

An inducible system for expression and validation of the specificity of short hairpin RNA in mammalian cells

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ABSTRACT

RNA interference (RNAi) by means of short hairpin RNA (shRNA) has developed into a powerful tool for loss-of-function analysis in mammalian cells. The principal problem in RNAi experiments is off-target effects, and the most vigorous demonstration of the specificity of shRNA is the rescue of the RNAi effects with a shRNA-resistant target gene. This presents its own problems, including the unpredictable relative expression of shRNA and rescue cDNA in individual cells, and the difficulty in generating stable cell lines. In this report, we evaluated the plausibility of combining the expression of shRNA and rescue cDNA in the same vector. In addition to facilitate the validation of shRNA specificity, this system also considerably simplifies the generation of shRNA-expressing cell lines. Since the compensatory cDNA is under the control of an inducible promoter, stable shRNA-expressing cells can be generated before the knockdown phenotypes are studied by conditionally turning off the rescue protein. Conversely, the rescue protein can be activated after the endogenous protein is completely repressed. This approach is particularly suitable when prolonged expression of either the shRNA or the compensatory cDNA is detrimental to cell growth. This system allows a convenient one-step validation of shRNA and generation of stable shRNA-expressing cells.

INTRODUCTION

RNA interference (RNAi) is an evolutionarily conserved gene-silencing process triggered by double-stranded RNAs (dsRNAs) (1). The use of RNAi as a technique for analyzing loss-of-function phenotypes has revolutionized research in mammalian cells. One way to induce RNAi in mammalian cells is by transfection of synthetic

small interfering RNAs (siRNAs). These siRNAs are 19-base-pair (bp) dsRNA with 2-nucleotide (nt) 3' overhangs (2), and mimic the structure of microRNA (miRNA) intermediates of the natural processing of longer dsRNA by RNase III. One strand of the siRNA or miRNA duplexes (called guide strand) is incorporated into the RNA-induced silencing complex (RISC), where it directs RISC to bind to complementary mRNA. It is believed that the other strand of the siRNA or miRNA (called passenger strand) is not incorporated into RISC and is destroyed. RISC cleaves the mRNAs at a site 10 nt upstream of the nucleotide complementing the 5'—most nucleotide of the guide strand, and the mRNA fragments are degraded by other nucleases, resulting in knockdown of expression (3).

An alternative way to induce RNAi in mammalian cells is by expression plasmids or viral vectors. A common approach involves the transcription by RNA polymerase III of short hairpin RNAs (shRNA). The shRNAs consist of a stem of 19–29 bp linked by a small terminal loop (4–6). The prevailing view is that shRNAs mimic the structure of a miRNA intermediate generated by the RNase III enzyme Droscha. Another RNase III enzyme called Dicer acts on the shRNAs to produce siRNA/miRNA duplexes, which are then loaded onto RISC to mediate silencing (7).

The use of shRNA offers several important advantages over siRNA (8). First, more delivery options are available for shRNA, including transfection, electroporation and infection with viral vectors. Second, substantially lower cost is required to generate shRNA than siRNA. Furthermore, while silencing using siRNA is inevitably transient, shRNA-expressing constructs can be stably integrated into the genome. Finally, while the effects of siRNA after delivery is constitutive, both constitutive and inducible systems can be used for shRNA after delivery.

It is generally accepted that the major problem of using shRNAs (as well as siRNAs) in experimentation is the possibility of off-target effects (9,10). Several methods are utilized to confirm the specificity of the RNAi results, including the use of shRNAs against irrelevant targets

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and the use of multiple shRNAs against the same gene. However, the ultimate control for shRNA experiment is the rescue of the RNAi effects by the expression of the target gene in a form refractory to the shRNA (11,12). This is usually achieved by introducing one or more silent point mutations to the region of the cDNA that is targeted by the shRNA.

The rescue of RNAi phenotypes using shRNA-resistant cDNA itself may present several problems. It is likely that individual cells may take up different amount of shRNA-versus cDNA-expressing constructs, triggering a spectrum of phenotypes within a population. Moreover, it is not trivial to obtain stable expression of both shRNA and cDNA at the same time. Here we describe a solution to the problems using a system that expresses both the shRNA and the rescue cDNA from the same plasmid. As the cDNA is under the control of an inducible promoter, the effects of the gene knockdown are effectively under conditional control. This considerably simplifies the generation of stable cell lines when prolonged expression of either the shRNA or the compensatory cDNA is detrimental to cell growth. The effectiveness of the pKAR system is demonstrated with cyclin A and MAD2.

MATERIALS AND METHODS

Materials

All reagents were obtained from Sigma-Aldrich (St. Louis, MO, USA) unless stated otherwise.

