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An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi

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ABSTRACT

RNA interference (RNAi) mediates gene silencing in many eukaryotes and has been widely used to investigate gene functions. A common method to induce sustained RNAi is introducing plasmids that synthesize short hairpin RNAs (shRNAs) using Pol III promoters. While these promoters synthesize shRNAs and elicit RNAi efficiently, they lack cell specificity. Monitoring shRNA expression levels in individual cells by Pol III promoters is also difficult. An alternative way to deliver RNAi is to use Pol II-directed synthesis of shRNA. Previous efforts in developing a Pol II system have been sparse and the results were conflicting, and the usefulness of those Pol II vectors has been limited due to low efficacy. Here we demonstrate a new Pol II system that directs efficient shRNA synthesis and mediates strong RNAi at levels that are comparable with the commonly used Pol III systems. In addition, this system synthesizes a marker protein under control of the same promoter as the shRNA, thus providing an unequivocal indicator, not only to the cells that express the shRNA, but also to the levels of the shRNA expression. This system may be adapted for *in vivo* shRNA expression and gene silencing.

INTRODUCTION

RNA interference (RNAi) can mediate sequence-specific post-transcriptional gene silencing in many eukaryotes. In RNAi, long double-stranded RNA or short hairpin RNA (shRNA) is cleaved to produce short RNA duplexes ~21–23 nt in length with 2 nt overhangs at the 3′ end (1,2). Dicer, an RNase III enzyme, catalyzes this cleavage (3). Together with R2D2 (a small RNA binding protein), dicer also facilitates the formation of RISC (RNA-induced silencing complex), which in mammalian cells minimally contains a short, single-stranded RNA and a protein Argonaute2 (4–6). RISC identifies, binds and cleaves the complementary RNA (7).

Because of its sequence specificity, RNAi has been widely used to silence specific genes for investigation of the gene functions. In mammalian cells, this is accomplished by introducing small interfering RNAs (siRNAs) (8) or short hairpin RNAs (shRNAs) into cells (9). The siRNAs are composed of two RNA strands with 19 nt duplexes and 2 nt overhangs at the 3′ ends. The shRNAs are usually delivered as gene constructs that are composed of a promoter, a short DNA sequence encoding an shRNA and a transcription termination sequence. The structure of shRNA mimics pre-micro RNAs (pre-miRNAs), which are endogenously encoded natural shRNAs that are synthesized and processed into miRNAs, which are single stranded. miRNAs also complex with Argonaute proteins to form RISC, which is capable of mediating both RNAi and translational repression (10).

Due to their potential for sustained RNAi, shRNAs have been often applied for *in vivo* gene silencing. They have been delivered using viral vectors or being directly injected into fertilized oocytes for making transgenic mice (11–13). Most constructs have used Pol III promoters, including U6, H1 and tRNA promoters (12,14,15). The advantages of these promoters are that they direct high levels of shRNA expression, which in turn mediates highly efficacious silencing. In addition, the gene construct is small and simple, and easily inserted into viral vectors. The limitation of these constructs is that these promoters have no cell specificity, and exceedingly high levels of shRNA expression increases the probability of off-target silencing and elicit non-specific effects such as interferon response and cellular toxicity (16,17).

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Compared with Pol III promoters, Pol II promoter-directed synthesis of shRNAs may be advantageous in some circumstances. For example, many cell- or tissue-specific Pol II promoters are known and have been successfully used in transgenic mice. These promoters may be used to direct cell- or tissue-specific gene silencing. Furthermore, while no evidence indicates that Pol III synthesizes miRNAs, some miRNAs are known to be downstream of Pol II promoters, are within known protein coding genes and are expressed by Pol II activity (18,19). Therefore, strategies using Pol II-directed synthesis of shRNA mimic the natural miRNA synthesis and could be an efficient RNAi strategy *in vivo*.

Limited attempts have been made to test the potential of shRNA-mediated silencing using Pol II promoters. Four groups have used the cytomegalovirus (CMV) promoter to synthesize shRNAs. Three used the CMV promoters to synthesize miRNAs that contained the shRNA but no protein-coding sequences (11,20,21). Among these two reported successful silencing (11,20), while one reported low efficacy (21). The fourth group placed the hairpin in the 3′-untranslated region (3′-UTR) of a GFP coding sequence and observed silencing (22). In a new study, a cell-specific promoter was used to synthesize shRNA and silencing was observed in a specific cell group (23).

