Control of specific gene expression in mammalian cells by co-expression of long complementary RNAs

Nham Tran[a,b], Mitch Raponi[c], Ian W. Dawes[b], Greg M. Arndt[a,*]

[a] *Johnson and Johnson Research Pty Ltd, 1 Central Ave, Australian Technology Park, Eveleigh, NSW 1430, Australia*
[b] *School of Biotechnology and Biomolecular Sciences, The University of New South Wales, Sydney, NSW 2052, Australia*
[c] *Molecular Diagnostics, Johnson & Johnson, 3210, Merryfield Row, San Diego, CA 92121, USA*

Received 15 June 2004; revised 30 July 2004; accepted 30 July 2004

Available online 9 August 2004

Edited by Takashi Gojobori

Abstract The use of long double-stranded RNA (dsRNA) for gene silencing in mammalian cells has generally been restricted to embryonic cell types and proposed to induce non-specific effects on gene expression in differentiated cells. In this study, we report that foreign and endogenous gene expression can be regulated in immortalised human cell lines by co-expression of long complementary RNAs with the potential to form dsRNA. The observed gene silencing effect was transferable to recipient control cells, occurred independently of cytoplasmic Dicer and produced an epi-allelic series of clones suitable for gene function studies. This complementary RNA co-expression approach permits the use of long complementary RNAs for regulating specific gene expression in mammalian cells.

Keywords: Mammalian cell; Double-stranded RNA; Gene silencing

1. Introduction

The introduction of double-stranded RNA (dsRNA) into a range of organisms induces both a potent and specific gene silencing effect [1,2]. This form of gene suppression by a dsRNA molecule was first observed in *Caenorhabditis elegans* and given the term RNA interference or RNAi [3]. Subsequent studies have shown that dsRNA is an effective inducer of gene silencing in a wide range of eukaryotic organisms and that the mechanism behind this form of gene regulation is most likely conserved throughout evolution. The molecular mechanism of RNAi has begun to be deciphered using biochemical and genetic approaches in different experimental systems. Presently, RNAi is postulated to involve both an initiation step and an effector step [4]. During the initiation phase, dsRNA is processed by the RNaseIII family nuclease Dicer to produce 21–23 nucleotide duplex small interfering RNAs (siRNAs) [5]. In the effector phase, these siRNAs are incorporated into a multiprotein complex called RNA-induced silencing complex (RISC) that targets transcripts by base pairing between one of the siRNA strands and the endogenous mRNA. A nuclease activity associated with the RISC complex then cleaves the mRNA–siRNA duplex thus targeting the cognate mRNA for destruction [6].

In general, RNAi-mediated interference of gene expression in mammalian cells is accomplished using synthetic siRNAs [5] or gene constructs expressing short hairpin RNAs (shRNAs) [7]. One alternative to shRNA constructs is transcription of siRNA sense and antisense strands via a convergent promoter system [8]. The use of longer dsRNA to reduce gene expression has been hampered by the presence of a unique global response mechanism. Unlike other organisms, mammalian cells exposed to dsRNA longer than 30 base pairs (bp) in length have been predicted to undergo a response mechanism involving activation of two key enzymes, dsRNA-activated protein kinase (PKR) and 2′-5′ oligoadenylate polymerase/RnaseL [9]. The activation of these enzymes leads to an inactivation of protein synthesis and eventually cell death via apoptosis. It was thus anticipated that the introduction of long dsRNA would activate this global response system. However, studies have shown that in both mouse pre-implantation embryos and undifferentiated embryonic stem cells and embryonic carcinoma cells, the use of in vitro generated long dsRNA was able to mediate specific gene silencing [10–12]. In addition, RNAi-like activity was demonstrated using gene constructs designed to encode long inverted repeat RNAs [13]. The primary reason provided for these observations was that these cell systems lack the generalised responses to dsRNA. The above results were encouraging but placed particular limitations on the utility of these approaches in mammalian cell types containing active and robust generalised dsRNA responses.

The most popular approach to gene silencing in non-mammalian cells is the use of long dsRNA. Processing of the long dsRNA produces a pool of siRNAs overcoming any issues associated with target RNA accessibility. Attempts to deliver long dsRNA to the cytoplasm of differentiated mammalian cells have been disappointing due to the activation of the interferon-like response [14]. Despite these observations, several lines of evidence suggest that dsRNAs longer than 30 bp exist in mammalian cells and that these species mediate gene silencing. First, a number of studies in mammalian cells using long antisense RNAs, expressed from genes integrated in the genome, mediate suppression by target RNA degradation, suggesting a dsRNA intermediate. Second, studies using sense

* Corresponding author. Fax: +61-2-8396-5811.
E-mail address: garndt@medau.jnj.com (G.M. Arndt).

Abbreviations: dsRNA, double-stranded RNA; siRNA, small interfering RNA; RISC, RNA-induced silencing complex; shRNA, short hairpin RNA; bp, base pair; ORF, open reading frame; RSV, rous sarcoma virus; CMV, cytomegalovirus; LTR, long terminal repeat

0014-5793/$22.00
doi:10.1016/j.febslet.2004.07.075

and antisense plasmids suggest that the presence of long dsRNAs can be tolerated in mammalian cells [15]. Third, gene-expressed self-complementary RNAs of 400 nucleotides have been shown to be effective at specific gene control in neuronal cells [16]. Finally, nuclear-directed long antisense RNA, or inverted repeat dsRNA, mediates specific gene silencing in cultured cells and mice without eliciting a global response [17]. Further evidence for extensive long dsRNA species in mammalian cells comes from sequencing of the human genome which revealed that as much as 8% of the expressed mRNAs have a corresponding antisense RNA complement of between 200 and 3000 bp [18]. These observations suggest that conditions exist in which long complementary RNAs can direct specific gene silencing in mammalian cells.