DNA constructs

pKAR1 was based on pUHD-P1/3C (13), which was in turn based on the tetracycline-inducible system pUHD10-3 (14) (a gift from Dr Hermann Bujard, University of Heidelberg, Germany), and mU6pro (5) (a gift from Dr David Turner, University of Michigan, MI, USA). The *Bam*H I-*Bam*H I fragment was first removed from pUHD-P1/3C. The resulting plasmid was cut with *Hind* III-*Pvu* II, and inserted with the *Hind* III-*Pvu* II fragment from mU6pro. *Bbs* I sites were then destroyed by mutagenesis using the oligonucleotides 5'CCCTTTCGTCTTTAGTCGAGTTT3', 5'CATAGAA GAGACCGGGACC3' and 5'GAGGCGAAGCTTCG GGCGGC3' (and their antisense). Mutation of the *Bbs* I site in the CMV promoter did not affect expression (our unpublished data). Specific shRNA constructs were created by annealing the following pairs of primers into *Bbs* I-*Xba* I of mU6pro or pKAR1: 5'TTTGGTAGCA GAGTTTGTGTACATTCAAGAGATGTACACAAA CTCTGCTACTTTTT3' and 5'CTAGAAAAAGTAGC AGAGTTTGTGTACATCTCTTGAATGTACACAAA CTCTGCTAC3' (corresponded to positions 823–841 of human cyclin A2 ORF); 5'TTTGGAGTCGGGACCA CAGTTTATTCAAGAGATAAACTGTGGTCCCGAC TCTTTTT3' and 5'CTAGAAAAAGAGTCGGGACCA CAGTTTATCTCTTGAATAAACTGTGGTCCCGAC TC3' (corresponded to positions 505–523 of human MAD2 ORF). Site-directed mutagenesis was carried out with QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Plasmids expressing FLAG-tagged cyclin A (15), GST-3C protease (13) and

histone H2B-GFP (16) were constructed or obtained from sources as previously described. Cyclin A resistant to the shRNA was created by introducing silent mutations using the oligonucleotide 5'CCAGAAGTAGCGGAATTCGTC TACATTACAGA3' and its antisense. The cyclin A shRNA (in mU6pro) was ligated into this plasmid using the *Hind* III-*Pvu* II sites to create FLAG-cyclin A/shRNA in pKAR1. The *Bam*H I fragment containing the puromycin-resistant gene (a gift from Katsumi Yamashita, Kanazawa University, Japan) was put into *Bam*H I-cut FLAG-cyclin A/shRNA in pKAR1 to generate FLAG-cyclin A/shRNA in pKAR1/PUR. MAD2 in CMV5 was a gift from Robert Benezra (Memorial Sloan-Kettering Cancer Center, NY, USA). The *Nco* I fragment was ligated into pUHD-P2 (15) to generate HA-MAD2 in pUHD-P2. Silence mutations were introduced using 5'GAGTCAGGTCCTCAGTTTA3' (and its antisense) to create a shRNA-resistant MAD2. The MAD2 shRNA (in mU6pro) was ligated into this plasmid using the *Hind* III-*Pvu* II sites to create HA-MAD2/shRNA in pKAR1.

Cell culture

HtTA1 cells are HeLa cells (human cervical carcinoma) expressing the tTA tetracycline repressor chimera (15). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) calf serum (Invitrogen Life Technologies, Carlsbad, CA, USA) in a humidified incubator at 37°C in 5% CO₂. Unless stated otherwise, cells were treated with the following reagents at the indicated final concentration: blasticidin (5 µg/ml), doxycycline (2 µg/ml), nocodazole (0.1 µg/ml), and puromycin (1 µg/ml). Cells were transfected with the calcium phosphate precipitation method (17). Cell-free extracts were prepared as previously described (18). For transient expression of shRNA-expressing plasmids, a plasmid expressing histone H2B-GFP and a blasticidine-resistant gene was cotransfected and cells were grown in a medium containing the blasticidin for 36 h to enrich the transfected cells. Selection medium was washed out and the cells were grown in normal medium for another 12 h. For generation of stable cell lines, cells were transfected with FLAG-cyclin A/shRNA in pKAR1/PUR and grown in the medium containing puromycin. After about two weeks of selection, individual colonies were isolated and propagated in the absence of puromycin. Individual clones were either mock-treated or exposed to doxycycline for 48 h before cell-free extracts were prepared. The knockdown of endogenous cyclin A and the inducible expression of FLAG-cyclin A were evaluated by immunoblotting for cyclin A.

