP-positive cells (Supplementary Fig. 1a).

25 Measure protein knockdown by western blot analysis, ELISA or functional assay (e.g., kinase assay).

▲ CRITICAL STEP RNA levels as measured by quantitative PCR do not always accurately report the level of protein reduction after shRNA expression (Supplementary Fig. 1b,c). We strongly encourage direct measurement of protein level.

Subcloning into cTGM ● TIMING 1 d

26 Digest miR30-containing plasmid and *Col1a1*-targeting vector (cTGM) with XhoI/EcoRI for 3–4 h at 37 °C.

Component	Amount per tube	Final
EcoRI buffer, 10×	5 µl	1×
BSA (10 mg ml^{-1}), 100×	0.5 µl	0.1 mg ml^{-1}
Plasmid DNA	10 µg (miR30 plasmid)/5 µg (cTGM)	
EcoRI (20,000 U ml^{-1})	1.5 µl	30 U
XhoI (20,000 U ml^{-1})	1.5 µl	30 U
DNase-free H_2O	Up to 50 µl	

27 Gel-extract the 110-bp shRNA fragment as described in Steps 3A(vi) and 3A(vii).

28 To the digested cTGM vector, add 10 U (1 µl) of CIP and incubate for 1 h at 37 °C.

29 Ligate the shRNA fragment with the cTGM targeting vector as detailed below:

Component	Amount per tube	Final
NEB ligation buffer, 10×	1 µl	1×
XhoI/EcoRI digested cTGM	100 ng	100 ng
XhoI/EcoRI digested 110-bp shRNA	4 ng	4 ng
NEB DNA ligase (2,000,000 U ml^{-1})	0.5 µl	1,000 U
DNase-free H_2O	Up to 10 µl	

30 Ligate and transform each reaction as described in Steps 9–11. Miniprep and screen three or four clones per shRNA for correct integration by sequencing with the miR30seq primer.

■ **PAUSE POINT** DNA can be stored indefinitely at −20 °C.

31 Retransform verified cTGM clones into XL10-GOLD or Top10 bacteria and streak out on an LB agar plate (+ ampicillin). Pick two clones into 4 ml of LB + ampicillin and culture them at 37 °C with shaking for 8–10 h (starter culture). Choose one clone and immediately transfer 1 ml of starter culture into 400 ml of LB containing ampicillin; culture for 14–18 h at 32 °C with shaking. The cTGM vector can be difficult to subculture in bacteria; we have obtained more consistent results when using freshly grown clones picked into starter culture and immediately expanded into a larger volume.

32 Prepare the cTGM vector using the Qiagen maxiprep kit according to the manufacturer's instructions. Wash the DNA pellet three times with 70% (vol/vol) ethanol to reduce salts for subsequent electroporation, and then resuspend the DNA in 10 mM Tris (pH 8.0) at a concentration of 2 µg $µl^{-1}$.

33 Sequence-confirm the identity of the maxiprep DNA with the miR30seq primer.

ES cell electroporation and selection ● TIMING 14–18 d

34 Thaw KH2 ES cells on top of the feeder layer (Box 1) in M15 medium (REAGENT SETUP) at least 3 d before electroporation, and longer if many electroporations are to be performed. Expand ES cells (on feeders) to generate enough cells for each electroporation (5×10^6 cells per electroporation).

35 Six hours before electroporation (cells should be 70–80% confluent), replace the M15 medium on ES cells.

36 Rinse the ES cells with PBS and add 1 ml of 0.25% (wt/vol) trypsin-EDTA.

37 Incubate the cells at 37 °C until they become detached from the plate (5–10 min). Tap the plate gently to help release the cells. Incubating in trypsin for longer than 15 min may decrease cell viability and reduce targeting efficiency.

38 For each electroporation condition, coat two wells of a 24-well plate with gelatin (as described in Box 1). These will be used to plate cells for testing electroporation efficiency.

39 Add 4 ml of M15 medium to inactivate the trypsin and triturate the cells with a 5-ml pipette 8–10 times to obtain a homogenous single-cell suspension.

40 Centrifuge the cells at 200*g* for 5 min at 4 °C; cells should remain on ice for the remainder of the electroporation procedure.

41 Resuspend the cells in 5 ml of cold PBS and centrifuge at 200*g* for 5 min at 4 °C.

42 Resuspend the cells at 5×10^6 cells per ml in cold PBS.

43 To an ice-cold 1.5-ml microcentrifuge tube, add 12.5 µl of pCAGs-FlpE (2 mg ml^{-1}), 25 µl of cTGM (2 mg ml^{-1}) and 900 µl of ES cells in PBS.

Component	Amount per tube (µl)	Final
pCAGs-FlpE (2 mg ml^{-1} in H_2O)	12.5	25 µg
cTGM (targeting vector in H_2O)	25	50 µg
KH2 ES cells (in PBS)	900	4.5×10^6 cells

44 Mix the cell suspension gently with a P1000 pipette, transfer it to an ice-cold 0.4-cm cuvette and incubate it on ice for 5 min.

45 Electroporate the mixture with two consecutive pulses at 400 V (125 µF) in a Bio-Rad Gene Pulser and immediately return the cuvette to ice.

46 Incubate the cuvette on ice for 10 min.

47 Add 1 ml of ES cell medium to each cuvette using a 2-ml pipette and gently mix the cell solution (cell mixture may be viscous because of dead cells releasing genomic DNA).

48 Add 100 µl of the electroporation mixture to one well of a gelatin-coated 24-well plate containing 900 µl of M15 medium, and to one well containing 900 µl of M15 and 1 µg ml^{-1} dox. Add the entire remaining volume (~1.8 ml) dropwise to a 10-cm plate containing feeders and 8 ml of M15 medium.

49 At 24 h after electroporation, Replace the medium on each of the 10-cm plates with fresh M15. It is not necessary to change the medium on the 24-well plate(s).

50 At 48 h after electroporation, trypsinize each well of the 24-well plate (± dox) and analyze by flow cytometry. If > 10% are GFP positive in the presence of dox, continue with Step 51. If < 10% of cells are positive, repeat electroporation (Steps 36–48). We routinely observe that electroporation efficiency > 10% reproducibly results in more than ten hygromycinresistant ES cell clones by day 10, of which more than 80% show single-site integration at the *Col1a1* locus.