In this study, we report on the development of a methodology for expressing long complementary RNAs in mammalian cells, with the potential to form dsRNA and regulate specific gene expression. We show that this approach can be used to suppress both a transgene and endogenous genes. The suppressive effect reduces both target mRNA steady state levels and the adundance of the encoded gene product. The observed regulatory event is dependent on the co-expression of complementary RNA strands, does not activate PKR or other cellular stress responses, is not dependent on Dicer for maintenance of gene silencing, and produces intermediate gene-specific siRNAs. Furthermore, the silencing effect is shown to be transferable to other mammalian cells in culture. We demonstrate that this strategy can be used to generate an ''epi-allelic'' series of mutants useful in functional analysis of specific genes. It is anticipated that this method will be extremely useful for controlling specific gene expression in mammalian cells.

2. Materials and methods

2.1. Construction of episomal expression vectors

Standard gene cloning methods were used to construct expression plasmids used in the present study. The plasmids used in the co-transfection experiments were based in the core episomal plasmids pREP7 (Invitrogen) or pEAK10(JJR) (Edge Biosystems). These plasmids are maintained within the nucleus and do not generally integrate into the genomic DNA. The sequences required for episomal plasmid maintenance are the Epstein Barr virus OriP and EBNA1 regions. The portion of the dEGFP target gene used to construct the sense and antisense dEGFP-expressing plasmids in pREP7 spanned positions 666 to 1749 in reference to the pd4EGFP-N1 (Clontech) sequence map. This region was PCR-amplified using pd4EGFP-N1 as a template and the following primers: 5′ TGA GGA TTC ACC GGT CGC CAC C**C**T GGT GAG CAA G 3′ and 5′ TGA GGA TTC ACA AAC CAC AAC TAG AAT GCA GTG 3′. The base change indicated by **C** was introduced to eliminate the ATG start codon and ensure that sense dEGFP RNA was not translated. The 1083 bp PCR product was digested with *Bam*HI and subcloned into the unique *Bam*HI site in pREP7 downstream of the RSV long terminal repeat (LTR) promoter in the sense and antisense orientations to produce pR7ctgES and pR7ctgEaS, respectively. The dEGFP insert in plasmid pJEAs was obtained by PCR amplifying the entire transcription unit of the dEGFP gene spanning positions 583 to 1749 (in reference to the pd4EGFP-N1 sequence map) using the following PCR primers: 5′ TCA GAT CCG CTA GCG CTA CCG GAC 3′ and 5′ ACA AAC CAC AAC TAG AAT GCA GTG 3′. This fragment was ligated to *Bam*HI adaptors created by annealing the following single-stranded oligonucleotides: 5′ TCT CTA GGG ATC CTC AGT CAG TCA GGA TG 3′ and 5′ CAT CCT GAC TGA CTG AGG ATC CCT AGA GAA TA 3′. The adaptor-ligated fragment was then digested with *Bam*HI and ligated into the unique *Bgl*II site in pEAK10(JJR) in the antisense orientation relative to the mammalian protein elongation factor 1α promoter to produce pJEAs. For construction of the plasmid pJctgES, the region of the dEGFP gene in pd4EGFP-N1 spanning positions 666 to 1749 was PCR-amplified using the forward primer 5′ TGA AGA TCT ACC GGT CGC CAC CCT GGT GAG CAA G 3′ and the reverse primer 5′ TGA GAA TTC ACA AAC CAC AACTAG AAT GCA GTG 3′. The *Bgl*II–*Eco*RI digested PCR product was directionally cloned in the sense direction downstream of the elongation factor 1α promoter of pEAK10(JJR) to produce pJctgES. The sense and antisense dEGFP genes contained on pR7ctgES, pR7ctgEaS, pJEAs, and pJctgES are indicated schematically in Fig. 1.

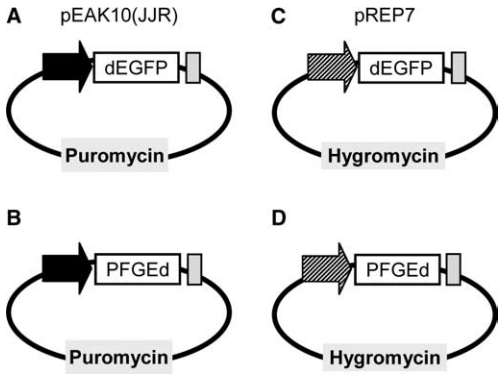

Fig. 1. Complementary RNA co-expression vectors specific for dEGFP. Two sets of episomal plasmids were constructed using pEAK10 (JJR) and pREP7 as the core vector backbones. (A) pJEctgES; (B) pJEAs; (C) pR7ctgES; (D) pR7ctgEaS. Plasmids pEAK10 (JJR) and pREP7 contain the elongation factor 1α (EF1α; filled arrrow) and Rous sarcoma virus (RSV; hatched arrows) promoters, respectively, controlling expression of dEGFP-specific RNAs. Selectable markers conferring resistance to either puromycin or hygromycin are indicated.

To construct the complementary RNA co-expression vectors specific for the endogenous TP53 gene, the open reading frame (ORF) was PCR-amplified from pORF53 (Invivogen) using the following forward and reverse primers: 5′ GCG CAA GCT TCT GGA GGA GCC GCA GTC ATG 3′ and 5′ GCG CAA GCT TTC AGT CTG AGT CAG GCC CCT 3′. This 1195 bp PCR product was digested with *Hin*dIII and subcloned in the antisense orientation downstream of the RSV LTR promoter in pREP7 and in the sense direction downstream of the elongation factor 1α promoter in pEAK10(JJR).

