Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles

Abdullah Ely, Tanusha Naidoo and Patrick Arbuthnot*

Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, University of the Witwatersrand Medical School, Private Bag 3, WITS 2050, South Africa

Received January 27, 2009; Revised May 11, 2009; Accepted May 12, 2009

ABSTRACT

Expressed polycistronic microRNA (miR) cassettes have useful properties that can be utilized for RNA interference (RNAi)-based gene silencing. To advance their application we generated modular trimeric anti-hepatitis B virus (HBV) Pol II cassettes encoding primary (pri)-miR-31-derived shuttles that target three different viral genome sites. A panel of six expression cassettes, comprising each of the possible ordering combinations of the pri-miR-31 shuttles, was initially tested. Effective silencing of individual target sequences was achieved in transfected cells and transcribed pri-miR trimers generated intended guide strands. There was, however, variation in processing and silencing by each of the shuttles. In some cases the monomers’ position within the trimers influenced processing and this correlated with target silencing. Compromised efficacy could be compensated by substituting the pri-miR-31 backbone with a pri-miR-30a scaffold. Inhibition of HBV replication was achieved in vivo, and in cell culture without disruption of endogenous miR function or induction of the interferon response. A mutant HBV target sequence, with changes in one of the guide cognates, was also silenced by the trimeric cassettes. The modular nature of the cassettes together with compatibility with expression from Pol II promoters should be advantageous for gene silencing applications requiring simultaneous targeting of different sites.

INTRODUCTION

The powerful and specific gene silencing that may be achieved by harnessing the RNA interference (RNAi) pathway is potentially useful for developing new therapies required to treat a variety of diseases. In addition, application of RNAi has utility for the study of gene function. Both synthetic and expressed sequences are being developed to activate RNAi (1). Exogenous expression cassettes achieve this by transcribing mimics of intermediates of the microRNA (miR) processing pathway (2). Short hairpin RNAs (shRNAs), which are typically expressed from Pol III promoters, simulate precursor miR (pre-miR) products of Drosha/DGCR8 processing. Primary miR (pri-miR) shuttles are analogues of nascent miR transcripts and their processing is compatible with expression from Pol II transcription regulatory elements (3–7). This important property provides the means of improving control of production of RNAi activators and thereby limiting unwanted off target effects caused by saturating the endogenous miR pathway (8). Pri-miR-like shuttles are also thought to effect superior silencing by simulating natural miR processing more closely. Processing of pri-miR shuttles by Drosha/DGCR8, which is bypassed by shRNAs, may improve entry into the RNAi pathway (4). pri-miR-30 was initially the most widely utilized (9–11) backbone, but other pri-miR shuttles such as miR-155 (6) miR-31 and miR-122 (3) have since been used successfully to generate exogenous RNAi effecters. The polycistronic arrangement of some naturally occurring miR clusters is an additional property that may be exploited to generate combinatorial multtargeting RNAi expression cassettes (4,6,7). This is particularly useful to improve knockdown efficacy and overcome attenuation of silencing caused by target site mutation as often occurs during chronic viral infection. Recently, the miR-106 (7) and miR-17-92 (4) polycistronic clusters have been used successfully to generate multiplexed anti-HIV-1 RNAi activators. To improve use of expressed multimeric RNAi effecters, a system that allows convenient assembly, modification to improve silencing efficacy and which causes knockdown without disrupting the endogenous miR pathways would be valuable. We demonstrate these attributes in a panel of anti-hepatitis B virus (HBV) Pol II trimeric pri-miR cassettes, which are

*To whom correspondence should be addressed. Tel: +27 (0)11 717 2365; Fax: +27 (0)11 717 2395; Email: Patrick.Arbuthnot@wits.ac.za

© 2009 The Author(s)
This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (http://creativecommons.org/licenses/by-nc/2.0/uk/) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.
capable of inhibiting viral replication in transfected cells and in vivo.