? TROUBLESHOOTING

51 At 48 h after electroporation, replace M15 medium on electroporated ES cells in 10-cm plates with M15 medium containing 140 µg ml^{-1} hygromycin B.

52 Select cells in hygromycin B-containing medium for 8–12 d, refreshing the medium every day. Note that correctly integrated hygromycin-resistant colonies are usually visible to the naked eye 7–8 d after electroporation, and should be picked by day 12 after electroporation. Some ES clones may appear after this time, although these usually do not show *Col1a1* targeting or GFP induction with dox.

? TROUBLESHOOTING

Picking and expanding ES cell clones ● TIMING picking clones 1 h, expanding 6–7 d

53 Select ES cell clones to be picked. Not all ES cell clones that survive hygromycin selection should be picked. Clones that show compact, regular and 3D morphology with bright edges should be chosen for further testing (Fig. 5a). In these clones, you should not be able to distinguish individual cell boundaries. Clones that are small with clear cell borders or show flattened 'fried egg' morphology are typical of differentiating clones and should not be picked (Fig. 5b). If possible, avoid picking clones that are directly adjacent to one another (Fig. 5b) in order to minimize the potential of expanding a 'mixed clone'.

? TROUBLESHOOTING

54 For each clone to be picked, add 100 µl of 0.5% (wt/vol) trypsin-EDTA to each well of a 96-well plate.

55 Rinse the plate containing the clones twice with room-temperature PBS (no Ca^{2+} /Mg^{2+}) and cover the cells with 5 ml of PBS.

56 By using a piece of nonreflective black paper underneath the plate to improve contrast, isolate each clone by mechanically dislodging and drawing the entire clone into a filtered P20 pipette tip with a small amount (~10 µl) of PBS.

57 Transfer each clone into one well of a 96-well plate containing 100 µl of 0.5% (wt/vol) trypsin-EDTA (prepared as above) and continue picking further clones. We routinely pick four to six clones per shRNA targeted.

58 Incubate each clone in trypsin-EDTA for 10–15 min, triturating 5–6 times in the middle of the incubation to break up clumps of cells.

59 To each well, add 100 µl of M15 medium and triturate 5–6 times to obtain a single-cell suspension.

60 Transfer the entire 200 µl to one well of a 24-well plate containing irradiated DR4 feeders and 800 µl of M15 medium + hygromycin B.

61 Maintain ES cells in hygromycin for a further 72 h, and then switch to M15 medium without antibiotic. Note that although they are resistant to hygromycin, ES cells generally perform better in culture in the absence of antibiotic selection.

62 Expand each ES clone into a 10-cm plate, and when they are 70–80% confluent, cryopreserve three or four vials of each clone (2–3 million cells per vial) in M15 freezing medium (REAGENT SETUP).

Testing ES cell clones ● TIMING 4–5 d

63 For each clone, resuspend 600,000 cells in 4.4 ml of M15 medium.

64 Plate 2 ml (250,000 cells) of the cell suspension onto irradiated DR4 feeders.

65 To the remaining volume, add 2.4 µl of 1 mg ml^{-1} dox (final concentration 1 µg ml^{-1}) and plate 2 ml onto irradiated DR4 feeders.

66 After 2 d, trypsinize and count the cells. Replate 250,000 cells (from each well) into a new well of a six-well plate coated with gelatin (no feeders).

67 Plate 500,000 untreated cells from each clone onto a 6-cm plate coated with gelatin (no feeders). These cells will be used to isolate genomic DNA for Southern blotting. Analyze the remaining cells (± dox) by flow cytometry to check the induction of GFP expression in the cells treated with dox. All dox-treated ES cells should show strong expression of GFP as measured by

microscopy (Fig. 5c) or flow cytometry (Fig. 5d,e). If the clone does not uniformly induce GFP, discard and repeat the targeting.

? TROUBLESHOOTING

68 Four days after the beginning of dox treatment, analyze knockdown (± dox) by western blotting or another appropriate functional assay.

69 Isolate the genomic DNA from ES cells on the 6-cm plate using the Qiagen blood/tissue harvest kit or equivalent genomic DNA extraction protocol.

Southern blot ● TIMING **3–4 d**

70 To a 1.5-ml tube, add the following:

Component	Amount per tube (µl)	Final
EcoRI buffer, 10×	5	1×
BSA (10 mg ml^{-1}), 100×	0.5	0.1 mg ml^{-1}
Genomic DNA (1 mg ml^{-1})	10	10 µg
EcoRI (20,000 U ml^{-1})	2.5	30 U
DNase-free H_2O	32	

71 Incubate the reaction overnight at 37 °C.

72 Add 0.5 µl of fresh EcoRI and incubate for a further 2 h to complete genomic DNA digestion.

73 Run 5 µl of the digest on a 0.7% (wt/vol) agarose gel. EcoRI-cut genomic DNA should show a high-molecular-weight smear (3 kb and above) and a prominent band at ~1,400 bp. Use remainder of the digest sample to perform the Southern blot.

74 Isolate a GFP probe for Southern blotting by digesting the cTGM vector as indicated below.

Component	Amount per tube (µl)	Final
NEB buffer 1, 10×	5	1×
BSA (10 mg ml^{-1}), 100×	0.5	0.1 mg ml^{-1}
cTGM (1 mg ml^{-1})	10	10 µg
NcoI (10,000 U ml^{-1})	1.0	10 U
AgeI (5,000 U ml^{-1})	2.0	10 U
DNase-free H_2O	31.5	

75 Run the digest on a 1% (wt/vol) agarose gel and excise the 700-bp band corresponding to GFP.

76 Purify the DNA using the Qiagen gel extraction kit and generate ^{32}P-labeled probe using the Stratagene PrimeIt II labeling kit according to the manufacturer's instructions.

77 Perform Southern blots by using the *Col1a1*-3′ probe (REAGENTS) and GFP probes on each individual ES cell clone as previously described[27]. Single

integration at the *Col1a1* locus will result in a single 4.04-kb band when hybridized with the GFP probe. The presence of a second band at any molecular weight indicates a second-site integration of the GFP-miR30 cassette (10–15% of clones show a second random integration). Any clones showing one or more integrations in addition to the *Col1a1* locus should not be used for mouse production. Correct integration at the *Col1a1* locus will result in two equal-intensity bands when hybridized with the *Col1a1*-3′ probe: 6.2 kb (wild-type) and 4.1 kb (targeted). Untargeted KH2 ES cells show two bands at 6.7 kb (FRT) and 6.2 kb (wild-type)[9].