Although these studies showed that pol II-directed synthesis of shRNA was useful for gene silencing, the efficacy of different constructs was not quantitatively compared, and therefore, the optimal construct design was not clear. Furthermore, the relative RNAi efficacy of these constructs in comparison with the pol III constructs was not known. In the experiments where the plasmid concentrations were reported, high ratios of the shRNA-synthesizing plasmid to the target plasmid, ranging from 8 to 1 to 80 to 1, had to be used to observe silencing (20,22), suggesting that those constructs were relatively inefficient compared with the Pol III-hairpin constructs.

In the present work, we tested several new strategies for Pol II-directed synthesis of shRNA and their silencing efficiency. Using the CMV promoter, we tested a series of variations of hairpins that mimic a human miRNA miR-30 structure (24). We found that the hairpins that adopted the authentic miR-30 structure mediated the most effective silencing. Furthermore, we placed this hairpin in an intron downstream of another Pol II promoter, the human ubiquitin C (UbC) promoter. This construct mediated silencing with the efficiency that was comparable with the widely used Pol III-hairpin constructs. Finally, we tested silencing using the same strategy against a different gene and demonstrated that this strategy can silence endogenous gene expression and function.

MATERIALS AND METHODS

Plasmid construction

The shRNA sequences are shown in the figures. To construct CMV promoter-driven shRNA vectors, DNA oligonucleotides were chemically synthesized and annealed to form double-stranded DNA fragments that were inserted between the restriction sites KpnI and EcoRI in the polylinker of the carrier vector pcDNA3 (Invitrogen). To construct the tandem shRNA vector, the first DNA oligonucleotide was inserted between KpnI and EcoRI, and the second between XbaI and NotI of pcDNA3 plasmid. The two shRNA stem–loop structures were separated by 100 nt sequence from the neomycin gene (Figure 1).

To construct human ubiquitin C (UbC) driven shRNA vector, the human ubiquitin C (UbC) promoter, including −350 bp upstream from transcription initiation site, first exon and the first intron (25), were cloned from the genomic DNA of human embryonic kidney 293 cells (HEK293) by PCR and inserted into pEGFP-N1 (Clonetech) to replace the CMV promoter. Two restriction sites KpnI (+530 bp) and EcoRI (+535 bp) were then introduced inside the first intron of the UbC sequence. The DNA oligonucleotides coding for the shRNAs were inserted between KpnI and EcoRI.

The U6-shRNA vector was constructed as described previously (26). To construct the H1-shRNA vector, human H1 promoter was cloned from the genomic DNA of HEK293 cells by PCR and placed into pUC19 vector.

To facilitate the analysis of shRNA efficacy, human SOD1 opening reading frame was inserted into the 3′ untranslated region 100 bp downstream from firefly luciferase opening reading frame in the pGL2 vector (Promega). All constructs were sequence-verified.

Cell culture and transfection

HEK293 cells were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Twenty-four hours before transfection, cells (70–90% confluence) were detached by trituration, transferred to 6-well or 24-well plates and cultured in 10% FBS-containing medium without antibiotics. The cells were transfected in the absence of serum with the transfection reagent lipofectamine-2000 (Invitrogen) according to manufacturer's instructions. Four hours later, the FBS was added back. The growth medium was changed every 24 h.

Neuroblastoma-spinal cord 34 (NSC-34) cells (27) were cultured and transfected similarly as the HEK293 cells. Twenty four hours after transfection of the Pol II-shRNA-EGFP vector, the cells were selected with 100 μg/ml zeocin (the zeocin-resistant gene was inserted into the shRNA-expressing vector). Individual colonies were picked and expanded. The expression levels of the transgene were evaluated by monitoring EGFP fluorescence. Cell lines with desired expression levels were maintained in DMEM containing 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 50 μg/ml zeocin.

Northern blotting analysis

HEK 293 cells were grown in 6-well plates and transfected with 2 μg of each plasmid per well. After 30 h of transfection, the cells were harvested and used for total RNA extraction with Trizol reagent (Sigma). Twenty micrograms of total RNA was fractionated on a 15% polyacrylamide gel and transferred onto HybondTM-N+ membrane (Amersham). After UV cross-linking, the membrane was probed with ^{32}P-labeled synthetic RNA oligonucleotide complementary to the antisense strand of the human SOD1 shRNA.

