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# Transgenic RNA Interference in Mice

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## Summary

RNA interference (RNAi) is a powerful tool in deciphering gene function. It has been used extensively, especially in cultured mammalian cells. We have shown that RNAi-induced gene silencing can be generated in mice. With conventional transgenic techniques, shRNA-expressing constructs can be introduced into one-cell mouse embryos. The transgenic animals so obtained exhibit reduced expression of the targeted genes. Furthermore, the knockdown effect can be transmitted through the germline in these animals. We describe a method of generating a transgenic RNAi mouse line.

**Key Words:** transgene; RNAi; gene silencing; shRNA; p57Kip2.

## 1. Introduction

Since the discovery of RNA interference ***(1)***, there have been some attempts to induce the gene silencing effect with long dsRNA in mice ***(2–4)***, but success was limited to pre-implanting embryos due to the PKR (protein kinase R) response in older embryos as seen in cultured cells. PKR is activated by dsRNA ***(5,6),*** and the activated PKR causes activation of NF-κ B and global gene silencing through blocking of translation, leading to cell death via apoptosis ***(7)***. This response is now largely circumvented through the use of short dsRNA or siRNA ***(8)***. More importantly, a stable RNAi effect in cultured cells can be achieved with vectors designed to express short hairpin RNA (shRNA) via pol III promoters ***(9)***, which paved the way to produce gene silencing in mice via transgenic technologies. Transgenic RNAi mice have been produced in two ways. One is to first generate mouse embryonic stem (ES) cell clones that show RNAi effect and then to use the ES cell clones to produce mice ***(10)***. The

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other is to infect or inject one-cell mouse embryos with lentiviruses carrying an shRNA expressing cassette ***(11)***. However, neither method is easily adaptable for routine lab use. We describe an example method that employs conventional pronuclear injection to generate RNAi mice. Note that a transgenic facility is required for this procedure.

## 2. Materials

### 2.1. Generation of pTshRNA Constructs

1. Oligodeoxynucleotides (Oligos) were synthesized by Sigma-Genosys (Woodlands, TX).
2. Enzymes: T4 Polynucleotide Kinase (PNK), T4 ligase, *Bgl* II, *Hin*d III, *Not* I, *Pvu* II, and the corresponding buffers are from New England Biolabs (Ipswich, MA).
3. Oligo annealing buffer: 10 m*M* of $MgCl_2$, 20 m*M* of Tris-HCl, pH 8.0.

### 2.2. Purification of DNA Fragment for Microinjection

1. Agarose gel: low melting point agarose from Sigma (#A2576) and prepared as 0.8% (w/v) in TAE.
2. DNA gel extraction reagent: QIAEX II Gel Extraction Kit (Qiagen, Valencia, CA).

### 2.3. Histology and Immunofluorescent Staining

1. Glass slides: Superfrost Plus from Fisher Scientific (Pittsburgh, PA).
2. Phosphate buffered saline (PBS): 8.0 g NaCl, 0.2 g KCl, 1.44 g $Na_2HPO_4$, 0.24 g $KH_2PO_4$, in 1 L of $H_2O$ with pH adjusted to 7.4.
3. Paraformaldehyde (PFA; Fisher Scientific): Prepare a 4% (w/v) solution in fresh 1X PBS for each use. A couple of sodium hydroxide pellets will help dissolve PFA. Adjust the pH to 7.4. Due to the toxicity of PFA, this solution should be prepared in a fume hood.
4. 10X antigen retrieval stock solution: 2.1 g of citric acid dissolved in 1 L of $H_2O$ with pH adjusted to 6.0.
5. Blocking and antibody dilution buffer: 2% BSA, 1X PBS, 0.1% Triton-X100.
6. Primary antibodies: polyclonal goat anti-p57 from Santa Cruz Biotech (M-20) and polyclonal rabbit anti-EGFP from Abcam (AB290).
7. Secondary antibodies: FITC-conjugated anti-goat IgG (Amersham Life Science Inc., Arlington Heights, IL).
8. Nuclear staining: 500 ng/mL of DAPI (4,6-diamidino-2-phenylindole) in PBS.
9. Mounting medium: anti-fade (H-1000, Vector Laboratory).
10. Hematoxylin and eosin staining: Hematoxylin (6765001) and eosin (6766007) are from Shandon Inc. (Pittsburgh, PA).

