A transgenic approach for RNA interference-based genetic screening in mice

Shaohua Peng*, J. Philippe York*, and Pumin Zhang*†

Departments of *Molecular Physiology and Biophysics and †Biochemistry and Molecular Biology, Baylor College of Medicine, Houston, TX 77030

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Genetic screening is the most powerful method through which to uncover gene function. It has been applied very successfully in lower organisms but seldom attempted in mammalian species because of their long generation time. In this study, we exploit RNA interference (RNAi) for its potential use in genetic screening in mice. We show that RNAi-induced gene knockdown can be generated through introducing small hairpin RNA-expressing constructs into the mouse as transgenes via conventional pronuclear injection. The knockdown effect can be transmitted for many generations in these transgenic animals. In a small-scale screening for developmental defects in the kidney, we uncovered a potential role of *Id4* in the formation of the renal medulla. Our results demonstrate the feasibility of using RNAi for genetic screening in mice.

Id4 | *p57^{kip2}* | cyclin D1 | kidney

RNA interference (RNAi) induced by short dsRNA has gained widespread application in biological research (1–3). The discovery of RNAi was rooted in the desire to alter gene function by an antisense approach in *Caenorhabditis elegans*. It was observed that introducing dsRNA homologous to a specific gene resulted in the posttranscriptional silencing of that gene (4). This dsRNA-induced gene silencing was termed RNAi. It occurs through two main steps. The dsRNA is initially recognized by an enzyme of the RNase III family of nucleases, named Dicer, and processed into small double-stranded molecules, termed small interfering RNA (siRNA) (5). siRNA is then incorporated into an inactive large multisubunit nuclease complex, RISC (RNA-induced silencing complex). RISC unwinds the siRNA in an ATP-dependent step and finds homologous target mRNAs by using the siRNA sequence as a guide and cleaves these mRNAs (6, 7).

The RNAi effect can be induced in mammalian cell cultures via transient transfection of double-stranded siRNA (1) or stable expression of small hairpin RNA (shRNA), which is processed to siRNA inside cells (8). This technical advancement has made it possible to perform loss-of-function genetic screens in cultured cells by using shRNA libraries (9, 10). However, there are limitations to the types of biological processes that can be studied in cultured cells. Many genes function in processes that cannot be recapitulated in cultured cells. Therefore, it is desirable to apply RNAi technology in the whole mouse, preferably in semihigh through-put fashion as has been done so successfully in *C. elegans* (11, 12).

Transgenic RNAi mice have been produced in two ways. One way has been to first generate mouse ES cell clones that show RNAi effect and then to use the ES cell clones to produce mice (13). The other is to infect or inject one-cell mouse embryos with lentiviruses carrying a shRNA-expressing cassette (14). However, both methods are not easily adaptable for routine laboratory uses, not to mention large-scale genetic screens. Here, we report the production of transgenic RNAi mice through conventional pronuclear injection of shRNA-expressing constructs. We performed a small-scale RNAi screen in mice aimed at identifying genes whose function are important for the development of the kidney.

Results

To make the generation of transgenic RNAi mice easier and faster for its potential use in large-scale genetic screens, we designed and constructed a transgenic RNAi vector (pTshRNA, Fig. 1A). The expression of shRNA from the vector is driven by the human H1 promoter (8). The genomic sequence (0.7 kb) of the mouse H1 gene 3' to its termination signal was placed downstream of the shRNA sequence. shRNA-encoding sequences were cloned into the vector as synthetic linkers. An EGFP expression cassette was also included in the vector to mark embryos that have received the transgenic DNA. The shRNA expression cassette and the EGFP cassette were purified away from the plasmid backbone and injected into one-cell mouse embryos. Fig. 1B shows a transgenic embryo that expresses GFP.