Luciferase assay

HEK293 cells were transfected in 24-well culture plates using 0.25 μg of either shRNA-expressing vector or empty pcDNA3 plasmid, 0.25 μg of firefly luciferase (Pp-luc) vector

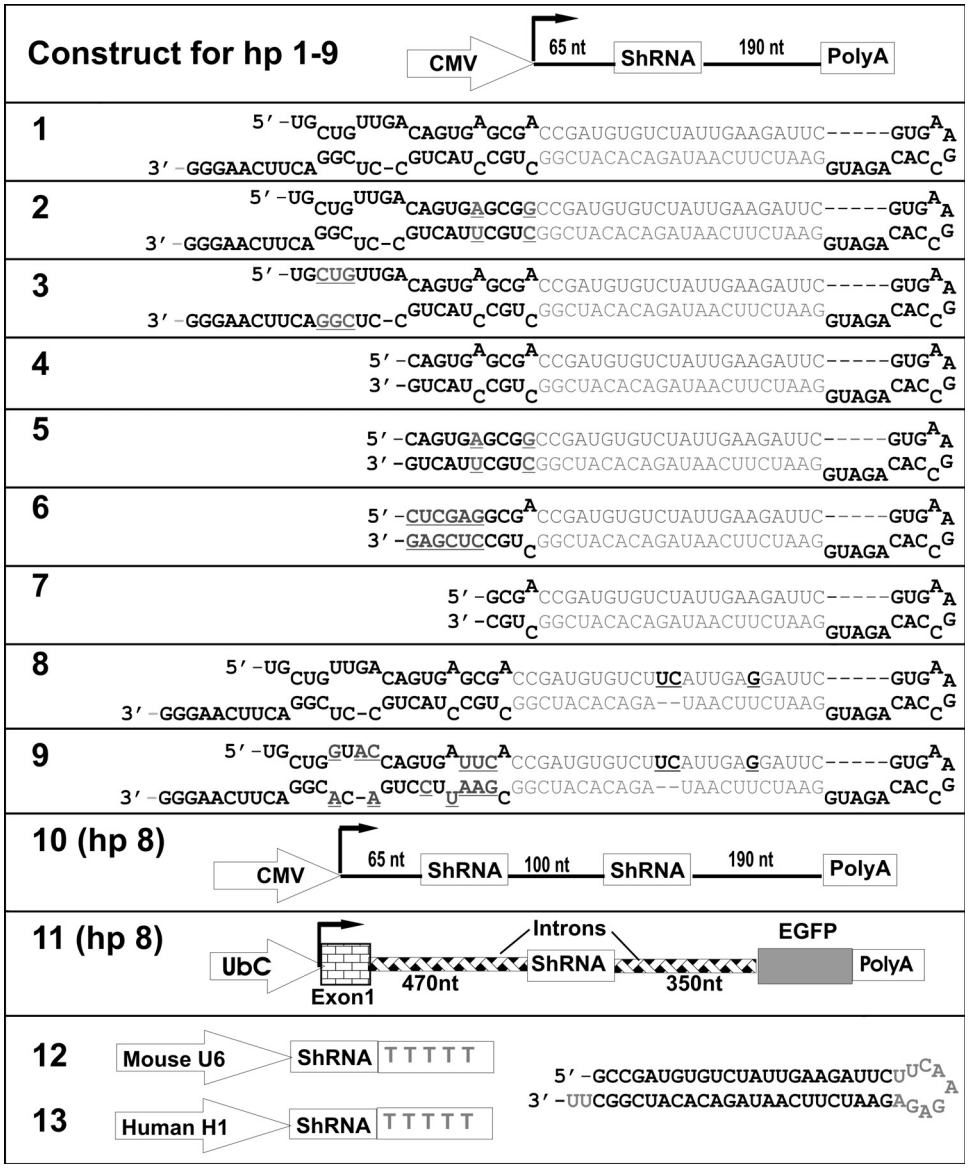

Figure 1. Design of the Pol II-driven hairpin vectors. In the first group of constructs, hairpins 1–9 were placed behind the CMV promoter. The hairpin structures were variations of human miR-30, which was represented by hairpin 8. The antisense strand of the miR-30 hairpin stem was replaced with the sequence that was complementary with the coding region of human SOD1 mRNA (light letters). The loop sequence of miR-30 was preserved since its variations have been investigated previously (20). Eight variations at the base of the hairpin stem were created (hp 1–7, 9). The sequences flanking either side of the hairpins shown here were derived from the neomycin-resistant gene (*neo*). Transcription was terminated using bovine growth hormone poly(A) sites. Construct 10 contained two tandem hairpins, both of which were hp 8. In construct 11, the CMV promoter was replaced with the ubiquitin C (UbC) promoter, which included the first intron. Hairpin 8 was inserted in the intron. For comparison, constructs 12 and 13 used U6 and H1 promoters, respectively, for the synthesis of the shRNA against SOD1.

with human SOD1 cDNA sequence at 3′-UTR (pGL2-luc-sod1), and 0.15 μg of renilla luciferase (Rr-luc) expressing vector pRL-TK as transfection control. The cells were harvested at 28 h after the transfection. The luciferase activity in the cleared cell lysate was measured with the Dual Luciferase assay kit (Promega, Madison, WI) using a Mediators Diagnostika (Vienna, Austria) PhL luminometer. The luciferase activity was defined as the ratio of Pp-luc activity from pGL2-control derivative to Rr-luc activity from pRL-TK. Thereafter, the relative luciferase activity was normalized to the control transfected with the empty vector pcDNA3 plus two luciferase-expressing vectors.