Flow cytometry

Cells were trypsinized and washed with phosphate-buffered saline (PBS). The cells were then fixed in ice-cold 80% ethanol and stained with a solution containing 40 mg/ml propidium iodide and 40 mg/ml RNaseA at 37°C for 30 min. Cell cycle distribution (for 10 000 cells) was analyzed using a FACSort machine (Becton-Dickinson). For bivariate analysis of DNA content and cyclin A expression, cells were harvested by trypsinization,

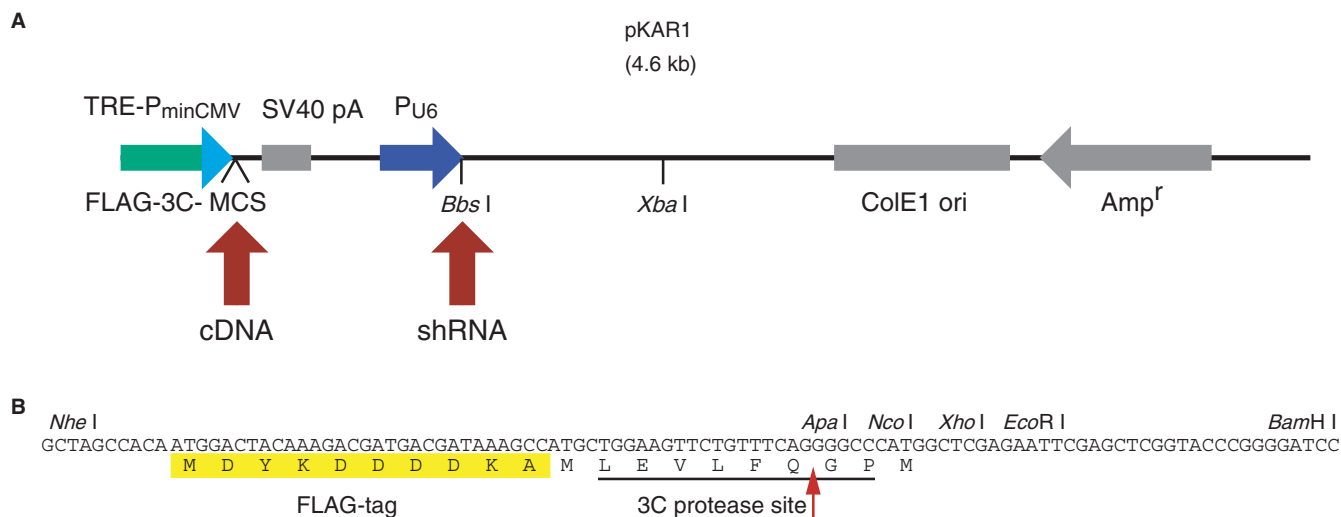


Figure 1. A vector for co-expression of shRNA and shRNA-resistant cDNA. (A) Schematic diagram of pKAR1. The various elements are shown to scale: TRE, tetracycline response element; P_{minCMV} , minimal immediate early cytomegalovirus (CMV) promoter; FLAG-3C-MCS, multiple cloning sites; SV40 pA, SV40 polyadenylation signal; P_{U6} , mouse U6 RNA promoter; Amp^r , ampicillin-resistant gene; ColE1 ori, ColE1 replication origin. The shRNA is first inserted between *Bbs*I-*Xba*I exactly as described for mU6pro (5), and the shRNA-resistant cDNA is cloned into the multiple cloning sites (see panel B). (B) The cDNA cloning region of pKAR1. The protein sequence of the N-terminal tag is shown. After the cloning of the shRNA, the cDNA can be inserted in frame into the unique *Apa*I, *Nco*I, *Xho*I, *Eco*R I and *Bam*H I sites. Un-tagged version can be cloned using the *Nhe*I site. The FLAG-tag is highlighted, the 3C protease recognition sequence is underlined, and the arrow indicates the site of cleavage.

fixed in 1% v/v paraformaldehyde for 5 min at 25°C, and resuspended in ice-cold MeOH for 10 min. The cell pellet was washed twice with PBST (PBS + 0.5% Tween + 0.05% w/v BSA), resuspended in the residue buffer, and incubated with 1 µg of monoclonal antibody E23 at 25°C for 60 min. The cells were washed twice with PBST, resuspended in the residue buffer, and incubated with 2.5 µl of FITC-conjugated rabbit anti-mouse IgG (DAKO, Glostrup, Denmark) at 25°C for 60 min. After washed twice in PBST, the cells were processed for propidium iodide staining and flow cytometry.

Antibodies and immunological methods

Monoclonal antibodies A17 against CDC2 (19), E23 against cyclin A2 (20), and M2 against FLAG tag (21) were obtained from sources as previously described. Monoclonal antibody V152 against cyclin B1 was a gift from Dr Julian Gannon and Dr Tim Hunt (Cancer Research UK, UK). Monoclonal antibody against MAD2 was obtained from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA). Immunoblotting was performed as previously described (18).

