INHIBITING HIV-1 GENE EXPRESSION AND REPLICATION WITH EXPRESSED LONG HAIRPIN RNAs

Sheena Meg Saayman

A thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand,

in fulfillment of the requirements for the degree of

Doctor of Philosophy

Johannesburg 2010

DECLARATION

I, Sheena Meg Saayman declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

……...

9th day of June, 2010

PUBLICATIONS AND PRESENTATIONS

The following articles and conference presentations arose from work presented in this thesis. The research articles may be found in the appendix.

Research Articles

Saayman S, Arbuthnot P and Weinberg M. Deriving four functional anti-HIV siRNAs from a single Pol III-generated transcript comprising two adjacent long hairpin RNA precursors. 2010. *Nucleic Acids Research.* In Press.

Saayman S, Arbuthnot P and Weinberg M. The design and expression of effective RNA Pol III-expressed long hairpin RNAs. 2010. In: *RNA Therapeutics: Function, Design and Delivery. Methods in Molecular Biology series, Humana Press, USA*. ISBN: 978-1-60761-656-6

Barichievy S, **Saayman S**, Arbuthnot P and Weinberg M. RNA Interference-Based Gene Expression Strategies Aimed at Sustained Therapeutic Inhibition of HIV. 2009. *Current Topics in Medicinal Chemistry*. 9(12): 1065-1078.

Saayman S, Barichievy S, Capovilla A, Morris K, Arbuthnot P and Weinberg M. The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. 2008. *PLoS ONE*. 3(7): e2602.

Barichievy S, **Saayman S**, von Eije K, Morris KV, Arbuthnot P and Weinberg MS. The Inhibitory Efficacy of RNA Pol III-Expressed Long Hairpin RNAs Targeted to Un-translated Regions of the HIV-1 5' Long Terminal Repeat. 2007. *Oligonucleotides*. 17(4): 419-432.

Conference Presentations

Saayman S, Arbuthnot P and Weinberg M. Deriving four predictive functional anti-HIV siRNAs from a single expressed Pol III-generated long hairpin RNA. 2010. South African Society of Biochemistry and Molecular Biology. Bloemfontein, South Africa.

Saayman S, Barichievy S, Arbuthnot P and Weinberg M. Deriving Predictive Functional Anti-HIV siRNAs from a Single Expressed Pol III-generated Long-Hairpin RNA. 2009. American Society of Gene Therapy. 12th Annual Meeting. San Diego, California, USA.

Saayman S, Barichievy S, Arbuthnot P and Weinberg M. Deriving two predictive functional siRNAs from a single expressed Pol III-generated long hairpin RNA. 2009. Keystone Symposia. The Biology of RNA Silencing. Victoria, British Columbia, Canada.

Barichievy S, **Saayman S**, and Weinberg M S. The primary microRNA background affects the targeting activity of different Pol III expressed anti-HIV guide sequences. 2008. American Society of Gene Therapy. 11th Annual Meeting. Boston, Massachusetts, USA.

Saayman S, Barichievy S, Crowther C, Morris K, Arbuthnot P and Weinberg M. Inhibiting HIV-1 Replication and Gene Expression with Expressed Long Hairpin RNAs. 2007. American Society of Gene Therapy. 10th Annual Meeting. Seattle, Washington, USA.

Saayman S, Arbuthnot P and Weinberg M. Inhibiting HIV-1 Replication and Gene Expression with Expressed Long Hairpin RNAs. 2007. University of the Witwatersrand AIDS Research Symposium. Johannesburg, South Africa.

ABSTRACT

The vast potential of the RNA interference (RNAi) pathway as a new tool for the development of therapeutic modalities has been quickly realised since its discovery in 1998. RNAi effector mimics have been developed to successfully silence an array of disease-causing genetic elements. However, because of the rapidly mutating genome of viruses such as the human immunodeficiency virus (HIV), inhibition of replication cannot be sustained with single RNAi effector mimics. Instead, a combinatorial approach is required, analogous to the cocktail of drugs necessary for successful highly active antiretroviral therapy (HAART). Pioneering studies utilizing long hairpin RNAs (lhRNAs) showed that the long double-stranded RNA stem region acts as a Dicer substrate and is processed into multiple siRNA species. This intrinsic combinatorial property of lhRNAs was exploited in this thesis by attempting to incorporate three non-contiguous potent siRNA sequences within a single lhRNA stem expressed from an RNA Pol III promoter. Although significant knockdown of three independent HIV target sequences was possible, the limitations of this approach became apparent when it was observed that human Dicer does not function efficiently as a multiple turnover enzyme. The generation of siRNA products therefore occurred in a gradient, with higher levels of siRNA produced from the base of the hairpin stem and decreasing quantities generated towards the loop. Modifications to the configuration of integrated siRNA sequences within the stem region enabled augmented RNAi activity of siRNAs in the second position of the hairpin stem. This led to the notion that further manipulation of the structural design of the stem duplex may improve efficacy of up to two siRNAs. Dual-targeting anti-HIV lhRNAs encoding only two highly effective siRNAs targeted against non-contiguous sites within the *tat*, *nef*, *LTR* and *int* viral genes were therefore propagated. The spatial arrangement of two siRNA sequences was extensively characterised within dual-targeting lhRNAs by inserting up to three random base pairs at the junctions of siRNA encoding sequences and 5 bp preceding the terminal loop sequence. A universally optimal hairpin design was identified which contained a single mismatched base pair between two 19 bp + 2 nt siRNA sequences, as well as a terminal extension. Two powerful dual-targeting lhRNA species, lhRNA-*tat-nef* +1 and lhRNA-*LTR-int* +1, each capable of producing two

potent anti-HIV siRNA products in equal quantities were selected for incorporation into a combinatorial RNAi system. These two effective dual-targeting lhRNAs were combined, adjacent to one another within a single RNA Pol III-expressed transcript to create a novel lhRNA-based combinatorial RNAi structure. This double lhRNA (dlhRNA) construct served as a precursor for four discrete highly functional RNAi effector sequences which were capable of simultaneously silencing four unique HIV target sites within the *tat*, *nef*, *LTR* and *int* genes. Furthermore, the ectopic expression of dlhRNAs did not elicit activation of the interferon response, nor did it cause saturation of the endogenous miRNA biogenesis pathway *in vitro*. In conclusion, the inherent combinatorial RNAi properties of long hairpin RNAs were evaluated and the detailed analysis is presented in this thesis. Structurally optimised dual-targeting lhRNAs subsequently formed the core components of a novel dlhRNA precursor which meets all the requirements for an effective combinatorial RNAi strategy and therefore holds great promise for mediating an effective and sustained gene therapy against HIV.

ACKNOWLEDGEMENTS

I wish first and foremost to extend my sincere gratitude to my supervisor Dr. Marco Weinberg. Marco, you are a remarkable person and scientist, and your consistent energy and enthusiasm for “the data” is truly inspirational. I want to express my deepest appreciation to you for your consistent supervision, for giving me such amazing opportunities and more especially for instilling inside me a passion for science.

I wish to thank my co-supervisor, Prof. Patrick Arbuthnot, for your unfailing support, encouragement and supervision, and for the privilege of conducting my research in the Antiviral Gene Therapy Research Unit (AGTRU). The experience in your lab has been incredible. I have to also thank you for the tremendous annual Christmas parties!

Thank you to my co-supervisor, Dr. Alexio Capovilla, for your supervision, assistance and advice with HIV-related research and for the use of the facilities in the HIV Pathogenesis Research Unit.

A massive thank you to all my fellow lab members of AGTRU, for technical assistance, advice, coffee breaks, chats and memorable cheese & wine evenings. You have been a fantastic group to work with.

A special thanks to my colleague Abdullah Ely, for your invaluable advice in the lab, your unfailing willingness to help, and for your ability to miraculously fix any computer glitch.

Thank you to Kevin Morris at the Scripps Research Institute in San Diego, Califoria for hosting me in your lab for an academic exchange programme and for giving me a taste of life in California. The research experience gained in your lab was invaluable.

Financial support in the form of bursaries and scholarships is gratefully acknowledged from The University of the Witwatersrand, The Antiviral Gene Therapy Research Unit, The German Academic Exchange Service (DAAD), The Poliomyelitis Research Foundation, The National Research Foundation and the Stella and Paul Loewenstein Charitable and Educational Trust.

To my incredible parents, thank you for your continuous love, support and encouragement throughout all of my student years.

To Ant, you have been there for me since the beginning of this journey and your love, encouragement, patience and support has kept me going through the good times and the bad. You have never stopped believing in me, and for that I cannot thank you enough.

Finally to the rest of my friends and family, your words of encouragement never went unnoticed.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

A	adenine
Ago	argonaute
AIDS	acquired immunodeficiency disease
AP	Antarctic phosphatase
ATP	adenosine triphosphate
BAF1	barrier to autointegration factor
bp	base pair
C	cytosine
CA	capsid
CCR5	chemokine (C-C motif) receptor 5
CDK	cyclin-dependent kinase
cDNA	complementary DNA
CMV	cytomegalovirus
cPPT	central polypurine tract
CRM1	chromosome region maintenance
CXCR4	chemokine (C-X-C motif) receptor 4
DGCR8	DiGeorge syndrome critical region gene 8
dlhRNA	double long hairpin RNA
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
dsRBD	double-stranded RNA binding protein
dsRNA	double-stranded RNA
DUF	domain of unknown function
e-shRNA	extended short hairpin RNA
EDTA	ethylenediaminetetraaceticacid
Endo-siRNA	endogenous small interfering RNA
Env	envelope
Exp5	exportin-5
FANA	fluoro-β-D-arabinonucleotide
FIV	feline immunodeficiency virus
G	guanine
Gag	group specific antigen
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFP	green fluorescent protein
gp	glycoprotein
GTP	guanosine triphosphate
HAART	highly active antiretroviral therapy
HBV	hepatitis B virus

HBx	hepatitis B virus X protein
HCV	hepatitis C virus
HIS	human immune system
HIV	human immunodeficiency virus
hRLuc	human *Renilla* luciferase
HSC	haematopoietic stem cell
hsp	heat shock promoter
IFN	interferon
IN	integrase
IPTG	Isopropyl-β-D-1-thiogalactopyranoside
kb	kilobase
LB	Luria Bertani broth
LEDGF	lens epithelium-derived growth factor
lhRNA	long hairpin RNA
LNA	locked nucleic acid
LTR	long terminal repeat
MA	matrix
MID	middle
miRNA	microRNA
mRNA	messenger RNA
NC	nucleocapsid
Nef	negative effector
NF-kB	nuclear factor kB
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside/nucleotide reverse transcriptase inhibitor
nt	nucleotide
OAS	oligoadenylate synthetase
OB	oligonucleotide / oligosaccharide-binding
ORF	open reading frame
OTE	off target effect
PAGE	polyacrylamide gel electrophoresis
PAK-1	serine/threonine protein kinase 1
P-body	processing body
PBS	phosphate buffered saline
PCR	polymerase chain reaction
P-TEFb	positive transcription elongation factor b
PACT	protein activator of PKR
PAZ	Piwi Argonaute Zwille/Pinhead
PBMC	peripheral blood mononuclear cell
PCAF	P300/CBP-associated factor
piRNA	Piwi-interacting RNA
PIWI	P-element induced wimpy testis
PKR	protein kinase R
PNK	polynucleotide kinase
Pol	polymerase
PR	protease

PRE	post-transcriptional regulatory element
Pre-miRNA	precursor microRNA
Pri-miRNA	primary microRNAs
qRT-PCR	quantitative real time reverse transcription PCR
Ran-GAP	Ran-specific GTPase activating protein
RanGDP	guanosine diphosphate-bound Ran
RanGTP	guanosine triphosphate-bound Ran
rde	RNAi deficient
Rev	regulator of virion gene expression
RHA	RNA helicase A
RIG-1	retinoic acid inducible gene 1
RISC	RNA induced silenced complex
RNA	Ribonucleic acid
RNAi	RNA interference
RSV	respiratory syncytial virus
RSV	rous sarcoma virus
RT	reverse transcriptase
SCID	severe combined immunodeficiency
SDS	sodium dodecylsulphate
SEM	standard error of the mean
shRNA	short hairpin RNA
SIN	self-inactivating
siRNA	small interfering RNA
snRNA	small nuclear RNA
ssRNA	single stranded RNA
TAR	transactivation response
Tat	transcriptional transactivator
TBE	Tris-Borate-EDTA
$TCID_{50}$	50% tissue culture infectious dose
tetO	tetracycline operator
tetR	tetracycline repressor
TGS	transcriptional gene silencing
TLR	toll-like receptor
TRBP	TAR RNA binding protein
tRNA	transfer RNA
U	uridine
UTR	untranslated region
VEGF	vascular endothelial growth factor
Vif	viral infectivity factor
Vpr	viral protein r
Vpu	viral protein u
X-Gal	5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside

CHAPTER 1

Introduction

1.1 Overview of the introduction

The discovery of the RNA interference (RNAi) pathway and its underlying mechanisms has offered researchers a new tool with which to develop novel therapeutics for the treatment of a wide array of diseases. This introductory chapter will explore the intricate network of factors associated with this fundamental gene silencing pathway and discuss various mechanisms through which the effectors of the pathway are mimicked for sequence specific gene silencing. The ability of RNAi effector mimics to inhibit disease-causing genetic elements and their consequent potential as therapeutic modalities will be emphasized. Given the lack of an effective vaccine or cure for infection by the human immunodeficiency virus (HIV), and the current pandemic caused by this virus, strategies for exploiting RNAi for the treatment of HIV/acquired immunodeficiency disease (AIDS) will form the focus of this thesis. The rapid evolution of HIV and its consequent ability to develop resistance to single RNAi effectors will be addressed, and the need for the simultaneous administration of multiple therapeutic modalities to effect prolonged viral inhibition will be highlighted. Finally, strategies to modify RNAi effector mimics for the simultaneous inhibition of multiple gene targets will be discussed, with the ultimate aim of designing and developing an RNAi-based combinatorial strategy for the effective long term inhibition of HIV gene expression and replication.

1.2 RNA interference

RNA interference (RNAi) is an evolutionary conserved eukaryotic gene silencing pathway and represents a fundamental system for intracellular gene expression regulation. This phenomenon was first observed in petunias when exogenously introduced transgenes were shown to suppress homologous gene expression, and was termed “co-suppression” (Napoli et al. 1990). Similar

observations were later made in the fungus *Neurospora crassa* and the phenomenon was termed "quelling" (Romano and Macino 1992). In 1998 Fire and Mello made the seminal discovery that double-stranded RNA (dsRNA) was the trigger for such silencing events and described the sequence specific silencing mechanism of RNAi in *Caenorhabditis elegans* (Fire et al. 1998).

The molecular mechanism underlying RNAi was largely elucidated by several studies conducted in *Drosophila melanogaster* cells (Hammond et al. 2000; Zamore et al. 2000; Elbashir et al. 2001b). RNAi was found to be mediated by small dsRNA species that are 21-25 bp in length, which are processed from longer dsRNA precursors by an RNase III endonuclease. These small interfering RNAs (siRNAs), together with protein co-factors were found to associate with endonucleases and members of the Argonaute protein family, to guide the cleavage of mRNA complementary to one of the strands of the siRNA, resulting in sequence specific degradation of target mRNA (Hammond et al. 2000; Zamore et al. 2000; Elbashir et al. 2001b).

1.3 Biogenesis of small RNAs in humans

Different classes of small RNAs have since been identified and can be distinguished on the basis of their biogenesis pathways, their silencing mechanisms and their cellular roles. To date, three classes of eukaryotic small RNAs have been classified: microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and Piwi-interacting RNAs (piRNAs) [reviewed in (Ghildiyal and Zamore 2009; Kim et al. 2009)]. For the purposes of this thesis I will focus on the biogenesis pathway of the miRNA class of small RNAs in humans (Figure 1.1). Today over 700 miRNAs have been identified in humans alone (miRBase accessed 2010). These small RNAs are derived from hairpin-like structures transcribed from the genome and regulate endogenous genes at the post-transcriptional level and in rare cases at the transcriptional level (Kim et al. 2008). To harness this pathway for the development of therapeutics, an understanding of the intricate biogenesis of miRNAs is imperative.

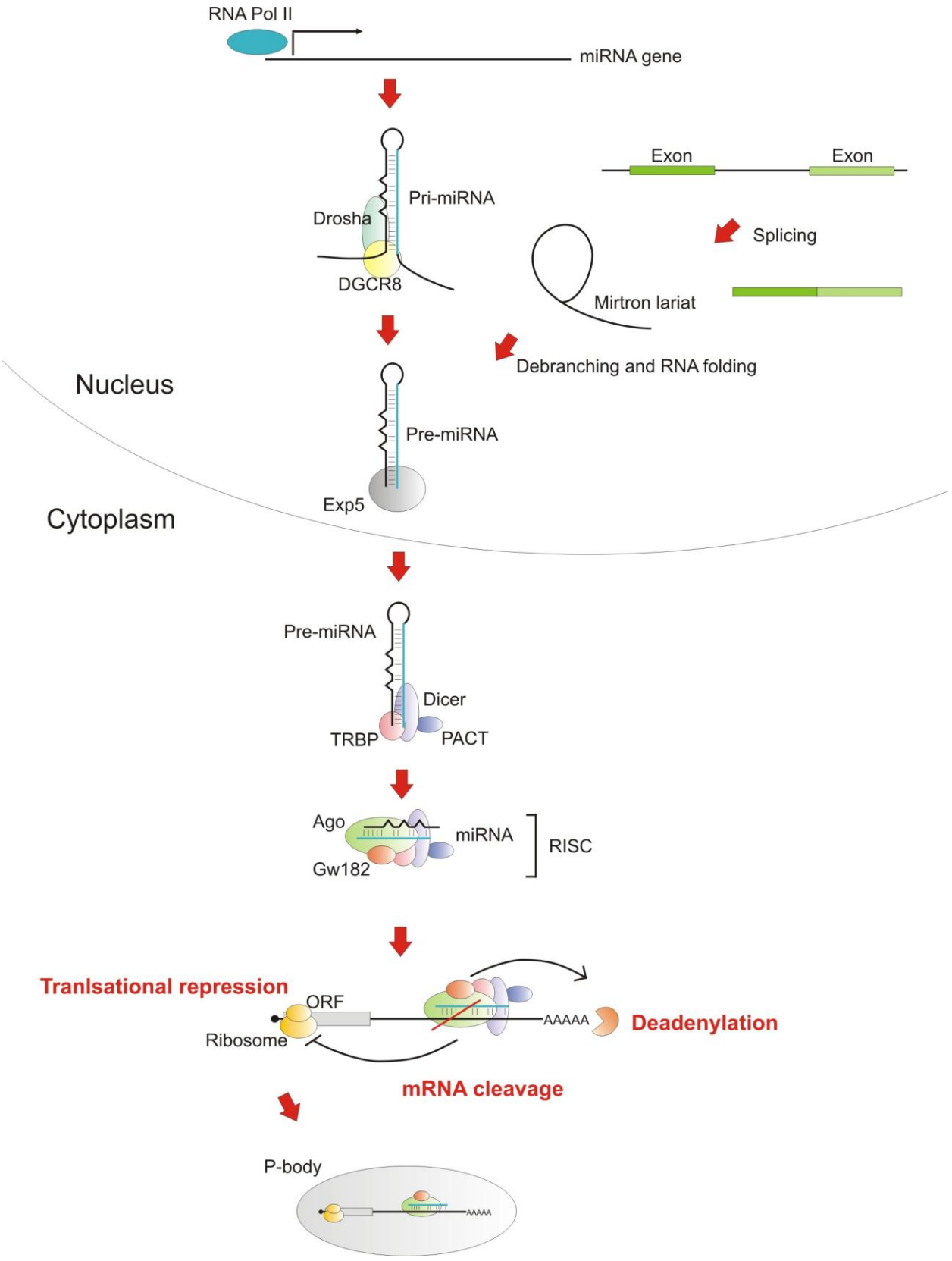

RNA Pol II
miRNA gene
Exon
Exon
Pri-miRNA
Drosha
DGCR8
Splicing
Mirtron lariat
Debranching and RNA folding
Nucleus
Pre-miRNA
Exp5
Cytoplasm
Pre-miRNA
Dicer
TRBP
PACT
Ago
miRNA
RISC
Gw182
Tranlsational repression
ORF
Ribosome
AAAAA
Deadenylation
mRNA cleavage
P-body
AAAAA

Figure 1.1: The endogenous mammalian miRNA biogenesis pathway. Genes encoding miRNAs are transcribed by RNA Pol II promoters to form stem loop structures termed Primary miRNAs (pri-miRNAs) which are flanked by ssRNA sequences. Pri-miRNAs are recognised and processed by the Drosha/DGCR8 microprocessor complex to form Precursor miRNAs (Pre-miRNAs) which are transported from the nucleus to the cytoplasm by exportin 5. Pre-miRNAs are recognised and cleaved in the cytoplasm by Dicer to yield miRNAs. Mature miRNAs assemble into a protein complex known as the RNA induced silencing complex (RISC), which guides the effector strand to a complementary region within the 3' UTR of a mRNA target where, depending on the degree of complementarity between the miRNA and mRNA target, can mediate translational suppression, deadenylation or mRNA cleavage. Translationally repressed mRNA targets are often stored in cytoplasmic processing bodies (P-bodies).

1.3.1 Expression and processing of primary miRNAs in the nucleus

Genes encoding miRNAs are scattered throughout the genome and may be found in intergenic regions, as well as within the introns of protein-coding genes, or the introns and exons of non-coding RNAs (Kim and Nam 2006). A large proportion of miRNAs have been found in clusters, usually of two or three functionally related miRNAs, and are transcribed as polycistronic units (Yuan et al. 2009). MicroRNA genes, with a few exceptions (Borchert et al. 2006), are transcribed by RNA Pol II promoters (Cai et al. 2004; Lee et al. 2004), which are responsible for the transcription of messenger RNAs and many small nuclear RNAs. Once transcribed, the RNA folds into a hairpin-like structure consisting of an imperfectly paired stem region of approximately 33 bp with an apical loop and single stranded 5' and 3' flanking sequences. The mature miRNA guide sequence is embedded in either of the 5' or the 3' arms of the hairpin duplex. Primary miRNA (pri-miRNA)-containing transcripts may comprise several kb and the pri-miRNA hairpins are excised from the transcript within the nucleus by the RNase III enzyme Drosha, to generate a precursor miRNA (pre-miRNA) (Lee et al. 2002b; Lee et al. 2003). For this cleavage reaction to occur, Drosha associates with a co-factor known as DiGeorge syndrome critical region gene 8 (DGCR8), to form the microprocessor complex (Gregory et al. 2004; Han et al. 2004; Landthaler et al. 2004). The complex catalyses the processing of the pri-miRNA stem to yield a pre-miRNA with a ~22 bp stem (Zeng et al. 2005; Han et al. 2006) (for further information regarding Drosha-mediated cleavage, see section 1.5.1). Another less common mechanism for the generation of pre-miRNA sequences is a Drosha/DGCR8-independent mechanism, first described in *Drosophila melanogaster* and *C. elegans* (Ruby et al. 2007) and later discovered in mammals (Berezikov et al.

2007; Babiarz et al. 2008). The miRNA is encoded by an intronic sequence which, following splicing and a debranching, closely resembles the structure of a pre-miRNA. These structures are termed mirtrons and in some cases may require exonucleolytic cleavage of extended 5' or 3' flanking tails before the convergence of the canonical and mirtronic miRNA biogenesis pathways (Babiarz et al. 2008).

1.3.2 Export of precursor miRNAs from the nucleus and cytoplasmic cleavage

Once nuclear processing of the pri-miRNAs is complete, pre-miRNAs require translocation to the cytoplasm. All non-coding RNAs are dependent on the karyopherin family of nucleocytoplasmic transport factors for export from the nucleus (Cullen 2003), and Exportin-5 (Exp-5) was defined as the karyopherin responsible for the transport of pre-miRNAs (Yi et al. 2003b; Bohnsack et al. 2004; Lund et al. 2004). Exportin-5 binds directly to the pre-miRNA in the presence of guanosine triphosphate (GTP)-bound Ran (RanGTP), and this Exp-5/RanGTP/pre-miRNA heteroternary complex is transported to the cytoplasm via nuclear pore complexes. In the cytoplasm, RanGTP is hydrolysed to form RanGDP, resulting in the release of the pre-miRNA (Cullen 2003; Kim 2004). It was reported that Exp-5 may bind to the entire stem region of pre-miR-30 thus protecting this miRNA against endonuclease degradation (Zeng and Cullen 2004). This was confirmed following the recent unveiling of the crystal structure of human Exp-5 (Okada et al. 2009). Exp-5 interacts with RanGTP and this duplex folds around the sugar-phosphate backbone of the dsRNA stem region of pre-miRNAs. Because both the 5' and 3' ends of the pre-miRNA are shielded within the Exp-5/RanGTP duplex, the pre-miRNA is completely protected against intracellular degradation (Okada et al. 2009).

The second processing reaction in the biogenesis of miRNAs occurs in the cytoplasm and is mediated by the RNase III enzyme Dicer (Bernstein et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). The Piwi Argonaute Zwille (PAZ) domain of the Dicer enzyme recognises and binds the terminal 3' overhang of the miRNA duplex, and the two RNase III domains catalyse the cleavage of the duplex approximately 22-25 nt away from the PAZ/RNA interaction, resulting in the removal of the

terminal loop sequence and the generation of a mature miRNA duplex (the structure and cleavage mechanism of Dicer is comprehensively addressed in section 1.5.1). Dicer-generated RNA duplexes are generally ~22 bp and contain distinctive characteristics such as 5' phosphate and 3' hydroxyl groups, and 2 nt 3' single stranded overhangs (Elbashir et al. 2001b; Elbashir et al. 2001c). Dicer may also associate with two RNA binding proteins: TAR RNA-binding protein (TRBP) (Chendrimada et al. 2005; Haase et al. 2005; Lau et al. 2009) and the protein activator of PKR (PACT) (Lee et al. 2006), which aid in facilitating assembly and later form part of the RNA-induced silencing complex (RISC).

1.3.3 RISC loading

Despite extensive research describing the assembly of the mature RISC complex, the intricacies of RISC loading are still debated. The prevailing mechanistic view is that Dicer interacts with TRPB and Ago2 to form the minimal RISC loading complex (Gregory et al. 2005), and the crystal structure of this complex has recently been solved (Wang et al. 2009b). The formation of the RISC loading complex facilitates the transfer of the miRNA duplex from Dicer to Ago2, although ultimately only a single strand (the guide strand) is retained in the mature/holo RISC complex which may comprise additional protein factors as well. The mechanism by which the passenger strand is removed remains a point of contention. During assembly of mature RISC, Ago2, which is the only human Ago protein known to have "slicer" activity (Liu et al. 2004; Meister et al. 2004), has been shown to cleave the passenger strand of the miRNA duplex in an ATP-independent manner, resulting in its dissociation from the RISC complex and probable degradation (Matranga et al. 2005; Rand et al. 2005; Leuschner et al. 2006). However, the identification of another ATP-dependent helicase, RNA helices A (RHA), which associates with the RISC loading complex during the assembly process, supports earlier notions that the miRNA duplex is unwound in an ATP-dependent manner before the incorporation of the guide strand into mature RISC (Robb and Rana 2007). Although Ago2 is the only human Ago protein which contains intrinsic slicer activity for the cleavage of mRNA targets, the remaining Ago proteins (Ago1, Ago3 and Ago4) still form part of mature RISC complexes. To complicate matters further, current work has just shown that Ago1 is also able to cleave the passenger strand of miRNA duplexes (Wang et al.

2009a), implicating Ago1 in the duplex dissociation process. It is a possibility that slightly different loading mechanisms proceed according to the nature of the RNA duplex, however further analysis is still essential to deduce the exact mechanism by which the active guide strand is incorporated into the mature RISC complex. The guide strand is in most cases selected based on thermodynamic stability of the miRNA duplex. The strand with the more unstably paired 5' end is typically retained whilst the unselected strand is removed (Khvorova et al. 2003; Schwarz et al. 2003). In addition to thermodynamic asymmetry, certain sequence elements have been shown to also play a distinct role in strand selection bias (Hu et al. 2009). A 5' U in the selected strand; a 5' C in the eliminated strand as well as a higher purine content of the selected strand and the consequent increase in the pyrimidine content of the unselected strand may all play a role in strand selection (Hu et al. 2009). These factors must therefore be recognised by the RISC loading complex, which determines the orientation of the miRNA duplex (Wang et al. 2009b). Until recently it has been largely assumed that the eliminated passenger strand is degraded upon dissociation from its duplex precursor. It has however been shown in *Drosophila* that the passenger strand may also be loaded onto Ago proteins to mediate mRNA target specific silencing (Czech et al. 2009; Okamura et al. 2009; Ghildiyal et al. 2010). This observed phenomenon remains to be confirmed in humans but given the conservation in the mechanisms underlying the RNAi pathway, this possibility should not be disregarded.

1.3.4 The silencing mechanism

The single stranded miRNA guide strand directs the RISC complex to its complementary RNA target, usually situated within the 3'UTR of an mRNA transcript. Nucleotides at positions 2-8 at the 5' end of the guide strand are known as the "seed" region and base pairing of the seed region to an mRNA target is both essential and sufficient to induce miRNA-mediated gene silencing (Lewis et al. 2003; Brennecke et al. 2005; Lewis et al. 2005). In addition to canonical 7-mer seed regions, 8-mer and 6-mer sites have also been identified with the latter exhibiting reduced efficacy (Lewis et al. 2005; Friedman et al. 2009). Although the remaining 3' sequence of the guide strand has been deemed inconsequential, central base pairing at positions 13-17 may supplement seed pairing or compensate

for a seed mismatch, thereby contributing to target recognition (Grimson et al. 2007) [For a comprehensive review of predicted miRNA targets see (Bartel 2009)]. Silencing of gene expression is effected by two primary mechanisms depending on the degree of complementarity between the miRNA guide strand and the mRNA target. Guide strands exhibiting perfect complementarity to their cognate target mRNA execute gene repression by Ago2-mediated cleavage of the target RNA (Yekta et al. 2004). This is uncommon for many mammalian miRNAs as the majority of miRNA guide strands only display partial complementarity with their mRNA targets resulting in translational suppression and consequent gene silencing. The underlying mechanism of translational repression is still disputed; however several mechanisms have been described and include: the inhibition of translational initiation (Kiriakidou et al. 2007; Mathonnet et al. 2007); elongation blockage or the dissociation of ribosomes (Petersen et al. 2006); co-translational degradation of the nascent polypeptide (Nottrott et al. 2006); or deadenylation resulting in decapping and the consequent degradation of the mRNA transcript (Wakiyama et al. 2007; Fabian et al. 2009) [reviewed in (Eulalio et al. 2008; Filipowicz et al. 2008; Chekulaeva and Filipowicz 2009)]. Furthermore, together with Ago and GW182 proteins, the miRNA guide strand-mRNA duplex may be sequestered into cytoplasmic foci known as processing bodies (P-bodies) which play a role in the degradation and storage of translationally repressed mRNAs (Liu et al. 2005; Parker and Sheth 2007) (Figure 1.1). It is intriguing to note that translational suppression of miRNAs may be a reversible process. Repression of CAT-1 mRNA by miR-122 in hepatoma cells was observed to be reversed under conditions of stress, and this derepression was accompanied by a release of repressed mRNA from P-bodies for active translation (Bhattacharyya et al. 2006).

1.4 Additional RNAi-related pathways in humans

Small interfering RNAs (siRNAs) represent a second class of small RNAs and these RNA duplexes closely resemble the structure of mature miRNAs. In contrast to the miRNA biogenesis pathway, siRNA biogenesis is Drosha independent. Double-stranded RNA transcripts are processed by Dicer in the cytoplasm to yield siRNAs. The siRNA/Dicer/TRBP complex is loaded onto Ago proteins and since the guide strand typically exhibits perfect complementarity with its mRNA target,

Ago2 cleaves the mRNA which is subsequently degraded. siRNAs were initially thought to be derived from Dicer-mediated cleavage of only exogenous dsRNA precursors such as viruses and transgene transcripts. More recently however, endo-siRNAs have been identified which are generated from endogenous sources such as convergent mRNA transcripts, natural sense-antisense duplexes, pseudogenes and transposons (Babiarz et al. 2008; Tam et al. 2008; Watanabe et al. 2008). The biological role of endo-siRNAs has yet to be elucidated [reviewed in (Ghildiyal and Zamore 2009; Kim et al. 2009)].

The class of small RNAs most recently identified is that of Piwi-interacting RNAs (piRNAs), which are derived from repeat sequences in the genome and function primarily in germ line cells [reviewed in (Ghildiyal and Zamore 2009; Kim et al. 2009)]. The biogenesis as well as the function of piRNAs remain somewhat unclear although piRNAs, which are distinctly longer than both miRNAs and siRNAs, appear to originate from single stranded precursors through a Drosha and Dicer independent mechanism (Vagin et al. 2006). As the name suggests, this class of small RNA associates with the PIWI subfamily of Ago proteins and has been shown to cause transcriptional silencing of transposons by establishing *de novo* DNA methylation in murine fetal testes (Aravin et al. 2007; Aravin et al. 2008; Kuramochi-Miyagawa et al. 2008).

Transcriptional gene silencing (TGS) is an RNAi-related pathway originally described in plants (Wassenegger et al. 1994; Mette et al. 2000) and later in fission yeast (Volpe et al. 2002) and Drosophila (Pal-Bhadra et al. 2002), whereby gene silencing is effected by epigenetic changes at the promoter region. Synthetic promoter-targeted small RNAs have since been used to induce TGS in human cells and although the underlying mechanism of TGS in mammalian cells remains unclear, the effect is largely mediated by DNA methylation at the promoter and methylation of histone 3 lysine 9 and 27 (Morris et al. 2004a; Castanotto et al. 2005; Weinberg et al. 2006). Interestingly, Ago1 and Ago2 (Janowski et al. 2006; Kim et al. 2006) as well as Dicer (Ting et al. 2008; Tan et al. 2009) have been implicated in the TGS pathway. In addition to exogenous small RNA-mediated TGS, it has been shown that endogenous miRNAs may be encoded by promoter regions and may mediate epigenetic changes and TGS of the downstream gene (Kim et al. 2008; Tan et al. 2009).

1.5 Key proteins for human RNAi pathways

Despite the existence of distinct classes of small RNAs which differ in both origin and function, their biogenesis pathways show some intersection not only with each other but with RNAi-related pathways, all of which share a common requirement for certain key RNAi proteins. These proteins include the argonaute proteins and in some cases, the RNase III enzymes Dicer and/or Drosha.

1.5.1 RNase III enzymes

Drosha and Dicer belong to the RNase III family of proteins and are both critical in the biogenesis of certain small RNAs. RNase III proteins are endonucleases which specifically catalyse the hydrolysis of dsRNA. The first RNase III enzyme was described in *E. coli* (Robertson et al. 1968) but has since been found ubiquitously in eukaryotes. Both Dicer and Drosha share a common double-stranded RNA-binding domain (dsRBD) as well as two ribonuclease (RNase III) domains which contain the catalytic sites necessary for cleavage of each of the RNA strands (Figure 1.2). In addition to the dsRBD and RNase III domains, Dicer also contains an N terminal helicase domain; a domain of unknown function (DUF 283) and a PAZ domain which is shared by Ago proteins (Figure 1.2).

Dicer is a crucial enzyme for the generation of mature RNAi effectors and a clear understanding of the mechanism of Dicer-mediated cleavage is fundamental for the design of RNAi-based therapeutic precursors, from which one or more effectors are derived in a Dicer-dependent manner. A model by which Dicer functions has in recent years been gradually pieced together. Three concurrent structural studies determined the three-dimensional crystal structures of the PAZ domains of *Drosophila* Ago 1 (Yan et al. 2003) and Ago 2 (Lingel et al. 2003; Song et al. 2003). The structure of the PAZ domain revealed a unique fold, exhibiting a similar topology to the folds found in oligonucleotide / oligosaccharide-binding (OB) proteins, which enable this family of proteins to bind single stranded nucleic acids. The binding surface of this PAZ domain fold was found to be conserved between Ago and Dicer PAZ domains and furthermore, positively charged residues residing on this surface suggested the possibility of binding negatively charged ligands such as nucleic acids.

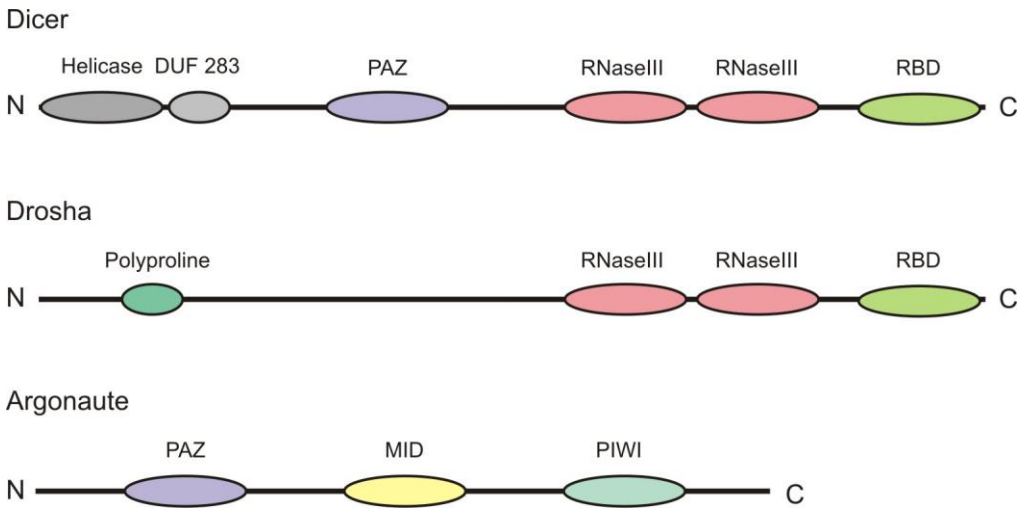


Figure 1.2: Schematic representation of the functional domains of the human RNase III enzymes Dicer and Drosha as well as of the Argonaute proteins. Human Dicer consists of N terminal helicase and DUF 283 domains, a PAZ domain, two RNase III domains and a dsRNA binding domain. Drosha also contains two RNase III domains and a dsRNA binding domain and in addition an N terminal proline rich region. Argonaute proteins share a PAZ domain with Dicer and also contain a MID and a PIWI domain.

Additional assays observed low affinity binding between the PAZ domain and single stranded RNA overhangs (Lingel et al. 2003; Song et al. 2003; Yan et al. 2003). The crystal structure of human Ago bound to a 9 bp RNA duplex confirmed that the PAZ domain of Ago and Dicer proteins contains a highly conserved RNA binding pocket which serves as the critical factor responsible for binding the 2 nt 3' overhang of siRNAs (Ma et al. 2004) (Figure 1.3). The conservation of the PAZ domain between Dicer and Ago proteins therefore makes sense, since both of these proteins recognise the 3' terminal structure of siRNAs/siRNA precursors for specific incorporation into the RNAi pathway. The interaction between PAZ and dsRNA is highly specific and the terminal structure as well as the sequence of the siRNA ends have a distinct effect on the position and efficiency of Dicer cleavage (Vermeulen et al. 2005). Terminal overhangs longer than 3 nt dramatically decreased the efficiency of Dicer processing and the overhang sequence, to a lesser extent, also affected Dicer processing with a CA overhang resulting in the most efficient cleavage and an AU overhang resulting in the poorest.

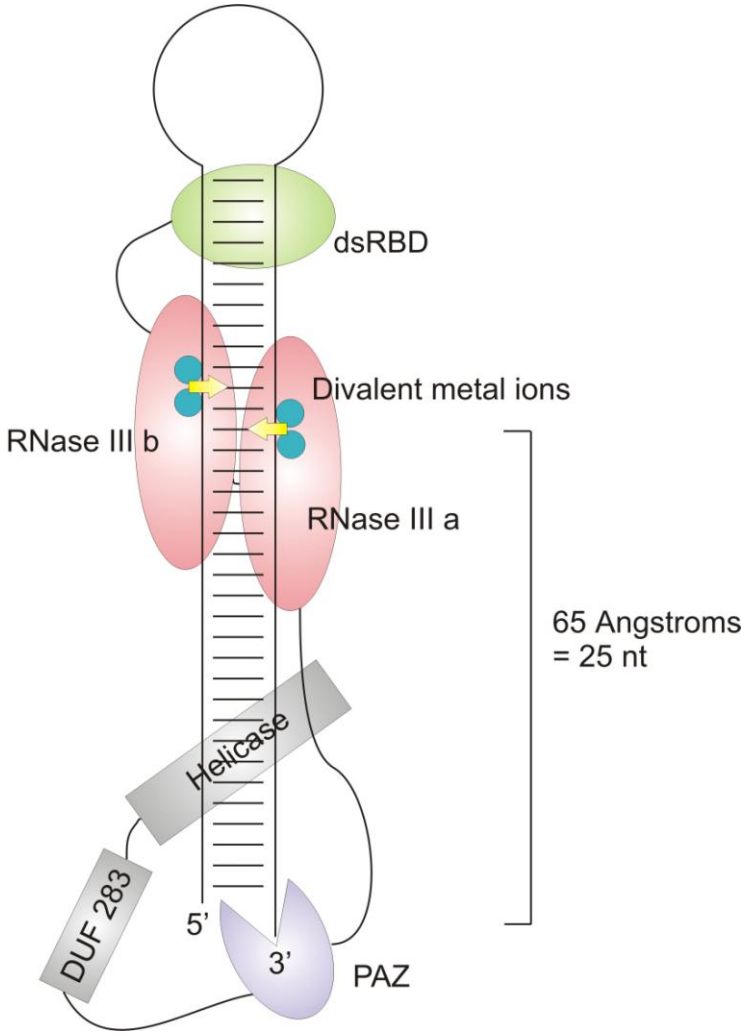


Figure 1.3: Schematic illustration of the proposed mechanism by which the functional domains of human Dicer interact with dsRNA. The PAZ domain recognises and binds to the 3' single stranded overhang and indirectly positions the two RNase III domains at a point along the RNA duplex approximately 25 nt away from the PAZ RNA binding pocket. The catalytic sites within each RNase III domain contain cation binding sites and the yellow arrows indicate approximate cleavage positions. The N terminal helicase domain is thought to position itself around the alpha helix which connects the PAZ and RNase III a domains.

The PAZ domain is connected to the two ribonuclease domains, termed RNase III a and b. Zhang et al. proposed a model in which the two RNase III domains form an intramolecular dimer, resulting in a single processing centre containing two catalytic sites which function independently of one another to each cleave one strand of the dsRNA duplex (Zhang et al. 2004) (Figure 1.3). This model was later confirmed by two studies which analysed the crystal structure of the intact *Giardia*

intestinalis Dicer (Macrae et al. 2006) and the *Aquifex aeolicus* RNase III (Gan et al. 2006). The Giardia Dicer enzyme contains a PAZ domain and two RNase III domains but lacks any N terminal or C terminal domains; however it is still capable of robust dicing activity producing ~25 nt products *in vitro*. The crystal structure of the intact enzyme showed that the PAZ domain shared a conserved 2 nt 3' RNA binding fold seen in the PAZ domains of Drosophila Ago 1 and 2. The PAZ domain is directly connected to the RNase III a domain by a long alpha helix which is surrounded by N terminal residues. The two RNase III domains are linked to form the internal heterodimer described by Zhang et al. Each catalytic site contains two cation binding sites to which divalent metal cations, necessary for hydrolysis, may bind (Figure 1.3). The length between the PAZ-RNA binding fold and the active site within RNase III was measured to be 65 Angstroms which is equivalent to 25 nt, corresponding to the length of *Giardia* Dicer products. This length, which is determined largely by the length of the connector alpha helix, is responsible for generating Dicer cleavage products which are consistently uniform in size (Macrae et al. 2006). Human Dicer cleavage products are slightly shorter (~21-23 nt), and this suggests that the alpha helix in human Dicer may also be slightly shorter.

Cleavage of dsRNA by these RNase III proteins therefore results in well defined products with distinct termini including a 5' monophosphate and a 2 nt 3' overhang, [reviewed in (MacRae and Doudna 2007)]. As mentioned previously, the N terminal of human Dicer consists of DExD/H-box helicase domain and the DUF 283 domain. The function of the DUF 283 domain is as yet unknown. It was proposed that this domain may bind dsRNA (Dlakic 2006), yet this notion was later disputed (Ma et al. 2008). The helicase domain contains binding sites for the direct interaction of Dicer with its accessory proteins: TRBP (Chendrimada et al. 2005; Haase et al. 2005) and PACT (Lee et al. 2006). Furthermore this domain was recently implicated in the ability of Dicer to process thermodynamically unstable RNA stems and in the multiple turnover kinetics of Dicer (Ma et al. 2008; Soifer et al. 2008) (discussed further in section 2.4). Although the mechanism by which Dicer mediates cleavage of dsRNA substrates has been largely elucidated, much work is still required to characterise optimal Dicer substrates and the ability of human Dicer to effectively process the structures of various RNAi effector mimics to yield single or multiple RNAi effectors. Drosha cleavage requires the action of a co-factor

known as DGCR8 which contains a proline binding domain and thus interacts directly with the polyproline domain of Drosha (Gregory et al. 2004).

Two mechanisms for Drosha-mediated cleavage have been proposed. Zeng et al. proposed a model whereby the microprocessor complex binds to the loop sequence of the pri-miRNA and Drosha subsequently cleaves the stem ~22 nt from the stem/loop junction (Zeng et al. 2005). In a conflicting model proposed by Han et al. DGCR8 is responsible for recognizing and binding to the junction between the single stranded flanking RNA sequences and the double-stranded stem region of the pri-miRNA. Drosha then catalyses the processing of the dsRNA stem 11 bp upstream from this junction. Both processing models require the presence of ~40 nt single stranded RNA flanking sequences for efficient Drosha-mediated cleavage (Han et al. 2006), and result in pre-miRNAs with stem lengths ~22 bp. The RNase III family of enzymes is responsible for the cleavage of a multitude of dsRNA substrates into functional small RNAs with unique biological roles. From their respective cleavage mechanisms described above, it is evident that the specificity of the Dicer and Drosha enzymes is conferred by their accessory domains (PAZ and polyproline domains respectively), which indirectly mediate the specific positioning of the relevant RNase III domains for precise cleavage of their substrate RNA.

1.5.2 Argonaute proteins

The Argonaute (Ago) proteins constitute the core component of the RNA induced silencing complex (RISC) and therefore play an indispensable role in the RNAi pathway [reviewed in (Hutvagner and Simard 2008)]. These proteins generally consist of three domains: a PAZ domain, MID domain and a PIWI domain (Figure 1.2). The PAZ domain, as previously mentioned, is shared by the Dicer enzyme and binds to the characteristic 2 nt 3' end of small Dicer-generated RNAs (Ma et al. 2004). The MID domain binds to the 5' phosphate of the small RNA. Additionally, the MID domain contains an MC motif, similar to the cap structure binding motif of the translation initiation factor eIF4E, and this MC motif has been shown to be able to bind to the cap of mRNA species and modulate translation

(Kiriakidou et al. 2007). The PIWI domain is located at the C-terminus of Ago proteins, and in some cleavage-competent Ago proteins such as mammalian Ago2, harbours a catalytic site for the endonucleolytic cleavage of the bound RNA (Liu et al. 2004; Meister et al. 2004). The Ago proteins are divided into two subfamilies which differ in the cells in which they are expressed and hence the small RNAs they associate with. The Ago subfamily of proteins is expressed ubiquitously and associate primarily with miRNAs and siRNAs whereas the PIWI subfamily of Ago proteins are expressed mainly in the germ cells and associate with piRNAs. The number of Ago proteins that are present varies between species, and in humans there are 4 Ago proteins and 4 PIWI proteins.

1.6 RNAi as a therapeutic

Since its discovery, the RNAi pathway has been widely exploited as a new tool in the development of novel therapeutics. The high specificity required for this gene silencing system provides an ideal framework to target disease causing genetic agents, and there are currently a number of clinical trials underway which harness different RNAi effector mimics to treat a variety of diseases including inherited disorders, viral infections such as HIV, hepatitis B virus (HBV) and hepatitis C virus (HCV) as well as different cancers [reviewed in (Castanotto and Rossi 2009)]. Mimics of intermediate RNAi effector molecules may be introduced at various stages of the miRNA/siRNA biogenesis pathways for gene specific silencing (Figure 1.4). These mimics are engineered to contain guide sequences perfectly complementary to gene targets of interest in order to mediate mRNA degradation.

1.6.1 Synthetic siRNA sequences

The most commonly administered RNAi effector mimics are chemically synthesised siRNAs which are typically 21mer RNA duplexes designed to mimic endogenous Dicer cleavage products. The guide strand of siRNAs is typically designed to be completely complementary to an mRNA target and thus results in Ago2-mediated mRNA cleavage (Figure 1.4). siRNA duplexes were first introduced into

Drosophila cells where they were shown to mediate target RNA degradation and this was the first demonstration that siRNA-mediated gene silencing could be uncoupled from the RNase III cleavage reactions of siRNA biogenesis (Elbashir et al. 2001b). Soon thereafter, exogenously introduced synthetic siRNAs were introduced into the cytoplasm of mammalian cell lines where they entered RISC and directed the cleavage and degradation of specific mRNA sequences (Elbashir et al. 2001a). The use of synthetic siRNAs offers a powerful tool for sequence specific gene silencing and these have been widely used to study gene function as well as in the development of potential therapeutics aimed at silencing disease associated RNA transcripts. Chemically synthesised siRNAs with longer duplexes (~27 bp) may act as Dicer substrates and have been shown to elicit a stronger gene silencing effect than conventional 21mer siRNAs, possibly because Dicer-generated products are incorporated into RISC more efficiently (Kim et al. 2005).

The chemical synthesis of siRNAs allows for the incorporation of certain chemical modifications within the duplex which may offer several advantages for the stability and efficacy of exogenously introduced siRNAs [reviewed in (Behlke 2008)]. Synthetic siRNAs are subject to degradation by serum nucleases in a cellular environment and therefore require chemical modifications for cellular stability. Nuclease protection, which does not significantly hamper the RNAi activity of the siRNA, may be increased by an array of intrinsic modifications which include the limited incorporation of: phosphorothioate linkages within backbone of the passenger (sense) or guide (antisense) strands (Chiu and Rana 2003; Choung et al. 2006), locked nucleic acids (LNA) (Elmen et al. 2005; Mook et al. 2007), or DNA bases (Hogrefe et al. 2006; Ui-Tei et al. 2008b). Another common means of increasing nuclease resistance is by introducing stabilizing moieties to the 2' position of the ribose such as 2'-O-methyl (Czauderna et al. 2003a; Morrissey et al. 2005; Choung et al. 2006), 2'-fluoro (Morrissey et al. 2005), or 2'-fluoro-β-D-arabinonucleotide (FANA) (Dowler et al. 2006) modifications. In addition to providing nuclease protection to siRNAs, 2'-O-methyl modifications as well as the substitution of DNA bases within the duplex have also been shown to significantly repress activation of an immune response (Judge et al. 2006; Robbins et al. 2007) (section 1.7.1). The strategic placement of 2'-O-methyl modifications and DNA bases may also served to reduce off target effects (OTEs) (section 1.7.2). More specifically, the addition of a 2'-O-methyl group to the 5' end of the passenger strand

blocks its incorporation into RISC thus preventing any OTEs caused by the unintentional incorporation of the passenger strand into the RNAi pathway (Chen et al. 2008). The substitution of the seed region of the guide strand with DNA bases allows for siRNA efficacy to be retained, whilst it prevents OTEs caused by seed region base pairing with miRNA target sites (Ui-Tei et al. 2008b). Finally, the conjugation of steroid or lipid groups to the siRNA duplex has the potential ability to enhance their cellular uptake (Lorenz et al. 2004; Soutschek et al. 2004), and other ligands such as antibody fragments (Song et al. 2005; Kumar et al. 2008) or aptamers (Chu et al. 2006; McNamara et al. 2006; Zhou et al. 2008b) may enable cell-specific siRNA uptake. The introduction of single or combinations of the modifications described above may therefore offer distinct advantages over expressed RNAi effectors, without impairing the efficacy and in some cases even enhancing the potency of the siRNA. The placement of introduced modifications should however be carefully considered as the position of such modifications within the sense or antisense strands may negatively or positively affect the RNAi activity of the siRNA (Czauderna et al. 2003a; Prakash et al. 2005).

The use of siRNAs can induce potent gene inhibition in a therapeutic context, especially when the targeted genes are confined to tissues amenable to topical or localized delivery. In support of this, clinical trials are already underway which use intravitreal injection of siRNAs targeting the vascular endothelial growth factor (*VEGF*) for the treatment of wet age-related macular degeneration (Shen et al. 2006; Singerman 2009). Furthermore, aerosolized siRNAs targeted against the respiratory syncytial virus (RSV) can be administered intranasally by inhalation and are also currently undergoing clinical trials (DeVincenzo et al. 2008). Systemic delivery of siRNA-based therapeutics is slightly more complicated and a number of obstacles have to be considered and overcome [reviewed in (Tseng et al. 2009)]. Although chemical modifications to siRNAs may increase their stability and biodistribution, the effects of exogenously introduced siRNAs are transient and targeted genes will regain expression within relatively short periods of time (Bartlett and Davis 2006). In a therapeutic setting, repeated administration will thus be necessary for sustained inhibition of gene expression. This may be feasible for the treatment of acute infections caused by viruses such as RSV, but is not practical for the treatment of chronic diseases such as acquired immunodeficiency disease (AIDS) caused by HIV, for which a more stable RNAi-based treatment modality is ideal.

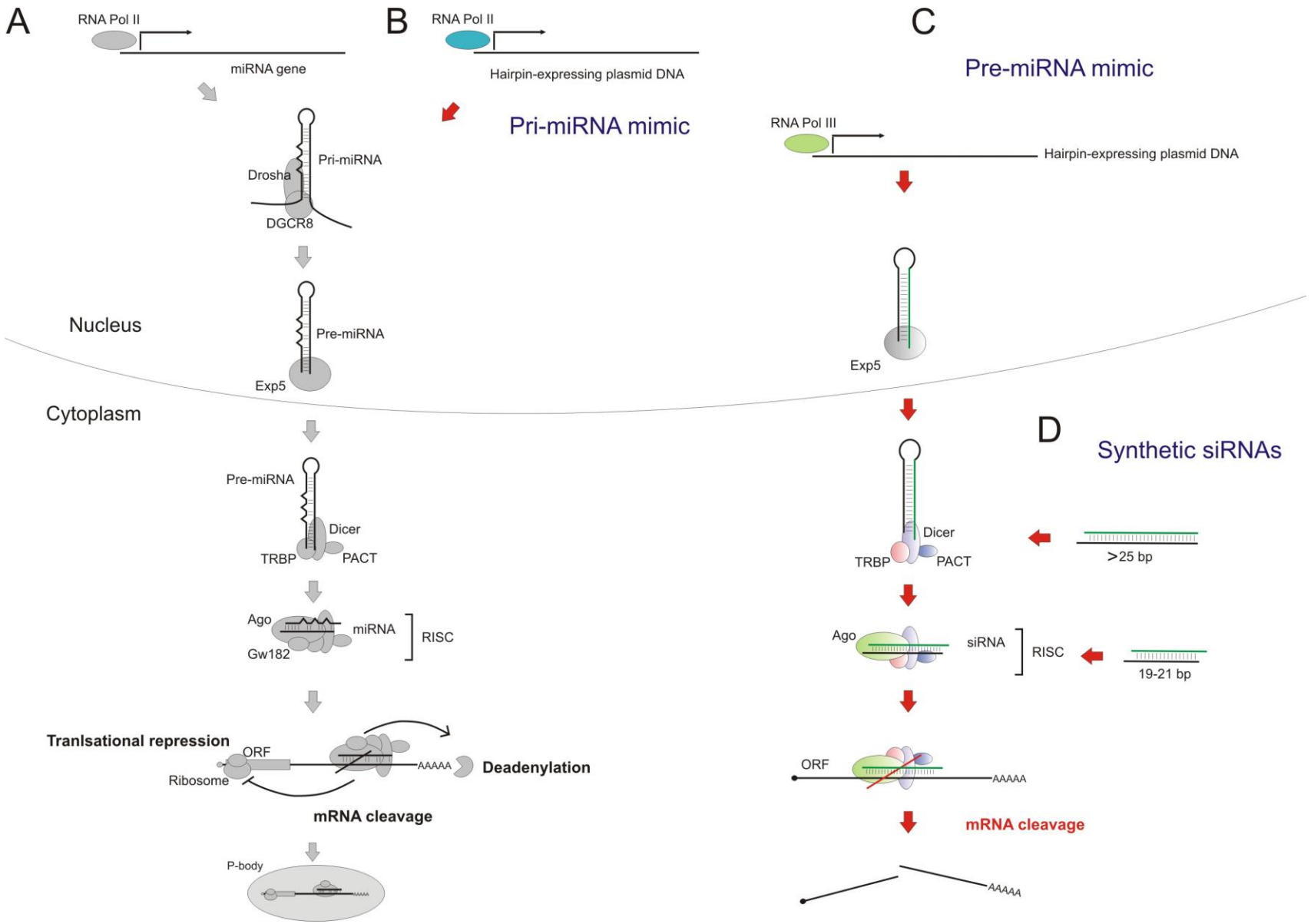

A
RNA Pol II
miRNA gene
Drosha
Pri-miRNA
DGCR8
Pre-miRNA
Exp5
Nucleus
Cytoplasm
Pre-miRNA
Dicer
TRBP
PACT
Ago
miRNA
Gw182
RISC
Tranlsational repression
ORF
Ribosome
AAAAA
Deadenylation
mRNA cleavage
P-body
B
RNA Pol II
Hairpin-expressing plasmid DNA
Pri-miRNA mimic
C
Pre-miRNA mimic
RNA Pol III
Hairpin-expressing plasmid DNA
Exp5
Dicer
TRBP
PACT
D
Synthetic siRNAs
>25 bp
Ago
siRNA
RISC
19-21 bp
ORF
AAAAA
mRNA cleavage
AAAAA

Figure 1.4: The introduction of miRNA precursor mimics into the miRNA biogenesis pathway as therapeutic modalities. Mimics may enter the pathway and exploit the endogenous RNAi machinery at various stages of the natural miRNA biogenesis pathway. **(A)** The natural endogenous miRNA biogenesis pathway (see Figure 1.1 for details). **(B)** Pri-miRNA mimics are expressed in the nucleus from DNA vectors and enter the canonical miRNA biogenesis pathway at the Drosha cleavage step. **(C)** shRNA or lhRNA expression cassettes are introduced into the nucleus and the inverted repeat sequences encoding the hairpins are generally transcribed by RNA Pol III promoters. Following transcription, the RNA folds into a stem loop structure resembling that of pre-miRNAs and is exported to the cytoplasm and processed by Dicer to form siRNAs which are incorporated into RISC. The guide strands of siRNAs are generally designed to be completely complementary to a target gene of interest, and Ago2 therefore mediates the cleavage of cognate mRNA species. **(D)** Synthetic siRNAs may be introduced into the cytoplasm of cells and depending on their length, may act as Dicer substrates bypass Dicer cleavage and enter directly into RISC.

1.6.2 Expressed RNAi activators

Pre-miRNA mimics

In contrast to chemically synthesised siRNAs, RNAi effector mimics may be introduced into the nucleus within a DNA vector for intracellular expression. RNA effector sequences are encoded within expressed transcripts as short inverted repeats that fold into stem loop structures which closely mimic the structure of endogenous pre-miRNAs and have been termed short hairpin RNAs (shRNAs) (Figure 1.4). Brummelkamp et al. first investigated the possibility of endogenously expressing RNAi effector sequences within a short hairpin structure. The RNA Pol III H1 promoter was used to drive expression of an shRNA encoding 19 nt guide and passenger strands separated by a loop sequence of 5, 7 or 9 nt, for the effective inhibition of the endogenous CDH1 gene (Brummelkamp et al. 2002). The RNA Pol III U6 snRNA promoter was subsequently used to express a similar shRNA structure targeted against the human lamin A/C gene (Paul et al. 2002). RNA Pol III promoters such as U6 or H1 are now routinely used to drive expression of shRNA sequences since they naturally express small RNAs endogenously and result in high levels of constitutively expressed small transcripts. The promoter elements for this class of RNA Pol III promoters are generally upstream from the transcription start site, allowing for transcription to occur at a purine residue in position 1. Pol III driven sequences therefore have a clear transcription start site and a termination signal consisting of ≥4 thymidine residues, thus

producing transcripts with well defined 5' and 3' termini. Furthermore, the resulting 3' 2-3 nt uridine overhang of Pol III-generated transcripts is analogous to the 3' overhangs characteristic of RNase III processed products, and therefore enables efficient export from the nucleus and allows shRNAs to be recognised in the cytoplasm as Dicer substrates. Dicer cleavage of shRNAs >19 bp yields a single mature siRNA which enters into RISC and guides the Ago-mediated target mRNA cleavage as previously described (Siolas et al. 2005) (Figure 1.4).

Ectopically expressed shRNAs have the potential to mediate gene silencing for longer periods than synthetic siRNAs since they are continually expressed, resulting in continual replenishment of RNA effector sequences. For this reason shRNAs are effective at a much lower dose than siRNAs and moreover, the assimilation of shRNAs into the endogenous RNAi pathway allows for more efficient RISC loading, further contributing to their enhanced efficacy (Rao et al. 2009). These RNAi precursor mimics have become powerful tools for the development of novel RNAi-based therapeutics to treat a variety of diseases. However shRNAs bear stems of ~22 bp and are thus able to generate only a single siRNA duplex following cleavage. Although therapeutically viable for the treatment of conserved pathological genes, a single therapeutic modality may not be sufficient for repression of rapidly mutating genes characteristic of viruses such as HIV and HCV (The emergence of viral escape mutants is discussed further in section 1.9.2).

Pri-miRNA mimics

A further drawback to the use of RNA Pol III expressed shRNAs is the potential for their constitutive expression at very high levels to result in unwanted cellular toxicities due to specific and non-specific off-target effects (section 1.7). RNA Pol II promoters drive the expression of most endogenous miRNAs and result in longer transcripts containing a 5' cap and poly (A) tail. In addition, Pol II promoters are advantageous for the expression of RNAi effector mimics due to their ability to drive inducible (Dickins et al. 2007; Wang et al. 2007; Yang and Paschen 2008) and tissue-specific (Nielsen et al. 2009; Rhee et al. 2009) expression. Exploitation of the properties of natural pri-miRNA transcripts for the delivery of therapeutic guide strands has therefore recently gained substantial

attention. Guide sequences of choice have been successfully incorporated into expression cassettes that encode mimics of natural pri-miRNAs for specific gene inhibition (McManus et al. 2002; Zeng et al. 2002; Ely et al. 2008; Ely et al. 2009). The maintenance of the parental miRNA structure is crucial for the downstream processing and efficacy of the guide strand (McManus et al. 2002; Zhou et al. 2005). The modified hairpin stem is therefore flanked by ~40 nt ssRNA on 5' and 3' ends, and the original loop sequence and symmetrical/asymmetrical mismatches are retained within the stem. Pri-miRNA mimics are Drosha substrates and unlike shRNAs, this upstream processing event is hypothesized to restrict over-production of pre-miRNAs thus preventing saturation of downstream pathway components. In comparative studies between shRNAs and pri-miRNA mimics (artificial miRNAs), shRNAs were shown to be substantially more potent than miRNAs, albeit due to their elevated expression levels (Boudreau et al. 2008). However, highly expressed shRNAs were also shown to cause saturation of the endogenous miRNA pathway and caused neurotoxicity whereas artificial miRNAs at the same effective dose caused no symptoms of saturation or toxicity (McBride et al. 2008; Boudreau et al. 2009) (further information regarding cellular toxicity caused by saturation effects is provided in section 1.7.3). To date, cellular toxicity as well as saturation of the RNAi pathway have not been caused by ectopic expression of pri-miRNA mimics (Ely et al. 2008; Ely et al. 2009; Keck et al. 2009). However, a limited number of miRNA 'scaffolds' have been studied for their abilities to effectively deliver therapeutic guide strands and furthermore, the contribution of the selected miRNA backbone to processing and silencing efficacies of the incorporated guide strands has yet to be determined.

1.6.3 Effective design of siRNAs and shRNAs

Regardless of the structure in which guide strands are introduced into target cells, certain parameters exist which define an effective guide strand. It is clearly evident from the large number of studies employing RNAi effector sequences that not all siRNAs, shRNAs, or miRNAs are equally effective. This emphasizes the need for careful planning which takes into account these factors which are integral for the design of guide sequences incorporated into RNAi effector precursors [reviewed in (Pei and Tuschl 2006; Hajeri and Singh 2009)]. There is currently a vast array of computational design

tools and algorithms in existence for the prediction of effective guide strands, many of which also aim to prevent potential off target effects. Although very helpful, none of these algorithms is infallible and the predicted guide strands still require validation experimentally. Outlined below are some of the common fundamental factors which should be considered when designing RNAi precursor mimics.

An asymmetrical duplex is key for the incorporation of the guide strand into RISC. This entails sequence asymmetry and the ensuing thermodynamic asymmetry, and the design should take into account that generally the strand with the more thermodynamically unstable 5' end is retained as the guide strand (Khvorova et al. 2003; Schwarz et al. 2003; Bramsen et al. 2009). Duplex stability should also be considered to allow for the passenger strand to be easily removed from the duplex, but simultaneously allow for stringent binding to the mRNA target to occur, and generally a guide strand with a GC content of 30-50% is optimal (Reynolds et al. 2004). Guide sequences should contain a 5' phosphate and a 2 nt 3' overhang, characteristic of Dicer products, for efficient incorporation into RISC. Furthermore the 3' overhang may be chemically modified for the enhanced or impaired selection into RISC of the guide and passenger strands respectively (Bramsen et al. 2009).

G:U mismatches may be introduced into the sense strand of dsRNA duplexes to facilitate propagation in *E.coli* and to aid in sequencing of long inverted repeat sequences (Akashi et al. 2005). Targets with strong secondary structures should be avoided, to permit accessibility of the guide stand to the miRNA target site (Overhoff et al. 2005; Patzel et al. 2005; Schubert et al. 2005). Finally, certain sequences at specified positions within the guide strand have been reported to potentially augment their silencing efficacy. Although some of these reports contradict one another, sequence preferences within the guide strand for which there is a consensus are briefly mentioned. Sequences which result in a thermodynamically unstable 5' end include a U or A residue at position 1 and a high A/U content in positions 1-7. In addition, an A or U base at position 10 decreases the stability within the duplex at the point of Ago2-mediated cleavage of both the passenger strand and the mRNA target (Reynolds et al. 2004; Takasaki et al. 2004; Jagla et al. 2005; Shabalina et al. 2006).

1.7 Safety concerns of RNAi-based therapeutics

The initial excitement following the discovery of the RNAi pathway and the rush to deliver RNAi-based therapeutics from bench to bedside has been met with some unforeseen challenges. Observations of non-specific gene silencing and cellular toxicities caused by the introduction of exogenous RNAi activators into the cellular environment have necessitated the reevaluation of the sequence, structure and delivery of RNAi activators, as well as the underlying molecular networks causing such non-specific effects.

1.7.1 Non-specific induction of the type-1 interferon response

Before the potential clinical applications of RNAi can be realised, crucial steps to avoid the induction of the innate immune response must be taken. Both siRNAs and shRNAs may be recognised intracellularly as foreign agents by different immune receptors present in the cytoplasm and in endosomal compartments, thus eliciting a non-specific type I interferon (IFN) response resulting in toxic side effects. Advances in our understanding of the mechanism by which siRNA and shRNA sequences/structures trigger such a response have allowed for the safer design of RNAi effectors capable of evasion of activators of the type 1 interferon response [reviewed in (Olejniczak et al.)].

The first cytoplasmic activator of the immune response to be identified was protein kinase R (PKR) (Roberts et al. 1976) which recognises long dsRNA generally 30-80 bp in length (Lemaire et al. 2008). Upon binding to dsRNA, PKR is autophosphorylated which led to the subsequent phosphorylation of the α subunit of the translation initiation factor eIF2, resulting in the global repression of protein translation (Dey et al. 2005). Oligoadenylate synthetase (OAS) is another cytoplasmic sensor of foreign agents which recognises long dsRNA 79-80 bp in length, to which the outcome is also a universal decrease in protein synthesis (Zilberstein et al. 1978). For this reason long dsRNAs (>30 bp) serving as precursors for siRNA production were considered unsafe due to their immunostimulatory potential and the use of shorter siRNA duplexes was therefore thought to represent a safer option.

Synthetic siRNAs however, were found to induce the IFN pathway by activating cytoplasmic retinoic acid inducible gene 1 (RIG-1) (Yoneyama et al. 2004), which activates a signaling cascade resulting in IFN and cytokine production. Upon further investigation it was found that only blunt-ended siRNAs activated RIG-1 whereas siRNAs designed to include 2 nt 3' overhangs, which are signature markers of endogenous Dicer cleavage products, bound but did not activate RIG-1 (Marques et al. 2006). RIG-1 also recognises 5' triphosphate groups which are found on many viral transcripts and thus should be excluded from the design of RNAi effectors (Hornung et al. 2006).

Furthermore, the mode of delivery of siRNAs may also play a role in immune activation. Synthetic siRNAs with or without 3' overhangs which are delivered by lipid complexes, are incorporated into endosomes where they are also capable of inducing an IFN response by activating toll-like receptors 3, 7 an 8 (TLR 3/7/8) which are localized within endosomal compartments (Robbins et al. 2006). TLR 7 and TLR 8 recognise certain G/U-rich immunostimulatory sequence motifs which include 5'-UGUGU-3' (Judge et al. 2005) and 5'-GUCCUUCAA-3' (Hornung et al. 2005). Although the absence of these sequence motifs does not guarantee the prevention of an immune response, every effort should be made to exclude these sequences from siRNA duplexes. As mentioned above (section 1.6.1), the inclusion of 2'-O-methyl groups at one or more bases within the siRNA duplex alleviates the immune activation by preventing activation of both TLR 7 and TLR 8 (Judge et al. 2006; Robbins et al. 2007). TLR 3, like TLR 7 and TLR 8, recognises foreign RNA and activates certain transcription factors which then promote the expression of pro-inflammatory cytokines (Sen and Sarkar 2005). However the activation of TLR 3 is sequence independent and 2'-O-methyl modified siRNAs do not prevent TLR 3 recognition. siRNAs introduced into the cell by electroporation, or derived from intracellularly expressed shRNAs, do not traverse the endosomal compartment and may therefore reduce the risk of immune stimulation through activation of toll-like receptors. Importantly, when synthetic siRNAs and expressed shRNAs, bearing the same duplex sequence, were compared for their immunostimulatory capacity in CD34+ derived immune cells including monocytes and dendritic cells, siRNAs induced a strong immune response whereas expressed shRNAs abrogated any immunostimulatory effects, thus providing a safer platform for gene specific knockdown (Robbins et al. 2006).

1.7.2 Sequence-specific off-target effects (OTEs)

Another concern for the therapeutic use of RNAi was raised when microarray profiling data showed off-target silencing of multiple genes in cells in which siRNAs had been introduced (Jackson et al. 2003; Scacheri et al. 2004). Potential off-target effects were originally predicted by looking for target matches in the genome complementary to the entire siRNA guide strand, however it was later proved that only partial complementarity of the guide sequence with endogenous cellular genes was required for off-target gene silencing (Lewis et al. 2005; Birmingham et al. 2006; Jackson et al. 2006). More specifically, base pairing between the seed region at position 2-8 of the 5' end of the guide strand, and the 3'UTR of an endogenous gene is sufficient to mediate the unintended translational suppression of that gene by mechanisms similar to those used by miRNAs. Measures to successfully prevent such off target effects have included chemical modifications to the duplex (section 1.6.1) as well as inclusion of a G:U wobble base pair in the seed region (Ui-Tei et al. 2008a). Prior to pre-clinical and clinical studies using siRNAs however, high throughput screening using predictive algorithms as well as microarrays and proteomic arrays (Selbach et al. 2008) should be executed, and potent siRNAs should be identified experimentally to minimize the effective dose required.

1.7.3 Saturation of the endogenous miRNA biogenesis pathway

Exogenously introduced RNAi precursors require, to varying extents, the endogenous RNAi machinery for their biogenesis and effect. Competition with natural miRNA precursors for certain vital components of the pathway may therefore ensue, leading to saturation of the endogenous miRNA biogenesis pathway and a subsequent disruption of natural miRNA function. An extreme case of cellular toxicity was observed in the livers of mice, following the administration of high doses of U6-expressed shRNAs, ultimately resulting in death (Grimm et al. 2006). The origin of such toxicity was found be the saturation of the exportin-5 karyopherin necessary for nuclear to cytoplasmic transport. Highly expressed shRNAs driven from the U6 promoter were also shown to cause severe toxicity in lentiviral-transduced human lymphocytes (An et al. 2006) as well as in the striata (McBride et al. 2008)

and cerebella (Boudreau et al. 2009) of mice. Cellular toxicity is in these cases was not necessarily attributable to export saturation but possibly saturation of other silencing factors. Castanotto et al showed that synthetic siRNAs as well as shRNAs could also actively compete with each other and with endogenous miRNAs for the selection incorporation into RISC (Castanotto et al. 2007). Constitutive expression of exogenously introduced RNAi precursors at high levels may therefore pose a significant risk for saturation effects and cellular toxicity. Since minor deregulation in endogenous miRNA function may lead to widespread perturbations in post transcriptional gene regulation, every effort should be made to identify potent RNAi activators which are able to mediate their effects at low doses.

1.8 HIV

HIV, the causative agent of AIDS, is a lentivirus belonging to the *Retroviridae* family and was first isolated in 1983 (Barre-Sinoussi et al. 1983; Gallo et al. 1983). Each virion contains two copies of the RNA genome which are reverse transcribed into cDNA which is then integrated into the human genome for expression and thus long term persistence. HIV has a very high replication rate and coupled with the lack of proof-reading ability of the viral reverse transcriptase, HIV is a rapidly evolving virus. More than 25 years following the discovery of this virus, a preventative vaccine or a therapeutic cure remain elusive. Although the success achieved in controlling viremia using a cocktail of chemically synthesised antiretroviral drugs has been invaluable; high cost, toxic side effects and the failure to remove latent infection have limited the success of these therapeutic modalities. Novel therapies against HIV are urgently required and studies have begun harnessing the RNAi pathway in an attempt to develop novel antiviral therapeutics to suppress HIV replication in the long term.

1.8.1 Current epidemic

The Human Immunodeficiency Virus Type 1 (HIV-1) epidemic is presently a major global health concern. As worldwide efforts to create awareness, prevention and treatment programs increase, so does the total number of people living with the virus. There were 2.7 million newly infected people in

2008 and 2 million AIDS deaths, bringing the total number of people living with HIV as reported at the end of 2008 to 33.4 million (UNAIDS 2008). While all countries are currently fighting the impact of this disease, sub-Saharan Africa, and Southern Africa in particular continue to bear the greatest burden of people infected with and affected by HIV. Just over 10% of the world's population inhabits sub-Saharan Africa, yet this region is home to 67% of people living with HIV-1 worldwide (UNAIDS 2008). In 2008, new infections in this region totalled more than those in all other regions of the world combined. In South Africa there are an estimated 5.7 million infected individuals which represents the largest number of individuals living with the virus in a single country. These patients are infected largely with HIV-1 subtype C, which accounts for 56% of all new infections worldwide (UNAIDS 2008).

1.8.2 HIV genome organization

The HIV genome is 9.8 kb in length and consists of nine partially overlapping genes (Figure 1.5 A) (Ratner et al. 1985; Sanchez-Pescador et al. 1985; Wain-Hobson et al. 1985). The *gag* (group-specific antigen); *pol* (polymerase) and *env* (envelope) genes encode mainly structural and enzymatic proteins. The pr55 Gag protein precursor is processed into the p17 matrix (MA); p24 capsid (CA) and p7 nucleic-acid binding (NC) structural proteins essential for assembly of viral particles (Ganser-Pornillos et al. 2008). The pr160 Gag-Pol protein precursor is processed into the reverse transcriptase (RT); protease (PR) and integrase (IN) enzymes which play important roles in the viral replicative cycle (Hill et al. 2005). Both the Gag and Gag-Pol polyprotein precursors are cleaved by the viral protease into their respective subunits (Figure 1.5 B). The gp160 Env protein is cleaved by cellular proteases into the surface gp120 and transmembrane gp41 subunits of the envelope glycoprotein which is necessary for binding to the CD4 primary receptor on host cells (Moulard and Decroly 2000). The *tat* (transcriptional transactivator) and *rev* (regulator of virion gene expression) genes encode two regulatory proteins. Tat binds to the TAR (transactivation response) element within the R domain of the 5' long terminal reapeat (LTR) to regulate transcription and Rev mediates the transport of singly spliced and unspliced viral transcripts from the nucleus to the cytoplasm (Jeang et al. 1991; Gait and Karn 1993; Hope 1999).

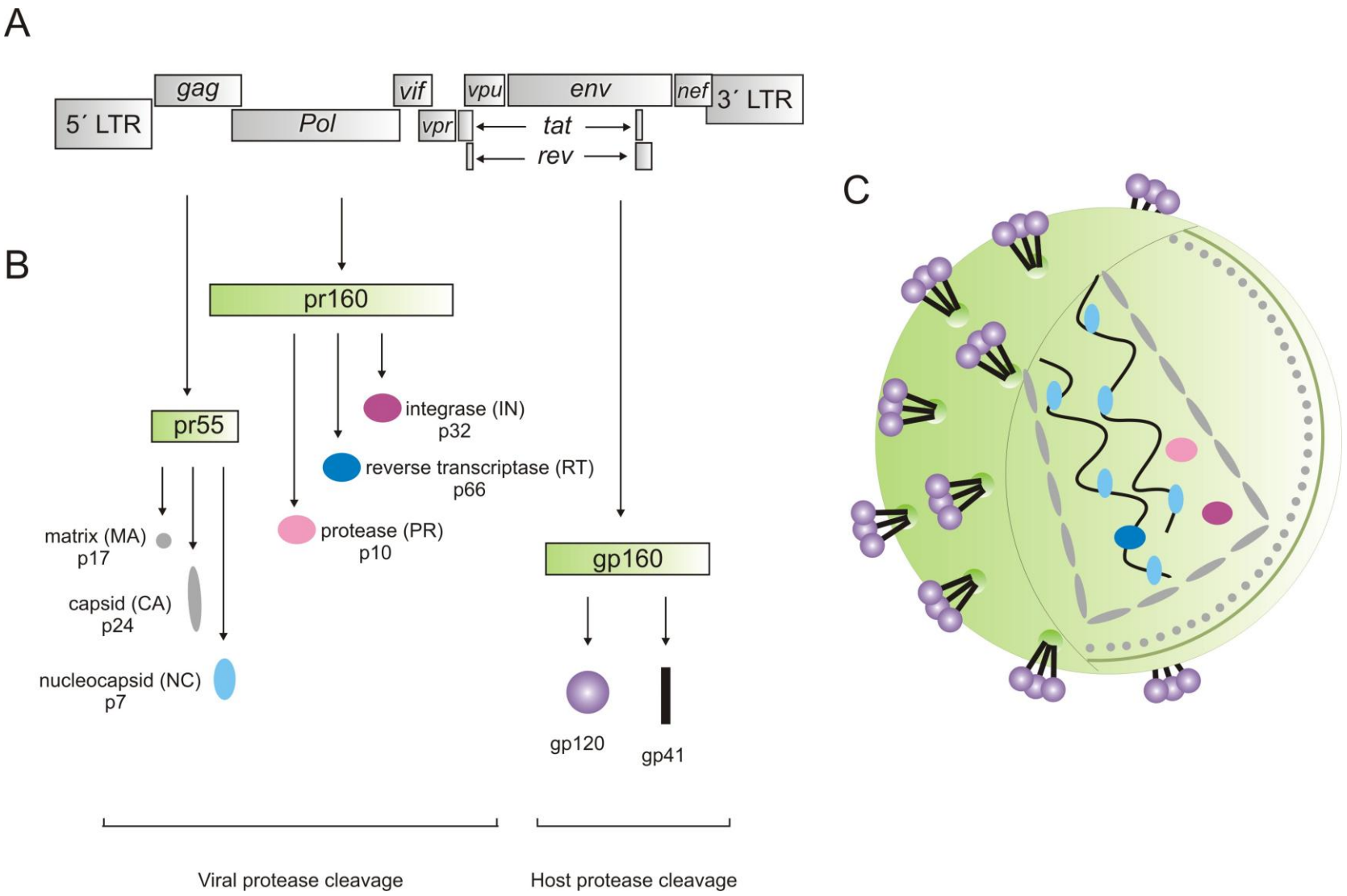


Figure 1.5: HIV genome organization. (A) A representation of the nine genes encoded by the HIV-1 genome. **(B)** The gag, pol and env genes encode protein precursors (pr55, pr160 and gp160) which require further processing by either viral or cellular proteases to generate structural proteins necessary for the formation of a mature virion **(C)**.

In addition to these five genes, HIV also encodes four accessory genes: *nef* (negative effector); *vif* (viral infectivity factor); *vpr* and *vpu* (viral proteins r and u) which represent critical virulence factors (Emerman and Malim 1998; Anderson and Hope 2004). Proteins coding sequences are flanked on either side by 5' and 3' LTRs. Each LTR is divided into 3 domains, namely the U3, R and U5 domains. The U3 region contains the basal promoter as well as an enhancer sequence and multiple transcription factor binding sites (Patarca et al. 1987; Roebuck and Saifuddin 1999). Transcription initiation occurs at the first nucleotide of the R region and this region also encodes the transcriptional response (TAR) regulatory stem loop structure to which Tat binds.

1.8.3 The replicative cycle of HIV-1

HIV infects cells of the immune system which express the relevant surface receptors necessary for interaction with the HIV transmembrane Env protein. HIV enters its target cells, which include CD4+ T cells; macrophages; monocytes; dendritic cells and microglia (He et al. 1997; Zaitseva et al. 1997; Rubbert et al. 1998; Ruiz et al. 1998), through interaction of the viral trimeric glycoprotein initially with the primary cellular CD4 receptor, and subsequently with the CCR5 or CXCR4 cellular chemokine co-receptors, [reviewed in (Doms and Trono 2000)]. The gp120 surface subunit of the Env protein initially engages with the cellular CD4 receptor leading to a conformational change in gp120 which allows it to bind to a co-receptor (Salzwedel et al. 2000). Viral tropism is largely determined by the co-receptor to which it binds. R5 strains use the CCR5 co-receptor and are able to infect CD4+T cells, macrophages and dendritic cells. X4 strains bind the CXCR4 co-receptor and only infect CD4+ T cells (Agrawal et al. 2009). Co-receptor binding then triggers the interaction of the gp41 transmembrane subunit of the Env protein with the host cell membrane. Fusion of the cellular and viral membranes ensues (Campbell and Hope 2008) and the viral core is released into the cellular environment and uncoated, releasing the viral genome (Dvorin and Malim 2003). The viral RNA genome is reverse transcribed into cDNA in the cytoplasm by viral RT (Harrich and Hooker 2002) and is then transported within the pre-integration complex consisting of viral cDNA; viral RT; matrix protein; integrase and Vpr,

to the nucleus. Nuclear localization signals on Vpr, integrase and matrix mediate import into the nucleus in both actively dividing and quiescent cells through the nuclear pores (Bukrinsky et al. 1993; Heinzinger et al. 1994; Gallay et al. 1997). Viral cDNA is subsequently integrated into the host genome, usually within active euchromatin (Schroder et al. 2002), to form the provirus. The provirus is flanked by the 5' LTR which serves as a promoter for transcription and the 3' LTR which provides the termination site. The synthesis of full length transcripts requires the interaction between the regulatory Tat protein and the TAR loop. In the absence of Tat, only short attenuated RNA transcripts are produced (Kao et al. 1987). Tat is a multiply spliced protein synthesised early in the viral life cycle and the interaction between Tat and the regulatory TAR loop is enhanced by the positive elongation factor (P-TEFb). Tat binds to the cyclin T1 subunit of P-TEFb and thereby recruits the cyclin dependent kinase 9 (CDK9) subunit to the LTR. CDK9 phosphorylates RNA Pol II, enabling the transition of initiation to elongation and the consequent synthesis of full length viral transcripts, [reviewed in (Jones and Peterlin 1994; Taube et al. 1999)].

Early phase transcripts encoding the Tat, Rev and Nef proteins are completely spliced and are exported from the nucleus by cellular machinery. Unspliced transcripts including genomic RNA and Gag-Pol precursors as well as incompletely spliced mRNAs encoding Env, Vif, Vpr and Vpu, require the interaction between the regulatory Rev protein and the Rev responsive element present within these transcripts for nuclear export into the cytoplasm, [reviewed in (Pollard and Malim 1998)]. Following translation, viral proteins together with two copies of the viral genome assemble into immature progeny virions within lipid rafts localized at the cell membrane. Env proteins are processed into their respective subunits which also gather at the cell membrane. Virions are released from infected cells through a budding process which results in virus particles coated with the host cell membrane containing trimeric Env glycoproteins embedded in the membrane. Maturation of progeny virions occurs following processing of the Gag and Gag-Pol polyprotein precursors extracellularly (Ganser-Pornillos et al. 2008).

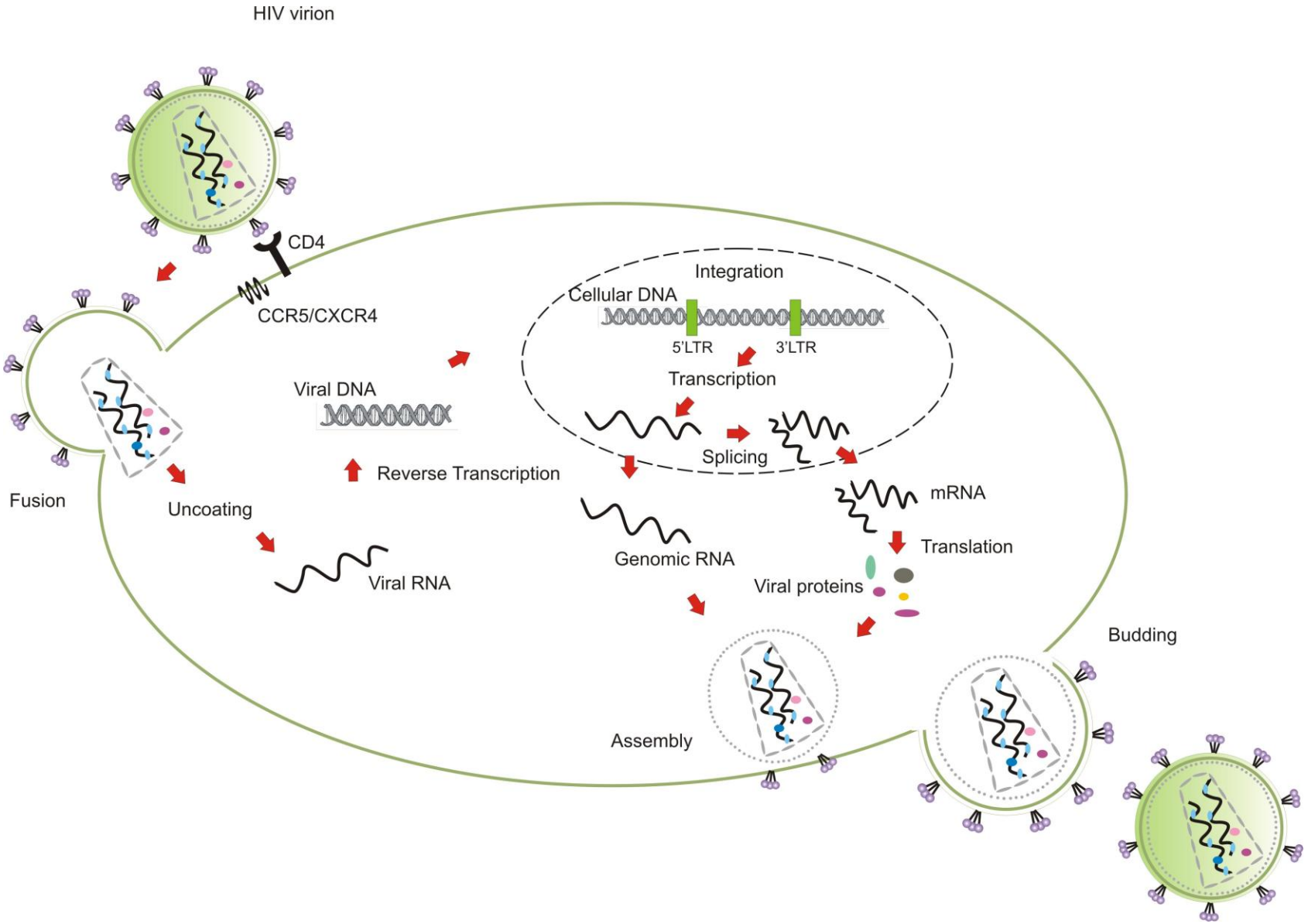

HIV virion
CD4
CCR5/CXCR4
Fusion
Uncoating
Viral RNA
Viral DNA
Reverse Transcription
Integration
Cellular DNA
5'LTR
3'LTR
Transcription
Splicing
mRNA
Translation
Viral proteins
Genomic RNA
Assembly
Budding

Figure 1.6: A basic representation of the central steps of the HIV replicative cycle. HIV virions attach to their target cells through binding of the gp120 Env protein primarily to the CD4, and subsequently to the CCR5 or CXCR4 host cell receptors. Receptor binding induces fusion of viral and cellular membranes resulting in the release of the viral core and subsequent release of the viral genome into the cytoplasm of the host cell. The viral RNA genome is reverse transcribed and then transported into the nucleus where the IN enzyme mediates integration of viral cDNA into the host genome. The integrated provirus serves as a template for the transcription of viral genomic RNA copies as well as viral mRNA which is exported to the cytoplasm for translation. Structural proteins and two copies of the RNA genome assemble into virion particles at the cellular membrane and are released from the cell by a budding process.

