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Methodology article

Expressing functional siRNAs in mammalian cells using convergent transcription

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Abstract

Background: The use of small interfering RNAs (siRNAs) as genetic inhibitors of gene expression has been shown to be an effective way of studying gene function in mammalian cells. Recently, different DNA vectors for expression of small hairpin RNAs (shRNAs) or co-expression of sense and antisense RNAs have been developed that direct siRNA-mediated gene silencing. One expression cassette design that has been used to express long sense and antisense RNAs in non-mammalian cell types is symmetric transcription using convergent promoters. However, convergent transcription as a way to generate functional siRNAs in mammalian cells has not been reported. This vector design permits the generation of expression constructs containing no repeat sequences, but capable of inducing RNA interference (RNAi)-mediated gene silencing.

Results: With the aim of simplifying the construction of RNAi expression vectors, we report on the production and application of a novel convergent promoter cassette capable of expressing sense and antisense RNAs, that form double-stranded RNA, and mediate gene silencing in mammalian cells. We use this cassette to inhibit the expression of both the EGFP transgene and the endogenous TP53 gene. The gene silencing effect is Dicer-dependent and the level of gene inactivation achieved is comparable to that produced with synthetic siRNA. Furthermore, this expression system can be used for both short and long-term control of specific gene expression in mammalian cells.

Conclusion: The experiments performed in this study demonstrate that convergent transcription can be used in mammalian cells to invoke gene-specific silencing via RNAi. This method provides an alternative to expression of shRNAs and co-expression of sense and antisense RNAs from independent cassettes or a divergent promoter. The main advantage of the present vector design is the potential to produce a functional siRNA expression cassette with no repeat sequences. Furthermore, the cassette design reported is ideal for both routine use in controlling specific gene expression and construction of randomised RNAi expression libraries for use in unbiased forward genetic selections.

Background

The introduction of double-stranded RNA (dsRNA) into a range of organisms induces both a potent and specific post-transcriptional gene silencing effect by directing degradation of homologous target RNAs. This form of gene suppression was first observed in *Caenorhabditis elegans* and termed RNA interference or RNAi [1]. Biochemical analysis of the mechanism of RNAi has indicated that the mediators of gene silencing are 21 base pair small interfering RNAs (siRNAs) generated from longer dsRNA by the RNAse III-like enzyme Dicer [2]. In mammalian cells, the use of long dsRNA has been restricted due to the proposed activation of an antiviral defense system that blocks protein translation leading to cell death [3]. Recently, this limitation to the application of RNAi in mammalian cells was overcome by the demonstration that chemically synthesised 21 base pair siRNAs, the effectors of RNAi, could be used in a wide range of human and mouse cell lines to induce gene silencing [4–6]. This approach for transiently controlling the expression of different target genes is fast becoming the method of choice for determining gene function in mammalian cells [7].

The transient nature of the gene silencing effect invoked by siRNAs and the prohibitively high costs of chemical synthesis have led to the development of DNA vectors capable of expressing siRNAs intracellularly. Expression cassettes have been developed using the endogenous U6 snRNA or H1 RNA polymerase III promoters to drive expression of sequence-specific small hairpin RNAs (shRNAs) that stably regulate gene expression in mammalian cells via RNAi [8–10]. As an alternative approach, some groups have used the co-expression of sense and antisense RNA strands from independent expression cassettes or a divergent cassette [11,12]. The use of convergent transcription from opposing promoters to induce RNAi-mediated gene inhibition has been reported in trypanosomes and Drosophila [13–15]. In these studies, a full-length cDNA sequence is positioned between two identical promoters such that independent transcription from each promoter produces a pool of sense and antisense RNAs capable of forming long dsRNA and undergoing processing to the effector siRNAs. The utility of this approach for inducing RNAi in mammalian cells has not been reported. It has been predicted that the expression of up to 8% of human genes may be influenced by antisense RNA or antisense transcription [16–19]. This suggests that convergent transcription does occur with a high frequency in the human genome. However, this form of transcription may regulate gene expression in cis by RNA polymerase collision or in trans via a RNAi-like mechanism [20]. In addition, to our knowledge, transcription in two directions across a small region of DNA has not been demonstrated to induce RNAi-mediated gene silencing in any organism.