Mouse production ● TIMING **21 d**

78 Produce mice by tetraploid embryo complementation using correctly targeted ES cell clones. You can follow the detailed protocol for the generation of mice by this method as previously described[28]; however, tetraploid complementation is a technically challenging procedure, and as such we recommend outsourcing it to an experienced transgenics facility. If tetraploid complementation is not available, standard blastocyst injection can be used effectively, and in our experience founder animals show 80–95% contribution from the KH2 donor cells and the GFP-shRNAmir transgene transmits efficiently to the F1 generation.

? TROUBLESHOOTING

shRNA-specific genotyping ● TIMING **4 h**

79 Design two forward primers with melting temperatures between 56 and 62 °C, which overlap the common loop region and the unique guide strand. The 3′ region of the forward primer should terminate within the shRNA unique sequence to avoid mispriming in the common downstream region. We recommend testing two alternate forward primers with each of two common reverse primers (RBG-R1 and RBG-R2).

80 Prepare genomic DNA from founder animals using the genomic DNA extraction kit or equivalent genomic DNA isolation compatible with PCR amplification.

81 Prepare the PCR master mix as described below and add 24 μl to each well of a 96-well PCR plate on ice.

Component	Amount per well (μl)	Final
DNase-free H_2O	16.5	
PCR buffer, 10×	2.5	1×
dNTPs (2.5 mM each)	2.5	0.25 mM
Forward (shRNA-specific) primer (10 μM)	1	0.4 μM
Reverse (common) primer (10 μM)	1	0.4 μM
Taq polymerase	0.5	

82 Add ~100 ng of genomic DNA to each well and cycle according to the protocol listed below.

Cycle number	Denature	Anneal	Extend
1	95 °C for 2:00		
2–35	95 °C for 0:30	56°C for 0:30	72 °C for 0:45
36			72 °C for 5:00

83 Run PCR product on a 2% (wt/vol) agarose gel. Exact product size will depend on the precise location of the forward primer; however, using common reverse primer RGB-R1 will generate a specific product of ~200 bp, and using RGB-R2 a product of ~250 bp.

Col1a1 genotyping

84 To distinguish homozygous versus heterozygous at the targeted *Col1a1* locus, prepare the PCR master mix as described below and add 24 μl to each well of a 96-well PCR plate on ice.

Component	Amount per well (μl)	Final
DNase-free H_2O	15.5	
PCR buffer, 10×	2.5	1×
dNTPs (2.5 mM each)	2.5	0.25 mM
ColA1 forward primer (10 μM)	1	0.4 μM
ColA1 reverse primer (10 μM)	1	0.4 μM
SAdpA reverse primer (10 μM)	1	0.4 μM
Taq polymerase	0.5	

85 Add ~100 ng of genomic DNA to each well and cycle according to the protocol listed below.

Cycle number	Denature	Anneal	Extend
1	95 °C for 2:00		
2–35	95 °C for 0:30	56 °C for 0:30	72 °C for 0:45
36			72 °C for 5:00

86 Run the PCR product on a 2% (wt/vol) agarose gel. This PCR generates a specific product of ~220 bp for the wild-type allele and a product of ~295 bp for the targeted allele.

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 2.

● TIMING

As outlined in Figure 1, the production of any shRNA transgenic mouse can be completed in as little as 14 weeks. However, in our experience, it is sometimes necessary to repeat the shRNA (PCR or linker) cloning to generate all designed shRNA constructs for testing. In addition, it is occasionally necessary to repeat ES cell targeting and/or submit a second ES

cell clone for mouse production (if the first clone fails to generate viable animals by tetraploid embryo complementation). Although these steps require additional time, given access to all necessary facilities the entire procedure should not take longer than 20 weeks.

ANTICIPATED RESULTS

We have successfully used this shRNA pipeline to produce more than 1,000 targeted ES cell clones and more than 50 different shRNA transgenic mouse strains. We have produced many different transgenic lines by using the TRE-based *Col1a1* targeting vector[4], and these mice breed at expected Mendelian ratios. In circumstances where we have targeted genes essential for proliferation[14], sterility in founder mice suggested that there may be 'leaky' shRNA expression in some tissue in adult mice, in this case the testis. Substituting the TRE promoter for a less leaky TREtight promoter eliminated founder sterility, although it still provided efficient gene silencing in many tissues, such as the skin and intestine[14]. However, it is important to note that in our experience the TREtight promoter does not drive the same level of expression as TRE in a number of tissues (kidney, lung, heart, liver and others) in the adult mouse when controlled by the same tTA. The reason(s) for this effect and strategies to improve inducible expression in all tissues remain an ongoing area of investigation in our laboratory. In all, we have developed three alternative shRNA targeting cassettes: TRE-GFP-miR30 (cTGM), TREtight-GFP-miR30 (cTtGM) and TREtight-turboRFP-miR30 (cTtRM) (Fig. 3). These vectors can be used interchangeably with respect to shRNA cloning and targeting, providing more flexibility for the user to customize transgenic mouse production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank C. Beard and R. Jaenisch (Whitehead Institute) for pBS31 flp-in and pCAGs-FLPe-Puro vectors, *Col1a1* 3′ probe and KH2 ES cells. We thank J. Bolden, A. Lujambio and J. White for advice on technical procedures and critical review of the manuscript. Thanks to L. Bianco, J. Coblentz, E. Earl and the Cold Spring Harbor Laboratory animal house staff. We gratefully acknowledge J. Simon, D. Grace and J. Cappellani for technical assistance, and members of the Lowe laboratory for advice and discussions. This study was supported by a Mouse Models of Human Cancer Consortium grant and a program project grant from the National Cancer Institute. P.K.P. was a Medical Science Training Program Fellow of Stony Brook University, L.E.D. is supported by a National Health & Medical Research Council of Australia overseas Biomedical Training Fellowship, C.M. was supported by a fellowship from the Deutsche Forschungsgemeinschaft and an American Association for Cancer Research– Astellas USA Foundation Fellowship in Basic Cancer Research, J.Z. was the Andrew Seligson Memorial Fellow, R.A.D. is a Victorian Endowment for Science, Knowledge and Innovation (VESKI) Fellow and G.J.H. and S.W.L. are Howard Hughes Medical Institute investigators.