1.8.4 Disease progression and current therapies for the treatment of HIV

HIV-1 infection consists of an initial acute phase followed by a chronic phase. The acute phase is characterised by an increase in viral RNA (viral load) and the consequent decline in CD4+ T cells (Clark and Shaw 1993). The activation of the immune system subsequently results in the suppression of viremia to a low steady state level termed the viral setpoint, and an increase in CD4+ T cells. During the chronic phase, viral loads as well as the number of CD4+ T cells may remain constant for several years with the patient remaining largely asymptomatic. However the steady replication of HIV eventually overwhelms the immune system, resulting in a gradual rise in viremia and a steady reduction in CD4+ T cells until the patient is severely immunocompromised, resulting in increased susceptibility to opportunistic infections and the development of AIDS.

Effective treatment and eradication of HIV continues to present a serious challenge in the struggle against infection with this virus. The first breakthrough in the development of anti-HIV therapeutics came in 1987, four years after the isolation of HIV. The first antiretroviral drug (Ezzell 1987) inhibited the viral reverse transcriptase enzyme by acting as a nucleoside analogue, thus preventing the complete synthesis of proviral DNA (Furman and Barry 1988). Additional nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) were subsequently developed (Gershon 1991); however the success of the NRTI class of drugs as a monotherapy was short lived. As a result of the replication kinetics of HIV, drug resistant viral variants quickly emerged to expose the limitations of a monotherapy to treat HIV infection. A need for drugs targeting alternative viral components was evident and the

class of protease inhibitor drugs was developed soon thereafter. These drugs inhibit viral protease thus preventing the production of mature viral proteins (Venaud et al. 1992). The advantages of a combination therapy were then realised following triple therapy or highly active antiretroviral therapy (HAART), initially using a combination of two NRTIs and a protease inhibitor (Hammer et al. 1997; Hirsch et al. 1999). Today there are 25 antiretroviral drugs available in 6 different classes (Zolopa 2010). Additional classes of antiretroviral drugs include the non-nucleoside class of reverse transcriptase inhibitors (NNRTIs) which function by direct binding and inhibition of the RT enzyme (de Bethune 2010); and most recently entry inhibitors which include CCR5 antagonists and fusion inhibitors (Tilton and Doms 2010); and integrase inhibitors which prevent integration of the provirus into the host chromosome (McColl and Chen 2010). A novel class of antiretroviral still undergoing clinical trial is that of the maturation inhibitors which inhibit cleavage of the capsid precursor (Temesgen and Feinberg 2006; Martin et al. 2008). The increased availability of drugs in recent years to include new generation drugs in existing classes, as well as new classes of antiretroviral drugs has greatly expanded the possibilities for modification of double and triple therapies.

1.8.5 Drawbacks associated with HAART

HAART has provided significant progress in the quest to decrease viral load and increase CD4+ and CD8+ cells in infected patients. Despite the significant decrease in morbidity and mortality offered by HAART, problems associated with these treatment regimens persist. Toxic side effects caused by antiretroviral drugs have been widely observed both in the short and long term and differ between drug classes and individual drugs. Short term adverse effects commonly include, but are not limited to, gastrointestinal effects such as nausea and diarrhoea as well fatigue, dizziness and rashes. In the long term, a variety of metabolic disorders such as lipodystrophy, hyperlipidaemia, diabetes mellitus and lactic acidaemia commonly occur; and in addition cardiovascular disease, renal dysfunction and hepatotoxicity have also been observed [reviewed in (Carr 2003; Mallon 2007; Hawkins 2010)]. In addition to severe side effects, and often a consequence thereof, patient compliance continues to pose a problem caused by complex regimens necessary for combination therapy (Gardner et al. 2008;

Simoni et al. 2008). The expense of distributing HAART, especially in developing countries where the pandemic is most rife is a major problem and finally the emergence of drug resistant strains to various antiretroviral drugs continues to represent the foremost setback in the development of an effective long term therapy [for a comprehensive review on the molecular mechanism underlying resistance to each drug class see (Menendez-Arias 2010)]. The extremely high replication rate of HIV coupled with its high mutation rate (Mansky 1996) may actively cause resistance to one or more drugs and furthermore, transmission of mutant viral strains may result in minority drug resistance in drug naïve patients which substantially increases the risk of first line treatment failure (Paredes et al. 2010). The continual need for the development of new drugs or new drug classes as well as new formulations and strategies for existing drugs is an expensive and tedious process involving further clinical trials and investigations into potential drug interactions between new and existing drugs. Resistance testing before treatment initiation is therefore ideal for an effective drug regimen to be formulated. Although theoretically possible, the reality is that in sub-Saharan Africa, access even to basic first line antiretroviral drugs is limited, and in the majority of cases the practicalities and cost involved in diagnosing patients with first line treatment failure and the subsequent administration of second line drug formulations is not feasible (Harries et al. 2010).

The persistence of latent viral reservoirs is another drawback of HAART since although viral replication may be effectively suppressed, current drug regimens are incapable of completely eradicating the virus. Despite long term containment of viremia in patients receiving HAART, a latent reservoir of virus was identified in resting CD4+ T lymphocytes from which replication competent viruses could be isolated (Finzi et al. 1997; Wong et al. 1997). The underlying molecular mechanisms of latency are extremely complex and the exact locations of these latent viral pools are still disputed. Memory CD4+ T cells are a well-defined reservoir and are thought to be the main source of residual viremia (Chun et al. 1995; Chun et al. 1998; Siliciano et al. 2003). Latency may occur when activated CD4+ T cells become infected with the virus, and soon thereafter make a transition into terminally differentiated memory T cells [reviewed in (Lassen et al. 2004; Dahl et al. 2010)]. Viral reservoirs are then able to persist and survive for years before potentially re-emerging, and to control viremia, drugs therefore need to be taken for life. Although HAART has been responsible for the transition of a fatal

infection into a treatable chronic disease, the limitations of this therapy are obvious and it remains an important medical objective to develop alternative novel, and cost effective therapeutic strategies

1.9 Exploitation of the RNAi pathway to develop novel therapeutics against HIV

The RNAi pathway is utilized in many species as a natural defense strategy against foreign genetic elements such as transposons and viruses (Ketting et al. 1999; Tabara et al. 1999; Mourrain et al. 2000). Intrinsic cellular defense properties of the RNAi pathway in mammals are still debated. However, the obvious interplay between components of the RNAi pathway and several human viral pathogens is intriguing (discussed further in section 5.5). Since this pathway has natural antiviral properties in certain species, the mechanism by which RNAi functions offers an attractive tool to exploit in the development of antiviral therapeutics in mammalian cells. In contrast to HAART, RNAi-based therapies have the potential to substantially simplify drug regimens, and furthermore, RNAi-based therapeutics may be designed to target any one of the viral genes thus eliminating the time consuming search for novel drug targets.

1.9.1 RNAi susceptible targets

Incoming viral genomic RNA

Theoretically, three approaches exist for inhibiting HIV-replication using RNAi and include directly targeting the viral genome, targeting host dependency factors or targeting newly synthesised viral messenger RNA. Each mature virion contains two copies of the viral RNA genome which, after fusion, are released into the cytoplasm for reverse transcription as described above (section 1.8.3). Inhibition of the incoming viral genomic RNA is an attractive target because synthesis of cDNA and establishment of the provirus is prevented, thus inhibiting the replicative cycle of HIV at a very early stage. Several pioneering studies utilizing RNAi to inhibit HIV replication reported successful inhibition

of incoming genomic RNA, using siRNA sequences targeted to various genomic sequences including *gag* (Capodici et al. 2002; Novina et al. 2002); *tat* and *rev* (Coburn and Cullen 2002); *nef* and *vif* (Jacque et al. 2002) and the viral LTR (Capodici et al. 2002; Jacque et al. 2002). However the efficient targeting of incoming RNA is controversial and several studies have reported that genomic RNA is not susceptible to RNAi, possibly due to its association with capsid, nucleocapsid, RT and possibly other proteins which render the genome inaccessible to the RNAi machinery (Hu et al. 2002; Surabhi and Gaynor 2002; Nishitsuji et al. 2004; Westerhout et al. 2006a). Hu et al. made use of siRNA sequences targeted against *gag* or *int* which were capable of inhibiting HIV >90%, and analysed the inhibition of early and late viral targets in isolation. While the accumulation of viral mRNA transcripts was effectively prevented, no degradation of genomic RNA was detected. Moreover, these results were replicated with another retrovirus, Rous sarcoma virus (RSV) (Hu et al. 2002).

Following these contradictory results, Westerhout et al. investigated the transduction efficiencies of lentiviral vectors in the presence or absence of HIV-specific shRNAs as a model of proviral integration. This study reported no difference in transduction efficiencies between control cells and cells stably expressing shRNAs targeted to different regions within the HIV-1 genome (Westerhout et al. 2006a). These results provide compelling evidence for inaccessibility of the viral genome to RNAi, and although the inhibition of incoming viral RNA is highly desirable for therapeutic purposes, the prevention of provirus integration seems unlikely.

Host dependency factors

A second possible strategy for the inhibition of HIV replication and an alternative approach for the prevention of viral entry is to suppress host cellular genes essential in the replicative cycle of HIV. In addition, inhibiting host dependency factors is an attractive strategy for preventing the emergence of viral escape mutants. The most frequently targeted host transcripts encode the primary CD4 receptor or the co-receptors CCR5 and CXCR4. Although the suppression of the CD4 receptor gene may result in potent inhibition of viral entry (Novina et al. 2002), complete inhibition of this host factor will abolish other intracellular roles it may play and therefore have detrimental effects on the host. Inhibition of the

CCR5 co-receptor is an equally effective strategy at preventing viral entry (Qin et al. 2003) and may offer a safer target since people homozygous for the mutant CCR5 allele are resistant to HIV-1 infection yet exhibit no consequent side effects (Liu et al. 1996; Samson et al. 1996). In a therapeutic context however, down regulation of a single co-receptor is insufficient due to the possible selection of minor populations of dual tropic strains and the possibility of co-receptor switching (Delobel et al. 2005; Moncunill et al. 2008), both of which will lead to an eventual viral infection. A combination of cellular receptors should therefore be targeted simultaneously.

Host factor targeting is not limited to host cell receptors and a variety of other host factors have been considered for down regulation to inhibit HIV infection. These factors include, but are not limited to, integration factors such as BAF1, Emerin and LEDGF/p75 (Maertens et al. 2003; Jacque and Stevenson 2006; Llano et al. 2006); transcription factors such as NF-kB, PAK-1 and cyclin T1 (Surabhi and Gaynor 2002; Chiu et al. 2004; Nguyen et al. 2006) and Furin which plays a role in the maturation of the Env protein (Nguyen et al. 2006). In a recent groundbreaking study, an siRNA screen was used to identify 250 HIV host dependency factors which are implicated in specific pathways of the viral life cycle (Brass et al. 2008). Similar studies have followed, also using large scale RNAi screens, and identified hundreds more potential host factors which are central to the establishment of viral infection (Konig et al. 2008; Zhou et al. 2008a; Kok et al. 2009). These studies have dramatically expanded the number of host dependency factors which may be targeted, however the results require further validation in T cells and moreover, to prevent disruption of natural endogenous cellular functions of these proteins, care should be taken to target only those genes dispensable to the host.

HIV mRNAs

The third and most obvious category of RNAi susceptible targets in HIV are the newly synthesised viral mRNA transcripts which are exported to the cytoplasm from the nucleus for translation. Transcripts encoding the Rev protein were amongst the first viral transcripts to be effectively silenced using RNAi effectors (Coburn and Cullen 2002; Lee et al. 2002a). Every possible HIV transcript has since been targeted for silencing using various RNAi effector mimics with varying

degrees of efficacy, but often inhibiting viral replication >90%. The drawback of this strategy is associated with the extraordinarily high rate of error of the viral RT enzyme. With an error rate of 3×10^{-5} (Mansky 1996), it is not surprising that HIV is a rapidly evolving virus, and in any infected cell, a pool of viral variants exist. It is thus essential to target conserved regions within viral genes to limit the emergence of viral mutants which are refractory to RNAi-mediated gene silencing. However, even when targeting conserved sites within the viral genome, mutations may arise under selective pressure, rendering the RNAi effector ineffective.

1.9.2 The emergence of viral escape mutants

Viral escape generally occurs through the development of mutations in the targeted sequence. A single mutation within the RNAi targeted site, especially the seed region, is sufficient to protect the virus from the silencing effects of RNAi effectors (Gitlin et al. 2002). The emergence of HIV escape mutants were first observed following inhibition of viral replication by stable expression of an shRNA targeted to the *tat* transcript. After 25 days of exposure of the virus to the shRNA, a point mutation within the shRNA target sequence emerged which abrogated the silencing effects of the shRNA (Boden et al. 2003a). Similarly, a stably expressed siRNA sequence targeted against the *nef* transcript was shown to cause potent suppression of viral replication, but only for 23 days, after which resistant viral variants arose. In this case escape mutants emerged due to substitutions or deletions which resulted in the modification or the deletion of the target sequence (Das et al. 2004). Westerhout et al. then analysed additional escape variants which emerged following exposure to the shRNA targeted against the *nef* gene (Westerhout et al. 2005). In five out of nine mutants, partial deletion of the target sequence occurred, three mutants acquired single or multiple substitutions within the target sequence, and in one mutant, a single nucleotide substitution upstream from the targeted region was found which also caused resistance to the RNAi effector. It was deduced that mutations occurring outside of the target region may result in an alternative structural conformation of the RNA which prevents siRNA binding and thus reduces RNAi efficacy (Westerhout et al. 2005).

The *nef* gene codes for a non-essential viral accessory protein, yet targeting of essential viral genes does not prevent the emergence of viral escape mutants either (Senserrich et al. 2008; von Eije et al. 2008). von Eije et al. performed a large scale analysis of viral evolution following exposure to shRNAs against the essential *gag, pol* and *tat/rev* genes. None of these shRNAs was able to abrogate viral escape completely, although in contrast to the deletions in the *nef* gene target, the sequence variation of these targets and thus viral escape was limited, presumably to prevent a compromise in viral fitness. Furthermore, a propensity for mutations in certain positions within the target sequences was observed. No escape at the termini of the 19 nt sequences was observed, (at positions 1, 2, 18 and 19) whilst the central region appeared more prone to mutation, suggesting that the terminal nucleotides are either inconsequential for RNAi-mediated silencing or that variation of these nucleotides will negatively affect viral fitness (von Eije et al. 2008).

Targeting conserved regions within essential viral genes may also aid in restricting the emergence of viral escape mutants. A series of shRNAs were designed to target either a poorly conserved region within *rev*, a *gag* site conserved only across subtype B viral strains, or a highly conserved *vif* sequence (Lee et al. 2005). Anti-*rev* shRNAs were capable of incredibly limited inhibition; anti-*gag* shRNAs effectively inhibited only subtype B viral strains whereas anti-*vif* shRNAs were effective against all viral isolates, thus highlighting the advantage of targeting widely conserved sequences. A further evasion mechanism was established following RNAi directed against the TAR loop within the viral LTR. HIV replication was blocked using an shRNA targeted to an RNAi susceptible region within the TAR loop, however resistant strains emerged which contained no mutations within the target site. Mutations were however found in alternative sites within the LTR promoter which caused an upregulation in transcription as a compensatory mechanism to overcome RNAi-mediated suppression, which served as a novel indirect means of escape (Leonard et al. 2008).

Finally, the HIV dosage to which shRNAs are exposed may play an important role in determining strong versus weak RNAi effectors. Nishitsuji et al. showed that while shRNAs targeted against integrase were effective at a low viral input, their anti-HIV efficacy was drastically reduced when the

viral dosage was increased (Nishitsuji et al. 2006). This data is supported by a comparative analysis of 20 shRNAs which used a dose response assay as one of the determinants for shRNA potency. It was shown that while elevated viral dosage will eventually overwhelm any shRNA, the viral input at which this occurs is indicative of the shRNA strength (von Eije et al. 2009).

Large-scale screens may identify potentially powerful siRNAs or shRNAs which upon extensive validation may yield a subset of strong, durable RNAi effectors (von Eije et al. 2009). However, HIV has evolved a versatile array of escape mechanisms and to persistently suppress viral replication with RNAi, a combination of single RNAi effectors, akin to HAART, is likely to be necessary.

1.10 Combinatorial RNAi

It is evident that when targeting rapidly evolving targets with single RNA effector molecules such as siRNAs or shRNAs, mutations within the target sequence will arise within relatively short periods following exposure to such effectors, leading to evasion of RNAi-mediated silencing. To circumvent this problem, multiple sites should be targeted simultaneously. The simultaneous introduction of multiple RNAi effectors is known as combinatorial RNAi and is of considerable importance when targeting viruses such as HIV. HIV causes chronic infections and thus requires long term treatment, and in addition is a highly mutable and thus represents a rapidly evolving target. Three major platforms currently exist for the simultaneous suppression of multiple gene targets, and all three strategies have been exploited for the inhibition of HIV. These approaches include multiple shRNA expression cassettes; polycistronic miRNA shuttles and long hairpin RNAs (lhRNAs) (Figure 1.7).

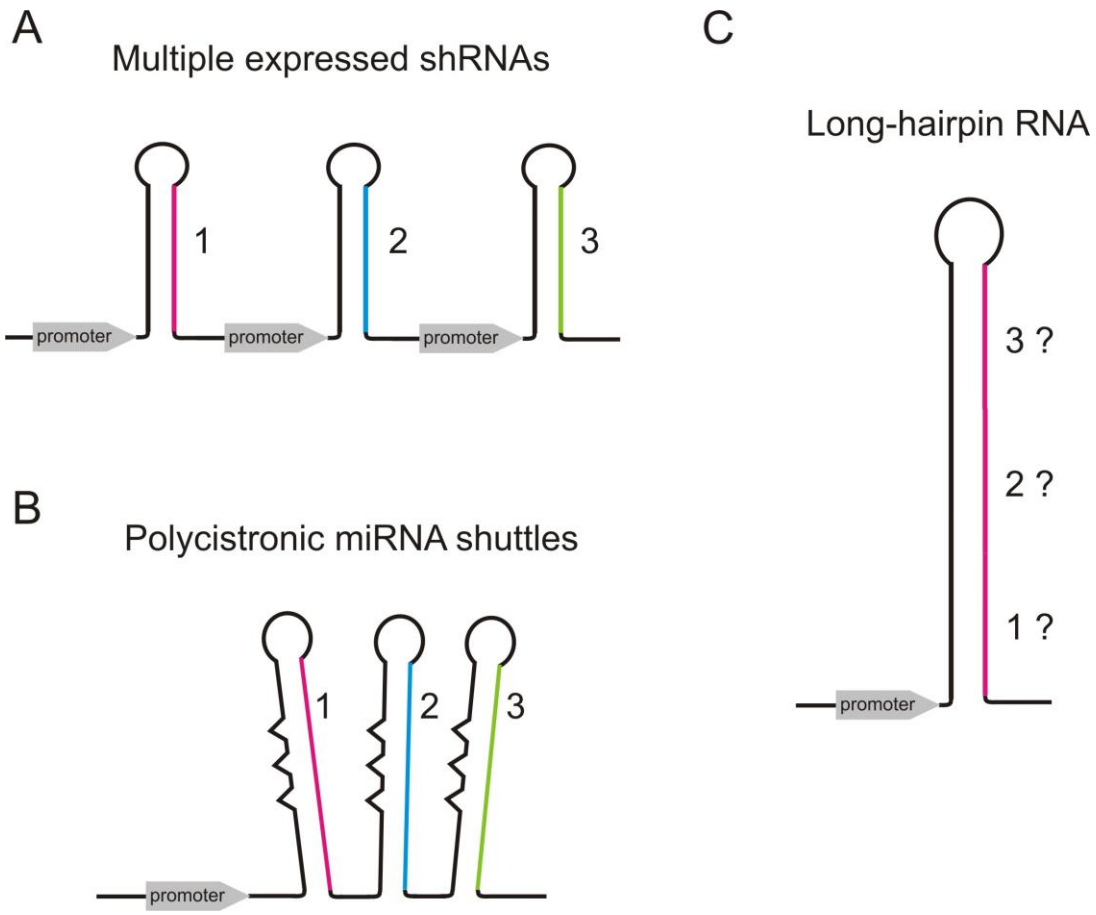


Figure 1.7: Schematic representation of currently utilized combinatorial RNAi strategies. These strategies include **(A)** multiple expressed shRNAs, where multiple siRNA sequences are expressed individually from multiple promoters, **(B)** polycistronic miRNA shuttles, in which multiple siRNA sequences are inserted into the natural pri-miRNA backbones within a polycistron, and **(C)** lhRNAs, from which the generation of multiple siRNAs has not been properly characterised.

1.10.1 Multiple expressed shRNAs

Multiple Pol III shRNA expression cassettes may be combined adjacent to one another within a single vector (Figure 1.7). The advantage of this approach is that previously characterised effective RNAi effector sequences may be combined into a single vector for the simultaneous inhibition of multiple gene targets. This strategy was investigated by Henry et al. who combined three H1 Pol III promoter-driven shRNAs targeted against two sites in the HCV genome and the host cell receptor

CD81, within a single DNA vector and showed effective suppression of all three targets (Henry et al. 2006). This method has also been successfully used to incorporate 3 (ter Brake et al. 2006) or 4 (Song et al. 2008) shRNAs targeted to HIV, and up to 6 shRNAs targeted against different proliferation and survival pathway factors for the treatment of cancer (Cheng et al. 2009). In addition, the effects of multiple shRNAs are reportedly additive, showing enhanced silencing of several targets in comparison to a single shRNA (Gonzalez et al. 2005; ter Brake et al. 2006; Gou et al. 2007; Song et al. 2008).

In an influential study by ter Brake et al., a lentiviral vector containing four anti-HIV shRNAs in tandem was able to suppress viral replication and prevent the emergence of escape mutants for an 80 day period. However, it was shown that when multiple shRNAs driven from the same promoter were cloned in tandem into a lentiviral delivery vector, recombination of repeat sequences caused deletions of one or more of the shRNA cassettes, and the use of multiple different promoters was therefore required (ter Brake et al. 2008). This was recently confirmed by McIntyre et al. who also showed significant deletion of individual shRNAs in a lentiviral vector containing up to six H1-driven shRNAs (McIntyre et al. 2009b). Despite the observed synergistic effect, the use of multiple shRNA expression cassettes in tandem has been observed to result in a decrease of mature guide strand production when compared to single shRNAs (ter Brake et al. 2006; ter Brake et al. 2008). Of greater concern regarding the use of multiple expressed shRNAs however, is the potential for saturating effects on the RNAi pathway. When multiple shRNAs are expressed from strong ubiquitous Pol III promoters, competition for strand incorporation into the RISC complex as well as for other components of the endogenous miRNA biogenesis pathway may occur, resulting in disrupted gene regulation and cell toxicity (Grimm et al. 2006; Castanotto et al. 2007; Boudreau et al. 2008) (section 1.7.3).

1.10.2 Polycistronic miRNA shuttles

A second combinatorial RNAi strategy combines multiple pri-miRNA mimics (described in section 1.6.2) within a single transcript (Chung et al. 2006; Ely et al. 2009). Chung et al. demonstrated that two tandem modified miR-155 precursors targeting two independent gene sequences could be

expressed from a single transcript. Encouragingly, the knockdown from each miRNA within the polycistronic transcript was comparable to that of single pri-miRNA mimics (Chung et al. 2006). Similarly, engineered polycistronic miRNAs containing trimeric expression cassettes have been successfully used for the inhibition of three different HBV target sequences (Ely et al. 2009). Three effective anti-HBV guide sequences were substituted into three miR-31 backbones in series or alternatively into three different tandem miRNA scaffolds including miR-31, miR-30a and miR-122. Each trimeric expression cassette was driven from a single CMV promoter and the parental miRNA structure and flanking sequences were consistently maintained. Although effective processing and potent knockdown of all three target sequences was achieved both *in vitro* and *in vivo*, variability of individual miRNA efficacy was observed which correlated with both the sequence and the position of each discrete pri-miRNA mimic (Ely et al. 2009).

Many miRNAs are also found to naturally occur in polycistronic clusters and these pri-miRNA precursor structures, if the secondary structures are conserved, may also be manipulated for the generation of multiple effector sequences (Aagaard et al. 2008; Liu et al. 2008). Liu et al. manipulated the naturally occurring mir-17-92 polycistron for the inhibition of HIV by inserting antiviral siRNAs into the endogenous polycistronic miRNA backbone. Silencing activity was enhanced in this multimeric system and efficient processing of four functional antiviral sequences capable of efficiently inhibiting HIV replication was achieved (Liu et al. 2008). The tri-cistronic miR-106b cluster has also been manipulated through substitution of the three endogenous miRNA sequences with siRNA sequences targeted against *tat* and *rev.* This construct was effective when tested against an HIV molecular clone; however this efficacy was significantly reduced when tested against a more virulent viral strain (Aagaard et al. 2008). Although both engineered and natural polycistronic miRNA shuttles represent a potentially viable combinatorial RNAi strategy, much characterisation is still required to determine the effects of different endogenous polycistronic miRNA shuttles on the efficacy and processing of effector sequences. Moreover the ability of such constructs to inhibit viral replication in the long term has yet to be demonstrated.

1.10.3 Long hairpin RNAs

A third strategy for the development of a combinatorial RNAi system, and the focus of this thesis, is the use of long hairpin RNAs. In non-mammalian cells, long dsRNA duplexes of approximately 500 bp have been shown to act as Dicer precursors for the production of a pool of siRNAs which are subsequently able to inhibit target gene expression specifically (Hamilton and Baulcombe 1999; Zamore et al. 2000; Elbashir et al. 2001b). It was then shown that recombinant human Dicer was also capable of processing long dsRNA *in vitro*, and generated ~22 bp siRNA fragments following incubation with a 130 bp dsRNA duplex (Zhang et al. 2002). Synthetic long dsRNA of ~500 bp have been shown to effect silencing of HIV-1 genes and to suppress viral replication in targeted mammalian cells (Park et al. 2002; Yamamoto et al. 2002). While these studies demonstrate the efficiency of Dicer and the RNAi pathway in mammalian cells, the use of synthetic long dsRNA remains a controversial issue since longer dsRNA duplexes >30 bp have been shown to induce non-specific silencing (Caplen et al. 2001; Elbashir et al. 2001a) by activating cytoplasmic PKR and causing an eventual repression of global protein translation (Clemens 1997).

Nonetheless, the use of expressed long dsRNA soon followed and the endogenous transcription of long dsRNA up to 1000 bp resulted in highly efficient, target specific silencing in mammalian cells (Paddison et al. 2002; Diallo et al. 2003; Yi et al. 2003a; Tran et al. 2004) which is supported by a later study which shows that endogenously expressed RNAi activators are not recognised by cytoplasmic sensors of dsRNA (Robbins et al. 2006). Tran et al. co-expressed long complementary single stranded RNAs from separate plasmids with the potential to form dsRNA and showed that when both RNA strands were expressed, siRNAs were produced from the annealed dsRNA which were capable of specific silencing of both endogenous and exogenous genes without activating PKR (Tran et al. 2004). The remaining studies utilized long dsRNA expressed as stem-loop, hairpin-like structures from the RNA Pol II cytomegalovirus (CMV) promoter. Hairpin stem duplexes were generally long and ranged from 500 to 1000 bp with loop/linker sequences of 6 to 700 nt and in all cases resulted in effective and specific gene silencing, again suggesting the absence of immune stimulation (Paddison et al. 2002; Diallo et al. 2003; Yi et al. 2003a). Diallo et al. used hairpin structures with a dsRNA stem region 800

bp in length, to completely inhibit the expression of specific endogenous genes. Furthermore, they showed the presence of 21 nt RNA species complementary to the silenced gene in targeted cells (Diallo et al. 2003). The constructs described above represented the first long hairpin RNAs (lhRNAs). Their ability to generate a pool of multiple siRNAs capable of gene specific silencing without prior identification of susceptible target sites, offers enormous potential for their development as combinatorial RNAi strategies. In a study by Konstantinova et al., a series of Pol II-expressed lhRNAs with stem lengths of 300 bp were constructed to target the HIV-1 *tat* and *nef* genes in an attempt to inhibit viral replication. However EFIα, 7tetO and HIV-1 LTR driven lhRNAs showed marginal anti-HIV-1 activity when compared to a Pol III-expressed shRNA targeting a corresponding sequence (Konstantinova et al. 2006). The use of expressed lhRNAs as novel treatment modality therefore requires further characterisation of the structural design of lhRNAs and the promoters that will drive their expression. In addition, validation of the reported sequence-specific gene silencing is necessary to eliminate possible non-specific knockdown effects.

Long hairpin RNAs driven by RNA Pol III promoters such as U6 and H1 were therefore developed soon thereafter as novel Dicer substrate precursors (Akashi et al. 2005; Nishitsuji et al. 2006; Watanabe et al. 2006; Weinberg et al. 2007). Pol III-expressed transcripts result in shorter defined transcripts with 2-3 nt 3' uridine overhangs akin to natural pre-miRNA 3' overhangs, and are therefore likelier Dicer substrates. Like shRNAs, lhRNAs mimic the structure of endogenous precursor microRNAs (pre-miRNAs). In contrast, the hairpin stem of Pol III-expressed lhRNAs is longer than that of shRNAs yet much shorter than those of the Pol II-expressed lhRNAs described above, and have typically ranged from 50-197 bp. RNA Pol III lhRNA transcripts have been used to inhibit the gene expression of both HIV-1 and HCV which are both rapidly evolving viruses. A noteworthy benefit afforded by the lhRNAs used against these viruses was their ability to maintain target gene suppression in the presence of emerging mutations which abrogated the effects of single shRNAs (Akashi et al. 2005; Nishitsuji et al. 2006). Akashi et al. showed the production of two discrete siRNA species from a 50 bp hairpin stem. The production of more than one unique siRNA enables a greater region to be targeted and thus provides a likely reason for the inhibition of HCV irrespective of genotypic variations, again highlighting the advantages of lhRNAs as a combinatorial RNA approach.

Prior to the initiation of the research described in this thesis, lhRNAs had been designed to target only long contiguous sequences and little research had sought to characterise the mechanism by which lhRNAs are processed or the siRNA products which are generated by Dicer cleavage. Evidence suggests that Dicer may generate multiple effective siRNAs from an lhRNA stem duplex, suggesting a possibility for incorporating the sequences of multiple siRNAs into the design of a single lhRNA. Processing of the hairpin stem would potentially enable the generation of a pool of siRNAs capable of simultaneously silencing multiple non-contiguous gene targets.

1.11 Objectives of this thesis

To sustain the inhibition of HIV replication effectively using RNAi-based therapeutic approaches, single RNAi effectors should be multimerised using one of the combinatorial RNAi strategies described above. Long hairpin RNAs (lhRNAs) represent an attractive system for the simultaneous derivation of multiple siRNAs from a single transcript, since theoretically, Dicer cleaves the dsRNA stem region at regular intervals to yield multiple siRNA duplexes. Prior to the initiation of this project however, the products of lhRNA cleavage had not been characterized, and this was due, in part, to the fact that lhRNAs had only ever been used against long contiguous sequences. The overall aim of this project was to characterise the potential of lhRNAs for use as a combinatorial RNAi strategy. This thesis aspired to demonstrate the possibility of incorporating the sequences of multiple unique siRNA sequences, targeted to non-contiguous sites, within a single lhRNA. The investigation sought to characterise the processing trends of lhRNAs encoding multiple discrete siRNA sequences and thus the ability of Dicer to generate multiple functional siRNAs simultaneously from a single expressed transcript. The final endeavor was to develop a novel lhRNA-based combinatorial RNAi strategy capable of effectively inhibiting highly mutable disease targets such as HIV.

The abovementioned specific objectives of the project were met as follows:

1. Long hairpin RNAs were designed to encode up to three unique highly effective siRNA sequences targeted against non-contiguous, conserved regions within the HIV-1 genome. A selection of candidate anti-HIV siRNA sequences were incorporated into lhRNA designs based on their antiviral efficacy in previously published studies. RNA Pol III lhRNA expression cassettes were subsequently generated for assessment of inhibitory efficacy *in vitro*.

2. The effect of sequential and spatial arrangement of putative siRNA sequences within the lhRNA duplex, on the RNAi activity afforded by each encoded siRNA sequence, was thoroughly investigated. Putative siRNA sequences were placed in each possible position within the hairpin stem to determine potential positional effects. Additionally, base pair spacing at siRNA encoding junctions was manipulated in lhRNAs encoding two or three siRNAs, to establish optimal structural features of an lhRNA stem duplex.

3. The processing of lhRNA duplexes incorporating two or three siRNAs, by the enzyme Dicer, was characterised and optimised for the generation of two, equally processed and fully functional siRNAs from a single hairpin duplex.

4. A novel lhRNA-based combinatorial RNAi system was designed to incorporate a combination of two optimally designed discrete dual-targeting lhRNAs. The ability of this unique RNAi precursor structure to be processed intracellularly was explored and the inhibitory efficacy of processed products was determined.

5. The ability of lhRNAs alone, or within a unique combinatorial system, to inhibit the replication of a primary HIV-1 subtype C isolate was assessed *in vitro*.

6. Potential off-target and non-specific effects caused by exogenously introduced lhRNA expression cassettes into mammalian cells were assessed. The activation of genes involved in the non-specific innate immune response was investigated and the potential saturation of the endogenous miRNA biogenesis pathway was determined by assessing derepression of both an endogenous and an exogenously introduced miRNA in lhRNA-transfected cells.

CHAPTER 2

The efficacy of generating three independent anti-HIV-1 siRNAs from a single Pol III-expressed long hairpin RNA.

2.1 Introduction

The use of Pol III-driven lhRNAs offers an attractive combinatorial RNAi strategy. However, extensive characterisation is still required to determine the mechanism as well as the efficiency by which these constructs are processed. Powerful gene silencing mediated by lhRNAs has been reported, however the RNAi activity contributed by different regions within the lhRNA stem has yet to be investigated. Preliminary evidence following further studies by others and us, characterising lhRNAs targeted to regions within HBV and HIV-1, suggests a possibility that lhRNA duplexes are not processed equally along the duplex, but rather that a bias exists for the cleavage of siRNAs at the base of the stem (Barichievy et al. 2007; Weinberg et al. 2007). Long hairpin RNAs with a stem length of 62 bp were assessed for their abilities to generate multiple siRNAs targeted against the *HBx* gene of HBV, and to silence minimal 21 nt targets spanning the duplex region. However a maximum of two processed siRNAs were observed. A third siRNA encoded by regions adjacent to the loop sequence was unable to be detected, which correlated with the poor RNAi inhibition from this region (Weinberg et al. 2007). Similarly lhRNAs targeted against the HIV LTR effected knockdown only of target sequences complementary to the base of the lhRNA stem (Barichievy et al. 2007). This phenomenon has not however been fully characterised and it is possible that the poor RNAi activity observed from siRNA sequences towards the loop of the hairpin stem was due to their ineffective guide sequences or inaccessibility of their target sequences, although northern blot analysis indicates a compromise not only of silencing efficacy but also of processing (Weinberg et al. 2007).

Efforts to harness lhRNAs as a tool for gene silencing prior to the initiation of this study, had only utilized hairpin stems targeting contiguous sequences of a specific gene (Akashi et al. 2005; Nishitsuji et al. 2006; Watanabe et al. 2006; Barichievy et al. 2007; Weinberg et al. 2007). This system mediates

silencing without prior identification of RNAi-susceptible target sites which is required for efficient silencing by single siRNA or shRNA guide sequences. However, rather than contiguous lhRNAs, which may produce multiple, yet only moderately effective siRNAs, lhRNAs may in theory be designed to incorporate the sequences of multiple highly effective siRNAs. This system would allow for the inclusion of previously characterised effective siRNA sequences within the hairpin stem, which once cleaved, yield guide strands able to target independent gene sequences, thus making lhRNAs an ideal tool for the development of a combinatorial RNAi strategy.

Dicer acts as a molecular ruler, and cleaves substrate RNA at an exact length from the site where it binds (Macrae et al. 2006). The products of human Dicer cleavage are generally ~22 nt, and in support of this, the siRNA species detected by Weinberg et al. in a mammalian cell line were ~22 nt in length (Weinberg et al. 2007). Therefore a stem of 63-75 bp should theoretically provide a substrate for 3 successive Dicer cleavage reactions and the subsequent generation of 3 discrete siRNAs. In this study, RNA Pol III-expressed lhRNAs 69 bp in length were designed to encode three 23 bp putative siRNA sequences targeted against three non-contiguous sites within the HIV genome previously shown to be highly susceptible to silencing by RNAi. Target sequences included conserved regions within the *tat* (Lee et al. 2002a), *rev* (Lee et al. 2002a) and *vif* (Lee et al. 2005) open reading frames, thus providing a platform for the simultaneous suppression of both early and late phase viral transcripts (section 1.8.3). A panel of lhRNAs was generated, each consisting of a different spatial arrangement of adjacent siRNA sequences. This allowed for putative siRNA sequences to be placed in each possible sequential position within the duplex to control for any potential positional effects on inhibitory efficacy of individual guide sequences. The work described in this chapter characterises the efficacy of expressed lhRNAs designed to generate up to three siRNAs targeted to non-contiguous sequences within the HIV-1 genome and highlights the advantages and current limitations of this strategy as a combinatorial RNAi tool.

2.2 Materials and Methods

2.2.1 Generation of plasmids encoding U6-driven lhRNA and shRNA sequences

U6-expressed lhRNA and shRNA sequences were constructed using a two-step PCR (Figure 2.3) which was adapted from a protocol originally used by Castanotto et al. to generate Pol III-driven shRNAs (Castanotto et al. 2002). In the first round of PCR, 10 ng of pTZU6+1, a plasmid containing the human U6 snRNA RNA Pol III promoter sequence (Bertrand et al. 1997) was used as a template. The universal U6 forward primer (Table 2.1) was complementary to the 5' end of the U6 promoter and was used for both rounds of PCR. It included a *Spe*I restriction enzyme recognition site to facilitate screening of correctly inserted clones. The reverse primer for the first round (R1) of PCR was complementary to 21 nt of the 3' end of the U6 promoter and contained a linker encoding the sense strand of the hairpin duplex as well as the loop sequence (Table 2.1). PCR reactions were carried out on a Mastercycler® (BioRad, CA, USA) and standard thermocycling conditions were used: initial denaturation at 95 °C for 5 minutes (min), followed by 30 cycles of denaturing at 95 °C for 30 seconds (sec), annealing at 55 °C for 30 sec and extension at 72 °C for 30 sec and a final extension step at 72°C for 10 min. In each 50 µl reaction, 10 pmol of each primer was used with the Expand High Fidelity^{PLUS} Taq polymerase kit (Roche, Basel, Switzerland) reagents: 1.5 mM $MgCl_2$; 200 µM dNTP mix; 2.5 U Expand HiFi^{PLUS} Taq polymerase and 1× Expand HiFi^{PLUS} Reaction Buffer (proprietary information). The first round of PCR generates the complementary sequence of the lhRNA sense strand as well as the loop sequence. For the second round of PCR, a 1:500 dilution of the round one amplicon (approximately 10 pg) was used as the template. The reverse primer sequence (R2) contained an 18-21 nt overlapping sequence with the loop region of the round one reverse primer and encodes the antisense strand of the lhRNA as well as 6 thymidine residues for Pol III transcriptional termination (Table 2.1). This overlap between each pair of reverse primers enables the extension of the PCR product to generate a U6 expressed lhRNA cassette with a transcription termination signal. The conditions for the second round PCR were the same as the first. PCR products were electrophoretically resolved on a 1.5% agarose gel run in Tris-Borate-EDTA (TBE) buffer (890 mM Tris base; 890 mM boric acid and 32 mM EDTA), and visualized on a UV transilluminator.

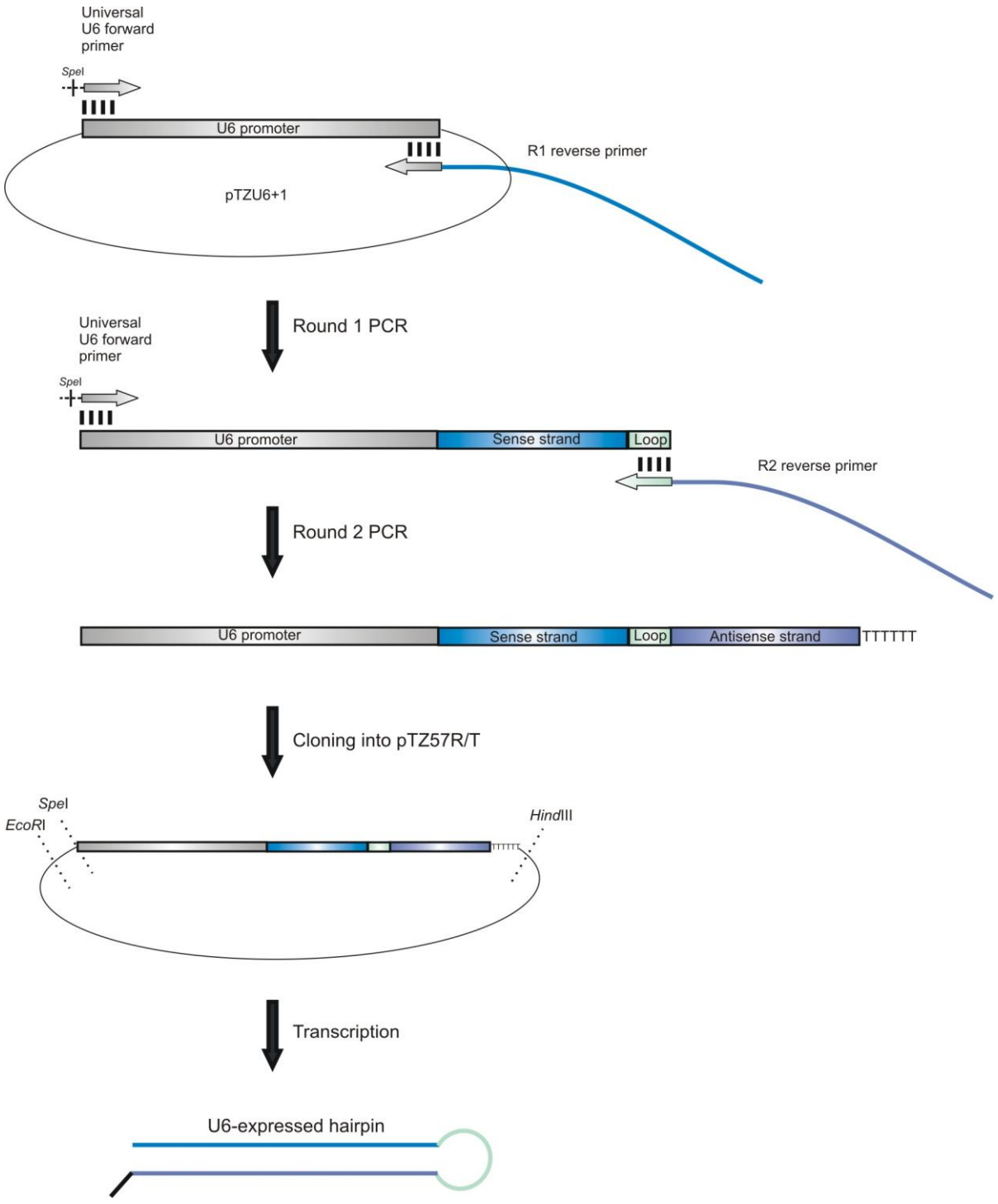


Figure 2.1: Schematic representation of the two step PCR cloning strategy used to generate lhRNA and shRNA expression cassettes. The first round of PCR generates an amplicon encoding the sense strand as well as the loop of the lhRNA. The second round amplicon encodes the sense, loop and antisense sequences and is cloned directly into the TA cloning vector pTZ57R/T. The inverted repeat sequences, once transcribed, fold back on themselves to form a hairpin duplex.

Table 2.1: Oligonucleotides used to generate lhRNAs encoding three putative siRNAs and corresponding shRNAs

Primer	Sequence (5'-3')	Length (nt)
U6 forward	CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG	37
lhRNA-*tat-rev-vif* R1	*CTTGAA***A**TGG**A**ATGT**A**TA**C**CTCT**A**AACAAGG**C**AGC**C**GAAGAG**A**CACAG**A**CAAGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCGGTG TTTCGTCCTTTCCACAA	94
lhRNA-*tat-rev-vif* R2	AAAAAAGCGGAGACAGCGACGAAGAGCTTGCCTGTGCCTCTTCAGCTACCTTGTTCAGAAGTACACATCCCACT*CTC TTGAA***A**TGG**A**ATGT**A**TA	94
lhRNA-*rev*-vif-*tat* R1	*CTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AACAAGG**C**AGC**C**GAAGAG**A**CACAG**A**CGGTG TTTCGTCCTTTCCACAA	94
lhRNA-*rev-vif-tat* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCTTGTTCAGAAGTACACATCCCACTTGCGGAGACAGCGACGAAGAGCT*CTC TTGA*AGC**C**CTTC**A**TC**A**C	94
lhRNA-*vif-tat-rev* R1	*CTTGA*AGG**C**AGC**C**GAAGAG**A**CACAG**A**CAAGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AACGGTG TTTCGTCCTTTCCACAA	94
lhRNA-*vif-tat-rev* R2	AAAAAAGTTCAGAAGTACACATCCCACTTGCGGAGACAGCGACGAAGAGCTTGCCTGTGCCTCTTCAGCTACCT*CTC TTGA*AGG**C**AGC**C**GAAGA	94
shRNA-*tat* R1	*CTCTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCGGTGTTTCGTCCTTTCCACAA	50
shRNA-*tat* R2	AAAAAAGCGGAGACAGCGACGAAGAGCT*CTCTTGA*AGC**C**CTTC**A**TC**A**C	48
shRNA-*rev* R1	*CTCTTGA*AGG**C**AGC**C**GAAGAG**A**CACAG**A**CGGTGTTTCGTCCTTTCCACAA	50
shRNA-*rev* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCT*CTCTTGA*AGG**C**AGC**C**GAAGA	48
shRNA-*vif* R1	*CTCTTGAA***A**TGG**A**ATGT**A**TA**C**CTCT**A**AACGGTGTTTCGTCCTTTCCACAA	50
shRNA-*vif* R2	AAAAAAGTTCAGAAGTACACATCCCACT*CTCTTGAA***A**TGG**A**ATGT**A**TA	48
lhRNA-*rev*-vif-*tat b* R1	*CTCTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AAC*AGG**C**AGC**C**GAAGAG**A**CACAG**A**CGG TGTTTCGTCCTTTCCACAA	95
lhRNA-*rev-vif-tat b* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCT*GTTCAGAAGTACACATCCCACTTGCGGAGACAGCGACGAAGAGCT*CTC TTGA*AGC**C**CTTC**A**TC	91
lhRNA-*rev*-vif-*tat c* R1	*CTCTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCTAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AAC*AGG**C**AGC**C**GAAGAG**A**CACAG**A**CG GTGTTTCGTCCTTTCCACAA	96

lhRNA-*rev-vif-tat c* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCT*GTTCAGAAGTACACATCCCACTTAGCGGAGACAGCGACGAAGAGCT*CT* *CTTGA*AGC**C**CTTC**A**TC**A**	93
lhRNA-*rev*-vif-*tat d* R1	*CTCTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCTTAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AAC*AGG**C**AGC**C**GAAGAG**A**CACAG**A**C GGTGTTTCGTCCTTTCCACAA	97
lhRNA-*rev-vif-tat d* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCT*GTTCAGAAGTACACATCCCACTTAAGCGGAGACAGCGACGAAGAGCT*C* *TCTTGA*AGC**C**CTTC**A**TC**A**	94
lhRNA-*rev*-vif-*tat e* R1	*CTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCAGTTAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AACA**G**C**A**GC**C**GAAGAG**A**CACAG**A**C GGTGTTTCGTCCTTTCCACAA	96
lhRNA-*rev-vif-tat e* R2	AAAAAAGCCTGTGCCTCTTCAGCTACC**GTTCAGAAGTACACATCCCACTTAACTGCGGAGACAGCGACGAAGAGC TC*TCTTGA*AGC**C**CTTC**A**TC**A**C	96
lhRNA-*rev*-vif-*tat f* R1	*CTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCTTAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AACAAGG**C**AGC**C**GAAGAG**A**CACAG**A**CGG TGTTTCGTCCTTTCCACAA	96
lhRNA-*rev-vif-tat f* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCTTGTTCAGAAGTACACATCCCACTTAAGCGGAGACAGCGACGAAGAGCT*C* *TCTTGA*AGC**C**CTTC**A**TC**A**C	96
lhRNA-*rev*-vif-*tat g* R1	*CTTGA*AGC**C**CTTC**A**TC**A**C**T**ATC**C**CCGCTTAA**A**TGG**A**ATGT**A**TAC**C**TCT**A**AACTAAGG**C**AGC**C**GAAGAG**A**CACAG**A**CG GTGTTTCGTCCTTTCCACAA	97
lhRNA-*rev-vif-tat g* R2	AAAAAAGCCTGTGCCTCTTCAGCTACCTTAGTTCAGAAGTACACATCCCACTTAAGCGGAGACAGCGACGAAGAGCT *CTCTTGA*AGC**C**CTTC**A**TC**A**C	97

Underlined sequences represent restriction endonuclease recognition sites. The loop sequence is italicized. Highlighted sequences indicate the overlapping regions between each pair of reverse primers. Bold letters indicate sites of G:U mismatch introductions in the sense strand. Double underlined letters indicate nucleotide spacers inserted between siRNA encoding sequences and asterisks indicate positions where nucleotide spacers have been deleted.

Round 2 amplicons that were visualized as a single band at the correct molecular weight on an agarose gel were ligated directly into the TA cloning vector pTZ57R/T using the InsTAclone™ PCR Cloning Kit (Fermentas, WI, USA). A 3:1 molar ratio of insert to vector (0,09 pmol insert:0.03 pmol vector) was ligated using 5 U T4 DNA ligase and T4 DNA ligase buffer (40 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT and 0.5 mM ATP [pH 7.8 at 25°C]) in a 20 µl volume and the reaction was incubated at room temperature overnight. A 7 µl aliquot of the ligation reaction was then used to transform 100 µl of chemically competent DH5α *E.coli* (Appendix A1.1). Transformed cells were plated on Luria Bertani agar plates containing ampicillin (Gibco, BRL, UK), X-Gal (5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside) (Sigma, MO, USA) and IPTG (Isopropyl-β-D-1-thiogalactopyranoside) (Roche, Basel, Switzerland) (Appendix A1.1) and incubated at 37°C overnight.

Individual white colonies were cultured in 3 ml of ampicillin-containing Luria Bertani broth (Appendix A1.1) at 37°C overnight with shaking. Plasmid DNA was then purified using the High Pure Plasmid Isolation Kit for small-scale (mini) preparations of purified plasmid DNA (Roche, Germany) (Appendix A1.2.1). Plasmids were screened for the presence of inserts and their relative orientation by digesting plasmid DNA with *EcoR*I and *Spe*I or with *Hind*III and *Spe*I (Fermentas, WI, USA) (Figure 2.1). Digested plasmids were resolved on a 3% agarose gel. Positive clones resulting in digested fragments of the desired molecular weight were verified by sequencing with M13 forward and reverse primers by automated cycle sequencing (Inqaba Biotech, South Africa), M13 forward: 5'-GTAAAACGACGGCCAG -3', M13 reverse: 5'- CAGGAAACAGCTATGAC -3'. The protocol for sequencing plasmids encoding lhRNAs included modifications in the cycle to reduce enzyme depletion and DMSO to stabilise the hairpin and lower melting temperatures. Clones with the correct sequence were cultured in 200 ml ampicillin-containing LB overnight for large-scale preparation of plasmid DNA using the QIAGEN Plasmid Maxi Kit (QIAGEN, CA, USA) (Appendix A1.2.2).

2.2.2 Dual luciferase fusion reporter plasmids

To determine knockdown induced by each putative siRNA encoded by the lhRNA sequences, a set of dual luciferase-based reporter vectors were constructed containing the individual target sequences of each siRNA or the target sequences of all three siRNAs in tandem. The target vectors were constructed using the psiCHECK^{TM}-2 (Promega, WI, USA) plasmid which comprises an HSV TK-driven Firefly luciferase cassette as well as an SV40-driven *Renilla* luciferase cassette with a multiple cloning site situated within the 3' UTR. Following insertion of a target sequence within the multiple cloning site, knockdown can be measured as a ratio of *Renilla* luciferase to Firefly luciferase expression. To insert the target sequences into the 3' UTR of the *Renilla* luciferase open reading frame, the psiCHECK^{TM}-2 (Promega, WI, USA) plasmid backbone was initially prepared by digestion with 15 U each of *Xho*I and *Not*I with Buffer O (50 mM Tris-HCl [pH 7.5 at 37°C]; 10 mM $MgCl_2$; 100 mM NaCl and 0.1 mg/ml BSA) (Fermentas, WI, USA) in a 50 µl volume for 1.5 hours at 37°C. The digested plasmid was then dephosphorylated by adding 5 U of Antarctic phosphatase (AP) (NEB, MA, USA) to the digestion reaction together with AP buffer (50 mM bis-tris-propane-HCl, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ pH 6.0) to a 60 µl total volume. The reaction was incubated at 37°C for a further 10 min. The phosphatase reaction was heat-inactivated for 15 min at 65°C followed by resolution of the linear psiCHECK vector backbone DNA on a 0.8% agarose gel. The double-digested vector backbone band was then excised and purified using the MinElute^{TM} Gel Extraction kit (QIAGEN, CA, USA) according to the manufacturer's instruction (Appendix A1.3).

The individual *tat*, *rev* and *vif* target sequences were generated by amplifying the desired target sequence from the HIV-1 subtype B molecular clone pNL4-3 (Adachi et al. 1986) with gene specific primers in a standard PCR reaction using the same thermocycling conditions and reagents described in 2.2.1. Each forward primer contained a 5' *Xho*I restriction site linker and each reverse primer contained a 5' *Not*I restriction site linker (Table 2.2). PCR amplicons were individually ligated to pTZ57R/T as described in 2.2.1. and clones were screened for inserts by digestion with *Xho*I and *Not*I

(Fermentas, WI, USA). The insert fragment was then excised from an agarose gel and purified using the MinElute™ Gel Extraction kit (QIAGEN, CA, USA) according to the manufacturer's instructions (Appendix A1.3).

To generate a combined target sequence containing the *tat*, *rev* and *vif* individual target sequences adjacent to one another, partially overlapping oligonucleotides (Table 2.2) (10 µM each) encoding the collective target sequence were treated with T4 Polynucleotide Kinase (PNK) (Fermentas, WI, USA) with Reaction buffer A (50 mM Tris-HCl [pH 7.6 at 25°C]; 10 mM $MgCl_2$; 5mM DTT; 0.1mM spermidine and 0.01 mM EDTA) for 30 min at 37°C. The oligonucleotides were annealed by slow cooling to room temperature following heating to 95°C for 5 min. The forward oligonucleotide contained a 5' *Xho*I restriction site linker and the reverse oligonucleotide contained a 5' *Not*I restriction site linker. To ligate the vector backbone to the target sequence inserts, 60 fmol (approximately 50 ng) of purified psiCHECK backbone fragment was ligated with 180 fmol of each digested PCR fragment or annealed dsDNA fragment, both containing 5' *Xho*I and 3' *Not*I cohesive ends, in a 20 µl reaction volume using 10 U T4 DNA ligase (NEB, MA, USA). A 10 µl aliquot of the ligation reaction was then used to transform 100 µl chemically competent DH5α *E.coli* as described previously. Transformed cells were plated onto ampicillin-containing agar plates and incubated overnight at 37°C. Purified plasmids from individually cultured colonies were screened by digestion with *Xho*I and *Not*I (Fermentas, WI, USA) and clones with the correct sized insert were subsequently verified by automated cycle sequencing (Inqaba Biotech, South Africa) using a forward primer specific to the *Renilla* luciferase ORF: 5' GAGGACGCTCCAGATGAAATG 3'.

Reporter vectors containing the HXB2 as well as the FV5 derived *vif* target sequences were constructed using a similar strategy to that described above. Complementary oligonucleotides (Table 2.2) were treated with PNK (Fermentas, WI, USA), annealed and cloned directly into the *Xho*I-*Not*I sites of the dephosphorylated psiCHECK™-2 linearised vector as previously described. An *Eco*RV site was incorporated into the overlapping region of each oligonucleotide to facilitate screening of plasmids containing inserted target sequences.

Table 2.2: Primers used to amplify target sequences for directional cloning into psiCHECK™-2

Primer	Sequence (5' to 3')	Length (nt)
tat target forward	GATCTCGAGAGTGTTGCTTTCATTGCCAA	29
tat target reverse	GATCGCGGCCGCGCATTACATGTACTACTTACTGCTT	37
rev target forward	GATCTCGAGAAGGTGGAGAGAGAGACAGA	29
rev target reverse	GATCGCGGCCGCCACCAATATTTGAGGGCTTC	32
vif target forward	GATCTCGAGATTTCAAGGAAAGCTAAGGA	29
vif target reverse	GATCGCGGCCGCAATGCCAGTCTCTTTCTCCT	32
tat-rev-vif target forward	GATCTCGAGGCGGAGACAGCGACGAAGAGCTTGCCTGTGCCTCTTCAGCTACC	53
tat-rev-vif target reverse	GATCGCGGCCGCGTGGGATGTGTACTTCTGAACAAGGTAGCTGAAGAGGCACAGGC	56
FV5 *vif* target forward	CTCGAGATATCGTTCAGAAGTACATATTCCATGC	34
FV5 *vif* target reverse	GCGGCCGCATGGAATATGTACTTCTGAACGATATC	35
HXB2 *vif* target forward	CTCGAGATATCGTTCAGAAGTACACATCCCACGC	34
HXB2 *vif* target reverse	GCGGCCGCGTGGGATGTGTACTTCTGAACGATATC	35

Underlined sequences represent the *Xho*I and *Not*I restriction endonuclease recognition sites in the forward and reverse primers respectively as well as the *EcoR*V restriction endonuclease recognition site within the overlapping region of forward and reverse oligonucleotides. Highlighted sequences indicate complementary overlapping regions between the pair of oligonucleotides.

2.2.3 Assessing the efficacy of expressed lhRNAs in cell culture

Transfection of cultured mammalian cells

The HEK293 cell line was maintained in Dulbecco's modified Eagle's medium (DMEM), (BioWhittaker, MD, USA) supplemented with 10% heat inactivated fetal calf serum (FCS), (Delta Bioproducts, Johannesburg, SA) at 37°C and 5% CO_2 as described in Appendix A1.4. Cells were seeded at 120 000 cells per well in 24-well tissue culture plates (Nunclon™ Δ Surface, Nunc, Denmark) 24 hours prior to transfection in antibiotic-free medium. Hairpin encoding plasmids (750 ng) together with dual luciferase target reporter plasmids (150 ng) were co-transfected in a 5:1 ratio. One hundred nanograms of plasmid pCI-eGFP (Passman et al. 2000), a plasmid expressing enhanced green fluorescent protein (GFP) from a CMV promoter, was also co-transfected to control for transfection efficiency. Each transfection was performed in triplicate. Transfections were carried out using 1 µl Lipofectamine™ 2000 (Invitrogen, CA, USA) per 1 µg DNA and 0.2× OptiMEM (Gibco, BRL, UK) (Appendix A1.5). Twenty four hours after transfection, culture medium was replaced and 48 hours after transfection cells were observed by fluorescence microscopy using the Axiovert 100 M microscope (ZEISS, Germany) to visualize GFP to ensure equal transfection efficiency.

Dual luciferase reporter assay

Reporter assays (Figure 2.2) were carried out 48 hours post transfection using the Dual-Luciferase® Reporter Assay System (Promega, WI, USA) according to the manufacturer's instructions. Culture medium was removed and cells were lysed with 100 µl Passive Lysis Buffer for 20 min with gentle agitation. Following lysis, 10 µl cell lysate was added to Costar® 96 well flat bottom assay plates (Corning Incorporated, NY, USA). Firefly and *Renilla* luciferase activity were measured independently using 50 µl 1× Luciferase Reagent II and Stop & Glo® solutions (proprietary information) respectively in a Veritas™ Microplate Luminometer (Turner Biosystems, CA, USA). All *Renilla* luciferase values were normalised to background Firefly luciferase values. The average expression ratio for pTZU6+1

(Bertrand et al. 1997), a control plasmid containing the U6 promoter with no RNAi effector sequence was set to 100%, and relative expression levels of other samples were calculated accordingly.

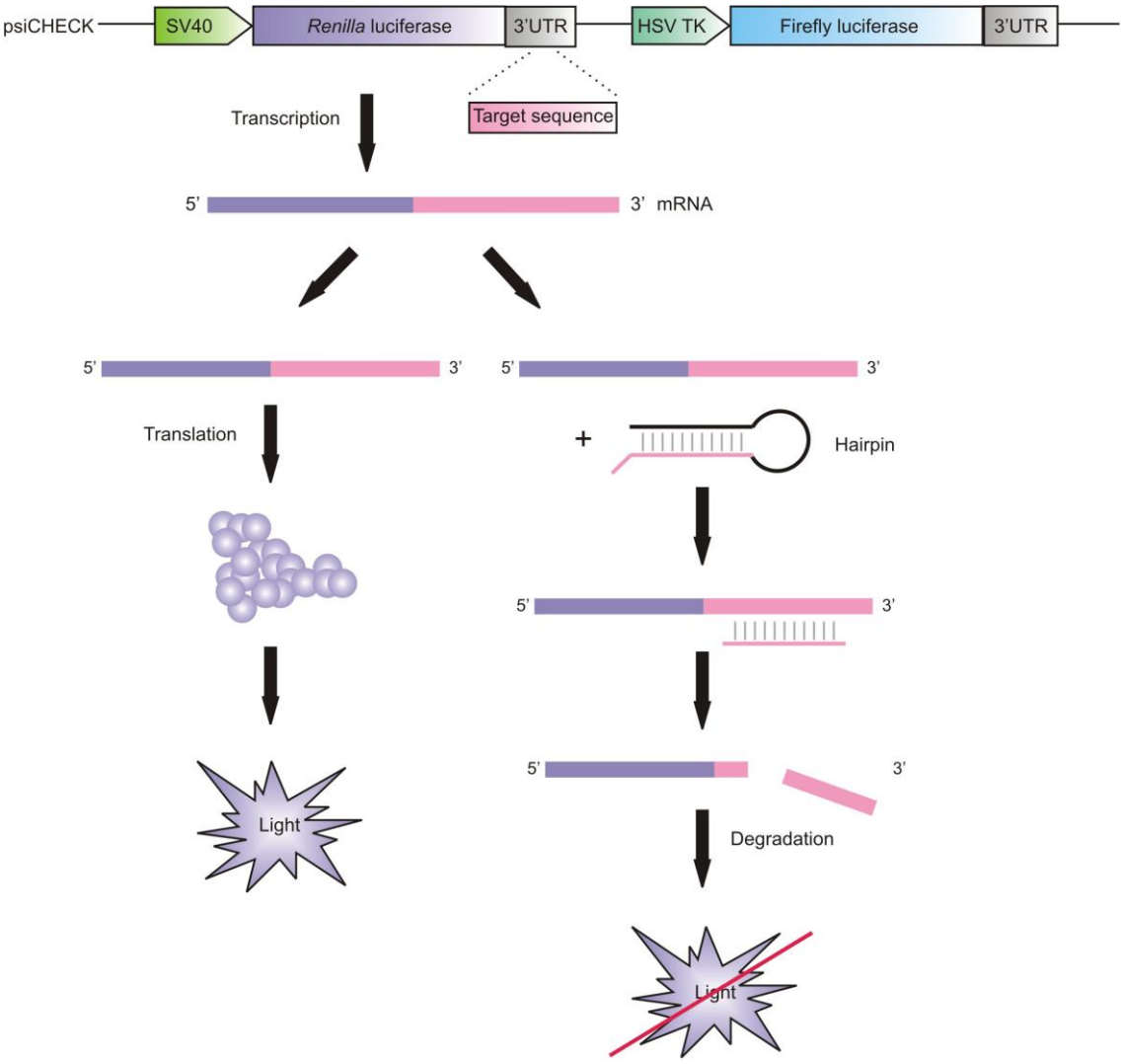


Figure 2.2: Diagrammatic representation of the dual luciferase reporter assay. A target sequence of choice is cloned into the 3' UTR of the human *Renilla* luciferase gene so that if targeted by a complementary shRNA or lhRNA the mRNA transcript is degraded and *Renilla* expression inhibited. *Renilla* luciferase activity is normalised to Firefly luciferase which is expressed independently within the same vector.

2.2.4 Detection of processed anti-HIV-1 hairpin sequences using polyacrylamide gel electrophoresis (PAGE) and northern blot analysis

HEK293 cells were maintained as described (Appendix A1.4). For northern blot analysis cells were seeded to approximately 70% confluence in 60 cm^2 Costar® tissue culture plates (Corning Inc, NY, USA) in antibiotic-free medium. Cells were transfected 24 hours later with 19 µg hairpin expression plasmids together with 1 µg pCI-eGFP as described in Appendix A1.5.

Forty eight hours after transfection, total RNA was extracted using TriReagent™ (Sigma, MO, USA) according to the manufacturer's instructions (Appendix A1.6). Standard RNA handling procedures to avoid RNase contamination were followed. Thirty micrograms of each RNA sample was resolved on a 15% polyacrylamide gel prepared at a 1:19 ratio of *bis*:acrylamide with 8 M urea and TBE buffer (890 mM Tris base; 890 mM boric acid and 32 mM EDTA). Decade Marker™ (Ambion, TX, USA) was used as a molecular weight marker and was prepared as per the kit's instructions (Appendix A1.7). Once total RNA was separated the polyacrylamide gel was stained with 10 mg/ml ethidium bromide (Sigma, MO, USA) for 5 minutes and visualized on a UV transilluminator (Kodak Gel Logic 200 Imaging System) to check RNA integrity and quality.

The RNA was transferred to Hybond-N+ positively charged nylon membrane (GE Healthcare, NJ, USA) using the Semi-dry Electroblotting Unit Z34,050-2 (Sigma, MO, USA) at 3.3 mA/cm^2 for one hour. RNA was then UV cross-linked to the membrane at 2000 × 100 µJ/cm^2 in a Ultra-Violet Products (UVP) UV cross linker (UVP, Inc., CA, USA) and then the membrane was baked at 80°C for 1 hour.

Oligonucleotide probes (20 µM) (Table 2.3) were labeled with γ-^{32}P-ATP (6000 Ci/mmol) (PerkinElmer, MA, USA) in 20 µl total volume using 5 U Polynucleotide Kinase (PNK) and PNK buffer (50 mM imidazole HCl (pH 6.6); 10 mM $MgCl_2$; 5 mM DTT; 0.1 mM spermidine and 0.1 mM EDTA) (Promega, WI, USA) at 37°C for 20 min. The reaction was diluted to 50 µl and purified by centrifugation through a sephadex G-25 column for 2 min at 2000 rpm.

Membranes were prehybridised in 10 ml/100 cm^2 of pre-warmed Rapid-Hyb buffer (GE Healthcare, NJ, USA) at 42°C for 20 min. Following pre-hybridisation the labelled probe was added to

the buffer and incubated with the membrane overnight at 42°C. Following overnight hybridisation, membranes were washed once with 0.1% SDS (sodium dodecylsulphate) and 5% SSC (NaCl, Na-citrate) (Sigma, MO, USA) in a volume of 50 ml at room temperature for 20 min and then subsequently washed twice with 0.1% SDS and 1% SSC in a volume of 50 ml at 42°C for 15 min each before exposure to Fuji x-ray film for 24-72 hours at -80°C. Membranes were stripped with 50 ml 1% SDS for 30 min at 80°C and then re-probed. A probe complementary to U6 snRNA was used to verify equal loading of each RNA sample.

Table 2.3: Oligonucleotide probes used to detect lhRNA guide sequences

Probe	Sequence (5' to 3')	Length (nt)
rev	GCCTGTGCCTCTTCAGCTACCT	23
vif	GTTCAGAAGTACACATCCCACTT	23
tat 1	GCGGAGACAGCGACGAAGAGCTT	23
tat 2	ACTTGCGGAGACAG	14
tat 3	GCGCGCGGAGACAG	14
U6 snRNA	TAGTATATGTGCTGCCGAAGCGAGCA	26

LNA nucleotides are underlined.

2.2.5 HIV viral challenge assay

U87.CD4.CCR5 cells were maintained in DMEM supplemented with 15% FCS, 1 µg/ml puromycin, 300 µg/ml G418, glutamine, penicillin and streptomycin as described in Appendix A1.4. Cells were seeded at 100 000 cells per well in 12-well tissue culture plates (Nunclon™ Δ Surface, Nunc, Denmark) 24 hours prior to transfection in antibiotic-free medium. Cells were then transfected with 900 ng hairpin expression plasmids or pTZU6+1 negative control together with 100 ng pCI-eGFP as described in Appendix A1.5.

FV5 (GenBank accession number DQ382363) is a primary HIV-1 CCR5-utilizing subtype C virus that was isolated from a drug-naïve HIV-positive AIDS patient admitted to the Johannesburg Hospital AIDS clinic, and propagated by standard PBMC co-culture techniques (HIV Pathogenesis Research Unit). The co-receptor tropism of FV5 was established genotypically by automated sequencing of the V3 loop of the viral *env* gene (GenBank® accession number 05ZAFV5), and confirmed phenotypically by MT-2 fusion assay (HIV Pathogenesis Research Unit).

Viral stocks were then expanded by infecting U87.CD4.CCR5 cells in 75 cm^2 tissue culture flasks (Corning Incorporated, NY, USA). Once cells reached approximately 60% confluence, culture medium was removed and 750 µl viral stock was suspended in 5 ml medium and added to the cells for overnight incubation under standard cell culturing conditions. After 24 hours culture medium was removed, wells were washed three times with phosphate buffered saline (PBS) to remove residual virus and 20 ml fresh medium was added to the cells. Cells were propagated for a further 72 hours before high titer culture supernatants were harvested and dispensed into 500 µl aliquots. The 50% tissue culture infectious dose ($TCID_{50}$) of the harvested supernatants was then determined as described in Appendix A1.9. The $TCID_{50}$ assay quantifies viable virus by measuring p24 antigen concentration (Appendix A1.8) following a dilution assay.

Twenty four hours after transfection, U87.CD4.CCR5 cells were infected with FV5 at a $TCID_{50}$ 1000. An aliquot of titered virus was serially diluted to a final $TCID_{50}$ 1000 per ml. Culture medium was then removed from transfected cells and 1 ml of the viral dilution was dispensed into each transfected well. Twenty four hours after infection cells were washed three times using PBS to remove residual virus from cultures and 1 ml fresh medium was added to each well. At days 0 (day of washing), and specified time points thereafter, 100 µl of supernatant was collected from each well and analysed by ELISA (Murex Biotech LTD, Dartford, UK) for p24 antigen production as a marker of viral replication.

2.2.6 Quantitative real-time reverse transcription PCR (qRT-PCR) to detect induction of interferon related genes

HEK293 cells were maintained as described in Appendix A1.4. Cells were seeded at 120 000 cells per well in 24-well tissue culture plates (Nunclon™ Δ Surface, Nunc, Denmark) 24 hours prior to transfection in antibiotic-free medium. Cells were transfected with 900 ng hairpin expression plasmids or poly (I:C) (Sigma, MO, USA) together with 100 ng pCI-eGFP as described in Appendix A1.5. Poly (I:C) is a synthetic double-stranded RNA analogue and was used as a positive control for induction of *IFN-β* expression.

To measure concentrations of IFN-related genes in cells transfected with lhRNA and shRNA expressing plasmids, total RNA was extracted from cells 48 hours after transfection using TriReagent™ (Sigma, MO, USA) according the manufacturer's instructions (Appendix A1.6). Standard RNA handling procedures to avoid RNase contamination were followed and RNA pellets were resuspended in 10 µl nuclease free water. RNA samples were DNase treated with 2 U DNase (Fermentas, WI, USA) for 60 min at 37°C after which DNase was inactivated during a 5 min incubation at 95°C. RNA was reverse transcribed using the Sensiscript® reverse transcription kit (QIAGEN, CA, USA). In a 10 µl reaction, 50 ng DNase treated RNA was incubated with 0.5 U Sensiscript RT; 1× RT buffer (proprietary information), 10 µM oligo-dT primer and 5 mM of each dNTP at 37°C for 60 min. Following reverse transcription *GAPDH* and *IFN-β* mRNA were amplified by real-time PCR using the SYBR® Green Jumpstart Taq Ready Mix kit (Sigma. MO, USA) in a Roche Lightcycler V.2. The 20 µl reaction consisted of 2 µl cDNA generated from the RT reaction described above, 10 µM of each primer and SYBR Green Ready Mix (0.5 U Taq DNA Polymerase; 10 mM Tris-HCl pH 8.3; 50 mM KCl; 3.5 mM $MgCl_2$; 0.2 mM each dNTP; stabilizers; internal reference dye and SYBR Green). The following primer sets were used to amplify *IFN-β* and *GAPDH* mRNA: *IFN-β* Forward: 5'- TCC AAA TTG CTC TCC TGT TGT GCT -3', *IFN-β* Reverse: 5'- CCA CAG GAG CTT CTG ACA CTG AAA A -3', *GAPDH* Forward: 5'- AGG GGT CAT TGA TGG CAA CAA TAT CCA -3' and *GAPDH* Reverse: 5'- TTT ACC AGA GTT AAA AGC AGC CCT GGT G -3'. Thermal cycling parameters consisted of a hot start for 30 seconds at 95 °C followed by 50 cycles of denaturation at 95°C for 10 sec, annealing at 58°C for

10 sec and extension at 72°C for 10 sec. Specificity of the PCR products was verified by melting curve analysis and *IFN-β* values were normalised against *GAPDH* values.

2.2.7 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (GraphPad, Software, Inc., CA, USA). Differences were considered significant when $p<0.05$ and was determined using either an unpaired Student's t-test or by ANOVA. The results of the $TCID_{50}$ assay were analysed using the Spearman-Karber statistical formula as described in Appendix A1.9.

2.3 Results

2.3.1 Design of anti-HIV-1 lhRNAs

Long hairpin RNAs (lhRNAs) with stem lengths of 69 bp, a 7 nt terminal loop sequence and a 2-3 nt terminus were designed to incorporate three independent anti-HIV siRNA sequences, such that three unique and non-overlapping 21-23 nt siRNAs could potentially be generated following three successive Dicer cleavage reactions (Figure 2.3). Sites targeted by each putative guide strand were based on previously published effective shRNAs which target conserved regions in the HIV-1 genome shown to be highly susceptible to RNAi. These target sites included an overlapping *tat*/*rev* site (Lee et al. 2002a) referred to hereafter as *tat*; an overlapping *rev*/*env* site (Lee et al. 2002a) referred to hereafter as *rev;* and a site within the *vif* ORF (Lee et al. 2005). Each lhRNA was designed to be transcribed from the U6 snRNA RNA Pol III promoter. RNA Pol III promoters naturally produce small transcripts which have a defined transcription start site and a termination signal consisting of ≥4 consecutive thymidine residues thus producing a well defined transcript. Furthermore, termination results in a 2 nt 3' overhang, which is optimal for Dicer recognition (Ma et al. 2004), nuclear export (Zeng and Cullen 2004) and the evasion of RIG-1 recognition (Marques et al. 2006). RNA Pol III transcripts therefore serve as ideal substrates for Dicer processing.

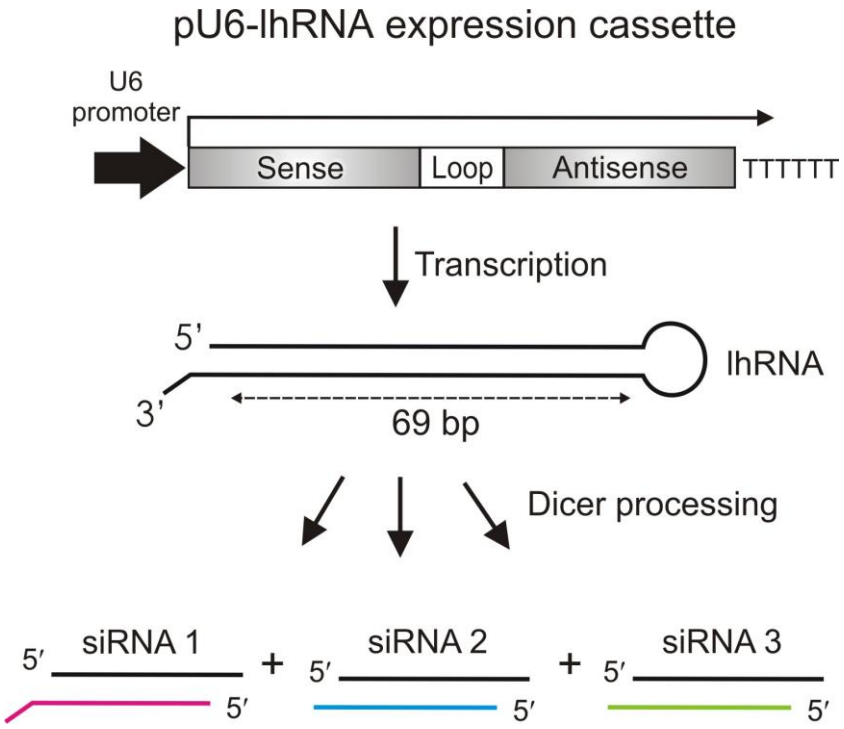


Figure 2.3: Schematic representation of an lhRNA expression cassette showing the upstream U6 promoter and the predicted lhRNA structure post transcription. Long hairpin RNAs were designed to have a 69 bp stem allowing for three predicted Dicer cleavage reactions and the subsequent generation of three siRNAs.

Because of the incorporation of independent siRNA sequences within the hairpin duplex, plasmids encoding three variants of the hairpin were constructed: lhRNA-*tat-rev-vif*, lhRNA-*rev-vif-tat* and lhRNA-*vif-tat*-rev, allowing for each unique siRNA sequence to be sequentially placed at each possible position within the hairpin duplex (Figure 2.4 A). It has been shown that human Dicer may favour the production of siRNAs positioned at the base of the stem (Barichievy et al. 2007; Weinberg et al. 2007), and the assessment of three lhRNA positional variants enables the assessment of any possible sequence or positional effects. G:U wobble base mismatches were introduced at regular intervals along the sense strand of the hairpin duplex (C is replaced by a T; A is replaced by a G in the DNA sequence) (Figure 2.4) to facilitate propagation of inverted repeat sequences in *E.coli* and sequencing of plasmids containing correctly cloned lhRNAs. Individual shRNAs matching the putative siRNA sequences included in the lhRNAs were constructed with corresponding G:U mismatches and loop sequences to act as positive controls (Figure 2.4 B).

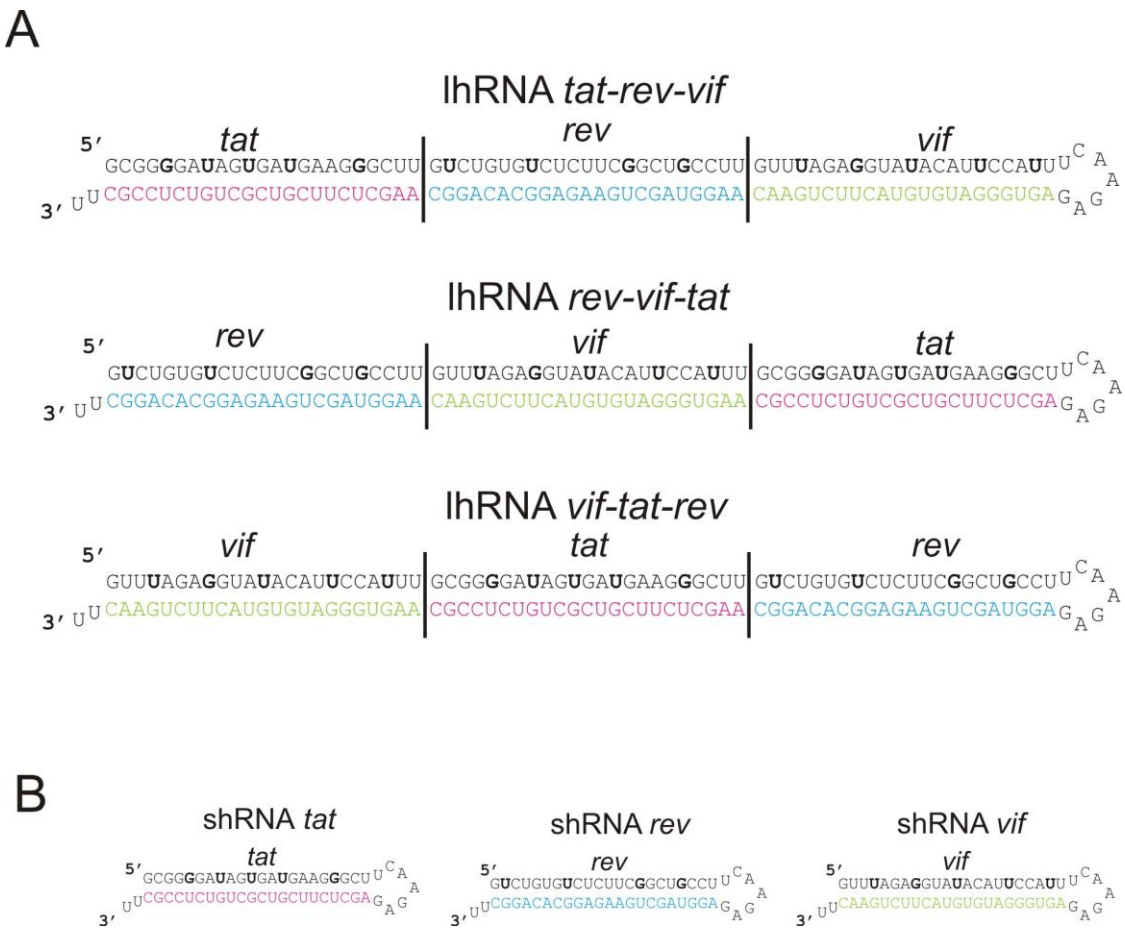


Figure 2.4: Design of anti-HIV lhRNAs incorporating three putative siRNAs. (A) Anti-HIV lhRNAs were designed to encode three 23 bp siRNA sequences targeted to the *tat*, *rev* and *vif* open reading frames of HIV-1. Three variants of the lhRNA were designed to allow for every possible spatial arrangement of putative siRNAs. Introduced G:U wobble base pairs are indicated in bold. **(B)** Individual shRNAs encoding corresponding siRNAs were designed to incorporate equivalent G:U mismatches and the same loop sequence.

2.3.2 Inhibitory efficacies of lhRNAs encoding three putative siRNAs

The long hairpin RNA sequences were assessed for their ability to inhibit the respective targets of each encoded siRNA *in vitro.* A non-specific lhRNA targeted against the TAR loop of HIV (Barichievy et al. 2007) was used as a negative control (lhRNA control) and this target sequence was not included in the psiCHECK reporter vectors. Target-specific knockdown was determined as a ratio of *Renilla*

luciferase to Firefly luciferase, and the values were normalised to pTZU6+1 (mock). When assessing knockdown of the individual target sequences, all of the lhRNA expression cassettes showed significant, yet variable inhibition of fusion gene activity (Figure 2.5 A). When only the *tat* target sequence was inserted downstream from the *Renilla* luciferase reporter gene, shRNA-*tat* mediated a 90% reduction in the *Renilla* to Firefly luciferase ratio while shRNA-*rev* and shRNA-*vif* showed no effect at all. This result was expected and confirms target specificity of control shRNAs. lhRNA-*tat-rev-vif* contains the *tat* siRNA sequence in the first position of the hairpin stem, distal from the loop sequence, and showed the greatest reduction (approximately 50%) in *Renilla-tat* fusion gene expression, whereas lhRNA-*vif-tat-rev* and lhRNA-*rev-vif-tat* were less effective. A similar trend was observed when assessing the inhibitory efficacies of hairpin expression cassettes against the individual *rev* and *vif* target sequences. The shRNAs showed target specific knockdown in all cases and the lhRNAs diminished target gene activity approximately 30-85%, with the strongest silencing effects consistently observed by the lhRNA containing the siRNA complementary to the target sequence positioned at the base of the hairpin stem. A gradual loss of RNAi activity was observed from siRNAs positioned in the second and third positions of the hairpin duplex. Inhibition of a specific target afforded by lhRNAs was also comparable to that of individual shRNAs only when the lhRNA contained the siRNA complementary to the target at the base of the hairpin stem. The non-specific lhRNA control showed no effect on *Renilla* to Firefly luciferase ratios as expected.

When all three target sequences were cloned in tandem downstream of the *Renilla* luciferase ORF to create a combined target, both shRNA and lhRNA constructs reduced *Renilla* to Firefly luciferase ratios by approximately 90% (Figure 2.5 B), which indicates an additive knockdown effect by the siRNAs within each lhRNA. A bias in RNAi activity was thus observed which correlates with the position of siRNA sequences within the hairpin stem and supports previous data using lhRNAs, (Barichievy et al. 2007; Weinberg et al. 2007), which also showed diminishing silencing efficacy from the base towards the loop of the hairpin stem.

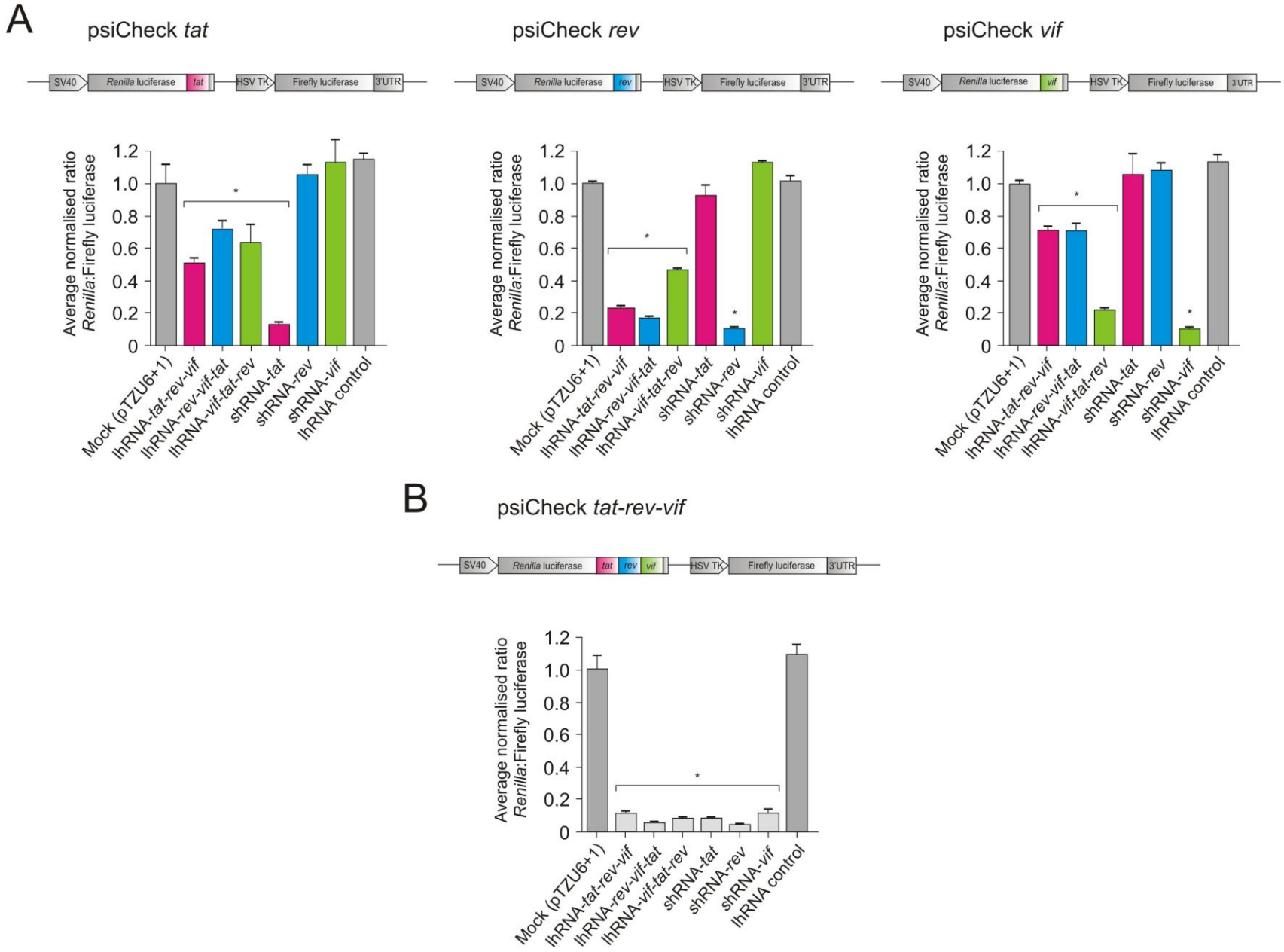

A
psiCheck tat
SV40
Renilla luciferase
tat
HSV TK
Firefly luciferase
3'UTR
Average normalised ratio
Renilla:Firefly luciferase
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Mock (pTZU6+1)
lhRNA-tat-rev-vif
lhRNA-rev-vif-tat
lhRNA-vif-tat-rev
shRNA-tat
shRNA-rev
shRNA-vif
lhRNA control
psiCheck rev
rev
psiCheck vif
vif
B
psiCheck tat-rev-vif

Figure 2.5: Inhibitory efficacy of lhRNAs encoding three putative siRNAs. Dual luciferase reporter assays were used to measure target knockdown following co-transfection of HEK293 cells with lhRNA expression plasmids together with psiCHECK reporter plasmids containing the *tat*, *rev* or *vif* specific target sequences **(A)**, or a combination of all of the target sequences in tandem **(B)**, inserted downstream of the *Renilla* luciferase open reading frame. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with pTZU6+1 (mock) (*, $p<0.05$, t-test relative to mock transfected control).