Immunoblots

HEK 293 cells were transfected twice in 6-well plates at 0 and 24 h, and split and plated onto 24-well plates at 48 h after the initial transfection. At 72 h, the cells were harvested for protein extraction. NSC-34 cell lines stably expressing the shRNA transgenes were collected from 6-well plates and pelleted. The cell pellets were lysed in ice-cold lysis buffer containing 0.4% NP-40, 0.2 mM Na_3VO_4, 20 mM HEPES (pH 7.9) and a cocktail of protease inhibitors (Complete-Mini, Sigma). Cell pellets were homogenized by short sonication and protein content in the cleared lysate was determined using the BCA assay (28). Fifty micrograms of

total proteins were resolved on 15% SDS–PAGE gel and blotted onto GeneScreen Plus membrane (Perkin Elmer). Proteins were detected using specific primary antibodies and SuperSignal kit, and photographed using the Kodak Digital Image Station 440CF. The primary antibodies were sheep anti-superoxide dismutase-1 (SOD1; 1:1000; BioDesign), rabbit anti-Mn superoxide dismutase (SOD2; 1:1000, Stressgen) and mouse anti-glyceraldehye-3-phosphate dehydrogenase (GAPDH; 1:10000; Abcam). After detection of either SOD1 or SOD2, the membrane was stripped for 30 min at 55°C in 100 mM β-mercaptoethanol, 2% SDS (w/v) in 62.5 mM Tris–HCl (pH 6.7) and used again for detecting immuno-reactivity of GAPDH, which served as loading control.

Cell viability assay

After two transfections and splitting as described above (see 'Immunoblot Analysis'), HEK 293 cells were grown in 24-well plates for another 24 h. After exposure to 300 μM H_2O_2 for 4 h, the cells were rinsed twice with PBS and cultured in normal medium containing 10% FBS for another 20 h (this minimized overestimation of cell death resulting from direct inhibition of mitochondria dehydrogenase by H_2O_2). Cell viability was determined using an MTS assay kit according to manufacturer's instructions (Promega). The CellTiter 96® AQueous One Solution Reagent was directly added to growth medium and incubated for 4 h before measuring absorbance at 495 nm with a 96-well plate reader. The absorbance of lysates was normalized to the average absorbance of the untreated cells. Statistical analysis was performed using ANOVA followed by Tukey post hoc test to compare group means.

Fluorescence activated cell sorting (FACS)

Mouse embryonic cell line NF-1 (ATCC) was maintained in DMEM medium containing 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. The cells were transfected with construct 11 (UbC-SOD2hp-EGFP) in the presence of 5% FBS. Forty-eight hours after transfection, the cells were separated into four groups using FACS based on their levels of eGFP fluorescence: group 1, 5.5–25 × 10^4 (arbitrary units); group 2, 2–7 × 10^3; group 3, 2–10 × 10^2; and group 4, 0–50. Approximately 1.5–2 million cells were collected for each group. The cells were lysed and immunoblotted for eGFP, SOD2 and GAPDH as described above.

RESULTS AND DISCUSSION

To find the optimal construct for Pol II-directed synthesis of shRNA, we made a series of variants of human miR-30 hairpin structure (Figure 1). We chose miR-30 as a model hairpin structure because its stem sequence could be substituted with other sequences that match different targets (20,29). Because the previous studies suggested that simplifying miR-30 structure by eliminating the mismatch, the bulge and most of the base sequence does not significantly alter the RNAi efficiency (20,29), we first constructed a similar hairpin and a series of its variations (Figure 1, constructs 1–7). These hairpins targeted the human SOD1 mRNA and were placed downstream from the CMV promoter in an mRNA transcript. This construct design was similar to several previously published Pol II-shRNA synthesizing constructs (20,29). In cotransfections with a report luciferase construct that contains the target sequence in its 3′-UTR, most of these hairpins (1–6 and 9) were expressed (Figure 2A) and mediated RNAi against the target (Figure 2B), albeit all at lowered efficiency compared with the construct that carried the unmodified miR-30 hairpin structure (Figure 1, construct 8; and Figure 2). The expression of construct 7 was not detected, nor was its RNAi efficacy (Figures 1 and 2). This lack of expression of shRNA in construct 7 is unlikely to be caused by RNA degradation as shown by ethidium bromide staining of this gel before transferring (data not shown). Rather, it might be caused by a lack of processing due to the relatively drastic change from the authentic miR-30 structure in this construct.