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Box 1 | Irradiated DR4 feeders and gelatin coating plates

Irradiated DR4 MEF feeder cells are produced from mice that have been engineered with resistance to four different antibiotics (puromycin, G418 (neomycin), hygromycin, 6-thioguanine)[30]. DR4 MEFs can be purchased from Open Biosystems (REAGENTS) or, alternately, MEFs can also be generated by harvesting cells from E13.5 embryos from DR4 transgenic mice as previously described[31].

Generating irradiated feeder cells ● TIMING 7 d (for 25 vials)

i. Thaw frozen DR4 MEFs and culture them in DMEM containing 10% (vol/vol) FCS (growth medium) for several (two or three) passages to obtain approximately 2×10^8 cells. If possible, culture MEFs in a low-oxygen incubator to delay the cell cycle arrest/ senescence response.

ii. Trypsinize MEFs and resuspend them in growth medium in one 50-ml conical tube.

iii. Irradiate the cells in suspension with 4,500 rads by using a γ-irradiator.

iv. Centrifuge the irradiated MEFs at 200*g* for 5 min at 4 °C.

v. Resuspend the cells in 25 ml of freezing medium (22.5 ml of FCS, 2.5 ml of DMSO) and cryopreserve 25 vials of irradiated feeders.

▲ CRITICAL STEP Each vial of 8 million feeders should cover the equivalent of 650–800 cm^2 or 8–10 10-cm plates, depending on cell viability. Each batch of cryopreserved feeders should be tested individually; 48 h after thawing the feeders should cover 90–100% of the surface of the plate.

Thawing the feeders

vi. Cover the surface of a culture plate with 0.1% (wt/vol) gelatin (in H_2O) and allow it to remain for 30 min at room temperature. Discard any excess liquid from the plate. Coat enough plates with 0.1% (wt/vol) gelatin to plate the feeders for ES cells expansion (if required), electroporation and selection (one or two plates per shRNA being targeted).

vii. Thaw the feeders rapidly in a water bath at 37 °C and immediately add growth medium (dropwise) up to a total volume of 10 ml.

viii. Centrifuge the cells at 200*g* for 5 min at 4 °C, resuspend them in feeder cell medium (REAGENT SETUP) and plate them at a density of ~10,000 cells per cm^2. Feeder plates are ready to use once the MEFs have covered 90–100% of the surface (usually 24–48 h after plating).

■ PAUSE POINT Feeder cells can be used for up to 10 d after plating if they are kept at 37 °C in a cell culture incubator maintained at 5% CO_2. Refresh the feeder medium after 5 d.

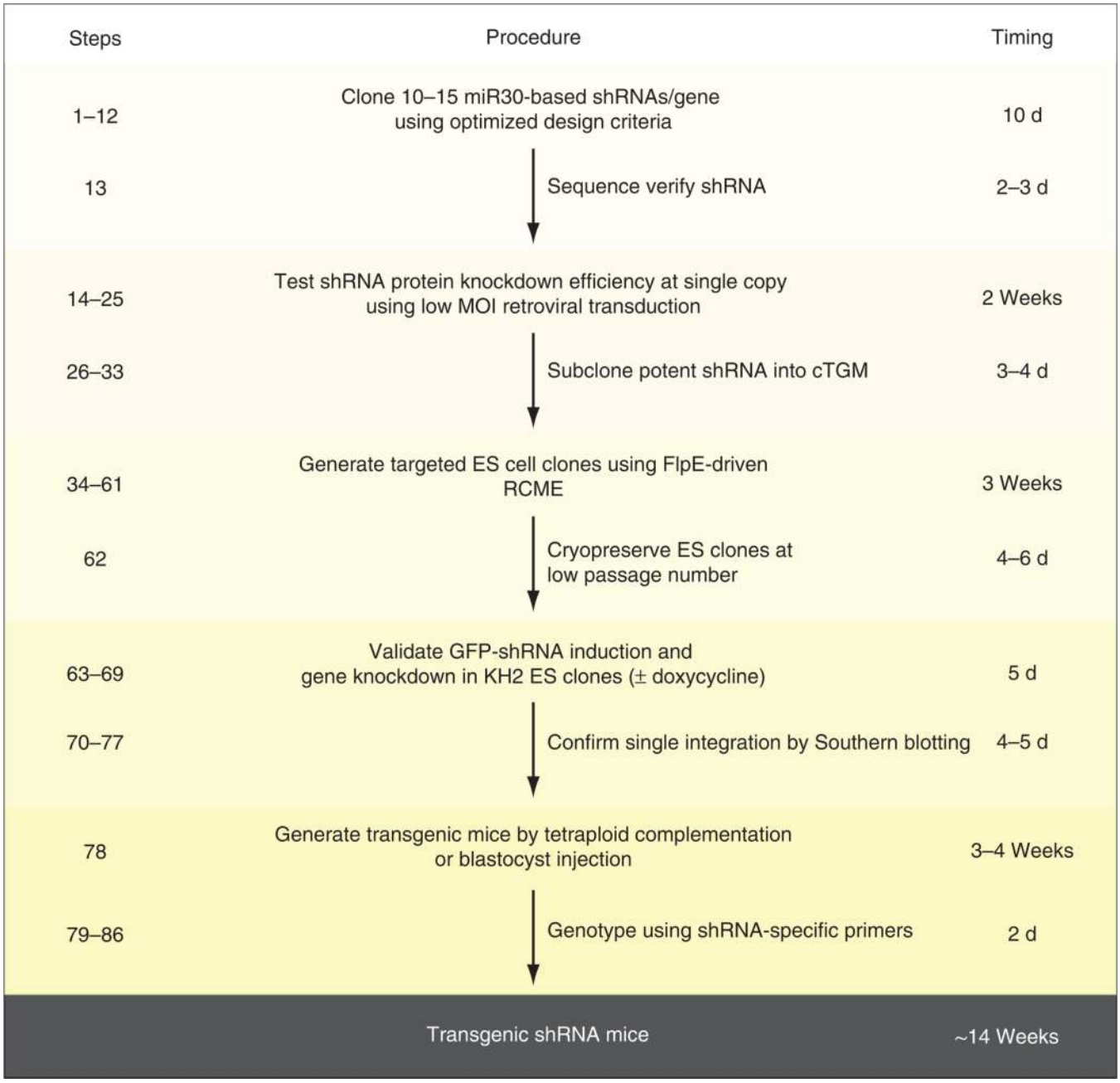

Figure 1.
Overview of transgenic shRNA mouse production. The figure shows the process to design, clone and test shRNAmirs for transgenic ES cells and mouse production. The expected time for completion of each stage is indicated on the right.

