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 Drosha. 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.
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 BamHI I-BamHI I fragment was first removed from pUHD-P1/3C. The resulting plasmid was cut with HindIII-Pvu II, and inserted with the HindIII-Pvu II fragment from mU6pro. BbsI sites were then destroyed by mutagenesis using the oligonucleotides 5’CCCTTTCTGCTTTAGTCGAGTTT3’ and 5’CATAGAA GAGACCGGAGGACC3’ and 5’GAGGCGAAGCTTCG GGCAGC3’ (and their antisense). Mutation of the BbsI 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 BbsI 5’-XbaI 1 of mU6pro or pKAR1: 5’TCTTGTAGCA GAGTTTGTGATCATTCAAGAGATGTACAAAA CTCTGCTACTTTT3’ and 5’CATAGAAAGATGC AGAGTTTGTGATCATTCAAGAGATGTACAAAA CTCTGCTACTC3’ (corresponded to positions 823–841 of human cyclin A 2 ORF); 5’TCTTGGAGTCGGGACCA CAGTTTATCTAAGATGCCATGTCCGAC TCCTTTT3’ and 5’CTAGAAAAAGATGCAGTTTGGAGTCGGGACCA CAGTTTATCTAAGATGCCATGTCCGAC 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’CCAGAAAGTAGCGGAATTCGTC TACATTACAAGA3’ and its antisense. The cyclin A shRNA (in mU6pro) was ligated into this plasmid using the HindIII-Pvu II sites to create FLAG-cyclin A/shRNA in pKAR1. The BamHI I fragment containing the puromycin-resistant gene (a gift from Katsumi Yamashita, Kanazawa University, Japan) was put into BamHI 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 Neo I fragment was ligated into pUHD-P2 (15) to generate HA-MAD2 in pUHD-P2. Silence mutations were introduced using 5’GAGTCAGGTCCTCATTTT3’ (and its antisense) to create a shRNA-resistant MAD2. The MAD2 shRNA (in mU6pro) was ligated into this plasmid using the HindIII-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 (5μg/ml), doxycycline (2μg/ml), nocodazole (0.1μg/ml), and puromycin (1μg/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 blasticidin-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 immuno-blotting 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 RNase A 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,
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 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 immuno-precipitated. 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
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 knocked 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 knocked down 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).
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).
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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