MATERIALS AND METHODS

miR expression plasmids

Anti-HBV pre-miR DNA shuttles were generated by annealing partly complementary pre-miR-31/5, -31/8, -31/9 and pre-miR30a/8 forward (F) and reverse (R) oligonucleotides, which was followed by primer extension to generate completely double-stranded DNA. The oligonucleotide sequences were pre-miR-31/5 F: 5'-GTA ACT CGG AAC TGG AGA GGG GTG AAG CCA AGT GCA CAC GGG TTG AAC TGG GAA CCA CG-3', pre-miR-31/8 F: 5'-CTG CTG TCA GAC AGG AAA GCC GTG AAT CGA CAG GTG GCA CAC GTC GTT CCC AGT TCA ACC CGT-3', pre-miR-31/31/9 R: 5'-CTG CTG TCA GAC AGG AAA GCC GTG AAT CGT TGA TGT GCA CAC GTC GTT GCA CAC GGG TTG AAC TGG GAA CGA CG-3', pre-miR-31/8/9 F: 5'-GTA ACT CGG AAC TGG AGA GGG GTG AAG CCA AGT GCA CAC GGG TTG AAC TGG GAA CCA CG-3', pre-miR-31/8/9 R: 5'-CTG CTG TCA GAC AGG AAA GCC GTG AAT CGT TGA TGT GCA CAC GTC GTT GCA CAC GGG TTG AAC TGG GAA CGA CG-3'.

miR target plasmids

To produce dual luciferase reporter plasmids containing sites individually targeted by pre-miR-31/5, -31/8, -31/9, -31/30a/8 and pre-miR-122/5 primers were designed to amplify HBV coordinates 1575–1599 (5'T), 1678–1702 (8'T) and 1774–1798 (9'T) (Genbank accession J02203). Oligonucleotide sequences, which also introduced a SpeI site at the 3' end of the amplicons, were 5'T F: 5'-CCG TGT GCA CCT CGG TTC CTG-3', 8'T F: 5'-CCG TGT GCA CCT CGG TTC CTG-3', 9'T F: 5'-CCG TGT GCA CCT CGG TTC CTG-3'. Successful ligation resulted in formation of pTZ pri-miR-31/5-8-9. The sequence encoding pri-miR-31/5 was then excised from pTZ pri-miR-31/9 with NheI and EcoRI and ligated to SpeI and EcoRI sites of pTZ pri-miR-31/5-8. Similarly, the sequence encoding pri-miR-31/9 was then excised from pTZ pri-miR-31/9 with NheI and EcoRI and ligated to SpeI and EcoRI sites of pTZ pri-miR-31/5-8. Successful ligation resulted in formation of pTZ pri-miR-31/5-8-9. Trimeric shuttle cassettes, containing pri-miR-31, pri-miR-30a and pri-miR-122 sequences, were formed by inserting combinations of pri-miR-31/5, pri-miR-31/8, pri-miR-30a/8, pri-miR-31/9 and pri-miR-122/5 downstream of the CMV immediate early promoter enhancer. A total of eight trimeric cassettes was generated (pri-miR-31/5-8-9, pri-miR-31/5-9-8, pri-miR-31/8-5-9, pri-miR-31/8-9-5, pri-miR-31/9-5-8, pri-miR-31/9-9-30a/30a/8 and pri-miR-122/5-31/9-30a/8). Six of the trimeric cassettes comprised pri-miR-31-derived sequences exclusively, while pri-miR-31/5-31/9-9-30a/8 and pri-miR-122/5-31/9-30a/8 also included pri-miR-30a and pri-miR-122 scaffolds. Similar cloning strategies were used to propagate each of the trimers. As an example, to generate the pri-miR-31/5-8-9 cassette, pri-miR-31/8 was excised from pTZ pri-miR-31/8 with NheI and EcoRI and ligated to pTZ pri-miR-31/5-9 that had been digested with SpeI and EcoRI to create pTZ pri-miR-31/5-8-9. Successfully ligated resulted in formation of pTZ pri-miR-31/5-8-9. The trimeric cassettes were excised with NheI and XbaI and inserted at equivalent sites of pCI-neo to generate the CMV panel of multimeric cassettes. Sequences were verified using standard automated dideoxy chain termination reactions.
CGA CTC 3' primers. Mutant bases of the target 5 sequence (underlined) were incorporated within the forward PCR primer. The resultant amplicon included RsFI and NotI sites at the 5' and 3' ends, respectively. After insertion into the pTZ57R/T PCR cloning vector (InstAclone™ PCR cloning Kit, Fermentas, MD, USA) and sequence verification, mHBx was excised with RsFI and NotI and then used to replace HBx in psiCHECK-HBx and generate psiCHECK-mHBx. Presence of a PsiI restriction digestion site (bold font in mHBx F primer), which is absent from wild-type HBx, was used to verify insertion of the intended mutant sequences. The HBV target plasmids, pCH-9/3091 (14) and pCH-FLuc (3), have been described previously.