RESULTS AND DISCUSSION

We constructed the pKAR (Knockdown And Rescue) plasmids based on an inducible system designed by Hermann Bujard's group (14), and a shRNA-expressing system originated from David Turner's group (5) (Figure 1A). The shRNA was expressed from a mouse U6 RNA promoter, and the rescue cDNA was expressed

under the control of doxycycline. The cDNA expressed from pKAR1 was engineered to fuse at the N-terminus with a FLAG-tag and a 3C protease cleavage site (Figure 1B). Due to the slight increase in size conferred by the epitope tag, both the endogenous protein to be silenced and the ectopically expressed version can be detected simultaneously. The tag also allowed the recombinant protein to be specifically detected or immunoprecipitated. Furthermore, the epitope tag can be removed using 3C proteases.

To evaluate if the pKAR system works in principal, we put it to test by targeting two genes: *cyclin A* and *MAD2*. Cyclin A plays critical roles in S phase and mitosis (22), and *MAD2* is an essential component of the spindle-assembly checkpoint (23). Oligonucleotides designed to express shRNAs against these genes were put behind the U6 promoter. The rescue *MAD2* and cyclin A cDNAs were subcloned under the control of tetracycline response element (TRE) (Figure 2A). Silence mutations were introduced into the regions that are targeted by the shRNAs, rendering their mRNAs to be resistant to the knockdown (Figure 2B). In this study, HeLa cells expressing the tTA tetracycline repressor chimera were used, so that the expression of the rescue cDNAs was repressed in the presence of doxycycline.

To determine if the endogenous cyclin A could be downregulated by the cyclin A/shRNA construct, cells were transfected with either control vectors or the cyclin A/shRNA construct. Figure 3A shows that the expression of cyclin A was effectively attenuated by the shRNA (lanes 1 and 2). As expected, FLAG-cyclin A (which exhibited a slightly slower gel mobility than the endogenous cyclin A) was expressed in the absence, but not the