### 2.4. Western Blotting

1. Protein quantification: Bradford assay using the concentrated dye from Bio-Rad Laboratory (Hercules, CA).

2. Primary antibodies: same as step 6 in **Section 2.3**.
3. Secondary antibodies: anti-goat and anti-rabbit IgG conjugated to horseradish peroxidase (Santa Cruz Biotechnology, Santa Cruz, CA).
4. Washing buffer (PBST): 0.1% (v/v) Tween 20 in 1X PBS.
5. Blocking buffer: 5% (w/v) nonfat dry milk in PBST.
6. 30% Acrylamide/Bis solution (Cat# 161-0157, Bio-Rad).
7. PVDF membrane (Bio-Rad).
8. Enhanced chemiluminescent (ECL) from Pierce Biotechnology, Inc. (Rockford, IL) and Bio-Max film (Kodak, Rochester, NY).

## 3. Methods

To knock down gene expression in mice, we designed a vector, pTshRNA (**Fig. 1**). The shRNA-coding sequence is cloned into *Hin*d III and *Bgl* II sites as head-to-head repeats separated by a 9-bp linker. The shRNA-expressing cassette together with the GFP marker is released from the vector via restriction digestion, gel-purified, and microinjected into one-cell embryos. The transgenic mice can easily be identified by their ubiquitous expression of GFP.

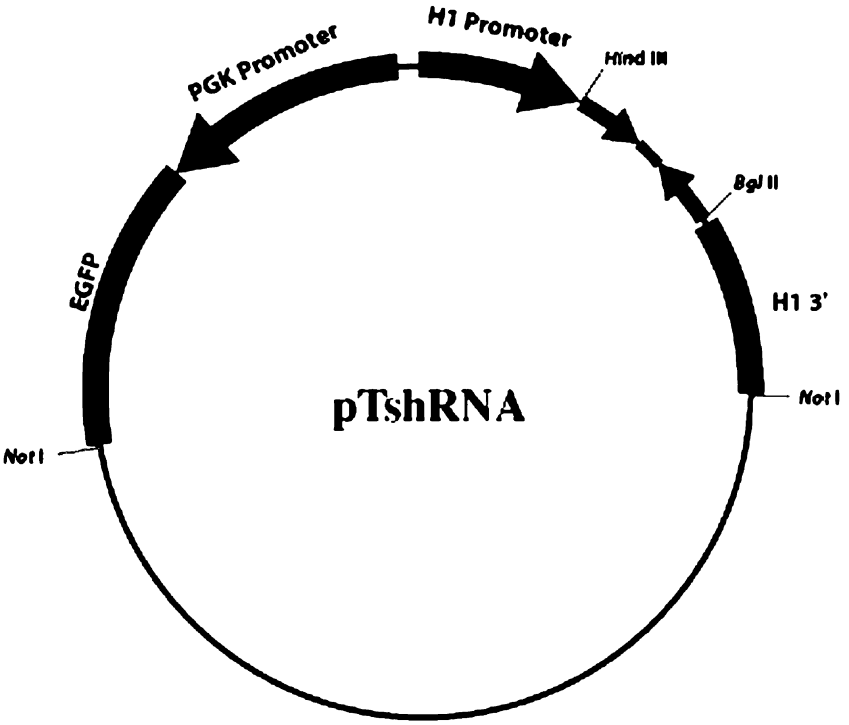


Fig. 1. Diagram of pTshRNA.

### 3.1. Generation of pTshRNA

1. shRNA is designed by scanning the target gene's cDNA for the presence of $AA(N)_{19}TT$ or $AA(N)_{19}AA$ or $AA(N)_{19}$ NN to choose a 19-nt sequence for RNAi targeting.
2. Synthesize four oligos with the following designs (*see* **Note 1**):

   F1: 5′ GATCCCC$(N_{1-19})_{sense}$TTCAAG 3′
   R1: 5′ TCTCTTGAA$(N_{1-19})_{antisense}$GGG 3′
   F2: 5′ AGA$(N_{1-19})_{antisense}$TTTTTGGAAA 3′
   R2: 5′ AGCTTTTCCAAAAA$(N_{1-18})_{sense}$ 3′

3. Phosphorylate R1 and F2. Mix 5.0 µL of 100 µM R1 or F2 with 2.5 µL of T4 DNA ligase buffer (NEB), 2.0 µL of T4 PNK, and 10.5 µL of $H_2O$. Incubate the mixture at 37 ºC for 30 min, and then heat to 75 ºC for 20 min to inactivate PNK.
4. Generate two linkers by annealing F1 to R1 and F2 to R2. Mix 10 µL of the R1 (F2) phosphorylation reaction, 2 µL of 100 µ*M* F1 (R2), and 8 µL of oligo annealing buffer together, heat to 85 ºC for 20 min, and slowly cool down to room temperature.
5. Ligate the two linkers with *Hin*d III- and *Bgl* II-digested pTshRNA vector in a three-way ligation reaction composed of 0.5 µL of each linker, 0.1 µg of vector, 2 µL of 10X T4 ligase buffer, 1 µL of T4 DNA ligase, and $H_2O$ to 20 µL. The ligation reaction is carried out at room temperature for 2 h or at 4 ºC overnight.
6. The correct construct is identified by restriction digestion (*see* **Note 2**) and sequence-verified. Sequencing primer: 5′ GTA GAA TTC GAA CGC TGA CG 3′.