2.2. Construction of dEGFP-expressing cell line

The derivative cell line expressing the dEGFP target gene was constructed by electroporating EcR293 cells (Invitrogen) with the plasmid pd4EGFP-N1 (Clontech) linearised with *Afl*II. The transfected cell population was selected in the presence of 500 μg/ml G418 and clones expanded and screened for dEGFP expression by fluorescence-activated cell sorting (FACs) analysis using the Becton Dickinson FACSORT. This cell line, expressing dEGFP under control of the cytomegalovirus (CMV) immediate early promoter, was previously shown to contain a single copy of the dEGFP expression cassette using Southern blot analyses [19].

2.3. Cell culture and methods

EcR293 human embryonic kidney cells (Invitrogen) and HCT116 colon carcinoma cells were maintained in DMEM containing 10% foetal calf serum and supplemented with glutamine, streptomycin and penicillin. To generate pooled populations containing two different episomal vectors, 2.5×10^6 dEGFP-expressing cells or HCT116 cells were electroporated with 2.5 μg of each plasmid. At 48 hours after transfection, cells were exposed to 0.7 μg/ml of puromycin (to select for pEAK10(JJR)-based plasmids) and 100 μg/ml hygromycin (to select for pREP7-based plasmids). Following 28 days of double selection, cells were then exposed to 500 μg/ml of G418. At five weeks post-electroporation, each of the selected pooled populations was characterised for dEGFP-mediated cell fluorescence, dEGFP protein level and steady-state level of dEGFP mRNA. To isolate individual clones, pooled populations were serially diluted. Transfection of mammalian cells with siRNAs was performed as previously described [8]. The Dicer-specific and nonsense control siRNA sequences were 5′-UGC

UUG AAG CAG CUC UGG A-3′ (sense) and 5′-GCG CGC TTT GTA GGA TTC G-3′ (sense), respectively.

2.4. Protein and RNA analyses

Cell lysates were prepared using RIPA buffer supplemented with protease inhibitors aprotonin (1 μg/ml), leupeptin (10 μg/ml) and DMSF (100 μg/ml). A total of 60 μg of total protein was loaded onto pre-cast 10% agarose Tris–HCl gels (BioRad). Proteins were separated by electrophoresis and transferred to PVDF membrane (Millipore). The antibodies used to detect specific proteins included: GFP mouse polyclonal (Clontech), PKR, PKR Phospho-rabbit polyclonal (Cell Signaling), p53, p21 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 (Santa Cruz), followed by visualisation using the luminol/enhancer chemiluminescent substrate (Amersham).

Northern blot analysis was used to determine the steady-state levels of dEGFP and p53 mRNAs. Total RNA isolated using Trizol reagent (Life Technologies) was separated on a 1% agarose–formaldehyde gel and transferred to a nylon charged membrane. DNA fragments containing the ORFs for either dEGFP or p53 were used as probes. Detection of small p53-specific RNAs was achieved using a hydrolysed p53 RNA probe as previously described [20]. This RNA was generated by in vitro transcription using pORF53 and the MegaScript Kit (Ambion) according to the manufacturer's instructions.

2.5. Media transfer experiments

To examine the effect of the culture medium on dEGFP-mediated cell fluorescence, control cells and cells co-expressing antisense and sense dEGFP RNA were each seeded in three media types: control cell conditioned medium, sense/antisense cell conditioned medium and unconditioned DMEM. After two and five days in each of these media, both control cells and cells co-expressing antisense and sense dEGFP RNA were assayed for cell fluorescence and dEGFP, p53 and β-actin protein levels.

3. Results

3.1. Co-expression of sense and antisense RNA suppresses transgene expression

A human embryonic kidney cell line stably expressing the dEGFP gene under control of the CMV immediate early promoter (and G418 resistant due to the presence of a linked neomycin phosphotransferase gene) was transfected with episomal plasmids containing either the gene conferring resistance to hygromycin or the gene conferring resistance to puromycin, and sense and antisense expression cassettes. The structure of the dEGFP-specific plasmids used to express antisense complementary to the target mRNA or sense RNA homologous to the target mRNA is outlined in Fig. 1. The ATG start codon in the sense gene was modified to prevent translation of the encoded sense RNA into dEGFP protein. Following co-transfection with the sense and antisense plasmids, cells containing both episomes and the target gene were selected using puromycin, hygromycin and G418. The control cells contained the two core vectors without antisense or sense genes, while the cells containing the antisense plasmid or sense plasmid only were co-transfected with the appropriate core vector containing the second selectable marker. In this way, all cells selected were resistant to puromycin, hygromycin and G418.

After selection, all co-transfectants were subcultured, grown and analysed for their cell fluorescence profile (Fig. 2A). Cells containing the vectors alone, the antisense plasmid alone or the sense plasmid alone did not display a reduction in dEGFP-mediated cell fluorescence. This outcome was observed using antisense or sense genes controlled by either the mammalian