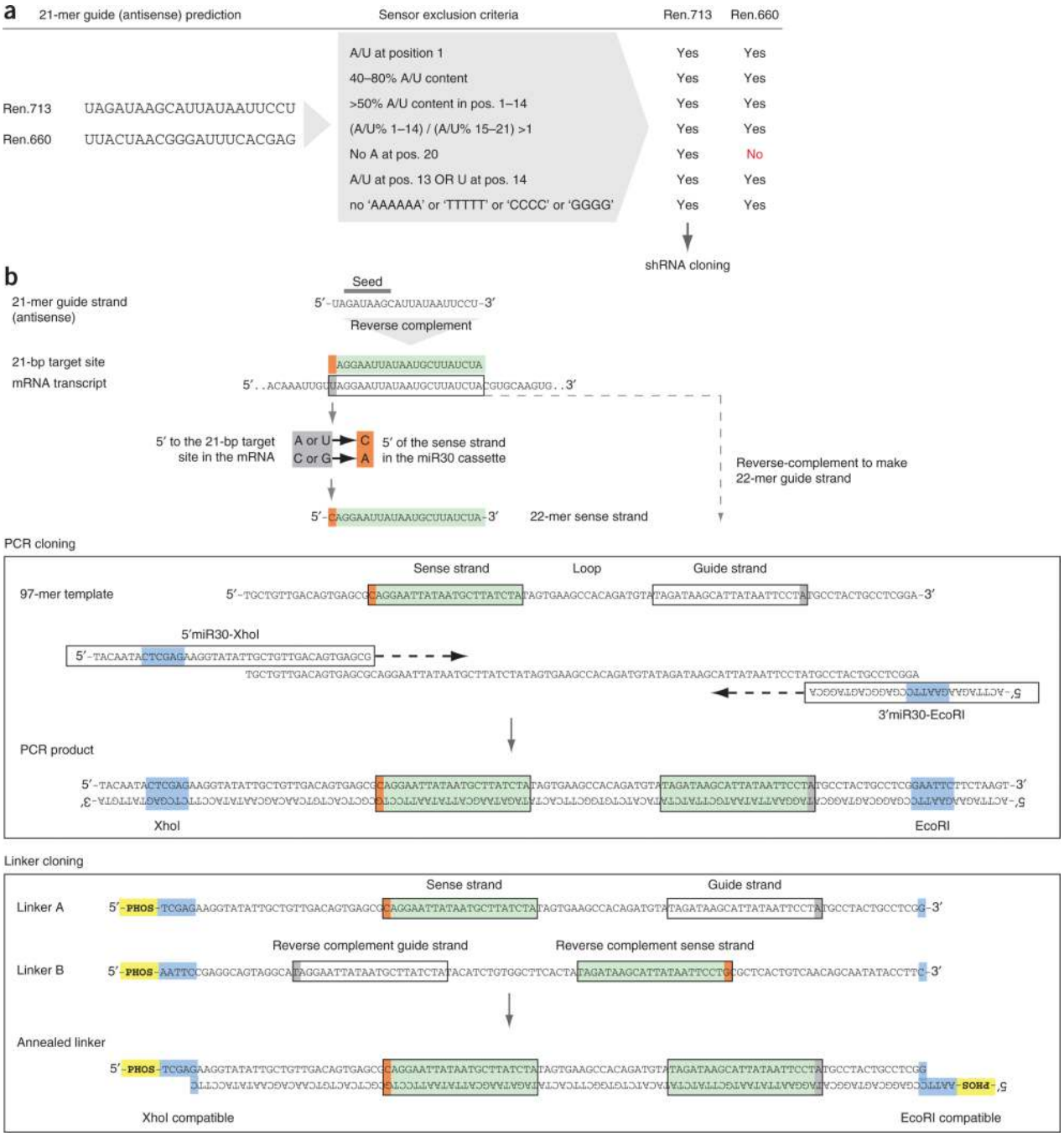

Figure 2.

shRNAmir design and cloning. (**a**) Example of shRNA guide strand predictions (from DSIR or similar tool) screened against a series of Sensor exclusion criteria (gray box). The example shows two potential 21-mer predictions targeting the *Renilla* luciferase cDNA. Each shRNAis given a numerical designation (e.g., Ren.713) that reflects the first nucleotide position of the target sequence in the mRNA transcript. Sequences that pass all criteria are selected for cloning and testing *in vitro*. (**b**) Schematic overview of the process to transform 21-mer guide strand predictions into miR30-based cloning templates for PCR (upper box) or linker (lower box) cloning. First, the 21-mer guide strand (gray) is reverse complemented to

generate the 21-mer sense strand (or 21-mer target site; green). To generate the appropriate shRNAmir template, the nucleotide immediately 5′ to the 21-mer sense strand (orange) is changed according to the nucleotide 5′ to the 21-mer target site in the mRNA transcript (gray); if the 5′ nucleotide in the mRNAis an A or U, the first base of the 22-mer sense strand becomes a C, and if the 5′ nucleotide in the mRNAis a C or G, the first base of the 22-mer sense strand becomes an A. The final 22-mer sense strand is then inserted into a 97-mer (PCR) or 110-mer (linker) cloning template (Table 1). The 22-bp guide strand is the exact reverse complement of the 22-bp target site. XhoI/EcoRI cloning fragments are then generated by PCR amplification using specific primers (Table 1) or by annealing two complementary oligonucleotides (linker cloning). Pos., position.