To determine whether RNAi efficiency might be further increased by including more sequences from the pri-miR-30 RNA that flank the hairpin, we tested a construct that contained 100 bp on either side of the miR-30 hairpin sequence

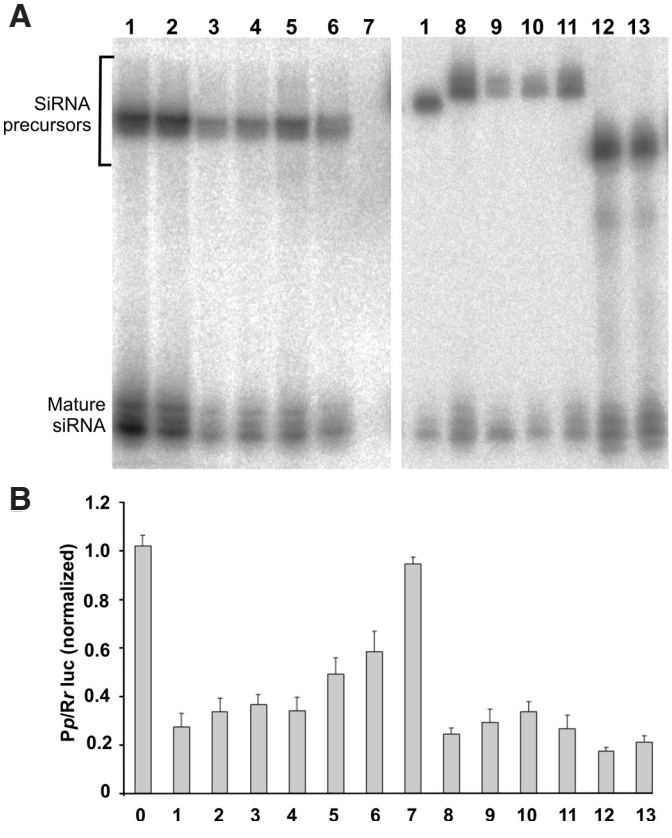

Figure 2. Comparisons in the levels of shRNA expression and in silencing efficacy. (**A**) Northern analysis of SOD1-shRNA expression under control of either Pol-II or Pol-III promoters. Each lane was loaded with equal amount of total RNA (20 μg/lane) extracted from HEK 293 cells transfected with indicated shRNA constructs. The equal loading was verified by staining the gel with ethidium bromide before transferring the RNA to the membrane (data not shown). Lane numbers correspond to the construct numbers shown in Figure 1. (**B**) Silencing activity of each construct. HEK 293 cells were transfected with Rr-luc-sod1 and Pp-luc plasmids together with various shRNA constructs against human SOD1 mRNA, located at 3′-UTR of firefly luciferase (Pp-luc) gene. Renilla luciferase (Rr-luc) plasmid served as transfection control. All luciferase activities were normalized to the value measured in the lysate from cells transfected with the empty vector pcDNA3 (carrier). The values are means with SD ($n = 6$). The construct numbers match with those in Figure 1.

from the pri-miR-30. In agreement with a previous report (30), the RNAi efficiency was not further enhanced from hairpin 8 (data not shown), suggesting that the authentic miR-30 hairpin 8 contains the essential elements for RNAi mediated by Pol II-synthesized shRNA.

Thus, it is noticeable from the above results that those hairpins with fewer structural alterations from miR-30 have RNAi efficiency closer to the hairpin with the authentic miR-30 structure (Figure 1, construct 8). For example, Hairpin 1 and 9, which had fewest structural alterations (Figure 1), mediated RNAi slightly less efficiently than hairpin 8 (Figure 2). In contrast, those with large structural alterations from miR-30 were far less effective or completely ineffective in RNAi (e.g. hairpins 6 and 7, see Figures 1 and 2). Interestingly, the least effective shRNAs share the lacking of the distal A/C bulge from the stem (Figures 1 and 2, hairpins 5–7), suggesting that this distal bulge is an important structural element of miR-30. Taken together, these results suggest that the authentic miR-30 structure is the most efficient in RNAi mediated by Pol II-synthesized shRNA in mammalian cells.