In this study, we report on the construction of a convergent promoter cassette capable of simultaneously expressing complementary sense and antisense RNAs that mediate gene silencing in mammalian cells through the RNAi pathway. We show that this vector design can be used to inhibit transgene and endogenous gene expression in a Dicer-dependent manner and without activating the dsRNA-dependent protein kinase PKR. This method of expressing functional siRNAs in mammalian cells can be used for both short and long-term regulation of specific gene expression. We discuss the utility of this siRNA expression vector format for routine regulation of specific gene expression and the construction of genome-wide RNAi libraries for forward genetic screening in mammalian cells.

Results

Suppression of dEGFP transgene expression using a convergent transcription cassette

In previous reports it was shown that shRNAs generated by transcription from a U6 snRNA promoter or short sense and antisense RNAs expressed from separate U6 expression cassettes could generate gene silencing through RNAi [8,9,21]. To develop a vector system for expressing siRNAs in mammalian cells through convergent transcription, we designed the convergent U6 promoter cassette indicated in Figure 1A. To determine the intracellular efficacy of this expression cassette for mediating specific gene silencing, we used the EGFP gene as a target. We constructed a U6 convergent expression vector containing a EGFP-specific insert (DualU6GFP) and co-transfected this plasmid with the pEGFP-N1 plasmid and the lacZ expression vector pSVβ into EcR293 embryonic kidney cells. Cells receiving DualU6GFP displayed a 40% reduction in cell fluorescence compared with cells transfected with the DualU6 control vector (data not shown).

To further examine the utility of the convergent U6 promoters, and the mechanism by which this vector regulated gene expression, we delivered the DualU6GFP plasmid to EcR293 cells containing a stably integrated destabilised EGFP (dEGFP) transgene [22]. As shown in figure 1B, cells transfected with DualU6GFP displayed a reduction in dEGFP-mediated cell fluorescence with the level of reduction in fluorescence equal to that of the synthetic EGFP siRNA at 48 h post-transfection. Consistent with the requirement for expression of the sense and antisense RNAs from DualU6GFP, gene silencing via this vector displayed a 24 h delay compared with a synthetic siRNA targeted to the same region of the dEGFP mRNA. The reduction in cell fluorescence exhibited by cells containing the DualU6GFP plasmid was confirmed using fluorescence microscopy (Figure 1C). As with the synthetic siRNAs, the residual population displaying cell