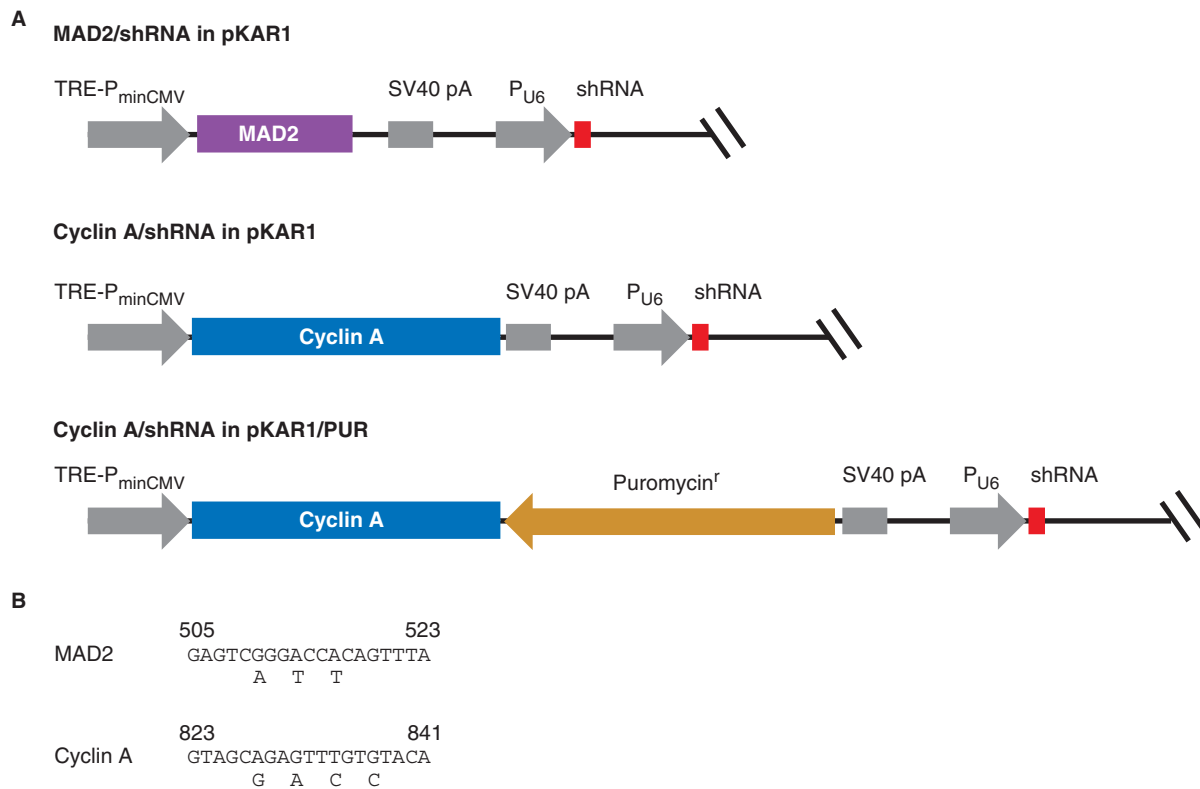


Figure 2. Schematic diagrams of constructs used in this study. (A) Plasmids containing MAD2 shRNA, cyclin A shRNA, shRNA-resistant MAD2, and shRNA-resistant cyclin A were constructed as indicated. A puromycin-resistant gene (which contained its own SV40 early promoter and SV40 polyadenylation signal) was also inserted into the cyclin A/shRNA construct. The various elements are shown to scale, and only a part of vector is shown (compare Figure 1). (B) The shRNA-targeted sequences and the silence mutations of MAD2 and cyclin A. The sequences in human MAD2 and cyclin A that are targeted by the shRNAs are shown. The numbers correspond to the nt positions in the open reading frames. The bases that were changed in generating the silent mutations are indicated below the sequences.

presence of doxycycline. The expression of the recombinant FLAG-cyclin A was also confirmed by immunoblotting for FLAG. Figure 3B shows that FLAG-cyclin A was suppressed by doxycycline progressively over the time course of the experiment. These results indicate that while the endogenous cyclin A could be silenced by the shRNA, the co-expressed recombinant cyclin A was refractory to the knockdown.

To further verify the versatility of the pKAR system, we performed the converse experiment by turning on the rescue cDNA after the endogenous protein was knock-down by the shRNA. Cells were transfected with MAD2/shRNA in pKAR1 in the presence of doxycycline to repress the expression of the recombinant MAD2. Figure 3C shows that the endogenous MAD2 was effectively knockdown by the procedure (lanes 1 and 2). Furthermore, the shRNA-resistant MAD2 (slightly larger than the endogenous protein because of the epitope tag) was induced robustly after the removal of doxycycline in the medium. Taken together, these data demonstrate that the rescue cDNAs could either be turned on or off after the endogenous proteins were silenced.

To generate cell lines that stably express cyclin A shRNA and the corresponding rescue cDNA, a puromycin-resistant gene was engineered into the cyclin A/shRNA construct (Figure 2). Cells were transfected

and selected in medium containing puromycin and in the absence of doxycycline. The basis of this was that the downregulation of cyclin A without a compensatory expression of the shRNA-resistant cyclin A would be cytotoxic. After selection, individual colonies were isolated and the knockdown of the endogenous cyclin A and the expression of the recombinant cyclin A were analyzed (Figure 4). We were able to generate cell lines that expressed FLAG-cyclin A, but were deficient in the expression of endogenous cyclin A. We also confirmed that the FLAG-cyclin A expression could be switched off with doxycycline.

To determine if fine adjustment of cyclin A expression can be achieved in the stable cell lines, cells were treated with different concentrations of doxycycline before harvested. Figure 5A shows that a range of cyclin A expression, from an undetectable level to a highly overexpressed level, was obtained by varying the doxycycline concentration. The expression of FLAG-cyclin A could be turned off relatively rapidly (Figure 5B). Our conclusion is that while the method is rather robust, the precise dose and time of doxycycline adopted for fine adjustment of the rescue protein will have to be determined empirically (which depends on the half-life of the protein and its levels relative to the endogenous protein).