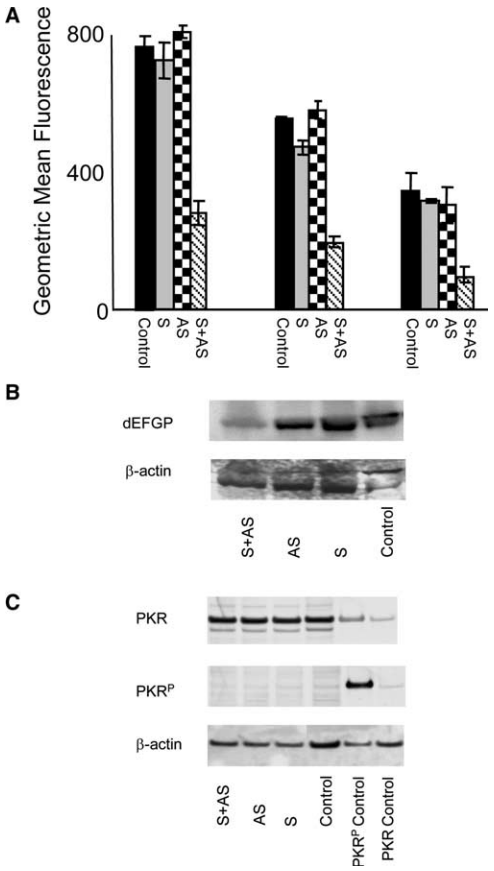

Fig. 2. Suppressing dEGFP gene expression by co-expression of long complementary RNAs. (A) Reduction of dEGFP-mediated cell fluorescence in EcR293 cells containing sense and antisense dEGFP-expressing plasmids. The three sets of histograms (control, S, AS, and S + AS) represent cells that are 50%, 70% and 90% confluent. Each histogram is the average of three experiments and error bars represent S.D. The abbreviations are as follows: control = vectors alone; S = sense vector; AS = antisense vector and S + AS = sense and antisense vectors. (B) Reduction of dEGFP protein levels in cells co-expressing long complementary RNAs. (C) Cells co-transfected with sense and antisense dEGFP episomal plasmids do not activate PKR. The steady-state levels of PKR (PKR), activated PKR (PKR^P) and β-actin is shown. The PKR control and PKR^P control samples represent HeLa cells untreated and treated with 0.1 μM of calyculin.

constitutive EF1α promoter or RSV LTR. In contrast, cells containing both the antisense and sense plasmids revealed an approximately 40–60% reduction in cell fluorescence mediated by the dEGFP target gene. These data show that co-expression of antisense and sense RNAs, in the presence of the target mRNA, is more effective at suppressing the cellular phenotype associated with expression of the target gene in human cells than using an antisense or a sense plasmid alone. This suggests that introducing two complementary RNAs, with the potential to form intermolecular dsRNA, into human cells can regulate the expression of a specific gene.

To examine the impact of co-expressing these complementary RNAs on the level of dEGFP protein, total protein was extracted from the above cells and analysed for the expression of the following gene products: dEGFP and β-actin (Fig. 2B). This analysis indicated that the level of dEGFP protein was reduced by 50% in cells co-expressing complementary RNAs.

In contrast, there was no reduction in dEGFP protein levels in cells expressing either sense or antisense RNA alone. This result indicated that the observed phenotypic change in cell fluorescence in the antisense and sense RNA co-expressing cells was due to the reduction in the steady-state level of dEGFP protein.

One of the potential limitations associated with using longer dsRNA in mammalian cells is the activation of PKR and the downstream effects [21]. In this study, we observed no abnormal changes in cell morphology and no significant decrease in cell density (data not shown). Furthermore, we were unable to detect the phosphorylated version of an active PKR in our cells expressing complementary RNAs (Fig. 2C). Studies have suggested that p53 is an important mediator of dsRNA-induced gene expression [22]. In the present study, we observed no increase in the steady-state levels of p53 protein and no activation of p53, as measured by the presence of p21, a downstream indicator of p53 transcriptional activity (data not shown). These results suggest that the presence of sense and antisense RNA-expressing plasmids within the same cell does not activate the global response mechanism or a general cellular stress signal mediated by p53 activation.

3.2. Complementary RNA co-expression produces clones with different levels of target gene expression

To examine the level of dEGFP gene suppression mediated within the pooled population, we selected clones containing sense, antisense or both sense and antisense dEGFP RNA-expressing plasmids. Analysis of cell fluorescence indicated that a greater number of clones containing the sense and antisense plasmids displayed reduced fluorescence. These clones could be arranged into three different classes based on the degree of reduction in cell fluorescence. Class I displayed no reduction, class II displayed intermediate levels and class III showed negligible levels of cell fluorescence (Fig. 3A). This method for regulating specific gene expression produces a series of single clones that display varying degrees of target gene expression – a most desirable tool for examining gene function.

One possible mechanism by which long complementary RNAs may operate is through the formation of long dsRNA that undergoes processing by the RNAse III-like enzyme Dicer to produce small dsRNAs that act as the effectors of gene silencing [23]. To determine whether Dicer was required for the maintenance of gene silencing, we used RNAi to reduce the expression of Dicer [24]. Reduction of Dicer in either class II or III clones did not reverse silencing of the dEGFP transgene, suggesting that the observed gene suppression was Dicer-independent (Fig. 3B). This result does not eliminate the possibility that a Dicer-like activity, not recognised by Dicer-specific siRNAs, was not involved [25].

3.3. The gene silencing effect induced by long complementary RNAs is transferable

It has been noted in earlier studies using dsRNA as a mediator of gene inactivation in non-mammalian cells that a proportion of the suppressive effect can be transferred to other cells in vivo [26] or in culture [27]. To examine the transferability of the dEGFP-specific dsRNA-mediated suppressive effect, we conducted a culture medium exchange experiment (Fig. 4A). Conditioned media from control cells and cells co-expressing antisense and sense dEGFP RNA were isolated and used to culture cells co-expressing antisense and sense dEGFP RNA and control cells, respectively. Control