2.3.3 Detection of processed siRNAs from lhRNA precursors

To analyse processing of anti-viral sequences generated from hairpin expression cassettes, PAGE northern blot analysis was carried out on total RNA extracted from HEK293 cells transfected with lhRNA or control shRNA expressing plasmids or with pTZU6+1 (mock). Figure 2.6 shows the signals obtained following hybridisation of a membrane containing immobilised RNA with three probes complementary to each of the guide strands of processed siRNAs, and exposure to X-ray film. Each probe was able to detect mature guide strands processed from shRNA controls and the guide strands generated from shRNA precursors were detected at a higher concentration than those produced from lhRNA transcripts. This indicates that Dicer processes shRNA substrates more efficiently than lhRNA substrates. In the context of lhRNAs, the *tat* and *vif* probes only detected siRNAs generated from the base position of the hairpin stem. The *rev* probe detected siRNAs that were processed from all three positions within the three lhRNAs, however, processing occurred in a gradient with most siRNA being generated from the base of the stem and decreasing quantities of siRNA generated from the loopside of the hairpin duplex. This observation provides an explanation for the gradient in knockdown efficiency seen in Figure 2.5. The processing of lhRNA duplexes by Dicer appears to yield minimal or undetectable quantities of siRNA from the second and third positions of the stem and minimal RNA activity is thus observed from these regions of the hairpin. This phenomenon is not due to inefficient transcription of long hairpin transcripts or their relative instability since all probes are able to detect full length lhRNA precursor RNA, but is likely to be a result of inefficient Dicer turnover for multiple cleavage reactions. As expected, no signal was obtained from RNA extracted from mock transfected cells and signals obtained following hybridisation with a probe complementary to U6 snRNA showed equal loading of each RNA sample (Figure 2.6).

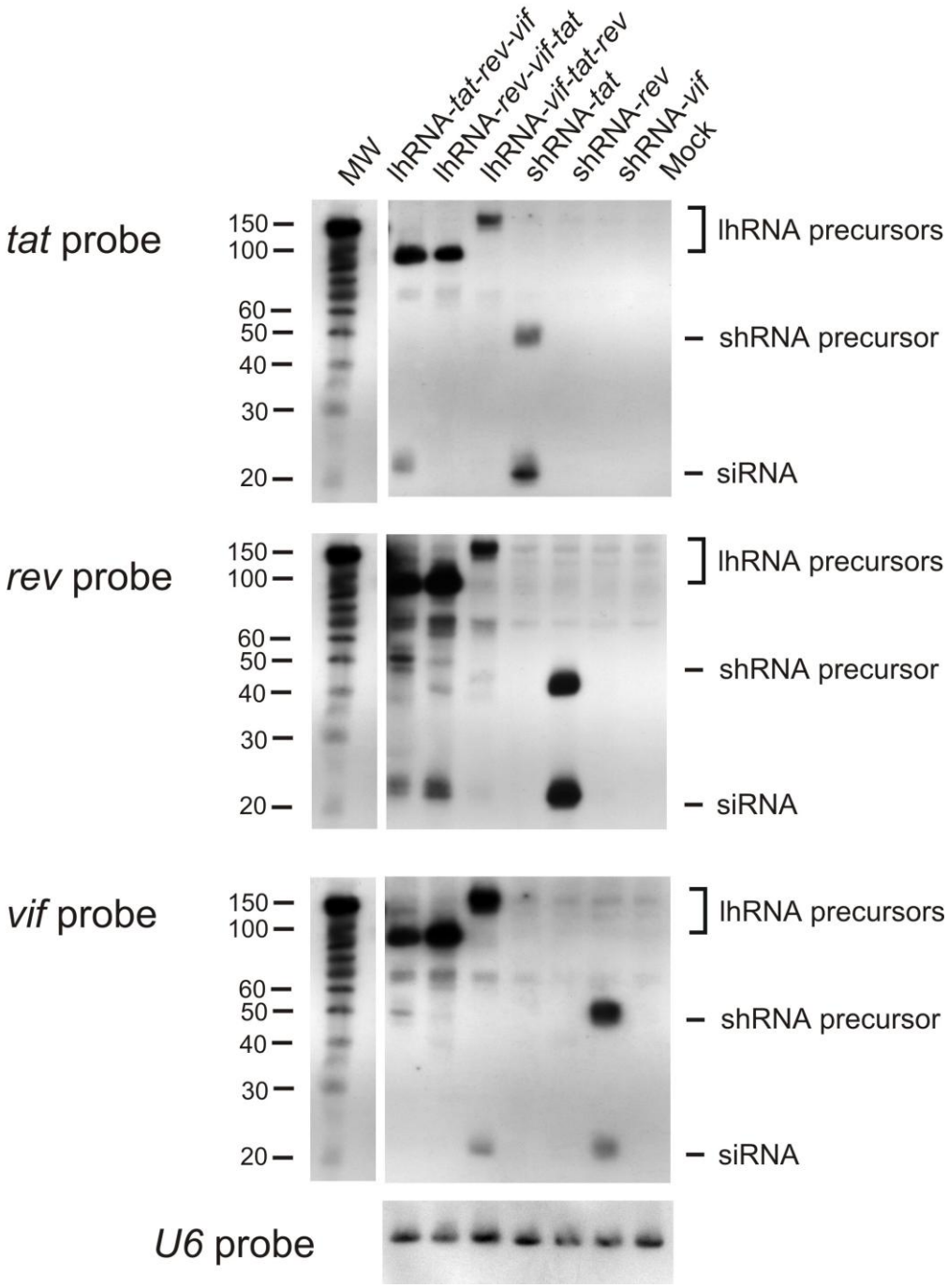


Figure 2.6: PAGE northern blot analysis of total RNA extracted from cells transfected with lhRNAs encoding three siRNAs. A single blot was hybridised with γ-^{32}P-ATP-labeled probes complementary to the mature guide strands of *tat*, *rev* and *vif* and exposed to x-ray film. lhRNA and shRNA precursor RNA as well as processed siRNAs are indicated. Decade Marker™ was used as a molecular weight marker (MW) and a probe complementary to U6 small nuclear RNA was used as a control to verify equal loading of each RNA sample.

Dicer is known to cleave its substrates inconsistently every 21-25 nt resulting in slightly varied RNA products (Macrae et al. 2006). To eliminate the possibility that mature siRNA products are not detected from the second and third position of the hairpin due to misalignment of the probe, two variables of the original *tat* probe (*tat* probe 1) were designed to span alternative possible mature *tat* guide sequences. These two probes, termed *tat* probe 2 and 3 were 14 nt in length and contained LNA (locked nucleic acid) nucleotides for higher affinity binding.

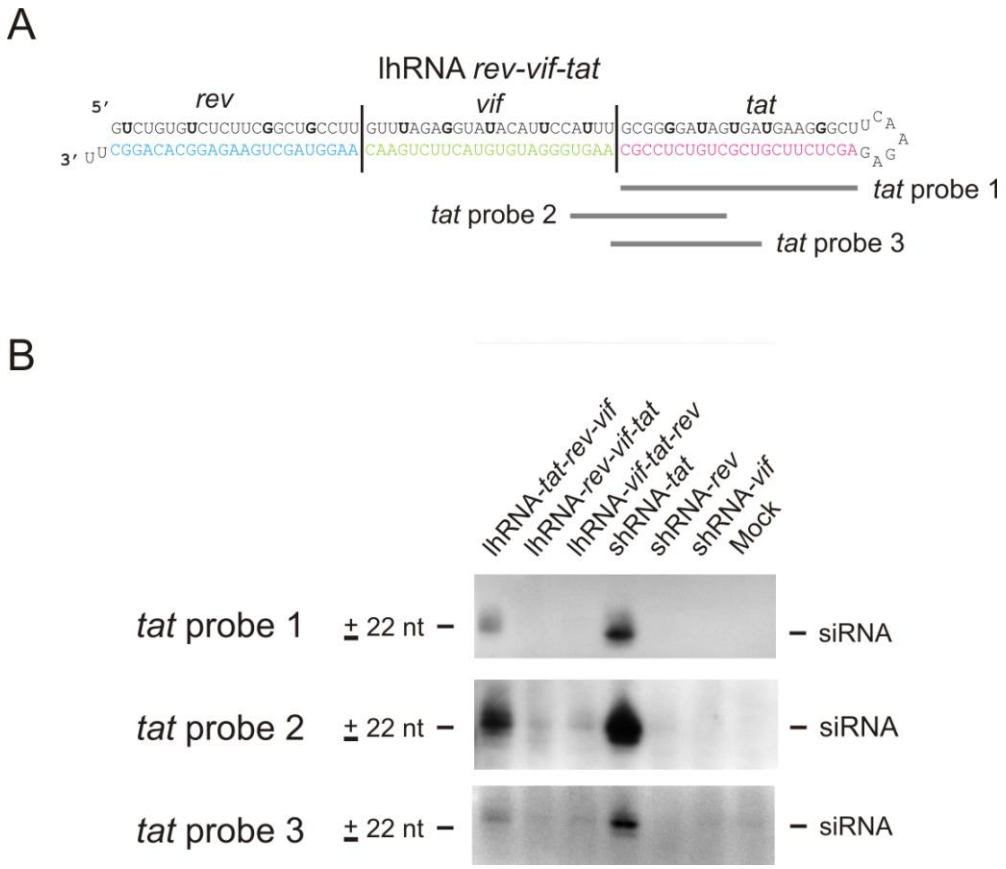


Figure 2.7: PAGE northern blot analysis of putative anti-*tat* guide sequences. (A) Three different probes were designed to span different regions of the putative *tat* siRNA guide strand. **(B)** Signals obtained from processed siRNAs approximately 22 nt in size are indicated.

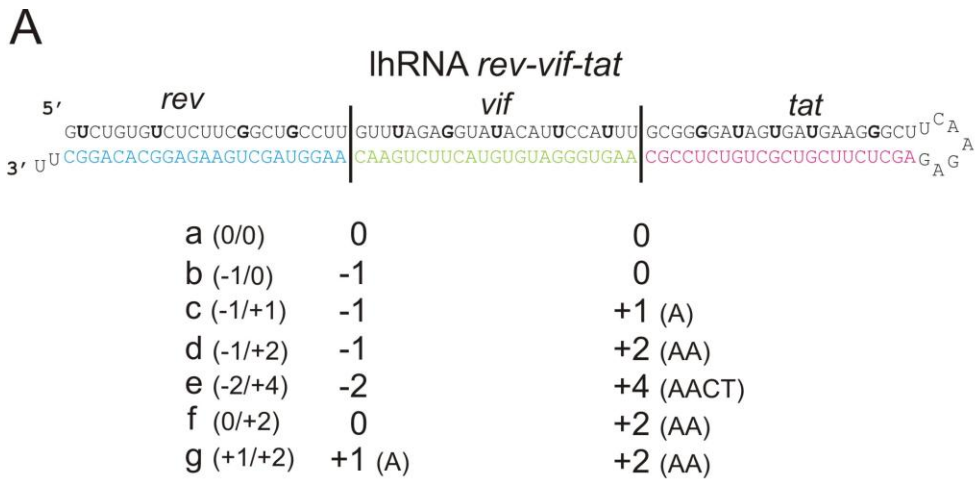
Figure 2.7 shows that regardless of the sequence of the *tat* probe, siRNA products were only observed from shRNA-*tat* and lhRNA-*tat-rev-vif* but were still not detected from the second and third position of lhRNAs. Therefore although Dicer products may vary slightly, there were no unpredicted *tat*

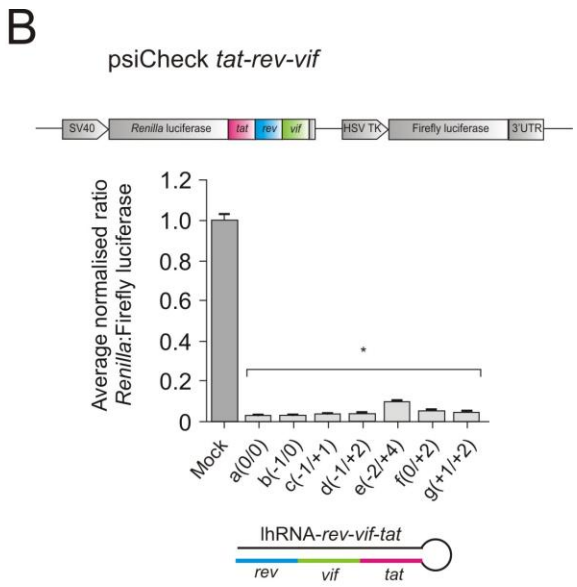
guide sequences generated from the second and third positions of the hairpin duplex. This result confirms the hypothesis that Dicer processing decreases in efficiency along the hairpin duplex yielding minimal siRNA products from regions adjacent to the loop sequence.

2.3.4 Effect of spacing between siRNA encoding sequences

It is clear from the data described above that Dicer does not process a long hairpin stem duplex with equal efficiency. Guide strand sequences of siRNAs positioned at the base of the stem are produced at higher concentrations than those positioned toward the loop sequence. It is also known however that Dicer does not consistently cleave its substrate at exactly the same position but instead cleaves its substrate every 21-25 nt (Macrae et al. 2006), thus generating processed products of slightly variable lengths. It was therefore hypothesized that modifications made to the nucleotide spacing between siRNA encoding sequences may affect the position of Dicer cleavage and potentially augment processing of siRNAs proximal to the loop sequence.

Six variables of the original lhRNA-*rev*-*vif*-*tat* (lhRNA-*rev*-*vif*-*tat* a) were therefore generated which contained 0-4 bp inserted or deleted at each siRNA encoding junction (between the *rev* and *vif* siRNAs or between the *vif* and *tat* siRNAs) and were termed lhRNA-*rev*-*vif*-*tat* b-g (Figure 2.8 A). This resulted in a series of lhRNAs with varied stem lengths of 68-72 bp and varied bp spacing between each siRNA encoding sequence. More specifically lhRNA-*rev*-*vif*-*tat* b contained a 1 bp deletion at the *rev*/*vif* junction; lhRNA-*rev*-*vif*-*tat* c contained a 1 bp deletion at the *rev*/*vif* junction and a 1 bp insertion at the *vif*/*tat* junction; lhRNA-*rev*-*vif*-*tat* d contained a 1 bp deletion at the *rev*/*vif* junction and a 2 bp insertion at the *vif*/*tat* junction; lhRNA-*rev*-*vif*-*tat* e contained a 2 bp deletion at the *rev*/*vif* junction and a 4 bp insertion at the *vif*/*tat* junction; lhRNA-*rev*-*vif*-*tat* f contained 2 bp insertion at the *vif*/*tat* junction; and lhRNA-*rev*-*vif*-*tat* g contained a 1 bp insertion at the *rev*/*vif* junction and a 2 bp insertion at the *vif*/*tat* junction (Figure 2.8 A).


A
lhRNA rev-vif-tat
rev
vif
tat
5′
3′ U
GUCUGUGUCUCUUCGGCUGCCUU
CGGACACGGAGAAGUCGAUGGAA
GUUUAGAGGUAUACAUUCCAUUU
CAAGUCUUCAUGUGUAGGGUGAA
GCGGGGAUAGUGAUGAAGGGCUU
CGCCUCUGUCGCUGCUUCUCGA
a (0/0) 0 0
b (-1/0) -1 0
c (-1/+1) -1 +1 (A)
d (-1/+2) -1 +2 (AA)
e (-2/+4) -2 +4 (AACT)
f (0/+2) 0 +2 (AA)
g (+1/+2) +1 (A) +2 (AA)


B
psiCheck tat-rev-vif
SV40
Renilla luciferase
tat
rev
vif
HSV TK
Firefly luciferase
3'UTR
Average normalised ratio Renilla:Firefly luciferase
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Mock
a(0/0)
b(-1/0)
c(-1/+1)
d(-1/+2)
e(-2/+4)
f(0/+2)
g(+1/+2)
lhRNA-rev-vif-tat
rev
vif
tat

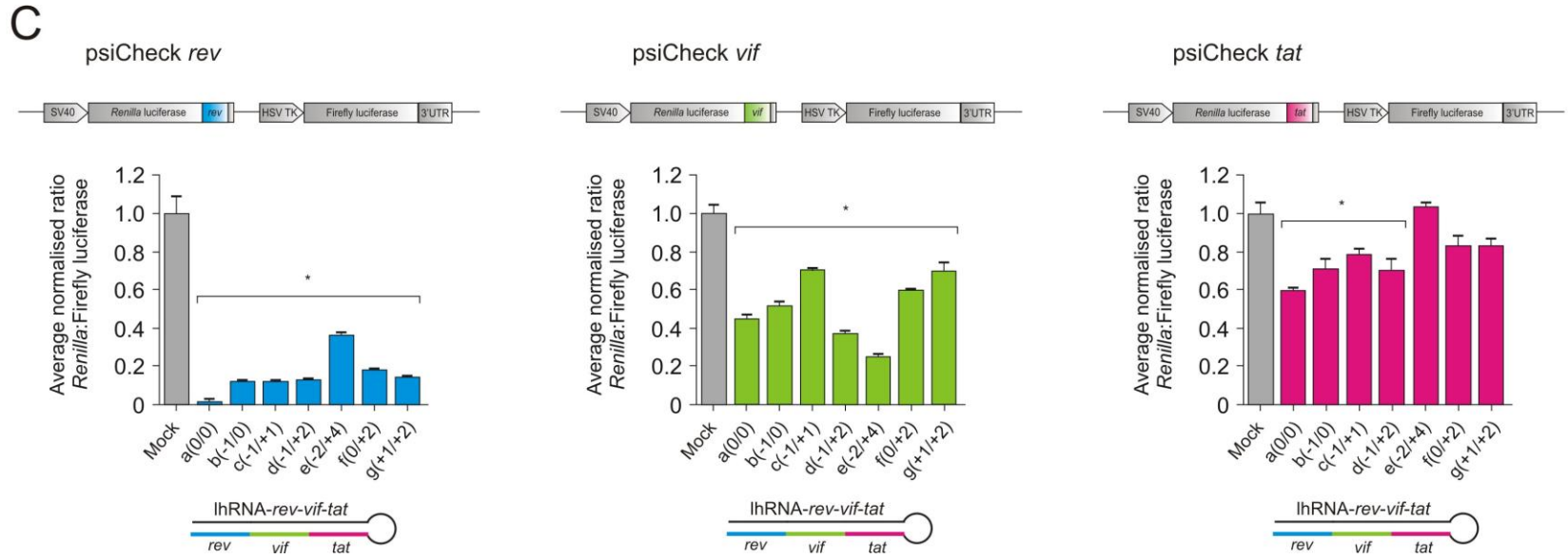

C
psiCheck rev
psiCheck vif
psiCheck tat
SV40
Renilla luciferase
rev
vif
tat
HSV TK
Firefly luciferase
3'UTR
Average normalised ratio Renilla:Firefly luciferase
1.2
1.0
0.8
0.6
0.4
0.2
0
*
Mock
a(0/0)
b(-1/0)
c(-1/+1)
d(-1/+2)
e(-2/+4)
f(0/+2)
g(+1/+2)
lhRNA-rev-vif-tat
rev
vif
tat

Figure 2.8: The effects of altered nucleotide spacing at the junctions of siRNA encoding sequences on knockdown efficacy of lhRNAs. Six variants of the lhRNA-*rev-vif-tat* hairpin containing up to 4 bp (the numbers and the sequence of the inserted bp with respect to the sense strand are indicated in brackets) inserted or deleted at each siRNA junction **(A)** were tested for their ability to inhibit psiCHECK reporter plasmids containing a combination of all of the target sequences in tandem **(B)** or each specific target sequence **(C)**, downstream of the *Renilla* luciferase (*h*RLuc) open reading frame. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with pTZU6+1 (mock) (*, $p<0.05$, t-test relative to mock transfected control).

These hairpin expression plasmids were transiently co-transfected with a psiCHECK dual luciferase target reporter plasmid containing the *tat*, *rev* or *vif* target sequence or a combination of all three target sequences downstream from the *Renilla* luciferase open reading frame and knockdown was determined as a ratio of *Renilla* luciferase: Firefly luciferase with values were normalised to pTZU6+1 (mock). When targeted against the psiCHECK *rev* target, all variants were able to decrease the *Renilla* to Firefly luciferase ratio approximately 90% with the exception of variant 'e' which was only capable of a 60% reduction suggesting that the 2 bp deleted at the *rev-vif* junction may be detrimental to Dicer processing of the first siRNA. Guide sequences targeted to the *vif* sequence were present in the second position of the hairpin variants, and inhibition of the *vif* target was significant yet variable. In certain variants such as 'e' and to a lesser extent 'd', knockdown from siRNAs in the second position was increased; however in 'c', 'f' and 'g' knockdown was weaker than that of the parent lhRNA. Modification of bp spacing did not appear to affect knockdown afforded by siRNAs in the third position and knockdown from these siRNAs was consistently poor (Figure 2.8 C). When the inhibitory efficacy of lhRNA-*rev-vif-tat* variants was tested against the combined target, an additive effect was again evident in all of the hairpins which inhibited *Renilla* luciferase expression more than 90%. The only variant whose knockdown was slightly less effective was 'e', a likely consequence of the decreased RNAi activity of the first position siRNA (Figure 2.8 B). These results suggest a possibility for improving RNAi activity from the first and second position siRNAs by optimising the spatial arrangement of these sequences along an lhRNA duplex. RNAi activity from the third position siRNA is unlikely however to be significantly improved.

Northern blot analysis was carried out on total RNA extracted from HEK293 cells transfected with the lhRNA-*rev-vif-tat* variants to determine the effect of nucleotide spacing on Dicer processing efficacy and the subsequent generation of mature guide sequences (Figure 2.9). Processed siRNAs from the first position of all the variant hairpins were detected at relatively similar quantities, albeit less than that processed from shRNA-*rev* and again with the exception of variant 'e' where negligible siRNA was detected. This provides an explanation for the decreased knockdown of the *rev* target by this construct and suggests that the modified spacing of this hairpin caused a decrease in Dicer processing of the first position of this construct. The possibility also remains that the 2 bp deletion in the 'e' variant alters the thermodynamic stability of the putative siRNA, possibly resulting in the incorporation of the sense strand into RISC and the elimination of the guide strand. One should also note that siRNA quantity is not necessarily always correlated with potency. While the 'a' variant shows considerably better inhibition of the *rev* target (Figure 2.8 C), elevated levels of siRNA are not detected when compared to the other variants (Figure 2.9). It is possible that the position of Dicer cleavage within the (0/0) configuration results in a guide strand with a more potent seed region or a 5' end which is more favourable for RISC incorporation, ultimately leading to increased levels of knockdown. A probe complementary to U6 snRNA showed equal loading of each RNA sample (Figure 2.9).

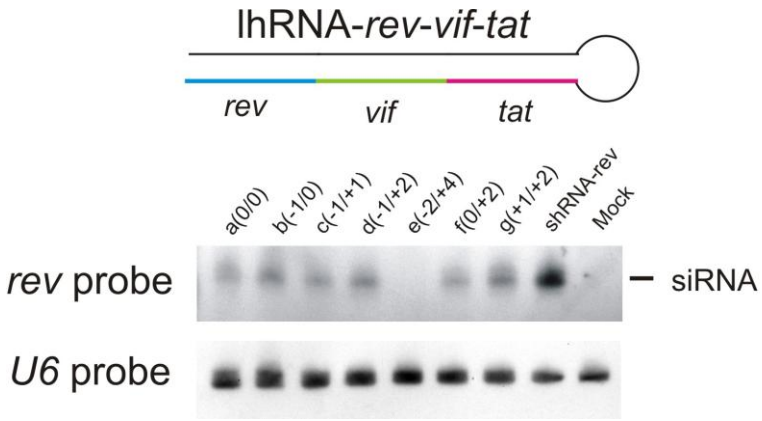


Figure 2.9: PAGE northern blot analysis of total RNA extracted from HEK293 cells transfected with lhRNA-*rev-vif-tat* and its spacing variants. Signals obtained following hybridisation of immobilised RNA to a γ-^{32}P-ATP-labeled probe complementary to the mature guide strand of the *rev* siRNA are shown. Processed siRNA products from the first position of all lhRNA variants as well as from shRNA-*rev* are indicated. A probe complementary to U6 small nuclear RNA was used as a control to verify equal loading of each RNA sample.

2.3.5 Inhibition of HIV-1 replication in infected cells in culture

Although lhRNAs were assessed for their inhibitory efficacies against reporter gene targets, and were shown to be capable of significant inhibition of reporter gene expression, this system is somewhat artificial in that only a single transcript bearing perfect complementarity to each siRNA sequence within the lhRNA is expressed from each reporter plasmid. In contrast, in the context of an HIV-infected cell population, a pool of viral variants is likely to exist. Therefore to determine the ability of lhRNAs to inhibit gene expression and replication of an infectious HIV-1 primary isolate *in vitro*, the HIV permissive cell line U87.CD4.CCR5 was transfected with lhRNA or shRNA expressing plasmids and subsequently challenged with the HIV-1 subtype C viral isolate FV5.

Concentration of p24 antigen present in supernatants was measured longitudinally on days 0, 3 and 5 following infection (Figure 2.10 B) and on day 6 both p24 antigen levels and viral RNA copies were determined (Figure 2.10 A). It is evident from Figure 2.10 A and B that shRNA-*tat* was the most effective inhibitor of viral replication followed closely by shRNA-*rev*, but shRNA-*vif* showed little to no inhibition of the virus. Since we have deduced that siRNAs positioned at the base of the stem contribute the majority of RNAi activity of the entire lhRNA, it was not unexpected in the case of the tested lhRNAs that lhRNA-*tat-rev-vif* showed the strongest knockdown of the virus followed by lhRNA-*rev-vif-tat*, and that lhRNA-*vif-tat-rev* demonstrated no inhibition of the virus which is likely to be a result of the ineffective shRNA-*vif* sequence positioned at the base of the hairpin. The fact that viral replication was not inhibited at all by lhRNA-*vif-tat-rev* again proves that the inhibitory activity of lhRNAs is primarily due to the RNAi activity of the guide sequence positioned at the base of the stem and that the second and third siRNA within a long hairpin stem do not compensate for an ineffective first position siRNA.

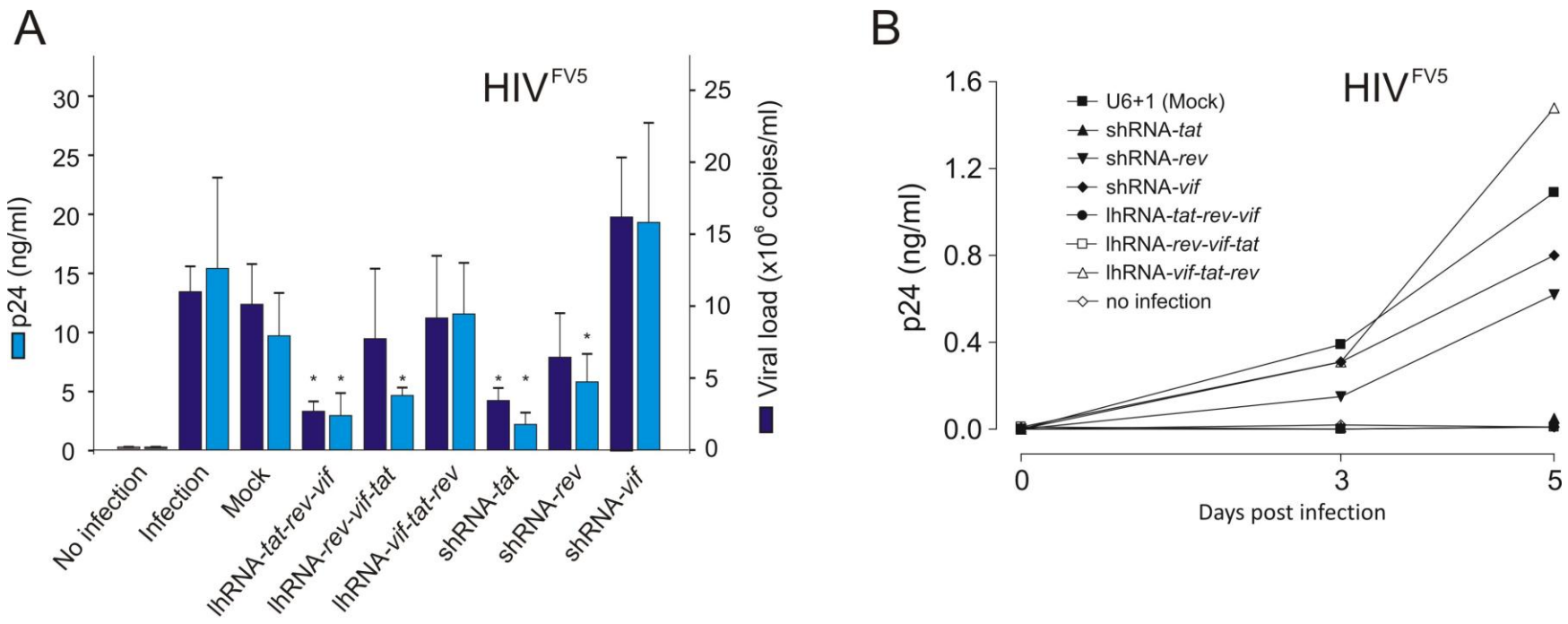


Figure 2.10: Inhibition of the HIV-1 subtype C FV5 viral isolate by lhRNAs encoding three siRNAs. U87.CD4.CCR5 cells were transfected with the indicated hairpin constructs and subsequently infected with the HIV-1 FV5 viral isolate at a $TCID_{50}$ 1000. Concentration of p24 antigen as well as viral RNA copies present in supernatants were measured 6 days post infection (*, $p<0.05$, t-test relative to mock transfected control) **(A)** and p24 levels were also measured longitudinally at specified time points **(B)**.

To determine whether imperfect sequence complementarity was the causative factor of the ineffective *vif* siRNA, the guide sequence was aligned with the *vif* sequence of the subtype B HXB2 virus and the subtype C FV5 virus. Although the guide sequence was perfectly complementary to the subtype B isolate, three G:U wobble base pairs were present between the guide strand sequence and the subtype C isolate. Furthermore all three of these misaligned bases were present within the seed region at the 5' end of the guide strand (Figure 2.11 A).

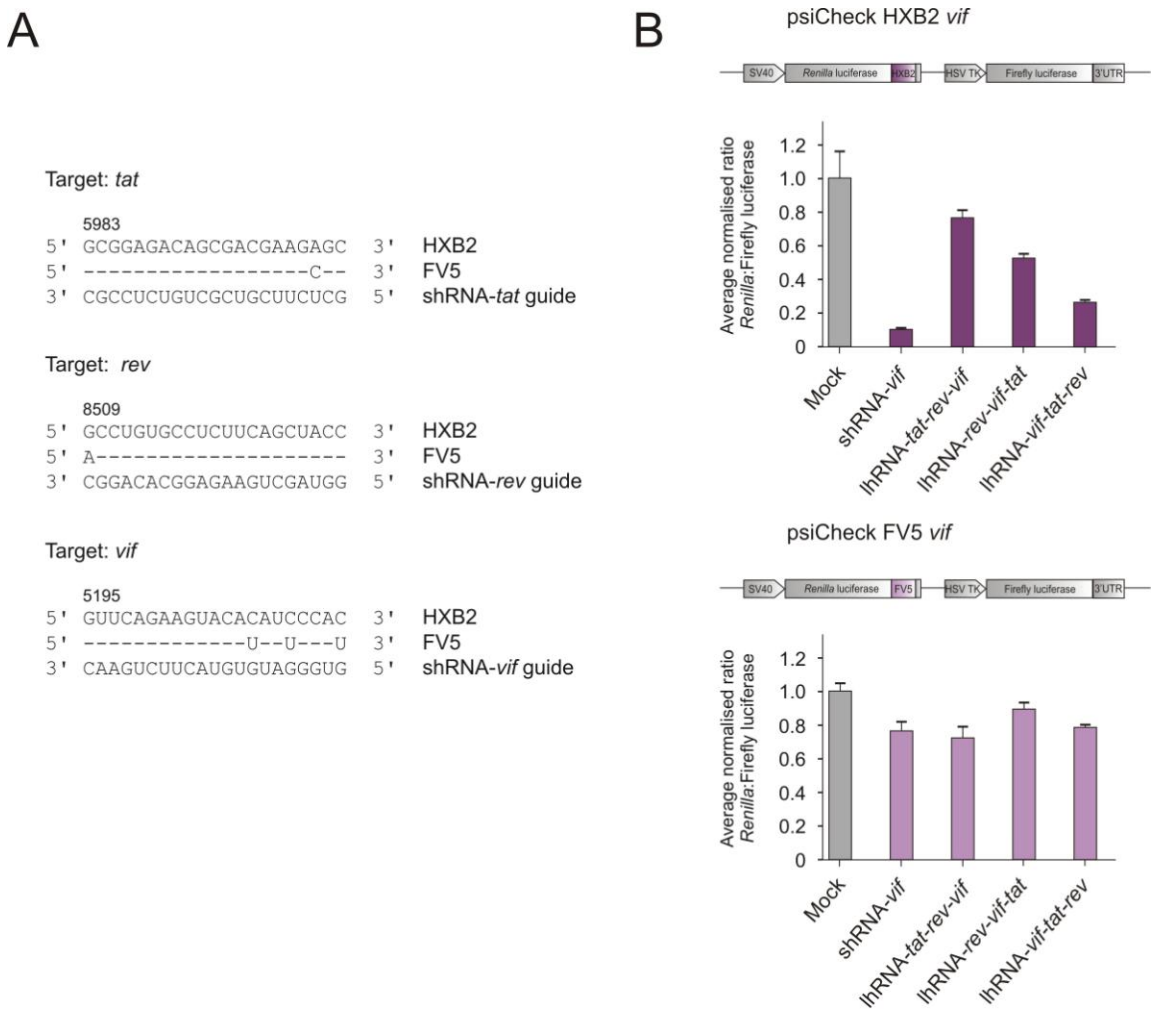


Figure 2.11: The conservation of the shRNA-*vif*-derived target site between FV5 and HXB2. **(A)** Sequence alignments of lhRNA- and shRNA-derived *tat*, *rev* and *vif* guide sequences with the complementary regions within FV5 and HXB2 HIV-1 isolates. Sequence numbering is based on isolate HXB2, accession K03455. **(B)** Inhibition of FV5 and HXB2 *vif* target reporter expression by shRNA-*vif* and the indicated lhRNAs. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with pTZU6+1 (mock).

To confirm whether these mismatches are the cause of the inefficiency of shRNA-*vif* and lhRNA-*vif-tat-rev*, the FV5 *vif* target and the HXB2 *vif* target sequences were cloned downstream of the *Renilla* luciferase ORF in a psiCHECK dual luciferase reporter plasmid. Plasmids expressing lhRNAs as well as shRNA-*vif* were transiently co-transfected together with the HXB2 or FV5 *vif* psiCHECK dual luciferase target reporter plasmids described above. Knockdown was determined as a ratio of *Renilla* luciferase: Firefly luciferase and values were normalised to pTZU6+1 (mock). Neither shRNA-*vif* or any of the lhRNAs were able to mediate knockdown of the FV5 *vif* target sequence however when tested against the HXB2 *vif* sequence, shRNA-*vif* was highly effective and the lhRNAs showed the expected knockdown trend with lhRNA-*vif-tat-rev* being the most effective followed by lhRNA-*rev-vif-tat* and lhRNA-*tat-rev-vif* showing negligible knockdown (Figure 2.11 B). This confirms that the presence of mismatched nucleotides between the *vif* guide strand and the target sequence rendered this siRNA ineffective against the FV5 viral isolate. This data also supports the results of the reporter gene knockdown assays showing that RNAi activity induced by lhRNAs is primarily mediated by siRNAs located at the base of the hairpin stem.

2.3.6 lhRNAs do not induce a non-specific immune response

The introduction of exogenous long dsRNA (>30 bp) into the cytoplasm of mammalian cells has been shown to elicit an interferon response by activating PKR and 2',5' OAS resulting non-specific gene silencing (section 1.7.1). It has since been shown that expressed RNAi effector sequences can evade cytoplasmic activators of the interferon pathway (Marques et al. 2006; Robbins et al. 2006). However the use of lhRNAs in a gene therapy context remains a controversial issue because of the potential of long dsRNA to elicit an immune response. To exclude the possibility of non-specific gene knockdown effects or toxicities caused by the induction of a non-specific interferon response following introduction of lhRNAs into the cellular environment, *IFN-β* mRNA concentrations were measured in cells transfected with lhRNAs, shRNA controls or the dsRNA analogue poly (I:C) which served as a positive control. None of the shRNA or lhRNA expression cassettes induced expression of *IFN-β*

relative to mock transfected cells as measured by qRT-PCR with samples normalised to *GAPDH* (Figure 2.12). Although further investigation into the activity of interferon pathway-related genes is required in immune cells such as monocytes and dendritic cells as well as *in vivo*, the use of expressed long hairpin RNAs does not appear to stimulate a type-1 interferon response in the HEK293 cell line, a result which is similar to previous reports using Pol III-expressed lhRNAs (Akashi et al. 2005; Barichievy et al. 2007).

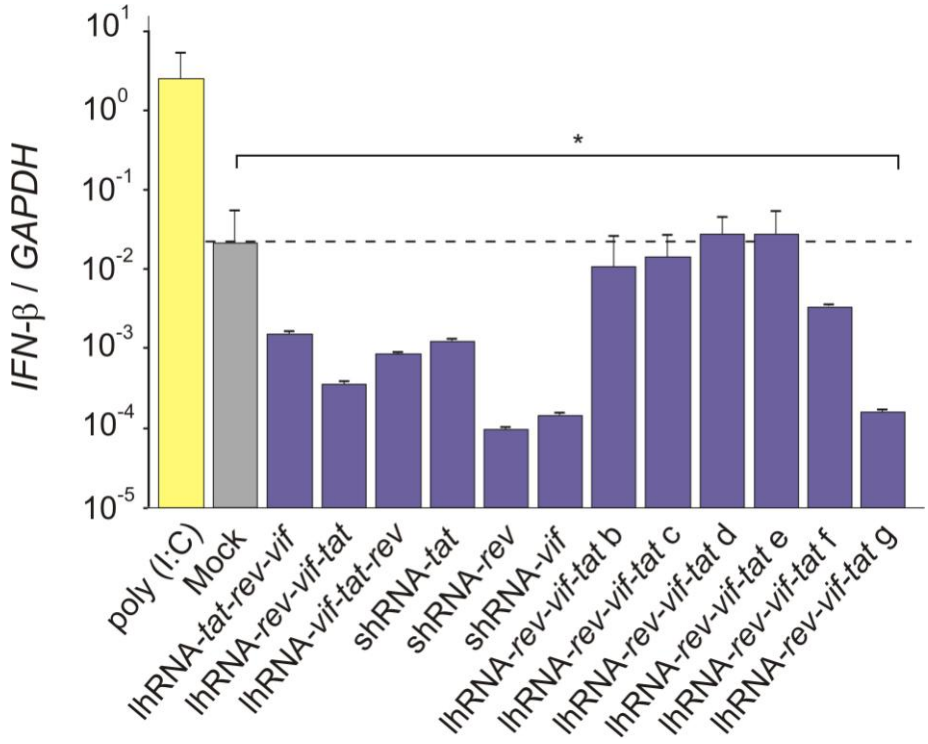


Figure 2.12: The potential induction of the IFN response in cells transfection with lhRNA expression cassettes. IFN induction was assessed by measuring *IFN-β* mRNA concentration in total RNA extracted from cells transfected with the indicated hairpin expression cassettes or with poly I:C which served as a positive control. Mean normalised ratios of *IFN-β* : *GAPDH* (±SEM) (n=3) are indicated as determined by qRT-PCR (*, $p<0.05$, t-test relative to poly (I:C) transfected control).

2.4 Discussion

The lhRNAs described in this Chapter were designed to encode three highly effective siRNA sequences within the dsRNA duplex, for the simultaneous targeting of three conserved non-contiguous sites within the HIV genome. It is worthy to note that the nomenclature of long hairpin RNAs is currently under debate because of the structural differences between the Pol III-expressed lhRNA constructs described here, and the much longer Pol II-driven lhRNAs originally described. The Berkhout laboratory refers to structures similar to those described in this thesis as extended-shRNAs (Liu et al. 2007; Liu et al. 2009), while in a recent review such structures were termed tandem hairpin RNAs (Sibley et al. 2010). These constructs have been defined here as Pol III-expressed long hairpin RNAs because it is felt that this term accurately describes, and correctly defines this class of hairpins.

Initially, lhRNAs expressed from Pol III promoters were only used against single contiguous sequences and the RNAi activity contributed by each processed siRNA was not characterised (Akashi et al. 2005; Nishitsuji et al. 2006; Watanabe et al. 2006). The incorporation of three discrete siRNA sequences within the hairpins used here allows for comparison of relative RNAi activity with that of individual shRNAs with corresponding guide sequences. The differential spatial arrangement of siRNAs within the hairpin duplex also enables the determination of potential positional effects on the inhibitory efficacies of individual siRNAs. Dual luciferase reporter assays to test the transient inhibitory efficacy of lhRNAs encoding three unique siRNAs showed that lhRNAs were capable of significantly inhibiting all target sequences but that the most effective knockdown was consistently observed from siRNAs generated from the base of the stem of the lhRNA. These lhRNA-generated siRNAs showed a decrease in target gene expression of up to 98%, comparable to that achieved by individual shRNAs targeting the same region (Figure 2.5). However guide sequences generated from the second and third positions of the duplex showed a gradual loss of RNAi activity. To corroborate this data, inhibition of FV5 viral replication was proven to be mediated primarily by the inhibitory activity of guide sequences positioned at the base of the stem (Figure 2.10). Northern blot analysis also detected an abundance of siRNA that was generated from the base of the hairpin stem but little to negligible quantities generated from the second or third positions of the duplex (Figure 2.6).

These results support previous studies which showed diminishing RNAi activity from the base towards the loop of contiguous lhRNAs with stems of 62 and 63 bp (Barichievy et al. 2007; Weinberg et al. 2007), and it is evident that this decrease in inhibitory activity is due to the decline in processing as Dicer proceeds along the duplex structure. A recent study by Sano et al. investigated the silencing efficacies as well as the processing trends of 50, 53 and 80 bp lhRNAs targeted to contiguous sequences within the *tat* and *rev* genes of HIV-1. They too observed a reduction in both knockdown efficacy and siRNA concentration for siRNAs in the third position, proximal to the loop sequence (Sano et al. 2008). In contrast to the observation of diminishing siRNA generation from lhRNAs in previous work studying contiguous hairpins (Barichievy et al. 2007; Weinberg et al. 2007; Sano et al. 2008), the lhRNAs used in this Chapter comprise unique siRNAs variably positioned within the duplex, and it was thus possible to also show that the consistently observed gradient effect is largely independent of the siRNA sequence.

A fundamental follow up study to that of Sano et al. investigated the role of the N terminal helicase domain of human Dicer in dsRNA processing. Dicer helicase mutants showed defective processing of thermodynamically unstable pre-miRNA substrates yet enhanced processing of thermodynamically stable lhRNA substrates (Soifer et al. 2008). Interestingly, when the processing of 50 bp and 80 bp lhRNAs was examined, Dicer helicase mutants generated a slight increase in the first siRNA, positioned at the base of the hairpin, and a 3-5 fold increase in the second and third siRNA. This suggests that the helicase domain of human Dicer has evolved to allow for the processing of thermodynamically unstable miRNA precursors and may represent the limiting factor for the cleavage of lhRNA substrates. In addition, Ma et al. also showed a substantial increase in the catalytic efficiency in helicase mutants, further implicating this domain in Dicer turnover (Ma et al. 2008). To further support these observations, Giardia Dicer, which lacks a helicase domain, is capable of multiple turnover kinetics (Zhang et al. 2002). The helicase domain is therefore thought to mediate the conformational changes in the Dicer enzyme following cleavage reactions for the release of mature miRNAs, thus restricting the multiple turnover kinetics of the enzyme. Therefore in the absence of a functional helicase domain, conformational changes are inhibited thus allowing for subsequent multiple cleavage reactions (Ma et al. 2008; Soifer et al. 2008).

Concurrent with this work, Liu et al. attempted to establish an optimal design for extended short hairpin RNAs (e-shRNAs), which are structurally similar to the Pol III-expressed lhRNAs described here. These e-shRNAs encoded two non-contiguous siRNAs targeted against the *pol* and *nef* genes of HIV-1 (Liu et al. 2007). It was also shown that e-shRNAs are processed from the base towards the loop of the hairpin and that there is a correlation between siRNA production and activity. Liu et al. then investigated the effect of spacing between each siRNA encoding sequence to potentially improve the inhibitory activity of the second siRNA. A 43 bp length was established as the minimal length of e-shRNAs for the production of two siRNAs, and 19 bp siRNA sequences separated by 4 bp and with a 2 bp terminal extension, was determined as the optimal hairpin design for the production of two effective siRNAs (Liu et al. 2007). Such spacing arrangements did not however prove optimal in the lhRNAs described in this Chapter suggesting that the siRNA sequence or specific sequence elements may play a role in optimal Dicer processing (Vermeulen et al. 2005). Nonetheless, minor modifications to the bp spacing at the junctions between siRNA encoding regions were investigated and it was shown that knockdown by siRNAs generated from the second position of the hairpin could be improved (Figure 2.8). Although it is difficult as yet to make gross generalizations regarding the optimal spatial arrangement of siRNA sequences within a long hairpin context, augmented knockdown from the first two siRNAs of the lhRNA duplex promises to be a feasible goal following further characterisation of lhRNAs, however this is unlikely to be the case for a third siRNA (Liu et al. 2007; Sano et al. 2008). This was again confirmed in a later study attempting to stack up to four siRNA sequences in an e-shRNA. Although a third siRNA was detected, it was present in reduced quantities, and hairpins encoding only two siRNAs again proved to be the most efficacious (Liu et al. 2009).

The potential of Pol III-driven lhRNAs to produce up to three unique siRNAs capable of inducing significant inhibition of non-contiguous sites within the HIV-genome has been clearly demonstrated. However an upper size limitation clearly exists which is associated with the inability of Dicer to act as a rapid turnover enzyme in mammalian cells. Mature RNAi products were consistently generated in a gradient from the base towards the loop of the hairpin stem, however minor spacing modifications were able to augment or hinder RNAi activity from siRNAs positioned further along the stem. It is therefore hypothesized that further manipulation and optimisation of the lhRNA design may result in efficient

Dicer cleavage of two functional siRNAs for effective multiple targeting. The concept explored in this study, of multiple unique siRNA production from a single expressed construct, is potentially an invaluable tool for the development of combinatorial RNAi strategies.

CHAPTER 3

Deriving two functional siRNAs from dual-targeting long hairpin RNAs.

3.1 Introduction

The ability of lhRNA duplexes to incorporate the sequences of multiple independent siRNAs capable of silencing more than one non-contiguous site has been clearly demonstrated. However, in support of previous studies (Barichievy et al. 2007; Weinberg et al. 2007; Sano et al. 2008), siRNAs positioned at the base of the hairpin duplex were cleaved with the greatest efficiency and were capable of inducing the strongest inhibitory activity of their cognate targets (>80%), while siRNAs in the second and third sequential positions within the hairpin duplex were poorly processed and in some cases contributed only minimal RNAi activity to the overall efficacy of the lhRNA (30-70%). The gradual decline in Dicer processing from the 3' overhang towards the terminal loop results in efficacious levels of a maximum of two siRNAs and therefore clearly suggests an upper size limit for expressed lhRNAs. Deriving more than two predicted functional siRNAs from an lhRNA scaffold remains difficult and requires a better understanding of the in situ processivity of human Dicer for such substrates.

Soifer et al. proposed that the helicase domain of human Dicer induces a conformational change in the enzyme following an initial cleavage reaction, thereby restricting subsequent processing of long dsRNA substrates (Soifer et al. 2008). Yet it was clearly observed here that by modifying the base pair spacing at siRNA junctions, the production of a second siRNA may be significantly augmented (Saayman et al. 2008). Furthermore, Liu et al. designed extended shRNAs (e-shRNAs) incorporating the sequences of two functional non-contiguous siRNAs, and a number of parameters pertaining to the optimal design of hairpins encoding two siRNAs were subsequently proposed (Liu et al. 2007). In support of the results described in section 2.3.4, the spacing at the junctions between siRNA encoding regions played an imperative role in the processing and thus RNAi activity induced by the second siRNA (Liu et al. 2007). A hairpin duplex length of 43 bp was observed to be the minimum length,

while a hairpin duplex of 44 bp was determined as the optimal length, for the production of two efficient guide strands. It is therefore possible to manipulate long dsRNA Dicer substrates for two subsequent cleavage reactions; however a general framework outlining the design of such substrates remains to be established. Although Liu et al. described optimal design principles for e-shRNAs encoding two siRNAs, these principles were based on the sequences of only two siRNAs utilized in their study. The PAZ domain of Dicer has exhibited a binding preference for specific sequences resulting in the preferred cleavage of certain substrates over others (Vermeulen et al. 2005). One cannot thus assume that the established parameters are universally applicable to all siRNA sequences. Moreover, the sustained generation of efficacious levels of a second siRNA when the lhRNA dose is reduced, has yet to be demonstrated.

To determine an optimal configuration for an efficacious lhRNA design, a series of hairpins encoding two putative siRNAs (dual-targeting lhRNAs) were designed and generated. Hairpins were designed to incorporate the sequences of two siRNAs targeted against independent sites within the HIV-1 genome. A total of four unique siRNA sequences were inserted at both positions of a hairpin duplex and their ability to be processed efficiently as well as their inhibitory activity in each position was investigated. The effects of base pair spacing between siRNA encoding regions, as well as before the loop sequence, on siRNA production and activity was explored. A maximum stem length for generating efficient lhRNAs capable of producing two highly-functional siRNAs from a single pol III-expressed lhRNA construct, regardless of the siRNA sequence was determined. Additionally, insight into the preferred length and structure of Dicer substrates was gained. Finally, two optimised and highly effective dual-targeting lhRNAs were identified and the possibility of integrating both hairpins into a single vector for combinatorial RNAi purposes was explored.

3.2 Materials and Methods

3.2.1 Generation of plasmids containing Pol-III expressed lhRNA and shRNA sequences

The procedure for generating dual targeting lhRNAs was similar to the method used to generate lhRNAs encoding three putative siRNA sequences described in 2.2.1. A panel of U6-driven lhRNA sequences encoding two putative siRNA sequences targeted to the *tat* and *nef* or *int* and *LTR* ORFs were constructed by a two-step PCR. In the first round of PCR, 10 ng pTZU6+1 was used as a template. The universal U6 forward primer described in 2.2.1 was used for both rounds of PCR. The reverse primers for the first round of PCR were complementary to 18-21 nt of the 3' end of the U6 promoter and contained a linker encoding the complementary sequence of the sense strand of the lhRNA as well as the loop sequence (Table 3.1). For the second round of PCR a 1:500 dilution of the R1 amplicon was used as the template for the reaction. The round two reverse primer sequences were designed to overlap the loop region of the round one reverse primer by 18-21 nt and contained a linker encoding the complementary sequence of the antisense strand of the lhRNA as well as 6 deoxyadenosines (Table 3.1). This overlap between each pair of reverse primers enabled the extension of the PCR product to generate a U6 expressed lhRNA cassette with a transcription termination signal. Control U6-driven shRNAs corresponding to the sequences included in the lhRNA expression constructs were generated by a single round of PCR using pTZU6+1 a template, and amplified with the universal U6 forward primer and specific reverse primers encoding the shRNA sequence (Table 3.1).

Table 3.1: Oligonucleotides used to generate dual-targeting lhRNAs and corresponding shRNAs

Primer	Sequence (5'-3')	Length (nt)
U6 forward	CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG	37
H1 forward	GGATCCTCGAGCGGCCGCTAGCAACGCTGACGTCATCAACCCG	43
7SK forward	GGATCCTCGAGCGGCCGCTAGCAGTATTTAGCATGCCCCACC	42
lhRNA-*tat-nef* +1 R1	CTGGGTCAGGGACATATTGTACTTCCAGCCAGACACTGCTCTTCATCACTATCCCCGCGGTGTTTCGTCCTTTCCACAA	79
lhRNA-*tat-nef* +1 R2	AAAAAAGCGGAGACAGCGACGAAGAGCGGTGCCTGGCTAGAAGCACAAGACGTCTGGGTCAGGGACATATT	71
lhRNA-*tat-nef* +2 R1	CTGGGTCAGGGACATATTGTACTTCCAGCCAGACACCTGCTCTTCATCACTATCCCCGCGGTGTTTCGTCCTTTCCACAA	80
lhRNA-*tat-nef* +2 R2	AAAAAAGCGGAGACAGCGACGAAGAGCGTGTGCCTGGCTAGAAGCACAAGACGTCTGGGTCAGGGACATATT	72
lhRNA-*tat-nef*-+3 R1	CTGGGTCAGGGACATATTGTACTTCCAGCCAGACACACTGCTCTTCATCACTATCCCCGCGGTGTTTCGTCCTTTCCACAA	81
lhRNA-*tat-nef* +3 R2	AAAAAAGCGGAGACAGCGACGAAGAGCGTGGTGCCTGGCTAGAAGCACAAGACGTCTGGGTCAGGGACATATT	73
lhRNA-*nef-tat* +1 R1	CTGGGTCAGGGACATGCTCTTCATCACTATCCCCGCTATTGTACTTCCAGCCAGACACGGTGTTTCGTCCTTTCCACAA	79
lhRNA-*nef-tat* +1 R2	AAAAAAGTGCCTGGCTAGAAGCACAAGGGCGGAGACAGCGACGAAGAGCACGTCTGGGTCAGGGACATGCT	71
lhRNA-*nef-tat* +2 R1	CTGGGTCAGGGACATGCTCTTCATCACTATCCCCGCCTATTGTACTTCCAGCCAGACACGGTGTTTCGTCCTTTCCACAA	80
lhRNA-*nef-tat* +2 R2	AAAAAAGTGCCTGGCTAGAAGCACAAGGTGCGGAGACAGCGACGAAGAGCACGTCTGGGTCAGGGACATGCT	72
lhRNA-*nef-tat* +3 R1	CTGGGTCAGGGACATGCTCTTCATCACTATCCCCGCACTATTGTACTTCCAGCCAGACACGGTGTTTCGTCCTTTCCACAA	81
lhRNA-*nef-tat* +3 R2	AAAAAAGTGCCTGGCTAGAAGCACAAGGTGGCGGAGACAGCGACGAAGAGCACGTCTGGGTCAGGGACATGCT	73
lhRNA-*LTR-int* +1 R1	CTGGGTCAGGGACATAACTAACCATTGCCTCCGGCTGTCCGAGAGATCTCTAATTACGGTGTTTCGTCCTTTCCACAA	79
lhRNA-*LTR-int* +1 R2	AAAAAAGTAACTAGAGACCTCTCAGACGGCCGGAGAGCAATGGCTAGTCACGTCTGGGTCAGGGACATAAC	71

lhRNA-*LTR-int* +2 R1	*C*TGGGTCAGG*GAC***A***T***A**ACTA**A**CCATTGC**C**CTCCGGC<u>CT</u>GTC**C**GAGAG**A**TCTCTA**A**TTACGGTGTTTCGTCCTTTCCACAA	80
lhRNA-*LTR-int* +2 R2	AAAAAAGTAACTAGAGACCTCTCAGAC<u>GT</u>GCCGGAGAGCAATGGCTAGTC*ACGTC*TGGGTCAGG*GAC***A***T***A**AC	72
lhRNA-*LTR-int* +3 R1	*C*TGGGTCAGG*GAC***A***T***A**ACTA**A**CCATTGC**C**CTCCGGC<u>ACT</u>GTC**C**GAGAG**A**TCTCTA**A**TTACGGTGTTTCGTCCTTTCCACAA	81
lhRNA-*LTR-int* +3 R2	AAAAAAGTAACTAGAGACCTCTCAGAC<u>GTG</u>GCCGGAGAGCAATGGCTAGTC*ACGTC*TGGGTCAGG*GAC***A***T***A**AC	73
lhRNA-*int-LTR* +1 R1	*C*TGGGTCAGG*GAC***A***T*GTC**C**GAGAG**A**TCTCTA**A**TTAC<u>T</u>**A**ACTA**A**CCATTGC**C**CTCCGGCGGTGTTTCGTCCTTTCCACAA	79
lhRNA-*int-LTR* +1 R2	AAAAAAGCCGGAGAGCAATGGCTAGTC<u>G</u>GTAACTAGAGACCTCTCAGAC*ACGTC*TGGGTCAGG*GAC***A***T*GTC	71
lhRNA-*int-LTR* +2 R1	*C*TGGGTCAGG*GAC***A***T*GTC**C**GAGAG**A**TCTCTA**A**TTAC<u>CT</u>**A**ACTA**A**CCATTGC**C**CTCCGGCGGTGTTTCGTCCTTTCCACAA	80
lhRNA-*int-LTR* +2 R2	AAAAAAGCCGGAGAGCAATGGCTAGTC<u>GT</u>GTAACTAGAGACCTCTCAGAC*ACGTC*TGGGTCAGG*GAC***A***T*GTC	72
lhRNA-*int-LTR* +3 R1	*C*TGGGTCAGG*GAC***A***T*GTC**C**GAGAG**A**TCTCTA**A**TTAC<u>ACT</u>**A**ACTA**A**CCATTGC**C**CTCCGGCGGTGTTTCGTCCTTTCCACAA	81
lhRNA-*int-LTR* +3 R2	AAAAAAGCCGGAGAGCAATGGCTAGTC<u>GTG</u>GTAACTAGAGACCTCTCAGAC*ACGTC*TGGGTCAGG*GAC***A***T*GTC	73
H1-lhRNA-*tat-nef* +1 R1	*C*TGGGTCAGG*GAC***A***T***A**TTGT**A**CTTC**C**AGCCAG**A**CAC<u>T</u>GCTCTTC**A**TC**A**CT**A**TC**C**CCGCGGGTCCGAGTGGTCTCATAC	79
H1-lhRNA-*tat-nef* +1 R3	GGAGATCTACTAGTAAAAAAGCGGAGACAGCGACG	35
7SK-lhRNA-*LTR-int* +1 R1	*C*TGGGTCAGG*GAC***A***T***A**ACTA**A**CCATTGC**C**CTCCGGC<u>T</u>GTC**C**GAGAG**A**TCTCTA**A**TTACGAGGTACCCAGGCGGCGCACA	79
7SK-lhRNA-*LTR-int* +1 R3	GGAGATCTACTAGTAAAAAAGTAACTAGAGACCTC	35
shRNA-*tat* R1	AAAAAAGCGGAGACAGCGACGAAGAGCTGGGTCAGGGCTCTTC**A**TC**A**CT**A**TC**C**CCGCGGTGTTTCGTCCTTTCCACAA	78
shRNA-*nef* R1	AAAAAAGTGCCTGGCTAGAAGCACAAGTGGGTCAGGATTGT**A**CTTCTAGCCAGGCACGGTGTTTCGTCCTTTCCACAA	78
shRNA-*LTR* R1	AAAAAAGTAACTAGAGATCCCTCAGACTGGGTCAGGGTCTGAG**A**GATCTCTAGTTACGGTGTTTCGTCCTTTCCACAA	78
shRNA-*int* R1	AAAAAAGCCGGAGAGCAATGGCTAGTGATTGGGTCAGGT**A**ACTA**A**CCATTGCTCTCCGGCGGTGTTTCGTCCTTTCCACAA	81

Underlined sequences signify restriction endonuclease recognition sites. Highlighted sequences indicate the overlapping regions between each pair of reverse primers. Italics represent the 5 nt terminal extension before the loop sequence indicated in red. Bold letters show sites of G:U mismatch introductions in the sense strand. Double underlined letters designate mismatched nucleotide spacing between siRNA encoding sequences.

To determine the effects of using alternative Pol III promoters to drive the expression of dual-targeting lhRNAs, lh*tat-nef* +1 and lh*LTR-int* +1 were regenerated to be driven off the RNA Pol III H1 and 7SK promoters respectively. H1-driven lh*tat-nef* +1 and 7SK-driven lh*LTR-int* +1 sequences were generated in a similar manner but using a three-step PCR (Figure 3.1). Either a plasmid expressing the human H1 promoter, or genomic DNA was used as respective templates. Forward primers complementary to the 5' end of the human H1 or 7SK RNA Pol III promoters were used for all three rounds of PCR, and both forward primers included *BamH*I; *Xho*I; *Not*I and *Nhe*I restriction endonuclease recognition sites to facilitate cloning into alternate vectors in the future (Table 3.1). Round one reverse primers were unique in that they overlapped the 5' end of the H1 or 7SK promoter sequence, and round two reverse primers were the same as those used for the generation of the respective U6-driven lhRNAs (Table 3.1). Round three reverse primers overlapped the 3' end and the termination signal of the respective lhRNAs and contained a linker with the *Spe*I (*Bcu*I) and *Bgl*II restriction endonuclease recognition sites (Table 3.1). Reagents and thermocycling conditions were the same as those described in 2.2.1. All the final PCR products were ligated directly into the TA cloning vector pTZ57R/T (Fermentas, WI, USA), and sequences were confirmed by standard procedures as described in 2.2.1.

To clone H1-lhRNA-*tat-nef* +1 and 7SK-lhRNA-*LTR-int* +1 head to tail into a single vector (pTZ57R/T) (Figure 3.2), correctly sequenced individual clones were first screened for orientation by digestion with *EcoR*I and *Not*I or *EcoR*I and *Bgl*II. The pTZ57R/T plasmid containing 7SK-lhRNA-*LTR-int* +1 in the reverse orientation was linearised by digesting 1 µg of the plasmid with 20 U *Spe*I and Tango^{TM} Buffer (33 mM Tris-acetate [pH7.5 at 37°C]; 10 mM Mg-acetate; 66 mM K-acetate and 0.1 mg/ml BSA) (Fermentas, WI, USA), in a 20 µl reaction at 37°C for two hours. Five units of Antarctic phosphatase (AP) (NEB, MA, USA) were then added to the digestion reaction together with AP buffer (50 mM bis-tris-propane-HCl, 1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$ pH 6.0) in a 30 µl total volume. The reaction was incubated at 37°C for a further 30 min. The phosphatase reaction was heat-inactivated for at 65°C for 15 min.

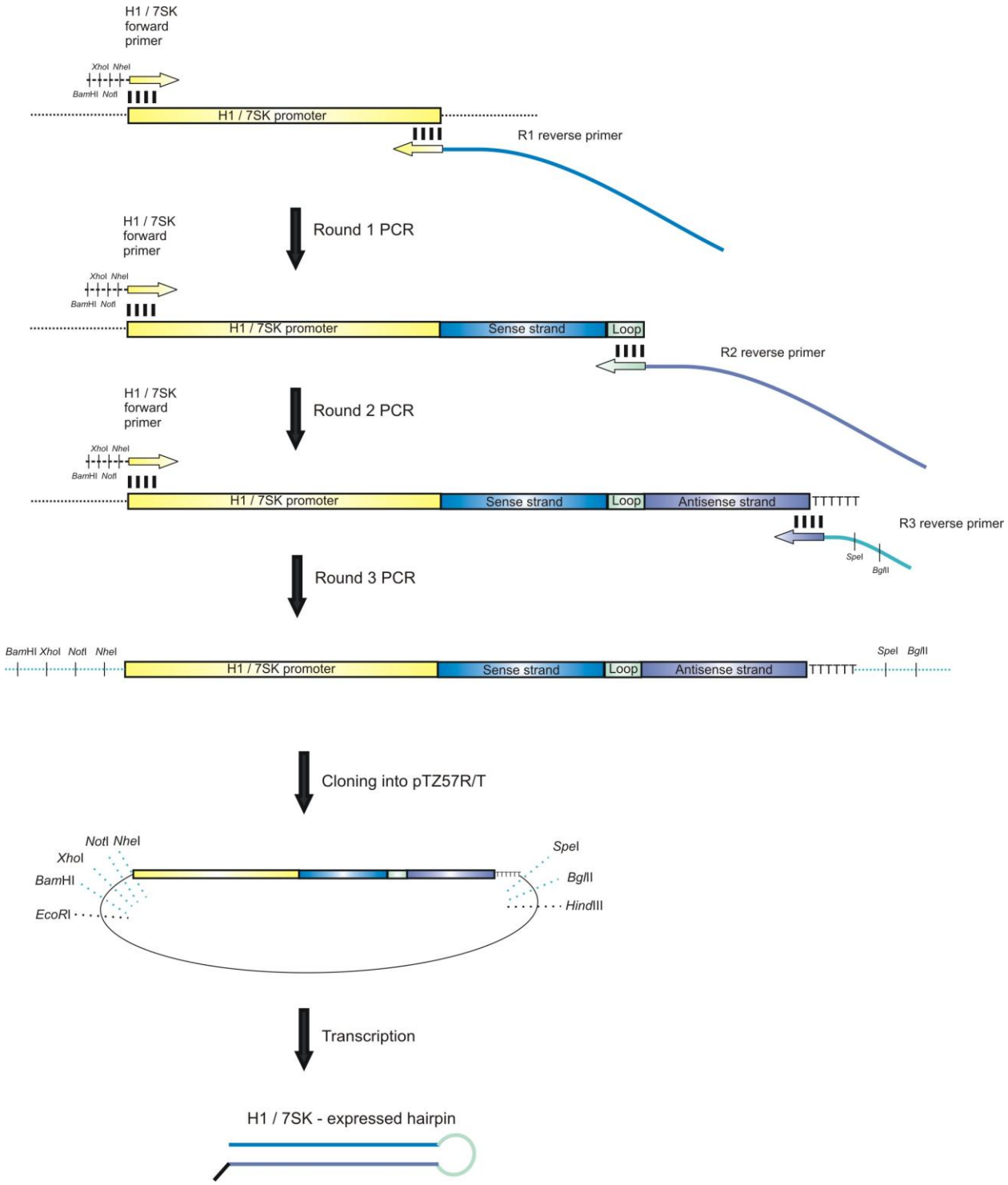


Figure 3.1: Schematic representation of the three step PCR cloning strategy used to generate H1-driven lh*tat-nef* +1 and 7SK-driven lh*LTR-int* +1 expression cassettes. The first two rounds of PCR proceed as described in 2.2.1. The third round of PCR produces inverted repeat sequences with a terminal linker containing *Spe*I and *Bgl*II restriction sites.

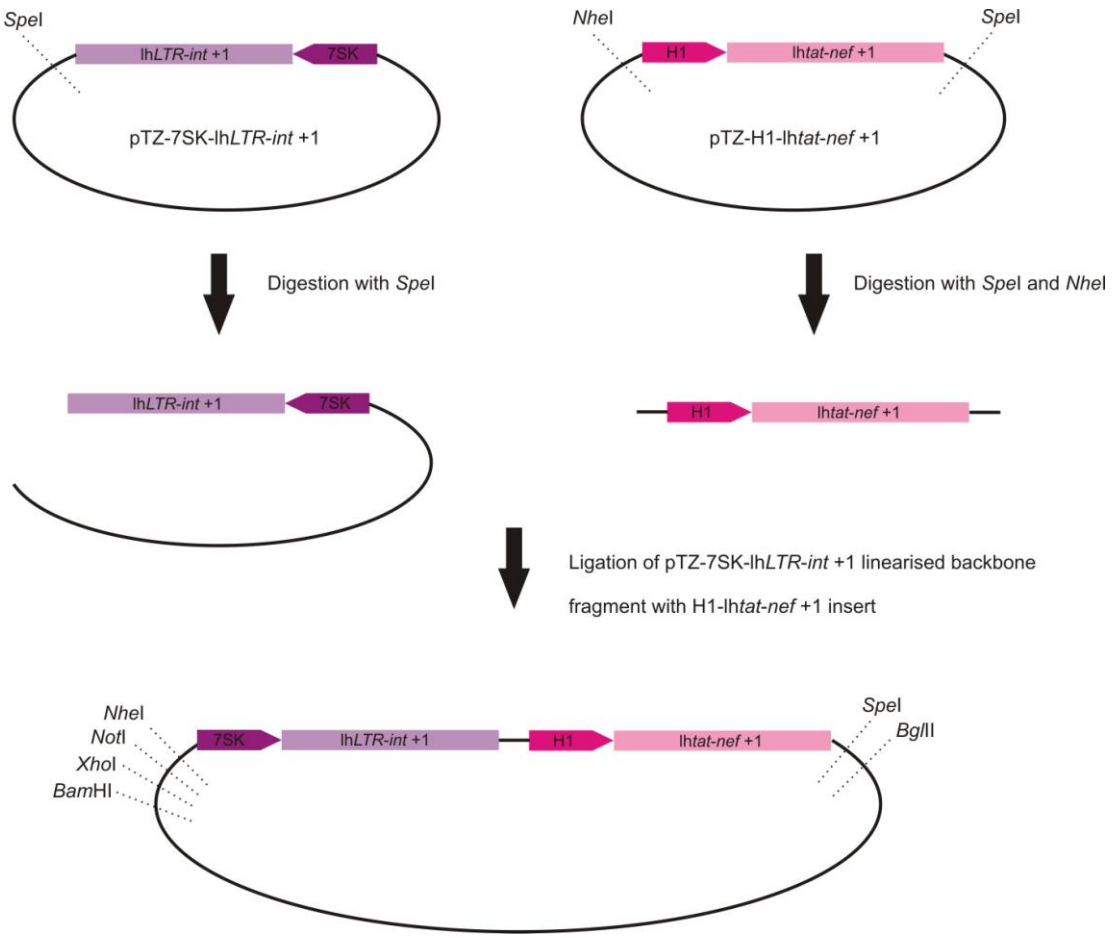


Figure 3.2: Schematic diagram illustrating the cloning strategy used to incorporate the H1-driven lh*tat-nef* +1 and 7SK-driven lh*LTR-int* +1 dual-targeting lhRNAs within a single pTZ57R/T plasmid vector. The pTZ-7SK-lhRNA-*LTR-int* +1 plasmid was linearised by digestion with *Spe*I and the H1-lhRNA-*tat-nef* +1 cassette was excised from the pTZ-H1-lhRNA-*tat-nef* +1 plasmid by digestion with *Nhe*I and *Spe*I. The H1-lhRNA-*tat-nef* +1-containing fragment was ligated into the linearised pTZ-7SK-lhRNA-*LTR-int* +1 plasmid backbone to generate a single plasmid containing the 7SK-lhRNA-*LTR-int* +1 and H1-lhRNA-*tat-nef* +1 expression cassettes.

The H1-lhRNA-*tat-nef* +1 insert in the forward orientation was excised by digesting 1 µg of the pTZ57R/T plasmid containing the insert with 10 U of each *Spe*I and *Nhe*I and Tango™ Buffer (33 mM Tris-acetate [pH7.5 at 37°C]; 10 mM Mg-acetate; 66 mM K-acetate and 0.1 mg/ml BSA) (Fermentas, WI, USA), in a 20 µl reaction at 37°C for 1 hour. Digested DNA was resolved on a 1% agarose gel and

the pTZ-7SK-lhRNA-*LTR-int* +1 linearised backbone fragment as well as the H1-lhRNA-*tat-nef* +1 insert fragment were excised from the gel and purified using the MinElute™ Gel Extraction kit (QIAGEN, CA, USA) according to the manufacturer's instructions (Appendix A1.3). The two purified fragments were ligated in a 1:1 ratio (approximately 50 ng of each) in a 20 µl reaction volume using 10 U of T4 DNA ligase (NEB, MA, USA). The ligation reaction was incubated at room temperature for 1 hour before transformation of chemically competent DH5α *E.coli* (Appendix A1.1). DNA isolated from individual clones was screened by digestion with *Xho*I and *Spe*I.

3.2.2 Dual luciferase fusion reporter plasmids

Individual target sequences complementary to the antisense strand of each putative siRNA were prepared by treating partially complementary oligonucleotides (Table 3.2) with polynucleotide kinase (Promega, WI, USA) and annealing them as described in 2.2.2. Each pair of overlapping oligonucleotides had an *Xho*I site at the 5' end of the forward oligonucleotide and a *Spe*I site at the 5' end of the reverse oligonucleotide. In addition, an *EcoR*V restriction site was incorporated into both the forward and reverse oligonucleotides within the overlapping region to facilitate screening of plasmids containing the inserted target sequence. A combined target sequence containing the *LTR*, *int*, *tat* and *nef* antisense target sequences in tandem, was prepared by a similar procedure using two sets of partially complementary oligonucleotides (Table 3.2) that contained a short overlapping region to facilitate the generation of a full length combined target sequence for insertion into psiCHECK.

The psiCHECK™-2 (Promega, WI, USA) plasmid backbone was prepared as described in 2.2.2. Dual luciferase psiCHECK target reporter plasmids were constructed by directly cloning the target sequences into the *Xho*I-*Not*I sites of the plasmid backbone such that the cloned sequences were within the 3' UTR of *Renilla* luciferase. The oligonucleotide sequences used to generate such target sequences are listed in Table 3.2.

Table 3.2: Oligonucleotides used to generate gene specific target sequences

Oligonucleotide	Sequence (5'-3')	Length (nt)
tat antisense target forward	TCGAGATATCGCTCTTCGTCGCTGTCTCCGCA	32
tat antisense target reverse	CTAGTGCGGAGACAGCGACGAAGAGCGATATC	32
nef antisense target forward	TCGAGATATCCTTGTGCTTCTAGCCAGGCACA	32
nef antisense target reverse	CTAGTGTGCCTGGCTAGAAGCACAAGGATATC	32
LTR antisense target forward	TCGAGATATCGTCTGAGAGGTCTCTAGTTACA	32
LTR antisense target reverse	CTAGTGTAACTAGAGACCTCTCAGACGATATC	32
int antisense target forward	TCGAGATATCGACTAGCCATTGCTCTCCGGCA	32
int antisense target reverse	CTAGTGCCGGAGAGCAATGGCTAGTCGATATC	32
LTR-int-tat-nef antisense target forward 1	TCGAGATATCAGTAACTAGAGATCCCTCAGACGAAGAGCCGGAGAGCAATGGCTAGTAGGCAGC	64
LTR-int-tat-nef antisense target reverse 1	**TCTCC**GCTGCCTACTAGCCATTGCTCTCCGGCTCTTCGTCTGAGGGATCTCTAGTTACTGATATC	65
LTR-int-tat-nef antisense target forward 2	**GGAGA**CAGCGACGAAGATGACGGTGCCTGGCTAGAAGCACAAGA	44
LTR-int-tat-nef antisense target reverse 2	CTAGTCTTGTGCTTCTAGCCAGGCACCGTCATCTTCGTCGCTG	43

Underlined sequences represent the *Xho*I and *Spe*I restriction endonuclease recognition sites in the forward and reverse primers respectively as well as *EcoR*V restriction endonuclease recognition sites within the overlapping region of forward and reverse oligonucleotides. Highlighted sequences indicate complementary regions between the pair of oligonucleotides and sequences in green represent overlapping regions.

3.2.3 Assessing the inhibitory efficacy of expressed lhRNAs in cell culture

Procedures for assessing the knockdown efficacy of dual-targeting lhRNAs against individual or combined psiCHECK target reporter plasmids were similar to those described in 2.2.3. HEK293 cells were cultured and transfected as previously described. Inhibition was initially assessed in cells transfected with 750 ng hairpin expression plasmid, 150 ng psiCHECK target reporter plasmid and 100 ng pCI-eGFP. For dose dependent inhibition assays, hairpin expression plasmids were transfected in diminishing quantities together with 150 ng psiCHECK target reporter plasmid and 100 ng pCI-e-GFP. The total amount of transfected DNA was kept constant at 1 µg and was made up with pUC19 (NEB, MA, USA) when hairpin expression plasmids were transfected at lower concentrations.

3.2.4 Detection of processed anti-HIV-1 hairpin sequences

Guide strands of siRNAs generated from dual targeting lhRNAs were detected according to the procedures described above (2.2.4). Membranes were however exposed to a phosphor plate which was scanned 3-7 days later using a Fujifilm FLA-7000 phosphorimager. Sequences of the probes complementary to the guide strands of *tat*, *nef*, *LTR* and *int* are as follows: *tat* probe 5'- GCG GAG ACA GCG ACG AAG AGC -3'; *nef* probe 5'– GTG CCT GGC TAG AAG CAC AAG –3'; *LTR* probe 5'– GTA ACT AGA GAC CTC TCA GAC –3' and *int* probe 5'– GCC GGA GAG CAA TGG CTA GTC –3'. The intensities of guide strands derived from dual-targeting lhRNA precursors were quantified relative to guide strands generated from shRNA controls using the Fuji ImageQuant software.

3.2.5 Statistical Analysis

Statistical analysis was carried out using the GraphPad Prism software (GraphPad, Software, Inc., CA, USA). Differences were considered significant when $p<0.05$ and was determined using one-way ANOVA followed by a Dunnett's Multiple Comparison post-test.

3.3 Results

3.3.1 Optimal design of dual-targeting lhRNAs

To establish a set of universally applicable guidelines for the optimal design of lhRNAs for the efficient production of two functional guide sequences, a series of anti-HIV-1 dual-targeting lhRNAs were designed to incorporate the sequences of four unique siRNA sequences complementary to conserved regions within the HIV-1 genome (Figure 3.3). These regions were selected because they have previously been shown to be highly susceptible to RNAi-mediated gene silencing. They include sequences within the *tat* (Lee et al. 2002a), *nef* (Westerhout et al. 2006b), *LTR* (Barichievy et al. 2007) and *int* (Nishitsuji et al. 2006) open reading frames. To assess the effect of siRNA sequence as well as siRNA position within the duplex, on Dicer processing, two panels of lhRNAs were generated (Figure 3.4). One panel of lhRNAs incorporated siRNA sequences against *tat* and *nef* while the remaining panel comprised siRNA sequences target to *int* and the *LTR.* Two sets of hairpins were present within each panel, allowing for each siRNA encoding sequence to be situated in both sequential positions of the hairpin duplex.

Human Dicer does not process siRNAs positioned at the loopside of stem duplexes with the same efficiency as it does siRNAs at the base of the stem duplex (Barichievy et al. 2007; Liu et al. 2007; Weinberg et al. 2007; Saayman et al. 2008; Sano et al. 2008). However, optimal spatial arrangement of the putative siRNA sequences within the duplex may result in augmented cleavage, and thus RNAi activity of a second siRNA sequence. Although gross generalizations regarding the most favourable siRNA spacing cannot yet be made, the insertion or deletion of bp at the siRNA junctions appears to enhance or reduce the efficiency of a second cleavage reaction. Liu et al. reported that 4 bp inserted between two 19 bp siRNAs sequences resulted in the production of two functional siRNA species (Liu et al. 2007). The effects of spacing between siRNA-encoding sequences within the dual-targeting lhRNAs studied in this Chapter, was thus investigated to ensure silencing efficacy from both siRNAs. Each set of hairpins therefore consisted of three lhRNAs with 1, 2 or 3 random mismatched bp inserted at the junction between siRNA encoding sequences of 21 bp. In

addition, another 5 bp were inserted adjacent to the loop sequence to eliminate the possibility of the second Dicer cleavage reaction occurring within the loop sequence thus yielding an ineffective second siRNA product. The lhRNA design approach described above resulted in four sets of hairpin duplexes each containing three dual-targeting lhRNAs with stem duplexes 48, 49 or 50 bp in length and with a 9 nt terminal loop (Figure 3.4). All lhRNAs were designed to be transcribed from the U6 snRNA Pol III promoter. G:U and U:G pairings were introduced at regular intervals along the sense strand of the duplex to facilitate enhanced propagation of inverted repeat sequences in *E.coli* as well as to assist with accurate sequencing of the lhRNAs.

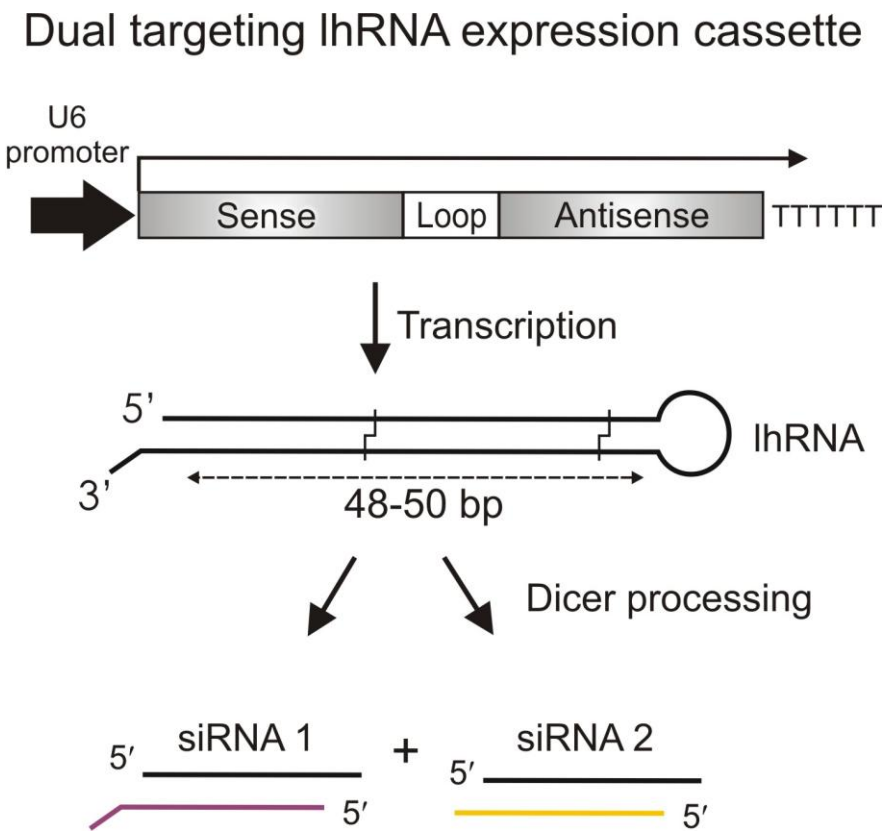


Figure 3.3: Schematic representation of a dual-targeting lhRNA expression cassette showing the upstream U6 promoter and the predicted lhRNA structure post transcription. Dual targeting lhRNAs were designed to incorporate two 21 bp siRNA sequences with variable spacing (1, 2 or 3 bp) at the junction and a 5 bp terminal extension. The resulting duplexes of 48, 49 or 50 bp allow for two predicted Dicer cleavage reactions and the subsequent generation of two siRNAs containing unique guide strands (indicated in colour).

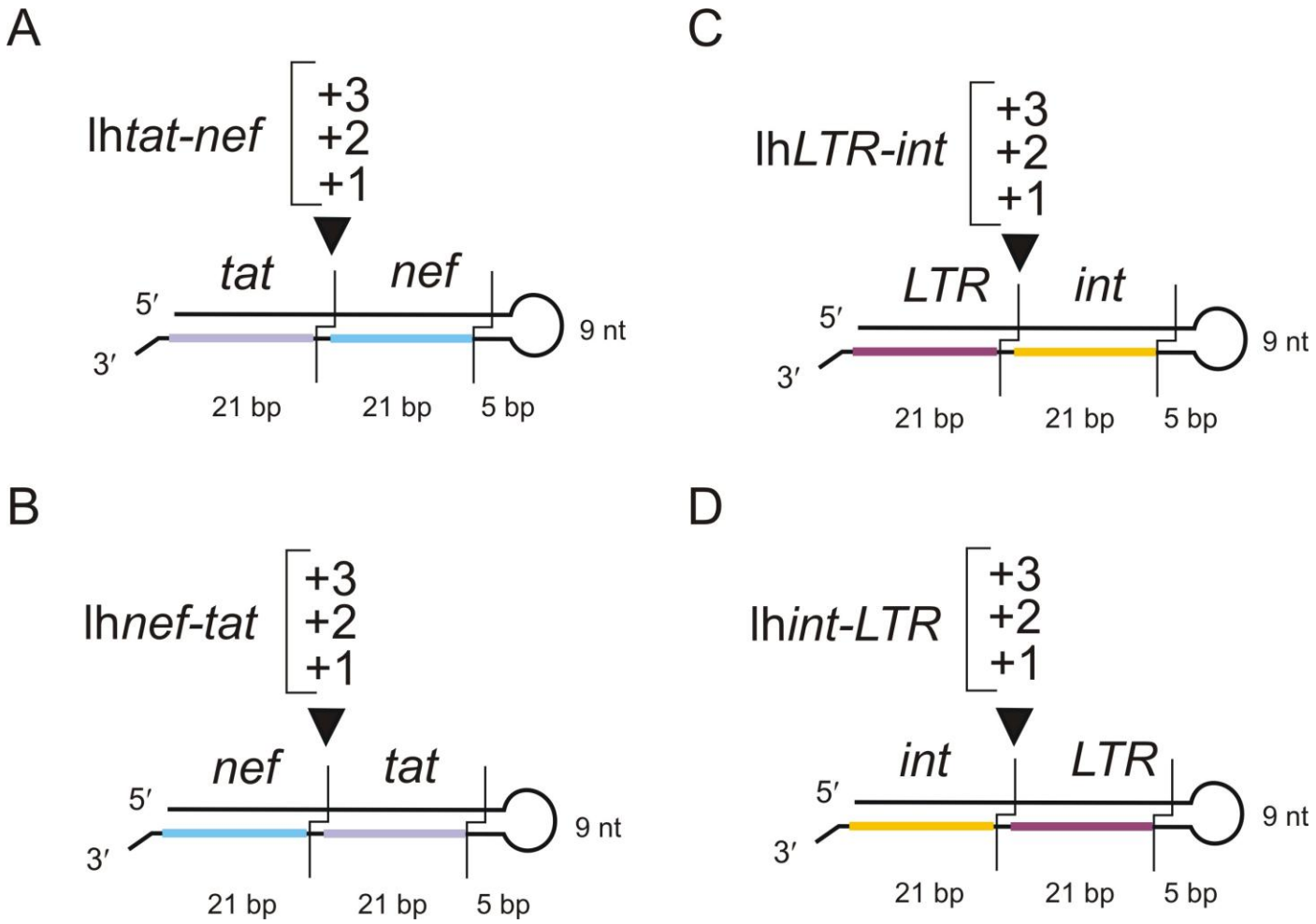
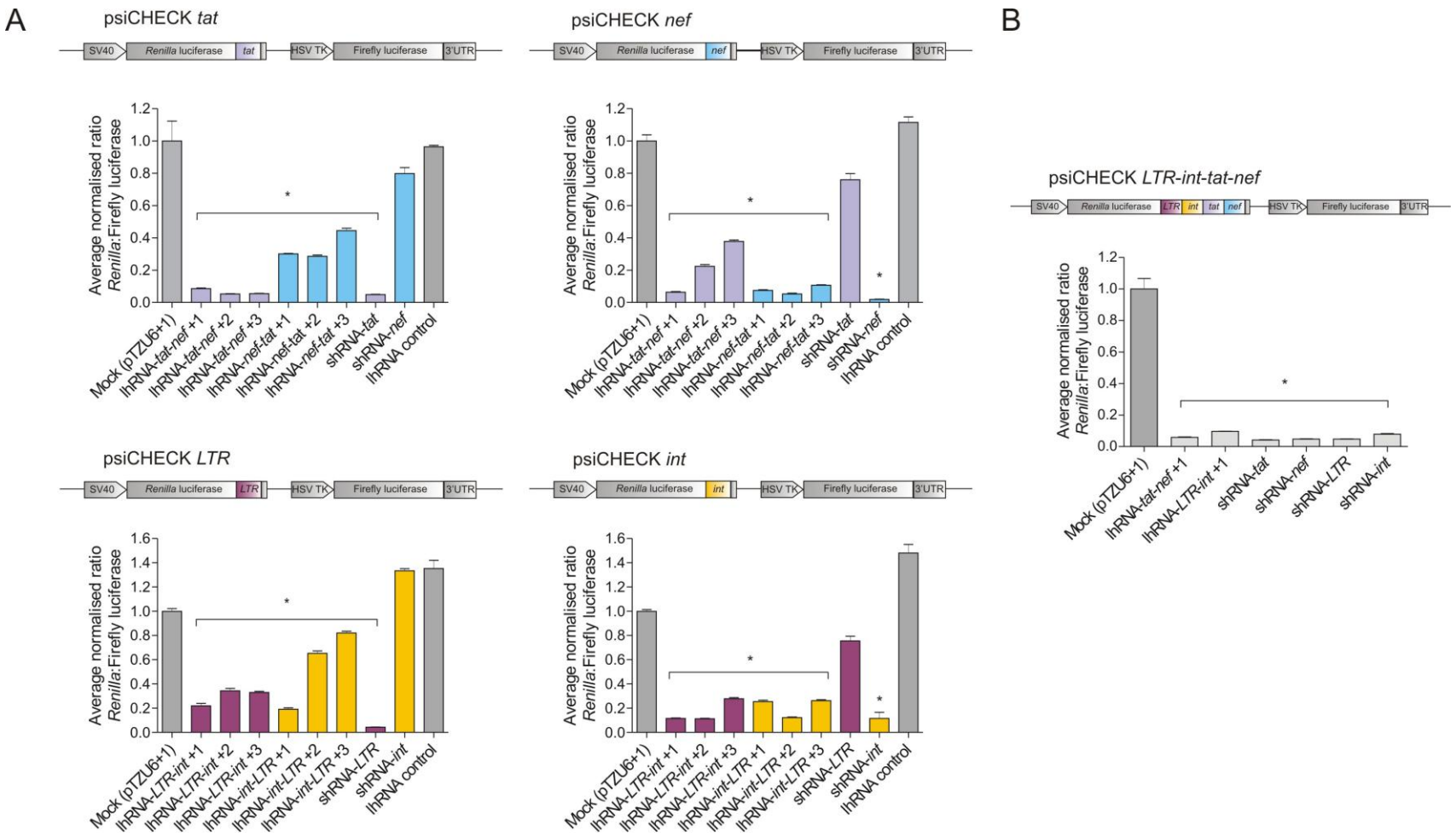


Figure 3.4: Design of anti-HIV dual-targeting lhRNAs. Panels of anti-HIV lhRNAs were designed to encode two 19 bp + 2 nt siRNAs targeted to the *tat* and *nef* genes **(A)** and **(B)** or the *LTR* and *int* genes **(C)** and **(D)**. siRNA sequences were positioned in both sequential positions of the hairpin duplex and within each set of lhRNAs, up to three random mismatched bp were inserted at the junction of siRNA encoding sequences. A terminal extension of 5 bp was inserted adjacent to the 9 nt loop sequence.