### 3.2. Purification of DNA Fragments for Microinjection

1. Digest 100 µg of TshRNA construct with *Not* I and *Pvu* II and separate on a 0.8% low melting point gel.
2. DNA fragments for microinjection are purified by using QIAEX II Gel Extraction Kit (Qiagen) (*see* **Note 3**).

### 3.3. Generation of Transgenic Mice

Inject the purified DNA fragments into mouse one-cell embryos in the transgenic core, a detailed description of which is beyond our scope.

### 3.4. Immunofluorescent Staining

1. Harvest embryos at desired developmental stages and fix in 4% PFA at 4 ºC overnight.
2. For dehydration, incubate the embryos sequentially in 50% ethanol for 1 h at room temperature, in 70% ethanol at 4 ºC overnight, in 95% ethanol for 1 h at room temperature, in 100% ethanol for 1 h at room temperature, and finally in 100% ethanol at 4 ºC overnight.

3. For embedding, incubate the embryos in 50% xylene and 50% ethanol for 1 h at room temperature, in xylene for 30 min (repeat once), in 50% xylene and 50% paraffin at 60 ºC for 1 h, in 100% paraffin for 1 h at 60 ºC (repeat twice), and place in molds.
4. Cut paraffin sections of 4-micron thickness and place them on Superfrost Plus glass slides.
5. Dewax the sections by incubating in xylene twice, 10 min each, and rehydrate by going through 100%, 95%, 70%, and 50% ethanol, and PBS, each for 10 min at room temperature.
6. To retrieve antigen for immunostaining, boil the sections twice for 15 min in 10 m*M* of sodium citrate solution (pH 6.0) using a microwave oven, and incubate in PBS three times at room temperature, 5 min each time.
7. Incubate the sections in blocking solution (2% BSA, 1X PBS, 0.1% Triton-X100) for 1 h at room temperature, followed by overnight incubation at 4 ºC with goat anti-p57 (M-20) antibodies (Santa Cruz Biotechnology) 1:20 diluted in the blocking solution.
8. Wash the sections with PBS three times at room temperature, 5 min each, followed by 1-h incubation at room temperature with FITC-conjugated secondary antibodies 1:100 diluted in the blocking solution. Wash again with PBS three times at room temperature, 5 min each.

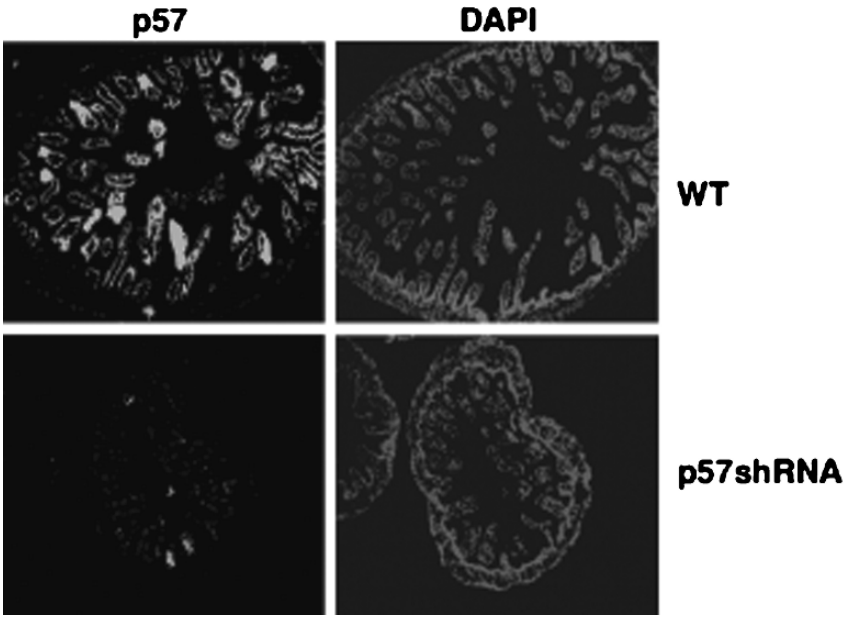


Fig. 2. Immunofluorescent staining of p57 in intestine. Magnification: 400X.

9. After counterstaining the nuclei with 500 ng/mL of DAPI, mount the sections with anti-fade medium (Vector Laboratory, Burlingame, CA).
10. Observe and photograph the immunostaining under a fluorescent microscope (we used a Nikon E800). An example of the staining is shown in **Fig. 2**.