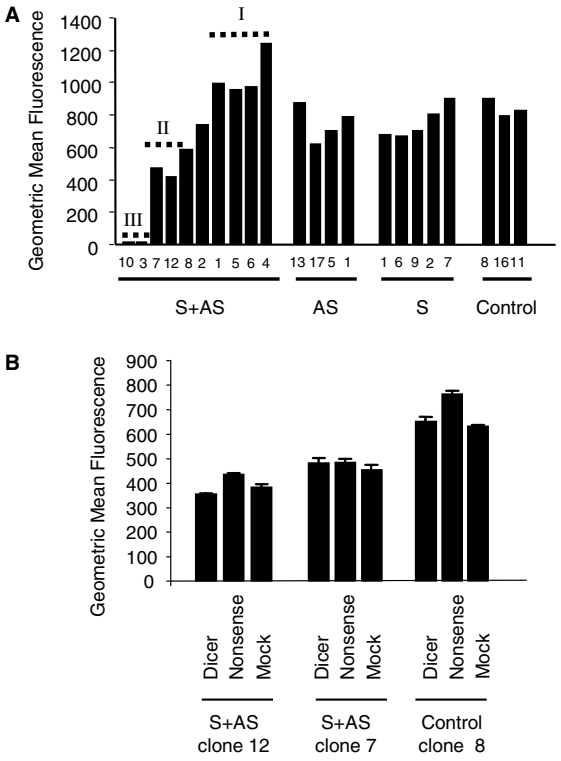

Fig. 3. Generation of an epi-allelic series of clones displaying different dEGFP target gene expression profiles. (A) Differential regulation of dEGFP-mediated cell fluorescence in cells containing sense and antisense plasmids. The geometric mean fluorescence for different EcR293 clones containing sense and antisense (S + AS), antisense (AS), sense (S) or vector (control) plasmids are shown. The clones containing sense and antisense plasmids are classified into three classes (I, II and III). (B) Gene silencing through complementary RNA co-expression is Dicer-independent. Representative class II clones (clones 12 and 7) and a control clone (clone 8) were transfected with either no siRNA (mock), Dicer siRNA (Dicer) or nonsense siRNA (nonsense) and monitored for geometric mean fluorescence. Each histogram is the average of three experiments and error bars represent S.D.

cells cultured in medium isolated from cells co-expressing antisense and sense dEGFP RNA displayed a reduction in dEGFP-mediated cell fluorescence (Fig. 4B). In contrast, the addition of conditioned control medium or unconditioned DMEM to these cells did not produce the same reduction in cell fluorescence, suggesting that the silencing effect was specific and did not result from depletion of nutrients. Similar trends were observed in sense and antisense plasmid-containing cells exposed to unconditioned DMEM and re-exposed to conditioned medium derived from sense and antisense RNA-expressing cells (Fig. 4B). These results suggest that the suppressive effect generated within human cells by co-expressing sense and antisense RNA was transferable to cells that had not been previously exposed to either the sense or antisense RNAs. The fact that the suppressive effect was not transferable to all cells within the original culture, or the recipient culture, suggests that other factors influence the transferability of the silencing effect between cells induced by complementary RNA co-expression.

To examine the specificity of the mobile silencing effect, the levels of the dEGFP protein were determined. As shown in Fig. 4C, only control cells receiving cultured medium from the

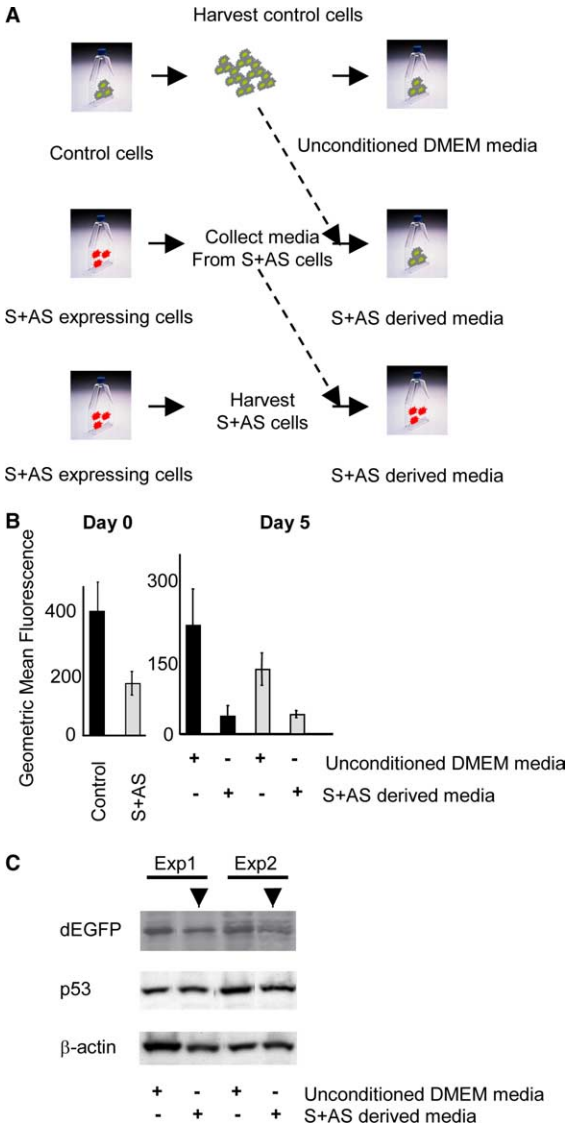

Fig. 4. Transferability of the specific gene-silencing signal induced by complementary RNA co-expression. (A) Overview of the media transfer experimental strategy. Control cells, containing vector alone, were harvested and exposed to unconditioned DMEM or conditioned medium collected from cells containing sense and antisense dEGFP plasmids (S + AS expressing cells). In addition, cells containing sense and antisense plasmids were re-exposed to their own conditioned medium. (B) Reduction in dEGFP-mediated cell fluorescence by conditioned medium. The geometric mean fluorescence for control cells and S + AS cells was determined at day 0 and 5 following exposure to either unconditioned DMEM or conditioned medium obtained from S + AS expressing cells (S + AS derived media). The black histograms indicated control cells and the grey histograms show the S + AS cells. The error bars represent S.D. (C) The transferable silencing signal is specific in recipient cells. The steady-state levels of dEGFP, p53 and β-actin proteins are shown in two independent experiments involving exposure of control cells to unconditioned DMEM or conditioned medium from S + AS cells (arrows).

sense and antisense plasmid-containing cells displayed a reduction in dEGFP protein levels. In contrast, no reduction in dEGFP protein was observed in cells grown in unconditioned DMEM or medium obtained from cells containing control plasmids. Furthermore, the specificity of the mobile signal was confirmed by the lack of effect on both p53 and β-actin protein levels. This suggests that the transferable silencing signal is specific for the dEGFP gene. Additional experiments will be required to determine the nature of the signalling molecule mediating this specific suppressive effect.