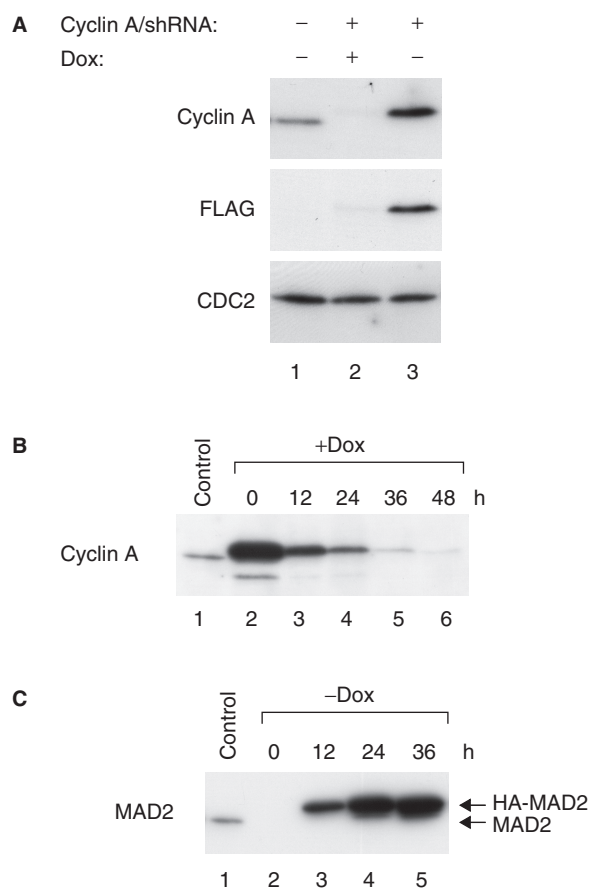


Figure 3. Knockdown and rescue by transient transfection of pKAR1 constructs. **(A)** Knockdown of endogenous cyclin A and expression of recombinant cyclin A by transient transfection. HtTA1 cells were transfected with either control vectors or cyclin A/shRNA-expressing plasmids. After enriching the transfected cells (Materials and Methods), over 90% of cells expressed a cotransfected GFP-tagged histone H2B (data not shown). Cells expressing cyclin A and shRNA were either mock-treated or treated with doxycycline for 48 h as indicated. Cell-free extracts were prepared and subjected to immunoblotting for cyclin A and FLAG. Uniform loading of lysates was confirmed by immunoblotting for CDC2. **(B)** Suppression of recombinant cyclin A expression by doxycycline. Cells were transfected with cyclin A/shRNA in pKAR1 as described in panel A. After doxycycline was applied, cell-free extracts were prepared at the indicated time points and subjected to immunoblotting for cyclin A. Lysates from control cells were loaded in lane 1. The lower band in lane 2 probably is a degradative product of FLAG-cyclin A. **(C)** Knockdown and rescue of MAD2. HtTA1 cells were transfected with either control vectors (lane 1) or MAD2/shRNA-expressing plasmids in the presence of doxycycline. After enriching the transfected cells, the MAD2- and shRNA-expressing cells were washed and grown in doxycycline-free medium. At the indicated time points, cell-free extracts were prepared and subjected to immunoblotting for MAD2.

Although the total cyclin A was reduced to a very low level when both the endogenous and the rescue cyclin A were repressed, it is conceivable that a minor portion of cells still expressed high levels of cyclin A. To examine this possibility, the abundance of cyclin A in individual cells was determined with flow cytometry. Several lines of evidence indicate that cyclin A is actively degraded during mitosis and G₁ phase (24). In agreement with this, two populations of cells with different cyclin A levels were detected with flow cytometry (Figure 6A).

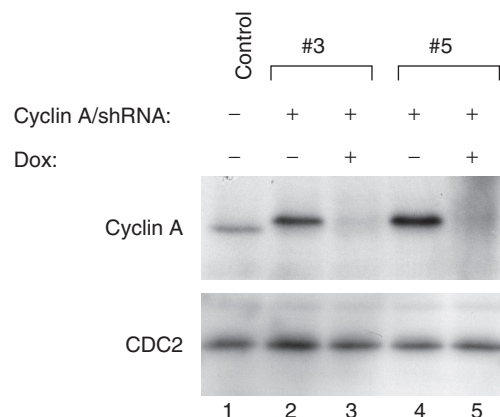


Figure 4. Knockdown and conditional rescue of cyclin A in stable cell lines. HtTA1 cells were transfected with cyclin A/shRNA in pKAR1/PUR and grown in puromycin-containing medium. After about two weeks of selection, individual colonies were isolated and were either mock-treated or treated with doxycycline for 48 h. Cell-free extracts were prepared and cyclin A was detected by immunoblotting. Extracts from control cells and two representative clones were loaded. Equal loading of lysates was confirmed by immunoblotting for CDC2.