Our results differ from a previous study, in which a Pol II synthesized hairpin similar to hairpin 7 was shown to be processed correctly to form mature miRNA and mediate RNAi similar to hairpins with the authentic miR-30 structure (20,29). The reason for this difference is not clear. One possibility is the difference in the mRNA sequences that harbor the hairpin between these two studies and these differences could lead to different processing of the transcripts. In terms of the RNAi efficiency, we note that in the previous experiments a high ratio of shRNA-synthesizing plasmid to the target plasmid (16:1 to 80:1) was used. Consequently, the RNAi effects might be saturated and the differences between the hairpins with the authentic miR-30 structure and other variations might not be revealed. In our experiments, the ratio of shRNA-synthesizing plasmid to the target plasmid was one to one.

Knowing that the authentic miR-30 hairpin structure was the best for the Pol II-directed hairpin expression and RNAi, we tested the efficacy of this hairpin by placing it in two additional constructs. In the first, we tried to enhance the RNAi efficacy by placing two hairpins targeting the same sequence in the human SOD1 gene downstream of the Pol II promoter (Figure 1, construct 10) in order to produce more of the shRNA. This reasoning has precedents since some miRNA genes are located on the same chromosome as clusters with intervals as short as a few nucleotides and are expressed with similar profiles (18,24). Such an organization suggests that these miRNA genes are expressed under control of the same promoter and their nascent transcripts are processed to become mature miRNAs. Recent experiments supported this notion (31). Surprisingly, construct 10 failed to enhance the expression of shRNA and siRNA (Figure 2A), resulting instead in a decrease in RNAi efficacy (Figure 2B).

In the second construct, we placed the hairpin downstream of a different Pol II promoter, the human ubiquitin C promoter. This promoter has been used for constructing genes that were placed in transgenic mice and has been shown to efficiently express transgenes ubiquitously in multiple transgenic lines (25,32). We placed the hairpin 8 in the first intron, which was part of the promoter cassette. Downstream of the first intron, we placed the eGFP open reading frame (Figure 1, construct 11). The rationale for this design was based on recent evidence that some miRNAs are located in the non-coding regions or introns of mRNAs for known genes (18). Since synthesis of both the shRNA and the eGFP depends on the same promoter, their expression in the same cells is assured. This provides a convenient way to monitor the cells in which the shRNAs are expressed, not only qualitatively, but also quantitatively. This UbC-shRNA-EGFP construct expressed shRNA at a similar level as the CMV promoter (construct 8) and mediated RNAi as efficiently (Figure 1, construct 11; and Figure 2). Overall, the Pol III promoters still expressed at higher levels of shRNA and mediated stronger target silencing (Figure 1, constructs 12 and 13; and Figure 2). However, the best Pol II constructs, 8 and 11, are approaching the levels of RNAi efficacy of the Pol III constructs (Figure 2).

Can the Pol II-hairpin construct silence the endogenously expressed genes? To answer this question, we transfected the most effective hairpin constructs into the HEK293 cells. Compared with the controls, the best hairpin constructs 8 and 11 significantly inhibited the endogenous SOD1 expression (Figure 3A). Construct 11a, which was promoted by the UbC promoter but without the eGFP open reading frame, inhibited SOD1 expression similarly as construct 11,