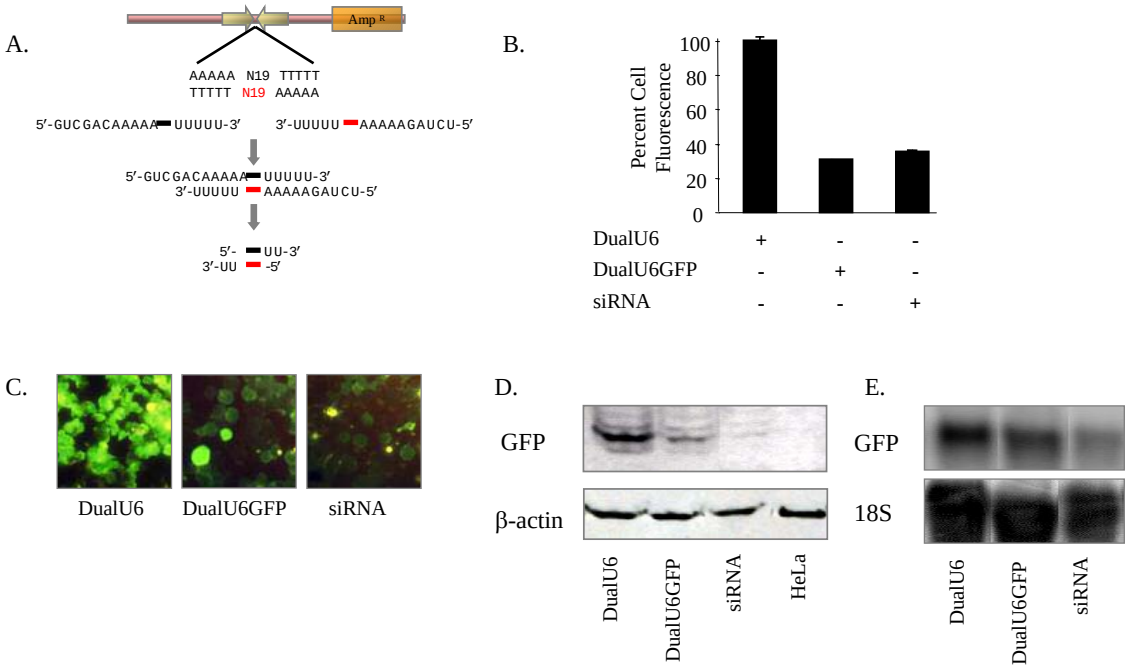

Figure 1

Strategy for generating intracellular siRNAs and effect of the expressed siRNAs on transgene expression. (**A**) The convergent U6 expression cassette encodes sense (black) and antisense RNAs (red) that terminate at directional termination sequences (represented as five consecutive thymidines [T]). The complementary RNAs anneal and undergo further Dicer-dependent processing to produce functional siRNAs. A U6 convergent expression vector containing an EGFP-specific insert (DualU6GFP) reduces dEGFP-mediated cell fluorescence as measured by (**B**) flow cytometry and (**C**) fluorescence microscopy. This same expression vector suppresses both (**D**) dEGFP protein levels and (**E**) dEGFP RNA levels. For Western analysis, β-actin protein levels were used as a loading control. For RNA analysis, the control for loading was 18s rRNA.

fluorescence most likely represents cells that have not been transfected with the expression plasmid.

It has been reported that shRNAs, or co-expression of small complementary antisense and sense RNAs, produce specific gene silencing by processing to siRNAs. To determine the mechanism of action of the DualU6GFP expression system, we examined transfected cells for dEGFP protein levels, dEGFP mRNA levels and the presence or absence of small RNAs encoded by the U6 convergent expression vector containing an EGFP-specific insert. Western analysis showed that the dEGFP protein levels were reduced in cells expressing the siRNA from the U6 convergent expression vector and that this effect was specific (Figure 1D). The level of suppression of the dEGFP protein was equivalent to that mediated by delivery of synthetic siRNAs. An examination of dEGFP target mRNA levels indicated that both the synthetic siRNAs and those expressed from the U6 convergent plasmid reduced target mRNA (Figure 1E). This latter result suggests that DualU6GFP produces siRNAs capable of mediating turnover of the target mRNA, an observation consistent with the mechanism of RNAi. To further confirm that the DualU6GFP plasmid maintains the potential to produce siRNAs, we identified the transcripts expressed from this plasmid using northern blot analysis. As shown in figure