**miR-16 sponge and dual luciferase target**

The U6-driven miR-16 sponge (15) was generated by cloning seven copies of an imperfectly complementary target of miR-16 into the U6 +27 sequence (16–18). A single copy of duplex DNA, comprising annealed oligonucleotides encoding a single copy of the miR-16 target site (miR-16S) with single nucleotide 3' A overhangs, was initially ligated to pTZ57R/T to create pTZ-miR-16S×1. The resulting target sequence included an XhoI site that was 5' of the target and Sall and NotI sites 3' of this sequence. Oligonucleotide sequences used to generate the inserts were miR-16S F: 5'-CTC GAG GCG CAA TAT TAT GTG CTG CTA GTC GAC GCC GCC GCA-3' and miR-16S R: 5'-GCC GGC GCC GCG TCG ACT AGC AGC ACA TAA TAT TGG CGC TCG AGA-3'. The miR-16S×1 sequence was restricted from pTZ-miR-16S×1R (insert in reverse orientation with respect to the β-galactosidase gene) with ApaI and PvuII and cloned into the ApaI and HincII sites of pGEM®-T Easy (Promega, WI, USA) to create pG-miR-16S×1. To generate vectors with tandem copies of the miR-16S sequence, pG-miR-16S×1 was digested with XhoI and ScaI and separately with Sall and Scal. The fragments containing the miR-16S sequence from each digestion were ligated to create pG-miR-16S×2, pG-miR-16S×3 and pG-miR-16S×4 were generated using similar procedures. Finally, the vectors containing three and four tandem copies of the miR-16S sequence were used to create pG-miR-16S×7.

The U6 +27 sequence (18) was produced using a two-step PCR of the human U6 promoter. U6 forward (U6 F, 5'-GAT CTC TAG AAA GGT CGG GCA GGA AGA GGG-3') and U6 +27 reverse 1 (U6 +27 R1, 5'-CTC GAG TAG TAT ATG TGC TGC CGA AGC GAC CAG GGT GTT TCC TTC TTA ACC-3') primers used in the first round of amplification. Amplicons from this reaction were used as template for the second round of PCR using the U6 +27 R2 primer (5'-GAT CAA AAA AGC GGA CCG AAC TCA GCC GCT TGC TGC AGT ATA TGT GCT G-3') and U6 F primer. The complete U6 +27 sequence was inserted into the PCR cloning vector pTZ57R/T to generate pTZ-U6 +27. The miR-16S×7 sequence was removed from pG-miR-16S×7 with XhoI and Sall and ligated to the XhoI site of pTZ-U6 +27 to produce the pTZ-U6-miR-16S×7 sponge plasmid. To generate the psiCHECK-miR-16T×7 target vector containing 7 miR-16 sites downstream of the Renilla luciferase ORF, the miR-16S×7 sequence was restricted from pG-miR-16S×7 with XhoI and NotI and inserted into equivalent sites of psiCHECK™-2 (Promega, WI, USA).

**Cell culture, transfection, northern blot analysis and dual luciferase assay**

Huh7 cells were cultured in DMEM (Lonza, Basel, Switzerland) supplemented with 10% fetal calf serum (Gibco BRL, UK). To determine the efficacy of individual pri-miR monomers in the context of multimeric cassettes, each trimeric plasmid (800 ng) was co-transfected with psiCHECK-5T, psiCHECK-8T or psiCHECK-9T (80 ng). Luciferase activity was assayed using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and Renilla luciferase to Firefly luciferase activity was determined. Silencing of mutant HBxs sequences was assessed using previously described psiCHECK-HBx and psiCHECK-mHBx dual luciferase reporter vectors. To assess HBV knockdown efficacy of the Pol III and Pol II pri-miR shuttles, Lipofectamine 2000™ (Invitrogen, CA, USA) was used to co-transfect 80 ng pCH-FLuc, 800 ng of the relevant pri-miR shuttle plasmid, together with effecter plasmid or vector control plasmid according to previously described methods (3). phRL-CMV (Promega, WI, USA), a plasmid constitutively expressing Renilla luciferase, was included in all transfections. Forty-eight hours after transfection cells were assayed for luciferase activity using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) and the ratio of Firefly luciferase to Renilla luciferase activity was calculated.