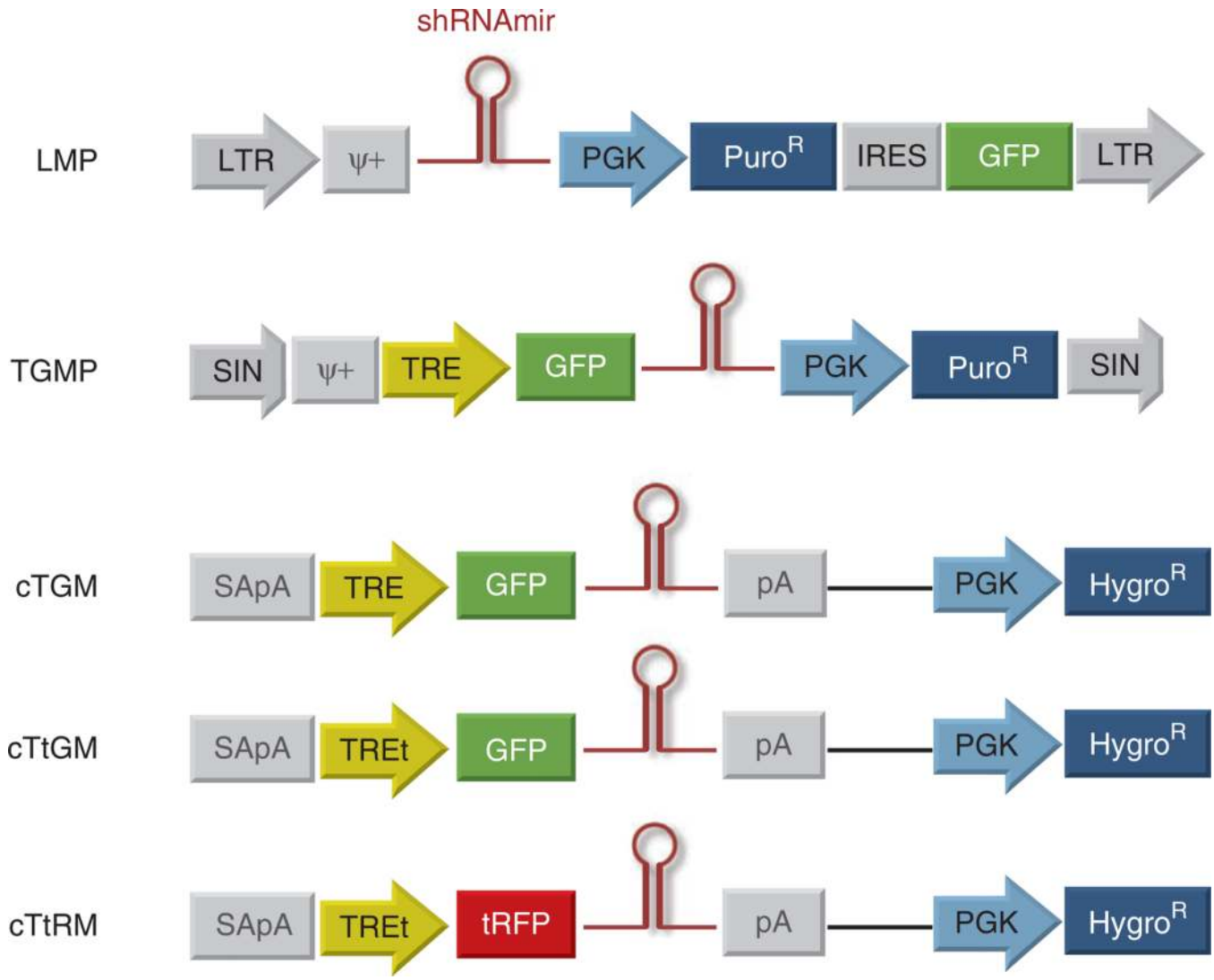

Figure 3.

Retroviral and targeting constructs. Schematic representation of miR30-based retroviral (LMP, TGMP) and *Col1a1*-targeting (cTGM, cTtGM and cTtRM) vectors used in this protocol. Constructs are shown as they appear after genomic integration. TGMP is cloned within a self-inactivating (SIN) retroviral backbone and when copied into the genome the 5′ LTR promoter activity is disrupted—represented by blunted arrows. The three *Col1a1*-targeting constructs shown differ only in the inducible promoter (TRE or TREtight) and fluorescent spacer before shRNAmir (GFP or turboRFP).