3.3.2 Inhibitory effects of expressed lhRNAs in cell culture

To assess the ability of variable length dual-targeting lhRNAs to inhibit the cognate targets of the respective incorporated siRNA sequences *in vitro*, HEK293 cells were transfected with dual-targeting lhRNAs from each panel in Figure 3.4 or with individual relevant shRNA expression plasmids serving as positive controls. These hairpin constructs were transiently co-transfected with a psiCHECK dual luciferase target reporter plasmid containing either the *tat*, *nef*, *LTR* or *int* target sequence or a combination of all four target sequences downstream from the *Renilla* luciferase open reading frame (Figure 3.3 A). A non-specific lhRNA targeted against the *HBx* gene of HBV (Weinberg et al. 2007) was used as a negative control (lhRNA control). Knockdown was determined as a ratio of *Renilla* luciferase: Firefly luciferase and values were normalised to pTZU6+1 (mock). Regardless of whether 1, 2 or 3 bp were inserted at the siRNA junction, or the relative orientation of siRNA sequences within the duplex, siRNAs at the base of the hairpin were consistently capable of suppressing their respective targets approximately 80-95%. This knockdown was comparable to the inhibition achieved by individual control shRNAs which, as expected, showed target specific knockdown (Figure 3.5 A). Interestingly however, silencing abilities of siRNAs in the second position of the hairpin duplex showed efficient knockdown when only one mismatched bp was present between siRNA encoding regions, while a gradual decrease in efficacy was seen with increasing spacing at the siRNA junction. The increased spacing did not however appear to affect inhibitory activity of siRNAs at the base of the duplex (Figure 3.5 A). This result was consistent among all four target sequences and suggests that putative siRNA sequences of 19 bp + 2 nt with one mismatched base pair inserted between them provides an optimal lhRNA design ensuring maximum RNA activity from both positions of the hairpin duplex. This also implies that 19 bp + 3 nt is the maximum length of an siRNA present within a long dsRNA duplex. Attention should be drawn to lh*tat-nef* +1 and lh*LTR-int* +1, both of which show >80% target inhibition by siRNAs generated from both positions of the hairpin. When tested against a psiCHECK reporter plasmid containing a combination of all four target sequences inserted in the 3' UTR of *Renilla* luciferase, lh*tat-nef* +1 and lh*LTR-int* +1 again showed potent inhibition of the combined target at levels comparable to those afforded by individual shRNAs (Figure 3.5 B).

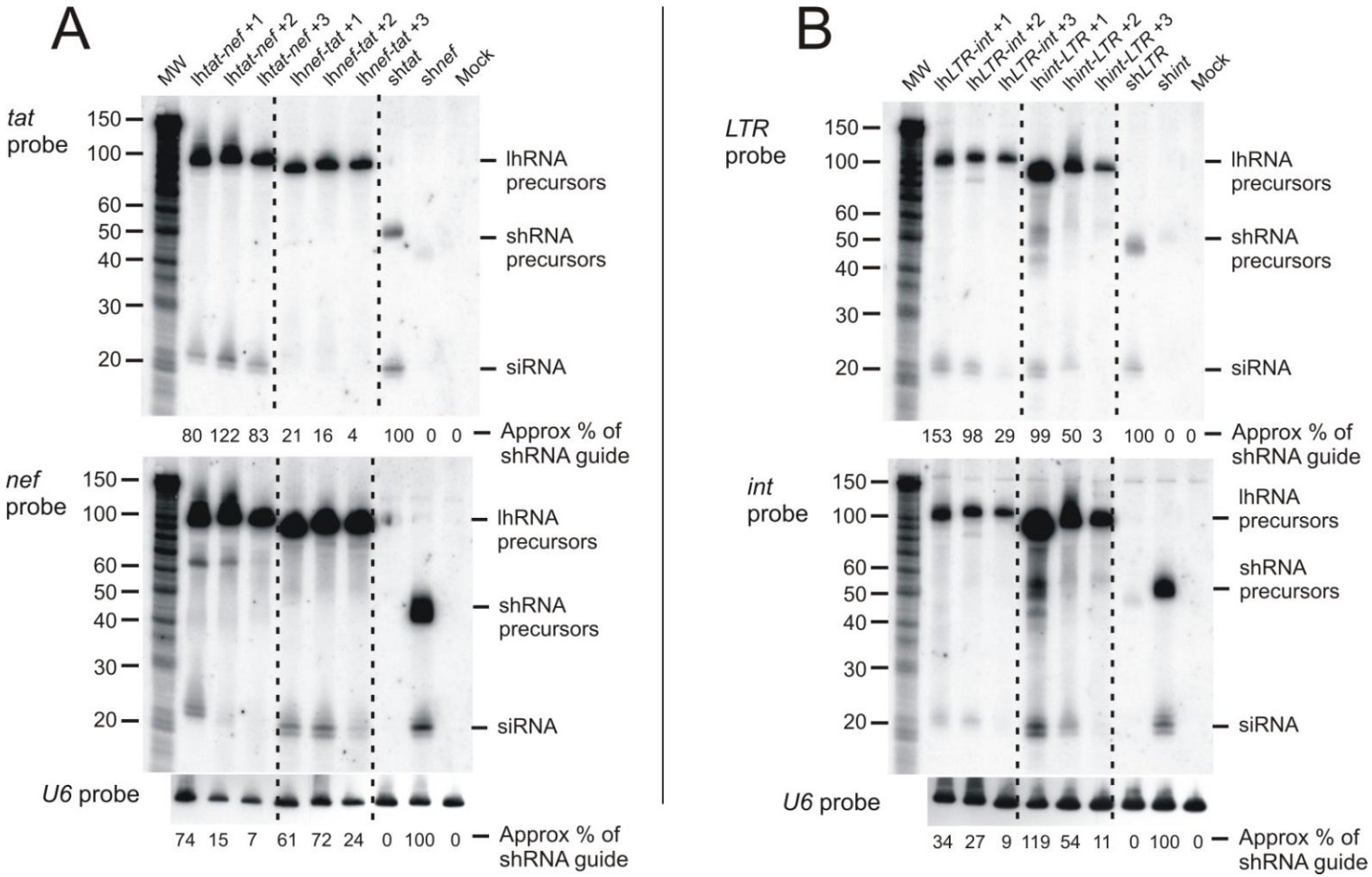


<u>Figure 3.5</u>: Inhibitory efficacy of dual-targeting lhRNAs. Dual luciferase reporter assays were used to measure target knockdown following co-transfection of HEK293 cells with dual-targeting lhRNA expression plasmids together with psiCHECK reporter plasmids containing each specific target sequence **(A)** or a combination of all of the target sequences in tandem **(B)**, inserted downstream of the *Renilla* luciferase (*h*RLuc) open reading frame. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with pTZU6+1 (mock) (*, p<0.05, one-way ANOVA relative to mock transfected control).

3.3.3 Detection of mature siRNAs generated from dual-targeting lhRNAs

To analyse processed anti-viral sequences from transfected hairpin expression cassettes, PAGE northern blot analysis was carried out on total RNA extracted from HEK293 cells transfected with dual-targeting lhRNA or control shRNA expression plasmids or with pTZU6+1 (mock). Figure 3.6 shows the signals obtained following hybridisation of membranes with four probes complementary to each of the guide strands of processed siRNAs. Confirming the results in Figure 3.5, northern blots showed a detectable signal from all dual-targeting lhRNAs when probed for the guide sequences of siRNAs positioned at the base of the stem. However, siRNAs at the base of the stem were only detected at comparable levels to shRNAs from lhRNAs when one or two bp were inserted at the junction. In the case of the *LTR* and *int* probes, minimal signals were detected from first position siRNAs where 3 bp were inserted at the siRNA junction. This contradicts the knockdown data (Figure 3.5) where spacing did not appear to affect RNAi activity from the base of the hairpin, and suggests that increased spacing may have a negative effect on the first Dicer cleavage reaction.

When probing for processed products from the second position of the hairpin, siRNAs were generally detected at lower concentrations. This decrease in siRNA production from the second position was observed in a gradient fashion with an inverse relationship to the bp spacing present at the junction between siRNA encoding sequences. When only one mismatched base pair was inserted at the junction, siRNAs in the second position were detected at similar levels to those of siRNAs at the base of the stem and the least signal reduction was observed. However when three mismatched base pairs were inserted at the siRNA junction, siRNAs in the second position were hardly detected, despite extended exposure of the blot. Where no signal was detected for siRNAs produced from the base of the stem (lh*int-LTR* +3), it was not unexpected that no signal was obtained for siRNAs at the top of the stem, since problems associated with the first Dicer cleavage reaction will invariably affect the second Dicer cleavage step.



<u>Figure 3.6</u>: PAGE northern blot analysis of total RNA extracted from cells transfected with the indicated dual targeting lhRNAs or shRNA controls. (A) and (B) represent two blots hybridised with labeled probes complementary to the guide strands of *tat* and *nef* **(A)** or *LTR* and *int* **(B)** and exposed to a phosporimaging plate. lhRNA and shRNA precursor RNA as well as processed siRNAs are indicated. Decade Marker™ was used as a molecular weight marker (MW) and a probe complementary to U6 small nuclear RNA was used as a control to verify equal loading of each RNA sample. The approximate band intensities (%) of generated guide sequences relative to the guide sequence derived from the relevant shRNA are indicated.

It should be noted that 2-3 bands differing in size by only 1 nt are often visible for a single siRNA which indicates that Dicer does not consistently cleave the duplex at the same position but rather generates siRNAs ranging in size from 19-22 nt. lh*tat-nef* +1 and lh*LTR-int* +1 were the most effective lhRNAs in producing siRNAs from the first position as well as from the second position in similar quantities. Signals obtained following hybridisation with a probe complementary to U6 snRNA show equal loading of each RNA sample (Figure 3.6).

3.3.4 Dose response inhibition of individual target sequences by lhtat-nef +1 and lhLTR-int +1

A structural lhRNA design has been identified which enables the efficient cleavage of dual-targeting lhRNAs into two distinctive siRNAs, both capable of highly significant inhibition of their cognate targets *in vitro*. The knockdown described in 3.3.2 however, was a result of lhRNAs transfected in a ratio of 5:1 (lhRNA expression cassette:target reporter plasmid), and although potent RNAi activity was observed throughout the hairpin duplex of both lh*tat-nef* +1 and lh*LTR-int* +1, it remained to be determined whether or not this potency was able to be sustained at lower concentrations. To determine the effective dose of the dual-targeting lhRNAs, as well as to investigate whether or not the RNAi activity from the second position of the hairpin duplex is decreased when lhRNAs are present at low concentrations, a dose response inhibition assay was carried out. Cells were transfected with decreasing concentrations of lhRNA expression plasmids, together with a fixed quantity of target reporter plasmid, and the inhibitory efficacies of decreased quantities of siRNAs from both positions of the hairpin duplex were determined.

It was found that both siRNAs generated from lh*tat-nef* +1 and lh*LTR-int* +1 were capable of maintaining effective target knockdown >60% from both positions of the hairpin duplex at concentration ratios as low as 0.005:1 (less than 1 ng) and 0.05:1 (7.5 ng) in the cases of lh*tat-nef* +1 and lh*LTR-int* +1 respectively (Figure 3.7). Therefore even when lhRNAs were present at very low concentrations, RNAi activity throughout the hairpin duplex remained highly effective. This is suggestive of a strong

dual siRNA response and also suggests that the design of the above mentioned lhRNAs allows for efficient Dicer processing along the entire duplex.

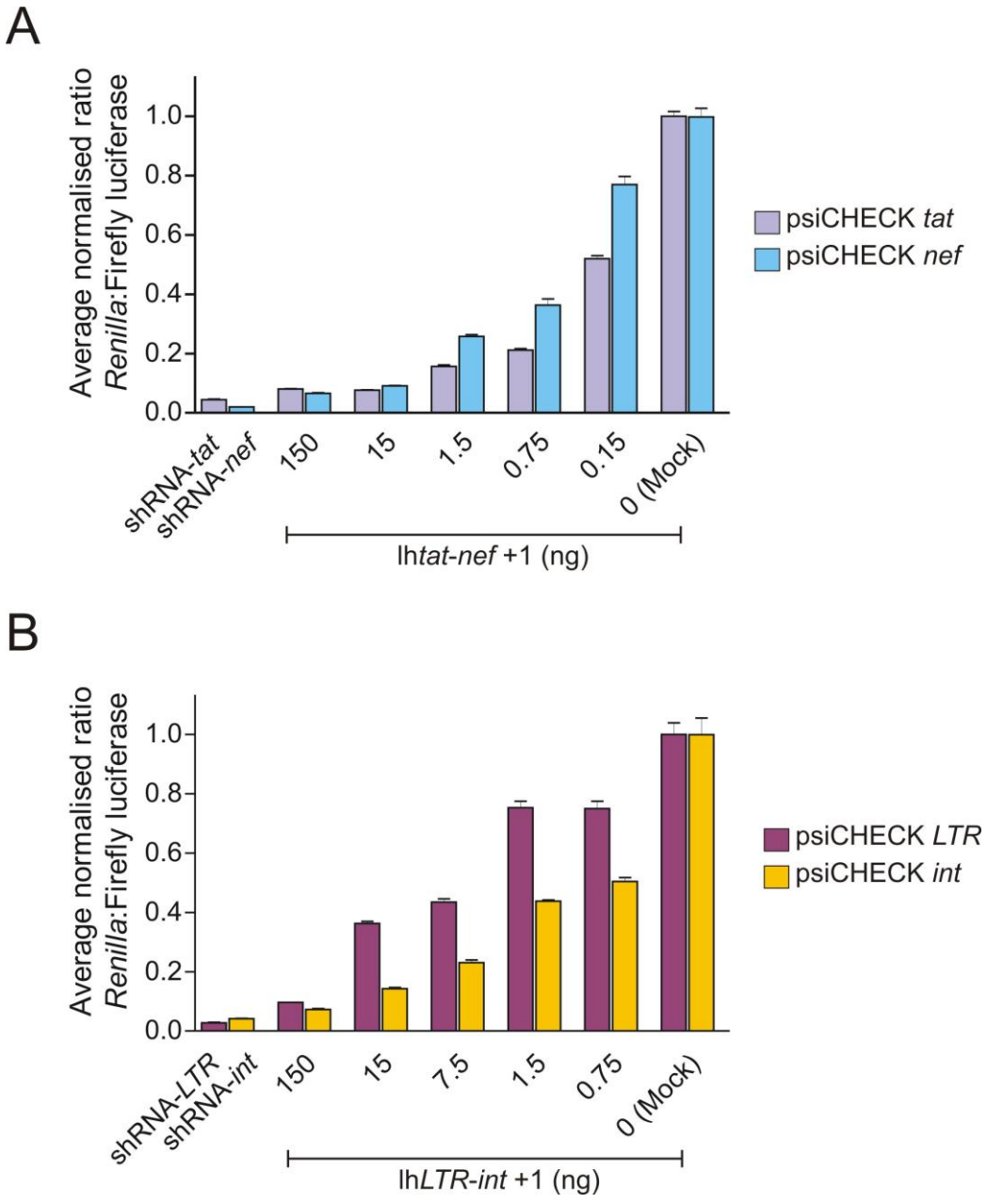


Figure 3.7: Dose response inhibition of individual targets by effective dual-targeting lhRNAs. Dual luciferase reporter assays were carried out to determine knockdown of the *tat* and *nef* targets **(A)** and *LTR* and *int* targets **(B)** following transfection with decreasing quantities of lh*tat-nef* +1 and lh*LTR-int* +1 respectively. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to mock transfected cells (0 ng).

Important to note however, is that although the four siRNA sequences selected for incorporation into the dual-targeting lhRNAs of this study showed equal inhibitory efficacies at high concentrations (Figure 3.5 and 3.7), when present at low concentrations it became evident that the RNAi activity afforded by each siRNA sequence was not equal (Figure 3.7). Within lh*tat-nef* +1, both the *tat* and *nef* siRNAs are highly potent, and at very low concentrations siRNA-*nef* is only slightly less effective than siRNA-*tat* and this is likely to be a result of siRNA-*nef* being located in the second position of the hairpin. Interestingly, the lh*tat-nef* +1 lhRNA is substantially more potent than lh*LTR-int* +1 and is able to maintain powerful knockdown at concentrations ten fold lower than lh*LTR-int* +1.

In the context of lh*LTR-int* +1, siRNA-*int* is situated in the second position of the hairpin duplex, yet displays stronger inhibition than siRNA-*LTR* when the hairpin is present at low quantities. However even though siRNA-*int* is stronger than siRNA-*LTR*, siRNA-*int* is capable of 50% target inhibition at a concentration five times that required by siRNA-*tat* for 50% target knockdown. This assay clearly reveals the strength of the *tat* and *nef* siRNAs and the relative weakness of the *LTR* siRNA in the context of dual-targeting lhRNAs, and indicates that at low concentrations, the inhibitory activity, even of highly effective siRNA sequences, is not uniform.

3.3.5 The use of alternative Pol-III promoters to drive expression of lhRNAs

Cellular toxicities may arise as a result of over-expression of RNAi effector molecules (An et al. 2006; Grimm et al. 2006) (section 1.7). The lhRNA and shRNA expression cassettes generated in this study have all been designed to be expressed from the human U6 snRNA Pol III promoter. As previously described, RNA Pol III transcripts act as ideal substrates for nuclear export as well as for Dicer processing. However because the U6 promoter is known to express transcripts at very high levels (Good et al. 1997), the activity of alternative RNA Pol III promoters was investigated. The simultaneous expression of multiple hairpins from a lentiviral vector requires the use of different promoters (ter Brake et al. 2008). Therefore for future combinatorial strategies harnessing the effective dual-targeting lhRNAs described in this Chapter, lh*tat-nef* +1 and lh*LTR-int* +1 sequences were

regenerated to be expressed from the human H1 or 7SK promoters respectively in individual expression plasmids as well as within a single vector. Both H1 and 7SK are RNA Pol III promoters which have previously been used to effectively drive the expression of hairpin RNAs (Brummelkamp et al. 2002; Czauderna et al. 2003b), albeit it at potentially lower levels than U6 (Makinen et al. 2006).

The effect of using alternate Pol III promoters H1 or 7SK to drive expression of lh*tat-nef* +1 and lh*LTR-int* +1 was assessed by a dual luciferase reporter assay following transfection of HEK293 cells with the various hairpin constructs. Very similar inhibition of each target was obtained when comparing the U6 driven transcripts to H1 or 7SK driven transcripts. When combining the two hairpin expression cassettes in a single vector, knockdown of the *tat* and *nef* targets remained highly potent and knockdown of the *LTR* and *int* targets was only minimally reduced (Figure 3.8). lhRNA-mediated inhibition therefore remained effective regardless of the Pol III promoter used.

Northern blot analysis was carried out on RNA extracted from cells transfected with the U6, H1 or 7SK expressed lhRNAs to determine expression levels from each promoter. When expressed from alternate Pol III promoters, similar siRNA concentrations were generated from the differently expressed lhRNAs except H1-lh*tat-nef* +1 which generated slightly more siRNA than U6-lh*tat-nef* +1. Slightly less siRNA was also generated from the lhRNAs when combined in a single vector (Figure 3.9). Important to note was the difference in hairpin precursor RNA concentration. H1 generated the least precursor RNA followed by U6 and then 7SK (Figure 3.9). The human H1 promoter thus appears to be the most effective in producing maximum siRNA with the least accumulation of precursor RNA. The use of the dual-targeting lhRNAs described in this Chapter could therefore be improved by using alternative, more efficient promoters. Furthermore, because these lhRNAs are effective at such low doses, the precursor accumulation seen in Figure 3.4 may also be decreased by using a lower concentration of these constructs. Moreover the strength of H1-lh*tat-nef* +1 and 7SK-lh*LTR-int* +1 was also maintained when expressed from within a single vector. This suggests that if used in combination, these two dual-targeting lhRNAs may potentially offer a basis for a combinatorial system effectively targeting four independent genes.

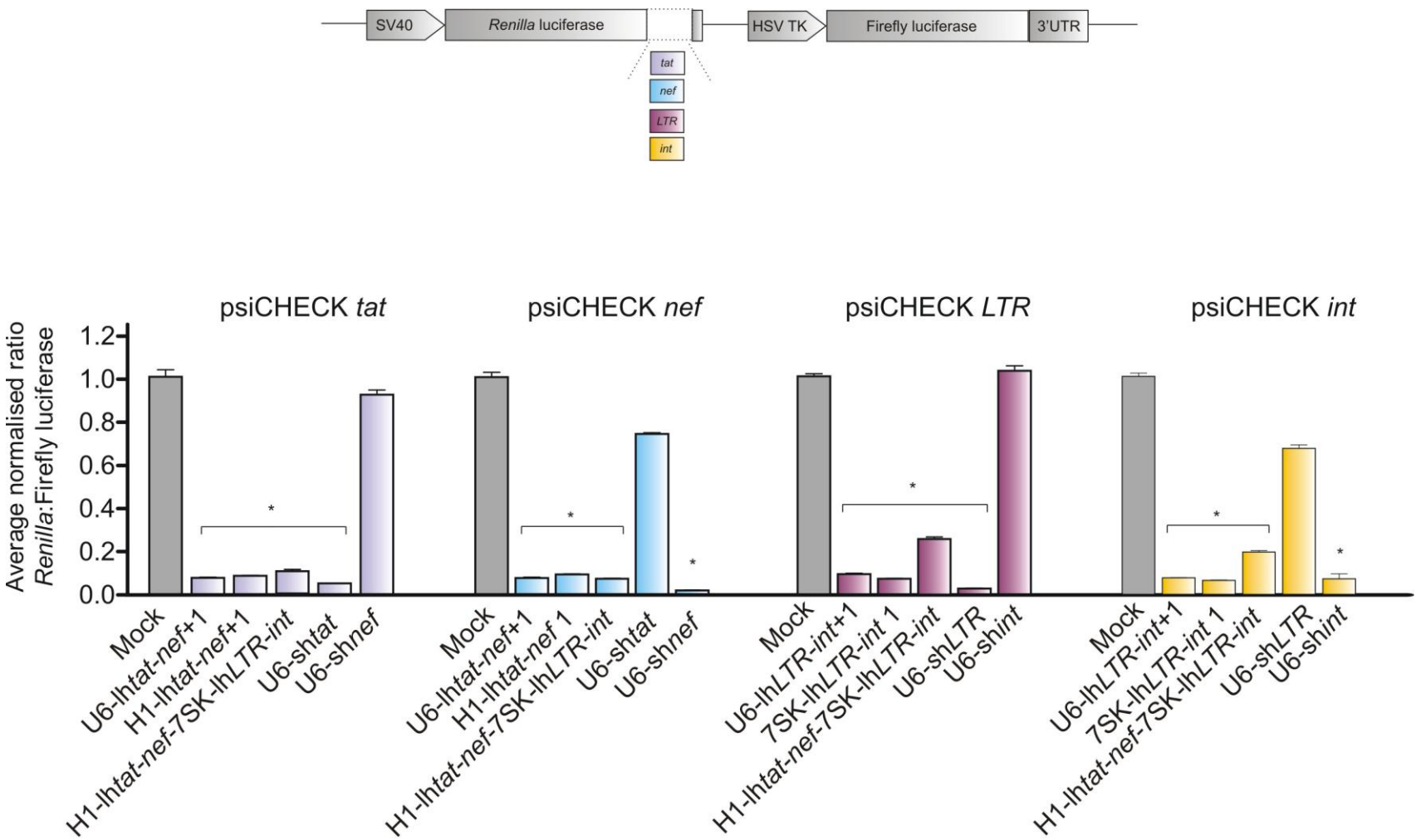


Figure 3.8: Effect of different RNA Pol III promoters on the inhibitory efficacy of lhRNAs. Dual luciferase reporter assays showing knockdown of each individual target by lhtat-nef+1 driven by the U6 or H1 promoter and lhLTR-int+1 driven by the U6 or 7SK promoter. H1-lhtat-nef-7SK-lhLTR-int represents a single vector consisting of two lhRNA expression cassettes. Individual U6 driven shRNAs were used as controls. Values represented are mean ratios of Renilla to Firefly luciferase (±SEM) and are normalised to cells transfected pTZU6+1 (mock) (*, $p<0.05$, one-way ANOVA relative to mock transfected control).

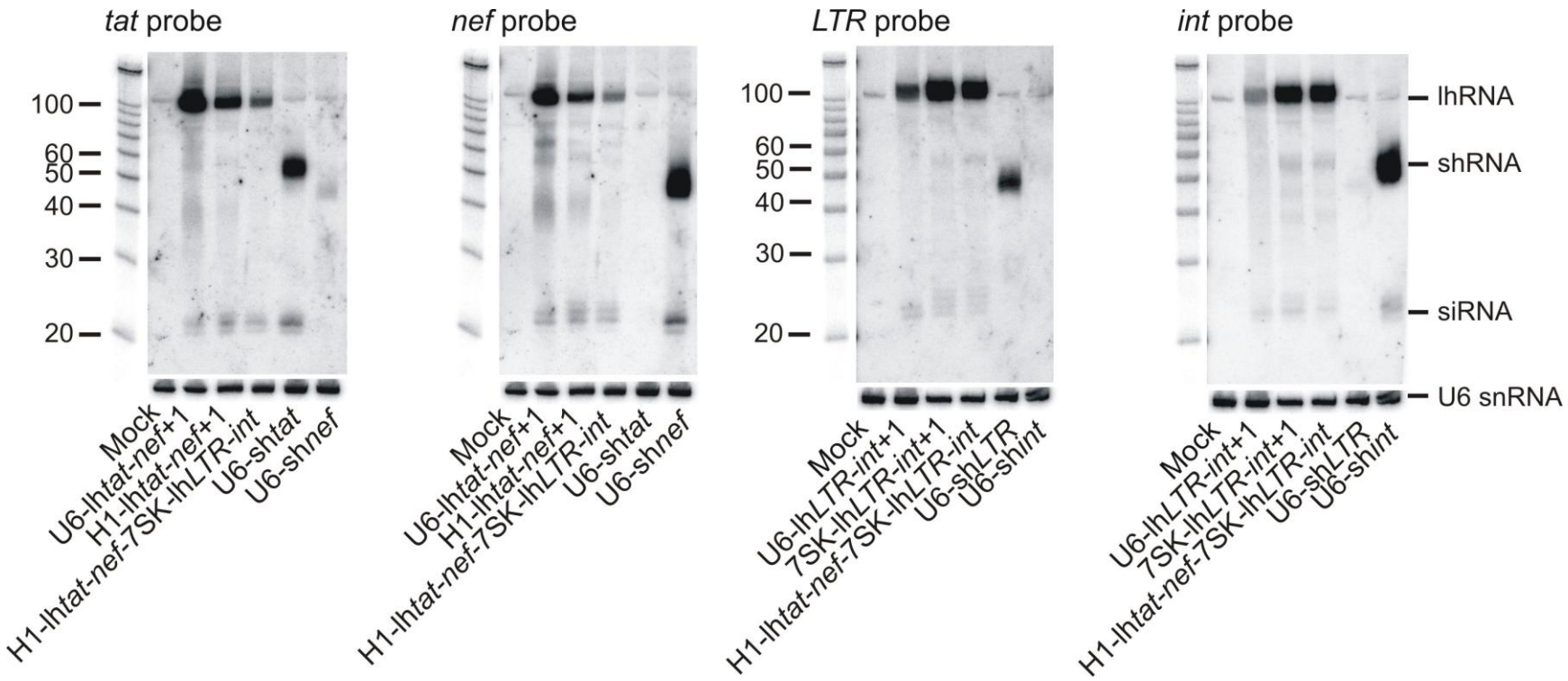


Figure 3.9: Effect of different RNA Pol III promoters on the intracellular processing of lhRNAs. PAGE northern blot analysis was carried out on total RNA extracted from cells transfected with the indicated transcripts. Labeled probes complementary to the guide strand of putative siRNAs targeting *tat*, *nef*, *LTR* and *int* were hybridised to immobilised RNA and exposed to a phosphorimaging plate. lhRNA and shRNA precursor RNA as well as processed siRNAs are indicated. Decade MarkerTM indicates fragment size and a probe complementary to U6 snRNA was used as a loading control.

3.4 Discussion

The catalytic activity of human Dicer is restricted to two efficient cleavage reactions, after which processivity rapidly declines (Barichievy et al. 2007; Liu et al. 2007; Weinberg et al. 2007; Saayman et al. 2008; Sano et al. 2008). Despite these repeated observations, little is known about the exact mechanism of action of human Dicer or the sequence and structure of an ideal Dicer substrate. Liu et al. recently attempted to optimise the design of hairpins encoding three and four siRNA sequences (Liu et al. 2009). Hairpins of 88 and 92 bp encoding four unique siRNAs had a decreased overall efficacy along the entire stem and suggest that the production of four siRNAs from a hairpin duplex is not feasible. Slightly shorter hairpins of 63 and 66 bp encoding three siRNAs were able to effectively inhibit all three target sites; however, mature siRNAs were yet again observed to be generated with greater efficacy from the base of the stem, thus yielding unequal quantities of siRNA products. Interestingly hairpins encoding three siRNAs containing a slightly longer terminal extension (5 bp vs. 2 bp) showed slightly enhanced activity from the second and third siRNAs. Once again hairpins of 43 bp encoding only two siRNAs were capable of consistently potent silencing from both positions of the hairpin duplex and exhibited the strongest inhibition of HIV-1 viral replication (Liu et al. 2009), once more implicating dual acting hairpins as ideal combinatorial hairpin substrates. This work therefore sought to characterise the inhibitory efficacy and processing trends of hairpin duplexes encoding two siRNAs. In addition, the effects on Dicer processing of siRNA sequence as well as of bp spacing at siRNA junctions and prior to the loop sequence were thoroughly investigated. A set of parameters have thus been proposed for the design of dual-targeting lhRNAs capable of producing two highly active siRNAs produced at similar quantities and effective at low doses.

Four effective siRNA sequences identified from previously published studies (Lee et al. 2002a; Nishitsuji et al. 2006; Westerhout et al. 2006b; Barichievy et al. 2007) were exploited in the design of the dual-targeting lhRNAs described here in order to identify an optimal sequence-independent design for dual-targeting hairpins . These lhRNAs were designed to incorporate the sequences of two of the above mentioned siRNAs in both positions of the duplex and with 1-3 mismatched bp inserted at the siRNA junction. A small terminal extension preceding the loop sequence has been shown to enhance

the RNAi activity of the second and third siRNA in 66 bp hairpins encoding three siRNAs (Liu et al. 2009). Each lhRNA had a 5 bp terminal extension inserted prior to the loop sequence to augment processing of the entire hairpin stem. The bp spacing inserted at the siRNA junctions proved to have a marked effect on the generation of siRNAs from the hairpin duplex and thus the knockdown efficacy of the lhRNAs. When only 1 mismatched bp was inserted at the junction between siRNA encoding sequences, the strongest inhibitory activity was induced from both positions of the hairpin duplex regardless of siRNA sequence or position. In the case of lh*tat-nef* +1 and lh*LTR-int* +1, PAGE northern blots indicated that siRNAs were also generated from both positions of the duplex in equal quantities. Furthermore RNAi activity from both positions of the duplex was maintained even when these lhRNAs were present at very low concentrations or when expressed from weaker RNA Pol III promoters. These results suggest that 19 bp + 2 nt siRNA sequences with 1 bp inserted at the junction in the context of a dual-targeting lhRNA constitutes an effective structure for optimal processing by Dicer. When 3 bp were inserted at the siRNA junction the knockdown afforded by siRNAs in the second position was markedly reduced, and northern blot analysis showed a decrease in siRNA production from both positions of the hairpin. The decrease in siRNA production from the second position was marked, to the point that no siRNA was detected at all. However this is not surprising if the first cleavage reaction is also being negatively affected, since Dicer processing begins at the open end of the duplex and proceeds towards the loop. Therefore if the first siRNA is not cleaved, the second siRNA will not be cleaved either. These results provide a guideline for the maximum length of a hairpin stem as well as a maximum length for each individual siRNA encoding sequence, for efficient Dicer processing. In the case of lh*LTR-int* +3, slightly less hairpin precursor RNA was detected by northern blot analysis which indicates that slightly longer transcripts may not be transcribed as efficiently by a Pol III promoter or that an lhRNA with a 3 bp insert is less stable. The fact that the 3 inserted bp are mismatched, thus creating a small bulge in the centre of the duplex may also play a role in the observed decline in Dicer processing. It has been shown that the N-terminal helicase domain of human Dicer plays a role in the preferred processing of thermodynamically unstable duplexes such as pre-miRNAs (Ma et al. 2008; Soifer et al. 2008), but that when this domain is mutated, cleavage of thermostable duplexes such as lhRNAs is enhanced (Soifer et al. 2008). However, shRNAs designed

to incorporate mismatches or small bulges within the stem, thus causing a reduction in thermodynamic stability, showed a marked reduction in processing regardless of the presence or absence of a mutated helicase domain (Soifer et al. 2008). The resulting bulge in dual-targeting lhRNAs with 3 bp inserted at the siRNA encoding junction may therefore be the limiting factor for efficient Dicer processing, and in support of previous studies, may affect not only the second but the first cleavage reaction too. It is therefore possible that the dramatic decrease observed in intracellular processing of lhRNAs with 3 bp inserted at the siRNA junction may be less pronounced if the inserted bp were flanking bp of the siRNA sequence.

In conclusion a general framework for the design of effective dual-targeting lhRNAs has been provided. It should be noted that both the sequence and spatial arrangement of siRNAs within a duplex affect the efficiency of Dicer processing, and that adjustments in the length of the duplex stem and the insertion of base pairs between each siRNA encoding sequence as well as prior to the loop sequence, may serve to augment RNAi activity throughout the stem. Two highly effective dual-targeting lhRNAs have been identified, namely lh*tat-nef* +1 and lh*LTR-int* +1 which together may generate four different siRNAs. These lhRNAs may be combined with other anti-HIV RNAi activators, or with conventional antiretroviral drugs to formulate a unique combination therapy for the treatment of HIV. Alternatively, the intrinsic combinatorial properties of these dual-targeting lhRNAs can be exploited in future endeavors to develop novel multiplexed RNAi modalities.

CHAPTER 4

Combining two effective dual-targeting lhRNAs within a single expression cassette as a novel combinatorial RNAi structure.

4.1 Introduction

The capacity for lhRNAs to encode multiple siRNA sequences has been exploited in the development of potential combinatorial RNAi strategies. However following extensive characterisation of lhRNAs, the limitations of such constructs have been clearly demonstrated. Efficacious quantities of only two siRNAs can be derived from a single lhRNA stem. However, a computational algorithm has predicted and experiments have confirmed that to prevent the emergence of HIV-1 viral escape mutants, four independent gene sequences should be simultaneously suppressed (Leonard and Schaffer 2005; ter Brake et al. 2008). Although optimised to produce two siRNAs efficiently, dual-targeting lhRNAs do not therefore represent a sufficient combinatorial approach for the prevention of resistance. However instead of using hairpins encoding three siRNAs, with only moderately efficacious levels of the third siRNA being produced, a combination of dual-targeting lhRNAs may offer a more efficient strategy for the simultaneous targeting of more than two gene sequences. It was shown in Chapter 3 that two effective dual-targeting lhRNAs can be expressed from a single vector for the suppression of four independent gene targets, however this strategy is analogous to the use of multiple expressed shRNAs and the same associated problems and limitations exist (section 1.10.1). Avenues need to be explored which investigate the feasibility of developing novel lhRNA-based combinatorial RNAi systems.

The combination of effective dual-targeting lhRNAs in tandem within a single expression cassette represents an attractive possibility, however the configuration of such a structure requires further exploration. During the initial quest to generate RNAi effector mimics following the demonstration that synthetic siRNAs could cause gene specific silencing in mammalian cells (Elbashir et al. 2001a), Leiral

and Sioud identified a bispecific siRNA structure configured with two shRNA structures separated by 8 nucleotides which essentially acted as a linker between the two shRNAs (Leirdal and Sioud 2002). This structure was transcribed *in vitro* by a T7 RNA polymerase and was shown to be processed intracellularly to yield RNA species correlating to the size of the individually incorporated shRNAs. It was hypothesized that an endoribonuclease was responsible for cleaving the single stranded RNA linker sequence to yield two functional shRNAs which were shown to effectively silence their cognate targets. This bispecific shRNA design was expanded upon soon thereafter by Anderson et al. who investigated the combinatorial properties of this unique structure to suppress two HIV-1 host cell receptors simultaneously (Anderson et al. 2003). Anderson et al. also showed that intracellular processing of this structure resulted in two discrete shRNA species capable of significant inhibition of complementary host cell receptor genes. Three different linker sequences were analysed for their role in the efficacy of these bispecific hairpin structures. Of the three sequences examined, only one resulted in optimal processing and gene silencing, indicating that the RNA sequence separating the two hairpin moieties plays an important role in the efficacy of such constructs. Although the bispecific shRNA structure as well as the mechanism by which it is processed, was not sufficiently characterised, these two studies suggest an interesting new approach to combining previously characterised lhRNAs.

Chapter three identified two unique highly effective lhRNAs, lh*tat-nef* +1 and lh*LTR-int* +1, each optimised for the generation of two potent siRNAs, for future incorporation into a multiplexed RNAi-based therapy. In this chapter a novel combinatorial RNAi structure is described that demonstrates the effects of tethering these two highly effective dual-targeting lhRNAs, adjacent to one another in a double-lhRNA expression cassette driven from a single RNA Pol III promoter. In addition, the ability of this novel structure to generate four independent functional siRNAs is revealed. These constructs did not induce an interferon response nor did they cause saturation of the endogenous miRNA biogenesis pathway and therefore represent a promising new strategy for the simultaneous suppression of multiple viral genes.

4.2 Materials and Methods

4.2.1 Generation of U6-driven double-lhRNA expression cassettes

Double-lhRNA expression cassettes were generated by the 2-step PCR method described in 2.2.1, using the existing dual-targeting lhRNAs (lh*tat-nef* +1 and lh*LTR-int* +1) as templates. A total of four rounds of PCR were therefore required for the production of a double-lhRNA sequence. Round one reverse primers were complementary to the last 18 nt and two thymine residues at the 3' terminal end of the dual-targeting lhRNA template. To generate the double-lhRNA: lh*LTR-int*-lh*tat-nef*, lhRNA-*LTR-int* +1 was used as a template and was amplified with the universal U6 forward primer and unique LITN R1 reverse primer: 5'- CTG GGT CAG GGA CAT ATT GTA CTT CCA GCC AGA CAC TGC TCT TCA TCA CTA TCC CCG CAA GTA ACT AGA GAC CTC TCA -3' (78 nt). The lhRNA-tat-nef +1 R2 primer (Table 3.1) was used as the reverse primer for the second round of PCR. To generate the double-lhRNA: lhtat-nef-lhLTR-int, lhRNA-tat-nef +1 was used as a template and amplified with the universal U6 forward primer and unique TNLI R1 reverse primer: 5'- CTG GGT CAG GGA CAT AAC TAA CCA TTG CCC TCC GGC TGT CCG AGA GAT CTC TAA TTA CAA GCG GAG ACA GCG ACG A -3' (76 nt). The lhRNA-*LTR-int* +1 R2 primer was used as the reverse primer for the second round of PCR. Individual shRNAs and dual-targeting lhRNAs described in Chapter 3 were used as positive controls.

4.2.2 Dual luciferase fusion reporter plasmids

Dual luciferase fusion reporter plasmids encoding the sequence targeted by the antisense strand of each putative siRNA were described in 3.2.2. To determine strand biasing, dual luciferase fusion reporter plasmids encoding the sequence targeted by the sense strand of each putative siRNA were also generated. These reporter plasmids were constructed using the same method described in 3.2.2 with partially complementary oligonucleotides (Table 4.1).

Table 4.1: Oligonucleotide sequences used to generate gene specific target sequences

Oligonucleotide	Sequence (5'-3')	Length (nt)
tat sense target forward	TCGAGATATCGCTCTTCGTCGCTGTCTCCGCA	32
tat sense target reverse	CTAGTGCGGAGACAGCGACGAAGAGCGATATC	32
nef sense target forward	TCGAGATATCCTTGTGCTTCTAGCCAGGCACA	32
nef sense target reverse	CTAGTGTGCCTGGCTAGAAGCACAAGGATATC	32
LTR sense target forward	TCGAGATATCGTCTGAGAGGTCTCTAGTTACA	32
LTR sense target reverse	CTAGTGTAACTAGAGACCTCTCAGACGATATC	32
int sense target forward	TCGAGATATCGACTAGCCATTGCTCTCCGGCA	32
int sense target reverse	CTAGTGCCGGAGAGCAATGGCTAGTCGATATC	32

Underlined sequences represent the *Xho*I and *Spe*I restriction endonuclease recognition sites in the forward and reverse primers respectively as well as *Eco*RV restriction endonuclease recognition sites within the overlapping region of forward and reverse oligonucleotides. Highlighted sequences indicate complementary overlapping regions between the pair of oligonucleotides.

4.2.3 Assessing the inhibitory efficacy of expressed lhRNA and dlhRNA constructs against sense and antisense targets in cell culture

Double-lhRNA expression cassettes were assessed for their ability to knockdown four individual target sites simultaneously in cell culture according to previously described procedures (section 2.2.3). Individual shRNAs as well as individual dual-targeting lhRNAs were used as positive controls. In addition to determining the knockdown of each individual target complementary to the antisense strand of each putative siRNA, inhibition of sequences complementary to the sense strand of each putative siRNA was also determined using the procedure described in 2.2.3 and the dual luciferase reporter plasmids described in 4.2.2.

4.2.4 Detection of processed anti-HIV-1 hairpin sequences from dlhRNAs

Guide strands of siRNAs generated from expressed double-lhRNAs were detecting using PAGE northern blot analysis according to the procedure described in 3.2.4. The antisense strand of each siRNA was detected using the oligonucleotide probes listed in 3.2.4. The passenger strands of putative siRNAs were detected using the following oligonucleotides complementary to the sense strand of each siRNA: *tat* sense probe 5'- GCT CTT CAT CAC TAT CCC CGC -3'; *nef* sense probe 5'– ATT GTA CTT CCA GCC AGA CAC –3'; *LTR* sense probe 5'– GTC CGA GAG ATC TCT AAT TAC –3' and *int* sense probe 5'– AAC TAA CCA TTG CCC TCC GGC –3'. The intensities of guide strands derived from dual-targeting lhRNA precursors were quantified relative to guide strands generated from shRNA controls using the Fuji ImageQuant software.

4.2.5 Suppression of HIV-1 subtype B and subtype C gene targets

HIV-1 FV5 challenge assay

To determine the extent of inhibition of HIV-1 subtype C primary isolate FV5, U87.CD4.CCR5 cells were seeded and transfected as described in 2.2.5. In this challenge assay however, the relative strength of the various hairpin constructs was determined by transfecting cells with two different concentrations of hairpin expressing plasmids. One set of cells was transfected with1 µg of each construct and the other set was transfected with only 100 ng of each construct. The challenge assay proceeded as described in 2.2.5.

Inhibition of gene targets in the pNL4-3.Luc.R-E- molecular clone

Hairpin expression cassettes were assessed for inhibitory efficacy of an HIV-1 subtype B target by determining knockdown of the pNL4-3 molecular clone. HEK293 cells were maintained and seeded for transfection as described in 2.2.3. Cells were co-transfected with each hairpin expression plasmid together with the molecular clone pNL4-3.Luc.R-E- (Connor et al. 1995; He et al. 1995). This

molecular clone has a Firefly luciferase gene inserted into the *nef* gene and is competent for only a single round of replication. Suppression of viral gene expression was therefore measured by levels of Firefly luciferase activity. Cells were transfected with one of three concentration ratios of hairpin expression plasmid to molecular clone: 5:1 (750 ng : 150 ng), 1:1 (150 ng : 150 ng), and 0.1:1 (15 ng :150 ng). Approximately 50 ng of phRL-CMV (Promega, WI, USA) was also transfected as a background luciferase control and 100 ng pCI-eGFP was co-transfected to control for transfection efficiency. At the lower hairpin concentrations, total DNA was made up with pTZU6+1. Dual luciferase reporter assays were carried out as described in 2.2.3 but Firefly luciferase values were normalised against background *Renilla* luciferase values.

4.2.6 Assessing the off target effects of lhRNA and double-lhRNA expression cassettes in vitro

Assay for derepression of an exogenously introduced miRNA

HEK293 cells were seeded at 120 000 cells per well in 24-well tissue culture plates (Nunclon™ Δ Surface, Nunc, Denmark) 24 hours prior to transfection in antibiotic-free medium as described in 6.1.5. To determine the effects of transfected hairpin constructs on the functioning of an exogenously introduced miRNA, cells were cotransfected with 100 ng pCMV miR-31 *HBx* (Ely et al. 2008), and 100 ng of psiCHECK *HBx*, together with the indicated quantity of hairpin construct. miR-31 HBx is a CMV-driven pri-miRNA shuttle vector based on the endogenous pri-miRNA-31. The guide sequence of this natural miRNA is substituted with an effective anti-HBV guide sequence targeted against the HBx ORF. The psiCHECK *HBx* plasmid expresses the cognate target of the guide strand downstream of the *Renilla* ORF. Transfections were carried out as described in 6.1.5 and 48 hours after transfection, a dual luciferase reporter assay was performed (2.2.3) to determine the ability of miR-31 *HBx* to inhibit its target in the presence of transfected hairpin expression cassettes.

Assay for derepression of an endogenous miRNA

To assess potential saturation of the endogenous miRNA pathway caused by transfected hairpin constructs, Huh7 cells were cotransfected with 80 ng psiCHECK-miR-16T×7 (Ely et al. 2009): a psiCHECK^{TM}-2 plasmid containing 7 miR-16 target sites downstream of the *Renilla* luciferase ORF; 750 ng hairpin expression plasmid or pTZ-U6-miR-16S×7 sponge plasmid (Ely et al. 2009): a plasmid containing 7 imperfectly complementary copies of a miR-16 target site; and 150 ng pCI-eGFP. Transfections and reporter assays were performed as described above to determine the ability of endogenous miR-16 to knock down psiCHECK-miR-16T×7 in the presence of the sponge plasmid or transfected hairpin expression cassettes.

Detection of non-specific immune response

To determine the induction of IFN response-related genes, HEK293 cells were maintained and seeded as described in 2.2.6. Cells were transfected with 900 ng of each hairpin expression plasmid or equivalent quantities of the dsRNA analogue poly (I:C) together with 100 ng pCI-eGFP per well. *IFN-β* mRNA was then quantified by reverse transcription of total RNA extracted from transfected cells and real-time PCR as described in 2.2.6.

4.2.7 Statistical analysis

Statistical analysis was carried out using the GraphPad Prism software (GraphPad, Software, Inc., CA, USA). Statistical difference was considered significant when $p<0.05$ and was determined using one-way ANOVA followed by a Dunnett's Multiple Comparison post-test.

4.3 Results

4.3.1 Design of U6-driven double-lhRNA (dlhRNA) expression cassettes

In order for the combinatorial capacity of lhRNAs to be realised, novel lhRNA-based RNAi strategies need to be explored. Two studies successfully used a bispecific shRNA structure for the generation of two independent shRNAs able to enter into the RNAi pathway (Leirdal and Sioud 2002; Anderson et al. 2003). Although it was suggested that this structure is likely to be cleaved by a single stranded ribonuclease at the site of the linker sequence, the mechanism by which these structures was processed was never fully characterised. However, the possibility of incorporating effective lhRNAs into a dual specific structure is highly attractive and offers enormous potential as a novel combinatorial RNAi approach. A similar structure was therefore adopted, modified, and investigated for its capacity to incorporate the dual-targeting lhRNAs already extensively described in Chapter 3.

Novel RNAi effector precursors were designed, which consisted of a combination of two previously identified highly effective dual-targeting lhRNAs, under the control of a single RNA Pol III promoter for the potential generation of multiple unique siRNAs from a single expressed transcript. Double-lhRNA (dlhRNA) cassettes were designed to be driven off a U6 promoter, yielding a double lhRNA structure comprising two optimised dual-targeting lhRNAs adjacent to one another. In contrast to previously described bispecific shRNAs which were separated by linker sequences of 8 nt, the two hairpin structures were separated by only two uridine residues; akin to a natural 2 nt 3' overhang ideal for Dicer recognition. Should this construct be recognised as an RNAi substrate precursor, the structure ought to allow for five Dicer cleavage reactions, yielding four independent effective siRNAs (Figure 4.1 A). Two dlhRNA cassettes were constructed based on the two most effective dual-targeting lhRNAs described in Chapter 3: lh*tat-nef* +1 and lh*LTR-int* +1, with each construct allowing each lhRNA to be in both the first and second position of the expression cassette (Figure 4.1 B).

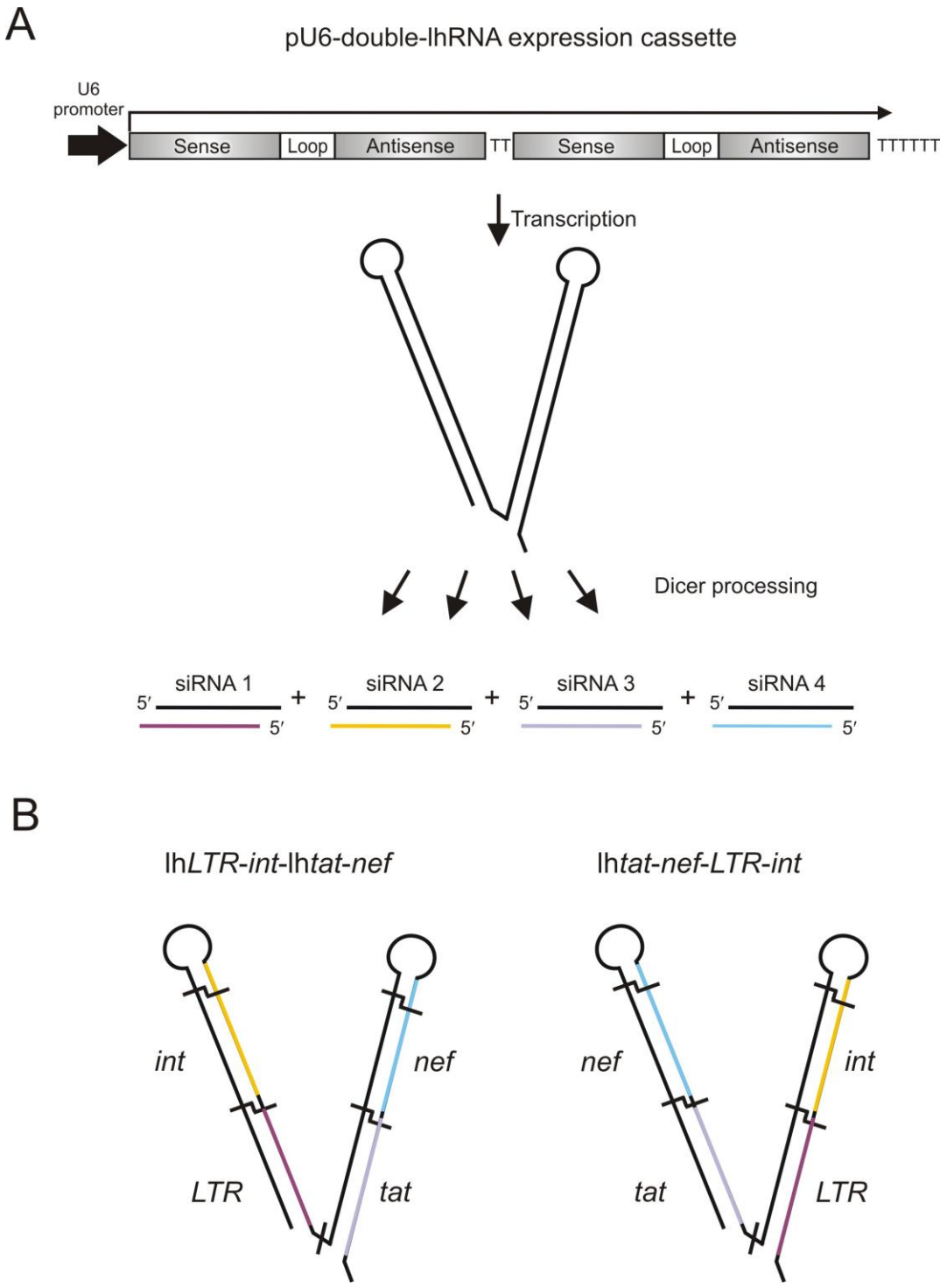


Figure 4.1: Schematic representation of a double-lhRNA expression cassette and the predicted double long hairpin structure post transcription. (A) Double-lhRNAs were designed to incorporate two effective dual-targeting lhRNAs adjacent to one another. The resulting structure allows for five predicted cleavage reactions and the subsequent generation of four siRNAs. **(B)** Two dlhRNAs: lhLTR-int-lhtat-nef and lhtat-nef-lhLTR-int were designed to incorporate a combination of two dual-targeting lhRNAs (lhtat-nef +1 and lhLTR-int +1) arranged in both possible sequential positions.

4.3.2 Detection of multiple siRNAs derived from a U6-driven double-lhRNA expression cassette

Double-long hairpin RNAs (dlhRNAs) represent a unique RNA structure and although each discrete hairpin entity contained a short 3' overhang, the capacity of these structures to act as RNAi precursors and thus Dicer substrates was unknown. Each dlhRNA has the potential to be processed to yield up to four unique siRNAs. Following generation of the two dlhRNAs expression cassettes, PAGE northern blot analysis was carried out on total RNA extracted from HEK293 cells transfected with plasmids expressing dlhRNA cassettes, to determine whether or not these novel structures were processed intracellularly, and to detect the potential generation of mature siRNAs.

Figure 4.2 shows the signals obtained following hybridisation with the same probes listed in 3.2.4. These probes were complementary to the antisense strand of each putative siRNA incorporated in the dlhRNA structure. All probes were able to detect dlhRNA precursor RNA from both dlhRNA constructs (lh*LTR-int*-lh*tat-nef* and lh*tat-nef*-lh*LTR-int*), which indicates that the dlhRNA sequences were effectively transcribed. All probes were also able to detect individual lhRNA precursor RNA of the same molecular weight as precursors detected in Figure 3.6, from the lh*LTR-int*-lh*tat-nef* dlhRNA cassette, indicating that both lhRNAs (lh*LTR-int* and lh*tat-nef*) are being processed from this particular dlhRNA and are present in the cell. The lhRNA in the second position of the dlhRNA cassette (lh*tat-nef*) was however detected at a much lower intensity than that in the first position (lh*LTR-int*). This suggests that either this lhRNA is not effectively cleaved and is thus present at a lower concentrations in the cell, or more likely that Dicer recognises the 3' overhang of the construct and thus processes the lhRNA in the second position first. This would initially result in two siRNAs derived from lh*tat-nef*, and an lh*LTR-int* lhRNA precursor which may then serve as a secondary Dicer substrate for the generation of two more siRNAs. All four mature siRNAs were detected from lh*LTR-int*-lh*tat-nef* and this is the first demonstration that four siRNAs can be successfully generated from a single U6-expressed lhRNA-based construct.

In the case of the dlhRNA lh*tat-nef*-lh*LTR-int*, a single lhRNA precursor was only detected by the *tat* and *nef* probes again indicating that the hairpin in the first position (lh*tat-nef*) is a bi-product of initial

Dicer cleavage reactions and therefore abundantly present in cells. However not only was the lh*LTR-int* precursor absent, mature siRNAs were also not detected with the *LTR* and *int* probes. Only the *tat* and *nef* probes were able to detect mature siRNAs from this construct. This was an unexpected observation which indicates that mature guide strands are not produced from the second hairpin of lh*tat-nef*-lh*LTR-int.* This may imply that siRNA cleavage products, or indeed the entire lhRNA precursor is unstable and perhaps degraded intracellularly following transcription. A second possibility to explain these observations is the phenomenon of strand biasing, whereby the sense (passenger) strand is functionally active and the antisense guide strand is degraded. Results observed in Figure 3.6 clearly show that the exact position of Dicer cleavage is variable. Although the concept of strand biasing is not yet well studied, it seems obvious that a slight shift in the position of Dicer cleavage may modify the thermodynamic stability of the siRNA duplex resulting in the unintended incorporation of the sense strand into RISC and the subsequent degradation of the antiviral antisense strand.

To eliminate the possibility of active passenger strands, oligonucleotide probes complementary to the passenger strand of each putative siRNA within the dlhRNA were used for northern blot analysis (Figure 4.3). As expected, precursor RNA of dlhRNAs, lhRNAs and shRNAs were detected, however no mature sense strands were detected from any of the hairpin constructs, indicating that siRNA passenger strands had indeed been degraded. Consistent with the signals detected with the antisense probes, no lh*LTR-int* precursor RNA was detected from the lh*tat-nef*-lh*LTR-int* dlhRNA with the sense probes confirming that this hairpin and products thereof were not present within the cell.

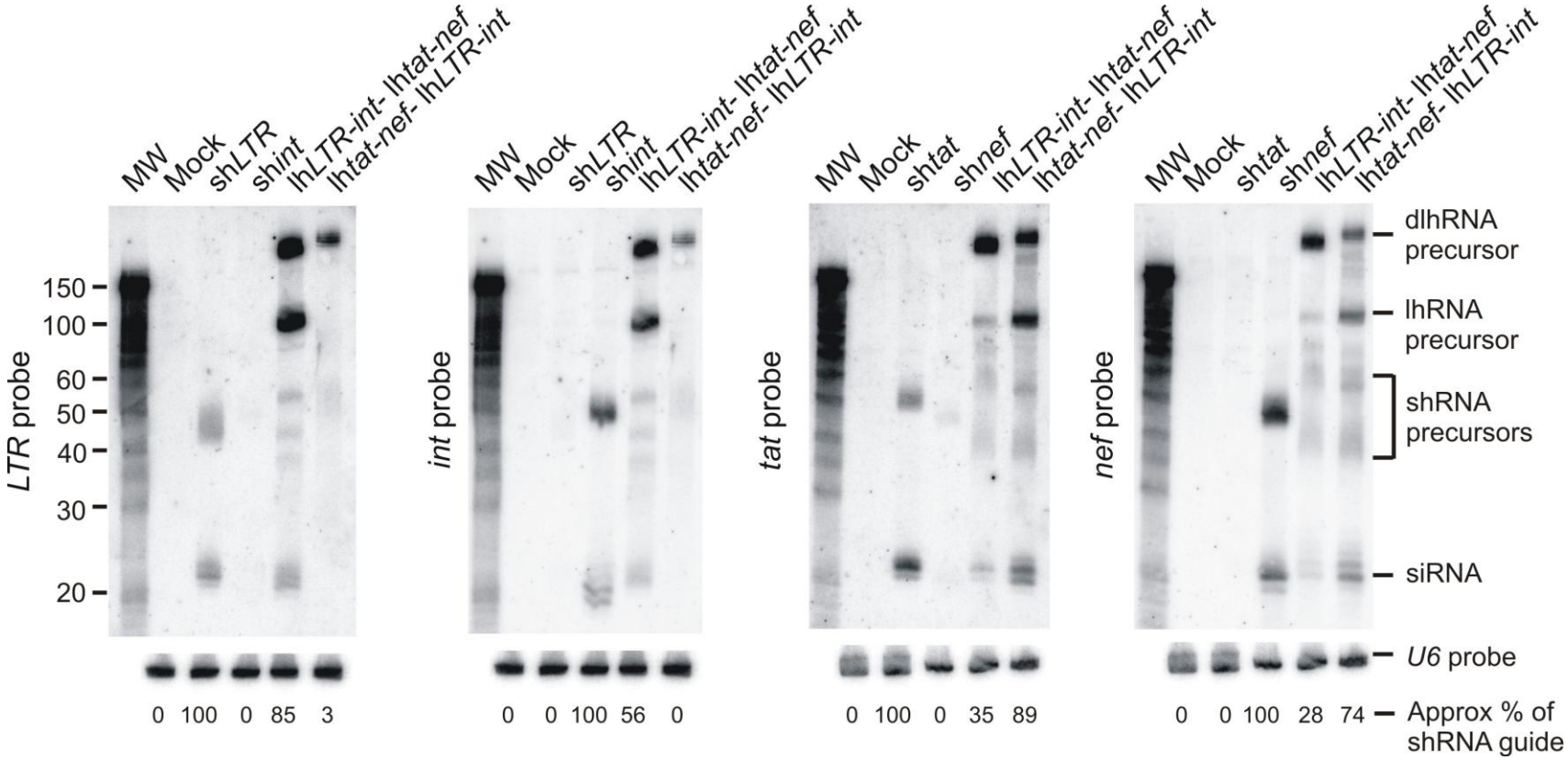


Figure 4.2: PAGE northern blot analysis to detect processed guide strands derived from dlhRNA precursors. PAGE northern blot analysis was carried out on total RNA extracted from cells transfected with double-lhRNA expression cassettes or with individual shRNA expression cassettes used as positive controls. Labeled probes complementary to the guide strand of *LTR, int, tat* and *nef* were hybridised to immobilised RNA and exposed to a phosphorimaging plate. Precursor hairpin RNA as well as processed siRNAs are indicated. Decade Marker™ indicates fragment size and a probe complementary to small nuclear U6 RNA was used as a loading control. The approximate band intensities (%) of generated guide sequences relative to the guide sequence derived from the relevant shRNA are indicated.

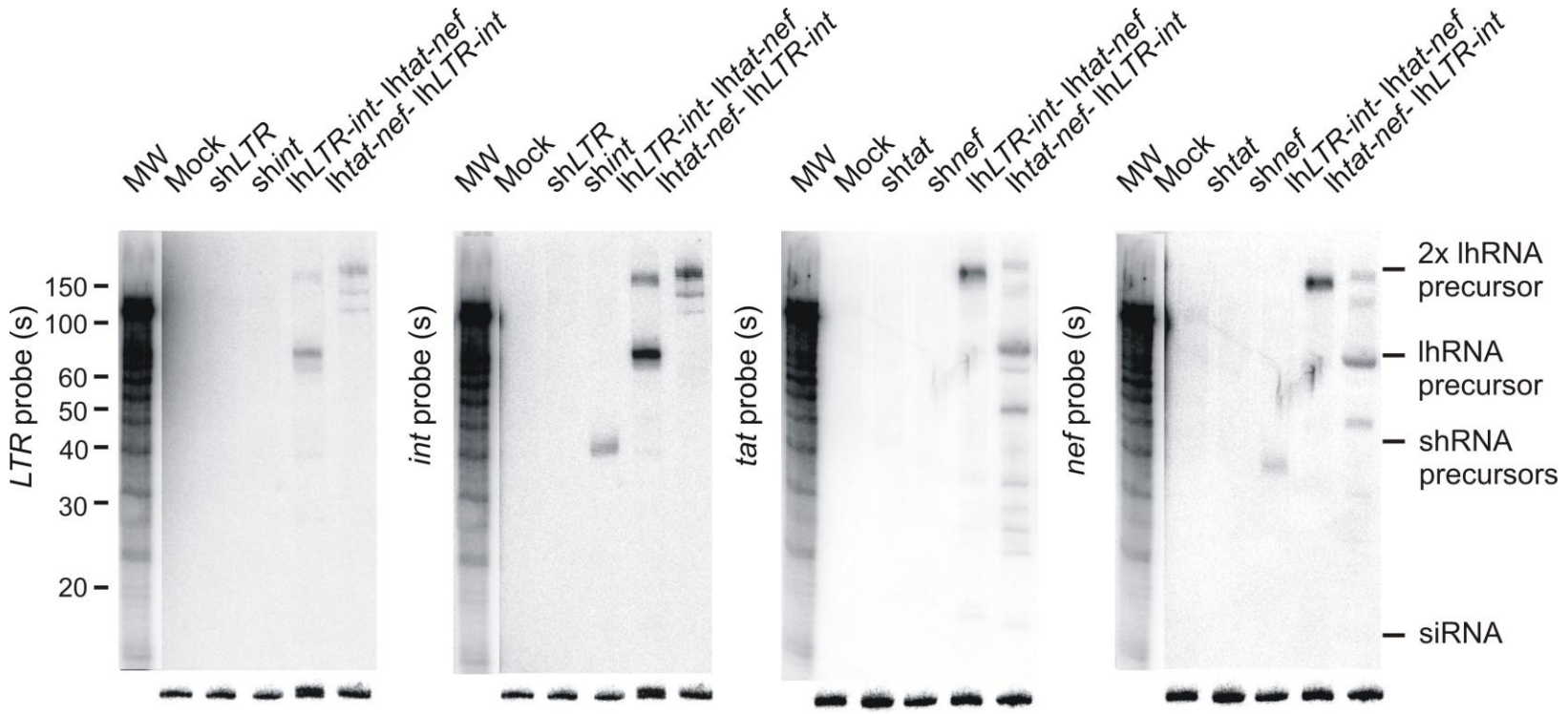


Figure 4.3: PAGE northern blot analysis to detect processed passenger strands derived from dlhRNA precursors. PAGE northern blot analysis was carried out on total RNA extracted from cells transfected with double -lhRNA expression cassettes or with individual shRNA expression cassettes used as positive controls. Labeled probes complementary to the passenger (sense) strand of *LTR, int, tat* and *nef* were hybridised to immobilised RNA and exposed to a phosphorimaging plate. Precursor hairpin RNA is indicated. Decade Marker™ indicates fragment size and a probe complementary to small nuclear U6 RNA was used as a loading control.

4.3.3 Inhibitory efficacy of processed guide sequences derived from double-lhRNA expression cassettes

The results in 4.3.2 show that up to four mature siRNAs may be generated from a single dlhRNA construct. The ability of these siRNAs derived from dlhRNA expression cassettes to knock down their cognate targets was subsequently investigated. RNAi activity was tested in a dual luciferase reporter assay using the psiCHECK reporter plasmids described in 3.2.2. The dlhRNA lh*LTR-int*-lh*tat-nef* was successfully able to elicit a 70-80% decrease in the *Renilla* to Firefly luciferase ratio of all four gene targets, comparable to the knockdown achieved by single dual-targeting lhRNAs (Figure 4.4). The observed RNAi activity implies that all four of the siRNAs derived from this construct were highly effective and active at similar levels. The production of siRNAs from dlhRNA precursors capable of similar RNAi activity to those generated from dual-targeting lhRNAs, also suggests that each of the precursors are processed by a similar mechanism. Only two mature siRNAs were derived from the dlhRNA lh*tat-nef*-lh*LTR-int* (Figure 4.2). Consistent with these findings, significant inhibition was observed only of the *tat* and *nef* targets (Figure 4.4), confirming that the second lhRNA of this construct and its encoded siRNAs are indeed inactive.

To determine whether the sense strand of each putative siRNA encoded by the dlhRNA constructs was actively being incorporated into RISC for target specific knockdown, target sequences complementary to each individual sense strand (sense targets) were inserted downstream of the *Renilla* ORF in dual luciferase reporter vectors and target knockdown was measured in a dual luciferase reporter assay. Consistent with the results in Figure 4.3 where no sense strands were detected by northern blot analysis, no inhibition >40% of the sense targets was observed by any of the siRNAs generated from the dlhRNA expression cassettes or from individual shRNA and lhRNA expression cassettes (Figure 4.5), suggesting that the sense strands of processed siRNAs were promptly degraded.

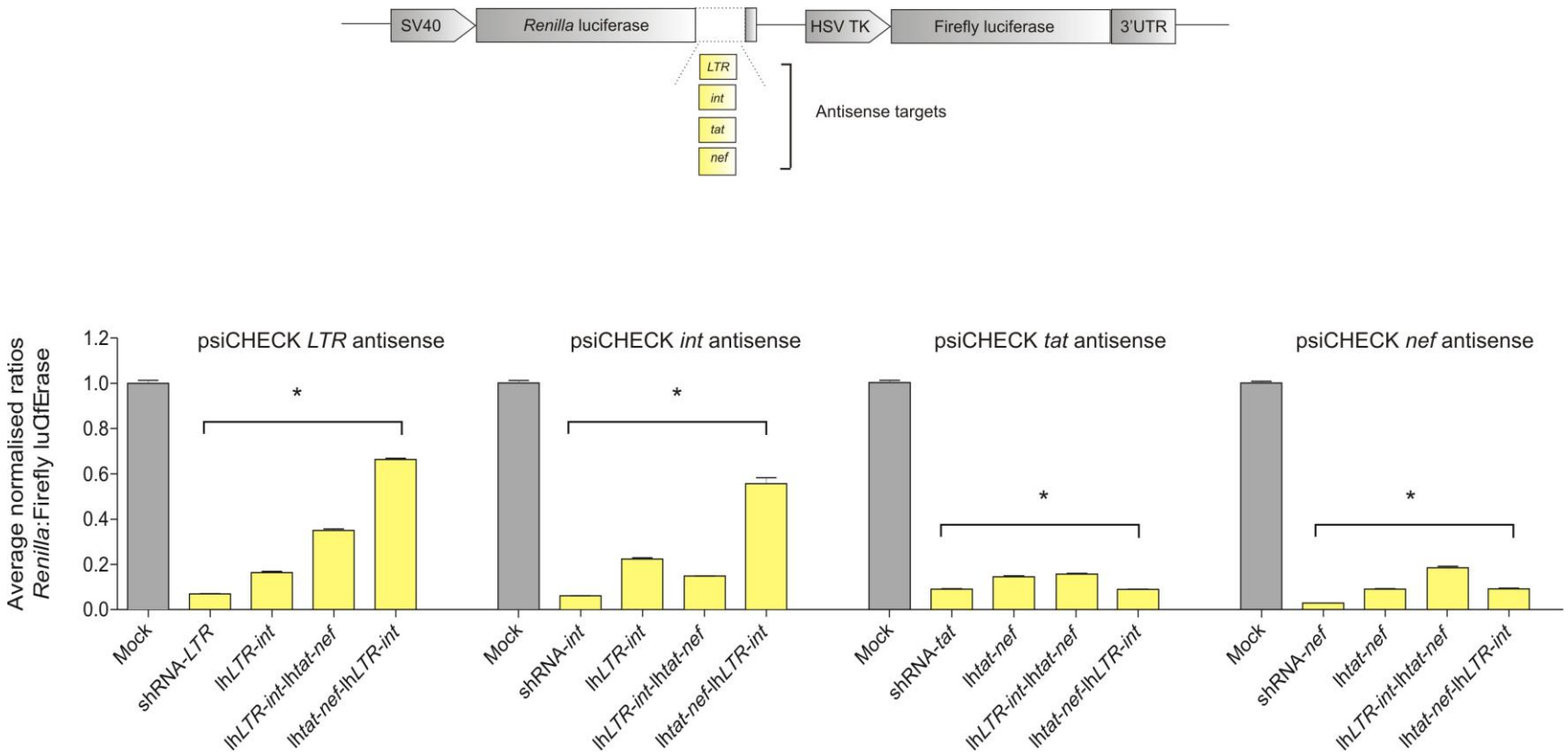


Figure 4.4: Dual luciferase reporter assays to measure inhibitory efficacies of guide strands derived from lhRNAs. Knockdown of target sequences complementary to the antisense strand of *LTR*, *int, tat* and *nef* when the target sequence was inserted downstream of the *Renilla* luciferase open reading frame are shown. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with a plasmid containing a U6 promoter only with no RNAi effector sequence (mock) (*, $p<0.05$, one-way ANOVA relative to mock transfected control).

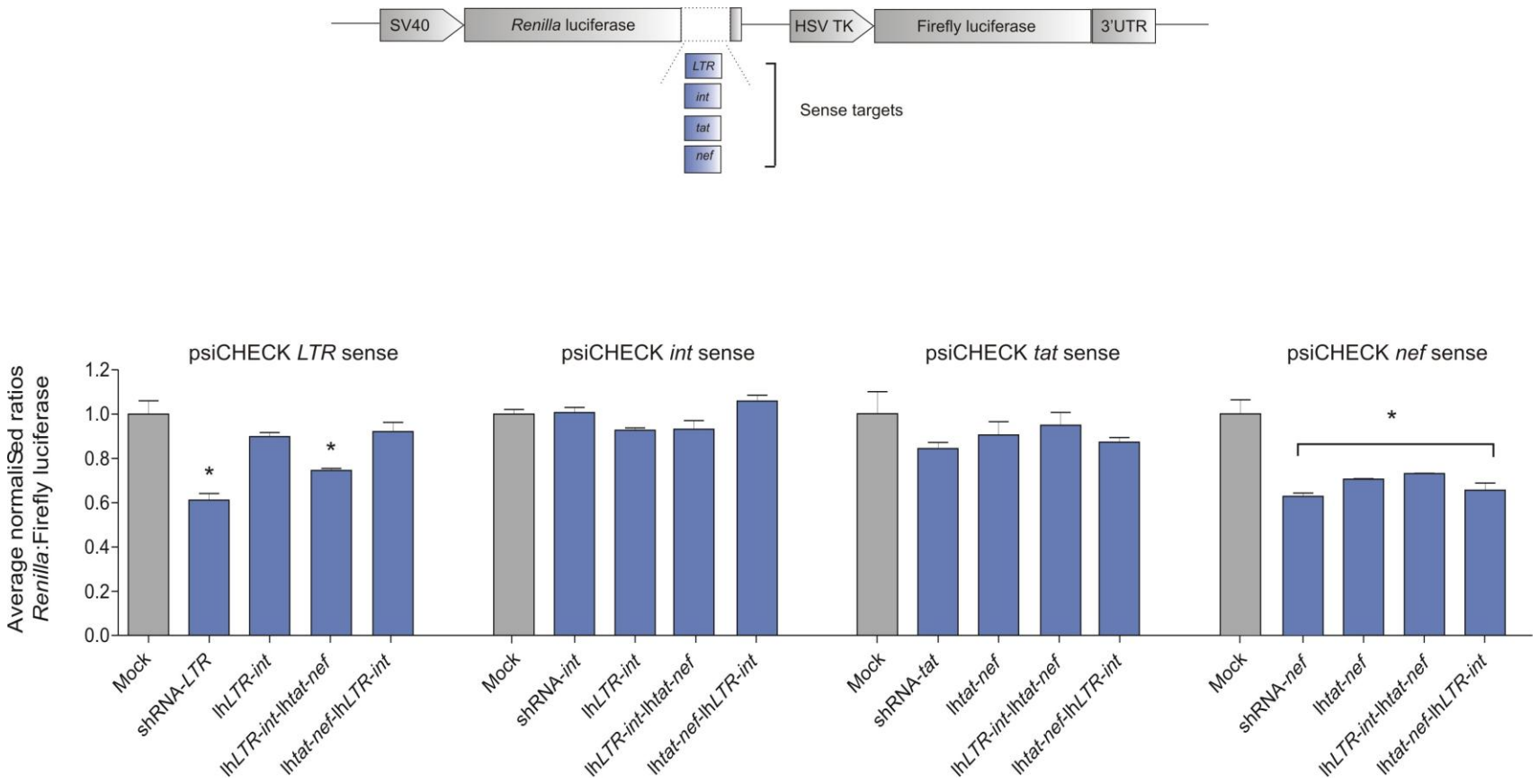


Figure 4.5: Dual luciferase reporter assays to measure inhibitory efficacies of passenger strands derived from lhRNAs. Knockdown of target sequences complementary to the sense strand (passenger strand) of *LTR*, *int, tat* and *nef* when the target sequence was inserted downstream of the *Renilla* luciferase open reading frame is shown. Values represented are mean ratios of *Renilla* to Firefly luciferase (±SEM) and are normalised to cells transfected with a plasmid containing a U6 promoter only with no RNAi effector sequence (mock) (*, $p<0.05$, one-way ANOVA relative to mock transfected control).

4.3.4 The efficacy of expressed lhRNAs and double-lhRNAs to protect cells against HIV-1 viral infection

The ability of the lhRNA and dlhRNA constructs described above, to inhibit the gene expression and thus replication of an HIV-1 viral isolate, was determined by challenging U87.CD4.CCR5 cells transfected with each of the hairpin constructs, with FV5, a primary subtype C HIV-1 viral isolate. The viral titer present in a natural infection is difficult to replicate in a challenge assay, therefore antiviral activity was quantified at two different concentrations of the various hairpin constructs to deduce their relative efficacies. The individual shRNAs targeted to the *LTR*, *tat* and *nef* sequences showed potent inhibition (>90%) of FV5 replication when present at both a high (1000 ng) and a low (100 ng) concentration. Unexpectedly however, the shRNA targeted to the *int* gene only inhibited viral replication approximately 60 % even when present at a high concentration (Figure 4.6 A). Since alignments showed that the siRNA sequence is perfectly complementary to the viral sequence, this lack of activity may be a result of inaccessibility of the target sequence. The emergence of mutations within the target site or the emergence of mutations in regions outside of the target site which affect viral replication as a compensatory mechanism (Leonard et al. 2008) may also potentially abrogate the effects of the siRNA-*int*.

When examining the inhibitory activity of single dual-targeting lhRNAs, lh*tat-nef*, comprising two highly effective siRNA sequences, suppressed viral replication almost completely. The viral inhibition achieved by this lhRNA was stronger than that of either individual shRNA suggesting an additive effect of the anti-*tat* and anti-*nef* siRNAs. The lh*LTR-int* dual-targeting lhRNA, comprising only one effective siRNA sequence, inhibited FV5 replication by approximately 50% when using a high concentration of hairpin. However, no viral inhibition was observed when the hairpin was present at a lower dose (Figure 4.6 A). A single effective siRNA within a lhRNA was therefore not sufficient to compensate for the ineffective siRNA, thus reducing the efficacy of the entire lhRNA.

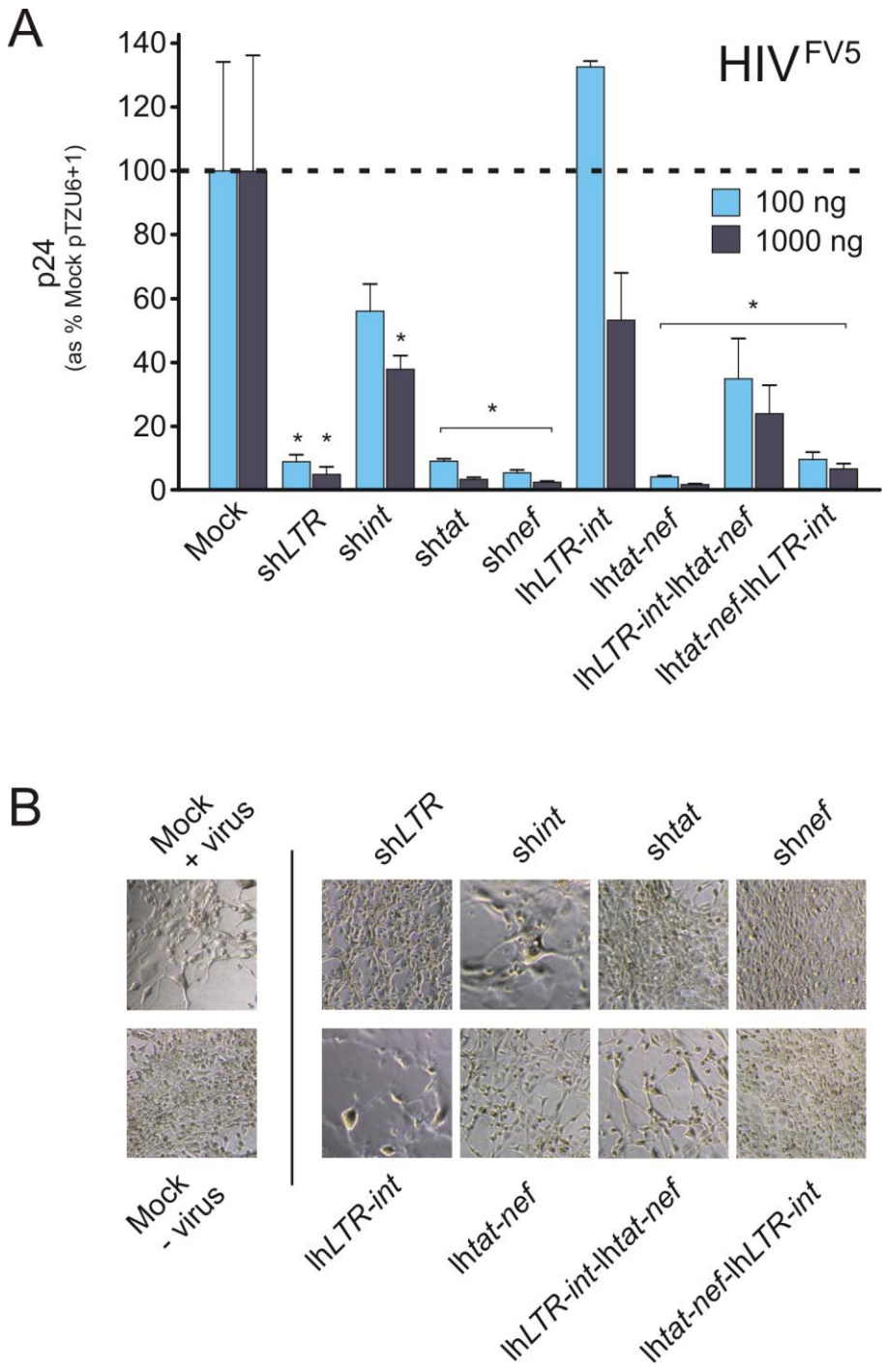


Figure 4.6: Inhibition of replication of an HIV-1 subtype C isolate. (A) U87.CD4.CCR5 cells were transfected with 100 ng or 1000 ng of the indicated hairpin constructs and subsequently infected with the HIV-1 FV5 viral isolate at a $TCID_{50}$ 1000. Concentration of p24 antigen present in supernatants was measured 72 hours post infection. Viral knockdown is expressed as a mean percentage p24 antigen of mock transfected cells ±SEM (*, $p<0.05$, one-way ANOVA relative to mock transfected control). **(B)** Cell morphology of infected U87 $CD4^+$ $CCR5^+$ cells indicating degrees of protection against viral infection by the indicated hairpin constructs.

We previously showed that the second lhRNA, lh*LTR-int* and its encoded siRNAs are not present in cells transfected with the dlhRNA lh*tat-nef*-lh*LTR-int*. However the potent lh*tat-nef*, placed in the first position of the dlhRNA was able to compensate for the inactive lh*LTR-int* and thus maintain silencing by the dlhRNA, not only at high concentrations but at low concentrations too (Figure 4.6 A). In cells transfected with the dlhRNA lh*LTR-int*-lh*tat-nef*, all four siRNAs were generated in similar quantities and were all active as seen in Figures 4.3 and 4.5 respectively. However lhLTR-*int* present in the first position, showed minimal RNAi activity and the inhibition observed by the dlhRNA is therefore likely to be a result of compensatory knockdown afforded by the effective lh*tat-nef* present in the second position of the dlhRNA. Of interest however, is that the compensatory effect contributed by lh*tat-nef* was much stronger when this lhRNA was placed in the first position of the dlhRNA indicating that the lhRNA placed in the first position of this novel structure is cleaved more efficiently and thus exhibits stronger RNAi activity that that of the lhRNA placed in the second position of the dlhRNA.

The cellular protection offered by the tested hairpin constructs was also determined visually by observing cell morphology using standard microscopy. Cell morphologies clearly correlated with the inhibitory efficacy of each of the hairpins. Cells transfected with effective hairpin constructs displayed a healthy phenotype whereas where in cells where minimal RNAi activity was observed, massive cell destruction and pathology was clearly visible (Figure 4.6 B).

In the event that mutations arose within the *int* region of the FV5 replicating strain, thus rendering this siRNA sequence ineffective, the protective ability of the hairpin constructs was also tested against the subtype B molecular clone, pNL4-3.Luc.R-E-. Additionally, inhibition was measured from three different concentrations of hairpin expression cassettes to further investigate the strength of the compensatory effects offered by the lh*tat-nef* hairpin. Knockdown of pNL4-3.Luc.R-E- was determined by a standard dual luciferase assay measuring Firefly luciferase values and normalizing them against background *Renilla* luciferase values. The efficacy trend of each of the hairpin constructs very closely mirrored that obtained against the FV5 viral isolate (Figure 4.7). Again sh*int* was ineffective against the pNL4-3.Luc.R-E- clone as was lh*LTR-int.* The potent lh*tat-nef* was effectively able to rescue the overall efficacy of the dlhRNA lh*tat-nef-LTR-int*, however in the context of the

dlhRNA lh*LTR-int-tat-nef*, this lhRNA could only rescue the overall efficacy of the dlhRNA when the dlhRNA was present at high concentrations, but this effect was clearly diminished at very low concentrations when we can assume negligible quantities of the second lhRNA are being processed.