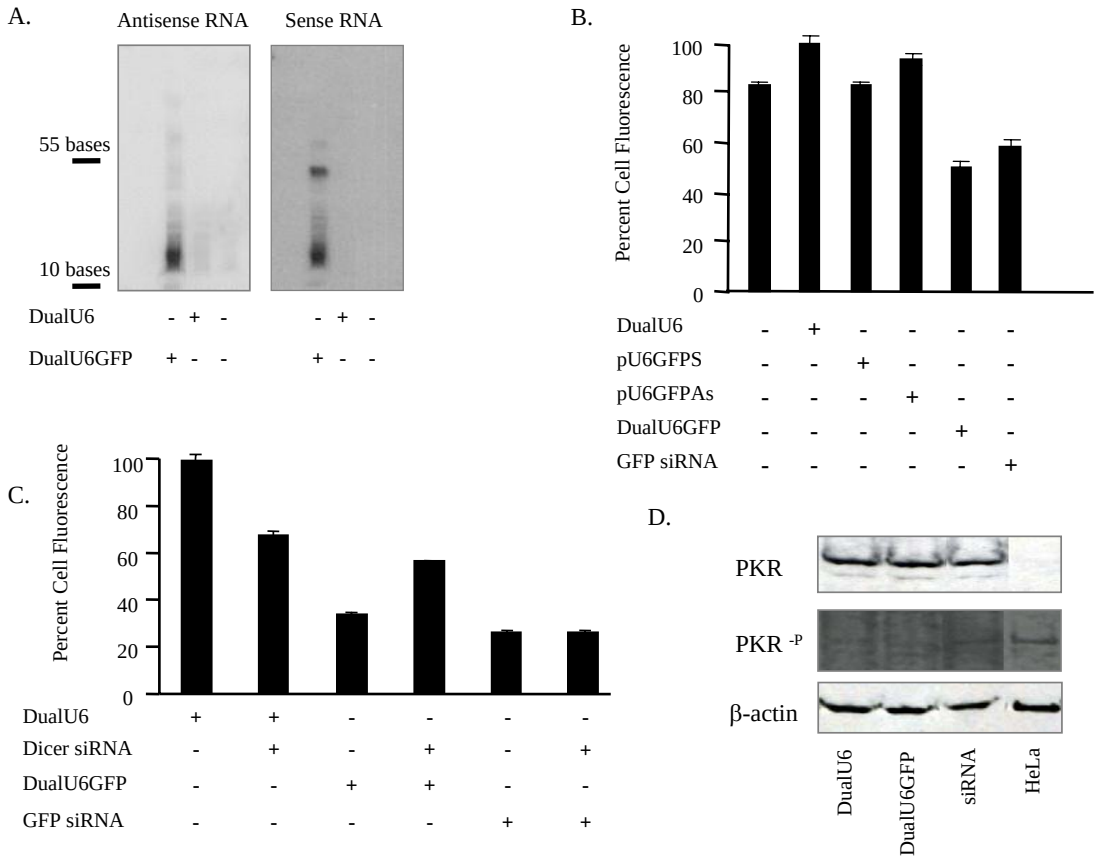

Figure 2

Gene suppression by complementary RNAs expressed from a U6 convergent cassette is Dicer-dependent. (**A**) The U6 convergent EGFP vector undergoes transcription in EcR293 cells to produce sense and antisense RNAs. (**B**) Suppression of target gene expression by the DualU6GFP vector requires the co-expression of both sense and antisense RNAs. (**C**) The DualU6GFP expression vector reduces dEGFP target gene expression in a Dicer-dependent manner. (**D**) Co-expression of complementary RNAs from the U6 convergent expression cassette does not activate PKR. Hela cells were treated with 0.1 μM calyculin A and serve as a positive control for activated PKR.

2A, bands of the expected length were observed only in cells containing the DualU6GFP plasmid and not in vector controls. In addition, using strand-specific probes, we were able to show that within the cells containing the U6 convergent EGFP vector both the antisense and sense RNAs were present. The sizes of the transcripts confirmed that the directional terminators were operative and that U6-directed transcriptional machinery efficiently truncated the antisense and sense transcripts within the convergent transcription unit. The above results indicate that the use of U6 convergent promoters in a single expression cassette can produce complementary sense and antisense RNAs that mediate specific gene suppression in a manner consistent with RNAi.

Convergent promoters necessary for gene suppression

To demonstrate the necessity for convergent U6 promoters in the DualU6GFP vector, and therefore the expression of both sense and antisense RNAs, to mediate suppression of the dEGFP target gene, we constructed derivatives of this plasmid containing only a single U6 promoter. These vectors were designated pU6GFPS and pU6GFPAs and were expected to encode small sense and antisense EGFP RNAs under control of the U6 promoter, respectively. Each of these plasmids was used to transiently transfect EcR293 cells expressing the dEGFP transgene. Cell populations were then analysed for dEGFP-mediated cell fluorescence. This analysis indicated that the expression of either sense or antisense EGFP strands alone was

insufficient to suppress the dEGFP gene, and that full inhibition of this target gene required the co-expression of both strands within the same cell (Figure 2B).

Convergent transcription induces RNAi

Given that the cells co-expressing the sense and antisense EGFP RNAs displayed many of the hallmarks of RNAi, we were interested in determining whether gene silencing occurred through formation of dsRNA. Toward this end, we utilised the Dicer siRNA as a tool to determine if the observed suppression was Dicer-dependent [23–25]. In this experiment, EcR293 cells expressing the dEGFP transgene were transfected with DualU6GFP in the presence and absence of the synthetic siRNA specific for Dicer. As shown in Figure 2C, the Dicer siRNA completely reversed the reduction in cell fluorescence mediated by the EGFP-specific U6 convergent plasmid. In contrast, cells transfected with both the synthetic EGFP- and Dicer-specific siRNAs still displayed a reduction of cell fluorescence, as the mechanism of synthetic siRNAs is Dicer-independent. These results suggest that the small sense and antisense RNAs encoded by DualU6GFP anneal to form dsRNA that is processed by Dicer into authentic siRNAs. It is most likely that gene silencing is then directed by these processed siRNAs.