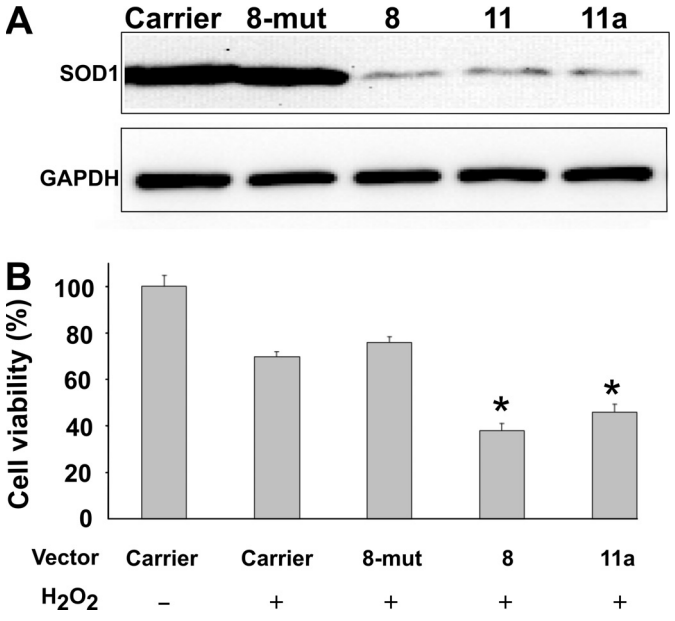

Figure 3. Pol II-directed synthesis of shRNA silenced the endogenous SOD1 expression and SOD1 function. (**A**) CMV and UbC promoter-directed synthesis of shRNAs knocked down the endogenous SOD1 in HEK 293 cells. HEK 293 cells were transfected with control and shRNA-expressing constructs and SOD1 levels were observed using western blot (see Methods). Each lane was loaded with 50 μg of total protein. After detecting SOD1, the same membrane was stripped and probed again with antibody recognizing glyceraldehye-3-phosphate dehydrogenase (GAPDH). Lanes were labeled with the construct numbers shown in Figure 1. Construct 8-mut had the hairpin sequence (antisense) 5′-GAAUCUUCAAGUCACACAUCGGC-3′, which contained 3 bp (underlined) that mismatched with the SOD1 target. Construct 11a was the same as construct 11 (Figure 1) but without EGFP open reading frame. (**B**) CMV and UbC promoter-directed synthesis of shRNAs sensitized HEK 293 cells to the toxicity of H_2O_2 (see Methods). Transfected cells were treated with 300 μM of H_2O_2 and the cell viability was measured using MTS assay. Mean values are from six independent experiments and error bars represent standard error of means. * indicates $P < 0.01$ when compared with carrier vector-transfected cells with or without H_2O_2 stimulation, or compared with 8-mut transfected cells.

indicating that eGFP did not interfere with the silencing activity of construct 11. To further confirm this silencing, we tested the function of SOD1 in these cells. Since a lack of SOD1 is known to enhance cell's vulnerability to H_2O_2 toxicity (33), we treated the cells with H_2O_2. Transfection with construct 8 and 11a significantly reduced the cell viability after the H_2O_2 treatment as compared with the control vectors (carrier vector and 8-mut) transfection (Figure 3B).

These experiments demonstrated that Pol II-directed synthesis of shRNA against SOD1 can mediate efficient RNAi. However, it was unclear whether this construct design was applicable to other target sequences and in different cell types. Furthermore, it remained unclear whether the eGFP expression provides a good indicator for the expression of the shRNA. To resolve these issues, we replaced the human SOD1 sequence in hairpin 8 with a sequence that target mouse Mn^{2+} superoxide dismutase (SOD2) (Figure 4A) and placed these hairpin in construct 11 (Figure 1). This construct along with the controls was transfected into NSC-34 cells. Because the transfection efficiency in these cells were lower (~50%) than in HEK293 cells (>90%), we selected clones that were transfected with the UbC-SOD2hp-eGFP vector. After expanding these clones, the SOD2 levels were measured. Different clones inhibited SOD2 expression differently (Figure 4B). Clone 3 inhibited SOD2 expression the most, followed by clones 5 and 4. The different levels of inhibition were correlated with the eGFP expression (Figure 4C, lower panels) and cellular toxicity. Clone 3 expressed the highest level of eGFP, followed by clones 5 and 4. Compared with the untransfected cells or cells transfected with the mutant