Northern blot analysis was performed on RNA extracted from cells transfected with the various miR-31 shuttle constructs according to previously described methods (3). The probes for the 5, 8 and 9 guide sequences were 5'-CCG TGT GCA CTT CGC TTC-3', 5'-CAA TGG CAA CGG ACC-3' and 5'-TAG GAG GCT GTA GGC ACA-3', respectively. Scanned autoradiographs were used to quantify guide bands using KODAK MI Software.

Knockdown of HBV replication was assessed in cells co-transfected with pCH-9-3091(14) and relevant RNAi effector plasmid. Forty-eight hours after transfection growth medium was harvested and HBsAg secretion measured by ELISA using the MONOLISA® HBs Ag ULTRA kit (Bio-Rad, CA, USA).

Activation of the interferon (IFN) response was assessed using previously described methods (19). Assays to assess saturation of the endogenous miR pathway were performed in Huh7 cells cotransfected with 80 ng psiCHECK-piR-16T×7 and 800 ng RNAi effector plasmids or miR-16 sponge plasmid. Luciferase assays were performed as described above.

**Assessment of efficacy of pri-miR-31 shuttles in vivo**

Mice were injected using the hydrodynamic injection procedure with a combination of 5 μg pCH-9-3091 (14), 5 μg of RNAi expression vector, 5 μg of control U6 (pTZ-U6 vector (12)) or CMV (pCI-neo, Promega, WI, USA).
Results
Design of trimeric pri-miR expression cassettes
Structure of the expression cassettes producing trimeric anti-HBV pri-miR-31 mimics is depicted schematically in Figure 1A. The pri-miR-31 backbone was initially selected as we have previously shown that single unit shuttles with this scaffold can be used to generate efficient Pol II anti-HBV expression cassettes (3). Sequences encoding the pri-miR-31 trimers were located within an exon and downstream of a CMV Pol II transcription controlling element and intron sequence. Trimeric cassettes were designed such that pre-miRs comprised 59 nt and were flanked by 51 nt of natural pri-miR-31-derived sequences (Figure 1B). According to this scheme, the mature anti-HBV miRs were predicted, using the MFold algorithm (20), to have a similar structure to that of naturally occurring pri-miR-31. To assess the modular nature of the cassettes, six different trimeric expression cassettes were generated using all possible ordering combinations of the three pri-miR-31 shuttles. Computer-based predictions indicated that the intended miR-31-like structures of the trimeric cassettes were energetically most favourable and similar for each of the six ordering combinations. The calculated ΔG values of each was approximately −195 kcal/mol.

Detection of processed pri-miR sequences and silencing of individual targets
To verify formation of individual guide sequences, northern blot analysis was carried out on RNA extracted from Huh7 liver-derived cells transfected with DNA-expressing pri-miR-31 shuttles (Figure 2A–C). Hybridization to a probe complementary to the intended miR-31/5 HBV guide showed heterogenous processing to form guide sequences of 20–22 nt in length. Guide strand 5 production was similar when generated from monomeric and trimeric cassettes and was not affected by shuttle position within the anti-HBV polycistron. As expected, measurement of relative guide band intensities showed that the 20–22 nt HBV anti-sense sequence was present in considerably higher amounts in cells transfected with U6 shRNA 5-expressing plasmid when compared to cells transfected with the pri-miR trimer shuttles. Specific knockdown of target 5 sequence, assessed using a dual luciferase reporter system, was similar and highly effective (~90%) for the U6 shRNA 5 and each of the pri-miR-31 shuttle expression cassettes (Figure 2D). Northern blot analysis to detect guide 8 revealed a single band of 21 nt (Figure 2B), which was distinct from the heterogenous mature pri-miR-31/5 sequences. Interestingly, no mature pri-miR-31/8 was detectable in RNA extracted from cells transfected with CMV pri-miR-31/9-8-5 and CMV pri-miR-31/5-9-8. This suggests that pri-miR-31/8 shuttle position within the trimer affects processing, and presence of pri-miR-31/9 immediately upstream of pri-miR-31/8 may be responsible for compromised guide 8 production. Assay of knockdown using a dual luciferase assay with miR-31/8 target alone, confirmed that knockdown of reporter expression correlated with detection of mature miR sequences (Figure 2E). Interestingly, guide produced from the U6 shRNA 8 cassette was slightly larger than that of the CMV miR-31-derived sequences. It is likely that the differences in secondary structure of the shRNA 8 and pri-miR-31/8 RNA, as well as the involvement of Drosha in miR shuttle processing, are responsible for generation of guide strands of different molecular weight. Analysis of silencing and processing of pri-miR-31/9 sequences showed less efficient knockdown (45–80%) of ORF containing pri-miR-31/9 target (Figure 2F), which correlated with lower efficiency of pri-miR-31/9 guide production (Figure 2C). Although there is variation in the efficiency of individual guide strand production and knockdown, these data indicate that the pri-miR-31 scaffold is useful for production of Pol II trimeric cassettes.
Incorporating a pri-miR-30a monomer scaffold improves silencing of HBV target 8