3.4. Long complementary RNAs suppress endogenous gene expression

To examine the utility of long complementary RNAs for controlling the expression of endogenous genes in mammalian cells, we chose the TP53 gene as a target. This gene encodes the p53 transcriptional activator protein and can be regulated by both synthetic and gene-expressed siRNAs [28]. Episomal plasmids encoding sense and antisense p53 RNAs were generated and combinations of these plasmids were delivered to HCT116 colon carcinoma cells. Following selection for cells containing antisense, sense or both expression plasmids, independent clones were isolated and examined for p53 protein levels. As observed for the dEGFP transgene, a larger number of the clones containing sense and antisense p53 plasmids

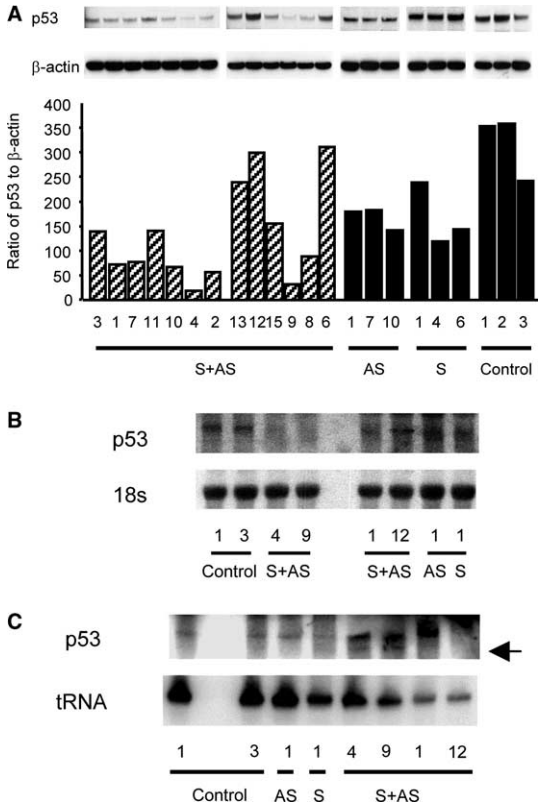

Fig. 5. Complementary RNA co-expression mediates specific reduction of the p53 protein in HCT116 colon cancer cells. (A) Western blot analysis of p53 and β-actin protein levels in stable clones containing sense and antisense, antisense, sense or control plasmids. The histograms represent the ratio of p53 to β-actin in each clone and clones are numbered on the horizontal axis. The p53:β-actin ratios for clones containing the sense and antisense p53 plasmids are indicated by hatched histograms. (B) Suppression of p53 protein correlates with p53 mRNA levels. Northern blot analysis of p53 mRNA and 18S rRNA in selected clones identified in (A). (C) Induction of gene silencing by long complementary RNA generates p53-specific small RNAs. Northern blot analysis of p53-specific small RNAs and $tRNA_{valine}$ in selected clones identified in A and B. The arrow indicates the small p53-specific RNAs in S + AS clones 4 and 9.

displayed a reduction in p53 protein compared with those containing vector alone, sense alone or antisense alone (Fig. 5A). In addition, as with the transgene target, the clones selected for maintenance of the sense and antisense p53 expression plasmids could be classified into three different groups based on the levels of p53 protein. These results demonstrate that co-delivery of episomal plasmids encoding long complementary RNAs can be used to regulate the expression of an endogenous gene in mammalian cells. Furthermore, this method results in the generation of an ''epi-allelic'' series of clones in which the specific target gene is expressed at different steady-state levels.

To further examine the underlying mechanism for long complementary RNA-mediated gene silencing, we performed RNA analyses of clones from the three different classes for the level of TP53 mRNA and the presence or absence of p53-specific small RNAs. The steady-state level of TP53 mRNA was found to correlate with the level of reduction of the p53 protein, suggesting that long complementary RNA co-expression mediated its silencing effect by reducing or eliminating target mRNA (Fig. 5B). In addition, those clones displaying the lowest level of p53 mRNA and protein also contained p53-specific small RNAs (Fig. 5C). These observations suggest that complementary RNA co-expression generates small gene-specific RNAs, the latter of which are considered a hallmark of RNAi.

4. Discussion

In this study, we report on the development of a method using episomal plasmids encoding long complementary RNAs to specifically suppress foreign and endogenous gene expression in mammalian cells. We demonstrate that suppression is dependent on the co-existence of plasmids encoding sense and antisense RNAs specific for the target gene sequence. The observed suppression of gene expression involves target RNA degradation, reduction in target protein levels and the production of gene-specific small RNAs, all molecular indicators of RNAi or a RNAi-like mechanism. Interestingly, the generation of small effector RNAs using this approach was not reduced by siRNA-mediated suppression of the RNAseIII-like enzyme Dicer. In addition, the gene silencing effect was shown to be transferable to other cells not previously exposed to the sense and antisense RNA-encoding episomal plasmids.