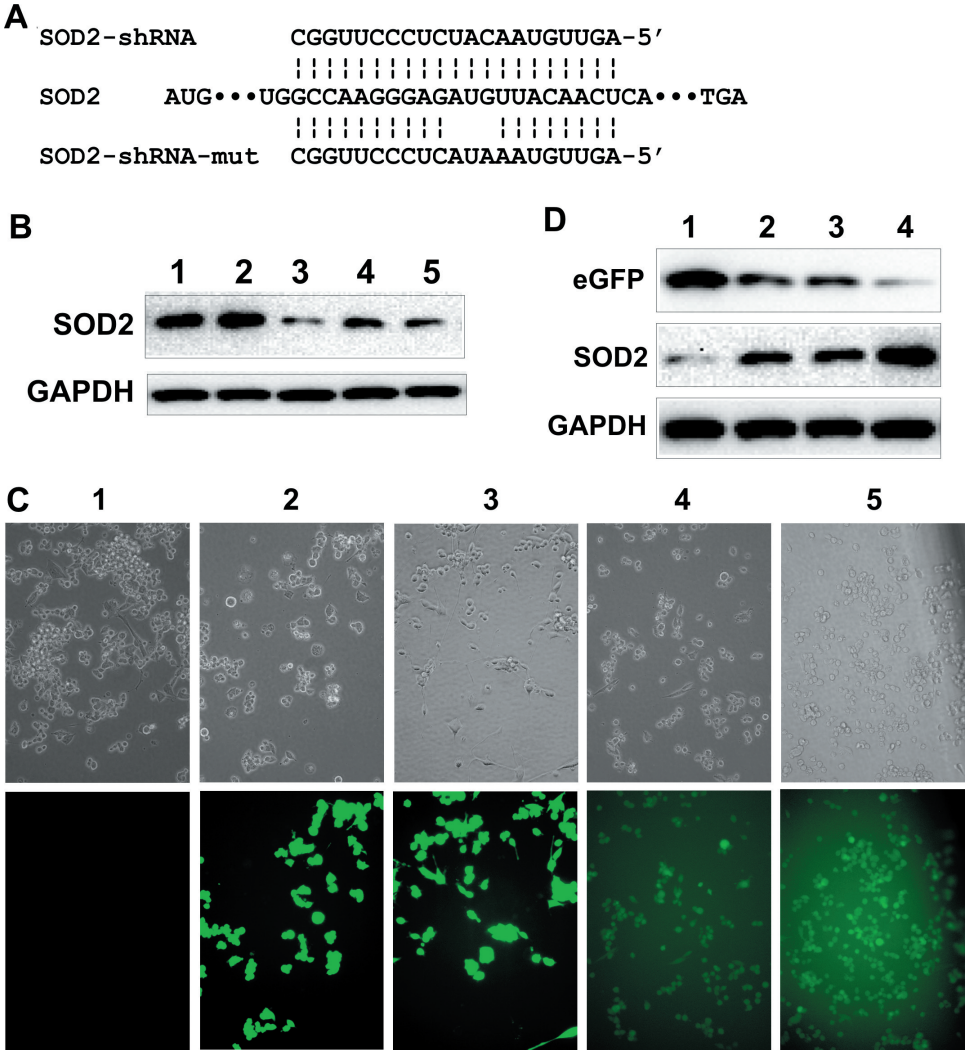

Figure 4. UbC-hp-EGFP provides a convenient indicator for shRNA expression. (**A**) The shRNA (antisense) sequence against Mn^{2+} superoxide dismutase (SOD2) was incorporated into the hairpin 8 structure and inserted into construct 11 (see Figure 1). A mutant hairpin construct that mismatched with SOD2 (SOD2-shRNA-mut) was also made as a control. (**B**) UbC-SOD2hp-EGFP expressed EGFP and knocked down endogenous SOD2 in stably transfected NSC34 cells. Western blot was similarly done as described in Figure 3 excepting using anti-SOD2 as the primary antibody. Lane 1, untransfected NSC 34 cells; lane 2, cells stably transfected with UbC-SOD2hpMut-EGFP; lane 3, cells transfected with UbC-SOD2hp-EGFP that express EGFP at high level; lanes 4 and 5, cells transfected with UbC-SOD2hp-EGFP that express EGFP at lower levels. (**C**) Morphology of NSC34 cells. NSC34 cells were photographed under a microscope using phase contrast (upper panels) and fluorescence (lower panels). The numbers correspond to those in (B). (**D**) NF-1 cells transfected with construct 11 were separated into four groups based on their eGFP fluorescence levels, from the highest in group 1 to the lowest in group 4 (see Methods). Proteins from these cell groups were immunoblotted for eGFP and SOD2 levels, with GAPDH as the loading control.

SOD2 hairpin (clone 2), all cell clones that expressed SOD2 shRNA were unhealthy, the degree of which appeared more severe in clones 3 and 5 than clone 4 (Figure 4C, upper panels). Clones 3 and 5 expressed more eGFP than clone 4 (Figure 4C, lower panels). All clones that expressed eGFP and SOD2 shRNA could not be maintained for the long term, probably due to the cellular toxicity of SOD2 knockdown.

To further confirm the quantitative correlation between the eGFP expression and the degree of silencing, we sorted cells that were transfected with construct 11 into four groups based on their eGFP expression levels. Proteins extracted from these cell groups were immunoblotted for eGFP and SOD2 (Figure 4D). The levels of eGFP were inversely correlated with the levels of SOD2, indicating that eGFP was a good indicator for the levels of shRNA expression and silencing efficacy.

Taken together, we have demonstrated that, by adopting the authentic miR-30 hairpin structure, Pol II can direct shRNA synthesis and mediate gene silencing with high efficiency comparable to Pol III. Therefore, this Pol II-hairpin design offers an alternative to Pol III constructs. Because the shRNA expression is under the control by same promoter as the indicator protein, the indicator protein indicates not only the cells that express the shRNA, but also the levels of the shRNA expression. This is especially valuable if an experiment desires to examine the effects of a gene expressed at different levels. Using construct 11, for example, one may separate cells with different levels of shRNA expression. Alternatively, one may establish the stably transfected cell lines that express different levels of shRNA using the sorted cells from the FACS, as demonstrated in Figure 4D. A further utility of this Pol II construct is that by replacing the ubiquitous Pol II promoter with various cell type-specific Pol II promoters, cell-specific gene silencing may be accomplished *in vivo*. In addition, various temporally inducible Pol II promoters, e.g. the tetracycline- or ecdysone-inducible promoters, can be used to temporally control gene silencing.

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Conflict of interest statement. None declared.

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