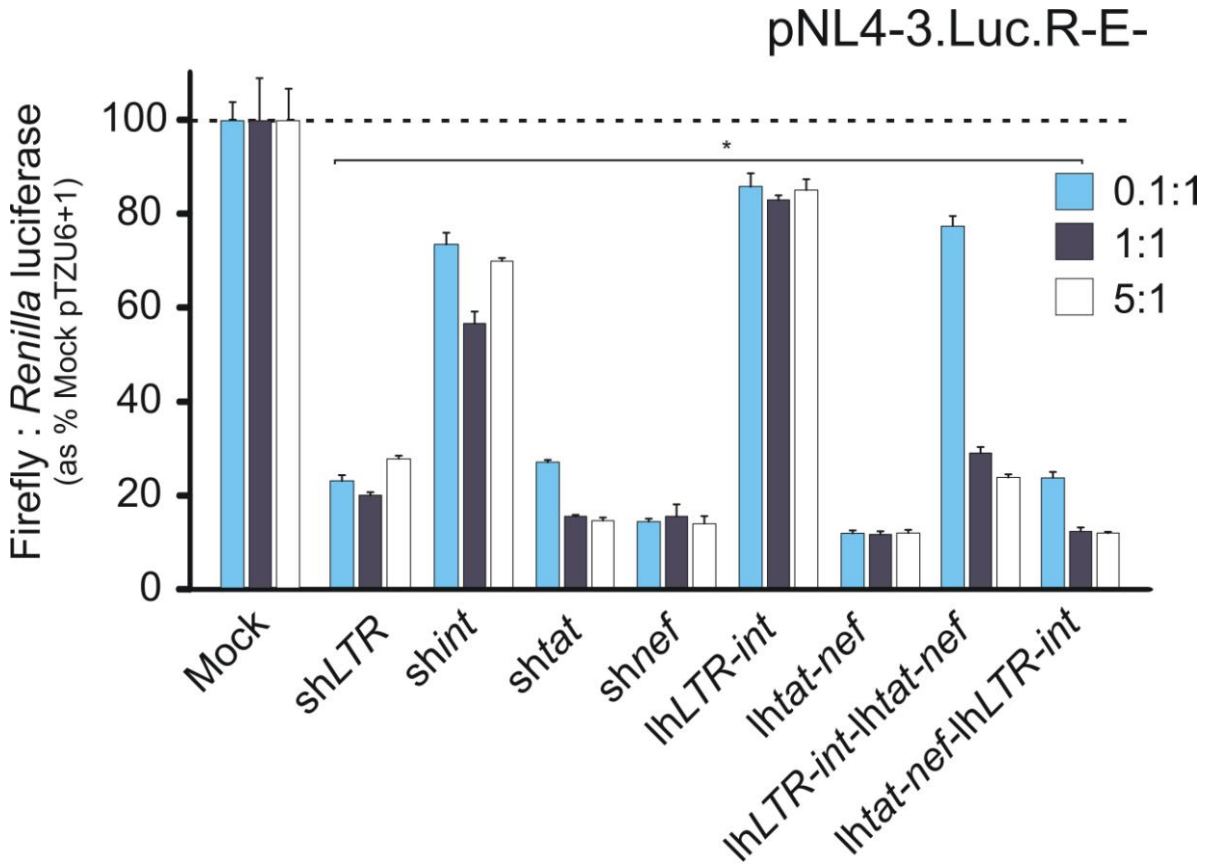


Figure 4.7: Inhibition of an HIV-1 subtype B molecular clone. HEK293 cells were transfected with 150 ng pNL4-3.Luc.R-E- together with 750 ng (5:1 ratio); 150 ng (1:1 ratio) or 15 ng (0.1:1 ratio) of the indicated hairpin constructs. Values represented are mean ratios of Firefly to *Renilla* luciferase (±SEM) expressed as a percentage of mock transfected cells (*, $p<0.05$, one-way ANOVA relative to mock transfected control).

4.3.5 Assessment of potential off target effects caused by exogenously introduced lhRNA and double-lhRNA expression cassettes in vitro

Saturation of the endogenous RNAi pathway

An important safety concern is the disruptive effect that lhRNA-based constructs may have on the endogenous cellular miRNA pathway (section 1.7). Expressed lhRNA constructs require the endogenous RNAi machinery for their biogenesis, and competition with natural miRNA precursors for certain components of the pathway may occur, leading to saturation of the miRNA biogenesis pathway and a subsequent disruption of natural miRNA function. To assess any such effects, two assays were carried out to determine saturation and the consequent derepression of both an endogenous and an exogenous miRNA, potentially caused by exogenously introduced RNAi sequences. In the first assay potential off target effects leading to the disruption in function of the natural endogenous miR-16 were investigated. The experiment was optimised for the Huh-7 cell line and these cells were co-transfected with hairpin expression plasmids together with a psiCHECK target reporter plasmid containing seven copies of a miR-16 target site cloned downstream from the *Renilla* luciferase open reading frame using a method previously described (Ebert et al. 2007; Ely et al. 2009). Endogenous miR-16 should be capable of inhibiting its cognate target thus causing a reduction in the *Renilla*:Firefly luciferase ratio. Potential saturation effects caused by the co-transfected hairpin constructs will result in disrupted miR-16 function and reduced silencing of the reporter gene. At a concentration of 5:1 (lhRNA expression cassette:target reporter plasmid), none of the lhRNAs showed any saturation effects on the natural endogenous miRNA pathway as seen by the ability of endogenous miR-16 to knock down its cognate psiCHECK target in the presence of transfected hairpins. However when the designed sponge, (a cassette expressing seven copies of an imperfectly matched miR-16 target) was co-transfected with the psiCHECK target, a disruption in miR-16 functioning, analogous to the effects of saturation, was clearly evident by the inability of endogenous miR-16 to no longer suppress its target (Figure 4.8 A).

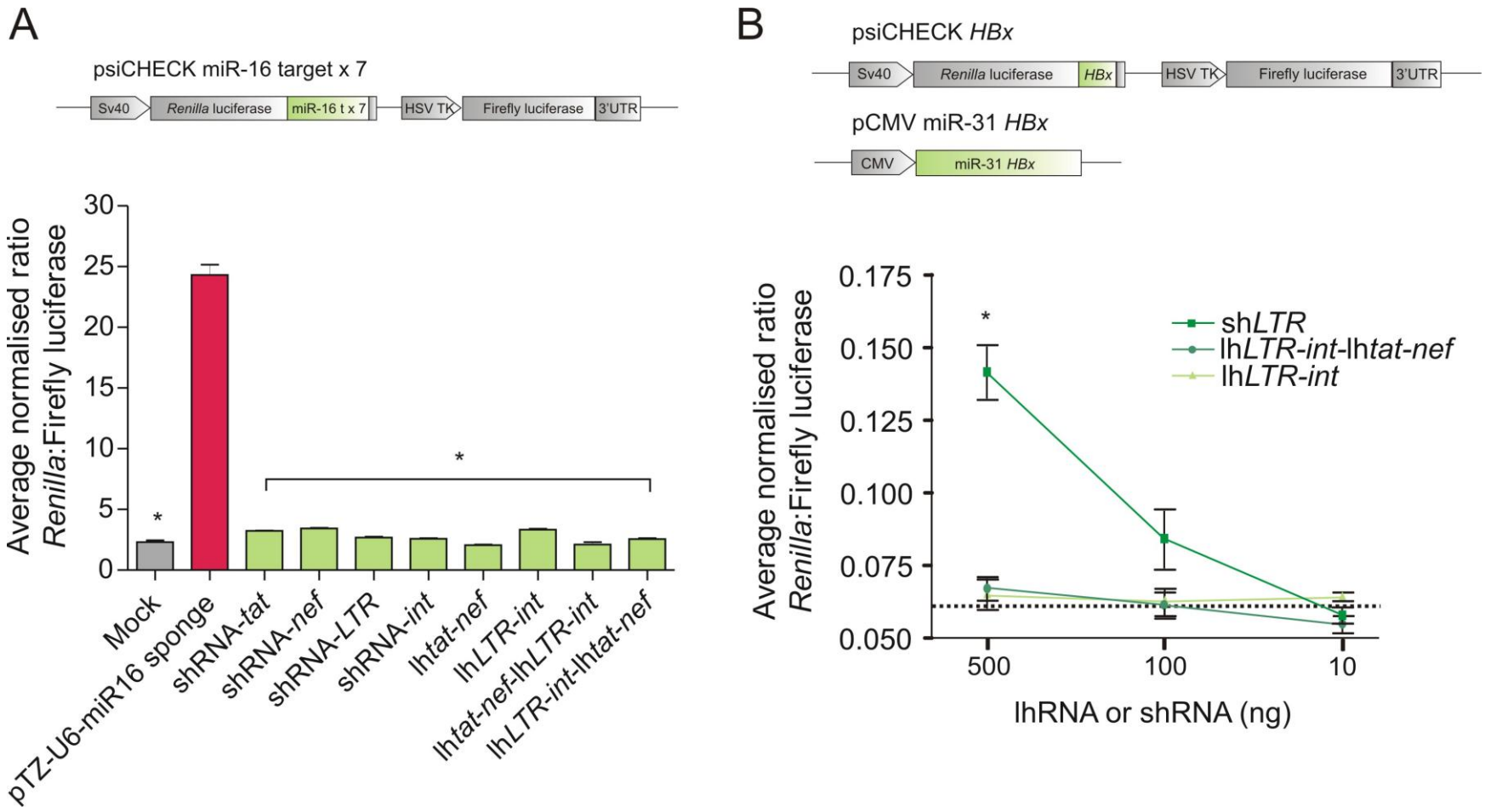

A
psiCHECK miR-16 target x 7
Sv40
Renilla luciferase
miR-16 t x 7
HSV TK
Firefly luciferase
3'UTR
Average normalised ratio
Renilla:Firefly luciferase
30
25
20
15
10
5
0
*
*
Mock
pTZ-U6-miR16 sponge
shRNA-tat
shRNA-nef
shRNA-LTR
shRNA-int
lhtat-nef
lhLTR-int
lhtat-nef-lhLTR-int
lhLTR-int-lhtat-nef
B
psiCHECK HBx
Sv40
Renilla luciferase
HBx
HSV TK
Firefly luciferase
3'UTR
pCMV miR-31 HBx
CMV
miR-31 HBx
Average normalised ratio
Renilla:Firefly luciferase
0.175
0.150
0.125
0.100
0.075
0.050
*
shLTR
lhLTR-int-lhtat-nef
lhLTR-int
500
100
10
lhRNA or shRNA (ng)

Figure 4.8: Assessment of potential saturation effects of exogenously introduced hairpin expression cassettes on the endogenous miRNA biogenesis pathway. (A) The effect of exogenously introduced hairpin expression cassettes on the function of endogenous miR-16 was analysed following co-transfection of a dual luciferase reporter plasmid containing 7 copies of the miR-16 target downstream of the *Renilla* luciferase ORF together with the indicated hairpin expressing plasmids or miR-16 sponge plasmid expressing 7 copies of an imperfectly matched miR-16 target. Mean ratios (±SEM) of *Renilla* to Firefly luciferase were used to determine derepression of miR-16 (*, $p<0.05$, one-way ANOVA relative to pTZ-U6-miR16 sponge control). **(B)** The effect of transfected hairpin expression cassettes on the function of an exogenously introduced miRNA was assessed by co-transfection of a plasmid expressing highly effective miR-31 *HBx* together with a dual luciferase reporter plasmid with the *HBx* target cloned downstream of the *Renilla* luciferase ORF, and increasing quantities of representative shRNA, lhRNA and multi-lhRNA expression cassettes. Mean ratios (±SEM) of *Renilla* to Firefly luciferase were used to determine derepression of miR-31 *HBx* (*, $p<0.05$, one-way ANOVA relative to mock transfected control).

Potential saturation effects were also eliminated using a second, more sensitive assay, to determine possible disruption in the functioning of an exogenously introduced miRNA. HEK293 cells were co-transfected with an exogenous pri-miRNA expression cassette containing an effective guide sequence targeted to a site within the HBV genome: pCMV miR-31 *HBx* and its cognate target plasmid: psiCHECK *HBx* in a 1:1 ratio. Co-transfection of these two plasmids at this ratio results in potent inhibition of the target as measured by the reduction in the ratio of *Renilla*:Firefly luciferase. The pCMV miR-31 *HBx* pri-miRNA expression cassette was introduced at its lowest functional concentration, and is thus present intracellularly at lower levels than endogenous miR-16 allowing for a more sensitive detection of derepression. Together with these plasmids, a representative shRNA, lhRNA or double-lhRNA expression plasmid was also transfected in diminishing quantities to determine any potential derepression as a result of exogenously introduced hairpin constructs. U6-driven sh*LTR* showed interference with miR-31 *HBx* mediated inhibition of its target when present at higher concentrations, however U6-driven lh*LTR-int* or U6-driven lh*LTR-int*-lh*tat-nef* showed no disruptive effects on the functioning of miR-31 *HBx* (Figure 4.8 B).

Induction of the type 1 interferon response

To exclude the possibility of non-specific effects caused by the induction of a non-specific type 1 interferon response, *IFN-β* mRNA concentrations were measured in transfected cells as described in section 2.3.6. None of the hairpin cassettes induced expression of *IFN-β* as measured by quantitative qRT-PCR with samples normalised to *GAPDH* (Figure 4.9). These results are very promising and suggest that expressed lhRNAs and dlhRNAs do not interfere with the natural gene regulation pathway nor stimulate the induction of the innate immune response in the above mentioned cell lines.

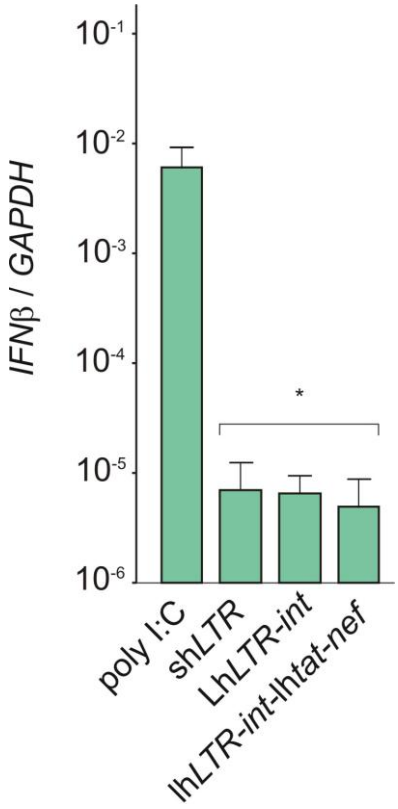


Figure 4.9: The potential induction of the IFN response in cells transfected with hairpin expression cassettes. IFN induction was assessed by measuring *IFN-β* mRNA concentration in total RNA extracted from cells transfected with the indicated representative shRNA, lhRNA and double-lhRNA expression cassettes or with poly I:C which served as a positive control. Mean normalised ratios of *IFN-β* : *GAPDH* (±SEM) are indicated as determined by quantitative RT-PCR (*, $p<0.05$, one-way ANOVA relative to poly I:C control).

4.4 Discussion

Certain limitations exist for most combinatorial RNAi strategies used to date and long hairpin RNAs are no exception. Advances in the development of a safe and efficient combinatorial RNAi strategy are therefore fundamentally important for the use of the RNAi pathway to inhibit rapidly evolving viruses such as HIV-1. To improve on the findings described in Chapter 2 and 3, a novel lhRNA-based approach was designed for the simultaneous production of four siRNAs from a single expressed transcript. The two highly effective and optimised dual-targeting lhRNAs: lh*tat-nef* +1 and lh*LTR-int* +1 (referred to as lh*tat-nef* and lh*LTR-int* respectively in this Chapter), identified in Chapter 3, were cloned adjacent to one another in both sequential positions to yield two double-long hairpin RNA (dlhRNA) structures: lh*tat-nef*-lh*LTR-int* and lh*LTR-int*-lh*tat-nef*, each driven by a single U6 snRNA RNA Pol III promoter. Four unique siRNAs were easily detected following processing of the dlhRNA lh*LTR-int*-lh*tat-nef*, and all four siRNAs sequences were capable of highly effective knockdown of their cognate targets, a very encouraging result in the quest for a combinatorial system able to suppress a minimum of four target sites simultaneously. Some variation was however evident in the quantities of the siRNAs products.

The mechanism of processing of these dlhRNA structures is unclear. If the PAZ domain of Dicer recognises the terminal 3' overhang (Ma et al. 2004), the lhRNA in the second position should be processed first, resulting in two siRNAs and a single dual-targeting lhRNA following the initial two Dicer cleavage reactions. The lhRNA bi-product would then serve as a Dicer substrate for the secondary cleavage reactions resulting finally in four unique siRNAs. This model is supported by the abundance of precursor RNA from the first position lhRNA present in the cell (Figure 4.2). However siRNAs produced from the lhRNA in the first position were present at greater concentrations suggesting that this hairpin was processed more efficiently. This may be a result of the lhRNA bi-product serving as a more favourable Dicer substrate than the dlhRNA precursor. However this represents only one model for the mechanism by which these novel structures are processed. It has been previously shown that efficient expression of siRNA sequences from the CMV Pol II promoter, required modification of the promoter such that the hairpin was positioned adjacent to the transcription initiation site (Xia et al.

2002). Giering et al. also showed that the position of the hairpin relative to the transcription initiation site plays an important role in the RNAi activity induced by such hairpin sequences (Giering et al. 2008). The 5' end of these Pol II-expressed transcripts had a greater effect on the efficacy of the hairpin than the 3' end, which consisted of either a minimal poly (A) tail or a U1 3' termination box (Xia et al. 2002; Denti et al. 2004; Giering et al. 2008). This suggests that the 5' end of hairpin sequences may play a stronger role in the docking of Dicer than the 3' 2 nt overhang present in Pol III transcribed hairpins, in which case the lhRNA sequence in the first position of the dlhRNA structures described in this Chapter should theoretically be processed first.

The sequence of the 3' ends of siRNAs has been shown previously to affect the binding affinity of Dicer for its substrate (Vermeulen et al. 2005). The siRNA sequence also appeared to play a role in the mechanism and efficiency by which these dlhRNA structures are processed. In the case of the dlhRNA lh*tat-nef*-lh*LTR-int* no siRNA products or lhRNA precursor RNA was detected from the hairpin in the second position (lh*LTR-int*) suggesting a problem with processing of this specific lhRNA from within a dlhRNA configuration. Since no precursor RNA was detected at all, it implies that the entire second lhRNA was degraded following cleavage from the first lhRNA. Intriguing structural elements within dlhRNAs allow for the possibility of processing by other RNase enzymes rather than Dicer, which may lead to unpredictable cleavage within the hairpin stem. However, these possibilities remain to be verified and further investigation into the mechanism by which dlhRNAs are processed is required before any such speculation can be confirmed.

The system described in this Chapter offers a potential lhRNA-based combinatorial RNAi strategy for the simultaneous silencing of four gene targets without inducing any obvious cellular toxicities. The unique dlhRNA structure does not appear to elicit an immune response and furthermore, the intracellular expression of dlhRNAs does not effect any derepression of endogenous or exogenously introduced miRNAs, indicative of no saturating effects on the endogenous miRNA biogenesis pathway. The inhibitory efficacy of this innovative construct coupled with its apparent safety suggests that it may be a good combinatorial RNAi candidate with which to pursue further work. The dlhRNAs used in this Chapter to test such a system incorporated shRNAs sequences with variable efficacy against HIV-1.

Although both dlhRNA constructs mediated significant inhibition of a primary isolate, the system may be improved upon by incorporating only highly effective sequences. The recent identification of a large panel of highly effective anti-HIV shRNAs should provide an ideal source from which to select potent candidates for incorporation into future dlhRNAs (McIntyre et al. 2009a). That being said, the incorporation of sub-optimal RNAi effector sequences in the current dlhRNAs provided a unique opportunity to gain fundamental insights into the mode of action of such constructs. It became evident that highly effective siRNAs within a dlhRNA are able to compensate for ineffective siRNA sequences and maintain inhibition of HIV replication in the short term. This is a convenient analogy to emerging mutations which render single siRNA sequences ineffective. Moreover, it suggests a possibility that should a mutation arise in a single target site, the emergence of viral escape mutants will be prevented as a result of the silencing abilities of the remaining siRNAs, a vital factor in successful combinatorial systems. Although further characterisation of the processing trends of such structures is required, the ability of these novel double-lhRNA constructs to produce four effective siRNAs will be of significant value for future combinatorial antiviral therapeutic applications.

CHAPTER 5

Discussion and Conclusions

5.1 General Discussion

The RNAi pathway has been extensively exploited for the development of therapeutic modalities. A wide variety of RNAi effector mimics has been designed for the successful inhibition of an array of disease causing genetic elements. However, the limitation of single RNAi effectors has been clearly shown when these have been used against rapidly evolving targets such as HIV. Under the selective pressure of a single siRNA sequence, HIV quickly develops mutations, usually within the targeted sequence, thus abrogating the effects of the RNAi-based therapeutic effector (section 1.9.2). The rapid evolution of resistant viral strains is a major problem when using a sequence specific therapeutic platform such as RNAi and a combination of RNAi effector sequences is therefore required to sustain inhibition of viral replication in much the same way as HAART. Successful multimerisation of single RNAi effector sequences into long hairpin RNA structures was described here as a possible RNAi-based combination therapy.

The possibility of simultaneously deriving multiple independent siRNAs from lhRNA stem duplexes of varying length and design was extensively investigated. The observed trends by which hairpin stems were processed allowed us to gain insight into the intrinsic functionality of human Dicer. The processing trends clearly implicated this enzyme as the limiting factor in multiple siRNA generation as a result of its inability to act efficiently as a multiple turnover enzyme *in vivo*. However, thorough characterisation of the spatial and sequential arrangement of guide sequences within the hairpin stem led to the identification of an optimal design for dual-targeting lhRNAs, ensuring the efficient generation of two highly functional guide sequences from each hairpin stem. The observations and conclusions drawn from Chapters 2 and 3 have greatly expanded upon the limited body of knowledge surrounding the capacity of lhRNAs to act as effective combinatorial RNAi precursors. Furthermore, the powerful

dual-targeting lhRNAs that were identified formed the foundation upon which the novel combinatorial double long hairpin structure was built.

To facilitate the development of a combinatorial RNAi strategy incorporating the structurally and sequentially optimised dual-targeting lhRNA species described in Chapter 3, a novel multiplexed double long hairpin RNA (dlhRNA) structure was described. The dlhRNA structure amalgamates the insight gained in Chapters 2 and 3 surrounding the properties of lhRNAs, into a unique lhRNA-based construct, which meets all the demands of a combinatorial RNAi-based therapy. Two effective dual-targeting lhRNAs were successfully tethered together under the control of a single Pol III promoter. The design of dlhRNAs is akin to that of engineered polycistronic transcripts (Chung et al. 2006) in that two hairpin structures are arranged in tandem within a single transcript. However, dlhRNA constructs contain no specific flanking regions, and furthermore, each hairpin incorporates the sequences of two effective guide strands instead of only one. Although further work is required to deduce the exact mechanism by which this novel RNA structure is processed, the PAGE northern blot data in Figure 4.2, when compared to that in Figure 3.6, suggest that this combinatorial RNAi precursor is processed by the endogenous RNAi machinery. Optimised dlhRNAs not only encompass the properties of previously described combinatorial RNAi platforms, but in addition include added features not yet described in multiplexed RNAi approaches. This unique configuration allows for the generation of four highly active RNAi guide sequences, which in turn are able to target four independent sites within the HIV-1 genome for specific inhibition. This strategically correlates with the number of targeted genes predicted to prevent the emergence of viral escape mutants (Leonard and Schaffer 2005; ter Brake et al. 2008). In addition, when dlhRNAs are efficiently processed, the active guide strands derived from dlhRNAs are generated in similar quantities unlike the unpredictable quantities of guide strands generated from polycistronic miRNA shuttles; or the variable quantities derived from multiple shRNAs expressed from different promoters. The incorporation of four powerful siRNA sequences into a dlhRNA will thus enable equal and potent knockdown of all four targeted genes. However since the siRNA sequence appears to play a role in dlhRNA processing, ongoing work aims to determine a set of parameters which will ensure consistent processing of these structures. Finally dlhRNA precursors are expressed from a single RNA Pol III promoter, thus reducing the risk of cellular toxicities and saturation

effects potentially caused by multiple Pol III expression cassettes, and eliminating the possibility of promoter occlusion.

The dlhRNA design therefore shows promise as a novel combinatorial RNAi strategy and further work which will characterise and optimise this unique system is worth pursuing. It now remains to be tested whether dlhRNAs will be able, as suggested, to suppress viral replication in the long term, without the emergence of viral escape mutants. Encouraging results in Figures 4.6 and 4.7 do however imply that in the event of arising mutations in one or even two target sequences, compensatory effects will ensure the maintenance of viral inhibition. To determine the ability of dlhRNA constructs to counteract the emergence of viral escape mutants, future work will include cloning dlhRNA expression cassettes into a suitable lentiviral delivery vector (discussed in section 5.2), which will be used to transduce an appropriate cell line for the stable expression of these antiviral constructs. Transduced cells will be infected with HIV-1 and monitored for viral breakthrough for 6-8 weeks by measuring viral RNA production or CA-p24 levels, in a similar procedure to those used to determine viral escape from single lhRNAs/e-shRNAs (Sano et al. 2008; Liu et al. 2009). In the event of viral breakthough, proviral DNA will be isolated from infected cells and the target sequences will be amplified, cloned into a suitable plasmid and sequenced to determine the genotypic variations of escape mutants. Hairpins encoding two and three siRNAs have been shown to suppress viral replication for 48 and 49 days respectively, demonstrating their distinct advantage over single shRNAs, to which the virus quickly became resistant (Sano et al. 2008; Liu et al. 2009). Although hairpins encoding two siRNAs have been observed to inhibit HIV-1 replication more potently than hairpins encoding three siRNAs (Liu et al. 2009), the latter hairpins provide an advantage against viral escape since they target a third site, thus making escape more difficult. However, when challenged with a higher viral dose, replication was detected even in cells containing a hairpin encoding three unique siRNAs (Liu et al. 2009), thus highlighting the importance of a combinatorial RNAi system able to target four unique sites. An intriguing point to note is that although viral breakthrough was eventually detected, viral escape mutations were scattered and did not emerge as the dominant genotype (Liu et al. 2009). Although this observation highlights the fact that HIV is able to escape by multiple routes, it

also suggests that in the event of emerging escape mutants; the fitness of these variants may be compromised.

Further measures to prevent the emergence of drug resistant viral strains may require the investigation of novel combination therapies. Given the success of HAART, the combination of lhRNA-based therapeutic effectors together with antiretroviral drugs may be an option worth exploring. Alternatively, the combination of dlhRNAs with alternative anti-HIV gene therapy modalities such as ribozymes, RNA decoys, transdominant rev proteins or U1 adaptors (Rossi et al. 2007; Reyes-Darias et al. 2008) remains a viable option. Nonetheless, for the potential of lhRNA-based combinatorial RNAi strategies, alone or as a component of a novel combination therapy, to be eventually realised in a clinical setting, two main focus areas still require a substantial amount of characterisation, namely the optimisation of *in vivo* delivery and ensuring the safety of such constructs.

5.2 Potential delivery vectors for lhRNA constructs

RNAi-based therapeutic applications require effective delivery platforms so that effector sequences may reach their target cells or tissues. An array of delivery strategies has been extensively investigated and include both viral and non-viral delivery vectors. Non-viral strategies have largely been used for the delivery of synthetic siRNA molecules and include but are not limited to cholesterol conjugates; polycation nanoparticles; positively charged antibodies and RNA aptamers [reviewed in (Castanotto and Rossi 2009; Whitehead et al. 2009)]. Expressed RNAi sequences are typically delivered within the backbone of viral vectors which are based on RNA viruses such as retroviruses and lentiviruses; or DNA viruses which include Herpes Simplex Virus, Adenovirus and Adenoassociated virus [reviewed in (Gonzalez-Rojas et al. 2010)]. HIV causes a chronic infection and thus requires long term treatment regimens to control viral loads. The lhRNA-based constructs described in this thesis need to be stably expressed to achieve effective viral suppression. Viral vectors, more specifically retrovirus based vectors, have therefore become the delivery vehicles of choice to deliver anti-HIV therapeutic effectors because of their inherent capabilities of integrating into

the host genome for the stable expression of transgenes. Lentiviral vectors are largely based on HIV, and this class of vectors has been widely used for the delivery of RNAi effector precursors as a result of their low immunogenicity; their ability to infect both dividing and non-dividing cells; and their efficient integration into host chromosomes which results in the stable long term expression of the delivered transgene [reviewed in (Vigna and Naldini 2000)].

Several major safety concerns exist for the use of HIV based delivery vectors and include the emergence of replication competent recombinants, recombination events with wild type HIV and the oncogenic potential of integrating vectors. These concerns have led to a number of advances in the biosafety features of these vectors (Dull et al. 1998; Kim et al. 1998; Zufferey et al. 1998), resulting in a safe and efficient HIV-1 based lentiviral vector strategy. However the incorporation of anti-HIV RNAi effector sequences leads to additional complications since viral sequences within the vector may become targets for the encoded siRNA sequences, leading to impaired vector production (ter Brake and Berkhout 2007). Encoded shRNAs have been shown to reduce transduction titers by targeting sequence elements within the HIV-1 vector backbone and more commonly sequences within the Gal-Pol transcript. The use of human codon-optimised Gag-Pol sequences is however able to largely abrogate interference of vector production caused by siRNA sequences targeted to sites within this transcript (Kotsopoulou et al. 2000; ter Brake and Berkhout 2007). The inhibition of Dicer using anti-Dicer siRNAs (Poluri and Sutton 2008) or the saturation of the RNAi machinery with an excess of random shRNAs (Liu et al. 2009) in the packaging cell line have also effectively overcome titer-related issues. Another approach to prevent vector targeting is to use non-primate lentiviral vectors such as feline immunodeficiency virus (FIV) (Poeschla et al. 1998) which are prepared by a similar method to HIV-based vectors, but bear little sequence homology with HIV and thus are resistant to the effects of anti-HIV RNAi sequences. It is also possible to use heterologous packaging to circumvent the restriction of antiviral targets, whereby HIV-1 vectors are efficiently packaged by FIV (Morris et al. 2004b).

Not only do lentiviral vectors serve as delivery vehicles for RNAi-based therapies, but it has been speculated that they may also provide a secondary means of protection against HIV infection. These

HIV-derived vectors have been termed conditionally replicating HIV lentiviral vectors and are capable of competing with wild type HIV in infected cells for both regulatory proteins such as Tat and Rev as well as for encapsidation (Dropulic et al. 1996; Bukovsky et al. 1999). These vectors are thus targeted to cells naturally infected with the virus and, in addition, this process reduces viral infection and spread, and results in vector mobilization enabling the spread of the vector to surrounding cells (Morris and Looney 2005). Although this may seem like an attractive strategy, the phenomenon does not occur with self-inactivating (SIN) vectors and therefore raises the initial safety concerns of lentiviral vectors.

Significant advances in the efficiency and safety of lentiviral vectors have in recent years been made, and in addition, strategies to overcome the inhibition of HIV-1-based lentiviral vectors by anti-HIV siRNA sequences have been successfully developed. Single anti-HIV long hairpin RNAs/e-shRNAs have been effectively packaged into lentiviral vectors to mediate long-term gene silencing in transduced cell lines (Sano et al. 2008; Liu et al. 2009). Nevertheless, optimised vectors for the delivery of complex combinatorial RNAi systems, such as those described in Chapter 4, will necessitate further exploration.

5.3 Future safety endeavors for lhRNA expression cassettes

The use of RNA Pol III promoters to drive the expression of RNAi effector mimics offers many advantages, and they are thus the promoters of choice (section 1.6.2). Nevertheless, several major drawbacks have been associated with the strong ubiquitous expression of Pol III transcripts and have been previously discussed (section 1.7.3). Although the lhRNA constructs described in this work showed no evidence of saturating the endogenous miRNA biogenesis pathway, every endeavor to minimize the effective dose of expressed RNAi effectors should be undertaken. A further safety concern related to RNAi-based therapeutics is the potential sequence specific off target effects mediated by partial complementarity of therapeutic effectors or their passenger strands, and cellular hexanucleotide mRNA sequences (Lewis et al. 2005; Birmingham et al. 2006; Jackson et al. 2006) (section 1.7.2). Off target effects are further complicated by combinatorial RNAi systems where the

generation of multiple guide sequences increases the risk of non-specific gene silencing. Furthermore, the inconsistency of the exact position of Dicer cleavage may result in slightly shifted guide sequences which serve to further increase the pool of guide strands, thus making the prediction of potential off target effects an almost impossible task.

A major advantage of the multimeric pri-miRNA shuttle combinatorial RNAi system, is its expression from RNA Pol II promoters, which enables inducible and tissue-specific expression. However, the use of alternative Pol III promoters, such as tRNA promoters, to drive lhRNA expression may also possibly alleviate certain non-specific effects. The tRNA family of promoters has been successfully used to drive expression of modified tRNA transcripts containing shRNA encoding sequences at their 3' termini (Boden et al. 2003b; Kawasaki and Taira 2003; Scherer et al. 2007). tRNA promoter-driven shRNAs are efficiently exported form the nucleus and effect target inhibition at similar or in some cases even enhanced levels to that of U6 or H1-expresed shRNAs (Boden et al. 2003b). tRNA transcripts are processed within the nucleus prior to export and it remains uncertain whether attached shRNAs are removed from the chimeric transcript by tRNAseZ^L within the nucleus as suggested by recent evidence (Bogerd et al. 2010), or whether Dicer is responsible for processing the shRNA from the transcript in the cytoplasm. A distinct advantage of tRNA-shRNA systems is that the nuclear karyopherin exportin-t is responsible for exporting tRNA products from the nucleus to the cytoplasm (Arts et al. 1998; Kutay et al. 1998). The potential export of shRNAs still attached to tRNAs by an alternative karyopherin to exportin-5 may circumvent the risk of saturating the natural miRNA nuclear export factor. Alternatively, the strong and ubiquitous Pol II U1 snRNA promoter has been used to effectively drive the expression of shRNAs (Denti et al. 2004). Furthermore, U snRNAs are exported from the nucleus by CRM1 (Fornerod et al. 1997) and this system therefore offers another approach to overcome saturation of the RNAi pathway at the point of nuclear export.

It is also possible to make use of inducible RNA Pol III promoters which have enabled the development of conditional RNAi systems, thus allowing for the spatial and temporal regulation of gene silencing. The development of an inducible Pol III promoter was initially demonstrated by Ohkawa and Taira who constructed an inducible derivative of the RNA Pol III U6 snRNA promoter to drive the

expression of an antisense RNA (Ohkawa and Taira 2000). The tet operator-repressor system which was well characterised in Pol II systems was adopted for the development of an inducible Pol III system. The tetracycline operator sequence (tetO) was inserted within the U6 promoter at different positions but was found to function effectively only when positioned between the TATA box and the transcription start site. This construct was then introduced into Hela cells expressing the Tet repressor (tetR) which, binds to tetO and blocks transcription in the absence of tetracycline. The addition of tetracycline thus results in the dissociation of tetR and the consequent transcription of the downstream sequence. This system represents a straight forward, rapid and reversible mechanism for the regulation of gene silencing afforded by Pol III-expressed RNAi effector sequences and was first employed for the expression of shRNAs by van der Wetering et al. to silence β-catenin in a colorectal cancer line cell (van de Wetering et al. 2003). The tetO sequence was inserted downstream of the TATA box of the H1 Pol III promoter and shRNA expression was derepressed by the addition of doxycycline. This method has since also been used to drive a number of siRNA sequences under the control of the U6 (Czauderna et al. 2003b; Matsukura et al. 2003) as well as the 7SK Pol III promoters (Czauderna et al. 2003b). This simple design has also been expanded upon and one such example was the construction of a lentiviral vector containing Tet-inducible U6 or H1 driven siRNA sequences targeted against Drosha or PKR, as well as CMV-driven tetR within the same all in one vector (Aagaard et al. 2007). Although this strategy offers a convenient approach for inducible RNAi, sustained basal promoter activity results in a “leaky” system and furthermore, this strategy offers no capacity for cell or tissue specific expression.

Fritsch et al. first reported the use of the CRE-*lox*-based approach for the inducible expression of H1-siRNA precursors (Fritsch et al. 2004). To prevent any alteration of the promoter sequence a neomycin cassette flanked by two *lox* sites was inserted between the sense and antisense strands of the siRNA. The neomycin sequence contained a termination signal preventing the transcription of full transcript. Upon addition of CRE recombinase, the neomycin cassette is eliminated and the remaining *lox* site acts as a loop sequence between the sense and antisense strands. The CRE-*lox* approach for conditional RNAi has been used for both the U6 and H1 Pol III promoters and the neomycin sequence may be replaced with any stuffer sequence (Kasim et al. 2004; Tiscornia et al. 2004; Ventura et al.

2004). Furthermore it has been shown that the lox-flanked cassette may also be inserted within the promoter between the distal and proximal site enhancers, thereby disrupting promoter activity (Coumoul et al. 2004). This strategy enables tight regulation of shRNA expression and tissue specific regulation has been shown in mice stably expressing CRE recombinase in certain cell populations (Coumoul et al. 2005), thus providing a platform for simultaneous regulation of gene silencing, both temporally and spatially. The drawback of this strategy however, is that the effects are largely irreversible, and in a therapeutic setting, the delivery of CRE recombinase to human cell populations for stable expression is not feasible.

The inducible expression systems described above have largely been used to investigate loss of function phenotypes in controlled cell populations. The potential of these systems to be manipulated for the controlled expression of RNAi-based therapeutic modalities in diseased cell populations remains an attractive possibility but which requires further characterisation. A clinically relevant inducible expression system has been described which makes use of a chimeric promoter comprising the RNA Pol II HIV LTR promoter fused to a minimal hsp70 promoter (Unwalla et al. 2004; Unwalla et al. 2006). The chimeric promoter is induced by Tat in HIV-infected cells, which binds to the TAR loop within the LTR promoter thereby recruiting PTEF-b to the promoter for the activation of transcription from the hsp70 promoter. The use of this system to drive expression of anti-HIV shRNAs results in an inducible therapeutic modality which is naturally activated only in HIV-infected cells. The incorporation of lhRNA-based combinatorial RNAi expression cassettes into a relevant and effective inducible expression system, coupled with an efficient delivery platform, may ultimately lead to a clinically relevant gene therapy.

5.4 Pre-clinical and clinical gene therapy

Given the associated safety concerns with RNAi-based therapies related to potential induction of an interferon response, the saturation of components of the endogenous miRNA biogenesis pathway, possible sequence-related off target effects, and delivery vector biosafety, a thorough pre-clinical

assessment of the efficacy and safety of combinatorial RNAi systems is absolutely essential. Experiments to exclude such non-specific effects in cell culture models *in vitro* are relatively straight forward and were described in section 4.3.5. The problem lies in the lack of a physiologically relevant animal model in which to perform pre-clinical evaluation studies of anti-HIV therapeutics. However in recent years, several mouse models have been developed which are appropriate for *in vivo* testing. Anti-HIV gene therapies may be administered through adoptive T cell immunotherapy, whereby T cells are isolated from a patient and subsequently transduced *ex vivo* with a viral vector carrying the therapeutic effector. Transduced T cells are then expanded before re-infusion into the patient. However the use of this technology against HIV has achieved variable success [reviewed in (Varela-Rohena et al. 2008)].

A more popular approach has been the *ex vivo* transduction of enriched populations of CD34+ haematopoietic stem cells for a protective immunotherapy. The first such study initially investigated whether haematopoietic stem cells (HSC) transduced with a lentiviral vector carrying a Pol III-expressed anti-*rev* siRNA, were able to retain their ability to differentiate into mature T lymphocytes and macrophages which are the primary targets of HIV infection (Banerjea et al. 2003). Not only were the transduced stem cells able to mature into both T cells and macrophages, but the macrophages derived from the transduced stem cells were resistant to HIV infection *in vitro*. Furthermore, transduced CD34+ cells were engrafted into severe combined immunodeficiency (SCID-hu) mice in order to reconstitute the thymocyte population absent in this mouse model. Normal maturation patterns of thymocytes were observed and when this cell population was challenged *ex vivo* with HIV, T cells showed protection against the virus. This fundamental study therefore showed that the reconstitution of a functional and protected immune system *in vivo* is possible, and suggests that a similar strategy could be adopted in humans. More recently, new and improved humanized mouse models have been developed and studied (Legrand et al. 2006). The human immune system (HIS) BALB/c RAG-2$^{-/-}$γc$^{-/-}$ mouse model which is a recombination activating gene (RAG) deficient mouse strain that also lacks the cytokine receptor gamma chain, efficiently sustains the development and maturation of all lymphoid cellular compartments and thus enables the experimentation with human T cells. Recently this model was used to engraft CD34+ HSC transduced with a lentiviral vector carrying

an shRNA targeted against *nef* (ter Brake et al. 2009). Following differentiation, mature CD4+ T cells were infected with HIV-1 *ex vivo*, and potent viral inhibition was observed, suggesting the successful reconstitution of HIV-1 resistant T cells. Although further experiments to challenge mice with HIV *in vivo* are still required, the use of murine models of HIV infection has led to significant progress in the development of clinically relevant RNAi-based therapeutics.

A little over ten years since the discovery of the RNAi pathway, clinical trials harnessing RNAi effector mimics for the treatment of a wide range of diseases are already well underway (Castanotto and Rossi 2009). The first RNAi-based clinical trial for the treatment of HIV is currently in progress. This Phase I trial is being conducted at The City of Hope National Medical Center in Duarte, California. A lentiviral vector containing three therapeutic modalities, one being a Pol III-expressed shRNA targeting the *tat*/*rev* common exon of HIV, was used for the efficient transduction of haematopoietic stem cells. Transduced cells were capable of differentiating into mature lymphocyte lineages upon engraftment into SCID-hu murine models, and furthermore, showed long-term resistance to HIV infection (Li et al. 2005; Li et al. 2006; Anderson et al. 2007). During the clinical trial, transduced stem cells were infused into four HIV positive individuals by autologous bone marrow transplantation for the treatment of AIDS related-lymphoma. This trial, of which the outcome is eagerly awaited, is a fundamental step forward in the race to deliver RNAi-based anti-HIV therapies in the clinic.

5.5 The complex interplay between HIV infection and the RNAi pathway

Expanding evidence suggests that an intricate relationship naturally exists between viral infection and the RNAi pathway. Intriguing data have shown that a number of viruses may either exploit the RNAi pathway to their advantage or alternatively activate mechanisms which repress the pathway. Furthermore, the expression levels of both viral and host derived miRNAs may be modulated during viral infection for host or viral gene regulation (Berkhout and Haasnoot 2006) and these factors should be considered when developing RNAi-based antiviral strategies.

Infection by HIV is not exempt from these observations and has been shown to interfere with components of the RNAi machinery for its own advantage. It may therefore be important to determine the effect of anti-HIV lhRNA-based therapeutic modalities on this natural interplay. The HIV Tat protein is able to directly interact with the helicase domain of Dicer and thus reduce processing of siRNA precursors *in vitro* (Bennasser and Jeang 2006). TRBP is an essential co-factor of Dicer (Chendrimada et al. 2005; Haase et al. 2005), yet this protein also binds to the TAR loop within the viral LTR to mediate transcriptional activation (Gatignol et al. 1991; Christensen et al. 2007). It was shown that ectopic expression of viral TAR RNA is able to sequester TRPB thus inhibiting RNAi (Bennasser et al. 2006). However since the RNAi pathway is still functional in cells infected with HIV, it is unlikely that the competition for TRBP represents a critical inhibitory factor. Moreover the TAR loop itself may act as a DICER substrate, although the role of this putative miRNA-like structure remains unknown (Klase et al. 2007; Ouellet et al. 2008). Besides the TAR loop acting as an alleged miRNA, HIV has been proposed to encode a miRNA precursor within its *nef* gene. The encoded miR-N367 targets a site at the 3' end of nef which overlaps the U3 region of the viral LTR and is thought to play a role in transcriptional regulation of the virus (Omoto et al. 2004; Omoto and Fujii 2005).

Cellular miRNAs have also been implicated in the HIV-RNAi interaction. The polycistronic miRNA cluster miR-17/92 is seen to be downregulated in HIV-infected cells. The histone acetyltransferase P300/CBP-associated factor (PCAF) is a co-factor for Tat and plays a role in processive viral transcription. Intriguingly, PCAF is a target for miR-17-5p and miR-20a, which are two miRNA components of the miR-17/92 cluster, suggesting an intricate interplay between HIV replication and miRNA-mediated gene regulation of host factors (Triboulet et al. 2007). Two more cellular miRNAs, miR-29a and miR-29b, are also involved in the regulation of viral replication (Ahluwalia et al. 2008; Nathans et al. 2009). These two miRNAs target a conserved site within the viral *nef* gene (Ahluwalia et al. 2008) and miR-29a has been shown to suppress HIV replication through accumulation of viral mRNA in P-bodies (Nathans et al. 2009).

Finally, it has been proposed that the interaction between HIV and the RNAi pathway may also contribute to the multifaceted mechanism underlying viral latency (discussed in section 1.8.5). In

resting CD+ T cells harbouring latent HIV, a cluster of five cellular miRNAs have been shown to be upregulated (Huang et al. 2007). These enriched cellular miRNAs inhibit HIV-1 protein translation through interactions with the 3' end of viral mRNA transcripts and therefore appear to play a pivotal role in HIV latency. The use of a panel of miRNA inhibitors effectively facilitated viral production in resting T cells and may potentially be used to aid in the purging of latent reservoirs (Han and Siliciano 2007; Huang et al. 2007). It is obvious from the studies described above that an RNAi-based HIV-host relationship exists, yet a better understanding of this interplay at the molecular level is imperative for the development of enhanced RNAi-based antiviral therapeutics.

5.6 Concluding remarks

The field of RNAi has grown substantially in recent years. This pathway has not only had a profound effect on functional genomics studies but moreover, has provided scientists with an exciting new tool with which to develop novel therapeutics. It is therefore no surprise that the discoverers of this fundamental gene regulation pathway, Andrew Fire and Craig Mello, were awarded the 2006 Nobel Prize in Physiology or Medicine. Interest in this field has continued to grow and so has the number of strategies used to exploit the effector molecules of this pathway. Mimics of intermediate RNAi effectors have diversified over the years and have recently included structures which enable the silencing of multiple targets simultaneously. Analogous to the combination therapy which constitutes HAART, combinatorial RNAi strategies have also been manipulated for the treatment of HIV. This thesis has focused on one such strategy, namely that of Pol III-expressed long hairpin RNAs, for the simultaneous derivation of multiple non-contiguous RNAi effector sequences. These constructs were extensively characterised and subsequently incorporated into a novel double long hairpin RNA structure, ideally suited for the long term inhibition of HIV gene expression and replication. The development of dlhRNAs has aimed to resolve many issues and limitations associated with current combinatorial RNAi approaches and although further improvement of the this novel approach is imminent, the foundation has been laid for the development of dlhRNA-based therapeutic modalities for the treatment not only of HIV, but of other viruses, particularly those with high rates of mutation

such as HCV. The work described in this thesis has substantially contributed to the ultimate achievement of an RNAi-based combinatorial therapy for the sustained inhibition of HIV.

REFERENCES

Aagaard, L., M. Amarzguioui, G. Sun, L. C. Santos, A. Ehsani, H. Prydz and J. J. Rossi (2007). A facile lentiviral vector system for expression of doxycycline-inducible shRNAs: knockdown of the pre-miRNA processing enzyme Drosha. *Mol Ther* **15**(5): 938-45.

Aagaard, L. A., J. Zhang, K. J. von Eije, H. Li, P. Saetrom, M. Amarzguioui and J. J. Rossi (2008). Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Ther* **15**(23): 1536-49.

Adachi, A., H. E. Gendelman, S. Koenig, T. Folks, R. Willey, A. Rabson and M. A. Martin (1986). Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. *J Virol* **59**(2): 284-91.

Agrawal, L., C. R. Maxwell, P. J. Peters, P. R. Clapham, S. M. Liu, C. R. Mackay and D. S. Strayer (2009). Complexity in human immunodeficiency virus type 1 (HIV-1) co-receptor usage: roles of CCR3 and CCR5 in HIV-1 infection of monocyte-derived macrophages and brain microglia. *J Gen Virol* **90**(Pt 3): 710-22.

Ahluwalia, J. K., S. Z. Khan, K. Soni, P. Rawat, A. Gupta, M. Hariharan, V. Scaria, M. Lalwani, B. Pillai, D. Mitra and S. K. Brahmachari (2008). Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology* **5**: 117.

Akashi, H., M. Miyagishi, T. Yokota, T. Watanabe, T. Hino, K. Nishina, M. Kohara and K. Taira (2005). Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Mol Biosyst* **1**(5-6): 382-90.

An, D. S., F. X. Qin, V. C. Auyeung, S. H. Mao, S. K. Kung, D. Baltimore and I. S. Chen (2006). Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol Ther* **14**(4): 494-504.

Anderson, J., A. Banerjea and R. Akkina (2003). Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. *Oligonucleotides* **13**(5): 303-12.

Anderson, J., M. J. Li, B. Palmer, L. Remling, S. Li, P. Yam, J. K. Yee, J. Rossi, J. Zaia and R. Akkina (2007). Safety and efficacy of a lentiviral vector containing three anti-HIV genes--CCR5 ribozyme, tat-rev siRNA, and TAR decoy--in SCID-hu mouse-derived T cells. *Mol Ther* **15**(6): 1182-8.

Anderson, J. L. and T. J. Hope (2004). HIV accessory proteins and surviving the host cell. *Curr HIV/AIDS Rep* **1**(1): 47-53.

Aravin, A. A., G. J. Hannon and J. Brennecke (2007). The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **318**(5851): 761-4.

Aravin, A. A., R. Sachidanandam, D. Bourc'his, C. Schaefer, D. Pezic, K. F. Toth, T. Bestor and G. J. Hannon (2008). A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol Cell* **31**(6): 785-99.

Arts, G. J., S. Kuersten, P. Romby, B. Ehresmann and I. W. Mattaj (1998). The role of exportin-t in selective nuclear export of mature tRNAs. *Embo J* **17**(24): 7430-41.

Babiarz, J. E., J. G. Ruby, Y. Wang, D. P. Bartel and R. Blelloch (2008). Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev* **22**(20): 2773-85.

Banerjea, A., M. J. Li, G. Bauer, L. Remling, N. S. Lee, J. Rossi and R. Akkina (2003). Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. *Mol Ther* **8**(1): 62-71.

Barichievy, S., S. Saayman, K. J. von Eije, K. V. Morris, P. Arbuthnot and M. S. Weinberg (2007). The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5' long terminal repeat. *Oligonucleotides* **17**(4): 419-31.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum and L. Montagnier (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). *Science* **220**(4599): 868-71.

Bartel, D. P. (2009). MicroRNAs: target recognition and regulatory functions. *Cell* **136**(2): 215-33.

Bartlett, D. W. and M. E. Davis (2006). Insights into the kinetics of siRNA-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucleic Acids Res* **34**(1): 322-33.

Behlke, M. A. (2008). Chemical modification of siRNAs for in vivo use. *Oligonucleotides* **18**(4): 305-19.

Bennasser, Y. and K. T. Jeang (2006). HIV-1 Tat interaction with Dicer: requirement for RNA. *Retrovirology* **3**: 95.

Bennasser, Y., M. L. Yeung and K. T. Jeang (2006). HIV-1 TAR RNA subverts RNA interference in transfected cells through sequestration of TAR RNA-binding protein, TRBP. *J Biol Chem* **281**(38): 27674-8.

Berezikov, E., W. J. Chung, J. Willis, E. Cuppen and E. C. Lai (2007). Mammalian mirtron genes. *Mol Cell* **28**(2): 328-36.

Berkhout, B. and J. Haasnoot (2006). The interplay between virus infection and the cellular RNA interference machinery. *FEBS Lett* **580**(12): 2896-902.

Bernstein, E., A. A. Caudy, S. M. Hammond and G. J. Hannon (2001). Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **409**(6818): 363-6.

Bertrand, E., D. Castanotto, C. Zhou, C. Carbonnelle, N. S. Lee, P. Good, S. Chatterjee, T. Grange, R. Pictet, D. Kohn, D. Engelke and J. J. Rossi (1997). The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *Rna* **3**(1): 75-88.

Bhattacharyya, S. N., R. Habermacher, U. Martine, E. I. Closs and W. Filipowicz (2006). Relief of microRNA-mediated translational repression in human cells subjected to stress. *Cell* **125**(6): 1111-24.

Birmingham, A., E. M. Anderson, A. Reynolds, D. Ilsley-Tyree, D. Leake, Y. Fedorov, S. Baskerville, E. Maksimova, K. Robinson, J. Karpilow, W. S. Marshall and A. Khvorova (2006). 3' UTR seed matches, but not overall identity, are associated with RNAi off-targets. *Nat Methods* **3**(3): 199-204.

Boden, D., O. Pusch, F. Lee, L. Tucker and B. Ramratnam (2003a). Human immunodeficiency virus type 1 escape from RNA interference. *J Virol* **77**(21): 11531-5.

Boden, D., O. Pusch, F. Lee, L. Tucker, P. R. Shank and B. Ramratnam (2003b). Promoter choice affects the potency of HIV-1 specific RNA interference. *Nucleic Acids Res* **31**(17): 5033-8.

Bogerd, H. P., H. W. Karnowski, X. Cai, J. Shin, M. Pohlers and B. R. Cullen (2010). A mammalian herpesvirus uses noncanonical expression and processing mechanisms to generate viral MicroRNAs. *Mol Cell* **37**(1): 135-42.

Bohnsack, M. T., K. Czaplinski and D. Gorlich (2004). Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. *Rna* **10**(2): 185-91.

Borchert, G. M., W. Lanier and B. L. Davidson (2006). RNA polymerase III transcribes human microRNAs. *Nat Struct Mol Biol* **13**(12): 1097-101.

Boudreau, R. L., I. Martins and B. L. Davidson (2009). Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Mol Ther* **17**(1): 169-75.

Boudreau, R. L., A. M. Monteys and B. L. Davidson (2008). Minimizing variables among hairpin-based RNAi vectors reveals the potency of shRNAs. *Rna* **14**(9): 1834-44.

Bramsen, J. B., M. B. Laursen, A. F. Nielsen, T. B. Hansen, C. Bus, N. Langkjaer, B. R. Babu, T. Hojland, M. Abramov, A. Van Aerschot, D. Odadzic, R. Smicius, J. Haas, C. Andree, J. Barman, M. Wenska, P. Srivastava, C. Zhou, D. Honcharenko, S. Hess, E. Muller, G. V. Bobkov, S. N. Mikhailov, E. Fava, T. F. Meyer, J. Chattopadhyaya, M. Zerial, J. W. Engels, P. Herdewijn, J. Wengel and J. Kjems (2009). A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res* **37**(9): 2867-81.

Brass, A. L., D. M. Dykxhoorn, Y. Benita, N. Yan, A. Engelman, R. J. Xavier, J. Lieberman and S. J. Elledge (2008). Identification of host proteins required for HIV infection through a functional genomic screen. *Science* **319**(5865): 921-6.

Brennecke, J., A. Stark, R. B. Russell and S. M. Cohen (2005). Principles of microRNA-target recognition. *PLoS Biol* **3**(3): e85.

Brummelkamp, T. R., R. Bernards and R. Agami (2002). A system for stable expression of short interfering RNAs in mammalian cells. *Science* **296**(5567): 550-3.

Bukovsky, A. A., J. P. Song and L. Naldini (1999). Interaction of human immunodeficiency virus-derived vectors with wild-type virus in transduced cells. *J Virol* **73**(8): 7087-92.

Bukrinsky, M. I., S. Haggerty, M. P. Dempsey, N. Sharova, A. Adzhubel, L. Spitz, P. Lewis, D. Goldfarb, M. Emerman and M. Stevenson (1993). A nuclear localization signal within HIV-1 matrix protein that governs infection of non-dividing cells. *Nature* **365**(6447): 666-9.

Cai, X., C. H. Hagedorn and B. R. Cullen (2004). Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. *Rna* **10**(12): 1957-66.

Campbell, E. M. and T. J. Hope (2008). Live cell imaging of the HIV-1 life cycle. *Trends Microbiol* **16**(12): 580-7.

Caplen, N. J., S. Parrish, F. Imani, A. Fire and R. A. Morgan (2001). Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc Natl Acad Sci U S A* **98**(17): 9742-7.

Capodici, J., K. Kariko and D. Weissman (2002). Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J Immunol* **169**(9): 5196-201.

Carr, A. (2003). Toxicity of antiretroviral therapy and implications for drug development. *Nat Rev Drug Discov* **2**(8): 624-34.

Castanotto, D., H. Li and J. J. Rossi (2002). Functional siRNA expression from transfected PCR products. *Rna* **8**(11): 1454-60.

Castanotto, D. and J. J. Rossi (2009). The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **457**(7228): 426-33.

Castanotto, D., K. Sakurai, R. Lingeman, H. Li, L. Shively, L. Aagaard, H. Soifer, A. Gatignol, A. Riggs and J. J. Rossi (2007). Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res* **35**(15): 5154-64.

Castanotto, D., S. Tommasi, M. Li, H. Li, S. Yanow, G. P. Pfeifer and J. J. Rossi (2005). Short hairpin RNA-directed cytosine (CpG) methylation of the RASSF1A gene promoter in HeLa cells. *Mol Ther* **12**(1): 179-83.

Chekulaeva, M. and W. Filipowicz (2009). Mechanisms of miRNA-mediated post-transcriptional regulation in animal cells. *Curr Opin Cell Biol* **21**(3): 452-60.

Chen, P. Y., L. Weinmann, D. Gaidatzis, Y. Pei, M. Zavolan, T. Tuschl and G. Meister (2008). Strand-specific 5'-O-methylation of siRNA duplexes controls guide strand selection and targeting specificity. *Rna* **14**(2): 263-74.

Chendrimada, T. P., R. I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura and R. Shiekhattar (2005). TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **436**(7051): 740-4.

Cheng, T. L., C. F. Teng, W. H. Tsai, C. W. Yeh, M. P. Wu, H. C. Hsu, C. F. Hung and W. T. Chang (2009). Multitarget therapy of malignant cancers by the head-to-tail tandem array multiple shRNAs expression system. *Cancer Gene Ther* **16**(6): 516-31.

Chiu, Y. L., H. Cao, J. M. Jacque, M. Stevenson and T. M. Rana (2004). Inhibition of human immunodeficiency virus type 1 replication by RNA interference directed against human transcription elongation factor P-TEFb (CDK9/CyclinT1). *J Virol* **78**(5): 2517-29.

Chiu, Y. L. and T. M. Rana (2003). siRNA function in RNAi: a chemical modification analysis. *Rna* **9**(9): 1034-48.

Choung, S., Y. J. Kim, S. Kim, H. O. Park and Y. C. Choi (2006). Chemical modification of siRNAs to improve serum stability without loss of efficacy. *Biochem Biophys Res Commun* **342**(3): 919-27.

Christensen, H. S., A. Daher, K. J. Soye, L. B. Frankel, M. R. Alexander, S. Laine, S. Bannwarth, C. L. Ong, S. W. Chung, S. M. Campbell, D. F. Purcell and A. Gatignol (2007). Small interfering RNAs against the TAR RNA binding protein, TRBP, a Dicer cofactor, inhibit human immunodeficiency virus type 1 long terminal repeat expression and viral production. *J Virol* **81**(10): 5121-31.

Chu, T. C., K. Y. Twu, A. D. Ellington and M. Levy (2006). Aptamer mediated siRNA delivery. *Nucleic Acids Res* **34**(10): e73.

Chun, T. W., D. Engel, M. M. Berrey, T. Shea, L. Corey and A. S. Fauci (1998). Early establishment of a pool of latently infected, resting CD4(+) T cells during primary HIV-1 infection. *Proc Natl Acad Sci U S A* **95**(15): 8869-73.

Chun, T. W., D. Finzi, J. Margolick, K. Chadwick, D. Schwartz and R. F. Siliciano (1995). In vivo fate of HIV-1-infected T cells: quantitative analysis of the transition to stable latency. *Nat Med* **1**(12): 1284-90.

Chung, K. H., C. C. Hart, S. Al-Bassam, A. Avery, J. Taylor, P. D. Patel, A. B. Vojtek and D. L. Turner (2006). Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res* **34**(7): e53.

Clark, S. J. and G. M. Shaw (1993). The acute retroviral syndrome and the pathogenesis of HIV-1 infection. *Semin Immunol* **5**(3): 149-55.

Clemens, M. J. (1997). PKR--a protein kinase regulated by double-stranded RNA. *Int J Biochem Cell Biol* **29**(7): 945-9.

Coburn, G. A. and B. R. Cullen (2002). Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J Virol* **76**(18): 9225-31.

Connor, R. I., B. K. Chen, S. Choe and N. R. Landau (1995). Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology* **206**(2): 935-44.

Coumoul, X., W. Li, R. H. Wang and C. Deng (2004). Inducible suppression of Fgfr2 and Survivin in ES cells using a combination of the RNA interference (RNAi) and the Cre-LoxP system. *Nucleic Acids Res* **32**(10): e85.

Coumoul, X., V. Shukla, C. Li, R. H. Wang and C. X. Deng (2005). Conditional knockdown of Fgfr2 in mice using Cre-LoxP induced RNA interference. *Nucleic Acids Res* **33**(11): e102.

Cullen, B. R. (2003). Nuclear RNA export. *J Cell Sci* **116**(Pt 4): 587-97.

Czauderna, F., M. Fechtner, S. Dames, H. Aygun, A. Klippel, G. J. Pronk, K. Giese and J. Kaufmann (2003a). Structural variations and stabilising modifications of synthetic siRNAs in mammalian cells. *Nucleic Acids Res* **31**(11): 2705-16.

Czauderna, F., A. Santel, M. Hinz, M. Fechtner, B. Durieux, G. Fisch, F. Leenders, W. Arnold, K. Giese, A. Klippel and J. Kaufmann (2003b). Inducible shRNA expression for application in a prostate cancer mouse model. *Nucleic Acids Res* **31**(21): e127.

Czech, B., R. Zhou, Y. Erlich, J. Brennecke, R. Binari, C. Villalta, A. Gordon, N. Perrimon and G. J. Hannon (2009). Hierarchical rules for Argonaute loading in Drosophila. *Mol Cell* **36**(3): 445-56.

Dahl, V., L. Josefsson and S. Palmer (2010). HIV reservoirs, latency, and reactivation: prospects for eradication. *Antiviral Res* **85**(1): 286-94.

Das, A. T., T. R. Brummelkamp, E. M. Westerhout, M. Vink, M. Madiredjo, R. Bernards and B. Berkhout (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol* **78**(5): 2601-5.

de Bethune, M. P. (2010). Non-nucleoside reverse transcriptase inhibitors (NNRTIs), their discovery, development, and use in the treatment of HIV-1 infection: a review of the last 20 years (1989-2009). *Antiviral Res* **85**(1): 75-90.

Delobel, P., K. Sandres-Saune, M. Cazabat, C. Pasquier, B. Marchou, P. Massip and J. Izopet (2005). R5 to X4 switch of the predominant HIV-1 population in cellular reservoirs during effective highly active antiretroviral therapy. *J Acquir Immune Defic Syndr* **38**(4): 382-92.

Denti, M. A., A. Rosa, O. Sthandier, F. G. De Angelis and I. Bozzoni (2004). A new vector, based on the PolII promoter of the U1 snRNA gene, for the expression of siRNAs in mammalian cells. *Mol Ther* **10**(1): 191-9.

DeVincenzo, J., J. E. Cehelsky, R. Alvarez, S. Elbashir, J. Harborth, I. Toudjarska, L. Nechev, V. Murugaiah, A. Van Vliet, A. K. Vaishnaw and R. Meyers (2008). Evaluation of the safety, tolerability and pharmacokinetics of ALN-RSV01, a novel RNAi antiviral therapeutic directed against respiratory syncytial virus (RSV). *Antiviral Res* **77**(3): 225-31.

Dey, M., C. Cao, A. C. Dar, T. Tamura, K. Ozato, F. Sicheri and T. E. Dever (2005). Mechanistic link between PKR dimerization, autophosphorylation, and eIF2alpha substrate recognition. *Cell* **122**(6): 901-13.

Diallo, M., C. Arenz, K. Schmitz, K. Sandhoff and U. Schepers (2003). Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures. *Oligonucleotides* **13**(5): 381-92.

Dickins, R. A., K. McJunkin, E. Hernando, P. K. Premsrirut, V. Krizhanovsky, D. J. Burgess, S. Y. Kim, C. Cordon-Cardo, L. Zender, G. J. Hannon and S. W. Lowe (2007). Tissue-specific and reversible RNA interference in transgenic mice. *Nat Genet* **39**(7): 914-21.

Dlakic, M. (2006). DUF283 domain of Dicer proteins has a double-stranded RNA-binding fold. *Bioinformatics* **22**(22): 2711-4.

Doms, R. W. and D. Trono (2000). The plasma membrane as a combat zone in the HIV battlefield. *Genes Dev* **14**(21): 2677-88.

Dowler, T., D. Bergeron, A. L. Tedeschi, L. Paquet, N. Ferrari and M. J. Damha (2006). Improvements in siRNA properties mediated by 2'-deoxy-2'-fluoro-beta-D-arabinonucleic acid (FANA). *Nucleic Acids Res* **34**(6): 1669-75.

Dropulic, B., M. Hermankova and P. M. Pitha (1996). A conditionally replicating HIV-1 vector interferes with wild-type HIV-1 replication and spread. *Proc Natl Acad Sci U S A* **93**(20): 11103-8.

Dull, T., R. Zufferey, M. Kelly, R. J. Mandel, M. Nguyen, D. Trono and L. Naldini (1998). A third-generation lentivirus vector with a conditional packaging system. *J Virol* **72**(11): 8463-71.

Dvorin, J. D. and M. H. Malim (2003). Intracellular trafficking of HIV-1 cores: journey to the center of the cell. *Curr Top Microbiol Immunol* **281**: 179-208.

Ebert, M. S., J. R. Neilson and P. A. Sharp (2007). MicroRNA sponges: competitive inhibitors of small RNAs in mammalian cells. *Nat Methods* **4**(9): 721-6.

Elbashir, S. M., J. Harborth, W. Lendeckel, A. Yalcin, K. Weber and T. Tuschl (2001a). Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **411**(6836): 494-8.

Elbashir, S. M., W. Lendeckel and T. Tuschl (2001b). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev* **15**(2): 188-200.

Elbashir, S. M., J. Martinez, A. Patkaniowska, W. Lendeckel and T. Tuschl (2001c). Functional anatomy of siRNAs for mediating efficient RNAi in Drosophila melanogaster embryo lysate. *Embo J* **20**(23): 6877-88.

Elmen, J., H. Thonberg, K. Ljungberg, M. Frieden, M. Westergaard, Y. Xu, B. Wahren, Z. Liang, H. Orum, T. Koch and C. Wahlestedt (2005). Locked nucleic acid (LNA) mediated improvements in siRNA stability and functionality. *Nucleic Acids Res* **33**(1): 439-47.

Ely, A., T. Naidoo and P. Arbuthnot (2009). Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Res*.

Ely, A., T. Naidoo, S. Mufamadi, C. Crowther and P. Arbuthnot (2008). Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Mol Ther* **16**(6): 1105-12.

Emerman, M. and M. H. Malim (1998). HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology. *Science* **280**(5371): 1880-4.

Eulalio, A., E. Huntzinger and E. Izaurralde (2008). Getting to the root of miRNA-mediated gene silencing. *Cell* **132**(1): 9-14.

Ezzell, C. (1987). AIDS drug gets green light. *Nature* **329**(6142): 751.

Fabian, M. R., G. Mathonnet, T. Sundermeier, H. Mathys, J. T. Zipprich, Y. V. Svitkin, F. Rivas, M. Jinek, J. Wohlschlegel, J. A. Doudna, C. Y. Chen, A. B. Shyu, J. R. Yates, 3rd, G. J. Hannon, W. Filipowicz, T. F. Duchaine and N. Sonenberg (2009). Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. *Mol Cell* **35**(6): 868-80.

Filipowicz, W., S. N. Bhattacharyya and N. Sonenberg (2008). Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet* **9**(2): 102-14.

Finzi, D., M. Hermankova, T. Pierson, L. M. Carruth, C. Buck, R. E. Chaisson, T. C. Quinn, K. Chadwick, J. Margolick, R. Brookmeyer, J. Gallant, M. Markowitz, D. D. Ho, D. D. Richman and R. F. Siliciano (1997). Identification of a reservoir for HIV-1 in patients on highly active antiretroviral therapy. *Science* **278**(5341): 1295-300.

Fire, A., S. Xu, M. K. Montgomery, S. A. Kostas, S. E. Driver and C. C. Mello (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**(6669): 806-11.

Fornerod, M., M. Ohno, M. Yoshida and I. W. Mattaj (1997). CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **90**(6): 1051-60.

Friedman, R. C., K. K. Farh, C. B. Burge and D. P. Bartel (2009). Most mammalian mRNAs are conserved targets of microRNAs. *Genome Res* **19**(1): 92-105.

Fritsch, L., L. A. Martinez, R. Sekhri, I. Naguibneva, M. Gerard, M. Vandromme, L. Schaeffer and A. Harel-Bellan (2004). Conditional gene knock-down by CRE-dependent short interfering RNAs. *EMBO Rep* **5**(2): 178-82.

Furman, P. A. and D. W. Barry (1988). Spectrum of antiviral activity and mechanism of action of zidovudine. An overview. *Am J Med* **85**(2A): 176-81.

Gait, M. J. and J. Karn (1993). RNA recognition by the human immunodeficiency virus Tat and Rev proteins. *Trends Biochem Sci* **18**(7): 255-9.

Gallay, P., T. Hope, D. Chin and D. Trono (1997). HIV-1 infection of nondividing cells through the recognition of integrase by the importin/karyopherin pathway. *Proc Natl Acad Sci U S A* **94**(18): 9825-30.

Gallo, R. C., P. S. Sarin, E. P. Gelmann, M. Robert-Guroff, E. Richardson, V. S. Kalyanaraman, D. Mann, G. D. Sidhu, R. E. Stahl, S. Zolla-Pazner, J. Leibowitch and M. Popovic (1983). Isolation of human T-cell leukemia virus in acquired immune deficiency syndrome (AIDS). *Science* **220**(4599): 865-7.

Gan, J., J. E. Tropea, B. P. Austin, D. L. Court, D. S. Waugh and X. Ji (2006). Structural insight into the mechanism of double-stranded RNA processing by ribonuclease III. *Cell* **124**(2): 355-66.

Ganser-Pornillos, B. K., M. Yeager and W. I. Sundquist (2008). The structural biology of HIV assembly. *Curr Opin Struct Biol* **18**(2): 203-17.

Gardner, E. M., S. Sharma, G. Peng, K. H. Hullsiek, W. J. Burman, R. D. Macarthur, M. Chesney, E. E. Telzak, G. Friedland and S. B. Mannheimer (2008). Differential adherence to combination antiretroviral therapy is associated with virological failure with resistance. *Aids* **22**(1): 75-82.

Gatignol, A., A. Buckler-White, B. Berkhout and K. T. Jeang (1991). Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science* **251**(5001): 1597-600.

Gershon, D. (1991). Green light for ddI. *Nature* **353**(6345): 589.

Ghildiyal, M., J. Xu, H. Seitz, Z. Weng and P. D. Zamore (2010). Sorting of Drosophila small silencing RNAs partitions microRNA* strands into the RNA interference pathway. *Rna* **16**(1): 43-56.

Ghildiyal, M. and P. D. Zamore (2009). Small silencing RNAs: an expanding universe. *Nat Rev Genet* **10**(2): 94-108.

Giering, J. C., D. Grimm, T. A. Storm and M. A. Kay (2008). Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol Ther* **16**(9): 1630-6.

Gitlin, L., S. Karelsky and R. Andino (2002). Short interfering RNA confers intracellular antiviral immunity in human cells. *Nature* **418**(6896): 430-4.

Gonzalez-Rojas, J., X. Abad and P. Fortes (2010). RNAi with Viral Vectors that Deliver Small Interfering RNAs. RNA Interference and Viruses. M. Martinez. Norfolk, UK, Caister Academic Press.

Gonzalez, S., D. Castanotto, H. Li, S. Olivares, M. C. Jensen, S. J. Forman, J. J. Rossi and L. J. Cooper (2005). Amplification of RNAi--targeting HLA mRNAs. *Mol Ther* **11**(5): 811-8.

Good, P. D., A. J. Krikos, S. X. Li, E. Bertrand, N. S. Lee, L. Giver, A. Ellington, J. A. Zaia, J. J. Rossi and D. R. Engelke (1997). Expression of small, therapeutic RNAs in human cell nuclei. *Gene Ther* **4**(1): 45-54.

Gou, D., T. Weng, Y. Wang, Z. Wang, H. Zhang, L. Gao, Z. Chen, P. Wang and L. Liu (2007). A novel approach for the construction of multiple shRNA expression vectors. *J Gene Med* **9**(9): 751-63.

Gregory, R. I., T. P. Chendrimada, N. Cooch and R. Shiekhattar (2005). Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. *Cell* **123**(4): 631-40.

Gregory, R. I., K. P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch and R. Shiekhattar (2004). The Microprocessor complex mediates the genesis of microRNAs. *Nature* **432**(7014): 235-40.

Grimm, D., K. L. Streetz, C. L. Jopling, T. A. Storm, K. Pandey, C. R. Davis, P. Marion, F. Salazar and M. A. Kay (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **441**(7092): 537-41.

Grimson, A., K. K. Farh, W. K. Johnston, P. Garrett-Engele, L. P. Lim and D. P. Bartel (2007). MicroRNA targeting specificity in mammals: determinants beyond seed pairing. *Mol Cell* **27**(1): 91-105.

Haase, A. D., L. Jaskiewicz, H. Zhang, S. Laine, R. Sack, A. Gatignol and W. Filipowicz (2005). TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. *EMBO Rep* **6**(10): 961-7.

Hajeri, P. B. and S. K. Singh (2009). siRNAs: their potential as therapeutic agents--Part I. Designing of siRNAs. *Drug Discov Today* **14**(17-18): 851-8.

Hamilton, A. J. and D. C. Baulcombe (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science* **286**(5441): 950-2.

Hammer, S. M., K. E. Squires, M. D. Hughes, J. M. Grimes, L. M. Demeter, J. S. Currier, J. J. Eron, Jr., J. E. Feinberg, H. H. Balfour, Jr., L. R. Deyton, J. A. Chodakewitz and M. A. Fischl (1997). A controlled trial of two nucleoside analogues plus indinavir in persons with human immunodeficiency virus infection and CD4 cell counts of 200 per cubic millimeter or less. AIDS Clinical Trials Group 320 Study Team. *N Engl J Med* **337**(11): 725-33.

Hammond, S. M., E. Bernstein, D. Beach and G. J. Hannon (2000). An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. *Nature* **404**(6775): 293-6.

Han, J., Y. Lee, K. H. Yeom, Y. K. Kim, H. Jin and V. N. Kim (2004). The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev* **18**(24): 3016-27.

Han, J., Y. Lee, K. H. Yeom, J. W. Nam, I. Heo, J. K. Rhee, S. Y. Sohn, Y. Cho, B. T. Zhang and V. N. Kim (2006). Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. *Cell* **125**(5): 887-901.

Han, Y. and R. F. Siliciano (2007). Keeping quiet: microRNAs in HIV-1 latency. *Nat Med* **13**(10): 1138-40.

Harrich, D. and B. Hooker (2002). Mechanistic aspects of HIV-1 reverse transcription initiation. *Rev Med Virol* **12**(1): 31-45.

Harries, A. D., R. Zachariah, J. J. van Oosterhout, S. D. Reid, M. C. Hosseinipour, V. Arendt, Z. Chirwa, A. Jahn, E. J. Schouten and K. Kamoto (2010). Diagnosis and management of antiretroviral-therapy failure in resource-limited settings in sub-Saharan Africa: challenges and perspectives. *Lancet Infect Dis* **10**(1): 60-65.

Hawkins, T. (2010). Understanding and managing the adverse effects of antiretroviral therapy. *Antiviral Res* **85**(1): 201-9.

He, J., Y. Chen, M. Farzan, H. Choe, A. Ohagen, S. Gartner, J. Busciglio, X. Yang, W. Hofmann, W. Newman, C. R. Mackay, J. Sodroski and D. Gabuzda (1997). CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. *Nature* **385**(6617): 645-9.

He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan and N. R. Landau (1995). Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J Virol* **69**(11): 6705-11.

Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson and M. Emerman (1994). The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. *Proc Natl Acad Sci U S A* **91**(15): 7311-5.

Henry, S. D., P. van der Wegen, H. J. Metselaar, H. W. Tilanus, B. J. Scholte and L. J. van der Laan (2006). Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. *Mol Ther* **14**(4): 485-93.

Hill, M., G. Tachedjian and J. Mak (2005). The packaging and maturation of the HIV-1 Pol proteins. *Curr HIV Res* **3**(1): 73-85.

Hirsch, M., R. Steigbigel, S. Staszewski, J. Mellors, E. Scerpella, B. Hirschel, J. Lange, K. Squires, S. Rawlins, A. Meibohm and R. Leavitt (1999). A randomized, controlled trial of indinavir, zidovudine, and lamivudine in adults with advanced human immunodeficiency virus type 1 infection and prior antiretroviral therapy. *J Infect Dis* **180**(3): 659-65.

Hogrefe, R. I., A. V. Lebedev, G. Zon, K. F. Pirollo, A. Rait, Q. Zhou, W. Yu and E. H. Chang (2006). Chemically modified short interfering hybrids (siHYBRIDS): nanoimmunoliposome delivery in vitro and in vivo for RNAi of HER-2. *Nucleosides Nucleotides Nucleic Acids* **25**(8): 889-907.

Hope, T. J. (1999). The ins and outs of HIV Rev. *Arch Biochem Biophys* **365**(2): 186-91.

Hornung, V., J. Ellegast, S. Kim, K. Brzozka, A. Jung, H. Kato, H. Poeck, S. Akira, K. K. Conzelmann, M. Schlee, S. Endres and G. Hartmann (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science* **314**(5801): 994-7.

Hornung, V., M. Guenthner-Biller, C. Bourquin, A. Ablasser, M. Schlee, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, S. Endres and G. Hartmann (2005). Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat Med* **11**(3): 263-70.

Hu, H. Y., Z. Yan, Y. Xu, H. Hu, C. Menzel, Y. H. Zhou, W. Chen and P. Khaitovich (2009). Sequence features associated with microRNA strand selection in humans and flies. *BMC Genomics* **10**: 413.

Hu, W. Y., C. P. Myers, J. M. Kilzer, S. L. Pfaff and F. D. Bushman (2002). Inhibition of retroviral pathogenesis by RNA interference. *Curr Biol* **12**(15): 1301-11.

Huang, J., F. Wang, E. Argyris, K. Chen, Z. Liang, H. Tian, W. Huang, K. Squires, G. Verlinghieri and H. Zhang (2007). Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nat Med* **13**(10): 1241-7.

Hutvagner, G., J. McLachlan, A. E. Pasquinelli, E. Balint, T. Tuschl and P. D. Zamore (2001). A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **293**(5531): 834-8.

Hutvagner, G. and M. J. Simard (2008). Argonaute proteins: key players in RNA silencing. *Nat Rev Mol Cell Biol* **9**(1): 22-32.

Jackson, A. L., S. R. Bartz, J. Schelter, S. V. Kobayashi, J. Burchard, M. Mao, B. Li, G. Cavet and P. S. Linsley (2003). Expression profiling reveals off-target gene regulation by RNAi. *Nat Biotechnol* **21**(6): 635-7.

Jackson, A. L., J. Burchard, J. Schelter, B. N. Chau, M. Cleary, L. Lim and P. S. Linsley (2006). Widespread siRNA "off-target" transcript silencing mediated by seed region sequence complementarity. *Rna* **12**(7): 1179-87.

Jacque, J. M. and M. Stevenson (2006). The inner-nuclear-envelope protein emerin regulates HIV-1 infectivity. *Nature* **441**(7093): 641-5.

Jacque, J. M., K. Triques and M. Stevenson (2002). Modulation of HIV-1 replication by RNA interference. *Nature* **418**(6896): 435-8.

Jagla, B., N. Aulner, P. D. Kelly, D. Song, A. Volchuk, A. Zatorski, D. Shum, T. Mayer, D. A. De Angelis, O. Ouerfelli, U. Rutishauser and J. E. Rothman (2005). Sequence characteristics of functional siRNAs. *Rna* **11**(6): 864-72.

Janowski, B. A., K. E. Huffman, J. C. Schwartz, R. Ram, R. Nordsell, D. S. Shames, J. D. Minna and D. R. Corey (2006). Involvement of AGO1 and AGO2 in mammalian transcriptional silencing. *Nat Struct Mol Biol* **13**(9): 787-92.

Jeang, K. T., Y. Chang, B. Berkhout, M. L. Hammarskjold and D. Rekosh (1991). Regulation of HIV expression: mechanisms of action of Tat and Rev. *Aids* **5 Suppl 2**: S3-14.

Jones, K. A. and B. M. Peterlin (1994). Control of RNA initiation and elongation at the HIV-1 promoter. *Annu Rev Biochem* **63**: 717-43.

Judge, A. D., G. Bola, A. C. Lee and I. MacLachlan (2006). Design of noninflammatory synthetic siRNA mediating potent gene silencing in vivo. *Mol Ther* **13**(3): 494-505.

Judge, A. D., V. Sood, J. R. Shaw, D. Fang, K. McClintock and I. MacLachlan (2005). Sequence-dependent stimulation of the mammalian innate immune response by synthetic siRNA. *Nat Biotechnol* **23**(4): 457-62.

Kao, S. Y., A. F. Calman, P. A. Luciw and B. M. Peterlin (1987). Anti-termination of transcription within the long terminal repeat of HIV-1 by tat gene product. *Nature* **330**(6147): 489-93.

Kasim, V., M. Miyagishi and K. Taira (2004). Control of siRNA expression using the Cre-loxP recombination system. *Nucleic Acids Res* **32**(7): e66.

Kawasaki, H. and K. Taira (2003). Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res* **31**(2): 700-7.

Keck, K., E. M. Volper, R. M. Spengler, D. D. Long, C. Y. Chan, Y. Ding and A. P. McCaffrey (2009). Rational design leads to more potent RNA interference against hepatitis B virus: factors effecting silencing efficiency. *Mol Ther* **17**(3): 538-47.

Ketting, R. F., S. E. Fischer, E. Bernstein, T. Sijen, G. J. Hannon and R. H. Plasterk (2001). Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. *Genes Dev* **15**(20): 2654-9.

Ketting, R. F., T. H. Haverkamp, H. G. van Luenen and R. H. Plasterk (1999). Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* **99**(2): 133-41.

Khvorova, A., A. Reynolds and S. D. Jayasena (2003). Functional siRNAs and miRNAs exhibit strand bias. *Cell* **115**(2): 209-16.

Kim, D. H., M. A. Behlke, S. D. Rose, M. S. Chang, S. Choi and J. J. Rossi (2005). Synthetic dsRNA Dicer substrates enhance RNAi potency and efficacy. *Nat Biotechnol* **23**(2): 222-6.

Kim, D. H., P. Saetrom, O. Snove, Jr. and J. J. Rossi (2008). MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc Natl Acad Sci U S A* **105**(42): 16230-5.

Kim, D. H., L. M. Villeneuve, K. V. Morris and J. J. Rossi (2006). Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat Struct Mol Biol* **13**(9): 793-7.

Kim, S. H., S. S. Yu, J. S. Park, P. D. Robbins, C. S. An and S. Kim (1998). Construction of retroviral vectors with improved safety, gene expression, and versatility. *J Virol* **72**(2): 994-1004.

Kim, V. N. (2004). MicroRNA precursors in motion: exportin-5 mediates their nuclear export. *Trends Cell Biol* **14**(4): 156-9.

Kim, V. N., J. Han and M. C. Siomi (2009). Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol* **10**(2): 126-39.

Kim, V. N. and J. W. Nam (2006). Genomics of microRNA. *Trends Genet* **22**(3): 165-73.

Kiriakidou, M., G. S. Tan, S. Lamprinaki, M. De Planell-Saguer, P. T. Nelson and Z. Mourelatos (2007). An mRNA m7G cap binding-like motif within human Ago2 represses translation. *Cell* **129**(6): 1141-51.

Klase, Z., P. Kale, R. Winograd, M. V. Gupta, M. Heydarian, R. Berro, T. McCaffrey and F. Kashanchi (2007). HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *BMC Mol Biol* **8**: 63.

Kok, K. H., T. Lei and D. Y. Jin (2009). siRNA and shRNA screens advance key understanding of host factors required for HIV-1 replication. *Retrovirology* **6**: 78.

Konig, R., Y. Zhou, D. Elleder, T. L. Diamond, G. M. Bonamy, J. T. Irelan, C. Y. Chiang, B. P. Tu, P. D. De Jesus, C. E. Lilley, S. Seidel, A. M. Opaluch, J. S. Caldwell, M. D. Weitzman, K. L. Kuhen, S. Bandyopadhyay, T. Ideker, A. P. Orth, L. J. Miraglia, F. D. Bushman, J. A. Young and S. K.

Chanda (2008). Global analysis of host-pathogen interactions that regulate early-stage HIV-1 replication. *Cell* **135**(1): 49-60.

Konstantinova, P., W. de Vries, J. Haasnoot, O. ter Brake, P. de Haan and B. Berkhout (2006). Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA. *Gene Ther* **13**(19): 1403-13.

Kotsopoulou, E., V. N. Kim, A. J. Kingsman, S. M. Kingsman and K. A. Mitrophanous (2000). A Rev-independent human immunodeficiency virus type 1 (HIV-1)-based vector that exploits a codon-optimized HIV-1 gag-pol gene. *J Virol* **74**(10): 4839-52.

Kumar, P., H. S. Ban, S. S. Kim, H. Wu, T. Pearson, D. L. Greiner, A. Laouar, J. Yao, V. Haridas, K. Habiro, Y. G. Yang, J. H. Jeong, K. Y. Lee, Y. H. Kim, S. W. Kim, M. Peipp, G. H. Fey, N. Manjunath, L. D. Shultz, S. K. Lee and P. Shankar (2008). T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* **134**(4): 577-86.

Kuramochi-Miyagawa, S., T. Watanabe, K. Gotoh, Y. Totoki, A. Toyoda, M. Ikawa, N. Asada, K. Kojima, Y. Yamaguchi, T. W. Ijiri, K. Hata, E. Li, Y. Matsuda, T. Kimura, M. Okabe, Y. Sakaki, H. Sasaki and T. Nakano (2008). DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev* **22**(7): 908-17.

Kutay, U., G. Lipowsky, E. Izaurralde, F. R. Bischoff, P. Schwarzmaier, E. Hartmann and D. Gorlich (1998). Identification of a tRNA-specific nuclear export receptor. *Mol Cell* **1**(3): 359-69.

Landthaler, M., A. Yalcin and T. Tuschl (2004). The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. *Curr Biol* **14**(23): 2162-7.

Lassen, K., Y. Han, Y. Zhou, J. Siliciano and R. F. Siliciano (2004). The multifactorial nature of HIV-1 latency. *Trends Mol Med* **10**(11): 525-31.

Lau, P. W., C. S. Potter, B. Carragher and I. J. MacRae (2009). Structure of the human Dicer-TRBP complex by electron microscopy. *Structure* **17**(10): 1326-32.

Lee, N. S., T. Dohjima, G. Bauer, H. Li, M. J. Li, A. Ehsani, P. Salvaterra and J. Rossi (2002a). Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol* **20**(5): 500-5.

Lee, S. K., D. M. Dykxhoorn, P. Kumar, S. Ranjbar, E. Song, L. E. Maliszewski, V. Francois-Bongarcon, A. Goldfeld, N. M. Swamy, J. Lieberman and P. Shankar (2005). Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood* **106**(3): 818-26.

Lee, Y., C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim and V. N. Kim (2003). The nuclear RNase III Drosha initiates microRNA processing. *Nature* **425**(6956): 415-9.

Lee, Y., I. Hur, S. Y. Park, Y. K. Kim, M. R. Suh and V. N. Kim (2006). The role of PACT in the RNA silencing pathway. *Embo J* **25**(3): 522-32.

Lee, Y., K. Jeon, J. T. Lee, S. Kim and V. N. Kim (2002b). MicroRNA maturation: stepwise processing and subcellular localization. *Embo J* **21**(17): 4663-70.

Lee, Y., M. Kim, J. Han, K. H. Yeom, S. Lee, S. H. Baek and V. N. Kim (2004). MicroRNA genes are transcribed by RNA polymerase II. *Embo J* **23**(20): 4051-60.

Legrand, N., K. Weijer and H. Spits (2006). Experimental models to study development and function of the human immune system in vivo. *J Immunol* **176**(4): 2053-8.

Leirdal, M. and M. Sioud (2002). Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochem Biophys Res Commun* **295**(3): 744-8.

Lemaire, P. A., E. Anderson, J. Lary and J. L. Cole (2008). Mechanism of PKR Activation by dsRNA. *J Mol Biol* **381**(2): 351-60.

Leonard, J. N. and D. V. Schaffer (2005). Computational design of antiviral RNA interference strategies that resist human immunodeficiency virus escape. *J Virol* **79**(3): 1645-54.

Leonard, J. N., P. S. Shah, J. C. Burnett and D. V. Schaffer (2008). HIV evades RNA interference directed at TAR by an indirect compensatory mechanism. *Cell Host Microbe* **4**(5): 484-94.

Leuschner, P. J., S. L. Ameres, S. Kueng and J. Martinez (2006). Cleavage of the siRNA passenger strand during RISC assembly in human cells. *EMBO Rep* **7**(3): 314-20.