It has been proposed that dsRNA greater than 30 base pairs in size induce a global response that results in activation of the double-stranded RNA-specific protein kinase PKR [3]. To eliminate PKR activation as being responsible for the gene silencing observed using this unique expression system, we examined the levels of both total PKR and activated PKR in EcR293 cells receiving the DualU6GFP plasmid. This analysis indicated that co-expression of the sense and antisense EGFP RNAs and formation of dsRNAs did not activate PKR (Figure 2D), suggesting that the observed gene silencing effect was specific and not related to this global response mechanism.

Stable gene regulation using a convergent promoter system

To examine the utility of the DualU6GFP expression system in long-term regulation of gene expression in mammalian cells, either the DualU6GFP plasmid, or the control vector, was co-delivered with pREP7 (containing the marker conferring resistance to hygromycin) to EcR293 cells expressing the dEGFP transgene. Following selection for cells stably maintaining the DualU6GFP plasmid, cells were examined for dEGFP-mediated cell fluorescence. This analysis demonstrated that cells containing the DualU6GFP plasmid displayed a significant (80%) reduction in cell fluorescence compared with cells receiving the control vector (Figure 3). This result indicates that the convergent expression cassette described can be used to mediate long-term regulation of gene expression in mammalian cells.

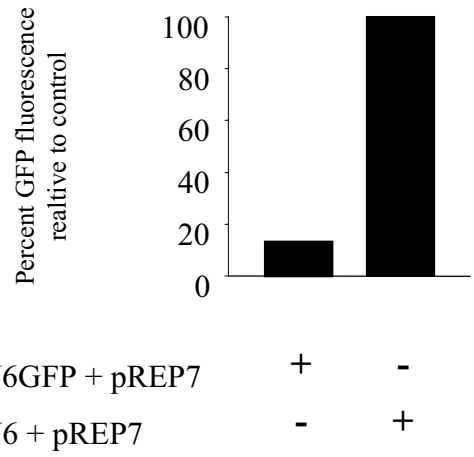

Figure 3
Stable suppression of dEGFP-mediated cell fluorescence. EcR293 cells containing a stable integrated dEGFP transgene were co-transfected with DualU6GFP and pREP7 and selected in 500 ug/ml hygromycin for two weeks. Cell fluorescence is reduced in stable pools of cells containing the DualU6GFP vector compared with those receiving the control DualU6 vector.

Convergent transcription regulates an endogenous gene

We next determined whether the U6 convergent promoter system could be used to control the expression of endogenous genes in mammalian cells. For this purpose, we chose as a target the TP53 gene that encodes the p53 tumor suppressor protein. To this end, we constructed a U6 convergent expression vector containing an insert encoding a p53-specific siRNA. The target site selected was identical to that reported earlier for synthetic p53-specific siRNAs [26]. The p53-specific U6 convergent expression plasmid, DualU6p53, was transfected into MDA MB 231 breast cancer cells and, at 48 h and 120 h post-transfection, cells were harvested and analysed for p53 protein levels. As shown in Figure 4A, delivery of the DualU6p53 plasmid into MDA MB 231 cells resulted in a significant and specific reduction of p53 protein, especially at 120 h. This result indicates that the U6 convergent promoter system can be used to effectively suppress the expression of endogenous genes through RNAi in mammalian cells.

Convergent transcription induces longer-term suppression of p53

To demonstrate the effectiveness of convergent transcription in mediating longer-term control of p53 gene