Sequence-specific properties of the individual anti-HBV pri-miR-31 shuttles as well as position of monomer shuttles within the trimers are likely to influence their processing and silencing efficiency. To assess the effect of pri-miR backbone scaffold sequences within the expression cassettes, silencing of target 8 sequences by an expanded panel of trimeric expression cassettes that included pri-miR-122/5 and pri-miR-30a/8 shuttles was measured (Figure 3). Each of the six pri-miR-31 trimers together with pCMV pri-miR-31/5-31/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 were co-transfected with psiCHECK 8T. As described before (Figure 2E), knockdown of Renilla luciferase activity was poor with cassettes containing pri-miR-31/5-9-8 and pri-miR-31/9-8-5. However, efficient inhibition of reporter gene activity was achieved with pCMV pri-miR-31/5-31/9-30a/8 and pCMV pri-miR-122/5-31/9-30a/8 (Figure 3B). This indicates that substitution of the pri-miR-31 scaffold with a pri-miR-30a backbone restores target 8 silencing. In addition, inclusion of the pri-miR-122/5 monomer, which we have previously shown to act efficiently against HBV (3), does not compromise silencing by the pri-miR-30a/8 monomer. Thus, in addition to allowing improved silencing efficiency by changing monomer positions within the trimers, the cassettes described here have the added advantage of permitting the changing of pri-miR shuttle backbones to compensate for any compromised silencing efficacy of pri-miR-31 scaffolds.

miR-mediated inhibition of markers of HBV replication in transfected cells and in vivo

Target sites of the individual miR cassettes are located within the HBV X (HBx) ORF (Figure 4A). This sequence is conserved, common to all viral transcripts and has been shown to be a good target for RNAi-based HBV silencing (19). A dual luciferase assay, in which the surface ORF of pCH-9-3091 was substituted with a Firefly luciferase ORF, demonstrated that each of the trimeric cassettes achieved good knockdown when all three cognates of the intended miR-31/5, miR-31/8 and miR-31/9 guides were present (Figure 4B). As an initial assessment of pri-miR-31 trimer-mediated inhibition of HBV replication, Huh7 liver-derived cells were transfected with the pCH-9-3091 HBV replication competent plasmid (14), together with the panel of RNAi expression plasmids. Secreted HBV surface antigen (HBsAg), which is a reliable indicator of HBV replication in our hands (3,19,21), was determined thereafter in the culture medium (Figure 4C). Knockdown of ~90% was achieved. This correlated with the inhibitory effect that was observed when using the dual luciferase reporter system to measure silencing of individual targets (Figure 2D–F) and also inhibition of a Firefly luciferase-HBx reporter gene construct (Figure 4B). To determine silencing of target genes in vivo in a model that simulates
HBV replication, mice were co-injected with an HBV replication competent plasmid together with a selection of vectors encoding pri-miR-31 shuttles using the hydrodynamic procedure (22). Significant knockdown of HBsAg was observed at Days 3 and 5 after the injection, and the effects appeared to be independent of promoter interference (Figure 4D). These findings confirm that trimeric pri-miR-31 shuttles are capable of silencing HBV replication and verify that they are active against transcripts that are produced during viral replication in vivo.

A potential advantage of employing multimeric cassettes to inhibit viral replication is that viral escape resulting from emergence of evading mutations is limited. A dual luciferase assay was undertaken to assess whether HBV target silencing occurred when mutations were introduced into the target 5' site of HBV (Figure 5A). Co-transfection of cells was carried out with each plasmid encoding the panel of eight trimeric expression cassettes together with wild-type or mutant HBx target. Silencing of reporter gene expression was achieved with all trimeric expression cassettes with the exception of mutant target silencing by CMV pri-miR-31/9-8-5. This was expected as these two expression cassettes are known to generate antiHBV guide 9 in low amounts and be defective with respect to guide 8 production (Figure 2). Importantly, mutant target silencing was restored in these cassettes by changing the pri-miR-31/8 monomer for a pri-miR-30a/8 unit, and confirms earlier observations (Figure 3) that target 8 silencing may be improved by substituting the pri-miR scaffold of its guide. Thus trimeric pri-miR cassettes are capable of efficiently silencing targets containing one mutant guide cognate and defective silencing may be overcome by changing the order of the miR units or scaffold sequence within the monomers.