Lewis, B. P., C. B. Burge and D. P. Bartel (2005). Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. *Cell* **120**(1): 15-20.

Lewis, B. P., I. H. Shih, M. W. Jones-Rhoades, D. P. Bartel and C. B. Burge (2003). Prediction of mammalian microRNA targets. *Cell* **115**(7): 787-98.

Li, M., H. Li and J. J. Rossi (2006). RNAi in combination with a ribozyme and TAR decoy for treatment of HIV infection in hematopoietic cell gene therapy. *Ann N Y Acad Sci* **1082**: 172-9.

Li, M. J., J. Kim, S. Li, J. Zaia, J. K. Yee, J. Anderson, R. Akkina and J. J. Rossi (2005). Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol Ther* **12**(5): 900-9.

Lingel, A., B. Simon, E. Izaurralde and M. Sattler (2003). Structure and nucleic-acid binding of the Drosophila Argonaute 2 PAZ domain. *Nature* **426**(6965): 465-9.

Liu, J., M. A. Carmell, F. V. Rivas, C. G. Marsden, J. M. Thomson, J. J. Song, S. M. Hammond, L. Joshua-Tor and G. J. Hannon (2004). Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **305**(5689): 1437-41.

Liu, J., M. A. Valencia-Sanchez, G. J. Hannon and R. Parker (2005). MicroRNA-dependent localization of targeted mRNAs to mammalian P-bodies. *Nat Cell Biol* **7**(7): 719-23.

Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup and N. R. Landau (1996). Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. *Cell* **86**(3): 367-77.

Liu, Y. P., J. Haasnoot and B. Berkhout (2007). Design of extended short hairpin RNAs for HIV-1 inhibition. *Nucleic Acids Res* **35**(17): 5683-93.

Liu, Y. P., J. Haasnoot, O. ter Brake, B. Berkhout and P. Konstantinova (2008). Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res* **36**(9): 2811-24.

Liu, Y. P., K. J. von Eije, N. C. Schopman, J. T. Westerink, O. Brake, J. Haasnoot and B. Berkhout (2009). Combinatorial RNAi against HIV-1 using extended short hairpin RNAs. *Mol Ther* **17**(10): 1712-23.

Llano, M., D. T. Saenz, A. Meehan, P. Wongthida, M. Peretz, W. H. Walker, W. Teo and E. M. Poeschla (2006). An essential role for LEDGF/p75 in HIV integration. *Science* **314**(5798): 461-4.

Lorenz, C., P. Hadwiger, M. John, H. P. Vornlocher and C. Unverzagt (2004). Steroid and lipid conjugates of siRNAs to enhance cellular uptake and gene silencing in liver cells. *Bioorg Med Chem Lett* **14**(19): 4975-7.

Lund, E., S. Guttinger, A. Calado, J. E. Dahlberg and U. Kutay (2004). Nuclear export of microRNA precursors. *Science* **303**(5654): 95-8.

Ma, E., I. J. MacRae, J. F. Kirsch and J. A. Doudna (2008). Autoinhibition of human dicer by its internal helicase domain. *J Mol Biol* **380**(1): 237-43.

Ma, J. B., K. Ye and D. J. Patel (2004). Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. *Nature* **429**(6989): 318-22.

MacRae, I. J. and J. A. Doudna (2007). Ribonuclease revisited: structural insights into ribonuclease III family enzymes. *Curr Opin Struct Biol* **17**(1): 138-45.

Macrae, I. J., K. Zhou, F. Li, A. Repic, A. N. Brooks, W. Z. Cande, P. D. Adams and J. A. Doudna (2006). Structural basis for double-stranded RNA processing by Dicer. *Science* **311**(5758): 195-8.

Maertens, G., P. Cherepanov, W. Pluymers, K. Busschots, E. De Clercq, Z. Debyser and Y. Engelborghs (2003). LEDGF/p75 is essential for nuclear and chromosomal targeting of HIV-1 integrase in human cells. *J Biol Chem* **278**(35): 33528-39.

Makinen, P. I., J. K. Koponen, A. M. Karkkainen, T. M. Malm, K. H. Pulkkinen, J. Koistinaho, M. P. Turunen and S. Yla-Herttuala (2006). Stable RNA interference: comparison of U6 and H1 promoters in endothelial cells and in mouse brain. *J Gene Med* **8**(4): 433-41.

Mallon, P. W. (2007). Pathogenesis of lipodystrophy and lipid abnormalities in patients taking antiretroviral therapy. *AIDS Rev* **9**(1): 3-15.

Mansky, L. M. (1996). The mutation rate of human immunodeficiency virus type 1 is influenced by the vpr gene. *Virology* **222**(2): 391-400.

Marques, J. T., T. Devosse, D. Wang, M. Zamanian-Daryoush, P. Serbinowski, R. Hartmann, T. Fujita, M. A. Behlke and B. R. Williams (2006). A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat Biotechnol* **24**(5): 559-65.

Martin, D. E., K. Salzwedel and G. P. Allaway (2008). Bevirimat: a novel maturation inhibitor for the treatment of HIV-1 infection. *Antivir Chem Chemother* **19**(3): 107-13.

Mathonnet, G., M. R. Fabian, Y. V. Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W. C. Merrick, E. Darzynkiewicz, R. S. Pillai, W. Filipowicz, T. F. Duchaine and N. Sonenberg (2007). MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. *Science* **317**(5845): 1764-7.

Matranga, C., Y. Tomari, C. Shin, D. P. Bartel and P. D. Zamore (2005). Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **123**(4): 607-20.

Matsukura, S., P. A. Jones and D. Takai (2003). Establishment of conditional vectors for hairpin siRNA knockdowns. *Nucleic Acids Res* **31**(15): e77.

McBride, J. L., R. L. Boudreau, S. Q. Harper, P. D. Staber, A. M. Monteys, I. Martins, B. L. Gilmore, H. Burstein, R. W. Peluso, B. Polisky, B. J. Carter and B. L. Davidson (2008). Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc Natl Acad Sci U S A* **105**(15): 5868-73.

McColl, D. J. and X. Chen (2010). Strand transfer inhibitors of HIV-1 integrase: bringing IN a new era of antiretroviral therapy. *Antiviral Res* **85**(1): 101-18.

McIntyre, G. J., J. L. Groneman, Y. H. Yu, A. Jaramillo, S. Shen and T. L. Applegate (2009a). 96 shRNAs designed for maximal coverage of HIV-1 variants. *Retrovirology* **6**: 55.

McIntyre, G. J., Y. H. Yu, A. Tran, A. B. Jaramillo, A. J. Arndt, M. L. Millington, M. P. Boyd, F. A. Elliott, S. W. Shen, J. M. Murray and T. L. Applegate (2009b). Cassette deletion in multiple shRNA lentiviral vectors for HIV-1 and its impact on treatment success. *Virol J* **6**: 184.

McManus, M. T., C. P. Petersen, B. B. Haines, J. Chen and P. A. Sharp (2002). Gene silencing using micro-RNA designed hairpins. *Rna* **8**(6): 842-50.

McNamara, J. O., 2nd, E. R. Andrechek, Y. Wang, K. D. Viles, R. E. Rempel, E. Gilboa, B. A. Sullenger and P. H. Giangrande (2006). Cell type-specific delivery of siRNAs with aptamer-siRNA chimeras. *Nat Biotechnol* **24**(8): 1005-15.

Meister, G., M. Landthaler, A. Patkaniowska, Y. Dorsett, G. Teng and T. Tuschl (2004). Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. *Mol Cell* **15**(2): 185-97.

Menendez-Arias, L. (2010). Molecular basis of human immunodeficiency virus drug resistance: an update. *Antiviral Res* **85**(1): 210-31.

Mette, M. F., W. Aufsatz, J. van der Winden, M. A. Matzke and A. J. Matzke (2000). Transcriptional silencing and promoter methylation triggered by double-stranded RNA. *Embo J* **19**(19): 5194-201.

miRBase (accessed 2010). The miRNA database, http://www.mirbase.org/.

Moncunill, G., M. Armand-Ugon, E. Pauls, B. Clotet and J. A. Este (2008). HIV-1 escape to CCR5 coreceptor antagonism through selection of CXCR4-using variants in vitro. *Aids* **22**(1): 23-31.

Mook, O. R., F. Baas, M. B. de Wissel and K. Fluiter (2007). Evaluation of locked nucleic acid-modified small interfering RNA in vitro and in vivo. *Mol Cancer Ther* **6**(3): 833-43.

Morris, K. V., S. W. Chan, S. E. Jacobsen and D. J. Looney (2004a). Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **305**(5688): 1289-92.

Morris, K. V., J. Gilbert, F. Wong-Staal, M. Gasmi and D. J. Looney (2004b). Transduction of cell lines and primary cells by FIV-packaged HIV vectors. *Mol Ther* **10**(1): 181-90.

Morris, K. V. and D. J. Looney (2005). Characterization of human immunodeficiency virus (HIV)-2 vector mobilization by HIV-1. *Hum Gene Ther* **16**(12): 1463-72.

Morrissey, D. V., J. A. Lockridge, L. Shaw, K. Blanchard, K. Jensen, W. Breen, K. Hartsough, L. Machemer, S. Radka, V. Jadhav, N. Vaish, S. Zinnen, C. Vargeese, K. Bowman, C. S. Shaffer,

L. B. Jeffs, A. Judge, I. MacLachlan and B. Polisky (2005). Potent and persistent in vivo anti-HBV activity of chemically modified siRNAs. *Nat Biotechnol* **23**(8): 1002-7.

Moulard, M. and E. Decroly (2000). Maturation of HIV envelope glycoprotein precursors by cellular endoproteases. *Biochim Biophys Acta* **1469**(3): 121-32.

Mourrain, P., C. Beclin, T. Elmayan, F. Feuerbach, C. Godon, J. B. Morel, D. Jouette, A. M. Lacombe, S. Nikic, N. Picault, K. Remoue, M. Sanial, T. A. Vo and H. Vaucheret (2000). Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. *Cell* **101**(5): 533-42.

Napoli, C., C. Lemieux and R. Jorgensen (1990). Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans. *Plant Cell* **2**(4): 279-289.

Nathans, R., C. Y. Chu, A. K. Serquina, C. C. Lu, H. Cao and T. M. Rana (2009). Cellular microRNA and P bodies modulate host-HIV-1 interactions. *Mol Cell* **34**(6): 696-709.

Nguyen, D. G., K. C. Wolff, H. Yin, J. S. Caldwell and K. L. Kuhen (2006). "UnPAKing" human immunodeficiency virus (HIV) replication: using small interfering RNA screening to identify novel cofactors and elucidate the role of group I PAKs in HIV infection. *J Virol* **80**(1): 130-7.

Nielsen, T. T., I. Marion, L. Hasholt and C. Lundberg (2009). Neuron-specific RNA interference using lentiviral vectors. *J Gene Med* **11**(7): 559-69.

Nishitsuji, H., T. Ikeda, H. Miyoshi, T. Ohashi, M. Kannagi and T. Masuda (2004). Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. *Microbes Infect* **6**(1): 76-85.

Nishitsuji, H., M. Kohara, M. Kannagi and T. Masuda (2006). Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. *J Virol* **80**(15): 7658-66.

Nottrott, S., M. J. Simard and J. D. Richter (2006). Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol* **13**(12): 1108-14.

Novina, C. D., M. F. Murray, D. M. Dykxhoorn, P. J. Beresford, J. Riess, S. K. Lee, R. G. Collman, J. Lieberman, P. Shankar and P. A. Sharp (2002). siRNA-directed inhibition of HIV-1 infection. *Nat Med* **8**(7): 681-6.

Ohkawa, J. and K. Taira (2000). Control of the functional activity of an antisense RNA by a tetracycline-responsive derivative of the human U6 snRNA promoter. *Hum Gene Ther* **11**(4): 577-85.

Okada, C., E. Yamashita, S. J. Lee, S. Shibata, J. Katahira, A. Nakagawa, Y. Yoneda and T. Tsukihara (2009). A high-resolution structure of the pre-microRNA nuclear export machinery. *Science* **326**(5957): 1275-9.

Okamura, K., N. Liu and E. C. Lai (2009). Distinct mechanisms for microRNA strand selection by Drosophila Argonautes. *Mol Cell* **36**(3): 431-44.

Olejniczak, M., P. Galka and W. J. Krzyzosiak Sequence-non-specific effects of RNA interference triggers and microRNA regulators. *Nucleic Acids Res* **38**(1): 1-16.

Omoto, S. and Y. R. Fujii (2005). Regulation of human immunodeficiency virus 1 transcription by nef microRNA. *J Gen Virol* **86**(Pt 3): 751-5.

Omoto, S., M. Ito, Y. Tsutsumi, Y. Ichikawa, H. Okuyama, E. A. Brisibe, N. K. Saksena and Y. R. Fujii (2004). HIV-1 nef suppression by virally encoded microRNA. *Retrovirology* **1**: 44.

Ouellet, D. L., I. Plante, P. Landry, C. Barat, M. E. Janelle, L. Flamand, M. J. Tremblay and P. Provost (2008). Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element. *Nucleic Acids Res* **36**(7): 2353-65.

Overhoff, M., M. Alken, R. K. Far, M. Lemaitre, B. Lebleu, G. Sczakiel and I. Robbins (2005). Local RNA target structure influences siRNA efficacy: a systematic global analysis. *J Mol Biol* **348**(4): 871-81.

Paddison, P. J., A. A. Caudy and G. J. Hannon (2002). Stable suppression of gene expression by RNAi in mammalian cells. *Proc Natl Acad Sci U S A* **99**(3): 1443-8.

Pal-Bhadra, M., U. Bhadra and J. A. Birchler (2002). RNAi related mechanisms affect both transcriptional and posttranscriptional transgene silencing in Drosophila. *Mol Cell* **9**(2): 315-27.

Paredes, R., C. M. Lalama, H. J. Ribaudo, B. R. Schackman, C. Shikuma, F. Giguel, W. A. Meyer, V. A. Johnson, S. A. Fiscus, R. T. D'Aquila, R. M. Gulick and D. R. Kuritzkes (2010). Pre-existing Minority Drug-Resistant HIV-1 Variants, Adherence, and Risk of Antiretroviral Treatment Failure. *J Infect Dis*.

Park, W. S., N. Miyano-Kurosaki, M. Hayafune, E. Nakajima, T. Matsuzaki, F. Shimada and H. Takaku (2002). Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. *Nucleic Acids Res* **30**(22): 4830-5.

Parker, R. and U. Sheth (2007). P bodies and the control of mRNA translation and degradation. *Mol Cell* **25**(5): 635-46.

Passman, M., M. Weinberg, M. Kew and P. Arbuthnot (2000). In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. *Biochem Biophys Res Commun* **268**(3): 728-33.

Patarca, R., C. Heath, G. J. Goldenberg, C. A. Rosen, J. G. Sodroski, W. A. Haseltine and U. M. Hansen (1987). Transcription directed by the HIV long terminal repeat in vitro. *AIDS Res Hum Retroviruses* **3**(1): 41-55.

Patzel, V., S. Rutz, I. Dietrich, C. Koberle, A. Scheffold and S. H. Kaufmann (2005). Design of siRNAs producing unstructured guide-RNAs results in improved RNA interference efficiency. *Nat Biotechnol* **23**(11): 1440-4.

Paul, C. P., P. D. Good, I. Winer and D. R. Engelke (2002). Effective expression of small interfering RNA in human cells. *Nat Biotechnol* **20**(5): 505-8.

Pei, Y. and T. Tuschl (2006). On the art of identifying effective and specific siRNAs. *Nat Methods* **3**(9): 670-6.

Petersen, C. P., M. E. Bordeleau, J. Pelletier and P. A. Sharp (2006). Short RNAs repress translation after initiation in mammalian cells. *Mol Cell* **21**(4): 533-42.

Poeschla, E. M., F. Wong-Staal and D. J. Looney (1998). Efficient transduction of nondividing human cells by feline immunodeficiency virus lentiviral vectors. *Nat Med* **4**(3): 354-7.

Pollard, V. W. and M. H. Malim (1998). The HIV-1 Rev protein. *Annu Rev Microbiol* **52**: 491-532.

Poluri, A. and R. E. Sutton (2008). Titers of HIV-based vectors encoding shRNAs are reduced by a dicer-dependent mechanism. *Mol Ther* **16**(2): 378-86.

Prakash, T. P., C. R. Allerson, P. Dande, T. A. Vickers, N. Sioufi, R. Jarres, B. F. Baker, E. E. Swayze, R. H. Griffey and B. Bhat (2005). Positional effect of chemical modifications on short interference RNA activity in mammalian cells. *J Med Chem* **48**(13): 4247-53.

Qin, X. F., D. S. An, I. S. Chen and D. Baltimore (2003). Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. *Proc Natl Acad Sci U S A* **100**(1): 183-8.

Rand, T. A., S. Petersen, F. Du and X. Wang (2005). Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **123**(4): 621-9.

Rao, D. D., J. S. Vorhies, N. Senzer and J. Nemunaitis (2009). siRNA vs. shRNA: similarities and differences. *Adv Drug Deliv Rev* **61**(9): 746-59.

Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K. Baumeister and et al. (1985). Complete nucleotide sequence of the AIDS virus, HTLV-III. *Nature* **313**(6000): 277-84.

Reyes-Darias, J. A., F. J. Sanchez-Luque and A. Berzal-Herranz (2008). Inhibition of HIV-1 replication by RNA-based strategies. *Curr HIV Res* **6**(6): 500-14.

Reynolds, A., D. Leake, Q. Boese, S. Scaringe, W. S. Marshall and A. Khvorova (2004). Rational siRNA design for RNA interference. *Nat Biotechnol* **22**(3): 326-30.

Rhee, S. W., J. R. Stimers, W. Wang and L. Pang (2009). Vascular smooth muscle-specific knockdown of the noncardiac form of the L-type calcium channel by microRNA-based short hairpin RNA as a potential antihypertensive therapy. *J Pharmacol Exp Ther* **329**(2): 775-82.

Robb, G. B. and T. M. Rana (2007). RNA helicase A interacts with RISC in human cells and functions in RISC loading. *Mol Cell* **26**(4): 523-37.

Robbins, M., A. Judge, L. Liang, K. McClintock, E. Yaworski and I. MacLachlan (2007). 2'-O-methyl-modified RNAs act as TLR7 antagonists. *Mol Ther* **15**(9): 1663-9.

Robbins, M. A., M. Li, I. Leung, H. Li, D. V. Boyer, Y. Song, M. A. Behlke and J. J. Rossi (2006). Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs in vitro. *Nat Biotechnol* **24**(5): 566-71.

Roberts, W. K., M. J. Clemens and I. M. Kerr (1976). Interferon-induced inhibition of protein synthesis in L-cell extracts: an ATP-dependent step in the activation of an inhibitor by double-stranded RNA. *Proc Natl Acad Sci U S A* **73**(9): 3136-40.

Robertson, H. D., R. E. Webster and N. D. Zinder (1968). Purification and properties of ribonuclease III from Escherichia coli. *J Biol Chem* **243**(1): 82-91.

Roebuck, K. A. and M. Saifuddin (1999). Regulation of HIV-1 transcription. *Gene Expr* **8**(2): 67-84.

Romano, N. and G. Macino (1992). Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. *Mol Microbiol* **6**(22): 3343-53.

Rossi, J. J., C. H. June and D. B. Kohn (2007). Genetic therapies against HIV. *Nat Biotechnol* **25**(12): 1444-54.

Rubbert, A., C. Combadiere, M. Ostrowski, J. Arthos, M. Dybul, E. Machado, M. A. Cohn, J. A. Hoxie, P. M. Murphy, A. S. Fauci and D. Weissman (1998). Dendritic cells express multiple chemokine receptors used as coreceptors for HIV entry. *J Immunol* **160**(8): 3933-41.

Ruby, J. G., C. H. Jan and D. P. Bartel (2007). Intronic microRNA precursors that bypass Drosha processing. *Nature* **448**(7149): 83-6.

Ruiz, M. E., C. Cicala, J. Arthos, A. Kinter, A. T. Catanzaro, J. Adelsberger, K. L. Holmes, O. J. Cohen and A. S. Fauci (1998). Peripheral blood-derived CD34+ progenitor cells: CXC chemokine receptor 4 and CC chemokine receptor 5 expression and infection by HIV. *J Immunol* **161**(8): 4169-76.

Saayman, S., S. Barichievy, A. Capovilla, K. V. Morris, P. Arbuthnot and M. S. Weinberg (2008). The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. *PLoS One* **3**(7): e2602.

Salzwedel, K., E. D. Smith, B. Dey and E. A. Berger (2000). Sequential CD4-coreceptor interactions in human immunodeficiency virus type 1 Env function: soluble CD4 activates Env for coreceptor-dependent fusion and reveals blocking activities of antibodies against cryptic conserved epitopes on gp120. *J Virol* **74**(1): 326-33.

Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart and M. Parmentier (1996). Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. *Nature* **382**(6593): 722-5.

Sanchez-Pescador, R., M. D. Power, P. J. Barr, K. S. Steimer, M. M. Stempien, S. L. Brown-Shimer, W. W. Gee, A. Renard, A. Randolph, J. A. Levy and et al. (1985). Nucleotide sequence and expression of an AIDS-associated retrovirus (ARV-2). *Science* **227**(4686): 484-92.

Sano, M., H. Li, M. Nakanishi and J. J. Rossi (2008). Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. *Mol Ther* **16**(1): 170-7.

Scacheri, P. C., O. Rozenblatt-Rosen, N. J. Caplen, T. G. Wolfsberg, L. Umayam, J. C. Lee, C. M. Hughes, K. S. Shanmugam, A. Bhattacharjee, M. Meyerson and F. S. Collins (2004). Short interfering RNAs can induce unexpected and divergent changes in the levels of untargeted proteins in mammalian cells. *Proc Natl Acad Sci U S A* **101**(7): 1892-7.

Scherer, L. J., R. Frank and J. J. Rossi (2007). Optimization and characterization of tRNA-shRNA expression constructs. *Nucleic Acids Res* **35**(8): 2620-8.

Schroder, A. R., P. Shinn, H. Chen, C. Berry, J. R. Ecker and F. Bushman (2002). HIV-1 integration in the human genome favors active genes and local hotspots. *Cell* **110**(4): 521-9.

Schubert, S., A. Grunweller, V. A. Erdmann and J. Kurreck (2005). Local RNA target structure influences siRNA efficacy: systematic analysis of intentionally designed binding regions. *J Mol Biol* **348**(4): 883-93.

Schwarz, D. S., G. Hutvagner, T. Du, Z. Xu, N. Aronin and P. D. Zamore (2003). Asymmetry in the assembly of the RNAi enzyme complex. *Cell* **115**(2): 199-208.

Selbach, M., B. Schwanhausser, N. Thierfelder, Z. Fang, R. Khanin and N. Rajewsky (2008). Widespread changes in protein synthesis induced by microRNAs. *Nature* **455**(7209): 58-63.

Sen, G. C. and S. N. Sarkar (2005). Transcriptional signaling by double-stranded RNA: role of TLR3. *Cytokine Growth Factor Rev* **16**(1): 1-14.

Senserrich, J., E. Pauls, M. Armand-Ugon, I. Clotet-Codina, G. Moncunill, B. Clotet and J. A. Este (2008). HIV-1 resistance to the anti-HIV activity of a shRNA targeting a dual-coding region. *Virology* **372**(2): 421-9.

Shabalina, S. A., A. N. Spiridonov and A. Y. Ogurtsov (2006). Computational models with thermodynamic and composition features improve siRNA design. *BMC Bioinformatics* **7**: 65.

Shen, J., R. Samul, R. L. Silva, H. Akiyama, H. Liu, Y. Saishin, S. F. Hackett, S. Zinnen, K. Kossen, K. Fosnaugh, C. Vargeese, A. Gomez, K. Bouhana, R. Aitchison, P. Pavco and P. A. Campochiaro (2006). Suppression of ocular neovascularization with siRNA targeting VEGF receptor 1. *Gene Ther* **13**(3): 225-34.

Sibley, C. R., Y. Seow and M. J. Wood (2010). Novel RNA-based Strategies for Therapeutic Gene Silencing. *Mol Ther*.

Siliciano, J. D., J. Kajdas, D. Finzi, T. C. Quinn, K. Chadwick, J. B. Margolick, C. Kovacs, S. J. Gange and R. F. Siliciano (2003). Long-term follow-up studies confirm the stability of the latent reservoir for HIV-1 in resting CD4+ T cells. *Nat Med* **9**(6): 727-8.

Simoni, J. M., K. R. Amico, C. R. Pearson and R. Malow (2008). Strategies for promoting adherence to antiretroviral therapy: a review of the literature. *Curr Infect Dis Rep* **10**(6): 515-21.

Singerman, L. (2009). Combination therapy using the small interfering RNA bevasiranib. *Retina* **29**(6 Suppl): S49-50.

Siolas, D., C. Lerner, J. Burchard, W. Ge, P. S. Linsley, P. J. Paddison, G. J. Hannon and M. A. Cleary (2005). Synthetic shRNAs as potent RNAi triggers. *Nat Biotechnol* **23**(2): 227-31.

Soifer, H. S., M. Sano, K. Sakurai, P. Chomchan, P. Saetrom, M. A. Sherman, M. A. Collingwood, M. A. Behlke and J. J. Rossi (2008). A role for the Dicer helicase domain in the processing of thermodynamically unstable hairpin RNAs. *Nucleic Acids Res* **36**(20): 6511-22.

Song, E., P. Zhu, S. K. Lee, D. Chowdhury, S. Kussman, D. M. Dykxhoorn, Y. Feng, D. Palliser, D. B. Weiner, P. Shankar, W. A. Marasco and J. Lieberman (2005). Antibody mediated in vivo delivery of small interfering RNAs via cell-surface receptors. *Nat Biotechnol* **23**(6): 709-17.

Song, J., A. Giang, Y. Lu, S. Pang and R. Chiu (2008). Multiple shRNA expressing vector enhances efficiency of gene silencing. *BMB Rep* **41**(5): 358-62.

Song, J. J., J. Liu, N. H. Tolia, J. Schneiderman, S. K. Smith, R. A. Martienssen, G. J. Hannon and L. Joshua-Tor (2003). The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. *Nat Struct Biol* **10**(12): 1026-32.

Soutschek, J., A. Akinc, B. Bramlage, K. Charisse, R. Constien, M. Donoghue, S. Elbashir, A. Geick, P. Hadwiger, J. Harborth, M. John, V. Kesavan, G. Lavine, R. K. Pandey, T. Racie, K. G. Rajeev, I. Rohl, I. Toudjarska, G. Wang, S. Wuschko, D. Bumcrot, V. Koteliansky, S. Limmer, M. Manoharan and H. P. Vornlocher (2004). Therapeutic silencing of an endogenous gene by systemic administration of modified siRNAs. *Nature* **432**(7014): 173-8.

Surabhi, R. M. and R. B. Gaynor (2002). RNA interference directed against viral and cellular targets inhibits human immunodeficiency Virus Type 1 replication. *J Virol* **76**(24): 12963-73.

Tabara, H., M. Sarkissian, W. G. Kelly, J. Fleenor, A. Grishok, L. Timmons, A. Fire and C. C. Mello (1999). The rde-1 gene, RNA interference, and transposon silencing in C. elegans. *Cell* **99**(2): 123-32.

Takasaki, S., S. Kotani and A. Konagaya (2004). An effective method for selecting siRNA target sequences in mammalian cells. *Cell Cycle* **3**(6): 790-5.

Tam, O. H., A. A. Aravin, P. Stein, A. Girard, E. P. Murchison, S. Cheloufi, E. Hodges, M. Anger, R. Sachidanandam, R. M. Schultz and G. J. Hannon (2008). Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **453**(7194): 534-8.

Tan, Y., B. Zhang, T. Wu, G. Skogerbo, X. Zhu, X. Guo, S. He and R. Chen (2009). Transcriptional inhibiton of Hoxd4 expression by miRNA-10a in human breast cancer cells. *BMC Mol Biol* **10**: 12.

Taube, R., K. Fujinaga, J. Wimmer, M. Barboric and B. M. Peterlin (1999). Tat transactivation: a model for the regulation of eukaryotic transcriptional elongation. *Virology* **264**(2): 245-53.

Temesgen, Z. and J. E. Feinberg (2006). Drug evaluation: bevirimat--HIV Gag protein and viral maturation inhibitor. *Curr Opin Investig Drugs* **7**(8): 759-65.

ter Brake, O. and B. Berkhout (2007). Lentiviral vectors that carry anti-HIV shRNAs: problems and solutions. *J Gene Med* **9**(9): 743-50.

ter Brake, O., P. Konstantinova, M. Ceylan and B. Berkhout (2006). Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol Ther* **14**(6): 883-92.

ter Brake, O., N. Legrand, K. J. von Eije, M. Centlivre, H. Spits, K. Weijer, B. Blom and B. Berkhout (2009). Evaluation of safety and efficacy of RNAi against HIV-1 in the human immune system (Rag-2(-/-)gammac(-/-)) mouse model. *Gene Ther* **16**(1): 148-53.

ter Brake, O., K. t Hooft, Y. P. Liu, M. Centlivre, K. J. von Eije and B. Berkhout (2008). Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol Ther* **16**(3): 557-64.

Tilton, J. C. and R. W. Doms (2010). Entry inhibitors in the treatment of HIV-1 infection. *Antiviral Res* **85**(1): 91-100.

Ting, A. H., H. Suzuki, L. Cope, K. E. Schuebel, B. H. Lee, M. Toyota, K. Imai, Y. Shinomura, T. Tokino and S. B. Baylin (2008). A requirement for DICER to maintain full promoter CpG island hypermethylation in human cancer cells. *Cancer Res* **68**(8): 2570-5.

Tiscornia, G., V. Tergaonkar, F. Galimi and I. M. Verma (2004). CRE recombinase-inducible RNA interference mediated by lentiviral vectors. *Proc Natl Acad Sci U S A* **101**(19): 7347-51.

Tran, N., M. Raponi, I. W. Dawes and G. M. Arndt (2004). Control of specific gene expression in mammalian cells by co-expression of long complementary RNAs. *FEBS Lett* **573**(1-3): 127-34.

Triboulet, R., B. Mari, Y. L. Lin, C. Chable-Bessia, Y. Bennasser, K. Lebrigand, B. Cardinaud, T. Maurin, P. Barbry, V. Baillat, J. Reynes, P. Corbeau, K. T. Jeang and M. Benkirane (2007). Suppression of microRNA-silencing pathway by HIV-1 during virus replication. *Science* **315**(5818): 1579-82.

Tseng, Y. C., S. Mozumdar and L. Huang (2009). Lipid-based systemic delivery of siRNA. *Adv Drug Deliv Rev* **61**(9): 721-31.

Ui-Tei, K., Y. Naito, K. Nishi, A. Juni and K. Saigo (2008a). Thermodynamic stability and Watson-Crick base pairing in the seed duplex are major determinants of the efficiency of the siRNA-based off-target effect. *Nucleic Acids Res* **36**(22): 7100-9.

Ui-Tei, K., Y. Naito, S. Zenno, K. Nishi, K. Yamato, F. Takahashi, A. Juni and K. Saigo (2008b). Functional dissection of siRNA sequence by systematic DNA substitution: modified siRNA with a DNA seed arm is a powerful tool for mammalian gene silencing with significantly reduced off-target effect. *Nucleic Acids Res* **36**(7): 2136-51.

UNAIDS (2008). Report on the global AIDS epidemic. Geneva, UNAIDS.

Unwalla, H. J., H. T. Li, I. Bahner, M. J. Li, D. Kohn and J. J. Rossi (2006). Novel Pol II fusion promoter directs human immunodeficiency virus type 1-inducible coexpression of a short hairpin RNA and protein. *J Virol* **80**(4): 1863-73.

Unwalla, H. J., M. J. Li, J. D. Kim, H. T. Li, A. Ehsani, J. Alluin and J. J. Rossi (2004). Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. *Nat Biotechnol* **22**(12): 1573-8.

Vagin, V. V., A. Sigova, C. Li, H. Seitz, V. Gvozdev and P. D. Zamore (2006). A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **313**(5785): 320-4.

van de Wetering, M., I. Oving, V. Muncan, M. T. Pon Fong, H. Brantjes, D. van Leenen, F. C. Holstege, T. R. Brummelkamp, R. Agami and H. Clevers (2003). Specific inhibition of gene expression using a stably integrated, inducible small-interfering-RNA vector. *EMBO Rep* **4**(6): 609-15.

Varela-Rohena, A., C. Carpenito, E. E. Perez, M. Richardson, R. V. Parry, M. Milone, J. Scholler, X. Hao, A. Mexas, R. G. Carroll, C. H. June and J. L. Riley (2008). Genetic engineering of T cells for adoptive immunotherapy. *Immunol Res* **42**(1-3): 166-81.

Venaud, S., N. Yahi, J. L. Fehrentz, N. Guettari, D. Nisato, I. Hirsch and J. C. Chermann (1992). Inhibition of HIV by an anti-HIV protease synthetic peptide blocks an early step of viral replication. *Res Virol* **143**(5): 311-9.

Ventura, A., A. Meissner, C. P. Dillon, M. McManus, P. A. Sharp, L. Van Parijs, R. Jaenisch and T. Jacks (2004). Cre-lox-regulated conditional RNA interference from transgenes. *Proc Natl Acad Sci U S A* **101**(28): 10380-5.

Vermeulen, A., L. Behlen, A. Reynolds, A. Wolfson, W. S. Marshall, J. Karpilow and A. Khvorova (2005). The contributions of dsRNA structure to Dicer specificity and efficiency. *Rna* **11**(5): 674-82.

Vigna, E. and L. Naldini (2000). Lentiviral vectors: excellent tools for experimental gene transfer and promising candidates for gene therapy. *J Gene Med* **2**(5): 308-16.

Volpe, T. A., C. Kidner, I. M. Hall, G. Teng, S. I. Grewal and R. A. Martienssen (2002). Regulation of heterochromatic silencing and histone H3 lysine-9 methylation by RNAi. *Science* **297**(5588): 1833-7.

von Eije, K. J., O. ter Brake and B. Berkhout (2008). Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J Virol* **82**(6): 2895-903.

von Eije, K. J., O. ter Brake and B. Berkhout (2009). Stringent testing identifies highly potent and escape-proof anti-HIV short hairpin RNAs. *J Gene Med* **11**(6): 459-67.

Wain-Hobson, S., P. Sonigo, O. Danos, S. Cole and M. Alizon (1985). Nucleotide sequence of the AIDS virus, LAV. *Cell* **40**(1): 9-17.

Wakiyama, M., K. Takimoto, O. Ohara and S. Yokoyama (2007). Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system. *Genes Dev* **21**(15): 1857-62.

Wang, B., S. Li, H. H. Qi, D. Chowdhury, Y. Shi and C. D. Novina (2009a). Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. *Nat Struct Mol Biol* **16**(12): 1259-66.

Wang, H. W., C. Noland, B. Siridechadilok, D. W. Taylor, E. Ma, K. Felderer, J. A. Doudna and E. Nogales (2009b). Structural insights into RNA processing by the human RISC-loading complex. *Nat Struct Mol Biol* **16**(11): 1148-53.

Wang, J., T. W. Theunissen and S. H. Orkin (2007). Site-directed, virus-free, and inducible RNAi in embryonic stem cells. *Proc Natl Acad Sci U S A* **104**(52): 20850-5.

Wassenegger, M., S. Heimes, L. Riedel and H. L. Sanger (1994). RNA-directed de novo methylation of genomic sequences in plants. *Cell* **76**(3): 567-76.

Watanabe, T., M. Sudoh, M. Miyagishi, H. Akashi, M. Arai, K. Inoue, K. Taira, M. Yoshiba and M. Kohara (2006). Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther* **13**(11): 883-92.

Watanabe, T., Y. Totoki, A. Toyoda, M. Kaneda, S. Kuramochi-Miyagawa, Y. Obata, H. Chiba, Y. Kohara, T. Kono, T. Nakano, M. A. Surani, Y. Sakaki and H. Sasaki (2008). Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* **453**(7194): 539-43.

Weinberg, M. S., A. Ely, S. Barichievy, C. Crowther, S. Mufamadi, S. Carmona and P. Arbuthnot (2007). Specific inhibition of HBV replication in vitro and in vivo with expressed long hairpin RNA. *Mol Ther* **15**(3): 534-41.

Weinberg, M. S., L. M. Villeneuve, A. Ehsani, M. Amarzguioui, L. Aagaard, Z. X. Chen, A. D. Riggs, J. J. Rossi and K. V. Morris (2006). The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells. *Rna* **12**(2): 256-62.

Westerhout, E. M., M. Ooms, M. Vink, A. T. Das and B. Berkhout (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res* **33**(2): 796-804.

Westerhout, E. M., O. ter Brake and B. Berkhout (2006a). The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference. *Retrovirology* **3**: 57.

Westerhout, E. M., M. Vink, P. C. Haasnoot, A. T. Das and B. Berkhout (2006b). A conditionally replicating HIV-based vector that stably expresses an antiviral shRNA against HIV-1 replication. *Mol Ther* **14**(2): 268-75.

Whitehead, K. A., R. Langer and D. G. Anderson (2009). Knocking down barriers: advances in siRNA delivery. *Nat Rev Drug Discov* **8**(2): 129-38.

Wong, J. K., M. Hezareh, H. F. Gunthard, D. V. Havlir, C. C. Ignacio, C. A. Spina and D. D. Richman (1997). Recovery of replication-competent HIV despite prolonged suppression of plasma viremia. *Science* **278**(5341): 1291-5.

Xia, H., Q. Mao, H. L. Paulson and B. L. Davidson (2002). siRNA-mediated gene silencing in vitro and in vivo. *Nat Biotechnol* **20**(10): 1006-10.

Yamamoto, T., S. Omoto, M. Mizuguchi, H. Mizukami, H. Okuyama, N. Okada, N. K. Saksena, E. A. Brisibe, K. Otake and Y. R. Fuji (2002). Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. *Microbiol Immunol* **46**(11): 809-17.

Yan, K. S., S. Yan, A. Farooq, A. Han, L. Zeng and M. M. Zhou (2003). Structure and conserved RNA binding of the PAZ domain. *Nature* **426**(6965): 468-74.

Yang, W. and W. Paschen (2008). Conditional gene silencing in mammalian cells mediated by a stress-inducible promoter. *Biochem Biophys Res Commun* **365**(3): 521-7.

Yekta, S., I. H. Shih and D. P. Bartel (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304**(5670): 594-6.

Yi, C. E., J. M. Bekker, G. Miller, K. L. Hill and R. H. Crosbie (2003a). Specific and potent RNA interference in terminally differentiated myotubes. *J Biol Chem* **278**(2): 934-9.

Yi, R., Y. Qin, I. G. Macara and B. R. Cullen (2003b). Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev* **17**(24): 3011-6.

Yoneyama, M., M. Kikuchi, T. Natsukawa, N. Shinobu, T. Imaizumi, M. Miyagishi, K. Taira, S. Akira and T. Fujita (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* **5**(7): 730-7.

Yuan, X., C. Liu, P. Yang, S. He, Q. Liao, S. Kang and Y. Zhao (2009). Clustered microRNAs' coordination in regulating protein-protein interaction network. *BMC Syst Biol* **3**: 65.

Zaitseva, M., A. Blauvelt, S. Lee, C. K. Lapham, V. Klaus-Kovtun, H. Mostowski, J. Manischewitz and H. Golding (1997). Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. *Nat Med* **3**(12): 1369-75.

Zamore, P. D., T. Tuschl, P. A. Sharp and D. P. Bartel (2000). RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* **101**(1): 25-33.

Zeng, Y. and B. R. Cullen (2004). Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. *Nucleic Acids Res* **32**(16): 4776-85.

Zeng, Y., E. J. Wagner and B. R. Cullen (2002). Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. *Mol Cell* **9**(6): 1327-33.

Zeng, Y., R. Yi and B. R. Cullen (2005). Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. *Embo J* **24**(1): 138-48.

Zhang, H., F. A. Kolb, V. Brondani, E. Billy and W. Filipowicz (2002). Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. *Embo J* **21**(21): 5875-85.

Zhang, H., F. A. Kolb, L. Jaskiewicz, E. Westhof and W. Filipowicz (2004). Single processing center models for human Dicer and bacterial RNase III. *Cell* **118**(1): 57-68.

Zhou, H., X. G. Xia and Z. Xu (2005). An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. *Nucleic Acids Res* **33**(6): e62.

Zhou, H., M. Xu, Q. Huang, A. T. Gates, X. D. Zhang, J. C. Castle, E. Stec, M. Ferrer, B. Strulovici, D. J. Hazuda and A. S. Espeseth (2008a). Genome-scale RNAi screen for host factors required for HIV replication. *Cell Host Microbe* **4**(5): 495-504.

Zhou, J., H. Li, S. Li, J. Zaia and J. J. Rossi (2008b). Novel dual inhibitory function aptamer-siRNA delivery system for HIV-1 therapy. *Mol Ther* **16**(8): 1481-9.

Zilberstein, A., A. Kimchi, A. Schmidt and M. Revel (1978). Isolation of two interferon-induced translational inhibitors: a protein kinase and an oligo-isoadenylate synthetase. *Proc Natl Acad Sci U S A* **75**(10): 4734-8.

Zolopa, A. R. (2010). The evolution of HIV treatment guidelines: current state-of-the-art of ART. *Antiviral Res* **85**(1): 241-4.

Zufferey, R., T. Dull, R. J. Mandel, A. Bukovsky, D. Quiroz, L. Naldini and D. Trono (1998). Self-inactivating lentivirus vector for safe and efficient in vivo gene delivery. *J Virol* **72**(12): 9873-80.

APPENDIX

A1 Laboratory Procedures

A1.1 E.coli Transformation of competent DH5α E.coli

A1.1.1 Competent DH5α

DH5α *E.coli* cells were grown to mid log phase in Luria Bertani broth (LB) [10 g/l Bacto-Tryptone (Oxoid, Hampshire, UK); 5 g/l Bacto Yeast extract (Oxoid, Hampshire, UK); 10 g/l sodium chloride (NaCl) (Merck, Darmstadt, Germany) dissolved in dH_2O and autoclaved at 121°C for 25 min at 1 kg/cm^2]. Cells were collected by centrifugation at 3 000×g for 15 min, resuspended in 5 ml PIPES transformation buffer (100 mM $CaCl_2$, 10 mM PIPES-Cl and 15% glycerol, pH 7.0, autoclaved and stored at -20°C), and incubated on ice for 20 min. Cells were collected by centrifugation at 1 000×g for 10 min, resuspended in 2 ml PIPES transformation and dispensed into 100 µl aliquots for storage at -70°C.

A1.1.2 Transformation

A 100 µl aliquot of competent DH5α was thawed on ice and approximately 0.5 µg plasmid DNA or 7 µl ligation reaction was added, gently mixed and incubated on ice for 30 min. Samples were then heat shocked at 42°C for 90 sec and returned to ice for a further 5 min. Positive transformants were selected by overnight growth at 37°C, on LB agar plates containing 100 µg/ml ampicillin.

For blue-white screening, ampicillin positive LB agar plates were spread with a volume of 40 µl of 5-bromo-4-chloro-3-indolyl-b-D-galactopyranoside (X-Gal) (20 mg/ml stock in dimethyl formamide;

Sigma, MO, USA), and a volume of 8µl of Isopropyl-β-D-1-thiogalactopyranoside (IPTG) (100 mg/ml aqueous solution; Roche, Germany). Plates were air-dried at 37°C for 20 min and stored at 4°C.

A1.2 Plasmid purification

A1.2.1 Small-scale preparations of purified plasmid

Small-scale purification of plasmid DNA was carried out using the High Pure Plasmid Isolation Kit for small-scale (mini) preparations of purified plasmid DNA (Roche, Germany). LB broth (3 ml) containing 100 µg/ml ampicillin was inoculated with a single transformed colony and incubated overnight at 37°C with shaking. Cultures were centrifuged at 3 000×g for 10 min, the supernatant was discarded and pellets were resuspended in 250 µl Suspension Buffer (50 mM Tris-HCl, 10 mM EDTA, pH 8.5 at 25°C) containing 2.5 mg RNase A. Once resuspended, 250 µl Lysis Buffer (0.2 M NaOH and 1% SDS) was added to the bacterial cells, incubated for 5 min at room temperature and then 350 µl chilled Binding Buffer (4 M guanidine hydrochloride, 0.5 M potassium acetate, pH 4.2) was added, incubated on ice for 5 min and centrifuged at maximum speed for 10 min. The supernatant was transferred to a High Pure filter tube and centrifuged at maximum speed for 1 min. The flowthrough was discarded and 700 µl Wash Buffer II (20 mM NaCl, 2 mM Tris-HCl, pH 7.5 at 25°C) was added and again centrifuged at maximum speed for 1 min. An additional 1 min centrifugation step was carried out following discarding of flowthrough and then 100 µl Elution Buffer (10 mM HCl, pH 8.5 at 25°C) was added to the filter tube, which was centrifuged at maximum speed for 1 min to elute the purified plasmid DNA into a nuclease-free tube.

A1.2.2 Large-scale preparations of purified plasmid

Large-scale preparation of plasmid DNA was carried out using the QIAGEN Plasmid Maxi Kit (QIAGEN, CA, USA). Approximately 200 ml LB broth was inoculated with a single transformed colony and incubated overnight at 37°C with shaking. Cultures were centrifuged at 3 000×g for 30 min and

bacterial pellets were resuspended in 10 ml Buffer P1 (50 mM Tris-HCl, pH 8.0; 10 mM EDTA) containing 100 µg/ml RNase A at a concentration of 100 µg/ml. Ten milliliters of Buffer P2 (200 mM NaOH; 1% SDS) was added to bacterial suspensions, mixed thoroughly and incubated at room temperature for 5 min. Ten milliliters of Buffer P3 (3 M Potassium acetate, pH 5.5) was then added to the lysate mixed thoroughly and transferred to a QIAfilter Cartridge and incubated for 10 min at room temperature. A QIAGEN-tip 500 was equilibrated by adding 10 ml Buffer QBT (750 mM NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol; 0.15% Triton® X-100) to the column and allowing it to drain through the column by gravity flow. The lysate from the QIA filter cartridge was then passed through the equilibrated column. The column was washed twice with 30 ml Buffer QC (1 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol) and then the DNA was eluted with 15 ml Buffer QF (1.6 M NaCl; 50 mM MOPS, pH 7.0; 15% Isopropanol). The DNA was precipitated by adding 10.5 ml isopropanol and centrifugation at 5 000×g for 60 min at 4°C. The pellet was air dried for 5-10 min and then resuspended in 100-200 µl nuclease-free water.

A1.3 DNA purification from agarose gels

DNA was extracted from agarose gels using the MinElute™ Gel Extraction kit (QIAGEN, CA, USA). DNA bands were excised from agarose gels and 3× volume of Buffer QG (composition not provided) was added to the gel fragment which was subsequently dissolved at 50°C. One volume of isopropanol (Merck, Darmstadt, Germany) was then added and the solution was transferred to a QIAquick spin column and centrifuged for 1 min at maximum speed. The flow through was discarded and 500 µl Buffer QG was added to the column and centrifuged again at maximum speed for 1 min. The flow through was again discarded and 750 µl Buffer PE (wash buffer, composition not provided) was then spun through the column. The column was then transferred to a clean tube and 10 µl Buffer EB (elution buffer, 10 mM Tris-HCl pH 8.5) was added to the column which was then centrifuged for 1 min at maximum speed to elute the purified DNA.

A1.4 Cell culture

The HEK293 and Huh7 cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM), (BioWhittaker, MD, USA) supplemented with 10% heat inactivated fetal calf serum (FCS), (Delta Bioproducts, Johannesburg, SA) at 37°C and 5% CO_2. U87.CD4.CCR5 cells were maintained in DMEM supplemented with 15% FCS; 1 µg/ml puromycin; 300 µg/ml G418; 100 U/ml penicillin; 100 µg/ml streptomycin; and glutamine at 37°C and 5% CO_2.

Cells were cultured in 75 cm^2 tissue culture flasks and were passaged every 3-4 days upon reaching 80-90% confluency. HEK293 cells were subcultured using phosphate buffered saline (PBS) (Gibco, BRL, UK) containing 0.01% EDTA. Culture medium was aspirated from the tissue culture vessel and 2-3ml saline was used to gently detach and suspend the cells. Huh7 and U87.CD4.CCR5 cells were passaged with 0.5× trypsin-EDTA solution (Gibco, BRL, UK). Culture medium was aspirated from the tissue culture vessel and cells were washed once with 3ml PBS before adding 1 ml trypsin-EDTA to the cells. Cells were then incubated at 37°C for approximately 5 minutes or until cells started to lift. Cells were gently suspended and an equal volume of medium was added to inactivate the trypsin. Cells were split to 20-30% confluency and 15 ml fresh medium was added to each 75 cm^2 flask.

A1.5 Transfections

Cells were seeded 24 hours prior to transfection by counting cells using the dye-exclusion haemocytometer method. A representative sample of the cells to be seeded was stained with trypan blue and counted on a haemocytometer. The number of viable cells in the sample was then calculated and seeded accordingly. In a 24-well tissue culture plate, HEK293 cells were seeded at 120 000 cells per well, Huh7 cells were seeded at 100 000 cells per well and U87.CD4.CCR5 cells were seeded at 50 000 cells per well in a volume of 500 µl of antibiotic-free medium.

Cells were transfected using Lipofectamine™ 2000 (Invitrogen, CA, USA) and OptiMEM (Gibco, BRL, UK). One microlitre of Lipofectamine™ 2000 was used per µg of DNA transfected and the total volume of DNA/ Lipofectamine™ 2000/OptiMEM was equal to one fifth of the total volume of plating medium. For a single well in a 24-well tissue culture dish, 1µg of DNA was added to 50 µl of OptiMEM in a microfuge tube. In a separate tube 1 µl Lipofectamine™ 2000 was added to 50 µl OptiMEM. Each solution was incubated at room temperature for 10 min and then the Lipofectamine™ 2000/OptiMEM mixture was added to the DNA/OptiMEM mixture and incubated at room temperature for a further 20 min. The DNA/ Lipofectamine™ 2000/OptiMEM mixture was then added to the cells. Total volumes were calculated and master mixes were prepared for each triplicate transfection. The number of cells plated, the volume of plating medium, the quantity of DNA transfected and the amount of Lipofectamine™ 2000/OptiMEM used was adjusted according to the surface area of the tissue culture vessel that cells were seeded in.

A1.6 RNA extraction from mammalian cells

Culture medium was aspirated off cell cultures and 1 ml TriReagent™ (Sigma, MO, USA) was added to each 60 cm^2 dish and incubated for 5 min at room temperature. Cell lysates were added to 1.7 ml eppendorf tubes containing 200 µl chloroform (Merck, Darmstadt, Germany), vortexed and centrifuged at 13 000 rpm for 30 min at 4°C. The clear top layer of the supernatant was removed and an equal volume of isopropanol (Merck, Darmstadt, Germany) was added, vortexed briefly and centrifuged again at 13 000 rpm for 30 min at 4°C. The supernatant was discarded and the pellet was air dried briefly before resuspension in ± 100 µl nuclease-free water. Volumes of TriReagent™ and chloroform used were adjusted accordingly for RNA extraction from smaller surface areas.

A1.7 Decade^{TM} Marker System

In a nuclease-free tube, 1 µl Decade Marker RNA (100 ng); 6 µl nuclease-free water; 1 µl 10× kinase reaction buffer; 1 µl [γ-^{32}P]ATP and 1 µl T4 polynucleotide kinase were mixed and incubated at 37°C for 1 hr. Following incubation 8 µl nuclease-free water and 2 µl 10× cleavage reagent were added to the reaction which was then incubated at room temperature for 5 min before adding 20 µl Gel Loading Buffer II. The mixture was heated at 95° for 5 min prior to loading.

A1.8 p24 antigen ELISA

HIV p24 antigen was measured by Murex HIV Antigen mAB (Murex Biotech LTD, Dartford, UK). One hundred µl Conjugate Working Solution I (biotinylated murine anti-p24 monoclonal antibodies, Proclin® preservative, bovine albumin, heat inactivated mouse serum and aggregated human IgG in phosphate buffer) was added to each pre-coated ELISA well together with 100 µl viral supernatant (diluted as necessary in culture medium). The samples were mixed by aspiration and incubated at 37°C for one hour. ELISA strips were washed five times with Wash Buffer (phosphate buffer and Proclin® preservative) using an automated plate washer (BioRad, CA, USA). Two hundred µl of Conjugate Working Solution II (peroxidase conjugate streptavidin, phosphate buffer, Proclin® preservative, bovine casein and bovine aprotinin) was then added to each well and incubated at 37°C for 30 min. ELISA strips were washed for a second time as described above and 200 µl Substrate Solution (tetramethylbenzidine, dimethyl sulphoxide, Thiomersal preservative, phosphate citrate buffer and hydrogen peroxide) was then added to each well followed by incubation at room temperature for 20 min. The reaction was then stopped by adding 50 µl 2 M H_2SO_4 (Merck, Darmstadt, Germany) to each well. Fluorescence was analysed at 450 nm using an automated plate reader (BioRad, CA, USA).

A1.9 $TCID_{50}$

U87.CD4.CCR5 cells were seeded at 10 000 cells per well in a 96-well flat-bottomed tissue culture plate 24 hours prior to infection. Cells were seeded only in the centre 24 wells as seen in the plate format in Table A1. The rest of the wells on the periphery of the plate were each filled with 200 µl PBS. On the day of infection, culture medium was aspirated off the cells and 50 µl fresh medium was added to each well containing cells. In a separate 96-well plate, eight serial four-fold dilutions of virus stock were carried out in triplicate ranging from 4^{-2} to 4^{-9} and then 150 µl of each dilution was added to the relevant wells bring the total volume of each well to 200 µl. The day following infection each well was washed three times with PBS and 200 µl fresh medium was added to each well. On day 7 following infection, supernatants were harvested and tested for p24 antigen as described in A1.8. A well was scored as positive if the absorbance at 450 nm was greater than the absorbance of the negative control + 0.050. The $TCID_{50}$ is calculated by the Spearman-Karber method, a statistical calculation where M = xk + d [0.5 - (1/n) (r)]. In the Spearman-Karber formula, xk = dose of the highest dilution; r = sum of the number of "-" responses; d = spacing between dilutions and n = number of wells per dilution.

Table A1: Plate format of a dilution assay used to calculate the $TCID_{50}$ of HIV viral isolates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
B	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
C	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	4^{-9}	PBS	PBS
D	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	4^{-9}	PBS	PBS
E	PBS	PBS	4^{-2}	4^{-3}	4^{-4}	4^{-5}	4^{-6}	4^{-7}	4^{-8}	4^{-9}	PBS	PBS
F	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
G	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS
H	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS	PBS

Nucleic Acids Research Advance Access published June 4, 2010

Nucleic Acids Research, 2010, 1–12
doi:10.1093/nar/gkq460

Deriving four functional anti-HIV siRNAs from a single Pol III-generated transcript comprising two adjacent long hairpin RNA precursors

Sheena Saayman, Patrick Arbuthnot and Marc S. Weinberg*

Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, University of Witwatersrand, Johannesburg, South Africa

Received March 7, 2010; Revised May 7, 2010; Accepted May 11, 2010

ABSTRACT

Several different approaches exist to generate expressed RNA interference (RNAi) precursors for multiple target inhibition, a strategy referred to as combinatorial (co)RNAi. One such approach makes use of RNA Pol III-expressed long hairpin RNAs (lhRNAs), which are processed by Dicer to generate multiple unique short interfering siRNA effectors. However, because of inefficient intracellular Dicer processing, lhRNA duplexes have been limited to generating two independent effective siRNA species. In this study, we describe a novel strategy whereby four separate anti-HIV siRNAs were generated from a single RNA Pol III-expressed transcript. Two optimized lhRNAs, each comprising two active anti-HIV siRNAs, were placed in tandem to form a double long hairpin (dlhRNA) expression cassette, which encodes four unique and effective siRNA sequences. Processing of the 3′ position lhRNA was more variable but effective multiple processing was possible by manipulating the order of the siRNA-encoding sequences. Importantly, unlike shRNAs, Pol III-expressed dlhRNAs did not compete with endogenous and exogenous microRNAs to disrupt the RNAi pathway. The versatility of expressed lhRNAs is greatly expanded and we provide a mechanism for generating transcripts with modular lhRNAs motifs that contribute to improved coRNAi.

INTRODUCTION

Exogenous RNA intermediates of the RNA interference (RNAi) pathway have become powerful tools for the development of reverse genetics approaches and novel therapeutics against a wide variety of diseases (1). Specifically, expressed short hairpin RNAs (shRNAs), which mimic precursor microRNA (pre-miRNA), are Dicer substrates of the mammalian miRNA pathway and have been extensively exploited for the production of effectors of the RNAi pathway (2). However, to prevent emergence of resistant viral mutants from highly mutable targets, such as those of human immunodeficiency virus (HIV) and hepatitis C virus (HCV), a combination of multiple short interfering RNA (siRNA) effectors is required (3,4). Therefore, an effective combinatorial RNAi (coRNAi) system aimed at generating active siRNA products, and which is capable of targeting multiple sites simultaneously, remains an important objective.

Harnessing the RNAi pathway to suppress multiple gene targets concurrently has been attempted using several different coRNAi strategies with varying success. The most common approach is to place shRNA expression cassettes adjacent to each other to achieve combined expression of multiple shRNAs (5–9). However, there are several limitations to combining multiple shRNA cassettes. Typically shRNAs are expressed from powerful constitutively active promoters (e.g. U6, H1 and U1 promoters). A coRNAi approach, with several shRNA expression cassettes in tandem, risks overwhelming the endogenous RNAi pathway. High levels of specific shRNAs may have serious and potentially fatal consequences, which are important for application to gene therapy (10). Another concern relates to the use of lentiviral vectors, which are often employed to deliver therapeutic anti-HIV RNAi sequences to T cells. These vectors are prone to recombining or deleting repeat sequences (11,12). Although this problem may be alleviated by simultaneous use of different promoters (8), such an arrangement of expressed silencing sequences would require careful empirical assessment to optimize expression of each shRNA. Other coRNAi approaches have made use of a combination of primary microRNA (pri-miRNA) shuttles, where siRNA guide sequences are placed within mimics of endogenous polycistronic pri-miRNAs (13–18). Although polycistronic pri-miRNA shuttles represent a promising coRNAi approach, efficient

*To whom correspondence should be addressed. Tel: +27 11 717 2561; Fax: +27 11 717 2395; Email: marc.weinberg@wits.ac.za

processing of multiple siRNAs from these pri-miRNA precursors is variable (13,15,18) and development of new methods of achieving coRNAi remains important.

Exploiting the action of endogenous Dicer to process long dsRNA templates and form multiple siRNA species is potentially a useful approach for achieving coRNAi. The most well-studied method is to employ Pol III promoters to generate transcripts with defined 5′- and 3′-termini that fold into long hairpins with duplex regions of between 30 and 100 bp (19–27). These lhRNAs, which have 2 nt 3′-OH overhangs have been shown to be intracellular Dicer substrates and produce multiple siRNA species (19–23). We and others have shown that siRNAs are processed by Dicer in a gradient of decreasing efficiency, which starts from the base of the dsRNA hairpin duplex and moves towards the apical loop (19,20,22,26).

Here we describe a novel lhRNA-based strategy whereby four independent siRNAs were produced in effective doses from a single RNA Pol III-expressed transcript. Two optimized dual-targeting lhRNAs were placed in tandem within a single transcript to form a double long hairpin RNA (dlhRNA) template. We show that the dlhRNA is processed into two lhRNAs, which in turn produce four active anti-HIV siRNAs. Moreover, there was no evidence of disruption of the endogenous miRNA pathway by dlhRNAs. This work describes a novel and safe dlhRNA approach to coRNAi and expands the versatility of expressed lhRNAs for applications requiring simultaneous silencing of multiple targets.

MATERIALS AND METHODS

Hairpin expression plasmids

The procedure for generating hairpin RNAs is a modification of the PCR-based method described by Castanotto *et al*. (28) and later adapted by ourselves to generate lhRNAs (20). A panel of twelve U6-driven lhRNAs encoding two putative siRNA sequences targeted to the HIV-1 *tat* and *nef* or *int* and *LTR* sequences was constructed using two rounds of PCR. Selection of target sites was based on previously reported effective knockdown of HIV-1 *tat* (29), *nef* (23), *int* (30) and *LTR* (20) sequences by shRNAs. Oligonucleotide sequences used in each of the amplification reactions are provided in Supplementary Table 1. During the first round of PCR a U6 promoter-containing plasmid DNA, pTZ-U6+1, was used as template (31). A universal U6 forward primer complementary to the 5′-end of the U6 promoter was used for both rounds of PCR. The reverse primers for the first round of PCR were complementary to 18–21 nt of the 3′-end of the U6 promoter and included sequences encoding the sense strand and loop of the lhRNA. The first round of PCR product was used as template for the second round of PCR. The round two reverse primer sequences were designed to hybridize to the loop-encoding region at the 3′ extremity of the first round PCR amplicon. These primers included the sequence encoding the antisense strand of the lhRNA as well as a RNA Pol III termination signal. Double-lhRNA expression cassettes were similarly generated using the two-round PCR method described above, but previously generated lhRNAs were used as templates. For dlhRNAs, round one reverse primers were complementary to the last 18 nt of the lhRNA template that would be positioned at the 5′-side. These primers included a spacer of two adenosine residues and a sequence encoding the sense strand of the 3′-lhRNA. To complete synthesis of the dlhRNA cassettes, round two reverse primers were employed that were the same as those used during the second round of PCR to generate lhRNA cassettes. To propagate the expression cassettes, the final PCR products were ligated directly to the TA cloning vector pTZ57R/T (Fermentas, WI, USA), and sequences were confirmed using standard procedures. Twelve lhRNA plasmids were generated: plh*LTR-int* +1, plh*LTR-int* +2, plh*LTR-int* +3, plh*int-LTR* +1, plh*int-LTR* +2, plh*int-LTR* +3, plh*tat-nef* +1, plh*tat-nef* +2, plh*tat-nef* +3, plh*nef*-tat +1, plh*nef*-tat +2 and plh*nef*-tat +3. Two dlhRNA plasmids were propagated: plh*LI-TN* and plh*TN-LI*. U6-driven shRNAs encoding siRNAs corresponding to those included in the lhRNA expression constructs were generated using a single round of PCR. The U6 promoter-containing plasmid DNA was used as template. Amplification with the universal U6 forward primer and specific reverse primers enabled formation of the shRNA-encoding sequences and these were inserted into pTZ57R/T (Fermentas) to generate: psh*LTR*, psh*int*, psh*tat* and psh*nef*. pH1-lh*tat-nef* +1 and p7SK-lh*LTR-int* +1 are plasmids expressing two lhRNAs from H1 and 7SK promoters, respectively. These transcriptional regulatory elements were incorporated using a similar procedure to that described above, but genomic DNA from HEK293 cells was used as a template. Forward and reverse primers used during the first round of PCR are listed in Supplementary Table 1. Second round reverse primers were the same as those used to generate U6-driven lhRNAs.

Target plasmids

Dual luciferase sense and antisense target plasmids were constructed by inserting annealed oligonucleotides encoding the HIV-1 targets into the 3′-UTR of the *Renilla* Luciferase cassette of the psiCheck-2 plasmid (Promega, WI, USA). After treatment with polynucleotide kinase (Promega) sticky end-containing duplex oligonucleotides were ligated to XhoI–NotI sites (sense targets) or XhoI–SpeI sites (antisense targets). Sequences of these oligonucleotides are provided in Supplementary Table 2. Identification of correct clones was facilitated by the introduction of an EcoRV site within each insert.

Cell culture and transfections

HEK293 and Huh7 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; BioWhittaker, MD, USA) supplemented with 10% heat inactivated fetal calf serum (FCS; Delta Bioproducts, Johannesburg, South Africa) at 37°C and 5% CO_2. U87.CD4.CCR5 cells were maintained in DMEM supplemented with 15% FCS, 1 μg/ml puromycin, 300 μg/ml G418, glutamine, penicillin

and streptomycin. Transfections were carried out using a ratio of 1 μl Lipofectamine2000 (Invitrogen, CA, USA) to 1 μg total DNA according to the manufacturer's instructions. Medium was changed 24 h after transfection and analyses were carried out a further 24 h thereafter. Co-transfecting a plasmid that constitutively produces enhanced green fluorescent protein (pCI-eGFP) followed by fluorescence microscopy was used to verify equivalent transfection efficiencies (32).

To evaluate the effects of the lhRNA- and shRNA-encoding plasmids on a reporter target, 120 000 HEK293 cells were seeded 24 h prior to transfection in each well of a 24-well culture dish. Cells were transfected with 150 ng target plasmid, 750 ng of lhRNA or shRNA encoding plasmid and 100 ng of pCI-eGFP, unless otherwise stated. For northern blot analysis HEK293 cells were seeded at 80% confluency in 10-cm culture dishes 24 h prior to transfection. Cells were transfected with 18 μg of hairpin-encoding plasmid and 1 μg pCI-eGFP. To determine the induction of interferon (IFN) response-related genes, HEK293 cells were seeded as described above and transfected with 900 ng of lhRNA or shRNA encoding plasmid and 100 ng pCI-eGFP per well. Control double-stranded RNA, Poly (I:C; Sigma, MO, USA), was transfected at equivalent amounts to the hairpin encoding plasmids. Measurement of IFN response gene mRNA concentrations were then determined using quantitative PCR according to previously described methods (33).

To assess saturation of the endogenous miRNA pathway caused by the lhRNA constructs, Huh7 cells were co-transfected with 80 ng psiCheck-miR-16T × 7 (18), 750 ng hairpin expression plasmid or pTZ-U6-miR-16S × 7 sponge and 150 ng pCI-eGFP. psiCheck-miR-16T × 7 is a psiCheck plasmid-containing 7 miR-16 target sites downstream of the *Renilla* luciferase ORF and pTZ-U6-miR-16S × 7 sponge includes a Pol III expression cassette that transcribes RNA containing seven copies of a miR-16 target site. To determine the effects of the lhRNA constructs on the silencing efficacy of an exogenous miRNA, HEK293 cells were cotransfected with 100 ng pCMV miR-31 *HBx,* 100 ng of psiCheck-HBx together with the hairpin constructs (18,34). pCMV miR-31 *HBx* encodes a miRNA shuttle that has a guide cognate in the X gene (*HBx*) of hepatitis B virus, and psiCheck-HBx is the reporter target vector.

Dual luciferase reporter assay

Firefly and *Renilla* luciferase activity was determined using a Veritas dual-injection luminometer (Turner Biosystems, C A, USA) according to the instructions of the manufacturer of the Dual-Luciferase Reporter kit (Promega). Target-specific *Renilla* luciferase expression was normalized to background Firefly luciferase expression. Average expression ratios for a control plasmid were set to 100%, and relative expression for other samples was calculated accordingly. Two independent experiments in triplicate were performed and the data were expressed as the mean ± SD.

Inhibition of gene targets in the full-length HIV luciferase reporter molecular clone

Hairpin expression cassettes were assessed for efficacy against a full-length HIV-1 target by determining knockdown in a HIV-1 molecular reporter pNL4-3.Luc.R-E-, as has been previously described (35,36). This molecular clone has a Firefly luciferase gene inserted into the *nef* gene and is capable of only a single round of replication. Suppression of viral gene expression was measured by determining Firefly luciferase activity. HEK293 cells (120 000) were co-transfected with 1:1 (150 ng:150 ng) ratio of hairpin expression plasmid to pNL4-3.Luc.R-E-. Approximately 50 ng of phRL-CMV (Promega), which encodes Renila luciferase, was used to control for transfection efficiency. Dual luciferase reporter assays were carried out as described above.

Northern blot analysis

Total RNA, extracted from HEK293 cells 48 h after transfection, was prepared using TriReagent™ (Sigma) according the manufacturer's instructions. Thirty micrograms of RNA was resolved on urea denaturing 15% polyacrylamide gels and blotted onto nylon membranes. Decade™ Marker (Ambion, TX, USA) was prepared according the manufacturer's instructions and run alongside the cellular RNA. Blots were hybridized to DNA oligonucleotides to detect products of hairpin processing. These probes were complementary to regions spanning the target sense or antisense sequences of the hairpins. Probes were labelled at their 5′-ends using [γ-^{32}P] ATP with T4 Polynucleotide kinase then purified using standard procedures. Northern blot hybridization and washing were carried out according to previously described methods (26). After analysis using a FLA-7000 phosphorimager (Fujifilm, Japan), blots were stripped and reprobed. An oligonucleotide probe that was complementary to U6 small nuclear RNA was used as a control to verify equal loading of cellular RNA. Sense probe oligonucleotide sequences were the following: *tat(S)* probe: 5′-GCG GAG ACA GCG ACG AAG AGC-3′, *nef(S)* probe: 5′-GTG CCT GGC TAG AAG CAC AAG -3′, *LTR(S)* probe: 5′-GTA ACT AGA GAC CTC TCA GAC-3′, *int(S)* probe: 5′-GCC GGA GAG CAA TGG CTA GTC-3′. Antisense probe oligonucleotide sequences were: *tat (AS)* probe: 5′-GCT CTT CGT CGC TGT CTC CGC-3′; *nef (AS)* probe: 5′-CTT GTG CTT CTA GCC AGG CAC-3′; *LTR (AS)* probe: 5′-GTC TGA GAG GTC TCT AGT TAC-3′; and *int (AS)* probe: 5′-GAC TAG CCA TTG CTC TCC GGC-3′.

RESULTS

Optimal design of Pol III-driven single lhRNA cassettes encoding two unique siRNA sequences

We and others have previously established that Pol III-expressed lhRNA sequences, which include a 2–3 nt uridine 3′-end overhang, can generate independent

effective siRNAs in cells (19,21). However, Dicer cleavage is initiated at the base of the hairpin duplex, and proceeds towards the loop with decreasing cleavage efficiency. Previously, Liu *et al.* (21) have shown that two rounds of Dicer cleavage can yield two effective siRNAs when lhRNAs are generated from a H1 Pol III promoter. The arrangement of each siRNA along a hairpin duplex was optimal for 44 and 45 bp stems (21), where siRNA precursors of 19 bp are separated by 3–4 bp. To avoid second Dicer cleavage occurring within the terminal loop, we generated lhRNAs with a longer 48–50 bp complementary duplex sequence and a 9 nt loop (Figure 1A). We designed several sets of U6 lhRNA expression plasmids that each encode two siRNA sequences. The intended siRNAs targeted four unique HIV-1 sites within *tat* (29), *nef* (23), *LTR* (20) and *int* (30), which have previously been shown to be susceptible to RNAi-mediated silencing (Figure 1A and B). In total, four different series of lhRNAs were generated: two sets of lhRNAs targeted against *tat* and *nef* and two sets targeted against *int* and the *LTR,* allowing for each siRNA-encoding sequence to be situated in the stem base or loop side positions of the hairpin duplex (Figure 1B). The position of these putative 19 bp+2 nt siRNAs were adjusted within each of the four lhRNA series by inserting 1, 2 or 3 symmetrical paired mismatched bases at the junction between first and second siRNAs (Figure 1B). In addition, four U6-expressing shRNA controls were generated for each target. All lhRNAs and shRNAs included G:U pairings that were introduced at regular intervals along the sense strand of the duplex. The antisense sequence was retained to ensure complete hybridization to each intended target RNA. The addition of G:U wobbles facilitated propagation of lhRNA expressing plasmids in *Escherichia coli* (24,25).

The processing and silencing efficacy of two siRNAs derived from a single U6-driven lhRNA in cell culture

To assess the ability of variable length dual targeting lhRNAs to inhibit their targets in cultured cells, HEK293 cells were transfected with lhRNAs (Figure 1B) or with individual shRNA expression plasmids. These hairpin constructs were transiently co-transfected with a psiCheck dual luciferase target reporter plasmid containing the *tat*, *nef*, *LTR* or *int* target sequence within the 3′-UTR of the human *Renilla* luciferase (hRLuc) transcript (top panel Figure 2A and B). A lhRNA targeted against the *HBx* gene of HBV (26) was used as a negative lhRNA control. Knockdown was determined according to the ratios of *Renilla* to Firefly luciferase activities and values were normalized relative to that obtained after co-transfection with the empty U6 vector pTZU6+1 (Mock). Regardless of the spacing at the siRNA junctions, or the relative arrangement of the siRNA sequence, siRNAs in the first position of the hairpin (at the base of the duplex) were consistently capable of suppressing their respective targets by ~80%, which is comparable to the knockdown achieved by individual control shRNAs. Target inhibition from the siRNA derived from the loopside position of the hairpin duplex was most efficient when only one mismatched base pair was present between siRNA encoding regions (+1 configuration). A gradual decrease in efficacy was observed with increased spacing at the junction of the first and second siRNAs (Figure 2A and B).

siRNA and hairpin-intermediates derived from long processed hairpin precursors were analysed by using small RNA northern blot hybridization carried out on total RNA extracted from transfected HEK293 cells. Figure 2C and D show representative blots using four probes complementary to each of the intended siRNA guide strands. The signal for siRNAs derived from the

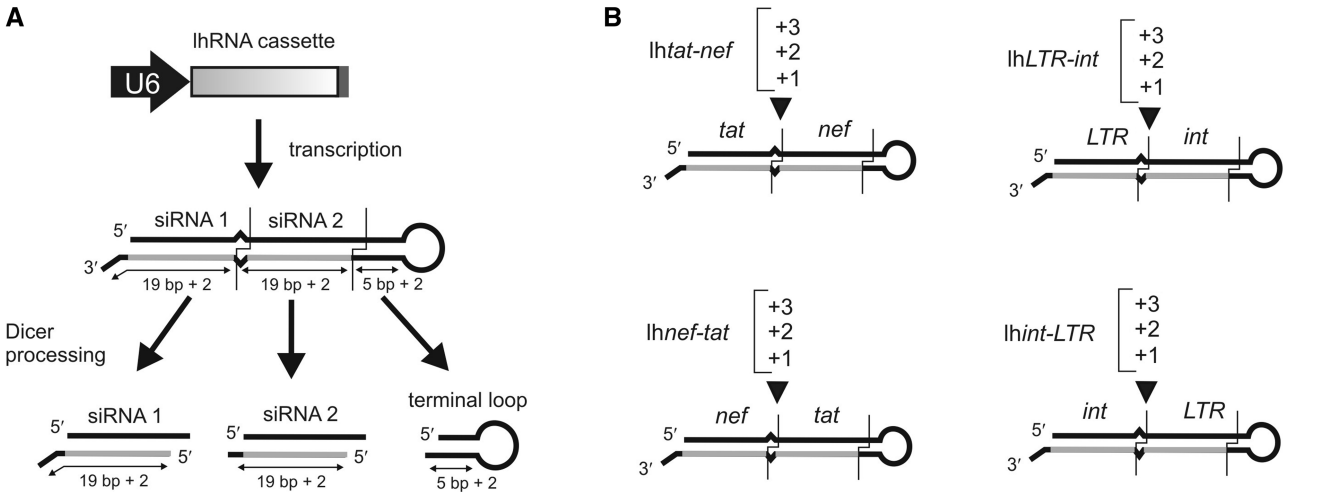


Figure 1. Design of dual targeting lhRNAs. (**A**) Schematic representation of an lhRNA expression cassette showing upstream U6 promoter and the predicted structures of the transcribed lhRNAs. Dual targeting lhRNAs encode two 19 bp +2 nt siRNAs and have a 5 bp +2 nt spacer before the loop sequence. (**B**) Four different series of 48–50 bp lhRNAs were generated that target *tat* and *nef* as well as *int* and *LTR*. Each 19 bp +2 nt siRNA-encoding sequence is positioned either in the stem or loopside of the lhRNA duplex and separated by 1, 2 or 3 mismatched paired bases.

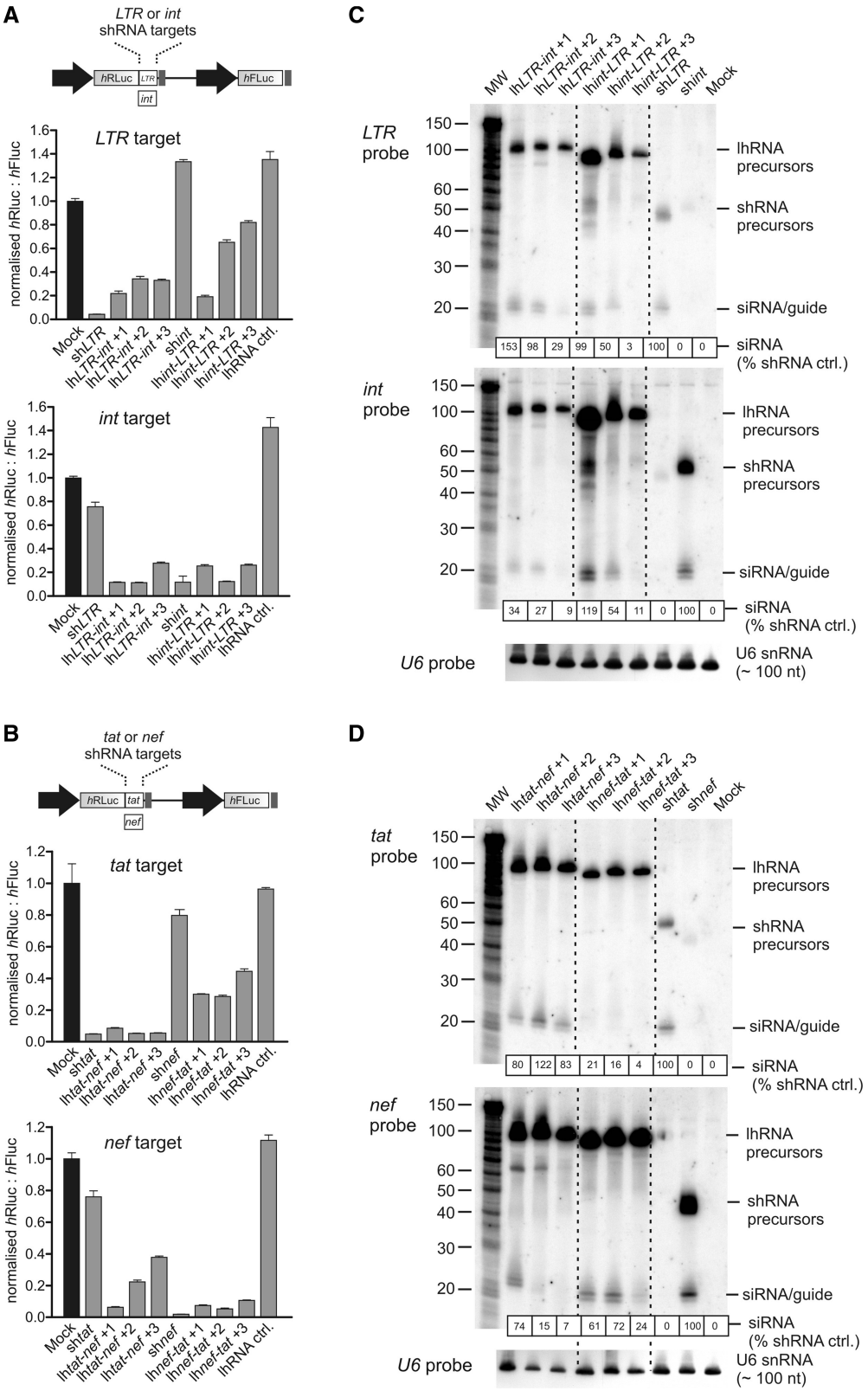


Figure 2. Knockdown efficacy and processing of dual targeting lhRNAs. Dual luciferase reporter assays showing knockdown of the *LTR* and *int* targets (**A**) and *tat* and *nef* targets (**B**) when the target sequence was inserted downstream of the *Renilla* luciferase (*h*RLuc) open reading frame. Values represented are mean ratios of *Renilla* to Firefly luciferase ($n = 3$, SEM) and are normalized to cells transfected with a plasmid containing a U6 promoter only with no RNAi effector sequence (mock). Small RNA (PAGE) northern blot analysis was carried out on total RNA extracted from cells transfected with the indicated transcripts. Labelled probes complementary to the guide strand of *LTR* and *int* (**C**) or *tat* and *nef* (**D**) were hybridized to immobilized RNA and exposed to a phosphorimaging plate. lhRNA and shRNA precursor RNA as well as processed siRNAs are indicated. The amount of processed guide strand is shown and normalized for each blot relative to the shRNA (set at 100). Decade Marker™ indicates fragment size and a probe complementary to small nuclear U6 RNA was used to detect U6 snRNA as a loading control.

first position of the lhRNAs with a +1 configuration was similar to that of the corresponding shRNA cassette. With the exception of the lh*tat-nef* series, the guide signal of the first position siRNA decreased with an increase in the mismatched paired bases inserted between the putative siRNAs of the hairpin duplex. For example, in the case of the *LTR* and *int* probes, very weak guide hybridization signals were detected when three mismatched bases were inserted between the siRNA-encoding regions. With the exception of lh*nef-tat*, processed products from the second position of the hairpin were easily detected after transfection of plasmids encoding lhRNAs with a +1 configuration. The *tat* siRNA from the lh*nef-tat* set was not detected for all spacing arrangements and correlates with poor knockdown that was observed with *tat* reporter targets (Figure 2A and B). The decrease in siRNA production from the second position was observed in a gradient fashion; an increase in mismatched paired bases at the junction between siRNA encoding sequences resulted in a decreased detection of processed second-position guide strand. This result is in accordance with the gradient in inhibition observed for the psiCheck reporter gene targets (Figure 2A and B) and previously reported for longer hairpins (19). When only one pair of mismatched bases was inserted at the junction, siRNAs in the second position were detected at similar levels to those of siRNAs at the base of the stem. It should be noted that 2–3 bands, differing in size by 1 nt, are often visible for single siRNA guide strands. This indicates that Dicer does not consistently cleave the duplex at the same position and therefore generates guide strands ranging in size from 19–22 nt. While the potency of individual shRNAs differ (in the order: shtat > shnef >> shint > shLTR), reporter gene inhibition was effective for both siRNAs along the duplex, even when transfecting with decreasing concentrations of hairpin-expressing plasmid (Supplementary Figure 1), using different Pol III promoters (H1 and 7SK), and when combining two different hairpin expression cassettes in a single vector (Supplementary Figure 2A). Thus lhRNAs lh*tat-nef* +1 and lh*LTR-int* +1 are optimally designed to allow for efficient processing along the entire hairpin duplex to produce a strong dual siRNA response.

The generation of four independent and effective siRNAs from a single U6-driven double lhRNA (dlhRNA) expression cassette

Placement of two shRNAs together within the same transcript has been shown to produce siRNAs targeted to two independent sites (37,38). Although Pol III-expressed shRNAs and lhRNAs consist of defined 5′- and 3′-termini that facilitate Dicer recognition and cleavage, shRNAs expressed from Pol II promoters produce longer 5′-leader and 3′-trailer sequences which often results in variable production of active siRNAs (39–41). To develop a system for generating four separate siRNAs from a single transcript, we combined the two most effective lhRNAs described here. lh*tat-nef* +1 and lh*LTR-int* +1 were placed in tandem to generate two dlhRNA expression constructs (Figure 3A). A 2-nt UU bridge was included between each lhRNA to mimic the 3′-overhang generated for a single Pol III-generated lhRNA. dlh*LI-TN* and dlh*TN-LI* included lhRNAs lh*LTR-int* +1 and lh*tat-nef* +1 in either the 5′-position or 3′-position of the dlhRNA transcripts, respectively (Figure 3B).

To characterize the processing of dlhRNAs, small RNA northern blot analysis was performed as before. Figure 3C shows the signals obtained following hybridization with antisense (AS) and sense (S) probes to detect siRNA guide and passenger strands. All probes detected the full-length dlhRNA transcripts from both dlhRNA constructs. However, only dlh*LI-TN* generated detectable processed precursors representing both lhRNAs. For dlh*TN-LI*, strong signals were detected for the 5′-position lhRNA precursor, lh*tat-nef* +1, with S and AS probes to *tat* and *nef*. No hybridization signal was detected by either S or AS probes to *LTR* and *int* for the 3′-position lh*LTR-int* +1, suggesting that this lhRNA precursor is rapidly degraded following initial Dicer cleavage of the dlhRNA transcript. For dlh*LI-TN*, the 5′-position lhRNA, lh*LTR-int* +1, was detected by S and AS probes targeted to *LTR* and *int*. The lhRNA in the 3′-position (lh*tat-nef* +1) was detected by S and AS *tat* and *nef* probes, but at a lower concentration than that of the 5′-position lhRNA precursor. Again this suggests that processing of the dlhRNA renders lhRNA at the 3′-position less stable. Nevertheless, all four siRNAs guide sequences were detected from dlh*LI-TN* demonstrating that a single dlhRNA construct is capable of successfully generating four independent siRNA guide strands.

To determine whether the guide strands were capable of effecting knockdown of defined HIV targets, a dual-luciferase reporter gene assay was performed as before. Double long hairpin RNA dlh*LI-TN* inhibited all four targets by 70–80% (Figure 3D), indicating that four effective siRNAs were generated from this dlhRNA and that a decreased amount of *tat* and *nef* guide strand derived from the second lhRNA did not affect knockdown under these conditions of transient transfection. As expected, dlh*TN-LI*, only inhibited *tat* and *nef* reporter targets, confirming that compromised processing of the second position lhRNA affects downstream guide strand production and subsequent target knockdown. To determine whether the passenger strand of each putative processed siRNA was active, dual-luciferase reporter constructs were generated that included AS targets. The passenger strands were largely ineffective, and guide strand formation was according to the intended bias.

Ability of the dlhRNAs to inhibit cognate targets within the context of a full-length HIV-1 sequence was also determined (Figure 4A and B). To quantify the anti-viral effects of these hairpins, we co-transfected the shRNA-, lhRNA- and dlhRNA-expressing plasmids with the HIV-1 molecular clone pNL4-3.Luc.E-R-. This reporter plasmid lacks functional *env* and the *nef* reading frame is substituted with a Firefly luciferase ORF (Figure 4A). Knockdown was measured by determining Firefly luciferase activity, which was normalized to activity of *Renilla* luciferase that was consitutively expressed from a co-transfected plasmid. Luciferase ratios were determined

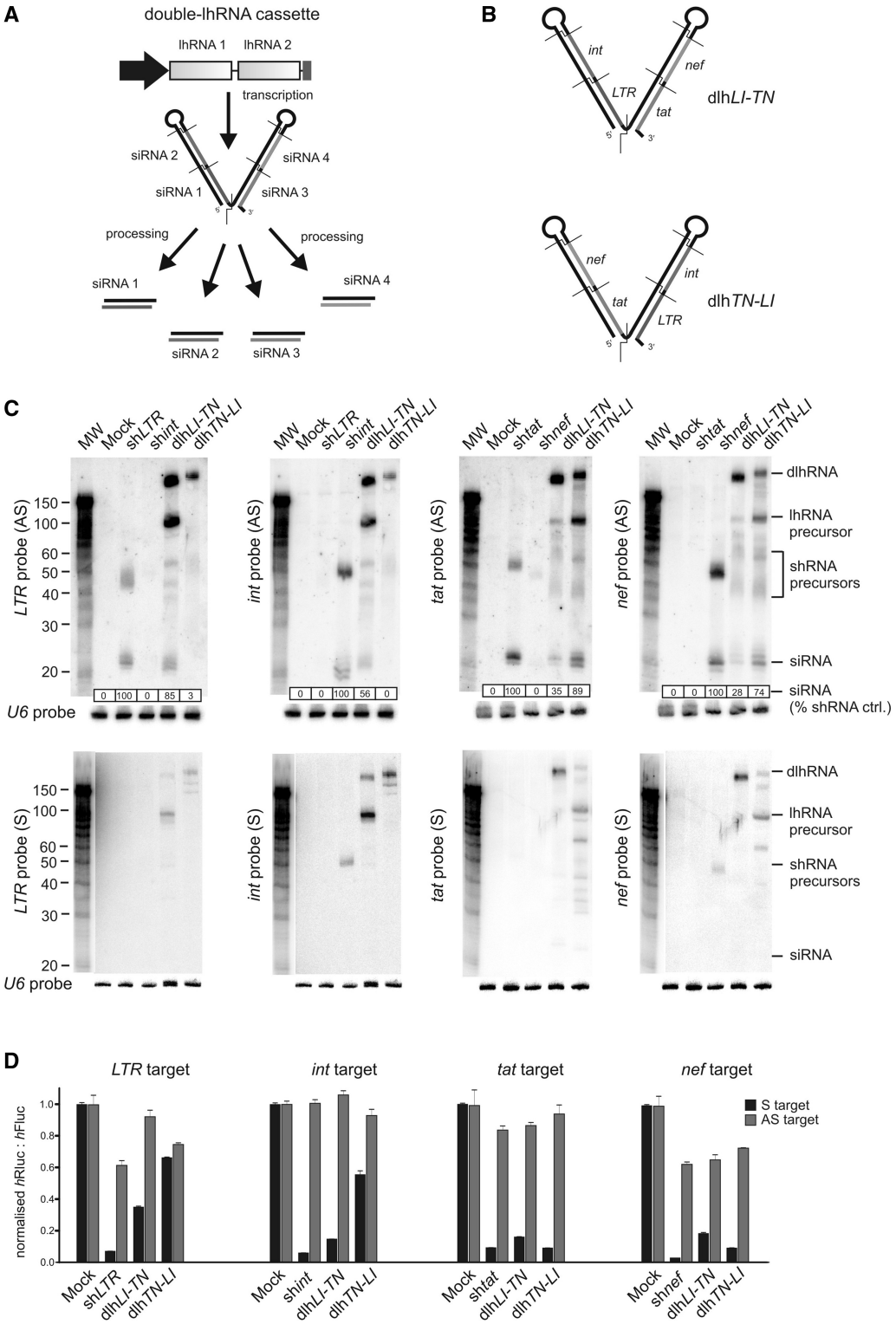


Figure 3. Generation of four effective individual siRNAs from a single dlhRNA transcript containing two adjacent lhRNAs. (**A**) Schematic representation of a dlhRNA expression cassette driven by a single promoter showing the predicted structure and derivation of four siRNAs. (**B**) Effective dual targeting long hairpin RNAs lh*tat-nef* +1 and lh*LTR-int* +1 were both combined in 5′ or 3′ positions within the dlhRNA transcript to generate lh*LI-TN* and lh*TN-LI*. (**C**) Low molecular weight northern blot analysis was carried out on total RNA extracted from cells transfected with the dlhRNA expression cassettes with individual targeting shRNAs used as positive controls. Labelled probes complementary to the guide and antisense strand of *LTR, int, tat* and *nef* were hybridized to immobilized RNA and exposed to a phosphorimaging plate. Precursor hairpin RNAs as well as processed siRNAs are indicated. The amount of processed guide strand is shown and normalized for each blot relative to the shRNA (set at 100). Decade Marker™ indicates fragment size and a probe complementary to small nuclear U6 RNA was used to detect U6 snRNA as a loading control. (**D**) Dual luciferase reporter assays showing knockdown of the sense (S) and antisense (AS) targets of *LTR*, *int, tat* and *nef* when the target sequence was inserted downstream of the *Renilla* luciferase open reading frame. Values represented are mean ratios of *Renilla* to Firefly luciferase ($n = 3$, ± SEM) and are normalized to cells transfected with a plasmid containing a U6 promoter only with no RNAi effector sequence (mock).