Unlike most other organisms, dsRNA of greater than 30 bp in length can induce the generalised interferon response in mammalian cells. This occurs by inactivation of the eukaryote initiation factor 2α by the dsRNA-dependent protein kinase PKR. In addition, cells also undergo activation of the 2′–5′OAS/RNAseL system leading to non-specific mRNA degradation. The end result arising from these combined pathways is cellular apoptosis. The presence of this generalised response has discouraged the use of longer dsRNA in mammalian cells and favoured the development of synthetic siRNAs and gene expression systems compatible with the expression of small hairpin RNAs [5,7,29]. In spite of this, attempts have been made to test longer dsRNA in mammalian cells as a trigger for RNAi with mixed results. In cell types lacking a PKR response system, in vitro transcribed or gene-expressed dsRNA was shown to be effective and specific in mediating gene silencing [11,30]. In contrast, delivery of in vitro transcribed long dsRNA to the cytoplasm of commonly used mammalian cells, in which the PKR response was expected to be more robust, appeared to induce sequence non-specific effects [31]. Despite these results, several lines of evidence have emerged suggesting that conditions exist in which longer dsRNA may be used in standard mammalian cell lines to induce gene-specific silencing. Gene constructs designed to express long inverted repeat dsRNA have been shown to control specific gene expression in CHO cells and mice [13]. Co-delivery of sense and antisense RNA-expressing plasmids leads to increased suppression of specific gene expression compared with antisense RNA alone [32]. Furthermore, conditional control of specific gene expression was accomplished by co-expression of 290 base sense and antisense RNAs in NIH 3T3 cells [33]. Interestingly, in all studies using gene-expressed longer dsRNAs, expression of the full-length RNA or sense and antisense RNAs has not been demonstrated, suggesting that these RNAs undergo rapid modification. In the present study, we have confirmed and extended the results from these studies by showing that co-expression of long complementary RNAs can be used to control the expression of specific genes in commonly used cell lines.

The premise behind the current report to controlling gene expression stems from earlier observations that introducing sense and antisense plasmids into eukaryotic cells enhances suppression of a target gene [34]. Co-expression of long sense and antisense RNAs occurs naturally in mammalian cells and, in most instances, these complementary RNAs are localised within the nuclear compartment [35]. Analysis of the human genome sequence for the presence of complementary RNA transcripts has identified a large number of potential RNA partners, some of which may act as regulators of RNA sequences having sequence complementarity [18]. This suggests that mammalian cells have mechanisms to deal with these long dsRNAs or use these as mediators of gene regulation. Although the precise mechanism by which long dsRNA results in gene-specific silencing remains to be elucidated, several lines of evidence from the present study suggest a nuclear step. Export of long dsRNA, formed by hybridisation between the co-expressed sense and antisense RNAs, to the cytoplasm would be expected to activate PKR and other global responses and induce cell death. As shown in this paper, PKR was not activated in cells showing gene-specific silencing and no differences in cell morphology or cell growth were observed. Attempts to reverse the gene-specific silencing effect by siRNA-mediated reduction of Dicer activity did not result in the loss of gene suppression by long dsRNA. This suggests that the generation of the observed small effector RNAs in cells co-expressing long complementary RNAs, and displaying gene knockdown, occurred in a Dicer-independent manner – for example, within the nucleus by a Dicer-like activity such as Drosha [25] or through the microRNA processing pathway [36]. In addition, the fact that Dicer has been reported as a cytoplasmic enzyme further supports a nuclear-based step. We propose that long dsRNA, if formed, is processed in the nucleus by a Dicer-like enzyme to produce gene-specific siRNAs that can act either in the nucleus or the cytoplasm to induce the degradation of complementary target mRNA. In this way, the mammalian cell can accommodate the presence of long dsRNA without inducing global responses. This is supported by a recent study aimed at deliberately localising long dsRNA to the nucleus to mediate tissue-specific knockdown in mice [37]. The recent

identification of potential antisense RNA sequences in the human genome has predicted that as much as 8% of genes may be influenced by antisense [18], with some suggesting that this may be an underestimate of the extent of antisense transcription [38]. Given that the average length of the overlaps in the sense-antisense pairs is 372 bp, the mammalian cell potentially contains a highly abundant sub-population of naturally occurring long dsRNAs, some or all of which may be modified or processed within the nucleus and block specific gene expression.

Studies in non-mammalian organisms have indicated that gene silencing mediated by dsRNA can be systemic and heritable, suggesting that the silencing signal is mobile and cell non-autonomous. In this way, dsRNA-mediated induction of gene silencing can lead to the establishment of RNAi in cells not directly exposed to the dsRNA. The spreading of the silencing effects mediated by RNAi has been shown in transgenic plants [39], *C. elegans* [3], and *Drosophila* S2 culture cells [27]. Little is known about the chemical nature of the signal, however, current candidates include siRNAs or long dsRNAs [40,41]. Genetic studies in *C. elegans* have identified a transmembrane protein encoded by the sid-1 gene that may act as a channel for the import of the mobile signal [42]. It is interesting to note that SID-1 homologues are present in mammalian cells, suggesting the possibility that RNAi-mediated gene silencing in these cells may include a cell non-autonomous component. In the present study, we demonstrate that conditioned medium isolated from cells containing sense and antisense episomal plasmids, but not control episomes, induces gene-specific suppression in cells not previously transfected with either plasmid. Exposure of the recipient cells to this medium did not result in the activation of PKR, indicating that gene silencing was not due to an interferon response. Furthermore, the observed level of suppression in the recipient cells was greater than in the original cells, raising the possibility that the mobile signal was possibly amplifiable. Both the presence of a sequence-specific mobile signal and a possible mechanism of amplification are consistent with the features used to define systemic silencing in other organisms. One alternative explanation is the release of episomal plasmids into the medium in the original culture and transfection of recipient cells with these plasmids. This scenario is highly unlikely, since plasmid uptake requires transfection reagents and the sense and antisense plasmids would need to enter the same cell to mediate gene silencing. The precise nature of the mobile silencing signal remains to be determined and the tissue culture model presented here should be useful in its characterisation.

In this study, we have shown for the first time that expression of long complementary RNAs from episomal plasmids mediates specific gene silencing in mammalian cells. This approach provides an alternative to other strategies for inducing dsRNA-mediated gene regulation with the benefit of sustained gene silencing, as opposed to transient suppression. In addition, the use of long RNA strands does not require pre-determination of the sensitive site for targeted regulation of gene expression, as required with siRNAs and gene expression methods using shRNAs [7]. A further advantage of using episomal vectors to transcribe either sense or antisense RNA is the flexibility to utilise plasmid segregation to conditionally convert from a suppressive phenotype to a normal phenotype in a relatively short time period. The generation of cell clones displaying varying levels of target gene expression provides an "epi-allelic" series useful in studying gene function [43]. It is anticipated that complementary RNA co-expression will provide a useful alternative approach to inducing RNAi (or RNAi-like)-mediated gene regulation in mammalian cells.