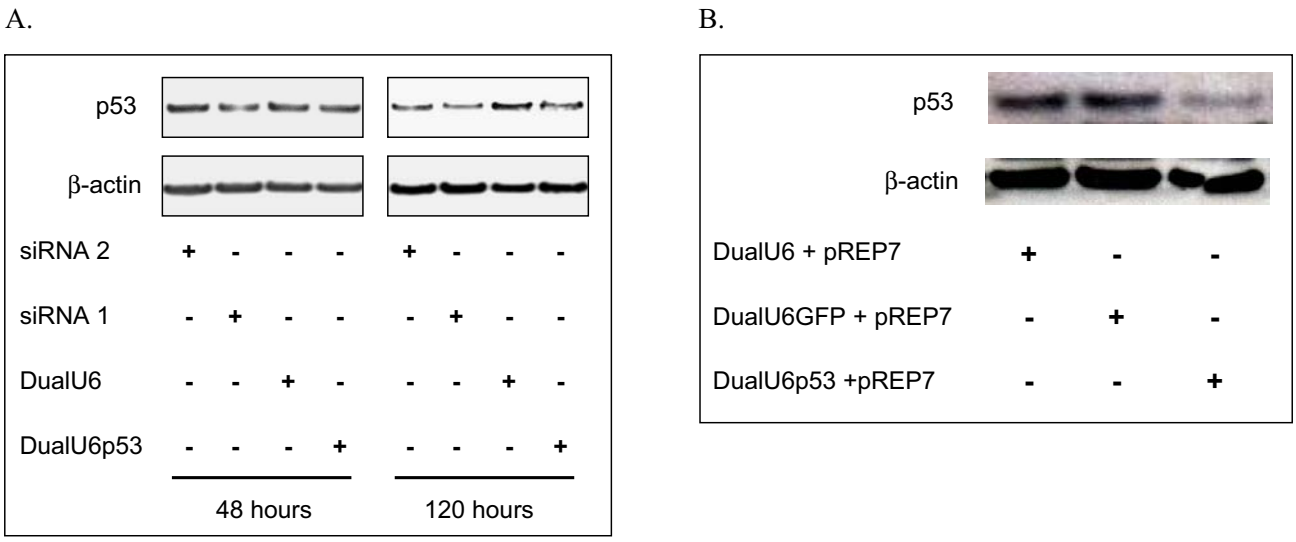

Figure 4

Suppression of p53 protein levels using a convergent U6 expression vector. (A) Transient suppression of p53 protein levels. Plasmids DualU6 and DualU6p53 or p53-specific siRNAs 1 and 2 were transfected into MDA MB 231 cells and, at 48 h and 120 h post-transfection, p53 and β-actin protein levels were determined using Western analysis. (**B**) Long term suppression of p53 protein levels. EcR293 cells containing a stably integrated dEGFP transgene were co-transfected with DualU6, DualU6GFP or DualU6p53 and pREP7. Following selection in hygromycin, cell populations were analysed for p53 and β-actin protein levels.

expression, we co-delivered the DualU6p53 plasmid with pREP7 (containing the hygromycin resistance gene) to EcR293 cells containing the dEGFP transgene. In addition, we also co-transfected these same cells with the DualU6GFP and pREP7. Each of these populations, and the vector alone with pREP7, were exposed to hygromycin selection for two weeks. Stable cells were selected and examined for p53 protein levels by Western blotting. This analysis indicated that cells containing the DualU6p53 plasmid showed a significant reduction in p53 protein levels compared with cells receiving the control vector or cells containing DualU6GFP (Figure 4B). This suggests that the observed suppression is sequence-specific and that long term regulation of endogenous gene expression can be achieved in mammalian cells using convergent transcription.

Discussion

In this report, we use convergent RNA polymerase III promoters to direct transcription of target gene-specific sense and antisense RNAs. This strategy has been used in trypanasomes and Drosophila for generating long dsRNA and inducing RNAi-mediated gene suppression [13–15]. However, the suitability of this vector design has not been tested in mammalian cells. The testing of convergent promoters to invoke RNAi requires consideration of features unique to RNAi-directed gene silencing in these cells. Firstly, it is possible that convergent transcription may not generate complementary RNAs, but instead truncated transcripts lacking homology due to collision between RNA polymerases [19]. Alternatively, transcription from opposing promoters may produce sense or antisense RNAs, but not both, due to domination by one promoter. Secondly, unlike most other organisms, the length of the complementary RNAs must be below 30 bp in size as longer dsRNAs have been reported to activate dsRNA-dependent protein kinase PKR and non-specific RNases. Finally, the efficiency of convergent transcription across a DNA insert of such limited size within mammalian cells is unknown and may impact on the synthesis of the complementary RNAs [17].

To examine this vector design we constructed and tested an expression cassette containing convergent U6 snRNA promoters flanking DNA inserts specific for a region of the cellular mRNA of interest. Data presented in this report indicates that convergent transcription of a short region of DNA specific to a target gene can be used to suppress its expression in mammalian cells. The gene silencing effect mediated by co-expression of sense and antisense RNAs was shown to be dependent on the level of Dicer, suggesting that these complementary RNAs form a dsRNA inter-

mediate that is processed by Dicer to functional siRNAs. Furthermore, the level of suppression observed using convergent transcription was comparable to that mediated by synthetic siRNAs directed against the same target site. This vector design was shown to confer both transient and long-term regulation of target gene expression without activating the antiviral global response.