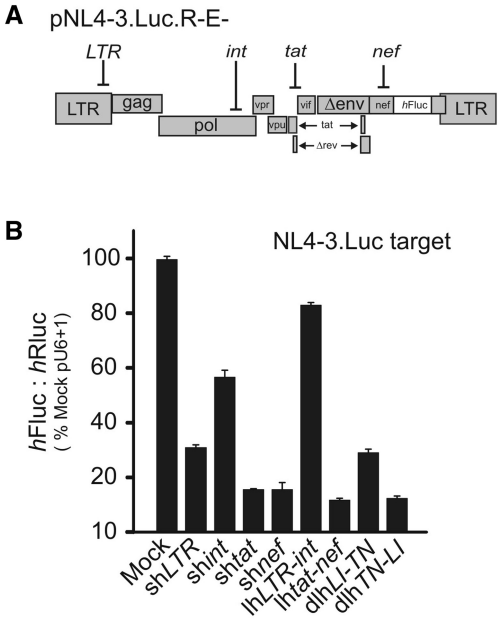


Figure 4. Inhibition of full-length HIV-1 molecular clone pNL4-3.Luc.R-E-. (**A**) The separate regions of the NL4-3.Luc sequence targeted by the four generated siRNAs are shown schematically. (**B**) HEK293 cells were transfected in a 1:1 ratio of pNL4-3.Luc.R-E- together with indicated hairpin constructs and trace amounts of *Renilla* luciferase plasmid phRL-CMV. The four siRNA-targeted regions are indicated above in the modified HIV genome of pNL4-3.Luc.R-E-. Values represented are mean ratios of Firefly luciferase normalized to *Renilla* luciferase ($n = 3$, ± SEM) and expressed as a percentage of mock (pU6 + 1) transfected cells (set at 100%).

relative to that of a mock control vector (Figure 4B). The individual shRNAs targeted to the *tat* and *nef* sequences showed potent luciferase inhibition (>80%), whilst shRNAs targeted to *LTR* and *int* were less effective (70% and 40% knockdown, respectively). This may reflect a lower potency of individual *LTR* and *int* siRNAs (Supplementary Figure 1). As expected, inhibition of the HIV-1 reporter by lhRNA*tat-nef* was similar to that achieved by each of the *tat* or *nef* shRNAs. However, the dual targeting hairpin lh*LTR-int* was only capable of inhibiting the HIV-1 reporter by ~20%. This confirms previous observations that *LTR* and *int* targets were silenced less effectively when produced from lh*LTR-int*. Importantly, both double lhRNA expression cassettes achieved good silencing of the reporter gene. However, dlh*TN-LI* achieved slightly better silencing than dlh*LI-TN*. This may be a result of better knockdown achieved by the more efficiently processed *nef*- and *tat*-targeting guides (Figure 3C). Collectively these data indicate that although there may be variability in the silencing efficacy of individual siRNAs, compensatory effects may result in good overall silencing which is a desirable property of coRNAi applications.

lhRNA cassettes do not disrupt independent RNAi-mediated gene silencing or stimulate the innate IFN response

An important safety concern of potentially therapeutic lhRNAs is the possible disruptive effect that they may have on the endogenous cellular miRNA pathway. Both exogenous siRNAs and shRNAs can compete with each other or with endogenous miRNAs for access to components of the RNAi machinery (42,43). However, RNAi activators that are present in lower concentrations, are less likely to saturate the RNAi pathway (34,43,44). To assess effects of lhRNA cassettes on independent RNAi, two assays were performed to determine whether lhRNA expression cassettes have any derepression effect on silencing caused by an endogenous or an exogenous miRNA. In the first assay, HEK293 cells were co-transfected in a 1:1 ratio with an exogenous miRNA shuttle, pCMV miR-31 *HBx* (targeted to a unique site within the HBV genome), and its cognate dual-luciferase reporter target plasmid, psiCheck HBx (34). Diminishing amounts of plasmids expressing shRNA, lhRNA or dlhRNAs relative to pCMV miR-31 *HBx* were co-transfected in ratios of 5:1, 1:1 and 0.1:1, respectively. U6 promoter-driven sh*LTR* showed significant derepression of miR-31 *HBx* mediated knockdown at ratios of 5:1 and 1:1 but not at a ratio of 0.1:1. However, equivalent ratios of U6 promoter-driven lh*LTR-int* or dlh*LI-TN* had no effect on miR-31 *HBx* knockdown efficacy (Figure 5A). In the second assay, Huh-7 cells were co-transfected with a panel of hairpin-expressing plasmids together with a dual-luciferase reporter plasmid containing seven copies of a natural miR-16 target site (18). A miRNA 'sponge' construct expressing seven copies of an imperfectly matched miR-16 target, pU6 miR16Tx7, significantly derepressed miR-16 silencing of its reporter cognate (Figure 5B). However, at a concentration of 5:1 (sh/lhRNA expression cassette:target reporter plasmid), none of the hairpin RNAs had any detectable disruptive effect on endogenous miR-16 silencing of its reporter cognate. Together, these results suggest that lhRNAs and dlhRNAs, although expressed from a potent U6 promoter, are less likely than shRNAs to compete with RNAi pathway components necessary for either exogenous or endogenous miRNA function. Although lhRNA and shRNA expression cassettes are expressed from U6 Pol III promoters, intracellular precursors and siRNA guide strands from lhRNA cassettes are present at lower concentrations than those derived from shRNA expression cassettes (Figure 3C). Lastly, to exclude the possibility of non-specific effects caused by the induction of an interferon response, *IFN-β* mRNA concentrations were measured in transfected cells. As previously shown for other Pol-III generated lhRNAs (21,22,26), quantitative qRT–PCR demonstrated that none of the hairpin cassettes induced expression of *IFN-β* (Figure 5C).

DISCUSSION

Despite extensive optimization of hairpin stem length, siRNA sequence, and the spatial arrangement of unique siRNAs along a lhRNA duplex, it seemed unlikely that expressed lhRNAs can be designed to produce more than two, possibly three, separate effective siRNAs (19,21,27). Deriving high concentrations of more than two independent functional siRNAs from an lhRNA scaffold remains

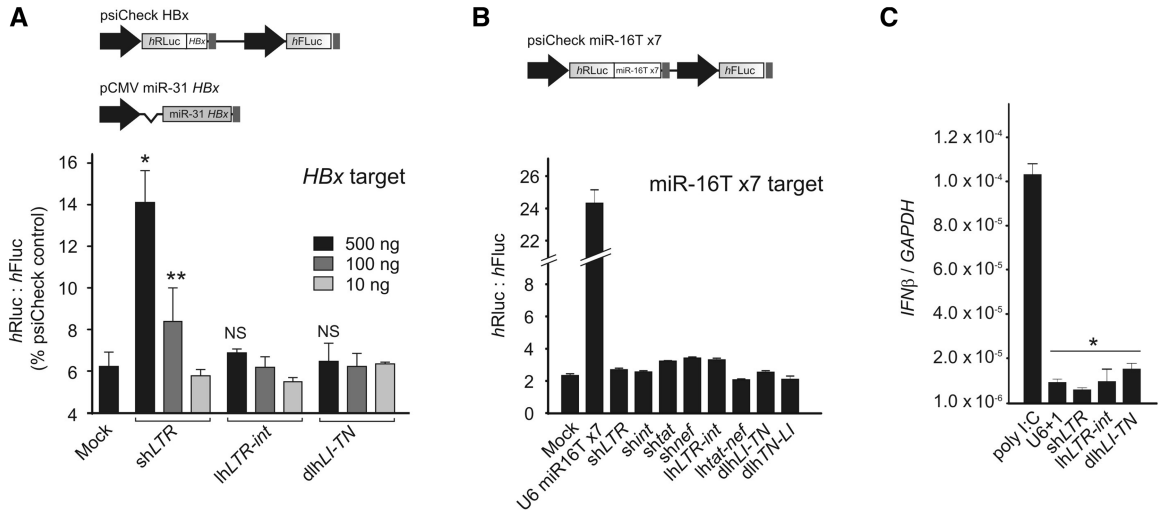


Figure 5. Assessment of non-specific effects mediated by long hairpin RNAs. (**A**) Analysis of effects of a shRNA, lhRNA and dlhRNA expression cassette on the repression of *HBx* target reporter sequence by an exogenously introduced miR-31 *HBx* shuttle using a dual luciferase assay. Co-transfection of reporter plasmid, containing an *HBx* target sequence downstream of the *Renilla* luciferase ORF, was carried out together with three different concentrations of RNAi expression cassettes and empty backbone plasmid (mock). Mean ratios of *Renilla* to Firefly luciferase (as a percentage of psiCheck2 empty backbone vector) were used to determine derepression of miR-31 *HBx*. Statistical significance was determined using a one-way ANOVA relative to mock transfected control (pU6 +1, $*P < 0.05$ and $**P < 0.001$). (**B**) The effect of hairpin expression cassettes on the function of endogenous miR-16 was analysed following co-transfection of a dual luciferase reporter plasmid containing seven copies of the miR-16 target downstream of the *h*Rluc ORF together with the indicated hairpin-expressing plasmids or miR-16 sponge plasmid expressing seven copies of an imperfectly matched miR-16 target. Mean ratios of *Renilla* to Firefly luciferase were used to determine derepression of miR-16. (**C**) The induction of the IFN response was assessed by measuring *IFN-β* mRNA concentration in total RNA extracted from cells transfected with the indicated shRNA, lhRNA and multi-lhRNA expression cassettes. Poly I:C served as a positive control. Mean normalized ratios of *IFN-β*:*GAPDH* ($n = 3$, ± SEM) determined by using quantitative RT–PCR are indicated. Statistical significance was determined using a one-way ANOVA relative to mock transfected control (pU6 +1, $*P < 0.001$).

difficult and requires new ways of exploiting the processing of dsRNA substrates by Dicer. To develop effective Pol III-driven lhRNAs expressing two functional siRNAs, we tested the effects of combining two 19 bp +2 nt siRNAs within a single 48–50 bp lhRNA duplex, and included up to three mismatched paired bases between each siRNA-encoding sequence. Liu *et al.* (21) showed that a single mismatched paired base between two effective siRNAs at the centre of the lhRNA stem is well tolerated and results in the same level of processing or siRNA activity within the context of a 43 bp lhRNA. In total, we established a panel of four unique anti-HIV siRNAs was used to generate 12 dual-targeting lhRNA structures. When tested against respective HIV targets, we observed an inverse correlation between siRNA silencing potency and increased spacing between each siRNA along the duplex. Optimal siRNA processing from lhRNAs occurred when only one mismatched paired base was placed between each 19 bp + 2 nt siRNA, and this was irrespective of siRNA position or sequence. These data are in accordance with previously published data reporting on dual-targeting lhRNAs (21), and is in accordance with predicted Dicer cleavage intervals of ~22 nt (20 bp +2 nt) in human cells (45). With some dual-acting lhRNAs, processing of the siRNA at the first position diminished when more mismatches were inserted in the lhRNA duplex at the junction of each the siRNAs. This is likely to be caused by an inhibitory effect on processing which is caused by bulges occurring at the Dicer cleavage sites (46). However, the +1 configuration did not affect Dicer processing through the lhRNA duplex. Importantly, we were able to identify two effective and optimized dual-targeting anti-HIV lhRNAs, lh*tat-nef* +1 and lh*LTR-int* +1, which together produce high levels of four siRNAs that inhibit their cognate targets. These lhRNAs were ideally optimized for inclusion into a single combinatorial dlhRNA expression cassette.

Based on HIV reverse-transcriptase error rates, it has been determined that a minimum of four separate HIV target sites should be targeted simultaneously to prevent the emergence of RNAi-resistant viral species (47,48). Therefore, it was encouraging that the dlhRNA design, with two highly effective lhRNAs joined together within a single expressed transcript, enabled accurate processing into four active anti-HIV siRNAs. Apart from polycistronic miRNA mimics (13–17), this is the first example of four active guide strands being derived from a single Pol III expression cassette, and provides a useful framework for generating effective coRNAi strategies against highly evolving viruses or multiple rogue genetic elements. Nevertheless, the mechanism by which these dlhRNA structures are processed is unclear. Although previous attempts to generate binary or dual shRNA-containing transcripts suggest that these duplexes can be processed into siRNA-sized products, the mechanism for their intracellular cleavage remains unexplained (37,38). Although we do not exclude involvement of other RNases in the processing of dlhRNA precursors,

it seems likely that Dicer is responsible for the initial cleavage to form lhRNA species. This is supported by evidence that Dicer is capable of cleaving hairpins with either 5′- and 3′-extensions, albeit less efficiently (39–41). The fact that the 3′-position lhRNA is present in reduced amounts (or degraded completely) suggests that, once cleaved, the 5′-position lhRNAs is initially protected by Dicer before being processed into shorter hairpin products. Since the intrinsic stability of the two lhRNAs is the same within the dlhRNA system, it remains odd that the 3′-position lhRNA in dlh*LI-TN* is processed but is degraded in the context of dlh*TN-LI*. The only difference appears to be the sequence presented for second round Dicer cleavage. Moreover, the 5′-terminus of the lh*LTR-int* within dlh*LI-TN* has a triphosphate moiety generated by Pol III transcription, a feature which is lacking for lh*LTR-int* in the context of dlh*TN-LI,* and which may add to the stability of lh*LTR-int*. However a clearer picture is likely to emerge with the study of more dlhRNA combinations comprising different lhRNAs.

Although coRNAi aims to induce strong silencing from multiple guide strands, high levels of shRNA produced from Pol III promoters are known to be associated with unwanted cellular toxicities. High levels of expressed shRNAs in the liver may cause fatality in mice as a result of saturation of the endogenous RNAi machinery (10). In addition, McBride and colleagues have observed toxicities caused by shRNA-based vectors in brain, which may have been caused by a buildup of guide strand RNA (49,50). Replacement of shRNAs with miRNA shuttles reduced neurotoxicities, suggesting that natural RNAi pathway precursors are less likely to interfere with endogenous miRNA functions. Although not fully understood, it is likely that highly expressed shRNAs abrogate the function of natural and exogenous miRNAs (10,34,43,44,51,52). Here, we have shown that both lhRNAs and dlhRNAs do not induce the same disruptive effects on endogenous miRNA that were observed after transfection of a U6-generated shRNA. This augurs well for the safety and potential therapeutic application of these constructs. Moreover, although there have been concerns about induction of the IFN response by long (>30 bp) duplex RNA, we did not detect any IFN-β mRNA activation for any of the hairpins tested. This confirms previously reported results from analysis of expressed lhRNAs (24,25,53), and suggests that intracellular transcription of dsRNA hairpins is less likely than exogenous synthetic RNA to activate the type 1 IFN response. This is explained by the fact that expressed lhRNAs, unlike transfected synthetic duplex RNA, do not traverse the endosome which contain Toll-like receptors that are typically activated by introduced siRNAs (54,55).

In conclusion, we show that RNA Pol III-expressed dlhRNA transcripts may be processed to generate four independent siRNAs that can effect significant knockdown of non-contiguous siRNA-susceptible regions of HIV-1. Although there is some variation in the processing efficiency of the 3′-lhRNA, effective coRNAi can be achieved. Importantly, the dlhRNA constructs described here do not appear to disrupt the natural miRNA pathway, which represents an important objective for their implementation as potential therapeutic agents. This design of dlhRNA cassettes improves on the limited versatility of expressed lhRNAs and provides a useful approach for generating transcripts with modular lhRNAs motifs that achieve effective coRNAi in mammalian cells.

FUNDING

South African National Research Foundation (NRF); South African Medical Research Council (MRC); Poliomyelitis Research Foundation (PRF); Sheena Saayman is a recipient of a Stella and Paul Loewenstein Studentship.

Conflict of interest statement. None declared.

REFERENCES

1. Castanotto,D. and Rossi,J.J. (2009) The promises and pitfalls of RNA-interference-based therapeutics. *Nature*, **457**, 426–433.
2. Rossi,J.J. (2008) Expression strategies for short hairpin RNA interference triggers. *Hum. Gene Ther.*, **19**, 313–317.
3. Henry,S.D., van der Wegen,P., Metselaar,H.J., Tilanus,H.W., Scholte,B.J. and van der Laan,L.J. (2006) Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. *Mol. Ther.*, **14**, 485–493.
4. ter Brake,O., Konstantinova,P., Ceylan,M. and Berkhout,B. (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol. Ther.*, **14**, 883–892.
5. McIntyre,G.J., Groneman,J.L., Tran,A. and Applegate,T.L. (2008) An infinitely expandable cloning strategy plus repeat-proof PCR for working with multiple shRNA. *PLoS ONE*, **3**, e3827.
6. Cheng,T.L., Teng,C.F., Tsai,W.H., Yeh,C.W., Wu,M.P., Hsu,H.C., Hung,C.F. and Chang,W.T. (2009) Multitarget therapy of malignant cancers by the head-to-tail tandem array multiple shRNAs expression system. *Cancer Gene Ther.*, **16**, 516–531.
7. Song,J., Giang,A., Lu,Y., Pang,S. and Chiu,R. (2008) Multiple shRNA expressing vector enhances efficiency of gene silencing. *BMB Rep.*, **41**, 358–362.
8. ter Brake,O., te Hooft,K., Liu,Y.P., Centlivre,M., von Eije,K.J. and Berkhout,B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol. Ther.*, **16**, 557–564.
9. Gonzalez,S., Castanotto,D., Li,H., Olivares,S., Jensen,M.C., Forman,S.J., Rossi,J.J. and Cooper,L.J. (2005) Amplification of RNAi–targeting HLA mRNAs. *Mol. Ther.*, **11**, 811–818.
10. Grimm,D., Streetz,K.L., Jopling,C.L., Storm,T.A., Pandey,K., Davis,C.R., Marion,P., Salazar,F. and Kay,M.A. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, **441**, 537–541.
11. Jetzt,A.E., Yu,H., Klarmann,G.J., Ron,Y., Preston,B.D. and Dougherty,J.P. (2000) High rate of recombination throughout the human immunodeficiency virus type 1 genome. *J. Virol.*, **74**, 1234–1240.
12. An,W. and Telesnitsky,A. (2001) Frequency of direct repeat deletion in a human immunodeficiency virus type 1 vector during reverse transcription in human cells. *Virology*, **286**, 475–482.
13. Aagaard,L.A., Zhang,J., von Eije,K.J., Li,H., Saetrom,P., Amarzguioui,M. and Rossi,J.J. (2008) Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Ther.*, **15**, 1536–1549.
14. Chung,K.H., Hart,C.C., Al-Bassam,S., Avery,A., Taylor,J., Patel,P.D., Vojtek,A.B. and Turner,D.L. (2006) Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. *Nucleic Acids Res.*, **34**, e53.

15. Liu,Y.P., Haasnoot,J., ter Brake,O., Berkhout,B. and Konstantinova,P. (2008) Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucleic Acids Res.*, **36**, 2811–2824.
16. Sun,D., Melegari,M., Sridhar,S., Rogler,C.E. and Zhu,L. (2006) Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques*, **41**, 59–63.
17. Zhou,H., Huang,C. and Xia,X.G. (2008) A tightly regulated Pol III promoter for synthesis of miRNA genes in tandem. *Biochim. Biophys. Acta*, **1779**, 773–779.
18. Ely,A., Naidoo,T. and Arbuthnot,P. (2009) Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Res.*, **37**, e91.
19. Saayman,S., Barichievy,S., Capovilla,A., Morris,K.V., Arbuthnot,P. and Weinberg,M.S. (2008) The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. *PLoS ONE*, **3**, e2602.
20. Barichievy,S., Saayman,S., von Eije,K.J., Morris,K.V., Arbuthnot,P. and Weinberg,M.S. (2007) The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5′ long terminal repeat. *Oligonucleotides*, **17**, 419–431.
21. Liu,Y.P., Haasnoot,J. and Berkhout,B. (2007) Design of extended short hairpin RNAs for HIV-1 inhibition. *Nucleic Acids Res.*, **35**, 5683–5693.
22. Sano,M., Li,H., Nakanishi,M. and Rossi,J.J. (2008) Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. *Mol. Ther.*, **16**, 170–177.
23. Nishitsuji,H., Kohara,M., Kannagi,M. and Masuda,T. (2006) Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. *J. Virol.*, **80**, 7658–7666.
24. Akashi,H., Miyagishi,M., Yokota,T., Watanabe,T., Hino,T., Nishina,K., Kohara,M. and Taira,K. (2005) Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Mol. Biosyst.*, **1**, 382–390.
25. Watanabe,T., Sudoh,M., Miyagishi,M., Akashi,H., Arai,M., Inoue,K., Taira,K., Yoshiba,M. and Kohara,M. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. *Gene Ther.*, **13**, 883–892.
26. Weinberg,M.S., Ely,A., Barichievy,S., Crowther,C., Mufamadi,S., Carmona,S. and Arbuthnot,P. (2007) Specific inhibition of HBV replication in vitro and in vivo with expressed long hairpin RNA. *Mol. Ther.*, **15**, 534–541.
27. Liu,Y.P., von Eije,K.J., Schopman,N.C., Westerink,J.T., Brake,O., Haasnoot,J. and Berkhout,B. (2009) Combinatorial RNAi against HIV-1 using extended short hairpin RNAs. *Mol. Ther.*, **17**, 1712–1723.
28. Castanotto,D., Li,H. and Rossi,J.J. (2002) Functional siRNA expression from transfected PCR products. *RNA*, **8**, 1454–1460.
29. Lee,N.S., Dohjima,T., Bauer,G., Li,H., Li,M.J., Ehsani,A., Salvaterra,P. and Rossi,J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol.*, **20**, 500–505.
30. Westerhout,E.M., Vink,M., Haasnoot,P.C., Das,A.T. and Berkhout,B. (2006) A conditionally replicating HIV-based vector that stably expresses an antiviral shRNA against HIV-1 replication. *Mol. Ther.*, **14**, 268–275.
31. Bertrand,E., Castanotto,D., Zhou,C., Carbonnelle,C., Lee,N.S., Good,P., Chatterjee,S., Grange,T., Pictet,R., Kohn,D. *et al.* (1997) The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA*, **3**, 75–88.
32. Passman,M., Weinberg,M., Kew,M. and Arbuthnot,P. (2000) In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. *Biochem. Biophys. Res. Commun.*, **268**, 728–733.
33. Carmona,S., Ely,A., Crowther,C., Moolla,N., Salazar,F.H., Marion,P.L., Ferry,N., Weinberg,M.S. and Arbuthnot,P. (2006) Effective inhibition of HBV replication in vivo by anti-HBx short hairpin RNAs. *Mol. Ther.*, **13**, 411–421.
34. Ely,A., Naidoo,T., Mufamadi,S., Crowther,C. and Arbuthnot,P. (2008) Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently in vitro and in vivo. *Mol. Ther.*, **16**, 1105–1112.
35. Connor,R.I., Chen,B.K., Choe,S. and Landau,N.R. (1995) Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. *Virology*, **206**, 935–944.
36. He,J., Choe,S., Walker,R., Di Marzio,P., Morgan,D.O. and Landau,N.R. (1995) Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. *J. Virol.*, **69**, 6705–6711.
37. Anderson,J., Banerjea,A. and Akkina,R. (2003) Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. *Oligonucleotides*, **13**, 303–312.
38. Leirdal,M. and Sioud,M. (2002) Gene silencing in mammalian cells by preformed small RNA duplexes. *Biochem. Biophys. Res. Commun.*, **295**, 744–748.
39. Giering,J.C., Grimm,D., Storm,T.A. and Kay,M.A. (2008) Expression of shRNA from a tissue-specific pol II promoter is an effective and safe RNAi therapeutic. *Mol. Ther.*, **16**, 1630–1636.
40. Xia,H., Mao,Q., Paulson,H.L. and Davidson,B.L. (2002) siRNA-mediated gene silencing in vitro and in vivo. *Nat. Biotechnol.*, **20**, 1006–1010.
41. Denti,M.A., Rosa,A., Sthandier,O., De Angelis,F.G. and Bozzoni,I. (2004) A new vector, based on the PolII promoter of the U1 snRNA gene, for the expression of siRNAs in mammalian cells. *Mol. Ther.*, **10**, 191–199.
42. Koller,E., Propp,S., Murray,H., Lima,W., Bhat,B., Prakash,T.P., Allerson,C.R., Swayze,E.E., Marcusson,E.G. and Dean,N.M. (2006) Competition for RISC binding predicts in vitro potency of siRNA. *Nucleic Acids Res.*, **34**, 4467–4476.
43. Keck,K., Volper,E.M., Spengler,R.M., Long,D.D., Chan,C.Y., Ding,Y. and McCaffrey,A.P. (2009) Rational design leads to more potent RNA Interference against hepatitis B virus: factors effecting silencing efficiency. *Mol. Ther.*, **17**, 538–547.
44. Castanotto,D., Sakurai,K., Lingeman,R., Li,H., Shively,L., Aagaard,L., Soifer,H., Gatignol,A., Riggs,A. and Rossi,J.J. (2007) Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res.*, **35**, 5154–5164.
45. Siolas,D., Lerner,C., Burchard,J., Ge,W., Linsley,P.S., Paddison,P.J., Hannon,G.J. and Cleary,M.A. (2005) Synthetic shRNAs as potent RNAi triggers. *Nat. Biotechnol.*, **23**, 227–231.
46. Soifer,H.S., Sano,M., Sakurai,K., Chomchan,P., Saetrom,P., Sherman,M.A., Collingwood,M.A., Behlke,M.A. and Rossi,J.J. (2008) A role for the Dicer helicase domain in the processing of thermodynamically unstable hairpin RNAs. *Nucleic Acids Res.*, **36**, 6511–6522.
47. Leonard,J.N. and Schaffer,D.V. (2005) Computational design of antiviral RNA interference strategies that resist human immunodeficiency virus escape. *J. Virol.*, **79**, 1645–1654.
48. ter Brake,O. and Berkhout,B. (2007) Lentiviral vectors that carry anti-HIV shRNAs: problems and solutions. *J. Gene Med.*, **9**, 743–750.
49. Boudreau,R.L., Martins,I. and Davidson,B.L. (2009) Artificial microRNAs as siRNA shuttles: improved safety as compared to shRNAs in vitro and in vivo. *Mol. Ther.*, **17**, 169–175.
50. McBride,J.L., Boudreau,R.L., Harper,S.Q., Staber,P.D., Monteys,A.M., Martins,I., Gilmore,B.L., Burstein,H., Peluso,R.W., Polisky,B. *et al.* (2008) Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc. Natl Acad. Sci. USA*, **105**, 5868–5873.
51. An,D.S., Qin,F.X., Auyeung,V.C., Mao,S.H., Kung,S.K., Baltimore,D. and Chen,I.S. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. *Mol. Ther.*, **14**, 494–504.
52. Ely,A., Naidoo,T. and Arbuthnot,P. (2009) Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Res.*, **37**, e91.
53. Weinberg,M.S., Ely,A., Barichievy,S., Mufamadi,S., Carmona,S. and Arbuthnot,P. (2007) Specific inhibition of HBV replication

in vitro and *in vivo* with expressed long hairpin RNA. *Mol. Ther.*, **15**, 534–541.
54. Marques,J.T., Devosse,T., Wang,D., Zamanian-Daryoush,M., Serbinowski,P., Hartmann,R., Fujita,T., Behlke,M.A. and Williams,B.R.G. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat. Biotechnol.*, **24**, 559–565.
55. Robbins,M.A., Li,M., Leung,I., Li,H., Boyer,D.V., Song,Y., Behlke,M.A. and Rossi,J.J. (2006) Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*. *Nat. Biotechnol.*, **24**, 566–571.

Chapter 11

Effective Pol III-Expressed Long Hairpin RNAs Targeted to Multiple Unique Sites of HIV-1

Sheena M. Saayman, Patrick Arbuthnot, and Marc S. Weinberg

Abstract

The RNA interference (RNAi) pathway has in recent years been exploited for the development of novel antiviral therapies. The emergence of viral escape mutants, however, is a major impediment to the use of RNAi effectors to treat highly mutable viruses such as HIV-1. A combinatorial approach is therefore required for long-term inhibition of gene expression. RNA Pol III-driven long hairpin RNA (lhRNA) duplexes can be cleaved several times by Dicer, yielding multiple functional siRNAs from a single construct. Here we describe a method for the generation of ectopically expressed U6-lhRNAs encoding three separate siRNA sequences targeting unique sites in HIV-1. This methodological overview will explain some crucial aspects of lhRNA design and cloning as well as facile experiments to determine their efficacy in cell culture.

Key words: Long hairpin RNA, lhRNA, RNA interference, Dicer, HIV-1, therapeutics.

1. Introduction

RNA interference (RNAi) is an evolutionary conserved pathway in eukaryotes whereby double-stranded RNA acts as an intracellular trigger to regulate gene expression (1). The RNAi pathway has been popularly used as a tool to silence genes and holds much promise as novel therapeutic approach aimed at suppressing specific cellular and viral genes at the post-transcriptional level. The therapeutic development of RNAi has been made possible by usurping elements of the endogenous mammalian microRNA (miRNA) biogenesis pathway (*see* **Chapter 14**) through exogenously introduced synthetic short interfering RNAs (siRNAs) (2)

M. Sioud (ed.), *RNA Therapeutics*, Methods in Molecular Biology 629,
DOI 10.1007/978-1-60761-657-3_11, © Humana Press, a part of Springer Science+Business Media, LLC 2010

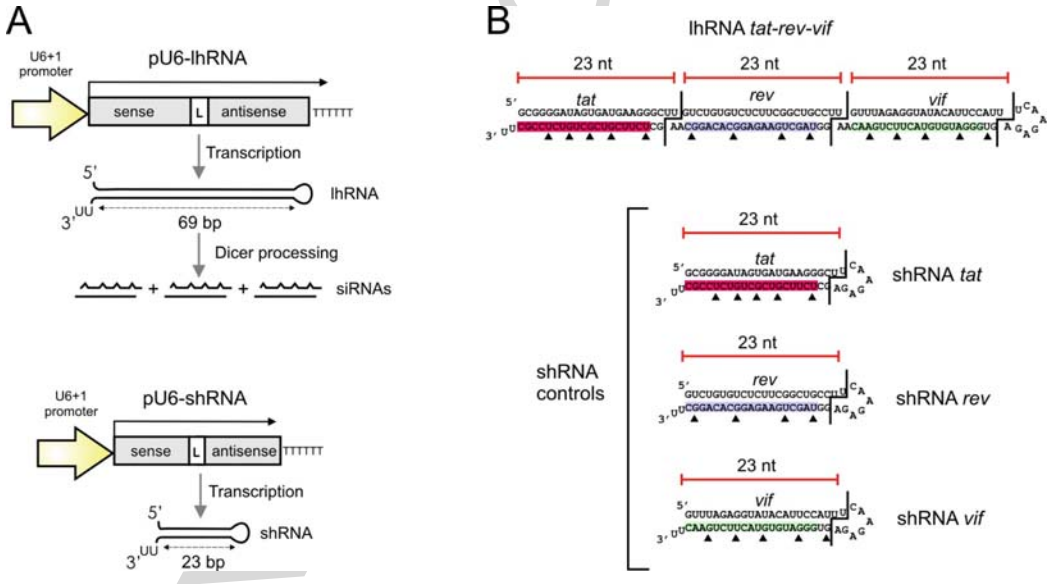
or through gene expression constructs which produce 21–29 bp short hairpin RNAs (shRNAs) (3, 4). These shRNA mimics of precursor miRNA are cleaved by the RNase III endonuclease Dicer to form effective siRNAs which unwind to load single-stranded RNA guide strands into an Argonaute-containing RNA-induced silencing complex (RISC). Loaded RISC complexes target cognate complementary mRNA sequences to mediate post-transcriptional gene silencing by Argonaute-2-mediated cleavage or translational suppression of targeted mRNAs (5).

A significant hurdle for the use of RNAi-based therapeutics has been the targeting of highly mutable sequences, such as genomic and sub-genomic RNAs from the human immunodeficiency virus type 1 (HIV-1). HIV replicates using an error-prone reverse transcriptase and has been shown to escape the silencing effects of expressed shRNAs by rapidly developing resistant viral variants (6–8). The emergence of such viral escape mutants is not unexpected since single-base mismatches between guide strand and target is enough to prevent silencing (6, 9). To circumvent this problem, efforts are underway to generate expressed RNAi effectors, which simultaneously target multiple sites within the viral genome (10–13). While a number of approaches are available to induce combinatorial silencing of viral sequences [reviewed in (14–16)], we have made use of RNA Pol III-expressed long hairpin RNAs (lhRNAs) with duplex stems encoding more than one putative siRNA sequence (17). In mammalian cells, introduction of synthetic dsRNA of greater than 30 bp leads to a strong innate immunostimulatory response (18). Although certain synthetic siRNA sequences were found to activate innate immunity (*see* **Chapter 3**), studies using expressed sequences have shown that safe and effective gene-specific silencing can be achieved and that these duplexes evade cytoplasmic activators of the type 1 interferon response (19, 20).

Long hairpin RNAs generated from Pol III promoters include 2–3 nt 3′-terminal uridine overhangs, which are produced by transcriptional termination. These 3′-overhangs facilitate export to the cytoplasm and allow binding of the Paz domain of human Dicer (hDicer) (21, 22). Processive cleavage of the lhRNA by hDicer then occurs from the open-ended stem to the loop of the hairpin duplex (23). Multiple successive siRNAs are produced by the intracellular processivity of hDicer in decreasing order of efficiency along the lhRNA duplex, resulting in the production of at least three non-overlapping functional siRNAs per lhRNA (17, 23–26). Long hairpin RNAs can be designed to target one contiguous sequence within the genome or, alternatively, can be made to incorporate multiple independent target sequences. The advantage of the latter is that separate mRNAs can be silenced simultaneously and previously characterized effective shRNA/siRNA sequences can be incorporated into a

single lhRNA. The optimal positioning of different 19 bp siRNA within an lhRNA duplex for efficient processing of successive siRNAs often requires empirically testing different sequence spacing arrangements at the junction of each siRNA. However, while no clear rules exist, we provide some design guidelines for optimally spacing effective 19 bp siRNA sequences along the length of an lhRNA duplex.

Here we present a method for cloning and generating 69 bp long hairpin RNAs expressed from the U6 snRNA promoter which encodes three separate anti-HIV siRNA duplexes (**Fig. 11.1a**). This method can be generally adapted for applications where multiple siRNAs are required for the simultaneous targeting of up to three separate and unique RNA sites. A number of different methodologies are available for producing shorter Pol III-expressed hairpin duplexes such as shRNAs. However, many of these are not easily adapted for generating lhRNAs. Owing to the increased length of the dsRNA duplex for lhRNAs and associated problems with subsequent PCR, cloning and sequencing of long inverted repeat sequences, we have relied on a modification of the two-round PCR protocol originally used by Castanotto et al. (27) to generate Pol III-expressed shRNAs. In addition



This figure will be printed in b/w

Fig. 11.1. Design of U6-expressed lhRNAs and shRNA controls targeting three sites of HIV-1. (**a**) Schematic illustration of lhRNAs and shRNAs comprising 69 and 23 bp duplexes, respectively. G:U pairings are indicated as a *corrugated* sense strand. A sequence of two terminal 3′-U residues is derived from the Pol III (U6) transcriptional termination signal. The intended mechanism of transcription and processing by Dicer of the lhRNAs to form three anti-HIV siRNAs is illustrated. (**b**) The sequence and predictive structure of the lhRNA, lhRNA *tat-rev-vif*, and each shRNA are shown. The order and special arrangement of the siRNA-encoded sequences within the lhRNA is indicated along the extent of the duplex. G:U and U:G pairings are indicated with an *arrowhead*. The putative guide strandsare *shaded* in *red*, *blue* and *green* for *tat*, *rev* and *vif* siRNAs, respectively.

to describing the design, PCR and cloning of U6-lhRNA cassettes, we describe a simple luciferase-based reporter gene assay for detecting the efficacy of each siRNA generated from the parental lhRNA duplex. Moreover, we provide a standard polyacrylamide gel electrophoresis (PAGE) northern blot protocol, which allows convenient quantitative detection of each siRNA and associated precursors generated from an lhRNA expression cassette.

2. Materials

2.1. PCR of lhRNA-Encoding DNA Templates

1. Expand High Fidelity^{PLUS} PCR kit (Roche) (*see* **Note 1**).
2. pTZU6+1 template plasmid (28) containing the human U6 snRNA RNA Pol III promoter.
3. PAGE-purified, synthesized oligodeoxynucleotide primers for PCR.
4. 1X Tris-acetate-EDTA (TAE) electrophoresis running buffer: 40 mM Tris, 1 mM ethylenediaminetetraacetic acid (EDTA), 20 mM glacial acetic acid. Store at room temperature.
5. InsTAclone^{TM} PCR Cloning Kit (Fermentas, WI, USA) which includes the plasmid pTZ57R/T. Store all kit components at –20°C.
6. Competent *Escherichia coli* DH5α bacterial cells. Aliquot and store at –70°C.
7. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). X-Gal should be dissolved in dimethylformamide at a concentration of 20 mg/mL and stored in the dark at –20°C.
8. Isopropyl-β-D-1-thiogalactopyranoside (IPTG). IPTG is dissolved in ddH_2O at a concentration of 100 mg/mL, filter sterilized and stored at –20°C.
9. Luria Bertani broth (LB): 10 g/L Bacto-Tryptone, 5 g/L bacto yeast extract, 10 g/L sodium chloride (NaCl) containing 1 μg/mL ampicillin. Autoclave prior to the addition of antibiotic and store at 4°C.
10. Agar plates containing 1 μg/mL ampicillin. Store inverted at 4°C.
11. High Pure Plasmid Isolation Kit for small-scale (mini) preparations of purified plasmid DNA.
12. *Spe*I, *Not*I, *Eco*RI and *Hin*dIII restriction enzymes (10 U/μL), supplied with recommended 10X buffers. Store at –20°C.
13. QIAGEN Plasmid Maxi Kit (for transfections).

14. Common restriction enzymes, each 5–10 U/μL, supplied with recommended 10X buffers. Store at –20°C.

2.2. Generation of Target Reporter Plasmids

1. psiCHECK™-2 vector.
2. Complementary forward and reverse oligodeoxynucleotides for annealing and directional cloning.
3. *Xho*I and *Not*I restriction enzymes (10 U/μL) supplied with recommended 10X buffers. Store at –20°C.
4. MinElute™ Gel Extraction Kit.
5. Antarctic phosphatase.

2.3. Cell Culture and Determination of Knockdown Efficiency by Dual Luciferase Reporter Assays

1. Human embryonic kidney 293 (HEK293) cell line.
2. Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal calf serum (FCS). Store at 4°C.
3. Phosphate-buffered saline containing 0.01% EDTA.
4. Lipofectamine™ 2000 transfection reagent.
5. OptiMEM.
6. Plasmid pCI-eGFP (29), a GFP-expression plasmid under control of the CMV promoter.
7. Dual-Luciferase® Reporter Assay System (Promega, WI, USA). Store the kit at –20°C, and once the luciferase assay substrate has been reconstituted, aliquot and store in the dark at –70°C.
8. Costar® 96 well flat bottom assay plates.
9. Veritas™ Microplate Luminometer.

2.4. Detection of IhRNA Processing by PAGE Northern Blots

1. TriReagent™ (Sigma, MO, USA). Store at 4°C. TriReagent™ should only be used in a fume hood.
2. A 15% polyacrylamide gel with 8 M urea (20 × 20 × 0.8 cm): 40% acrylamide solution (19:1 acrylamide:bisacrylamide) 18.5 mL; 10X TBE 5 mL; urea 24.03 g; ammonium persulphate 20 mg; *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED) 15 μL (*see* **Note 2**).
3. Loading buffer: 95% formamide, 0.025% xylene cyanol, 0.025% bromophenol blue, 0.025% sodium dodecyl sulphate (SDS), 18 mM EDTA.
4. 10X Tris-borate-EDTA buffer (TBE): 890 mM Tris–HCl, 890 mM boric acid and 20 mM EDTA, pH 8.0. Store at room temperature and discard if a precipitate begins to form.
5. Decade™ Marker kit (Ambion, TX, USA). Components include Decade Marker RNA (100 ng/μL in 10 mM

Tris–HCl, pH 7.0), T4 polynucleotide kinase (10 U/μL), 10X kinase reaction buffer, 10X cleavage reagent, gel loading buffer and nuclease-free water. Store the Decade Marker RNA at –70°C, the cleavage reagent at room temperature and all the other reagents at –20°C.

6. Semi-dry blotter.
7. Hybond-N^+ nylon membrane.
8. Ultra-Violet Products (UVP) UV cross-linker.
9. Rapid-hybridization buffer.
10. γ^{32}P-ATP (6,000 Ci/mmol).
11. T4 polynucleotide kinase (PNK), supplied with 10X PNK buffer.
12. DNA oligonucleotides (approximately 18–21 nt) complementary to the antisense strand of each siRNA sequence (*see* **Note 3**).
13. Sephadex columns. These can be prepared using 5 g Sephadex® G-25 in 50 mL TE buffer. Insert 0.5 cm nylon fibre into a 1 mL syringe and then add 1 mL of sephadex/TE solution. Spin at 2,000*g* for 2 min (*see* **Note 4**).
14. Hybridization incubator with a rotisserie.
15. Sodium dodecyl sulphate, 1% solution in water. Store at room temperature. However if a precipitate begins to form, the solution may be heated to re-dissolve precipitate.
16. 20X Sodium chloride sodium citrate (SSC) buffer: 3 M NaCl, 0.3 M sodium Citrate dehydrate, pH 7.0.
17. Medical x-ray film.
18. Phosphorimaging plates.
19. Phosphorscanner.

3. Methods

3.1. Generating U6-Driven lhRNA Vectors Targeting Three Unique Sequences Within HIV-1

3.1.1. The Design of U6-Driven lhRNA Cassettes

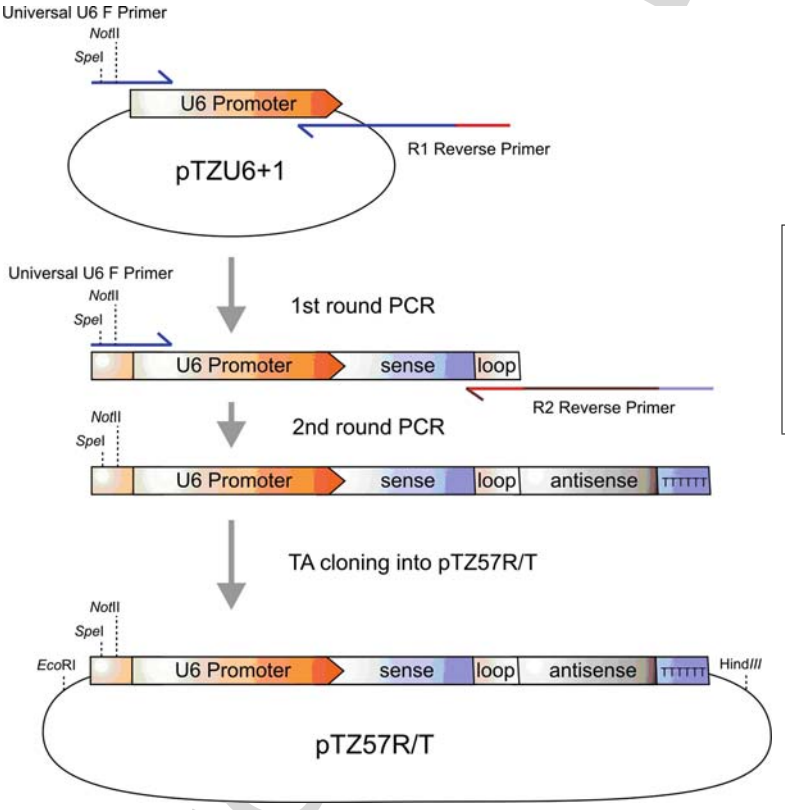
Here we provide some design guidelines for optimally spacing three effective anti-HIV 19 bp siRNA sequences along the length of a U6-driven lhRNA duplex. We also describe important design features which need to be considered for constructing and cloning a plasmid containing an lhRNA expression cassette ex. The lhRNA comprises a stem of 69 bp and a loop of seven nucleotides. The lhRNA is designed to be transcribed from a U6 RNA Pol III promoter such that three 21–23 bp siRNAs can potentially be generated by hDicer cleavage (**Fig. 11.1a, b**). We chose to incorporate the sequences of three unique anti-HIV-1 shRNAs targeting Tat/Rev (referred to as *tat*) (30), Rev/Env

(*rev*) (30) and Vif (*vif*) open reading frames (31) (**Fig. 11.1b**). Some of the design criteria are described below:

1. A single 69 bp lhRNA duplex encodes three separate 19 bp siRNAs (for *tat, rev* and *vif*, respectively). However, since intracellular Dicer cleavage occurs approximately every 22–23 nucleotides along the dsRNA duplex, for optimal Dicer cleavage, each 19 bp siRNAs with two 3′-terminal nucleotides is spaced every 23 nt from the base of the stem (*see* **Fig. 11.1b**) (*see* **Note 5**).
2. The guide strands for each siRNA are placed sequentially in the 3′-arm of the lhRNA duplex. To avoid confusion, the sense strand always represents the 5′-arm such that transcription occurs in the following direction: sense–loop–antisense.
3. Wobble base mismatches (G:U or U:G bp) are introduced into the sense strand of the lhRNA duplex (C is replaced by a T; A is replaced by a G in the DNA sequence) at regular intervals (every 4–8 bp) (**Fig. 11.1b**). These mismatches greatly facilitate PCR of the lhRNA expression cassette, cloning of inverted repeat sequences in *E. coli* and later sequencing of clones (*see* **Note 6**).
4. Individual short hairpin RNAs (shRNAs) with corresponding G:U mismatches serve as positive controls for each siRNA generated from the lhRNA duplex (**Fig. 11.1b**).
5. We chose a random 7 nt loop sequence: 5′-UCAAGAG-3′. A longer loop provides a unique anchor for two rounds of PCR (**Fig. 11.1b** and **Section 3.1.2b**) (see **Note 7**).

3.1.2. Generation of Expressed lhRNAs by a Two-Step PCR

1. U6-expressing lhRNAs are constructed using a two-step PCR which was adapted from Castanotto et al. (27) (**Fig. 11.2**). In the first round of PCR, 10 pg of pTZU6+1 is used as a template.
2. The universal U6 forward primer, 5′-CTA ACT AGT GGC GCG CCA AGG TCG GGC AGG AAG AGG G-3′, is complementary to the 5′-end of the U6 promoter and is used for both rounds of PCR. It includes *Spe*I and *Not*I sites to facilitate later screening of correctly inserted clones (*see* **Section 3.2**).
3. The reverse primer for the first round (R1) of PCR for the lhRNA (*tat-rev-vif*) is complementary to 18–21 nt of the 3′-end of the U6 promoter and contains a linker encoding the *tat-rev-vif* siRNA sequences: lhRNA *tat-rev-vif* (R1) 5′-CTT GAA ATG GAA TGT ATA CCT CTA AAC AAG GCA GCC GAA GAG ACA CAG ACA AGC CCT TCA TCA CTA TCC CCG CGG TGT TTC GTC CTT TCC ACA A-3′.



This figure will be printed in b/w

Fig. 11.2. A two-step PCR strategy for producing a U6-driven lhRNA cassette for cloning in the TA vector pTZ57R/T.

4. We use standard thermocycling conditions: 95°C for 5 min, followed by 30 cycles of denaturing at 95°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s. With the Expand High Fidelity Taq polymerase kit, 10 pmol of each primer is used in a 50 μL reaction. The first round of PCR produces the complementary sequence of the lhRNA sense strand as well as of the loop sequence.
5. For the second round of PCR, a 1:500 dilution of the round one PCR product (approximately 10 pg) is used as the template. The U6 forward primer is used again and the reverse primer sequence (R2) is designed to have 18 nt of overlapping sequence with the loop region of the round one reverse primer. The R2 primer encodes the complementary sequence of the antisense strand of the lhRNA as well as six thymidine (uridine) residues for Pol III-transcriptional termination (**Fig. 11.2**). The primer for round two is lhRNA *tat-rev-vif* (R2) lhRNA *tat-rev-vif* R2 5′-AAA AAA GCG GAG ACA GCG ACG AAG AGC TTG CCT GTG CCT CTT CAG CTA CCT TGT TCA GAA GTA CAC ATC CCA CTC TCT TGA AAT GGA ATG TAT A-3′. This

AQ5

overlap between each pair of reverse primers enables the extension of the PCR product to generate a U6-expressed lhRNA cassette with a transcription termination signal.

6. The conditions for the second round PCR are the same as the first, except for the additional 10 min extension step at completion of thermal cycling. This facilitates cloning into the TA vector, pTZ57R/T.
7. Load 5 μL of each PCR product on a 1.5% agarose gel in 1X TAE buffer, resolve electrophoretically and visualize on a UV transilluminator.

3.1.3. Plasmid Propagation of lhRNA Expression Cassettes

1. If a single band is visualized representing the expected size of the round two PCR product on the agarose gel (*see* **Section 3.1.2**), it can be ligated directly into the TA cloning vector pTZ57R/T (*see* **Note 8**). The ligation reaction is set up by adding together: 4 μL round two PCR product, 1 μL pTZ57R/T, 4 μL 5X ligation buffer and 1 μL (5 U) T4 DNA ligase. The volume is made up to 20 μL with water. The reaction can be left at room temperature overnight.
2. The ligation reaction (7 μL) is used to transform 100 μL competent DH5α cells before plating on ampicillin-containing agar plates.
3. Agar plates contain 40 μL of X-Gal stock and 8 μL of IPTG stock for blue-white screening. Plates containing transformed bacteria are left at 37°C overnight.
4. Pick white colonies and grow in 3 mL of ampicillin-containing LB at 37°C overnight for mini-preparation of plasmid DNA using the Roche high-pure miniprep kit.
5. Screen plasmids for presence of inserts and orientation by digesting plasmid DNA with *Eco*RI and *Spe*I, and *Hin*dIII and *Spe*I, respectively (**Fig. 11.2**). Run digested plasmids on a 3% agarose gel (*see* **Note 9**).
6. Sequence positive clones with M13 forward and reverse primers. Since errors are commonly incorporated in the synthesis of long oligonucleotides, a number of clones may need to be sequenced.

3.2. Generation of Target Reporter Plasmids

1. A standard PCR reaction is used to amplify approximately 200 bp of the gene containing the desired target sequences for *tat*, *rev* and *vif* using 10 pmol each of gene-specific primers and 10 pg of HIV-1 subtype B plasmid, pNL4-3, as the template. The forward primer contains a 5′-*Xho*I restriction site linker, while the reverse primer contains a 5′-*Not*I restriction site linker.

2. PCR products for *tat*, *rev* and *vif* are individually ligated into pTZ57R/T as described above (**Section 3.2**). Plasmid pTZ57R/T, containing the target sequence, is screened by digestion with *Xho*I and *Not*I and the insert is then excised from an agarose gel and purified using the MinElute Gel Extraction kit according to the manufacturer's instructions. Fragments are eluted from the column in 20 μL of TE buffer.
3. To insert target sequences in the 3′-UTR of the *Renilla* luciferase open reading frame, the psiCHECK plasmid backbone is prepared by digestion with 15 units (1.5 μL) each of *Xho*I and *Not*I for 1.5 h at 37°C in a 50 μL volume. One microlitre (5 U) of Antarctic phosphatase (AP) is added to the digestion reaction together with 10X AP buffer and water to 60 μL total volume. The reaction is incubated at 37°C for a further 10 min. The phosphatase reaction is heat-inactivated for 15 min at 65°C followed by resolving the linear psiCHECK vector backbone DNA on a 0.8% agarose gel. The double-digested vector backbone band is excised and purified using the MinElute Gel Extraction kit. Fragments are eluted from the column in 20 μL of TE buffer.
4. To ligate vector backbone to digested PCR fragments, 60 fmol (approximately 50 ng) of purified psiCHECK backbone fragment is ligated with 180 fmol of each *tat*, *rev* and *vif* fragment in a 20 μL reaction volume containing 1 μL (10 U) of T4 DNA ligase and 1X ligase buffer.
5. The ligase reaction is used to transform bacteria as described previously: 10 μL of ligation reaction is used to transform 100 μL of chemically competent *E. coli*; the reaction is plated onto ampicillin-containing agar plates and incubated overnight at 37°C.
6. Colonies are screened by digestion with *Xho*I and *Not*I and clones with the correct sized insert are subsequently sequenced using a forward primer specific to the *Renilla* luciferase ORF: 5′-GAG GAC GCT CCA GAT GAA ATG-3′.

3.3. Cell Culture and Knockdown Assays

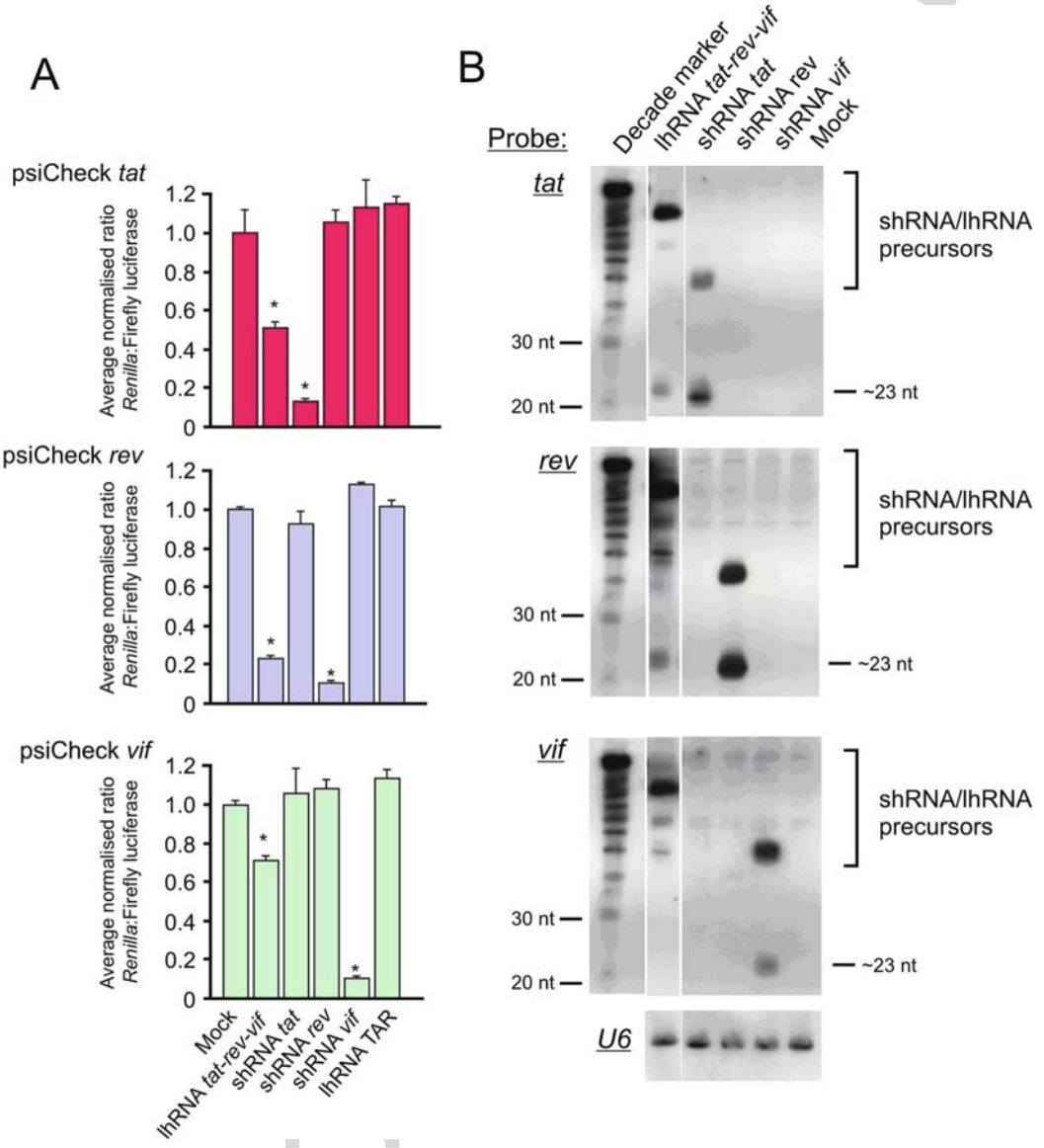
1. The HEK293 cell line is maintained with DMEM supplemented with 10% heat-inactivated FCS at 37°C and 5% CO_2. Cells are passaged using PBS containing EDTA.
2. Seeding of 120,000 cells per well in 24 well plates is carried out 24 h prior to transfection. lhRNA-encoding plasmid (750 ng) together with target reporter plasmid (150 ng) are co-transfected in a 5:1 ratio (*see* **Note 10**). A 100 ng of plasmid pCI-eGFP is also co-transfected to control for

transfection efficiency. Each transfection should be performed in triplicate. Transfections are carried out using 1 μL lipofectamine 2000 per 1 μg DNA and 0.2X optiMEM.

3. Twenty-four hours post-transfection media should be replaced and the dual luciferase reporter assay is carried out a further 24 h later according to the manufacturer's instructions. All *Renilla* luciferase values are normalized against background firefly luciferase values. The average expression ratio for a control plasmid containing the U6 promoter with no hairpins is set to 100%, and relative expression levels of other samples are calculated accordingly (**Fig. 11.3a**).

3.4. PAGE Northern Blot Hybridization

1. Cells are cultured and transfected as previously described. For northern blot analysis cells are seeded to approximately 70% confluence in 10 cm culture plates and transfected 24 h later with 18 μg hairpin construct. Forty-eight hours post-transfection, total RNA is extracted using TriReagent according to the manufacturer's instructions. Standard RNA-handling procedures to avoid RNase contamination should be followed.
2. Prepare a 15% polyacrylamide gel with a 1:19 ratio of bis:acrylamide with 8 M urea and 1X TBE.
3. Ambion Decade Marker should be prepared as per the kit's instructions.
4. Add an equal volume of loading dye to 30 μg of each RNA sample, heat at 80°C for 5 min and then return sample to ice before loading.
5. To warm the apparatus prior to the loading of RNA samples, the gel should be pre-electrophoresed in 0.5X TBE buffer at 200 V for 30 min.
6. Resolve RNA samples and labelled markers at constant voltage (200–300 V) and run the gel until the bromophenol blue band migrates to within 1 cm from the bottom of the gel.
7. Transfer to a positively charged membrane (Hybond-N^+) using a semi-dry blotter. For the transfer, the gel and membrane are tightly placed between six layers of 0.5X TBE-soaked chromatography paper and the current applied for 1 h.
8. UV cross-link the RNA to the membrane at 2,000 × 100 μJ/cm^2.
9. Bake the membrane at 80°C for 1 h.
10. Prehybridize the membrane in 10 mL of pre-warmed rapid-hyb buffer at 42°C for 20 min.



This figure will be printed in b/w

Fig. 11.3. Knockdown of individual reporter gene targets and intracellular processing of lhRNA and shRNA-expressing plasmids in transfected HEK293 cells. (**a**) Average normalized *Renilla*:firefly luciferase activity determined 48 h after transfecting HEK293 cells with the psiCHECK *tat-rev-vif* target together with each individual shRNA control and an irrelevant lhRNA control (lhRNA TAR) (*$p < 0.5$, for experiments conducted in triplicate). (**b**) Northern blot analysis of RNA extracted from HEK293 cells that had been transfected with lhRNA *tat-rev-vif* and shRNA-expressing plasmids. The blot was probed with an oligonucleotide that was complementary to putative *tat*, *rev* and *vif* guide sequence. The blot was stripped and re-probed with an oligonucleotide complementary to U6 snRNA to control for equal RNA loading.

11. Label 2 μL oligo probe (20 μM) with fresh γ-^{32}P-ATP in a 20 μL total volume using 1 μL PNK (5 U) and 2 μL 10X PNK buffer. Dilute to 50 μL and spin through a freshly prepared sephadex G-25 column. Add the labelled

probe to the hybridization buffer and leave rotating at 42°C overnight.

12. Wash membrane once with 0.1% SDS and 5X SSC in a volume of 50 mL at room temperature for 20 min. Wash membrane twice with 0.1% SDS and 1X SSC in a volume of 50 mL at 42°C for 15 min each (*see* **Note 11**).
13. Place membrane in cling wrap and expose membrane to x-ray film for 24–72 h before developing film. The membrane can also be exposed to a phosphor plate and scanned using a phosphor-imager (e.g. Fuji FLA-7000).
14. Strip membrane in 50 mL 1% SDS at 80°C for 30 min. The membrane can then be re-probed as described above.
15. Once stripped, the membrane is re-probed using a 20-mer oligonucleotide antisense to the U6 snRNA. This serves as an ideal loading control.

4. Notes

1. We also find standard Taq polymerase easily amplifies long hairpins but does introduce mismatches which need to be screened by sequencing. The use of high-fidelity thermostable polymerases may improve sequence fidelity but often hampers the generation PCR products useful for subsequent cloning. Nevertheless, we have used the Expand High Fidelity^{PLUS} PCR System (Roche) to produce lhRNA-encoded PCR fragments.
2. Ammonium persulphate should be made up fresh each time. Therefore make up small quantities at a time. Alternatively, small 500 μL aliquots can be frozen for up to 3 months.
3. Locked nucleic acid (LNA) oligonucleotide probes may be used for increased specificity.
4. The sephadex G-25 should be hydrated by overnight agitation in 50 mL TE buffer. Briefly centrifuge the solution at 4,000 rpm for 2 min, discard the supernatant and add another 50 mL TE buffer. Repeat this two to three times before adding a final volume of TE buffer.
5. If the lhRNA is designed to encode more than two siRNAs, the third siRNA may not be processed efficiently or may be present at too low a concentration to be an effective inhibitor. Although efficient production of all siRNAs is not always guaranteed, there are modifications that may be employed to improve the yield of the third siRNA. The

processing of multiple siRNAs in a single duplex may be augmented by empirically testing different spatial arrangement of siRNAs along the hairpin stem. Although gross generalizations regarding the most favourable siRNA spacing cannot yet be made, improvements can be achieved by inserting or deleting random base pairs at each siRNA junction as well as before the loop sequence.

6. Wobble mismatches can be strategically used to improve the thermodynamic asymmetry of each siRNA, thereby facilitating correct guide strand incorporation into RISC. Moreover, there is some anecdotal evidence to suggest that wobble base pairs help prevention of the induction of the IFN response by masking protein kinase R (PKR) recognition (32).
7. There is some evidence to suggest that increasing the duplex length at the loop side of the duplex (by 5 bp or more) may improve cleavage of the third siRNA (26). However, this has not been tested by us.
8. If non-specific bands are visible on the agarose gel, purify the desired PCR fragment from the gel and then ligate 10 μL gel-purified DNA into the TA cloning vector.
9. Resolve bands properly on a high percentage agarose gel (e.g. 3% gel). Clones with minor deletions and insertions can be detected on the gel and screened out.
10. Lower ratios of hairpin to target can be used without significantly affecting efficient target knockdown.
11. If high levels of background are present on the film, wash steps can be repeated.

References

1. Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., and Mello, C.C. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature*, **391**, 806–811.
2. Elbashir, S.M., Lendeckel, W., and Tuschl., T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Develop*, **15**, 188–200.
3. Paddison, P.J., Caudy, A.A., Bernstein, E., Hannon, G.J., and Conklin, D.S. (2002) Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. *Genes Dev*, **16**, 948–958.
4. Brummelkamp, T.R., Bernards, R., and Agami, R. (2002) A system for stable expression of short interfering RNAs in mammalian cells. *Science*, **296**, 550–553.
5. Filipowicz, W., Bhattacharyya, S.N., and Sonenberg, N. (2008) Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? *Nat Rev Genet*, **9**, 102–114.
6. von Eije, K.J., ter Brake, O., and Berkhout, B. (2008) Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J Virol*, **82**, 2895–2903.
7. Boden, D., Pusch, O., Lee, F., Tucker, L., Shank, P.R., and Ramratnam, B. (2003) Promoter choice affects the potency of HIV-1 specific RNA interference. *Nucl Acids Res*, **31**, 5033–5038.
8. Das, A.T., Brummelkamp, T.R., Westerhout, E.M., Vink, M., Madiredjo, M., Bernards,

R., and Berkhout, B. (2004) Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. *J Virol*, **78**, 2601–2605.

9. Schwarz, D.S., Ding, H., Kennington, L., Moore, J.T., Schelter, J., Burchard, J., Linsley, P.S., Aronin, N., Xu, Z., and Zamore, P.D. (2006) Designing siRNA that distinguish between genes that differ by a single nucleotide. *PLoS Genet*, **2**, e140.
10. ter Brake, O., 't hooft, K., Liu, Y.P., Centlivre, M., von Eije, K.J., and Berkhout, B. (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol Ther*, **16**, 557–564.
11. Liu, Y.P., Haasnoot, J., Ter Brake, O., Berkhout, B., and Konstantinova, P. (2008) Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron. *Nucl Acids Res*, **36**, 2811–2824.
12. Aagaard, L.A., Zhang, J., von Eije, K.J., Li, H., Saetrom, P., Amarzguioui, M., and Rossi, J.J. (2008) Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Ther*, **53**, 1536–1549.
13. Li, M., Li, H., and Rossi, J.J. (2006) RNAi in combination with a ribozyme and TAR decoy for treatment of HIV infection in hematopoietic cell gene therapy. *Ann NY Acad Sci*, **1082**, 172–179.
14. Grimm, D. and Kay, M.A. (2007) Combinatorial RNAi: a winning strategy for the race against evolving targets? *Mol Ther*, **15**, 878–888.
15. Haasnoot, J. and Berkhout, B. (2009) Nucleic acids-based therapeutics in the battle against pathogenic viruses. *Handb Exp Pharmacol*, **189**, 243–263.
16. Scherer, L., Rossi, J.J., and Weinberg, M.S. (2007) Progress and prospects: RNA-based therapies for treatment of HIV infection. *Gene Ther*, **14**, 1057–1064.
17. Saayman, S., Barichievy, S., Capovilla, A., Morris, K.V., Arbuthnot, P., and Weinberg, M.S. (2008) The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA. *PLoS ONE*, **3**, e2602.
18. Manche, L., Green, S.R., Schmedt, C., and Mathews, M.B. (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. *Mol Cell Biol*, **12**, 5238–5248.
19. Marques, J.T., Devosse, T., Wang, D., Zamanian-Daryoush, M., Serbinowski, P., Hartmann, R., Fujita, T., Behlke, M.A., and Williams, B.R. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. *Nat Biotechnol*, **24**, 559–565.
20. Robbins, M.A., Li, M., Leung, I., Li, H., Boyer, D.V., Song, Y., Behlke, M.A., and Rossi, J.J. (2006) Stable expression of shRNAs in human $CD34^+$ progenitor cells can avoid induction of interferon responses to siRNAs in vitro. *Nat Biotechnol*, **24**, 566–571.
21. Lund, E. and Dahlberg, J.E. (2006) Substrate selectivity of exportin 5 and Dicer in the biogenesis of microRNAs. *Cold Spring Harb Symp Quant Biol*, **71**, 59–66.
22. Macrae, I.J., Zhou, K., Li, F., Repic, A., Brooks, A.N., Cande, W.Z., Adams, P.D., and Doudna, J.A. (2006) Structural basis for double-stranded RNA processing by Dicer. *Science*, **311**, 195–198.
23. Weinberg, M.S., Ely, A., Barichievy, S., Crowther, C., Mufamadi, S., Carmona, S., and Arbuthnot, P. (2007) Specific inhibition of HBV replication in vitro and in vivo with expressed long hairpin RNA. *Mol Ther*, **15**, 534–541.
24. Barichievy, S., Saayman, S., von Eije, K.J., Morris, K.V., Arbuthnot, P., and Weinberg, M.S. (2007) The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5′ long terminal repeat. *Oligonucleotides*, **17**, 419–431.
25. Soifer, H.S., Sano, M., Sakurai, K., Chomchan, P., Saetrom, P., Sherman, M.A., Collingwood, M.A., Behlke, M.A., and Rossi, J.J. (2008) Role for the Dicer helicase domain in the processing of thermodynamically unstable hairpin RNAs.. *Nucl Acids Res*, **36**, 6511–6522.
26. Sano, M., Li, H., Nakanishi, M., and Rossi, J.J. (2008) Expression of long anti-HIV1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. *Mol Ther*, **16**, 170–177.
27. Castanotto, D., Li, H., and Rossi, J.J. (2002) Functional siRNA expression from transfected PCR products. *RNA*, **8**, 1454–1460.
28. Bertrand, E., Castanotto, D., Zhou, C., Carbonnelle, C., Lee, N.S., Good, P., Chatterjee, S., Grange, T., Pictet, R., Kohn, D., Engelke, D., and Rossi, J.J. (1997) The expression cassette determines the functional activity of ribozymes in mammalian cells by controlling their intracellular localization. *RNA*, **3**, 75–88.
29. Passman, M., Weinberg, M., Kew, M., and Arbuthnot, P. (2000) In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis

Bx sequence in cultured cells. *Biochem Biophys Res Commun*, **268**, 728–733.

30. Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P., and Rossi, J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat Biotechnol*, **20**, 500–505.
31. Lee, S.K., Dykxhoorn, D.M., Kumar, P., Ranjbar, S., Song, E., Maliszewski, L.E., Francois-Bongarcon, V., Goldfeld, A., Swamy, N.M., Lieberman, J., and Shankar, P. (2005) Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood*, **106**, 818–826.
32. Akashi, H., Miyagishi, M., Yokota, T., Watanabe, T., Hino, T., Nishina, K., Kohara, M., and Taira, K. (2005) Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Mol Biosyst*, **1**, 382–390.

Chapter 11

Q. No.	Query
AQ1	The sentence "Although in mammalian cells, . . .(18) Although certain. . ." has been changed to two sentences with the first one reading "In mammalian cells. . .". Please check if the edit is ok.
AQ2	The printed version of the book will have only b/w figures. Please rephrase the caption without the mention of color.
AQ3	Please check the edit of "cassette. ex" to "cassette ex" in the sentence "We also describe important design features. . .".
AQ4	Please clarify what is meant by Section. 3.1.2b as such a section does not exist.
AQ5	Please clarify whether "lhRNA tat-rev-vif R2" in the sentence "The primer for round two. . ." can be deleted or alternatively it can be separated from the earlier sentence by a period (.).

RNA Interference-Based Gene Expression Strategies Aimed at Sustained Therapeutic Inhibition of HIV

Samantha Barichievy, Sheena Saayman, Patrick Arbuthnot and Marc S. Weinberg*

Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, University of Witwatersrand, Johannesburg, South Africa.

Abstract: The naturally-occurring RNA interference (RNAi) pathway represents a powerful tool for the sequence-specific post-transcriptional silencing of gene expression. By exploiting the endogenous mammalian RNAi pathway, several expression-based strategies have been developed to inhibit human immunodeficiency virus (HIV) gene expression and replication. This approach potentially has utility as a protective 'therapeutic vaccine' of virus-susceptible lymphocytes. In this review we discuss new developments aimed at improving efficacy and delivery of novel RNAi-based gene expression antiviral strategies. Particular attention is given to advances in combinatorial gene expression systems that prevent the emergence of RNAi-resistant virus by simultaneously targeting multiple HIV targets. Potential usefulness of silencing host factors that are required for viral replication is also discussed. These approaches form the basis for a number of promising ongoing and future clinical trials aimed at providing an effective, safe and prolonged single-intervention therapy for HIV/AIDS.

Keywords: HIV, RNAi, shRNA, Gene therapy.

1. INTRODUCTION

Recent estimates indicate that globally there are over 33 million people infected with human immunodeficiency virus type 1 (HIV-1) [1]. The epidemic is particularly severe in sub-Saharan Africa which accounted for 67% of all infected individuals and 72% of AIDS deaths in 2007 [1]. Current therapies aimed at inhibiting HIV replication include the use of drugs that target viral reverse transcriptase and protease. More recently, viral entry inhibitors and drugs that counter integrase have been introduced. These agents, particularly in combination as a cocktail of highly active antiretroviral therapy (HAART), have had an important positive impact on the morbidity and mortality of HIV-related illness [2]. However, current treatment regimens have significant limitations. These include toxicities, resistance and the inability to eradicate a latent infection. In addition, most therapeutic agents have been developed to target HIV-1 subtype B, which affects individuals predominantly in Western Europe and North America, and therefore these drugs are not necessarily tailored to meet the therapeutic burden of HIV in sub-Saharan Africa, where subtype C infection is common. These concerns, along with ensuring patient compliance with treatment and the high cost of improved treatment regimes, have prompted the search for innovative and globally-effective therapies to counter HIV infection.

The discovery by Fire, Mello and colleagues that double-stranded RNA can effect powerful inhibition of gene expression has spawned many important biotechnological advances [3]. Harnessing this highly-conserved biological pathway, known as RNA interference (RNAi), is potentially very useful for advancing antiviral gene therapy [3, 4]. Importantly, earlier development of nucleic acid-based technologies, such as antisense and ribozymes, established a foundation for accelerated progression of RNAi research from the laboratory bench to the clinic. Indeed, today there is a flurry of activity in testing the antiviral potential of RNAi-based approaches. This review will focus on recent advances in RNAi-based HIV therapy, with particular emphasis on the mechanism of action of novel gene expression-based strategies that are intended for long-term single-intervention clinical applications. Anti-HIV gene therapy approaches have unique hurdles to overcome before they are ready for clinical use. Eliminating latent infection, efficient delivery of antiviral effectors and RNAi-induced viral escape are some of the important considerations. Drug resistance may be solved by inhibiting multiple targets and new strategies aimed at usurping natural RNAi structures to generate safe combinatorial RNAi modalities will be highlighted. Recently published genome-wide screens have identified many host factors that are required for viral replication. Advances in the silencing of these cellular genes as a treatment approach and means of preventing viral escape, will also be discussed. Recent and ongoing preclinical and clinical developments in the field are evaluated before concluding with a discussion of the future prospects of RNAi-based antiviral therapies.

2. THE MAMMALIAN RNAI PATHWAY

In mammals, RNAi is triggered by double-stranded RNA (dsRNA), which is processed into short RNAs of ~20-30 nucleotides in length. These short RNAs associate with members of the Argonaute (Ago) family of proteins to regulate gene expression at the transcriptional and post-transcriptional level. RNAi plays an important role in many fundamental cellular functions and has increased our appreciation for the major role of small non-coding RNAs in

*Address correspondence to this author at the Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, University of Witwatersrand, Private Bag 3, Wits 2050, Johannesburg, South Africa; Tel: +27-11-717-2561; Fax: +27-11-717-2395;
E-mail: marc.weinberg@wits.ac.za

1568-0266/09 $55.00+.00

biology. However, it is activation of RNAi with exogenous regulators of gene expression, which makes their discovery particularly important for therapeutic application.

RNAi-based therapeutic approaches have been made possible by appropriating elements of the endogenous mammalian microRNA (miRNA) biogenesis pathway to achieve post-transcriptional gene silencing (PTGS) (Fig. **1**). MiRNAs represent a class of short ~22 nucleotide (nt) RNAs derived from longer hairpin RNA-containing precursors that are found in single or polycistronic clusters and in exonic or intronic regions of coding and non-coding mRNA [reviewed in [5, 6]]. RNA Pol II transcripts containing short stem-loop structures called primary microRNAs (pri-miRNAs) are processed in two successive steps. The first occurs in the nucleus where the hairpin-like structure of the pri-miRNA is recognised and cleaved by the microprocessor complex, which comprises the RNAse III enzyme Drosha and its partner, DiGeorge critical region 8 protein (DGCR8). A shorter 70-80 nt hairpin duplex known as a precursor-microRNA (pre-miRNA) is produced by the microprocessor complex [7, 8]. In rare cases, short intronic sequences may be processed by the spliceosome to form pre-miRNA hairpins or "mirtrons" without requiring Drosha cleavage [9-12]. Once formed, pre-miRNAs containing a 2 nt 3′ hydroxyl overhang are exported from the nucleus to the cytoplasm by the nuclear karyopherin exportin-5 [13, 14]. The second processing step occurs in the cytoplasm where the pre-miRNA is cleaved by the RNase III enzyme Dicer to produce a ~22 base pair, staggered miRNA/anti-miRNA duplex [15, 16]. Dicer and its binding partner, TAR RNA-binding protein (TRBP) [17], loads one of the strands, referred to as the mature miRNA or "guide strand", into the RNA-induced silencing complex (RISC). In its simplest form RISC comprises Argonaute 2 (Ago2) and Dicer/TRBP [18, 19]. The other strand of the RNA duplex, known as the "passenger strand", may be cleaved within RISC by the RNase H-like Piwi domain of Ago2 [20-22]; or simply released from the complex. Selection of the guide strand is based on asymmetric thermodynamic stability of the RNA duplex ends [23]. The strand with the weaker paired bases at the 5′ end of the duplex is preferentially incorporated into RISC as a guide [reviewed in [24, 25]].

The mature miRNA guide strand associates with Ago2-containing RISC and acts as a guide by targeting 2-7 nucleotide "seed regions" of the 3′ untranslated regions (3′ UTRs) of mRNAs [26]. Complementary matches of the guides with 3′ UTRs induce translation suppression by several known mechanisms: transcriptional cleavage, blocking of ribosomal function, deadenylation or shunting of mRNAs to transcriptionally inactive cytoplasmic P bodies [reviewed in [27, 28]]. Guide strands with near-perfect sequence complementarity to their cognate targets, which is a feature of short interfering RNAs (siRNAs), effect gene silencing by Ago2-mediated post-transcriptional cleavage of targeted RNAs. Mammalian miRNAs rarely bind their targets completely and hybridization of the seed region alone is sufficient for translational suppression [29]. RNAi guide sequences may additionally associate with Ago1 in a RNA-induced transcriptional silencing complex (RITS) to induce transcriptional gene silencing (TGS). This is characterized by the targeting of siRNAs or short antisense RNAs to promoter elements and resultant transcriptional inhibition is caused by induction of silent-state epigenetic modifications of DNA and associated nucleosomes (Fig. **1**) [30-33]. As siRNA-directed TGS elicits more permanent epigenetic modifications, TGS may offer a more sustained inhibition of gene expression than PTGS. This represents an exciting and important new approach to therapeutic HIV gene silencing.

Recently, other RNAi-based pathways have been uncovered, which make use of specific dsRNA substrates. It is premature to speculate on what role these newer RNAi pathways may have in generating novel therapeutics. In the germline, a number of short 24-31 nt RNAs are associated with Piwi-family proteins or Piwi-interacting RNAs (piRNAs) (Fig. **1**) [reviewed in [34]] and originate from repeat-rich regions of the genome. piRNAs are processed through a distinct Dicer-independent mechanism [35] and cause transcriptional silencing of transposons by establishing *de novo* DNA methylation in murine fetal testes [36-38]. In recent studies of mouse oocytes and embryonic stem (ES) cells, an abundant class of endogenous siRNAs or endo-siRNAs was discovered. These RNAs are thought to be derived from transcripts with long inverted repeats or from convergent and divergent transcripts of pseudogenes or transposons (Fig. **1**) [12, 39, 40]. Apart from blocking retro-transposition, little is known about the function of endo-siRNAs. However, the ubiquity of overlapping transcripts suggests that there are more endo-siRNAs with their putative RNA targets that are present within the genome.

3.EXOGENOUS ACTIVATORS OF RNAI

There is a myriad of exogenous dsRNA triggers of the RNAi pathway that have been introduced into cells to effect post-transcriptional degradation of mRNAs. The most common form of silencing is typically achieved by administering chemically synthesized siRNAs. While synthetic siRNAs are not the focus of this review, it is worth pointing out that these species do have some advantages over expressed RNAi effectors. siRNAs can be chemically modified to improve their efficacy *in vivo*. This may be achieved by increasing resistance to serum nucleases, enhanced guide strand function, attenuation of innate immunostimulation, improved pharmacokinetic properties and the addition of targeting ligands for uptake by specific cells *in vivo* [reviewed elsewhere in [4, 41]]. Expressed RNAi activators however have other advantages which are discussed below.

Most RNAi expression approaches generate hairpin duplexes that mimic natural miRNA precursors, such as pri-miRNAs and pre-miRNAs (Fig. **2**). Expressed effectors differ from their synthetic counterparts by providing a more sustained suppression of the target RNA through the continued renewal of RNAi precursors. RNAi expression cassettes are also compatible with highly efficient viral vectors, which may allow exploitation of the cell-specific targeting, integration and expression characteristics of the recombinant viruses. Short hairpin RNA (shRNA)-encoding sequences typically mimic pre-miRNAs and are widely used in gene-based silencing platforms. These are usually inserted downstream of a RNA Pol III promoter, such as H1 and U6, to generate transcripts with defined 5′ and 3′ termini. This allows for efficient nuclear export and processing by Dicer in

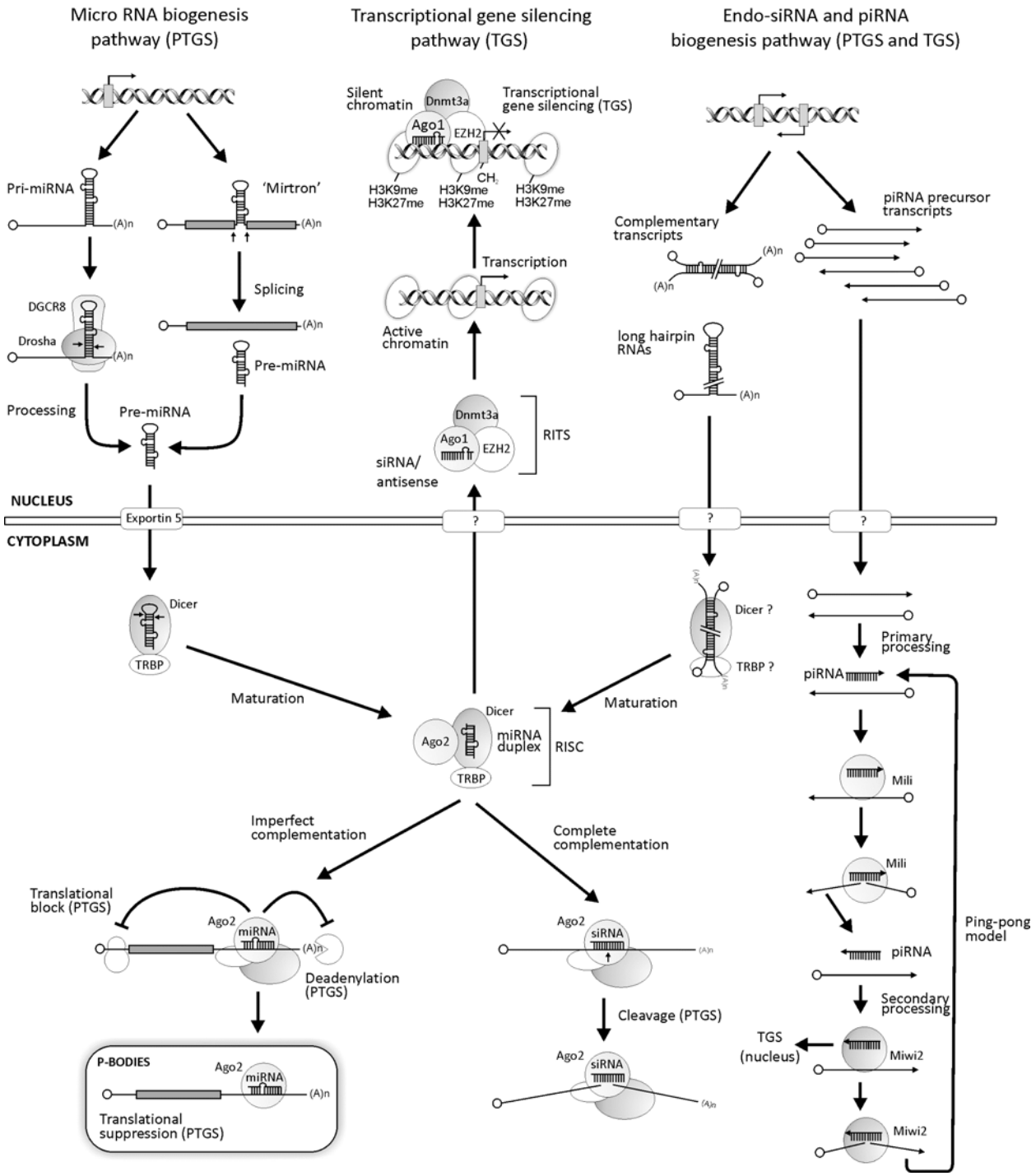


Fig. (1). Mammalian RNAi regulatory pathways. Pri-miRNAs of approximately 100 nt are microRNAs precursors and are transcribed by Pol II. Processing in the nucleus by Drosha/DGCR8 produces shorter hairpin duplexes known as a 70-80 nt pre-miRNAs. Some short intronic sequences, or "mirtrons", can be directly processed by the spliceosome to form pre-miRNA-like hairpins without requiring Drosha cleavage. Pre-miRNAs are exported from the nucleus to the cytoplasm by the exportin-5 following which recognition and cleavage by Dicer/TRBP produces a ~22 base pair, staggered miRNA duplex with 2 nt 3′ overhangs. Dicer/TRBP loads one of the strands, the "guide strand", into RISC and directs cleavage or translational suppression of cognate RNA targets. RNA-induced trancriptional gene silencing (TGS) in mammals is effected by a short guide antisense RNA guide strand in the RITS complex, which is directed to promoter sequences in the nucleus. TGS may require the presence of low-copy promoter-derived transcripts to direct silent heterochromatin marks (H3K9 and/or H3K27 methylation) and DNA methylation at the targeted locus [149, 150]. Endogenous siRNAs (endo-siRNAs) are derived from long hairpin sequences and complementary transcripts which are processed by Dicer into siRNAs. Piwi-interacting RNAs (piRNAs) are 24-31 nt short RNAs derived from single-stranded precursors that are expressed from transposons or genomic repeat elements in the germline. In the "ping-pong model", primary piRNAs interact with the Piwi protein Mili to cleave a transcript that generates a piRNA for incorporation into Miwi2, which in turn cycles back to produce new Mili-interacting piRNAs [151].