Exclusion of non-specific effects induced by pri-miR-31 trimer cassettes

Verification that the pri-miR-31 trimer cassettes are indeed non-toxic and induce gene silencing by an RNAi-mediated mechanism is important to establish. To address this, disruption of the endogenous miR pathway and stimulation of the innate IFN response by pri-miR-31 trimer cassettes were assessed. Measurement of IFN-β mRNA concentration in cells transfected with trimer expression cassettes showed no elevation of this transcript, indicating that little or no immunostimulation is caused by IFN pathway induction (Figure 6). To assess disruption of the endogenous miR pathway, we adapted the recently described method that utilizes miR sponges as a control to verify derepressive effects of endogenous miR.
A dual luciferase reporter vector was generated in which seven copies of an imperfectly matched endogenous miR-16 target were inserted downstream of the Renilla luciferase ORF (Figure 7A). Perturbations in miR-16 translational suppression could be detected sensitively by measuring Renilla/Renilla luciferase reporter gene activity in addition to a plasmid constitutively expressing Renilla luciferase. Results are given as ratios of Firefly to Renilla luciferase activity. Column-labelled negative represents data from transfections that excluded the pCH-FLuc plasmid. Column-labelled negative represents data from transfections that excluded the pCH-9/3091 HBV plasmid. (D) Silencing of HBV replication in vivo. Serum concentration of HBsAg was measured at Days 3 and 5 after hydrodynamic injection of mice with replication-competent pHBV plasmid together with indicated anti-HBV expression cassettes. Column-labelled negative represents data from transfections that excluded the pCH-9/3091 HBV plasmid.

DISCUSSION

function (15). A dual luciferase reporter vector was generated in which seven copies of an imperfectly matched endogenous miR-16 target were inserted downstream of the Renilla luciferase ORF (Figure 7A). Perturbations in miR-16 translational suppression could be detected sensitively by measuring Renilla/Firefly luciferase reporter gene activity. miR-16 was selected for this assay as it is expressed in a variety of tissues (23) and can be conveniently used to determine disruption of natural miR function. A miR sponge expression cassette that encodes seven tandemly repeated miR-16 target sites was used to control for endogenous miR derepression (Figure 7B). Analysis revealed that co-expression of each of the trimeric constructs within transfected cells did not cause derepression of miR-16 inhibition of its cognate in the reporter fusion sequence (Figure 7C). The Pol II promoter-controlled expression of trimeric anti-HBV miR-31 shuttles, therefore, cause no detectable toxicity that results from IFN response induction or disruption of the endogenous miR pathway.

The powerful gene silencing that can be achieved by harnessing RNAi has facilitated development of new approaches to inhibition of pathology-causing genes and the study of gene function (1). Although synthetic siRNAs have been favoured as RNAi activators for many such applications, use of expressed silencing sequences has several advantages. These include achievement of sustained knockdown, compatibility with recombinant viral vectors and evasion of some of the immunostimulatory properties of exogenous synthetic sequences (24). Convenient expression of silencing sequences that efficiently target multiple...
sites would be a particularly useful attribute to enhance knockdown and counter evading target mutations. Achieving this without causing unintended off target effects and needing to utilize complex systems that require multiple expression cassettes is desirable. The engineered polycistrons described here provide a suitable method to attain these objectives. Cassettes were generated using pri-miR-31-, pri-miR-30a- and pri-miR-122-derived modules, which were combined as trimers and expressed from a Pol II promoter. Efficient processing of the shuttles and silencing of HBV cognates was observed without evidence for disruption of the endogenous miR pathway.