All DNA vectors used to express shRNAs or co-express sense and antisense RNAs in mammalian cells to induce RNAi contain repeated sequences specific to the gene of interest [8,9,12]. The presence of inverted repeats has been reported to affect the stability of these vectors and may possibly impact on the longer-term regulation by RNAi. This situation is further complicated when the inverted repeat sequences are contained within retroviral vectors [27–30]. In this paper, we use two opposing U6 promoters for testing the utility of symmetrical transcription as a method of inducing RNAi in mammalian cells. This design eliminates the presence of repeat sequences derived from the target gene. Replacement of one U6 promoter with an H1 promoter would produce a siRNA expression cassette lacking repeat sequences. This design is expected to increase both the cloning efficiency and the stability of RNAi expression vectors.

The system described in this report provides a novel alternative expression modality to shRNA-expressing plasmids for gene silencing in mammalian cells. This opposing promoter system forms the basis for generating randomised RNAi libraries in which random double-stranded DNA oligonucleotides can be introduced between the convergent U6 promoters. The expansion of this design to include two different RNA polymerse III promoters in opposing orientations, with random oligonucleotide sequences between the convergent promoters, would produce a randomised RNAi library expressing functional siRNAs in mammalian cells and containing no inverted repeat sequences. Such genome-wide RNAi libraries would be useful for performing forward genetic screens similar to those reported using randomised ribozyme libraries [31–33] and universal peptide libraries [34]. A significant advantage in using randomised RNAi libraries, over other nucleic acid-based libraries, in forward genetic approaches in mammalian cells would be the identification of 21 bases of complete sequence complementarity to the intracellular target RNA that is linked to the modified cellular phenotype. This length of sequence conservation could be used to more effectively identify candidate genes using homology-based search tools. In addition, these sequences could be chemically synthesised and used as tools for further validation of the identified targets or as potential therapeutics.

Conclusions

The present study introduces a novel method for expressing functional siRNAs capable of directing specific gene silencing in mammalian cells through RNAi. The vector design provides a feasible alternative to other DNA vectors used for expressing siRNAs, with the added benefit of eliminating the incorporation of inverted repeat sequences. As highlighted above, this DNA vector has a number of advantages for the generation of randomised RNAi expression libraries suitable for genome-wide somatic cell genetics.

Methods

Constructs and siRNAs

To construct DualU6 containing convergent U6 promoters, the primers 5'-GCG CAA GCT TAT AGG GAA TTC GAG CTC GGT A-3', and 5'-GCG CTC TAG AGG TGT TTC GTC CTT TCC ACA A-3' were used to PCR amplify the U6+1 promoter region from pTZ(U6+1) [9] and the resulting amplicon cloned as a XbaI-HindIII fragment into pTZ(U6+1). The inserts encoding the sense and antisense RNAs were designed to include target-specific sequences (in bold below) flanked by two directional transcription terminators composed of five thymidines. The oligonucleotides used to construct DualU6GFP were 5'-TCG ACA AAA A**CG GCA AGC TGA CCC TGA AG**T TTT T-3' and 5'-CTA GAA AAA **CTT CAG GGT CAG CTT GCC G**TT TTT G-3', while the following were used to construct DualU6p53: 5'-TCG ACA AAA A**GA CTC CAG TGG TAA TCT AC**T TTT T-3' and 5'-CTA GAA AAA **GTA GAT TAC CAC TGG AGT C**TT TTT G-3'. These oligonucleotides were synthesised (Sigma Genosys, Sydney, Australia), annealed and cloned into the SalI and XbaI sites of DualU6. The control vectors pU6GFPS and pU6GFPAs were constructed by deleting a single U6 promoter from DualU6GFP by HindIII-XbaI and HincII-AflIII double digests, respectively.

In transient transfection experiments aimed at testing the DualU6GFP, the target plasmid used was pEGFP-N1 which contains the EGFP reporter under control of the CMV immediate early promoter (Clontech, Palo Alto, CA). The vector pSVβ, encoding the β-galactosidase protein, was co-transfected with the EGFP siRNA and the pEGFP-N1 plasmid to act as a transfection control. The β-galactosidase activity was assayed using the β-galactosidase enzyme assay system according to the manufacturer's instructions (Promega, Madison, WI).