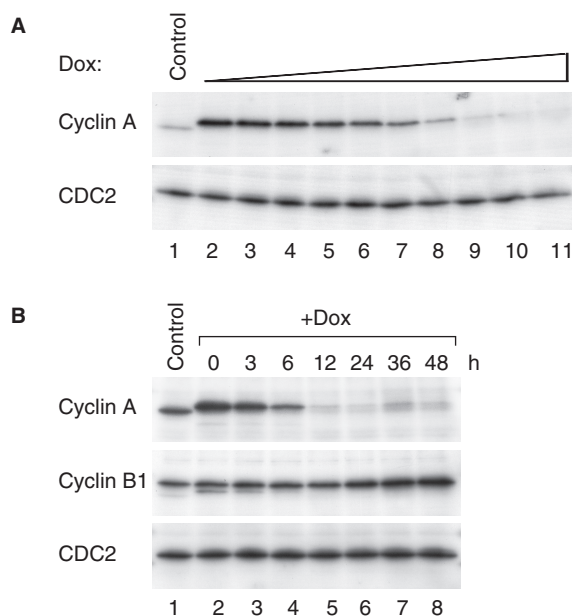


Figure 5. Regulation of cyclin A expression in stable cyclin A/shRNA-expressing cells. **(A)** Dose-dependent repression of cyclin A by doxycycline in stable cyclin A/shRNA-expressing cells. A cyclin A/shRNA-stable cell line (clone 11) was treated with different doses of doxycycline (from lanes 2–11: 1.3, 2.6, 3.3, 4.4, 6.6, 13.3, 40, 400 and 2000 ng/ml) for 48 h. Cell-free extracts were prepared and the expression of cyclin A was detected by immunoblotting. Extracts from control cells were loaded in lane 1 and CDC2 analysis was included to assess protein loading and transfer. **(B)** Time-dependent repression of cyclin A by doxycycline in stable cyclin A/shRNA-expressing cells. A cyclin A/shRNA-stable cell line (clone 3) was exposed to 2 µg/ml of doxycycline and harvested at the indicated time points. Cell-free extracts were prepared and were subjected to immunoblotting for cyclin A and cyclin B1. Extracts from control cells were loaded in lane 1 and uniform loading of lysates was confirmed by immunoblotting for CDC2.