Acknowledgements: The authors acknowledge Allison Arndt for her technical assistance in generating a subset of the dEGFP expression plasmids.

References

[1] Bosher, J.M. and Labouesse, M. (2000) Nat. Cell. Biol. 2, E31–E36.
[2] Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Methods 26, 199–213.
[3] Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E. and Mello, C.C. (1998) Nature 391, 806–811.
[4] Hutvagner, G. and Zamore, P.D. (2002) Curr. Opin. Genet. Dev. 12, 225–232.
[5] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[6] Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) Nature 404, 293–296.
[7] Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J. and Conklin, D.S. (2002) Genes Dev. 16, 948–958.
[8] Tran, N., Cairns, M.J., Dawes, I.W. and Arndt, G.M. (2003) BMC Biotechnol. 3, 21.
[9] Clemens, M.J. (1997) Int. J. Biochem. Cell Biol. 29, 945–949.
[10] Wianny, F. and Zernicka-Goetz, M. (2000) Nat. Cell Biol. 2, 70–75.
[11] Yang, S., Tutton, S., Pierce, E. and Yoon, K. (2001) Mol. Cell. Biol. 21, 7807–7816.
[12] Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Proc. Natl. Acad. Sci. USA 27, 27.
[13] Ui-Tei, K., Zenno, S., Miyata, Y. and Saigo, K. (2000) FEBS Lett. 479, 79–82.
[14] Clemens, M.J. and Elia, A. (1997) J. Interferon Cytokine Res. 17, 503–524.
[15] Aoki, Y., Cioca, D.P., Oidaira, H., Kamiya, J. and Kiyosawa, K. (2003) Clin. Exp. Pharmacol. Physiol. 30, 96–102.
[16] Gan, L., Anton, K.E., Masterson, B.A., Vincent, V.A., Ye, S. and Gonzalez-Zulueta, M. (2002) J. Neurosci. Methods 121, 151–157.
[17] Yi, C.E., Bekker, J.M., Miller, G., Hill, K.L. and Crosbie, R.H. (2003) J. Biol. Chem. 278, 934–939.
[18] Yelin, R. et al. (2003) Nat. Biotechnol. 21, 379–386.
[19] Raponi, M., Dawes, I.W. and Arndt, G.M. (2000) Biotechniques 28, 838–840, 842, 844.
[20] Hamilton, A.J. and Baulcombe, D.C. (1999) Science 286, 950–952.
[21] Williams, B.R. (1999) Oncogene 18, 6112–6120.
[22] Hummer, B.T., Li, X.L. and Hassel, B.A. (2001) J. Virol. 75, 7774–7777.
[23] Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Nature 409, 363–366.
[24] Hutvagner, G., McLachlan, J., Pasquinelli, A.E., Balint, E., Tuschl, T. and Zamore, P.D. (2001) Science 293, 834–838.
[25] Filippov, V., Solovyev, V., Filippova, M. and Gill, S.S. (2000) Gene 245, 213–221.
[26] Bosher, J.M., Dufourcq, P., Sookhareea, S. and Labouesse, M. (1999) Genetics 153, 1245–1256.
[27] Caplen, N.J., Fleenor, J., Fire, A. and Morgan, R.A. (2000) Gene 252, 95–105.
[28] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Cancer Cell 2, 243–247.
[29] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Science 296, 550–553.
[30] Paddison, P.J., Caudy, A.A. and Hannon, G.J. (2002) Proc. Natl. Acad. Sci. USA 99, 1443–1448.
[31] Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A. (2001) Proc. Natl. Acad. Sci. USA 98, 9742–9747.
[32] Aoki, Y., Cioca, D., Oidaira, H., Kamiya, J. and Kiyosawa, K. (2003) Clin. Exp. Pharmacol. Physiol. 30, 96–102.

[33] Wang, J., Tekle, E., Oubrahim, H., Mieyal, J.J., Stadtman, E.R. and Chock, P.B. (2003) Proc. Natl. Acad. Sci. USA 100, 5103–5106.
[34] Raponi, M. and Arndt, G.M. (2003) Nucleic Acids Res. 31, 4481–4489.
[35] Kumar, M. and Carmichael, G.G. (1998) Microbiol. Mol. Biol. Rev. 62, 1415–1434.
[36] Lee, Y., Jeon, K., Lee, J.T., Kim, S. and Kim, V.N. (2002) Embo J. 21, 4663–4670.
[37] Shinagawa, T. and Ishii, S. (2003) Genes Dev. 17, 1340–1345.
[38] Carmichael, G.G. (2003) Nat. Biotechnol. 21, 371–372.
[39] Palauqui, J.C., Elmayan, T., Pollien, J.M. and Vaucheret, H. (1997) EMBO J. 16, 4738–4745.
[40] Klahre, U., Crete, P., Leuenberger, S.A., Iglesias, V.A. and Meins Jr., F. (2002) Proc. Natl. Acad. Sci. USA 99, 11981–11986.
[41] Hamilton, A., Voinnet, O., Chappell, L. and Baulcombe, D. (2002) EMBO J. 21, 4671–4679.
[42] Winston, W.M., Molodowitch, C. and Hunter, C.P. (2002) Science 295, 2456–2459.
[43] Hemann, M.T., Fridman, J.S., Zilfou, J.T., Hernando, E., Paddison, P.J., Cordon-Cardo, C., Hannon, G.J. and Lowe, S.W. (2003) Nat. Genet. 33, 396–400.