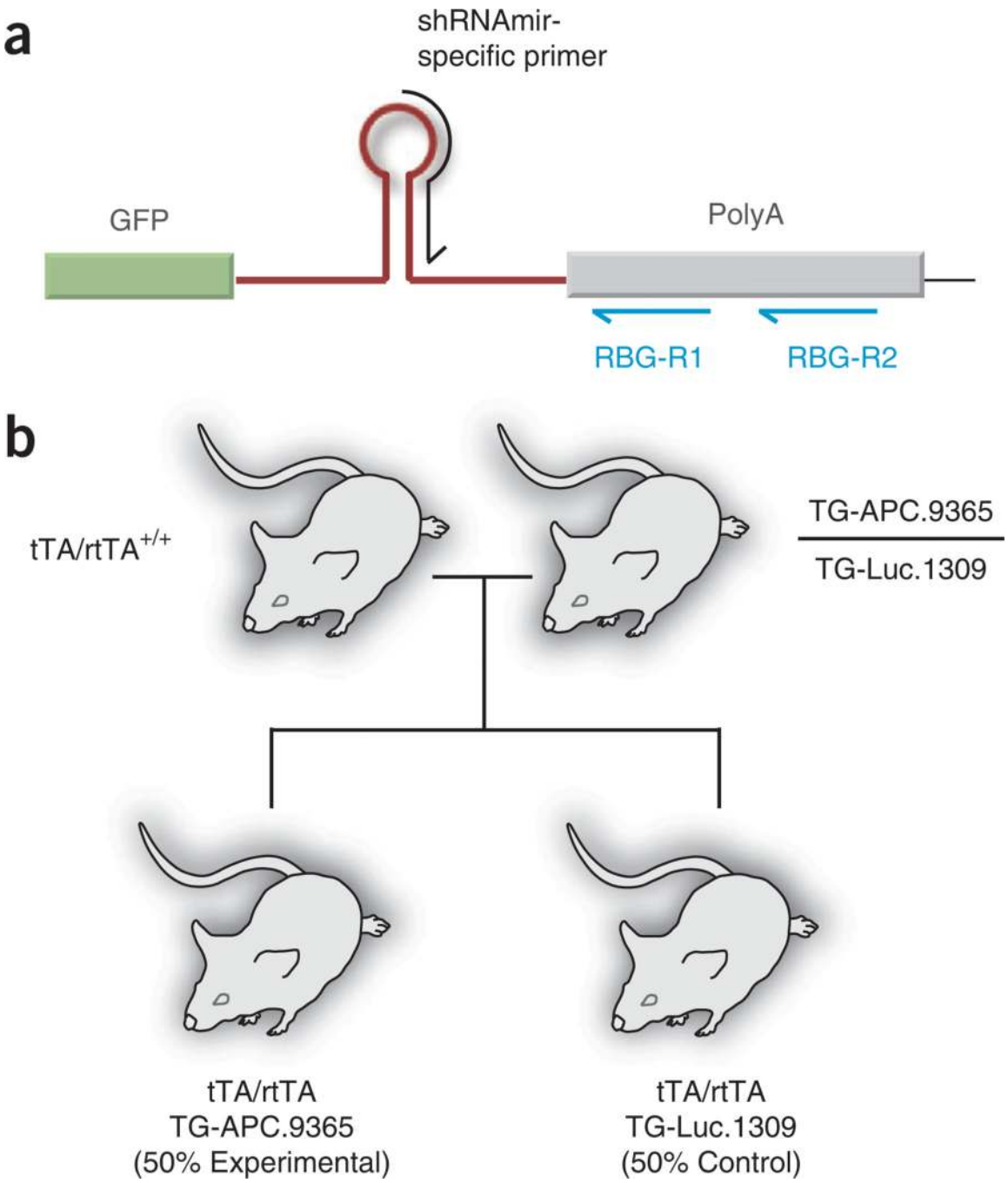

Figure 4.

shRNA genotyping and transgenic breeding. (**a**) Schematic representation of transgenic *Col1a1*-shRNAmir genotyping approach. A specific forward primer, designed to overlap the loop and guide strand of the shRNA, is used in combination with one of two common primers (RBG-R1 and RBG-R2) to generate shRNA-specific PCR product. (**b**) Breeding strategy to generate littermate control animals. Compound homozygous animals carrying one experimental shRNAmir (e.g., TG-APC.9365) and one control shRNAmir (e.g., TG-Ren.713), both at the *Col1a1* locus, are crossed to mice carrying a tet-transactivator (tTA/rtTA) to generate F_1 animals that carry either the experimental or control shRNAmirs.

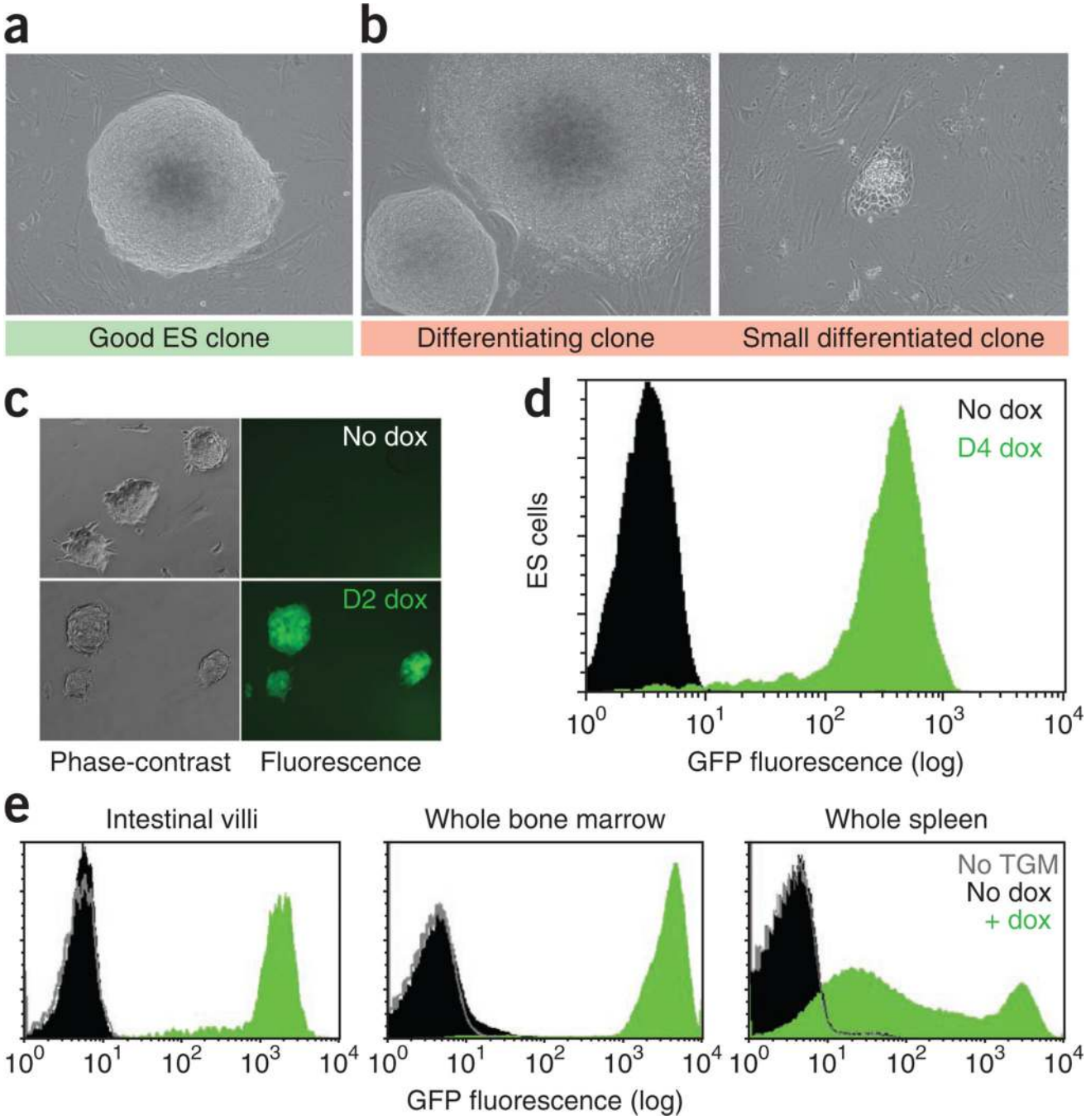

Figure 5.