To test the efficacy of pTshRNA in knocking down gene expression, we generated an shRNA construct to target *p57^{KIP2}* (15). Embryos injected with the construct were harvested and analyzed at embryonic day 18.5 without establishing transgenic lines first. Two GFP-positive embryos were obtained that did not show any gross abnormalities. *p57* is expressed in a number of tissues during mouse development (15, 16). We analyzed its expression in the nontransgenic littermates and the GFP-positive embryos by immunofluorescence staining. The expression of *p57* was reduced in the eye lens (Fig. 2A), the kidney (Fig. 2B), and other tissues (data not shown) of the transgenic embryos. In the kidney, the reduction in expression was so strong that the expression of *p57* was undetectable with the immunostaining method. A similarly strong reduction of *p57* expression was seen in glomeruli as well (data not shown). However, no reduction of expression in the differentiating chondrocytes was observed (Fig. 2C). It is unclear whether this lack of RNAi effect was caused by the inability of chondrocytes to perform RNAi because of missing key components of the process or a result of failed expression of the shRNA. These cells did express GFP (data not shown), however, indicating the transgenes were not shut down in these cells. Similar results were obtained in both GFP-positive embryos. Next, we targeted another cell cycle-related gene, *cyclin D1*, for transgenic knockdown. As shown in Fig. 3, there was a clear reduction in *cyclin D1* expression in the transgenic retina. These results demonstrate that shRNA expression constructs delivered by the conventional transgenic technique can produce RNAi effect in mice.

To determine whether the RNAi effect observed in the injected embryos can be transmitted through germ line, we established a transgenic line that carried the *p57* shRNA construct and examined the expression of *p57* in the embryos derived from this line. We found a similar knockdown of *p57* expression in this transgenic line as in the transgenic embryos obtained transiently. To assess the RNAi effect more quantitatively, we dissected selected tissues out of embryonic day 18.5

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; shRNA, small hairpin RNA.

†To whom correspondence should be addressed. E-mail: pzhang@bcm.tmc.edu.

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Fig. 1. Generation of the transgenic shRNA vector. (A) Diagram of pTshRNA. (B) A GFP-positive embryo produced by using pTshRNA.

embryos derived from a cross between a transgenic male and a nontransgenic female, and the expression of GFP and *p57* in the tissues was analyzed by Western blotting. Significant reductions in the expression of *p57* were seen in the skeletal muscle, lung, and small intestine (Fig. 4). The reduction was less pronounced in the adrenal gland (Fig. 4). We have bred the *p57* shRNA transgenic line up to four generations and have not observed a reduction in the RNAi effect. Despite the observed RNAi effect, we did not observe any developmental defects in either *p57* or *cyclin D1* shRNA transgenic mice as seen in the respective knockout mice (16–18), indicating that the residual amount of

these two genes' product is sufficient to support the normal developmental processes in which they are involved.

Having demonstrated the feasibility of generating transgenic RNAi mice with conventional technology, we next wanted to perform genetic screens to identify genes that are required for developmental processes involved in the formation of the kidney. To avoid the cost of establishing and maintaining transgenic lines, we used F₀ embryos obtained directly from pronuclear injections. As a positive control for this approach, we chose to target *Pax6*, because it is haploinsufficient for development. A reduction of 50% in its expression through transgenic RNAi should produce the phenotypes seen in *Pax6*^{+/-} mice. The phenotypes include small eyes and a reduced body size (19). As expected, the transgenic *Pax6* RNAi embryo harvested at 18.5 days after the injection of the shRNA construct displayed a smaller body size (Fig. 5A) and the eyes were smaller as well (Fig. 5B). Histological analysis of the eye also indicated defects in the lens (data not shown).

Target genes for screening were chosen from our microarray experiment that identified genes that are highly expressed in the developing kidney (P.Z., unpublished results). Two transgenic shRNA constructs targeting different regions of a gene were generated for each gene (see Table 1 for the name of the genes and the target sequences), and each construct was injected once (into ≈50 one-cell embryos). Around 30% of the embryos survived the injection, and 10% of the survivors were transgenic. The transgenic embryos were identified under an epi-fluorescent dissecting microscope. GFP-negative (as control) and GFP-positive embryos from the same litter were fixed and processed for histological examination. If no GFP-positive embryos for both constructs of a gene were obtained, there could be two reasons. One could simply be that the injection was

Fig. 2. Silencing the expression of *p57*^{KIP2} with transgenic shRNA. Sections from embryonic day 18.5 transgenic and nontransgenic embryos were immunostained for the expression of *p57* in the ocular lens (A), the medulla of the kidney (B), and the costal bone (C). (Magnifications: ×400.)

Fig. 3. Reducing *cyclin D1* expression with transgenic shRNA. Sections from embryonic day 18.5 transgenic and nontransgenic (Non-TG) embryos were immunostained for the expression of *cyclin D1* in the retina. (Magnifications: ×400.)

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