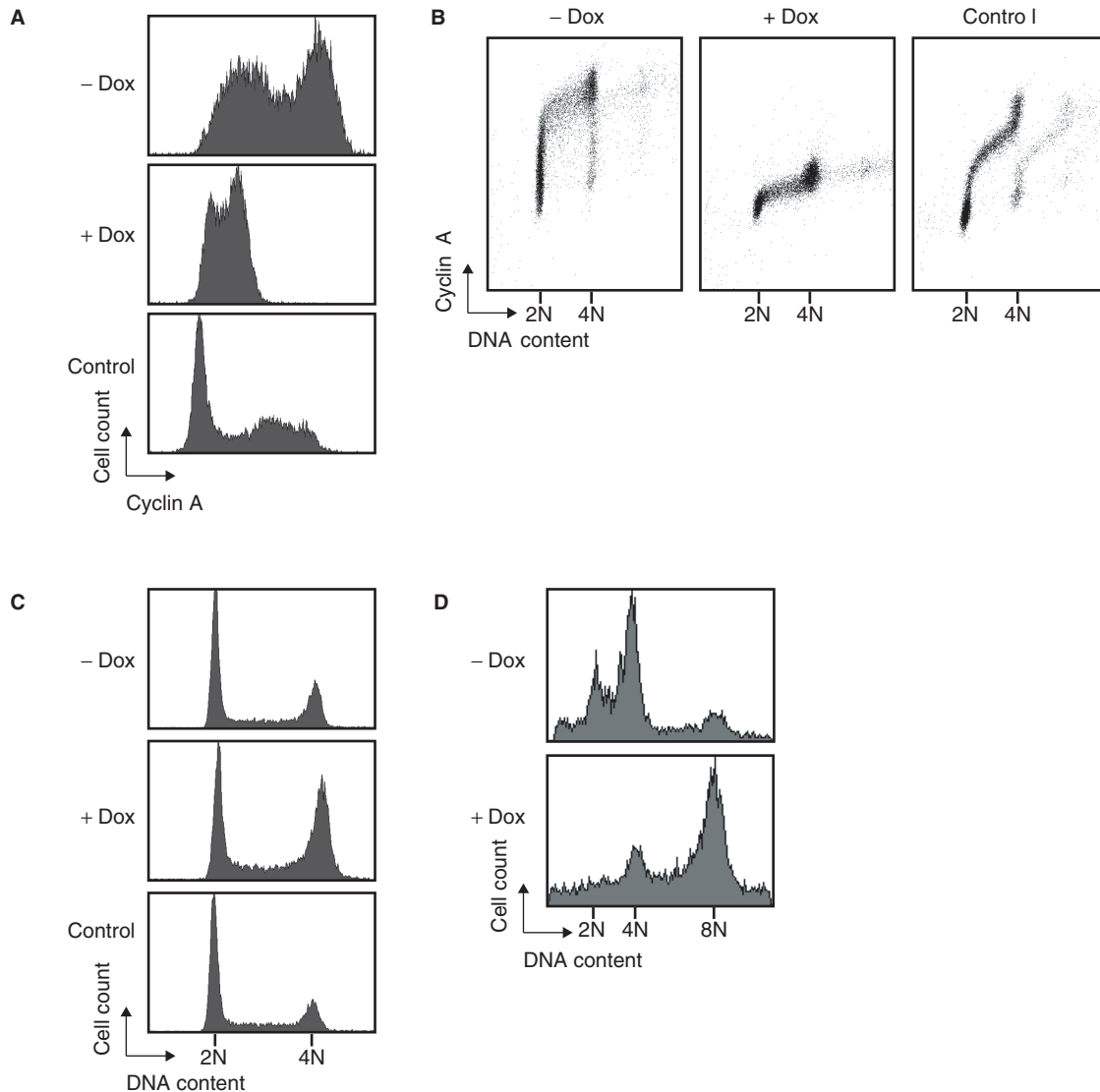


Figure 6. Manifestation of knockdown phenotypes after the removal of the rescue cyclin A or MAD2. (A) Cyclin A is silenced by shRNA in the whole cell population. Stable cyclin A/shRNA-expressing cells (clone 3) were either mock-treated or treated with doxycycline for 48 h. The cells were then fixed, stained with a monoclonal antibody against cyclin A, and processed for flow cytometry analysis. The parental HtTA1 cells were also analyzed as a control. (B) Cyclin A is repressed by shRNA throughout the cell cycle. Stable cyclin A/shRNA-expressing cells were either mock-treated or exposed to doxycycline for 48 h. The cells were then fixed, stained with a monoclonal antibody against cyclin A and propidium iodide, and subjected to bivariate flow cytometry analysis. The parental HtTA1 cells were also analyzed as a control. The positions of the 2N and 4N DNA contents are indicated. (C) Knockdown of cyclin A delays the cell cycle at G₂/M. Stable cyclin A/shRNA-expressing cells were either mock-treated or treated with doxycycline for 48 h. The cells were then fixed, stained with propidium iodide, and processed for flow cytometry analysis. The parental HtTA1 cells were also analyzed as a control. The positions of the 2N and 4N DNA contents are indicated. (D) Knockdown of MAD2 abolishes the spindle-assembly checkpoint. Cells were transfected with MAD2/shRNA in pKAR1. Plasmids expressing GFP-histone H2B constitutively were cotransfected to allow the identification of transfected cells. The cells treated with nocodazole, and were either mock-treated or treated with doxycycline for 44 h. The cells were then fixed, stained with propidium iodide, and processed for flow cytometry analysis of the GFP-positive cells. The positions of the 2N, 4N and 8N DNA contents are indicated.

Doxycycline reduced the expression of cyclin A in the entire population, suggesting that cyclin A was not only eliminated in selected cells. After staining with propidium iodide, bivariate analysis further indicated that cyclin A was reduced in different phases of the cell cycle (Figure 6B).

Cells expressing cyclin A shRNA and the compensatory cDNA together displayed a relatively normal cell cycle profile (Figure 6C). In marked contrast, a prominent G₂/M

delay was introduced after FLAG-cyclin A was repressed. In agreement with this, cyclin B1 (which normally accumulates during the G₂ phase and mitosis) also increased after the elimination of cyclin A (Figure 5B). Detailed analysis of the cyclin A knockdown phenotypes will be described elsewhere. This brief analysis serves to illustrate that the cytostatic phenotypes from shRNA can be conditionally rescued in stable cell lines, underscoring the usefulness of the pKAR system.

To further validate the effectiveness of the system for knockdown and rescue, the spindle-assembly checkpoint was analyzed after the knockdown of MAD2. Cells were transfected with MAD2/shRNA in pKAR1 and the expression of the compensatory MAD2 was either induced or suppressed with doxycycline. As expected, cells co-expressing MAD2 shRNA and the rescue MAD2 were blocked with 4N DNA contents after treatment with the spindle-disrupting drug nocodazole (Figure 6D). In marked contrast, cells expressing MAD2 shRNA in the absence of compensatory MAD2 failed to be arrested by nocodazole, and continue to re-replicate their DNA. These data indicate that MAD2 knockdown phenotypes could be conditionally rescued with the pKAR system.

In summary, we have devised a vector that can express shRNA and the respective rescue cDNA together. The pKAR1 vector provides a convenient way to subclone shRNA and the rescue cDNA, as well as for the generation of stable cell lines. This system is particularly useful when the prolonged expression of either the shRNA or the compensatory cDNA is cytotoxic. In the first scenario, the shRNA can be allowed to completely knockdown the endogenous proteins in the presence of the rescue cDNA; the rescue cDNA can then be turned off to attain the knockdown phenotypes. In the second scenario, the rescue cDNA can be turned on only after the endogenous proteins are completely eliminated. Here we have used a cell line expressing the tTA tetracycline repressor chimera, so that the expression of the rescue cDNA was repressed by doxycycline. Likewise, cell lines expressing the reverse tTA (25) can also be adopted to turn on the rescue cDNA with doxycycline. Other applications of the method include the conditional removal of the rescue protein for a defined period of time before the rescue protein is restored. We found that the pKAR system is particularly suitable for generation of stable cell lines. Probably due to the toxicity of both cyclin A/shRNA and cDNA, we found that all the colonies isolated were inevitably without endogenous cyclin A and expressing FLAG-cyclin A. Furthermore, clones that grew at a normal rate tended to express the rescue cyclin A at a level similar to that in control cells (our unpublished data). Thus this method appears to have an additional advantage of isolating clones that express the rescue protein at a comparable level to the endogenous protein.

In conclusion, the pKAR system allows a convenient one-step validation of shRNA and generation of stable shRNA-expressing cells.

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Conflict of Interest Statement. None declared.

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