### 3.5. Western Blotting

1. Homogenize various tissues collected from WT and p57 shRNA transgenic mice (from the same litter by genotyping under a fluorescent dissecting microscopy) on ice in imidazole buffer: 50 m*M* of imidazole–HCl, pH 7.4, 5 m*M* of EGTA, pH 8.0, 100 m*M* of NaCl, 0.5% Triton X-100, 1 m*M* of dithiothreitol (DTT) plus protease inhibitors (Complete Protease Inhibitor Cocktail Tablet, Roche). The homogenates are spun at the maximum speed of a refrigerated microcentrifuge for 10 min at 4 ºC to remove debris.
2. Determine the protein concentration with the Bradford protein assay method using BSA as standard.
3. Mix a total of 60 µg of protein with sample buffer and run on a 10% PAGE-SDS gel with 80 V for stacking and 120 V for resolving.
4. Transfer proteins in the gel onto PVDF membrane (Bio-Rad) with a Bio-Rad semidry transfer apparatus at 10 V for 60 min.

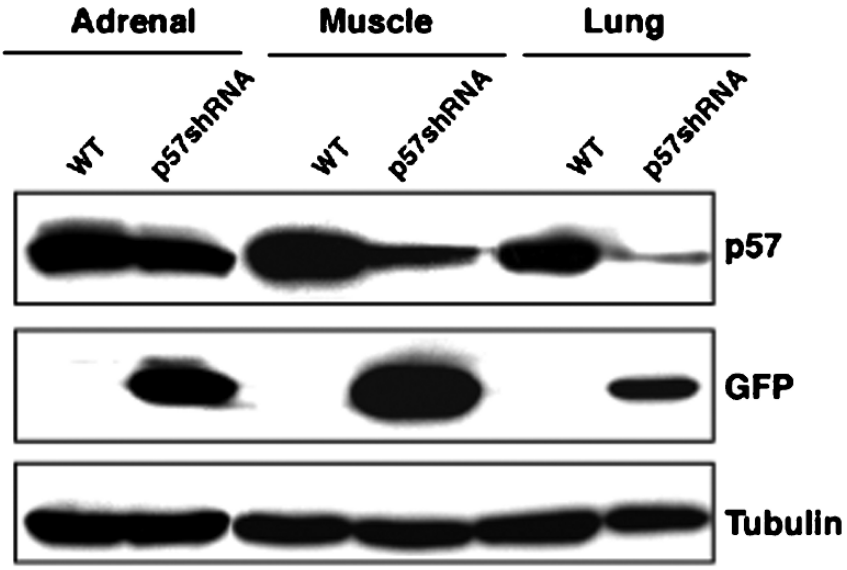


Fig. 3. Western blot analysis of p57 and GFP expression in wild-type and p57shRNA transgenic mice.

5. Following the transfer, wash the PVDF membrane briefly in $H_2O$ and then block in 5% nonfat dry milk/PBST (PBS plus 0.1% Tween 20) at room temperature for 1 h.
6. Incubate the membrane at 4 ºC overnight with primary antibody diluted in the blocking buffer, followed by PBST wash three times, 15 min each.
7. Incubate the membrane with the secondary antibody (1:3,000) in blocking buffer for 1 h at room temperature, and wash again with PBST three times, 15 min each.
8. Prepare the ECL reagent during the final wash. Warm 2-mL aliquots of each portion of the reagent separately to room temperature.
9. Bring the membrane (in PBST) and the ECL reagents to the darkroom. Once the final wash is discarded, mix the ECL reagents together. Immediately add to the membrane and rock by hand to ensure even coverage.
10. Remove membrane from the ECL reagents, gently dry with Kim-Wipes, and expose to Kodak film for desirable time. An example is shown in **Fig. 3**.

## 4. Notes

1. One can synthesize two long oligos (64-mer) instead of the four shorter ones:
   a. 5′ GATCCCC$(N_{1-19})_{sense}$TTCAAGAGA$(N_{1-19})_{antisense}$TTTTTGGAAA 3′
   b. 5′ AGCTTTTCCAAAAA$(N_{1-19})_{sense}$TCTCTTGAA$(N1\text{-}19)_{antisense}$GGG 3′

   Oligos a and b are annealed to generate a single linker to be ligated to pTshRNA. However, PAGE purification is advised for these long oligos. Use of the shorter oligos eliminates this step and the associated cost.
2. Once the linkers are ligated to pTshRNA, the *Hin*d III site is destroyed, but *Bgl* II remains. Correct constructs will show no digestion by *Hin*d III and a single cut by *Bgl* II.
3. Purification of DNA fragments for microinjection has to be done carefully to avoid contaminating the DNA with the beads, which will block injection needles. Extra centrifugation of the DNA is required sometimes to remove the last bits of solid particles. Also, check with your transgenic core and follow its instructions on DNA preparation.

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