Picking ES cell clones and induction of GFP in transgenic cells. Phase-contrast images showing an example of a good ES cell clone (**a**) and two examples of bad or differentiated ES cell clones that should not be picked for further analysis (**b**). The left panel of **b** shows an example of a morphologically good clone (lower left) adjacent to a differentiated clone (upper right). If possible, we suggest avoiding this good clone to minimize the chance of isolating a mixed clone. (**c**) Phase-contrast and fluorescence images of *Col1a1*-targeted transgenic KH2 ES cells carrying a control (TG-Ren.713) shRNAmir. Two days after dox treatment (1 μg ml^{-1}), ES cells show strong GFP expression, as measured by microscopy.

(**d**) Flow cytometry analysis of targeted ES cells 4 d after dox treatment. (**e**) Flow cytometry analysis of single-cell populations derived from intestine (left), whole bone marrow (middle) and whole spleen (right) cells. Samples represent nontransgenic (gray line) and double-transgenic CAGs-rtTA3/TG-Ren.713 mice either untreated (black fill) or dox treated (green fill). Intestine and bone marrow show quite uniform GFP expression, whereas spleen shows heterogeneous expression of the GFP-shRNAmir cassette.

TABLE 1

Primers.

Primer	Use	Working concentration	Sequence
5′miR30-XhoI	PCR cloning	10 μM	5′-TACAATACTCGAGAAGGTATATTGCTGTTGACAGTGAGCG-3′
3′miR30-EcoRI	PCR cloning	10 μM	5′-ACTTAGAAGAATTCCGAGGCAGTAGGCA-3′
97-mer template	PCR cloning	0.02 ng μl^{-1}	5′-TGCTGTTGACAGTGAGCG-**22merSense**-TAGTGAAGCCACAGATGT A-**22merGuide**-TGCCTACTGCCTCGGA-3′
Linker A (5′-phosphorylated), 110 bp	Linker cloning	1 μg μl^{-1}	5′**phos**-TCGAGAAGGTATATTGCTGTTGACAGTGAGCG-**22merSense**-TAGTGAAGCCACAGATGTA-**22merGuide**-TGCCTACTGCCTCGG-3′
Linker B (5′-phosphorylated)	Linker cloning	1 μg μl^{-1}	5′**phos**-AATTCCGAGGCAGTAGGCA-**RC22merGuide**-TACATCTGTGG CTTCACTA-**RC22merSense**-CGCTCACTGTCAACAGCAATATACCTTC-3′
miR30seq	Sequencing	0.5 μM	5′-TGTTTGAATGAGGCTTCAGTAC-3′
shRNA-specific forward	Genotyping	10 μM	See Step 57
RBG-R1	Genotyping	10 μM	5′-GAAAGAACAATCAAGGGTCC-3′
RBG-R2	Genotyping	10 μM	5′-CACCCTGAAAACTTTGCCCC-3′
ColA1 forward	Genotyping	10 μM	5′-AATCATCCCAGGTGCACAGCATTGCGG-3′
ColA1 reverse	Genotyping	10 μM	5′-CTTTGAGGGCTCATGAACCTCCCAGG-3′
SAdpA reverse	Genotyping	10 μM	5′-ATCAAGGAAACCCTGGACTACTGCG-3′

Abbreviations: 5′Phos, the oligonucleotide should be phosphorylated at the 5′ end; 22merSense, unique 22mer sense strand (see Figure 2); 22merGuide, unique 22mer guide strand (see Figure 2); RC22merSense, reverse complement of ‘22merSense’; RC22merGuide, reverse complement of ‘22merGuide’.

TABLE 2

Troubleshooting table.

Step	Problem	Cause	Solution
4A(iii)	PCR produces an extra (higher) band	Primer may be self-priming	Increasing the primer concentration to 0.6 μM (3 μl) and lowering the template to 0.01 or 0.005 ng can improve yield of the 131-bp product. The additional band does not interfere with subsequent cloning, as it is removed during gel purification
13	No colonies on the ligation plate	Linkers are not phosphorylated	Order phosphorylated linkers for cloning or phosphorylate using T4 polynucleotide kinase
		Vector is not digested with both enzymes	Check that both enzymes cleave the vector by single-digest controls
		CIP is not removed from vector DNA	Purify linearized vector backbone using Qiagen PCR purification kit
	Too many colonies on the control plate	Vector backbone is not fully digested/uncut DNA present	Repeat the XhoI/EcoRI digest, check the cutting efficiency of each enzyme individually by control single digests
		Vector backbone is not dephosphorylated	Repeat the CIP treatment, use fresh CIP enzyme
	Multiple shRNA cassettes in the miR30 vector	Concentration of 110-bp shRNA fragment too high in the ligation	Repeat the ligation and reduce shRNA fragment (insert) concentration. Do not increase insert/vector molar ratio above 5:1
50	ES cell electroporation efficiency is low	Low-quality DNA	Wash DNA thoroughly with 70% (vol/vol) ethanol (at least three times) to remove excess salts before resuspending in H_2O
		Reduced cell viability	Ensure that the ES cells to be transfected are subconfluent (70–80%) and have fresh medium 4–6 h before electroporation. Keep cells on ice before and immediately after electroporation
52	No clones survive selection	Failure to induce RMCE in the ES cells	Sometimes this is due to a lack of FlpE expression. Check the quality of the pCAGs-FlpE plasmid and repeat electroporation and selection
53	ES cells differentiate	Poor-quality feeder cells	Prepare new irradiated feeder cells; make sure they are growing exponentially when irradiated and frozen. Do not use feeders that have been sitting in the incubator for more than 10 d
		Medium is exhausted	Do not let cells become overconfluent (> 90%). Ensure medium is changed daily
67	Clones do not induce GFP	Nontargeted clones	Do not pick clones that develop late in hygromycin selection. Clones that reach picking size only after 12–14 d of selection are usually not correctly targeted and do not induce GFP after dox treatment
78	ES clones do not produce viable founder animals	Bad/differentiated ES cell clone	Repeat the injection for transgenic production using a different ES clone. Ensure that the ES cell clone grows well in culture and does not show signs of marked differentiation (Fig. 5b). Check chromosomal integrity (and ploidy) of the ES clone; more than 75% should show a normal karyotype
			Use two-inhibitor medium. Although we have not required the use of 2i (MEK and GSK3 inhibitor) medium, others have shown that it can substantially improve the likelihood of obtaining tetraploid-derived founder animals[29]