Detailed analysis revealed variation in efficacy of individual units that was dependent on specific sequences of the monomers as well as their position within the engineered polycistrons. Despite simultaneous production of three RNAi effectors from a single transcript, the mature guide sequences were not formed in equimolar amounts. Although knockdown of single targets may be compromised as a result of poor processing of individual guides, the modular nature of the cassettes facilitates improvement of defective silencing. Rearranging the order of the pri-miR units, which is not easily achieved with polycistronic miR cluster mimics, may restore function of individual miR shuttles. In addition, the cassettes described here allow improvement of efficacy to be achieved by

Figure 5. Dual luciferase reporter assay to detect silencing of wild-type HBx and mutant HBx target sequences using pri-miR expression cassettes. (A) The dual luciferase reporter vectors include the entire wild-type or mutant HBx target sequence downstream of the Renilla luciferase ORF. Guide 5 sequence is indicated in red, and mutations within its mutant HBx cognate are indicated in blue. (B) Assessment of knockdown efficacy of trimer shuttles using the psiCHECK-based dual luciferase reporter gene assay in which wild-type HBx or mutant HBx had been inserted downstream of the Renilla ORF. Data are represented as mean ratios of Renilla to Firefly luciferase activity (±SEM) and are normalized relative to the mock-treated cells.

Figure 6. Assessment of IFN response induction by miR-expression cassettes. IFN-β mRNA concentrations were determined in HEK293 cells, which were transfected with the indicated miR-encoding cassettes, or with poly(I–C) as positive control. RNA was extracted from the cells 24h later and then subjected to quantitative RT-PCR to determine concentrations of IFN-β and GAPDH mRNA. Means (±SEM) of the normalized ratios of IFN-β to GAPDH mRNA concentrations are indicated from three independent experiments.
substituting poorly acting pri-miR-31 scaffolds with other backbone shuttles, such as those derived from pri-miR-30a and pri-miR-122. The sequence-specific differences in individual guide processing and target knock-down that we observed are not surprising but currently difficult to explain. Although computer-predicted structures of the shuttles were similar, empirical characterization of the processing of expressed RNAi effecters remains critically important.

Previous investigations have demonstrated that a combinatorial approach to knockdown of HIV-1 replication augments silencing and prevents the emergence of viral escape mutants (25). It has been calculated that four optimally acting individual antiviral guide sequences are required to prevent HIV-1 escape from RNAi (26), and anti-HIV-1 RNAi activators have been designed accordingly (4,7). Unlike with HIV-1, the HBV genome comprises overlapping ORFs with embedded viral cis elements (27). This highly compact arrangement of the genome restricts ability of HBV to mutate without compromising its replication fitness. The number of RNAi effecters within a combinatorial cassette that is required to prevent emergence of HBV escape mutants is not established, but it is likely to be fewer than the four that are required for HIV-1. Nevertheless, although only three pri-miR-31 shuttles were tested in the polycistronic cassettes described here, it is likely that a larger number of monomeric modules can be accommodated.

An important concern for the development of RNAi-based therapy is avoidance of off target effects. Unintended consequences may result from disruption of endogenous miR functions and silencing of normal cellular genes that have partial sequence complementarity to exogenous RNAi activators. We have shown that endogenous miR-16-mediated repression of a target reporter fusion is unaffected by expression of the Pol II pri-miR-31 trimer cassettes. Generating multiple silencing sequences from an engineered polycistronic cassette potentially increases the likelihood of causing unintended off target effects. Interaction of a guide seed region, comprising nucleotides 2–8 from the 5′ end, is potentially sufficient to effect translational suppression of a cellular target. This weak sequence restraint emphasizes the importance of utilizing potent expressed silencing sequences that are effective at low concentration, and also restricting the expression of RNAi effecters to target tissues.

Compatibility of polycistronic pri-miR shuttles with expression from Pol II promoters, and efficacy that is equivalent to that of shRNA transcribed from a U6 promoter, are useful features that may be harnessed to diminish unintended effects. Compared to Pol III promoters, Pol II transcription regulatory elements have greater versatility that facilitates production of mature RNAi effecters without perturbing endogenous miR function. In the case of developing RNAi-based HBV therapy, expression cassettes containing liver-specific promoters that are induced by target-encoded transcription activators, e.g. the HBV X protein (28,29), should facilitate transcription regulation with consequent attenuation of off target effects.

**FUNDING**

The Sixth Research Framework Programme of the European Union, Project RIGHT (LSHB-CT-2004-005276), from CANSA; the South African National Research Foundation (NRF GUN 68339 and 65495), ESASTAP; and the South African Poliomyelitis Research Foundation. Funding for open access charge: University of the Witwatersrand.

Conflict of interest statement. None declared.
REFERENCES