In the production of stable pooled populations containing the DualU6 vector control or DualU6GFP, each plasmid was co-transfected into EcR293 cells (containing an integrated dEGFP transgene) in a 10:1 molar ratio with the episomal plasmid pREP7 (Invitrogen, Carlsbad, CA).

This plasmid contains a positive selectable marker conferring resistance to hygromycin.

The RNA oligonucleotides used to form the siRNAs were synthesised by Dharmacon Research Inc (CO, USA) and the sequences were: GFP, 5'-CGG CAA GCU GAC CCU GAA G dTdT (sense); p53(siRNA1), 5'-GAC UCC AGU GGU AAU CUA C dTdT (sense); and p53(siRNA2), 5'-GCA UGA ACC GGA GGC CCA U dTdT (sense). The sequence of the Dicer-specific siRNA used was as reported in [24]. All RNA oligonucleotides were annealed with their corresponding antisense strands as described [4].

Cell culture and transfection

Mammalian cells used in this study included the human embryonic kidney (HEK) cell line EcR293 (Invitrogen, CA, USA) and the human breast cancer cell line MDA MB 231. The construction of the EcR293 cell line expressing the dEGFP gene has been described [22]. EcR293 cells and their derivatives were maintained in DMEM containing 10% fetal calf serum supplemented with glutamine, streptomycin and penicillin. MDA MB 231 cells were grown in RPMI containing 10% fetal calf serum supplemented with glutamine.

Cells were seeded into six well plates 24 h prior to transfection. For all transfections, a total of 4 μg of plasmid DNA or 60 nM of siRNA was delivered using Lipofectamine 2000 (Invitrogen, CA, USA)) according to the manufacturer's instructions. Cells were harvested at 24 h and 48 h for flow cytometry analysis of EGFP expression (Becton Dickinson, USA). Fluorescent microscopy was performed using a fluorescence microscope (Nikon, Japan) with a B-2H filter cube.

RNA analysis

Total RNA was isolated using Trizol (Invitrogen, CA, USA) and immobilised onto nylon membrane (Invitrogen, CA, US) for detection using standard probe hybridisation. For the detection of small antisense and sense RNAs encoded by DualU6GFP, the following oligonucleotides were end-labelled and hybridised to these membranes at 37°C for 1 h: 5'-TCG ACA AAA ACG GCA AGC TGA CCC TGA AGT TTT T-3' or 5'-CTA GAA AAA CTT CAG GGT CAG CTT GCC GTT TTT G-3'. For the detection of dEGFP mRNA, the ORF of dEGFP was digested with HindIII and XbaI from the vector pd4EGFP-N1 (Clontech, USA). For the detection of 18S the following oligonucleotide 5'-CTGCAGCAACTTTAATATACGCTATTGGAGCTGGAAT-TACCAAAAAAAAA-3' was used as a specific probe. For these two sequences they were labelled using the Megaprime kit (Amersham, USA). All hybridisations were performed using ExpressHyb (Clontech, USA) according to the manufacturer's instructions. Membranes were analysed using a phosphorimager (Molecular Dynamics, USA) and an ImageQuant software package (Molecular Dynamics, USA).

Western analysis

Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotonin (1 μg/ml), leupeptin (10 μg/ml) and DMSF (100 μg/ml). Total protein was loaded onto 4–12% Bis-Tris agarose gels (Invitrogen, CA, USA), separated by electrophoresis and transferred to polyvinylidene fluoride (PVDF) membrane. The antibodies used for detection of specific proteins included: GFP, mouse polyclonal (Clontech), PKR monoclonal (Cell Signaling), PKR phospho rabbit polyclonal (Cell Signaling), p53 mouse monoclonal (Oncogene Research Products) or β-actin mouse monoclonal (Sigma) antibodies. Secondary antibody detection was performed using either the goat anti-mouse horseradish peroxidase (HRP)-linked or the goat anti-rabbit HRP (SantaCruz), followed by visualisation using the luminol/enhancer chemiluminescent substrate (Amersham Pharmacia Biotech, Piscataway, NJ).

List of abbreviations

siRNA: small interfering RNA; shRNA: small hairpin RNA; RNAi: RNA interference; dsRNA: double-stranded RNA.

Author's contributions

N.T. performed the described experiments and drafted the figures for the manuscript. M.C. and I.D. participated in the experimental design and the assessment of the results. G.A. is the principal investigator of the lab and participated in the study's design, development and coordination. All authors read and approved the final manuscript.

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