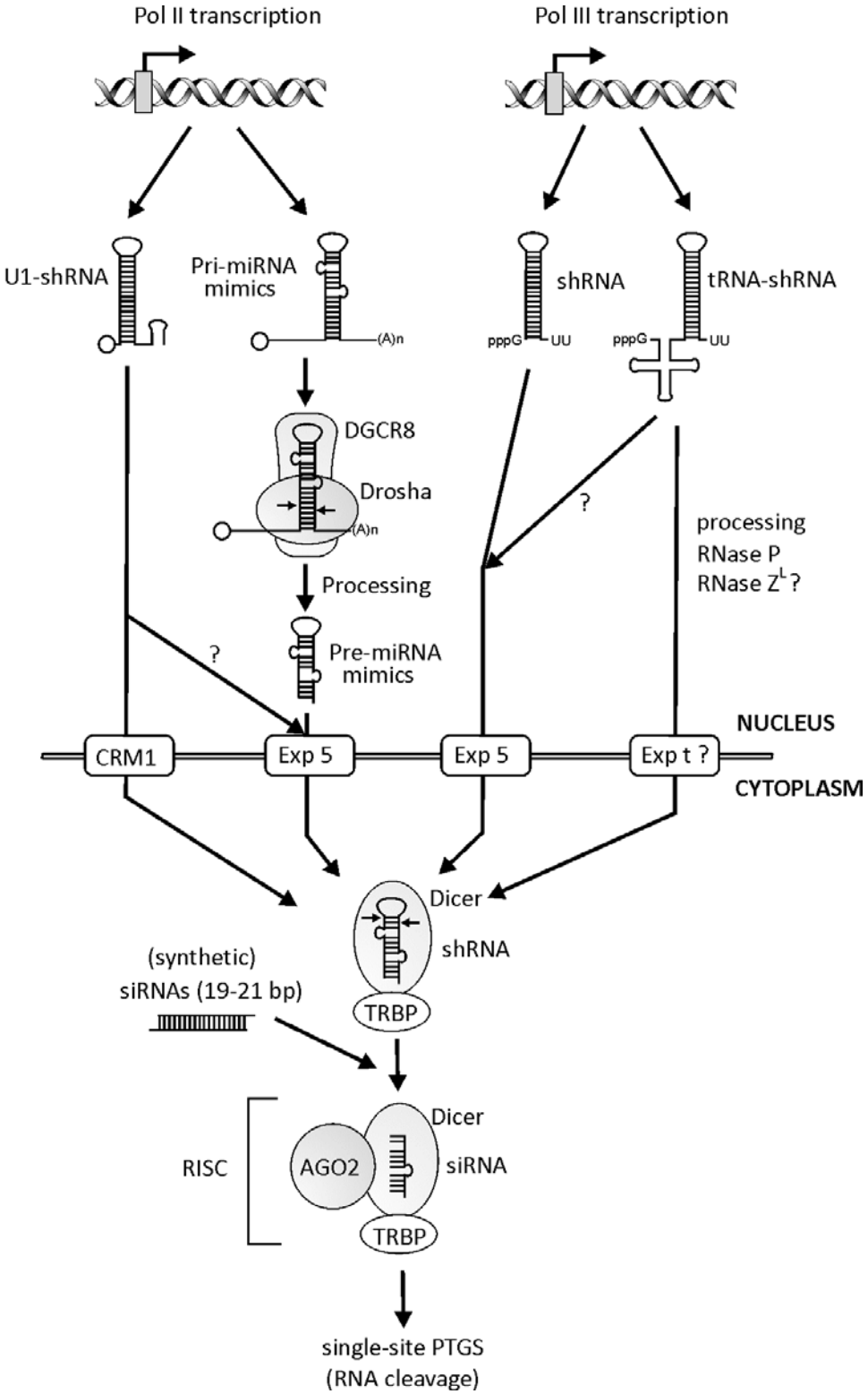


Fig. (2). Exogenous RNAi-mediated gene silencing. RNA Pol II-derived transcripts introduce hairpin duplexes which structurally mimic mono or polycistronic pri-miRNAs which are recognised and processed by Drosha/DGCR8 to form pre-miRNA-like hairpins. These hairpins are cleaved by Dicer/TRBP following export *via* exportin-5. The Pol II-generated U1 short hairpin RNA (shRNA) transcripts, which contain a 3′ terminal B-box, structurally mimic pre-miRNAs but are likely to be exported by the CRM1 and exportin-5 pathways prior to Dicer/TRBP cleavage [152]. RNA Pol III promoters express shRNAs with defined 5′ and 3′ termini. U6 or H1-derived shRNAs, like pre-miRNAs, have 2 nucleotide 3′ overhangs and exit the nucleus *via* exportin-5. $tRNA^{Lys3}$ and $tRNA^{Val}$ Pol III promoters may also be used to produced tRNA-shRNAs for processing in the nucleus by 5′ and 3′ tRNA processing enzymes prior to export [43, 153]. If unprocessed by RNase Z^L, tRNA-shRNAs may exit the nucleus *via* exportin-t [154]. Synthetic siRNAs can be introduced for direct loading into Ago2-RISC.

the cytoplasm. Other transcriptional regulatory elements, such as U1 Pol II and tRNA Pol III promoters, have also been used (Fig. **2**). Transcripts derived from these cassettes have the advantage of utilising a different nuclear export pathway to that of endogenous miRNAs. This is important to minimise disruption of the function of the naturally occurring RNAi activators [42-44]. Most Pol III promoters have the drawback of being constitutively active and not being amenable to tight transcriptional regulation. Pol II promoters, which can be regulated more easily by cell-specific environmental factors, have recently become more popular for the expression of pri-miRNA mimics (discussed later).

4. RNAI-MEDIATED TARGETING OF HIV RNAS

4.1. Incoming Viral Genomic RNA as a Target

Incoming, uncoated HIV RNA is an enticing target for RNAi-mediated inhibition. Blocking viral replication at this stage would prevent proviral integration and subsequent viral gene expression and replication. There is still some debate as to whether incoming RNA is accessible to RNAi-mediated attack. Several early studies suggested that incoming RNA could be targeted using siRNAs [45-47]. Incoming viral RNA, represented by two viral RNA genomes, is continually bound by proteins such as p17 matrix, integrase and reverse transcriptase [48]. This association may limit access of the RNAi machinery to the target HIV genomic RNA. Also, following membrane fusion between the host cell and virion, the viral core particle is only partially dissolved while the RNA genome is reverse transcribed and transported towards the nucleus. Furthermore, reverse transcription usually occurs within 6 hours of viral entry into lymphocytes [49], providing a very short window of opportunity for RNAi-mediated inhibition of incoming viral RNA. Indeed two thorough investigations using synthetic siRNAs and shRNAs against a wide range of targets indicate that the newly-released viral genome remains encapsidated or partially-encapsidated during reverse transcription, thus blocking access to guide strand-loaded RISC [50, 51]. These studies indicate that targeting of viral mRNAs is a better approach to achieving RNAi-mediated HIV inhibition.

4.2. Post-Transcriptional Inhibition of HIV mRNAs and Viral Escape

Targeting of viral mRNA species transcribed from integrated provirus has been more successful and RNAi strategies have been used against every coding and non-coding genomic sequence to inhibit viral replication. The *rev* open reading frame (ORF) was one of the first viral genes to be targeted using siRNAs and shRNAs [46, 52]. These initial investigations showed that viral transcripts generated early during the life cycle were accessible to RNAi-mediated inhibition, but not all sites were susceptible to silencing. Additional information on HIV target site accessibility was gathered using siRNAs/shRNA against the Tat-responsive (TAR) stem-loop RNA structure of the 5′ long terminal repeat (LTR). The TAR target was found to be refractory to RNAi knockdown and emphasizes the importance of potential inhibition of gene silencing by secondary-structure RNA folding [47, 53, 54].

Single nucleotide substitutions within target mRNA, especially in the seed region, are sufficient for viral protection from RNAi effectors [55]. In one example, a shRNA targeted to *tat* mRNA became ineffective after 25 days and viral genome analysis showed that a single base substitution within *tat* was responsible for the resistance [56]. Similarly, escape mutants appeared following several weeks of exposure to a shRNA targeting the *nef* mRNA. Further analysis revealed that base substitutions or deletions within *nef* modified the shRNA target [57]. These two examples involve targeting of non-essential viral genes but designing siRNAs against essential genes may also result in emergence of resistance [58, 59]. Even though *env* and *rev* ORFs overlap, viral breakthrough does occur. Inhibition of infection with three different HIV primary isolates in the presence of a single siRNA targeted to overlapping *env*/*rev* mRNA was not absolute. Escape mutants bearing two point mutations in the target sequence evolved readily [58]. The use of shRNAs targeted to essential viral sequences did not arrest all viral escape, although there was restriction of escape possibilities. Mutations were limited to non-silent changes that presumably allowed RNAi resistance while maintaining viral fitness [59]. Viral escape from RNAi does not always involve target sequence mutations. Sequencing of two viral mutants replicating in the presence of shRNAs targeted to *nef* mRNA revealed substitution mutations upstream of the siRNA target. Resistance was caused by alterations in viral mRNA secondary structures which probably prevented access of activated RISC complex to its cognate [60]. Even more unexpected is the emergence of escape through the modulation of transcription, Mutations in non-targeted LTR promoter sequences have been shown to compensate for RNAi-mediated inhibition by upregulating viral gene transcription [61]. Notwithstanding these additional factors associated with resistance, much of the focus has shifted to targeting conserved vital sequences within the HIV genome, since HIV is less able to mutate these sites without loss of fitness [59, 62-66]. Further evidence of the extent of RNAi tolerance to sequence polymorphisms emerged from a study using expressed shRNAs against three targets: a highly variable *rev* sequence, a *gag* site conserved across subtype B strains only, and a *vif* sequence conserved across all subtypes [64]. *Rev*-specific shRNAs inhibited the least number of isolates, regardless of subtype. The *gag*-specific shRNAs protected against subtype B virus alone and the anti-*vif* shRNAs were effective against all isolates [64]. These results suggest that many separate strains of HIV may be silenced by targeting conserved sequences. It appears that the sequence space for the virus to find resistance is limited and many of the sequence changes in conserved target sites are found within natural variants of the viral population [59]. It will be interesting to determine the fitness costs *in vivo* that are associated with escaping the effects of RNAi.

Experiments by Nishitsuji *et al* also revealed that HIV dosage plays a key role in determining whether RNAi-mediated inhibition is effective. Specifically, shRNAs designed to target HIV integrase showed some inhibition of HIV replication when shRNA-expressing CD4+ T cells were challenged with virus at a low dose of infection [53]. This effect was abrogated during long-term (22 days) culture or following a high-dose infection [53]. By increasing the

dosing regimen of virus infection on T-cells stably expressing anti-HIV shRNAs, von Eije *et al* were able to identify robust shRNA-mediated inhibition by comparing the sustained inhibitory effects of each shRNA on its respective cognate target [67]. This suggests that although highly conserved viral sequences allow for the discovery of many effective anti-HIV siRNAs/shRNAs [66, 68, 69], there remains a need to validate observations with analysis of the long-term inhibitory effects against actively evolving HIV infection [59].

5. HOST CELL TARGETS OF HIV

Although using RNAi to target viral sequences is important, it is clear that targeting viral sequences alone may not be sufficient to ensure long-term viral suppression. Since, HIV requires a large number of cellular host factors for successful replication, these are potential therapeutic targets for RNAi-mediated inhibition. A significant advantage of targeting host genes is that these are not under selective pressure, and therefore to gain resistance, HIV would need to evolve an entirely new set of cellular associations to bypass the requirement for an important host factor.

The major CD4 receptor required for HIV docking was one of the first host cell sequences targeted using siRNAs [70]. While these results were promising, there has been a shift away from targeting CD4. This is a result of the ubiquitous presence of the receptor on various host cells and its function in normal immune pathways. The chemokine co-receptors required for HIV entry are more attractive alternatives and there have been numerous studies describing RNAi-mediated suppression of chemokine (c-c motif) receptor 5 (CCR5) [71-73] and CXC chemokine receptor 4 (CXCR4/Fusin) [71, 74]. CCR5 was particularly enticing as a deletion mutant allele was found to be present at a frequency of 0.092 in Caucasian populations, and individuals homozygous for the deletion are highly resistant to M-tropic HIV infection [75]. Unfortunately this strategy is inadequate for complete protection against HIV-1 as T-tropic and dual tropic strains can still gain access to cells. One study using HIV-2 showed CXCR4 to be sufficient for entry even in the absence of CD4 [76] and furthermore, although less common, there exist primary HIV isolates that utilize CCR3 and CCR2b as co-receptors to gain access to CD4+ cells [77, 78]. RNAi-mediated inhibition of expression of other proteins involved in HIV transcription, such as NFκβ, cyclin T1 and CDK9, have also been shown to down-regulate viral replication [79, 80]. Differences in the viral life cycle in T cells and in macrophages may present a challenge to development of RNAi-based HIV therapy. Specifically, in T cells, nascent viral mRNAs transcribed from the provirus are the most attractive targets for RNAi as they are shuttled to the cytoplasm by Rev. However, in macrophages new virions do not assemble and bud at the plasma membrane. Instead viruses may bud into endocytic organelles and accumulate in these vacuoles before being released as an exosome. The advantage for HIV is that exosomes escape immune surveillance but importantly these organelles are also inaccessible to antiviral therapies and RNAi-based gene silencing [81]. Thus the identification of host factors that are required for HIV infection, and which are common to all cells infectable by the virus, has become more important for the development of gene expression-based antiviral silencing approaches.

Advances in high-throughput screens using robotic instrumentation and genome-wide siRNA libraries have resulted in novel reverse genetics applications to monitor host-pathogen interactions. Last year alone, three influential papers were published describing the use of large-scale RNAi screens to uncover host dependency factors (HDFs) required for HIV replication (Brass, Dykxhoorn *et al.* 2008; Konig, Zhou *et al*. 2008; Zhou, Xu *et al*. 2008). All three studies examined approximately 20 000 cellular genes, and used between three and six siRNAs to target each gene. König *et al* focused exclusively on those genes involved during the initial phases of viral infection and subjected the data to thorough, integrative and robust analysis (Konig, Zhou *et al*. 2008). In this study, a genome-wide siRNA array with related cellular toxicities was used to generate a foundation dataset, which was compared to functional, transcriptional and biochemical data collected from protein interaction and gene expression databases. Compiling relevant information required an ontogeny-based pattern algorithm to create a 'decision matrix' to prioritize HDFs involved in early replication steps of HIV (Konig, Zhou *et al.* 2008). Brass *et al* identified HDFs associated with the nuclear pore complex, various transcription factors, Golgi-associated proteins, trans-membrane transporters, glycosylation enzymes and autophagy components (Brass, Dykxhoorn *et al.* 2008). König *et al* identified over forty HDFs that regulate uncoating of the viral capsid, as well as fifteen factors involved in nuclear entry of the viral pre-integration complex (PIC) (Konig, Zhou *et al*. 2008). Zhou *et al* catalogued genes with well characterized roles in HIV replication (CD4, CXCR4, Rev-mediated transport interactions) as well as previously unlinked HDFs, including factors associated with oxidative phosphorylation and adipokine pathways (Zhou, Xu *et al*. 2008). Surprisingly little concordance existed in the 200-300 HDFs identified for each of the three studies, with no single gene in common between all three datasets. Importantly the results were generated using different HIV vectors, siRNA design algorithms, separate array formats, and unequal culturing efficacies and culture periods. Despite these limitations, the genes identified in all three studies have established novel potentially druggable targets. However, further investigation will be required to verify a genuine role of HDFs in a physiological context (e.g. HIV infection of primary T cells) before they may be classified as good targets. Of course another important consideration in developing this approach is making sure that silencing of cellular genes does not have unintended toxic effects.

6. COMBINATORIAL RNAI AGAINST HIV

The rapid emergence of viral resistance to single gene silencing sequences has prompted development of multiple or combinatorial RNAi-based approaches which are akin to HAART. Multitarget gene silencing has an advantage over traditional HAART in that there are many conserved RNAi-susceptible sequences within the HIV-1 genome. At this stage it is premature to predict the long-term efficacy of multiple targeting methods, however it has been proposed that assuming equal and effective inhibition, the simul-

taneous targeting of at least four RNAi-susceptible sequences may be sufficient to prevent or, at the very least, severely delay the emergence of RNAi-resistant variants [82-84]. There are several strategies for inhibiting multiple target sites on HIV-1 using RNA-based effector sequences. Highlighted below are some of the promising combinatorial approaches based on use of expressed RNAi activators.

6.1.Multiple shRNA Expression

Short hairpin RNA expression cassettes using RNA Pol III promoters (eg. U6, H1, tRNA and VA1) can be placed adjacent to each other within a single delivery vector for the combined expression of multiple shRNAs or effector RNAs (Fig. **3A**). Early studies showed that the simultaneous targeting of both wild-type HIV and RNAi-escape mutant variants by two or more shRNAs in tandem has limited usefulness, serving only to reduce the efficacy of the anti-wild-type shRNA through diminished access to the targeted site [53]. Strategies which have targeted up to three separate conserved sites have yielded more promising results [85-87]. However, most show the emergence of viral escape in culture, although at a delayed rate when compared to single shRNAs alone. In a seminal study, ter Brake and colleagues used a single lentiviral vector to produce stably transduced T cells containing four tandem anti-HIV shRNA expression cassettes [69]. This multi-shRNA approach prevented emergence of escape mutants over an 80-day period in culture. However it was noted that some degree of background viral replication occurred in the presence of the inhibiting multi-RNAi effectors, which was attributed to the lack of silencing of incoming RNA and incomplete viral RNA suppression. This study assessed efficacy against a single molecular clone of HIV. It remains to be determined whether sustained repression of a more diverse viral population, such as primary virus from an infected individual, is possible. Additionally, ter Brake *et al* made use of different promoters to produce each shRNA, since multiple copies of the U6 promoter were subject to deletion during lentiviral recombination [69]. It has been noted that the additive effect of using multiple promoters in tandem results in an overall decrease in guide strand production [69, 87]. Also, a combinatorial system with heterogenous promoters produces different amounts of each RNAi hairpin precursor and derived effector sequences. Theoretical computational analyses of escape kinetics highlight the importance of ensuring efficient delivery to HIV-1 susceptible cells and potent silencing by each individual RNAi effector of a multitargeting system [82].

6.2. Long Hairpin-Based RNAs

An alternative approach to using multiple expression cassettes is to exploit the endogenous action of Dicer to process long dsRNA templates into multiple siRNA species. This has been a controversial application as the main concern of working with longer (> 30 bp) dsRNAs in mammalian cells is the potential activation of innate immune response [88, 89]. However, several studies have now shown that expressed dsRNAs, including long hairpin RNA (lhRNA), are unlikely to induce unwanted immunotoxicities [90-94]. Importantly, unlike with transfection of synthetic RNAi activators, expressed lhRNAs do not traverse the endosome, and therefore avoid activation of toll-like receptors (TLRs) located in this subcellular compartment. The most well-studied approach to generating expressed lhRNAs is the use Pol-III promoters to generate duplexes of between 30 and 100 bp with the 2 nt 3′ overhangs that are required for nuclear export by the exportin-5 complex (Fig. **3B**). These lhRNAs have been shown to be intracellular Dicer substrates and produce multiple siRNA species that are capable of simultaneously inhibiting different HIV targets [53, 90, 95-97]. To date, two studies have investigated the potential of lhRNAs to prevent RNAi-resistance [53, 97]. However, only the study by Sano *et al*, show sustained antiviral activity of lhRNAs when targeted to the overlapping *tat*/*rev* region of HIV. Importantly, both Sano *et al* and Nishitsuji *et al* used lhRNAs targeted to a single continuous sequence, making it very difficult to identify the relative inhibitory contributions of each siRNA generated from the lhRNA. We and others have shown that siRNAs are processed by Dicer in a gradient of decreasing efficiency, starting from the base of the dsRNA hairpin duplex and moving towards the apical loop [90, 94, 95, 97]. This phenomenon also occurs when the arrangement of separate unique functional siRNAs along the lhRNA duplex is opitmized [95]. It thus seems unlikely that expressed lhRNAs can be made to produce more than two siRNAs at effective functional doses [95, 96], suggesting that two or more lhRNA-expression cassettes may be needed to induce sustained viral suppression and prevent viral escape.

6.3. Polycistronic miRNA Precursor Mimics

The main drawback associated with use of Pol III-mediated expression of shRNAs and lhRNAs is the powerful and constitutive production of RNAi effectors. This is especially a concern when using multiple shRNA expression cassettes, where many tandem Pol-III promoters are typically used. *In vivo* experiments in mice show that U6 shRNAs are capable of inducing severe toxicities by blocking the endogenous RNAi pathway [98-100]. In the most extreme case, high doses of shRNAs produced in the liver after expression from adeno-associated virus vectors induced acute liver toxicity and mortality in mice [98]. The toxic effect resulted from saturation of the exportin-5 karyopherin, which is responsible for transporting pre-miRNA from the nucleus to the cytoplasm. These observations have subsequently been confirmed by experiments which examined the long-term effects of U6 and H1-expressed shRNAs in lentiviral-transduced human primary peripheral blood-derived T lymphocytes (PBLs) that were transplanted into immune-deficient mice [100]. Observations suggest that the transcriptionally stronger U6 promoter, and not the H1 promoter, is responsible for causing unwanted toxicities. Further systematic experiments in cell culture show that the introduction of multiple shRNAs from combinatorial expression cassettes blocks RISC loading of individual siRNAs [101]. To prevent these saturating effects on the endogenous RNAi pathway, much of the recent attention in combinatorial RNAi has focused on exploiting the properties of natural microRNA precursor biogenesis (Fig. **3C**). At present there is no evidence that pri-miRNA shuttles, even if

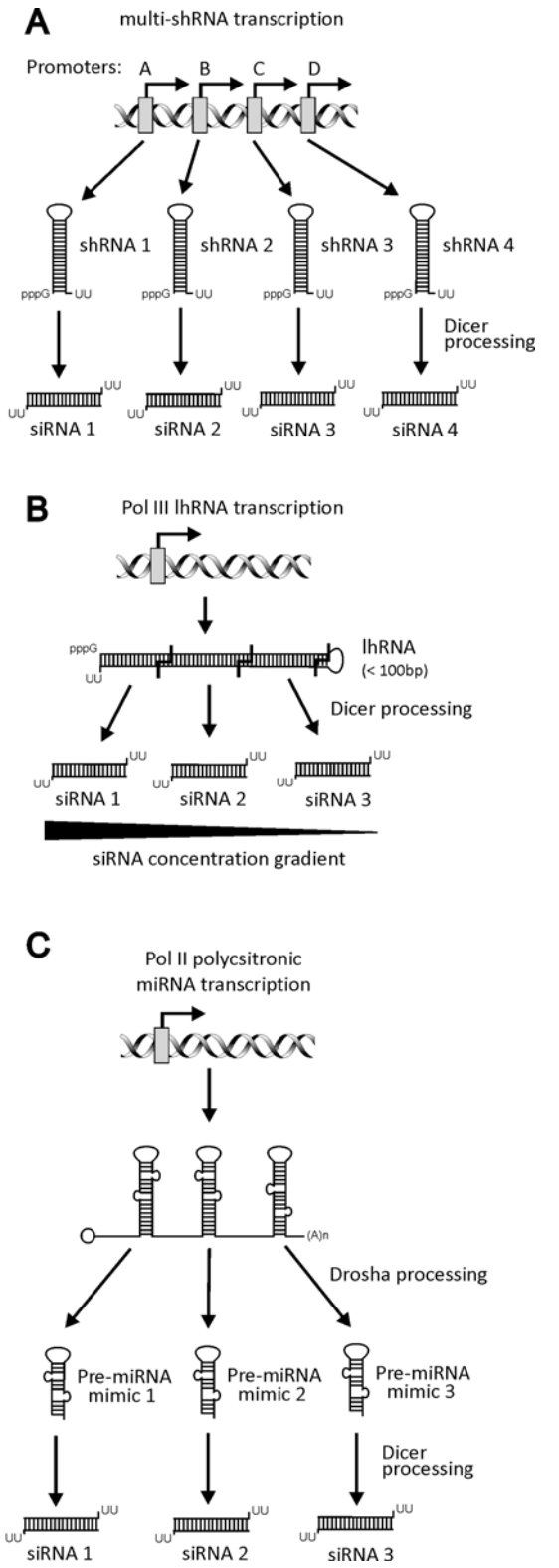


Fig. (3). Combinatorial RNAi-based expression approaches. **A.** Multiple shRNA expression cassettes, expressed from Pol III promoters (or off the U1 Pol II promoter) each produce shRNAs with defined 3′ and 5′ termini for export and processing by Dicer into independent siRNAs. **B.** lhRNAs, are processed sequentially by Dicer to produce up to 3 siRNAs. **C.** Polycistronic pri-miRNA mimics rely on processing by Drosha/DGCR8 to produce multiple pre-miRNAs that are independent Dicer substrates.

highly expressed, can saturate the endogenous RNAi pathway [102-105]. MiRNAs also have other advantages for developing combinatorial RNAi approaches. Since pri-miRNAs are expressed from Pol II promoters, tissue-specific or inducible gene regulation is possible, allowing for greater control of dosage and cell-specific expression. Most importantly, many natural pri-miRNAs are found in polycistronic configurations which is a feature exploited for the generation of multiple RNAi effectors against HIV [106-108]. Lui *et al* was the first to show that a lymphocyte-specific polycistronic miRNA cluster (miR-17-92 cluster) could be used to generate five anti-HIV targeting miRNA mimics [108]. Not all combinations of polycistronic miRNAs were effectively processed from their precursors, however strong short-term inhibition of HIV was observed in stably-transduced T cells challenged with HIV [108]. In a similar study, Aagaard *et al* showed that three anti-HIV miRNA mimics could be independently expressed within the framework of a natural polycistonic cluster of three pri-miRNAs (miR106b-miR-93-miR-25 cluster) [106]. When the polycistronic miRNA cassette was expressed in T-lymphocyte cell lines and challenged with two different strains of HIV, each processed miRNA mimic effectively suppressed the replication of the subtype B molecular clone HIV-LAI, but suppressed the more pathogenic subtype B lab strain HIV-IIIB less effectively [106]. It remains to be shown whether combinatorial approaches based on polycistronic miRNA mimics can prevent viral escape in the long term. Moreover, at this stage, further investigation is needed to address important questions relating optimization in generating miRNA mimics from their endogenous polycistronic scaffolds. For example, which endogenous pri-miRNA backbone structure represents the best scaffold for guide sequence substitution? Does the choice of backbone affect the guide strand efficacy, and does each backbone function equally well when placed adjacent to each other in a polycistronic fashion? Is the choice of backbone affected by cell-specific factors for proper processing, and if so, can this be exploited for therapeutic use?

7. POTENTIAL UNWANTED TOXICITIES

An important consideration in advancing use of RNA-based gene therapy approaches is the potential to induce unwanted off target effects (OTEs). These may result from innate immunostimulation and non specific interaction of RNAi effectors with host cellular sequences. The intra-cellular presence of duplexed RNA may activate the innate immune system. Specifically the cytoplasmic receptors dsRNA dependent protein kinase (PKR) and retinoic-acid-inducible gene-I (RIG-I), leading to a type 1 Interferon (IFN) response [reviewed in [109]]. Over 300 interferon-stimulated genes (ISGs) are activated by increased circulating levels of cytokines IFN-α and β [110]. In particular, the activation of 2'-5′-oligoadenylate synthetases (2-5-OAS) leads to general translational arrest, resulting in apoptosis *via* the non-specific degradation of cellular mRNA by activated RNase L [reviewed in [109, 111]]. Exogenously introduced RNAs (ssRNAs and dsRNAs) are also capable of interacting with different endosomal Toll-like receptors (TLRs), leading to a signaling cascade that activates the IFN pathway. However, it is important to note that expressed duplexed RNA can

evade detection by TLRs when expressed in CD34+ progenitor hematopoietic stem cells [93]. The therapeutic use of RNAi may also be complicated by induction of sequence-specific OTEs. Unintended binding of the guide strand seed regions to complementary cellular sequences may lead to translational inhibition [112, 113]. Cellular hexanucleotide mRNA sequences that may interact with potentially therapeutic guides are widespread, which makes it difficult to predict true OTEs using computational algorithms. In the future, it is likely that transcriptional and proteomic array data will be necessary to exclude off-target toxicities associated with a particular siRNA guide sequence. Ideally, to limit the non specific silencing therapeutic sequences should be expressed in specific target tissues, potent and effective at a low concentration. Analyses of the global effects on gene expression become even more important when using multiple targeting approaches. This is especially the case for long duplex RNAs where it is often difficult to predict the variety of guide sequences generated from RNAi effectors.

8. HIV MODULATES THE RNAI PATHWAY (OR VICE VERSA)

There is a number of intriguing studies which suggest a delicate interplay between the endogenous RNAi pathway and HIV replication. Specifically, the viral transactivator Tat appears to directly inhibit Dicer processing of siRNA precursors *in vitro* [114]. Moreover, there is possibly a direct association between viral transcriptional activation and the RNAi processing pathway since Dicer requires its binding partner, TRBP, for functional RNAi [17]. TRBP, like Tat, also binds the HIV-1 TAR loop to ensure viral transcriptional activation [115-117]. Sequestration of TRBP by the HIV-1 TAR loop may thus inhibit Dicer activity, thereby reducing the overall effectiveness of RNAi [118]. Nevertheless, the role of both Tat and TRBP as RNAi inhibitory factors remains controversial. A global decrease in cellular microRNAs upon HIV infection should be expected but this has not been widely observed using large-scale genomic screens [119, 120]. Nevertheless, in a thorough study, Triboulet *et al* have reported that HIV infection downregulates members of the miR-17-92 polycistronic miRNA cluster. This affects the Tat co-factor, P300/CBP-associated factor (PCAF), which is responsible for RNA Pol II elongation and processive viral transcription [120]. Although these results suggest a direct link between cellular miRNAs and increased HIV activation, at this stage it remains unclear as to what causes a decrease in miR-17-19 levels upon infection.

There is the possibility that HIV encodes its own miRNAs or siRNAs [121-123]. However with only fragmentary data available and without a clear understanding of the mechanism by which these miRNAs/siRNAs might function, little can be inferred about the role of these putative effector sequences. Nevertheless, Omoto *et al* have shown that HIV *nef* encodes a miRNA (miR-N367) that appears to affect transcription of viral mRNAs through interactions with the negative response element (NRE) of the U3 LTR region [122, 123]. Also, two human miRNAs, miR-29a and miR-29b, detected in infected peripheral lymphocytes were shown to inhibit *nef* expression and HIV replication *in vitro* [124] and *in vivo* [125]. The TAR stem-loop region has been shown to be a substrate for Dicer cleavage but a function of this putative viral miRNA remains inconclusive [126, 127]. Lastly, a cluster of host miRNAs are activated in latently-infected CD4+ T cells [128]. These miRNAs appear to contribute to viral latency by targeting sequences within the 3′ UTRs of most HIV mRNA species. Interestingly, one suggested mode of therapy is to inhibit latency-associated miRNAs with antisense inhibitors. This may activate dormant virus and allow conventional therapies to eradicate the reservoir of latently-infected cells [128, 129].

9. THE THERAPEUTIC APPLICATION OF ANTI-HIV RNAI

9.1. Pre-Clinical Studies

Current gene therapy protocols have focused mainly on achieving long term suppression of HIV by using retroviral or lentiviral vectors that generate stably expressed RNAi transgenes. More recently, HIV-based lentiviral vectors have been used for delivery and stable integration of transgenes in hematopoietic cells, such as CD4+ T cells or CD34+ haematopoietic progenitor stem cells (HSCs). The aim of this approach has been to provide a protective and reconstituted immune system *in vivo* (reviewed in [130]). In a seminal paper, primary CD34+ HSCs transduced with anti-*rev* shRNAs underwent successful differentiation to form mature macrophages or T cells [131]. These *in vitro* data were further assessed using a humanised mouse model of HIV infection. In this model lentiviral vector-transduced human haematopoetic stem cells were used to reconstitute the T-lymphoid compartment of SCID (severe combined immunodeficiency) mice that do not produce these immune cells [131, 132]. Engraftment of RNAi-transduced HSCs into the mice (SCID-hu mice) resulted in reconstitution of thymocytes that matured and responded to mitogenic stimulation as expected for normal T cells. Significant viral resistance was observed following *ex vivo* HIV challenge of differentiated, transduced macrophages and T cells [131]. While useful, the SCID-hu mouse model has low engraftment levels with limited systemic spread of HIV following infection, and this has prompted research aimed at further modifying SCID mice. One model is the non-obese diabetic/SCID (NOD/SCID) mouse which harbours a complete null mutation in the common cytokine receptor γ chain that abrogates interleukin 2 binding (NOD/SCID/IL2rγ^{-}) [133]. In these mice T and B cell development is disrupted as the γ chain receptor is required for innate immunity [134]. In a recent elegant study, a single-chain antibody specific for the T-cell specific CD7 receptor was conjugated to several anti-HIV siRNAs *via* an arginine tag [135]. Impressively, anti-*vif* and anti-*tat* siRNA-antibody conjugates were introduced into NOD/SCID/IL2rγ^{-} and SCID-hu mice and prevented HIV infection and CD4+ T cell loss without any observed toxicities. Using a mouse model derived from a recombination activating gene (RAG)–deficient strain that also lacks a γ chain receptor (BALB-Rag2$^{-/-}$γ^{-}), HSCs tranduced with a lentivector encoding an anti-HIV *nef* shRNA showed significant inhibition of HIV replication [136]. All of the mouse models described above have demonstrated their utility for *ex vivo* engraftment and

immune reconstitution of T cells that are protected from the cytopathic effects of HIV, providing a foundation for similar approaches in human patients. Using a non-human primate model, An *et al* were able to report successful stable T-cell expression of shRNAs directed to CCR5 in rhesus macaques [137]. Following transplantation of rhesus HSCs, consistent downregulation of CCR5 expression was observed in mature T cells for up to 14 months. These cells were less susceptible to simian immunodeficiency virus (SIV) infection when assessed *ex vivo* [137]. Although physiologically-relevant models of HIV are lacking, success with murine models of HIV infection has led to progress towards clinical trial assessment of RNAi-based anti HIV therapy. Advances of these investigations are briefly described below.

9.2. Clinical Studies

A number of anti-HIV RNAi effectors have all been tested in human phase 1 or 2 clinical trials with varying efficacies depending on the transfer vector used and whether T cells or haematopoetic progenitor cells were transduced [138-141]. The first example of RNAi in a stem cell setting is currently being conducted by John Rossi and collaborators at the City of Hope Medical Center in Duarte, California. This trial involves a pilot study of transduced HSCs in AIDS lymphoma patients to assess the safety and therapeutic feasibility of using a lentiviral vector encoding three anti-HIV modalities, including a U6-expressed shRNA targeted to the *tat*/*rev* overlapping region [4, 142]. This clinical trial was initiated when a lentiviral construct expressing the anti-HIV transgene was shown to successfully transduce primary HSCs and induce long-term inhibition of HIV replication in these cells [143]. Transduced cells were additionally able to differentiate normally into different lymphocyte lineages without evidence of untoward effects. Protected HSCs gave rise to phenotypically normal T cells that were HIV resistant when engrafted into SCID-hu mice [144]. For the human trial, bone marrow lymphocytes were removed from four patients and cultured *ex vivo* to enrich progenitor cells. Following enrichment, the haematopoietic precursors were transduced with the lentiviral vector before autologous reintroduction into patients who had undergone bone marrow ablation [142]. Outcomes from these studies are keenly awaited and will be of importance for refinement of this exciting new technology.

10. CONCLUSIONS AND FUTURE PROSPECTS

Just over 10 years have passed since RNAi was discovered and already RNAi-based gene expression strategies are being tested in a clinical setting. Although much has been achieved, a complete understanding of the mammalian RNAi pathway, and its influences on cellular biology, remains an important focus of research. RNAi belongs to a paradigm where cellular function and gene activity are integrally linked to short (and long) non-coding RNAs. These newly-discovered RNAs are found throughout the genome and we are only now beginning to understand their roles in the cell. The influence of the intricate network of small RNAs on gene expression is complex, and the effects of exogenous therapeutic sequences on normal gene function will require rigorous characterisation. Careful regulation of RNAi effector dose and targeted delivery of therapeutic sequences will be important to avoid unintended disruption of endogenous RNAi pathways.

Some RNAi-related pathways such as TGS are being considered seriously as potential antiviral modalities. Using synthetic and expressed antisense RNAs, we and others have previously reported that HIV transcription can be blocked when targeting the LTR promoter of the integrated provirus, resulting in permanent inhibition of gene expression [33, 145-147]. This approach was recently shown to be feasible in T cells transduced with replication-competent lentiviruses expressing the anti-HIV TGS-inducing transgene [148]. If specific viral or cellular genes can be permanently silenced, this may alleviate the need for continuous expression of the therapeutic RNA inhibitor and has enormous potential for therapeutic application. However, as with other emerging RNA-based therapies, the safety and sustained inhibitory potential of TGS-based RNAi will need to be evaluated comprehensively.

The rapid progress of RNAi -based HIV therapy has been stimulated by important improvements in the efficacy and safety of modern lentiviral vectors, as well as better animal models of HIV infection. This has enabled rigorous pre-clinical testing and proof-of-concept studies. In human trials conducted thus far, we know that transduction of HSCs can be accomplished safely and therefore the outcome of phase 2 efficacy studies is eagerly anticipated. Future clinical trial results will soon determine whether introduction into CD34+ HSCs of expressed RNAi activators results in a) protection and possibly immune reconstitution of all derived haemato-poietic lineage cells; and b) effectively achieves long-term viral suppression in HIV/AIDS patients. Although it is currently unclear how AIDS may be cured by harnessing RNAi, the potential of gene therapy for countering HIV infection is exciting. The technology has the potential to improve the quality of life and lifespan of infected indivi-duals and to relegate HIV to an easily-managed chronic infection. Momentum in the field is now considerable and this is likely to overcome existing hurdles. Prospects for advancing RNAi-based therapy for HIV infection are exciting and important developments in the field will no doubt be realised during the coming years.

AKNOWLEDGMENTS

Work in the authors' laboratory is supported by funding from the Sixth Research Framework Programme of the European Union, Project RIGHT (LSHB-CT-2004-005276), the South African National Research Foundation (NRF), ESASTAP, CANSA, the Medical Research Council (MRC) and Poliomyelitis Research Foundation (PRF).

REFERENCES

[1] UNAIDS. 2008 *Report on the global AIDS epidemic*, UNAIDS: Geneva, **2008**, 1-360.

[2] Palella, F. J., Jr.; Delaney, K. M.; Moorman, A. C.; Loveless, M. O.; Fuhrer, J.; Satten, G. A.; Aschman, D. J.; Holmberg, S. D. Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N. Engl. J. Med*, **1998**, *338*, 853-860.

[3] Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. C. Potent and specific genetic interference by double-

stranded RNA in Caenorhabditis elegans. *Nature* **1998**, *391*, 806-811.

[4] Castanotto, D.; Rossi, J. J. The promises and pitfalls of RNA-interference-based therapeutics. *Nature* **2009**, *457*, 426-433.

[5] Eulalio, A.; Huntzinger, E.; Izaurralde, E. Getting to the root of miRNA-mediated gene silencing. *Cell* **2008**, *132*, 9-14.

[6] Stefani, G.; Slack, F. J. Small non-coding RNAs in animal development. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 219-230.

[7] Han, J.; Lee, Y.; Yeom, K. H.; Kim, Y. K.; Jin, H.; Kim, V. N. The Drosha-DGCR8 complex in primary microRNA processing. *Genes Dev.* **2004**, *18*, 3016-3027.

[8] Denli, A. M.; Tops, B. B.; Plasterk, R. H.; Ketting, R. F.; Hannon, G. J. Processing of primary microRNAs by the Microprocessor complex. *Nature* **2004**, *432*, 231-235.

[9] Ruby, J. G.; Jan, C. H.; Bartel, D. P. Intronic microRNA precursors that bypass Drosha processing. *Nature* **2007**, *448*, 83-86.

[10] Okamura, K.; Hagen, J. W.; Duan, H.; Tyler, D. M.; Lai, E. C. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. *Cell* **2007**, *130*, 89-100.

[11] Berezikov, E.; Chung, W. J.; Willis, J.; Cuppen, E.; Lai, E. C. Mammalian mirtron genes *Mol. Cell* **2007**, *28*, 328-336.

[12] Babiarz, J. E.; Ruby, J. G.; Wang, Y.; Bartel, D. P.; Blelloch, R. Mouse ES cells express endogenous shRNAs, siRNAs, and other Microprocessor-independent, Dicer-dependent small RNAs. *Genes Dev.* **2008**, *22*, 2773-2785.

[13] Yi, R.; Qin, Y.; Macara, I. G.; Cullen, B. R. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. *Genes Dev.* **2003**, *17*, 3011-3016.

[14] Lund, E.; Guttinger, S.; Calado, A.; Dahlberg, J. E.; Kutay, U. Nuclear export of microRNA precursors. *Science* **2004**, *303*, 95-98.

[15] Bernstein, E.; Caudy, A. A.; Hammond, S. M.; Hannon, G. J. Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature* **2001**, *409*, 363-366.

[16] Hutvagner, G.; McLachlan, J.; Pasquinelli, A. E.; Balint, E.; Tuschl, T.; Zamore, P. D. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* **2001**, *293*, 834-838.

[17] Chendrimada, T. P.; Gregory, R. I.; Kumaraswamy, E.; Norman, J.; Cooch, N.; Nishikura, K.; Shiekhattar, R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. *Nature* **2005**, *436*, 740-744.

[18] Liu, J.; Carmell, M. A.; Rivas, F. V.; Marsden, C. G.; Thomson, J. M.; Song, J. J.; Hammond, S. M.; Joshua-Tor, L.; Hannon, G. J. Argonaute2 is the catalytic engine of mammalian RNAi. *Science* **2004**, *305*, 1437-1441.

[19] MacRae, I. J.; Ma, E.; Zhou, M.; Robinson, C. V.; Doudna, J. A. *In vitro* reconstitution of the human RISC-loading complex. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 512-527.

[20] Diederichs, S.; Haber, D. A. Dual role for argonautes in microRNA processing and posttranscriptional regulation of microRNA expression. *Cell* **2007**, *131*, 1097-1108.

[21] Matranga, C.; Tomari, Y.; Shin, C.; Bartel, D. P.; Zamore, P. D. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. *Cell* **2005**, *123*, 607-620.

[22] Rand, T. A.; Petersen, S.; Du, F.; Wang, X. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. *Cell* **2005**, *123*, 621-629.

[23] Khvorova, A.; Reynolds, A.; Jayasena, S. D. Functional siRNAs and miRNAs exhibit strand bias *Cell* **2003**, *115*, 209-216.

[24] Hutvagner, G.; Simard, M. J. Argonaute proteins: key players in RNA silencing. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 22-32.

[25] Rana, T. M. Illuminating the silence: understanding the structure and function of small RNAs. *Nat. Rev. Mol .Cell Biol.* **2007**, *8*, 23-36.

[26] Lewis, B. P.; Shih, I. H.; Jones-Rhoades, M. W.; Bartel, D. P.; Burge, C. B. Prediction of mammalian microRNA targets. *Cell* **2003**, *115*, 787-798.

[27] Filipowicz, W.; Bhattacharyya, S. N.; Sonenberg, N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight.? *Nat. Rev. Genet.* **2008**, *9*, 102-114.

[28] Flynt, A. S.; Lai, E. C. Biological principles of microRNA-mediated regulation: shared themes amid diversity. *Nat. Rev. Genet* **2008**.

[29] Brennecke, J.; Stark, A.; Russell, R. B.; Cohen, S. M. Principles of microRNA-target recognition. *PLoS Bio.* **2005**, *3*, e85.

[30] Kim, D. H.; Saetrom, P.; Snove, O., Jr.; Rossi, J. J. MicroRNA-directed transcriptional gene silencing in mammalian cells. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 16230-16235.

[31] Kim, D. H.; Villeneuve, L. M.; Morris, K. V.; Rossi, J. J. Argonaute-1 directs siRNA-mediated transcriptional gene silencing in human cells. *Nat. Struct. Mol. Biol.* **2006**, *13*, 793-797.

[32] Morris, K. V.; Chan, S. W.; Jacobsen, S. E.; Looney, D. J. Small interfering RNA-induced transcriptional gene silencing in human cells. *Science* **2004**, *305*, 1289-1292.

[33] Weinberg, M. S.; Villeneuve, L. M.; Ehsani, A.; Amarzguioui, M.; Aagaard, L.; Chen, Z. X.; Riggs, A. D.; Rossi, J. J.; Morris, K. V. The antisense strand of small interfering RNAs directs histone methylation and transcriptional gene silencing in human cells.*Rna* **2006**, *12*, 256-262.

[34] Kim, V. N.; Han, J.; Siomi, M. C. Biogenesis of small RNAs in animals .*Nature Rev. Mol. Cell Biol.* **2009**, *10*, 126-139.

[35] Vagin, V. V.; Sigova, A.; Li, C.; Seitz, H.; Gvozdev, V.; Zamore, P. D. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* **2006**, *313*, 320-324.

[36] Aravin, A. A.; Hannon, G. J.; Brennecke, J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* **2007**, *318*, 761-764.

[37] Aravin, A. A.; Sachidanandam, R.; Bourc'his, D.; Schaefer, C.; Pezic, D.; Toth, K. F.; Bestor, T.; Hannon, G. J. A piRNA pathway primed by individual transposons is linked to de novo DNA methylation in mice. *Mol. Cell* **2008**, *31*, 785-799.

[38] Kuramochi-Miyagawa, S.; Watanabe, T.; Gotoh, K.; Totoki, Y.; Toyoda, A.; Ikawa, M.; Asada, N.; Kojima, K.; Yamaguchi, Y.; Ijiri, T. W.; Hata, K.; Li, E.; Matsuda, Y.; Kimura, T.; Okabe, M.; Sakaki, Y.; Sasaki, H.; Nakano, T. DNA methylation of retrotransposon genes is regulated by Piwi family members MILI and MIWI2 in murine fetal testes. *Genes Dev.* **2008**, *22*, 908-917.

[39] Tam, O. H.; Aravin, A. A.; Stein, P.; Girard, A.; Murchison, E. P.; Cheloufi, S.; Hodges, E.; Anger, M.; Sachidanandam, R.; Schultz, R. M.; Hannon, G. J. Pseudogene-derived small interfering RNAs regulate gene expression in mouse oocytes. *Nature* **2008**, *453*, 534-538.

[40] Watanabe, T.; Totoki, Y.; Toyoda, A.; Kaneda, M.; Kuramochi-Miyagawa, S.; Obata, Y.; Chiba, H.; Kohara, Y.; Kono, T.; Nakano, T.; Surani, M. A.; Sakaki, Y.; Sasaki, H. Endogenous siRNAs from naturally formed dsRNAs regulate transcripts in mouse oocytes. *Nature* **2008**, *453*, 539-543.

[41] Behlke, M. A. Chemical modification of siRNAs for *in vivo* use.*Oligonucleotides* **2008**, *18*, 305-319.

[42] Kato, Y.; Taira, K. Expression of siRNA from a single transcript that includes multiple ribozymes in mammalian cells. *Oligonucleotides* **2003**, *13*, 335-343.

[43] Scherer, L. J.; Frank, R.; Rossi, J. J. Optimization and characterization of tRNA-shRNA expression constructs. *Nucleic Acids Res.* **2007**, *35*, 2620-2628.

[44] Denti, M. A.; Rosa, A.; Sthandier, O.; De Angelis, F. G.; Bozzoni, I. A new vector, based on the PolII promoter of the U1 snRNA gene, for the expression of siRNAs in mammalian cells. *Mol. Ther* **2004**, *10*, 191-199.

[45] Capodici, J.; Kariko, K.; Weissman, D. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. *J. Immunol.* **2002**, *169*, 5196-5201.

[46] Coburn, G. A.; Cullen, B. R. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. *J. Virol* **2002**, *76*, 9225-9231.

[47] Jacque, J. M.; Triques, K.; Stevenson, M. Modulation of HIV-1 replication by RNA interference. *Nature* **2002**, *418*, 435-438.

[48] Dvorin, J. D.; Malim, M. H. Intracellular trafficking of HIV-1 cores: journey to the center of the cell. *Curr. Topics Microbiol. Immunol.* **2003**, *281*, 179-208.

[49] Zack, J. A.; Arrigo, S. J.; Weitsman, S. R.; Go, A. S.; Haislip, A.; Chen, I. S. Y. HIV-1 entry into quiescent primary lymphocytes: molecular analysis reveals a labile, latent viral structure. *Cell* **1990**, *61*, 213-222.

[50] Westerhout, E. M.; ter Brake, O.; Berkhout, B. The virion-associated incoming HIV-1 RNA genome is not targeted by RNA interference. *Retrovirology* **2006**, *3*, 1-9.

[51] Gao, Y.; Lobritz, M. A.; Roth, J.; Abreha, M.; Nelson, K. N.; Nankya, I.; Moore-Dudley, D. M.; Abraha, A.; Gerson, S. L.; Arts, E. J. Targets of small interfering RNA restriction during human

immunodeficiency virus type 1 replication. *J. Virol* **2008**, *82*, 2938-2951.

[52] Lee, N. S.; Dohjima, T.; Bauer, G.; Li, H.; Li, M. J.; Ehsani, A.; Salvaterra, P.; Rossi, J. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. *Nat. Biotechnol* **2002**, *20*, 500-505.

[53] Nishitsuji, H.; Kohara, M.; Kannagi, M.; Masuda, T. Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. *J. Virol* **2006**, *80*, 7658-7666.

[54] Yoshinari, K.; Miyagishi, M.; Taira, K. Effects on RNAi of the tight structure, sequence and position of the targeted region. *Nucleic Acids Res* **2004**, *32*, 691-699.

[55] Gitlin, L.; Karelsky, S.; Andino, R. Short interfering RNA confers intracellular antiviral immunity in human cells.*Nature* **2002**, *418*, 430-434.

[56] Boden, D.; Pusch, O.; Lee, F.; Tucker, L.; Ramratnam, B. Human immunodeficiency virus type 1 escape from RNA interference. *J. Virology* **2003**, *77*, 11531-11535.

[57] Das, A. T.; Brummelkamp, T. R.; Westerhout, E. M.; Vink, M.; Madiredjo, M.; Bernards, R.; Berkhout, B. Human immunodeficiency virus type 1 escapes from RNA iInterference-mediated inhibition. *J. Virology*, **2004**, *78*, 2601-2605.

[58] Senserrich, J.; Pauls, E.; Armand-Ugón, M.; Clotet-Codina, I.; Moncunill, G.; Clotet, B.; Esté, J. A. HIV-1 resistance to the anti-HIV activity of a shRNA targeting a dual-coding region. *Virology* **2008**, *372*, 421-429.

[59] von Eije, K. J.; ter Brake, O.; Berkhout, B. Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. *J Virol*, **2008**, *82*, 2895-2903.

[60] Westerhout, E. M.; Ooms, M.; Vink, M.; Das, A. T.; Berkhout, B. HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. *Nucleic Acids Res.* **2005**, *33*, 796-804.

[61] Leonard, J. N.; Shah, P. S.; Burnett, J. C.; Schaffer, D. V. HIV evades RNA interference directed at TAR by an indirect compensatory mechanism. *Cell Host Microbe*, **2008**, *4*, 484-494.

[62] Chang, L. J.; Liu, X.; He, J. Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1 *Gene Therapy* **2005**, *12*, 1133-1144.

[63] Han, W.; Wind-Rotolo, M.; Kirkman, R. L.; Morrow, C. D. Inhibition of human immunodeficiency virus type 1 replication by siRNA targeted to the highly conserved primer binding site. *Virology* **2004**, *330*, 221-232.

[64] Lee, S. K. Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. *Blood* **2005**, *106*, 818-826.

[65] Morris, K. V.; Chung, C. H.; Witke, W.; Looney, D. J. Inhibition of HIV-1 replication by siRNA targeting conserved regions of gag/pol *RNA. Biology* **2004**, *1*, 114-117.

[66] **Naito, Y.; Nohtomi**, K.; Onogi, T.; Uenishi, R.; Ui-Tei, K.; Saigo, K.; Takebe, Y. Optimal design and validation of antiviral siRNA for targeting HIV-1 *Retrovirolog*, **2007**, *4*, 80.

[67] von Eije, K. J.; ter Brake, O.; Berkhout, B. Stringent testing identifies highly potent and escape-proof anti-HIV short hairpin RNAs. *J. Gene Med.* **2009**, *11*, 459-467.

[68] **McIntyre, G. J.; Groneman**, J. L.; Yu, Y. H.; Jaramillo, A.; Shen, S.; Applegate, T. L. 96 shRNAs designed for maximal coverage of HIV-1 variants. *Retrovirology* **2009**, *6*, 55.

[69] ter Brake, O.; t Hooft, K.; Liu, Y. P.; Centlivre, M.; von Eije, K. J.; Berkhout, B. Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. *Mol .Ther.* **2008**, *16*, 557-564.

[70] Novina, C. D.; Murray, M. F.; Dykxhoorn, D. M.; Beresford, P. J.; Riess, J.; Lee, S.; Collman, R. G.; Lieberman, J.; Shankar, P.; Sharp, P. A. siRNA-directed inhibition of HIV-1 infection. *Nature Med.* **2002**, *8*, 681-686.

[71] Martinez, M. A.; Gutierrez, M. I.; Armand-Ugon, M.; Blanco, J.; Parera, M.; Gomez, J.; Clotet, B.; Este, J. A. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. *AIDS* **2002**, *16*, 2385-2390.

[72] Qin, X.; An, D. S.; Chen, I. S. Y.; Baltimore, D. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5 *Proceedings of the Nat. Acad. Sci. USA* **2003**, *100*, 183-188.

[73] Song, E.; Lee, S.; Dykxhoorn, D. M.; Novina, C.; Zhang, D.; Crawford, K.; Cerny, J.; Sharp, P. A.; Lieberman, J.; Manjunath, N.; Shankar, P. Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. *J. Virol.* **2003**, *77*, 7174-7181.

[74] Anderson, J.; Banerjea, A.; Planelles, V.; Akkina, R. Potent suppression of HIV type 1 infection by a short hairpin anti-CXCR4 siRNA. *AIDS Res. Human Retroviruses*, **2003**, *19*, 699-706.

[75] Samson, M.; Libert, F.; Doranz, B. J.; Rucker, J.; Liesnard, C.; Farber, C.; Saragosti, S.; Lapoumeroulie, C.; Cognaux, J.; Forceille, C.; Muyldermans, G.; Verhofstede, C.; Burtonboy, G.; Georges, M.; Imai, T.; Rana, S.; Yi, Y.; Smyth, R. J.; Collman, R. G.; Doms, R. W.; Vassart, G.; Parmentier, M. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR5 chemokine receptor gene. *Nature* **1996**, *382*, 722-725.

[76] Endres, M. J.; Clapham, P. R.; Marsh, M.; Ahuja, M.; Davis Turner, J.; McKnight, A.; Thomas, J. F.; Stoebenau-Haggarty, B.; Choe, S.; Vance, P. J.; Wells, T. N. C.; Power, C. A.; Sutterwala, S. S.; Doms, R. W.; Landau, N. R.; Hoxie, J. A. CD4-independent infection by HIV-2 is mediated by Fusin/CXCR4. *Cell* **1996**, *87*, 745-756.

[77] Choe, H.; Farzan, M.; Sun, Y.; Sullivan, N.; Rollins, B.; Ponath, P. D.; Wu, L.; Mackay, C. R.; LaRosa, G.; Newman, W.; Gerard, N.; Gerard, C.; Sodroski, J. The b-chemokine receptors CCR3 and CCR5 facilitate infection by primary HIV-1 isolates. *Cell* **1996**, *85*, 1135-1148.

[78] Doranz, B. J.; Rucker, J.; Yi, Y.; Smyth, R. J.; Samson, M.; Peiper, S. C.; Parmentier, M.; Collman, R. G.; Doms, R. W. A dual-tropic primary HIV-1 isolate that uses fusin and the b-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. *Cell* **1996**, *85*, 1149-1158.

[79] Chiu, Y. L.; Cao, H.; Jacque, J. M.; Stevenson, M.; Rana, T. M. Inhibition of human immunodeficiency virus type 1 replication by RNA interference directed against human transcription elongation factor P-TEFb (CDK9/CyclinT1). *J. Virology*, **2004**, *78*, 2517-2529.

[80] Surabhi, R. M.; Gaynor, R. B. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. *J. Virology*, **2002**, *76*, 12963-12973.

[81] Verani, A.; Gras, G.; Pancino, G. Macrophages and HIV-1: dangerous liaisons. *Mol. Immunol.*, **2005**, *42*, 195-212.

[82] Leonard, J. N.; Schaffer, D. V. Computational design of antiviral RNA interference strategies that resist human immunodeficiency virus escape. *J. Virol* . **2005**, *79*, 1645-1654.

[83] Berkhout, B.; Haasnoot, J. The interplay between virus infection and the cellular RNA interference machinery. *FEBS Lett.* **2006**, *580*(12),2896-2902

[84] Berkhout, B.; ter Brake, O. Towards a durable RNAi gene therapy for HIV-AIDS. *Expert Opin Biol.Ther.* **2009**, *9*, 161-70.

[85] Anderson, J.; Banerjea, A.; Akkina, R. Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4 and CCR5 confer HIV-1 resistance. *Oligonucleotides* **2003**, *13*, 303-312.

[86] Song, J.; Giang, A.; Lu, y.; Pang, S.; Chiu, R. Multiple shRNA expressing vector enhances efficiency of gene silencing. *BMB Reports* **2007**, *41*, 358-362.

[87] ter Brake, O.; Konstantinova, P.; Ceylan, M.; Berkhout, B. Silencing of HIV-1 with RNA interference: a multiple shRNA approach. *Mol Ther* **2006**, *14*, 883-92.

[88] Ui-Tei, K.; Zenno, S.; Miyata, Y.; Saigo, K. Sensitive assay of RNA interference in Drosophila and Chinese hamster cultured cells using firefly luciferase gene as target. *FEBS Lett.* **2000**, *479*, 79-82.

[89] Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature* **2001**, *411*, 494-498.

[90] Barichievy, S.; Saayman, S.; von Eije, K. J.; Morris, K. V.; Arbuthnot, P.; Weinberg, M. S. The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5′ long terminal repeat. *Oligonucleotides* **2007**, *17*, 419-431.

[91] Akashi, H.; Miyagishi, M.; Yokota, T.; Watanabe, T.; Hino, T.; Nishina, K.; Kohara, M.; Taira, K. Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. *Mol. Biosyst* **2005**, *1*, 382-390.

[92] Konstantinova, P.; ter Brake, O.; Haasnoot, J.; de Haan, P.; Berkhout, B. Trans-inhibition of HIV-1 by a long hairpin RNA expressed within the viral genome. *Retrovirology* **2007**, *4*, 15.

[93] Robbins, M. A.; Li, M.; Leung, I.; Li, H.; Boyer, D. V.; Song, Y.; Behlke, M. A.; Rossi, J. J. Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*. *Nat. Biotechnol* **2006**, *24*, 566-571.

[94] Weinberg, M. S.; Ely, A.; Barichievy, S.; Crowther, C.; Mufamadi, S.; Carmona, S.; Arbuthnot, P. Specific inhibition of HBV replication *in vitro* and *in vivo* with expressed long hairpin RNA *Mol. Ther.* **2007**, *15*, 534-541.

[95] Saayman, S.; Barichievy, S.; Capovilla, A.; Morris, K. V.; Arbuthnot, P.; Weinberg, M. S. The efficacy of generating three independent anti-HIV-1 siRNAs from a single U6 RNA Pol III-expressed long hairpin RNA.*PLoS ONE* **2008**, *3*, e2602.

[96] Liu, Y. P.; Haasnoot, J.; Berkhout, B. Design of extended short hairpin RNAs for HIV-1 inhibition. *Nucleic Acids Res.* **2007**, *35*, 5683-93.

[97] Sano, M.; Li, H.; Nakanishi, M.; Rossi, J. J. Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. *Mol .Ther.* **2008**, *16*, 170-177.

[98] Grimm, D.; Streetz, K. L.; Jopling, C. L.; Storm, T. A.; Pandey, K.; Davis, C. R.; Marion, P.; Salazar, F.; Kay, M. A. Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature* **2006**, *441*, 537-541.

[99] McBride, J. L.; Boudreau, R. L.; Harper, S. Q.; Staber, P. D.; Monteys, A. M.; Martins, I.; Gilmore, B. L.; Burstein, H.; Peluso, R. W.; Polisky, B.; Carter, B. J.; Davidson, B. L. Artificial miRNAs mitigate shRNA-mediated toxicity in the brain: implications for the therapeutic development of RNAi. *Proc. Natl. Acad. Sci. USA* **2008**, *105*, 5868-5873.

[100] An, D. S.; Qin, F. X.; Auyeung, V. C.; Mao, S. H.; Kung, S. K.; Baltimore, D.; Chen, I. S. Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes *via* lentiviral vectors. *Mol .Ther*. **2006**, *14*, 494-504.

[101] Castanotto, D.; Sakurai, K.; Lingeman, R.; Li, H.; Shively, L.; Aagaard, L.; Soifer, H.; Gatignol, A.; Riggs, A.; Rossi, J. J. Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. *Nucleic Acids Res.* **2007**, *35*, 5154-5164.

[102] Boudreau, R. L.; Martins, I.; Davidson, B. L. Artificial MicroRNAs as siRNA Shuttles: Improved Safety as Compared to shRNAs *In vitro* and *In vivo*. *Mol.Ther*. **2008**. *17*(1)169-75.

[103] Ely, A.; Naidoo, T.; Mufamadi, S.; Crowther, C.; Arbuthnot, P. Expressed anti-HBV primary microRNA shuttles inhibit viral replication efficiently *in vitro* and *in vivo*. *Mol .Ther*. **2008**, *16*, 1105-1112.

[104] Keck, K.; Volper**,** E. M.; Spengler, R. M.; Long, D. D.; Chan, C. Y.; Ding, Y.; McCaffrey, A. P. Rational Design Leads to More Potent RNA Interference Against Hepatitis B Virus: Factors Effecting Silencing Efficiency. *Mol .Ther*. **2008**. *17*(3),538-547.

[105] Ely, A.; Naidoo, T.; Arbuthnot, P. Efficient silencing of gene expression with modular trimeric Pol II expression cassettes comprising microRNA shuttles. *Nucleic Acids Res.* **2009**. *37*(13),e91. Epub 2009 May 27.

[106] Aagaard, L. A.; Zhang, J.; von Eije, K. J.; Li, H.; Saetrom, P.; Amarzguioui, M.; Rossi, J. J. Engineering and optimization of the miR-106b cluster for ectopic expression of multiplexed anti-HIV RNAs. *Gene Ther*. **2008**.

[107] Chung, K. H.; Hart, C. C.; Al-Bassam, S.; Avery, A.; Taylor, J.; Patel, P. D.; Vojtek, A. B.; Turner, D. L. Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155 *Nucleic Acids Res.* **2006**, *34*, e53.

[108] Liu, Y. P.; Haasnoot, J.; ter Brake, O.; Berkhout, B.; Konstantinova, P. Inhibition of HIV-1 by multiple siRNAs expressed from a single microRNA polycistron *Nucleic Acids Res*. **2008**, *36*, 2811-2824.

[109] Garcia-Sastre, A.; Biron, C. A. Type 1 interferons and the virus-host relationship: a lesson in detente *Science* **2006**, *312*, 879-882.

[110] de Veer, M. J.; Sledz, C. A.; Williams, B. R. Detection of foreign RNA: implications for RNAi *Immunol. Cell Biol.* **2005**, *83*, 224-228.

[111] Karpala, A. J.; Doran, T. J.; Bean, A. G. Immune responses to dsRNA: implications for gene silencing technologies *Immunol. Cell Biol.* **2005**, *83*, 211-216.

[112] Jackson, A. L.; Burchard, J.; Leake, D.; Reynolds, A.; Schelter, J.; Guo, J.; Johnson, J. M.; Lim, L.; Karpilow, J.; Nichols, K.; Marshall, W.; Khvorova, A.; Linsley, P. S. Position-specific chemical modification of siRNAs reduces "off-target" transcript silencing *Rna* **2006**, *12*, 1197-1205.

[113] Lin, X.; Ruan, X.; Anderson, M. G.; McDowell, J. A.; Kroeger, P. E.; Fesik, S. W.; Shen, Y. siRNA-mediated off-target gene silencing triggered by a 7 nt complementation *Nucleic Acids Res.* **2005**, *33*, 4527-4535.

[114] Bennasser, Y.; Jeang, K. T. HIV-1 Tat interaction with Dicer: requirement for RNA. *Retrovirology* **2006**, *3*, 95.

[115] Gatignol, A.; Lainé, S.; Clerzius, G. Dual role of TRBP in HIV replication and RNA interference: viral diversion of a cellular pathway or evasion from antiviral immunity.? *Retrovirology* **2005**, *2*, 1-16.

[116] Christensen, H. S.; Daher, A.; Soye, K. J.; Frankel, L. B.; Alexander, M. R.; Laine, S.; Bannwarth, S.; Ong, C. L.; Chung, S. W.; Campbell, S. M.; Purcell, D. F.; Gatignol, A. Small interfering RNAs against the TAR RNA binding protein, TRBP, a Dicer cofactor, inhibit human immunodeficiency virus type 1 long terminal repeat expression and viral production. *J. Virol.* **2007**, *81*, 5121-5131.

[117] Gatignol, A.; Buckler-White, A.; Berkhout, B.; Jeang, K. T. Characterization of a human TAR RNA-binding protein that activates the HIV-1 LTR. *Science* **1991**, *251*, 1597-600.

[118] Bennasser, Y.; Yeung, M. L.; Jeang, K. HIV-1 TAR RNA subverts RNA interference in transfected cells through sequestration of TAR RNA-binding protein, TRBP. *J. Biological Chem.* **2006**, *281*, 27674-27678.

[119] Lin, J.; Cullen, B. R. Analysis of the interaction of primate retroviruses with the human RNA interference machinery *J. Virol.* **2007**, *81*, 12218-12226.

[120] Triboulet, R.; Mari, B.; Lin, Y. L.; Chable-Bessia, C.; Bennasser, Y.; Lebrigand, K.; Cardinaud, B.; Maurin, T.; Barbry, P.; Baillat, V.; Reynes, J.; Corbeau, P.; Jeang, K. T.; Benkirane, M. Suppression of microRNA-silencing pathway by HIV-1 during virus replication *Science* **2007**, *315*, 1579-1582.

[121] Bennasser, Y.; Le, S. Y.; Yeung, M. L.; Jeang, K. T. HIV-1 encoded candidate micro-RNAs and their cellular targets *Retrovirology* **2004**, *1*, 43.

[122] Omoto, S.; Fujii, Y. R. Regulation of human immunodeficiency virus 1 transcription by nef microRNA *J. Gen. Virol* **2005**, *86*, 751-755.

[123] Omoto, S.; Ito, M.; Tsutsumi, Y.; Ichikawa, Y.; Okuyama, H.; Brisibe, E. A.; Saksena, N. K.; Fujii, Y. R. HIV-1 nef suppression by virally encoded microRNA. *Retrovirology* **2004**, *1*, 44.

[124] Ahluwalia, J. K.; Khan, S. Z.; Soni, K.; Rawat, P.; Gupta, A.; Hariharan, M.; Scaria, V.; Lalwani, M.; Pillai, B.; Mitra, D.; Brahmachari, S. K. Human cellular microRNA hsa-miR-29a interferes with viral nef protein expression and HIV-1 replication. *Retrovirology* **2008**, *5*, 1-25.

[125] Nathans, R.; Chu, C. Y.; Serquina, A. K.; Lu, C. C.; Cao, H.; Rana, T. M. Cellular microRNA and P bodies modulate host-HIV-1 interactions. *Mol. Cell* **2009**, *34*, 696-709.

[126] Klase, Z.; Kale, P.; Winograd, R.; Gupta, M. V.; Heydarian, M.; Berro, R.; McCaffrey, T.; Kashanchi, F. HIV-1 TAR element is processed by Dicer to yield a viral micro-RNA involved in chromatin remodeling of the viral LTR. *BMC Mol .Biol*. **2007**, *8*, 63.

[127] Ouellet, D. L.; Plante, I.; Landry, P.; Barat, C.; Janelle, M. E.; Flamand, L.; Tremblay, M. J.; Provost, P. Identification of functional microRNAs released through asymmetrical processing of HIV-1 TAR element *Nucleic Acids Res*. **2008**, *36*, 2353-2365.

[128] Huang, J.; Wang, F.; Argyris, E.; Chen, K.; Liang, Z.; Tian, H.; Huang, W.; Squires, K.; Verlinghieri, G.; Zhang, H. Cellular microRNAs contribute to HIV-1 latency in resting primary CD4+ T lymphocytes. *Nat. Med*. **2007**, *13*, 1241-1247.

[129] Han, Y.; Siliciano, R. F. Keeping quiet: microRNAs in HIV-1 latency. *Nat. Med*. **2007**, *13*, 1138-1140.

[130] Morris, K. V.; Rossi, J. J. Lentivirus-mediated RNA interference therapy for human immunodeficiency virus type 1 infection. *Human Gene Therapy* **2006**, *17*, 479-486.

[131] Banerjea, A.; Li, M.; Bauer, G.; Remling, L.; Lee, N.; Rossi, J. J.; Akkina, R. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and

CD34+ progenitor cell-derived macrophages. *Molecular Therapy* **2003**, *8*, 62-71.

[132] Akkina, R. K.; Rosenblatt, J. D.; Campbell, A. G.; Chen, I. S.; Zack, J. A. Modeling human lymphoid precursor cell gene therapy in the SCID-hu mouse. *Blood* **1994**, *84*, 1393-1398.

[133] Ishikawa, F.; Yasukawa, M.; Lyons, B.; Yoshida, S.; Miyamoto, T.; Yoshimoto, G.; Watanabe, T.; Akashi, K.; Shultz, L. D.; Harada, M. Development of functional human blood and immune systems in NOD/SCID/IL2 receptor gamma chain null mice. *Blood* **2005**, *106*, 1565-1573.

[134] Watanabe, S.; Ohta, S.; Yajima, M.; Terashima, K.; Ito, M.; Mugishima, H.; Fujiwara, S.; Shimizu, K.; Honda, M.; Shimizu, N.; Yamamoto, N. Humanized NOD/SCID/IL2R gamma null mice transplanted with hematopoietic stem cells under nonmyeloablative conditions show prolonged life spans and allow detailed analysis of human immunodeficiency virus type 1 pathogenesis. *J. Virology* **2007**, *81*, 13259-13264.

[135] Kumar, P.; Ban, H.; Kim, S.; Wu, H.; Pearson, T.; Greiner, D. L.; Laouar, A.; Yao, J.; Haridas, V.; Habiro, K.; Yang, Y.; Jeong, J.; Lee, K.; Kim, Y.; Kim, S. W.; Peipp, M.; Fey, G. H.; Manjunath, N.; Shultz, L. D.; Lee, S.; Shankar, P. T cell-specific siRNA delivery suppresses HIV-1 infection in humanized mice. *Cell* **2008**, *134*, 1-10.

[136] ter Brake, O.; Legrand, N.; von Eije, K. J.; Centlivre, M.; Spits, H.; Weijer, K.; Blom, B.; Berkhout, B. Evaluation of safety and efficacy of RNAi against HIV-1 in the human immune system (Rag-2-/-cc -/-) mouse model. *Gene Therapy* **2009**, *16*, 148-153.

[137] An, D. S.; Donahue, R. E.; Kamata, M.; Poon, B.; Metzger, M.; Mao, S. H.; Bonifacino, A.; Krouse, A. E.; Darlix, J. L.; Baltimore, D.; Qin, F. X.; Chen, I. S. Stable reduction of CCR5 by RNAi through hematopoietic stem cell transplant in non-human primates. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13110-5.

[138] Kohn, D. B.; Bauer, G.; Rice, C. R.; Rothschild, J. C.; Carbonaro, D. A.; Valdez, P.; Hao, Q.; Zhou, C.; Bahner, I.; Kearns, K.; Brody, K.; Fox, S.; Haden, E.; Wilson, K.; Salata, C.; Dolan, C.; Wetter, C.; Aguilar-Cordova, E.; Church, J. A clinical trial of retroviral-mediated transfer of a rev-responsive element decoy gene into CD34(+) cells from the bone marrow of human immunodeficiency virus-1-infected children. *Blood* **1999**, *94*, 368-371.

[139] Mitsuyasu, R. T.; Merigan, T. C.; Carr, A.; Zack, J. A.; Winters, M. A.; Workman, C.; Bloch, M.; Lalezari, J.; Becker, S.; Thornton, L.; Akil, B.; Khanlou, H.; Finlayson, R.; McFarlane, R.; Smith, D. E.; Garsia, R.; Ma, D.; Law, m.; Murray, J. M.; von Kalle, C.; Ely, J. A.; Patino, S. M.; Knop, A. E.; Wong, P.; Todd, A. V.; Haughton, M.; Fuery, C.; Macpherson, J. L.; Symonds, G. P.; Evans, L. A.; Pond, S. M.; Cooper, D. A. Phase 2 gene therapy trial of an anti-HIV ribozyme in autologous CD34+ cells *Nature Medicine*, **2009**, *15*, 285-292.

[140] Woffendin, C.; Ranga, U.; Yang, Z.; Xu, L.; Nabel, G. J. Expression of a protective gene prolongs survival of T cells in human immunodeficiency virus-infected patients. *Proc. Natl. Acad.Sci. USA* **1996**, *93*, 2889-2894.

[141] Macpherson, J. L.; Boyd, M. P.; Arndt, A. J.; Todd, A. V.; Fanning, G. C.; Ely, J. A.; Elliott, F.; Knop, A.; Raponi, M.; Murray, J.; Gerlach, W.; Sun, L. Q.; Penny, R.; Symonds, G. P.; Carr, A.; Cooper, D. A. Long-term survival and concomitant gene expression of ribozyme-transduced CD4+ T-lymphocytes in HIV-infected patients. *J. Gene Med.* **2005**, *7*, 552-564.

[142] Li, M.; Li, H.; Rossi, J. J. RNAi in combination with a ribozyme and TAR decoy for treatment of HIV infection in hematopoietic cell gene therapy. *Ann. N Y Acad. Sci.* **2006**, *1082*, 172-179.

[143] Li, M. J.; Kim, J.; Li, S.; Zaia, J.; Yee, J. K.; Anderson, J.; Akkina, R.; Rossi, J. J. Long-term inhibition of HIV-1 infection in primary hematopoietic cells by lentiviral vector delivery of a triple combination of anti-HIV shRNA, anti-CCR5 ribozyme, and a nucleolar-localizing TAR decoy. *Mol. Therapy.* **2005**, *12*, 900-909.

[144] Anderson, J.; Li, M.; Palmer, b.; Remling, L.; Li, s.; Yam, p.; Yee, j.; Rossi, J. J.; Zaia, j.; Akkina, R. Safety and efficacy of a lentiviral vector containing three anti-HIV genes—CCR5 ribozyme, Tat-Rev siRNA, and TAR decoy—in SCID-hu mouse–derived T cells. *Molecular Therapy*, **2007**, *15*, 1182-1188.

[145] Weinberg, M. S.; Barichievy, S.; Schaffer, L.; Han, J.; Morris, K. V. An RNA targeted to the HIV-1 LTR promoter modulates indiscriminate off-target gene activation. *Nucleic Acids Res.* **2007**, *35*(21),7303-7312

[146] Lim, H. G.; Suzuki, K.; Cooper, D. A.; Kelleher, A. D. Promoter-targeted siRNAs induce gene silencing of simian immunodeficiency virus (SIV) infection *in vitro*. *Mol Ther*, **2008**, *16*, 565-570.

[147] Suzuki, K.; Juelich, T.; Lim, H.; Ishida, T.; Watanebe, T.; Cooper, D. A.; Rao, S.; Kelleher, A. D. Closed chromatin architecture is induced by an RNA duplex targeting the HIV-1 promoter. region *J. Biol. Chem.* **2008**, *283*, 23353-23363.

[148] Turner, A. M.; De La Cruz, J.; Morris, K. V. Mobilization-competent Lentiviral Vector-mediated Sustained Transcriptional Modulation of HIV-1 Expression *Mol. Ther.* **2009**, *17*, 360-368.

[149] Hawkins, P. G.; Santoso, S.; Adams, C.; Anest, V.; Morris, K. V. Promoter targeted small RNAs induce long-term transcriptional gene silencing in human cells. *Nucleic Acids Res.* **2009**, *37*, 2984-2995.

[150] Han, J.; Kim, D.; Morris, K. V. Promoter-associated RNA is required for RNA-directed transcriptional gene silencing in human cells. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 12422-12427.

[151] Ghildiyal, M.; Zamore, P. D. Small silencing RNAs: an expanding universe. *Nat. Rev. Genet* **2009**, *10*, 94-108.

[152] Fornerod, M.; Ohno, M.; Yoshida, M.; Mattaj, I. W. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell* **1997**, *90*, 1051-1060.

[153] Kawasaki, H.; Taira, K. Short hairpin type of dsRNAs that are controlled by tRNA(Val) promoter significantly induce RNAi-mediated gene silencing in the cytoplasm of human cells. *Nucleic Acids Res* **2003**, *31*, 700-707.

[154] Rodriguez, M. S.; Dargemont, C.; Stutz, F. Nuclear export of RNA. *Biol .Cell* **2004**, *96*, 639-655.

Received July 7, 2009 Accepted July 14, 2009

OPEN ACCESS Freely available online

PLoS one

The Efficacy of Generating Three Independent Anti-HIV-1 siRNAs from a Single U6 RNA Pol III-Expressed Long Hairpin RNA

Sheena Saayman[1], Samantha Barichievy[1], Alexio Capovilla[2], Kevin V. Morris[3], Patrick Arbuthnot[1], Marc S. Weinberg[1]*

1 Antiviral Gene Therapy Research Unit, Department of Molecular Medicine and Haematology, University of Witwatersrand, Johannesburg, South Africa, **2** HIV Pathogenesis Lab, Department of Molecular Medicine and Haematology, University of Witwatersrand, Johannesburg, South Africa, **3** Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California, United States of America

Abstract

RNA Interference (RNAi) effectors have been used to inhibit rogue RNAs in mammalian cells. However, rapidly evolving sequences such as the human immunodeficiency virus type 1 (HIV-1) require multiple targeting approaches to prevent the emergence of escape variants. Expressed long hairpin RNAs (lhRNAs) have recently been used as a strategy to produce multiple short interfering RNAs (siRNAs) targeted to highly variant sequences. We aimed to characterize the ability of expressed lhRNAs to generate independent siRNAs that silence three non-contiguous HIV-1 sites by designing lhRNAs comprising different combinations of siRNA-encoding sequences. All lhRNAs were capable of silencing individual target sequences. However, silencing efficiency together with concentrations of individual lhRNA-derived siRNAs diminished from the stem base (first position) towards the loop side of the hairpin. Silencing efficacy against HIV-1 was primarily mediated by siRNA sequences located at the base of the stem. Improvements could be made to first and second position siRNAs by adjusting spacing arrangements at their junction, but silencing of third position siRNAs remained largely ineffective. Although lhRNAs offer advantages for combinatorial RNAi, we show that good silencing efficacy across the span of the lhRNA duplex is difficult to achieve with sequences that encode more than two adjacent independent siRNAs.

Citation: Saayman S, Barichievy S, Capovilla A, Morris KV, Arbuthnot P, et al. (2008) The Efficacy of Generating Three Independent Anti-HIV-1 siRNAs from a Single U6 RNA Pol III-Expressed Long Hairpin RNA. PLoS ONE 3(7): e2602. doi:10.1371/journal.pone.0002602

Editor: Sheila Mary Bowyer, National Institute for Communicable Diseases, South Africa

Received October 28, 2007; **Accepted** June 6, 2008; **Published** July 2, 2008

Funding: SS holds a postgraduate scholarship from the Ernst and Ethel Eriksen Trust and German Academic Exchange (DAAD). This work was supported by grants from the National Research Foundation (NRF), Medical Research Council (MRC), Poliomyelitis Research Foundation (PRF), and from funding under the Sixth Research Framework Programme of the European Union, Project RIGHT (LSHB-CT-2004-005276).

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: marc.weinberg@wits.ac.za

Introduction

RNA Interference (RNAi) is a highly conserved biological pathway in eukaryotes where gene silencing is mediated by a double-stranded RNA (dsRNA) trigger [1]. Exploitation of the RNAi pathway has lead to fundamental new tools for genetics research and for sequence-specific therapeutic approaches aimed at suppressing rogue cellular genes or viral-associated RNAs. RNAi has traditionally been induced in mammalian cells through the exogenous introduction of synthetic short interfering RNAs (siRNAs) [2], or through the use of RNA Pol III or Pol II gene constructs which express 21–29 bp short hairpin RNAs (shRNAs) [3–6]. Expressed short hairpins resemble pre-microRNAs (pre-miRNAs), which are part of the endogenous microRNA (miRNA) pathway [7,8]. The targeting of highly mutable sequences, such as genomic and sub-genomic RNAs from infectious agents, remains a significant hurdle for the use of RNAi-based therapeutics. In particular, the human immunodeficiency virus type 1 (HIV-1), which replicates using an error-prone reverse transcriptase, has been shown to escape the silencing effects of shRNAs. Resistant viral variants emerge easily in cell culture experiments, even when targeting highly conserved sequences [9–11]. Effective targeting of rapidly evolving targets requires a combinatorial approach, which is analogous to Highly Active Antiretroviral Therapy (HAART) [reviewed by [12,13]].

The targeting of many sites simultaneously using RNAi has been attempted with multiple shRNA expression units, where each unit is expressed from a RNA Pol III promoter [14–16] or RNA Pol II promoter [17]. Similarly, concatenated miRNA mimics expressed from a single RNA Pol II promoter have been shown to suppress simultaneously up to three separate target sequences [6,18,19]. Although RNAi-mediated silencing in lower eukaryotes can be achieved efficiently by introducing precursor dsRNAs comprising more than 150 base pairs (bp), intracellular presence of dsRNA of greater than 30 bp leads to a strong innate immunostimulatory response, which is mediated by dsRNA-activating protein kinase (PKR) and 2′–5′ oligoadenylate synthetase [20]. A re-evaluation of long dsRNA greater than 30 bp in mammalian cells has shown that safe and effective gene-specific silencing can be achieved when dsRNA is expressed from DNA-based expression cassettes [21–26]. Although a complete characterization of how intracellular dsRNAs are discriminated remains to be established, intracellularly expressed dsRNA seem capable of evading cytoplasmic activators of the type 1 interferon response

[27,28]. A natural potential advantage of longer dsRNAs is that processing by the RNAse III endonuclease Dicer theoretically allows for the generation of multiple siRNAs, providing a mechanism of combinatorial targeting of rapidly-evolving RNAs. The silencing caused by lhRNAs may also be more effective than that resulting from a single unique siRNA derived from an individual shorter (<30 bp) expressed shRNA.

Akashi et al. showed that (~50 bp) long hairpin RNAs (lhRNAs) expressed from $tRNA^{Val}$ and U6 RNA Pol III promoters generated multiple siRNAs [24]. We and other have shown that similar constructs were capable of suppressing Hepatitis B Virus (HBV) [26], Hepatitis C Virus (HCV) [29] and HIV-1 [30–33] targets. To date, lhRNAs capable of producing more than two independent siRNAs have only been used against contiguous target sequences. Since ~60 bp hairpin RNAs should be capable of providing a substrate for at least three catalytic reactions involving Dicer, we have examined the possibility of introducing three distinct non-contiguous target sequences that, if processed by Dicer, are capable of generating highly-effective independent siRNA species. To determine the ability of targeting disparate regions in the HIV-1 genome, we generated a panel of ~69 bp U6-lhRNA expression cassettes, each consisting of a different arrangement of three adjacent 21-mer putative siRNA sequences. The siRNA sequences chosen were previously characterized as highly effective anti-HIV-1 shRNAs targeted to Tat/Env, Tat/Rev and Vif open reading frames [4,34]. We show that all combinations of lhRNAs were capable of significant knockdown against individual target sequences. However, silencing efficiency together with individual siRNA concentrations diminished from stem base to loop side along the length of the duplex. We present here a thorough characterisation of the efficacy of expressed lhRNAs designed to generate multiple siRNAs targeted to non-contiguous siRNA-susceptible regions of HIV-1.

Results

Design of anti HIV-1 lhRNA-expressing plasmids

Three sites that have been previously shown to be effective for RNAi-mediated inhibition of HIV-1 were selected. These include shRNAs targeted to sites within two separate overlapping reading frames of the HIV-1 genome: Tat/Rev (*tat*) and Rev/Env (*rev*) [4]. The third site includes a sequence within the Vif open reading frame (*vif*) [34] (Fig. 1A). Long hairpin RNAs of approximately 69 bp (with a 5 nt terminal loop) were designed to be transcribed from a U6 RNA Pol III promoter such that three 21–23 bp siRNAs could potentially be generated by Dicer cleavage (Fig. 1B). LhRNA and shRNA expression cassettes were designed to encode siRNA precursors targeted to each of these 3 HIV-1 sites (Fig. 1C). G:U wobble base pairs were included at regular intervals by adjusting sequences in the sense strand to facilitate propagation of the lhRNA-encoding cassettes in *E. coli*. To control for changes in strand bias, similar G:U mismatches were made to each shRNA (Fig. 1C). These changes have previously been shown to have no impact on RNAi knockdown efficacy [24,26] and may help in suppressing the innate immune response to dsRNA [24]. The lhRNAs were therefore intended to be capable of serving as substrates to form siRNAs against each of the *tat*, *rev* and *vif* HIV-1 targets. By targeting three viral sites simultaneously, the lhRNAs have a possible therapeutic benefit of limiting viral escape.

Although this is an important theoretical advantage, there is some evidence to suggest that Dicer processing of lhRNAs may not be equal across the span of the duplex [26,33] and that Dicer favours the production of siRNAs generated from the hairpin stem base. Thus, to assess the importance of the position of the *tat*, *rev* and *vif* siRNA-encoding sequences within the anti HIV-1 lhRNAs, their efficacy when ordered as first, second or third within the stem duplex was assessed.

Assessing anti HIV-1 efficacy of expressed lhRNA sequences in cell culture

Initially, to assess efficacy against HIV-1 *in vitro*, HEK293 cells were cotransfected with lhRNA-or shRNA-expressing vectors together with the dual luciferase psiCheck vector encoding a reporter/HIV-1 fusion gene (Fig. 2A). Four target vectors were generated, which each included *tat*, *rev*, *vif* or a combination of *tat-rev-vif* HIV-1 21-mer targets downstream of the *Renilla* luciferase open reading frame (ORF). Measurement of *Renilla*:Firefly luciferase allowed convenient and accurate measurement of the *in situ* efficacy of the hairpin sequences. When using the luciferase reporter that includes all three HIV-1 targets (psiCheck *tat-rev-vif*), highly effective knockdown of approximately 90% was achieved for each of the lhRNA- and shRNA-encoding plasmids (Fig. 2B). When the *tat* sequence alone was inserted downstream of the *Renilla* luciferase reporter gene, the shRNA *tat* vector was capable of 90% inhibition of reporter gene expression, and as expected, the shRNA *rev* and shRNA *vif* vectors caused no decrease in reporter fusion gene activity (Fig. 2C). lhRNA expression cassettes diminished *Renilla-tat* gene activity by approximately 30–50% and lhRNA *tat-rev-vif* was the most efficient. When similar assessment was carried out on the psiCheck *rev* and psiCheck *vif* targets, the shRNAs induced specific silencing and lhRNAs with the siRNA-encoding sequences located at the base of the stem duplex were most efficient. A 63 bp lhRNA targeted to the HIV-1 TAR loop, showed no inhibitory activity against any of the targets. These data support our previous observations using lhRNAs to inhibit HBV [26] and HIV [31,33] replication and indicate that there is a bias of silencing efficiency that diminishes from the base of the stem to loop side of the duplex RNA.

Detection of processed antiviral hairpin sequences

The spacing arrangement of each individual siRNAs within the long hairpin duplex is such that 4 "neutral" bases were placed between each 21-mer sequence. This arrangement was recently determined as optimal for two siRNAs placed within an extended shRNA [32]. To analyse primary transcripts and processed products of the anti HIV-1 hairpin expression cassettes a northern blot hybridisation was carried out. RNA was extracted from transfected HEK293 cells and Figure 3A shows the signals obtained after hybridisation to probes that were complementary to the putative mature processed *tat*, *rev* or *vif* siRNA guide strands. Mature products of each of the shRNA expression cassettes were detectable as bands of approximately 22–23 nt in size. Processing of the shRNA primary transcripts to produce siRNA appeared to occur more effectively than that of lhRNA expression cassettes for guide strands at the base of the duplex, which may be due to better recognition by Dicer for the shRNA than the lhRNA (Fig. 3A). The band representing precursors for construct lhRNA *vif-tat-rev* was larger than the precursor bands of the other two lhRNAs, suggesting that read-through transcription is occurring beyond the polyT termination signal. Detection with the vif probe suggests that the production of siRNAs were not impaired for lhRNA *vif-tat-rev* (at least in the first position). For the lhRNAs, guide strands derived from the duplex stem base region of the lhRNA expression cassettes were present in highest concentration, while those that originated from the second and third positions of the lhRNA stem were only detected for the lhRNA *rev-vif-tat* construct, in decreasing order of concentration. It is possible that the probes are not detecting the second or third siRNA because of

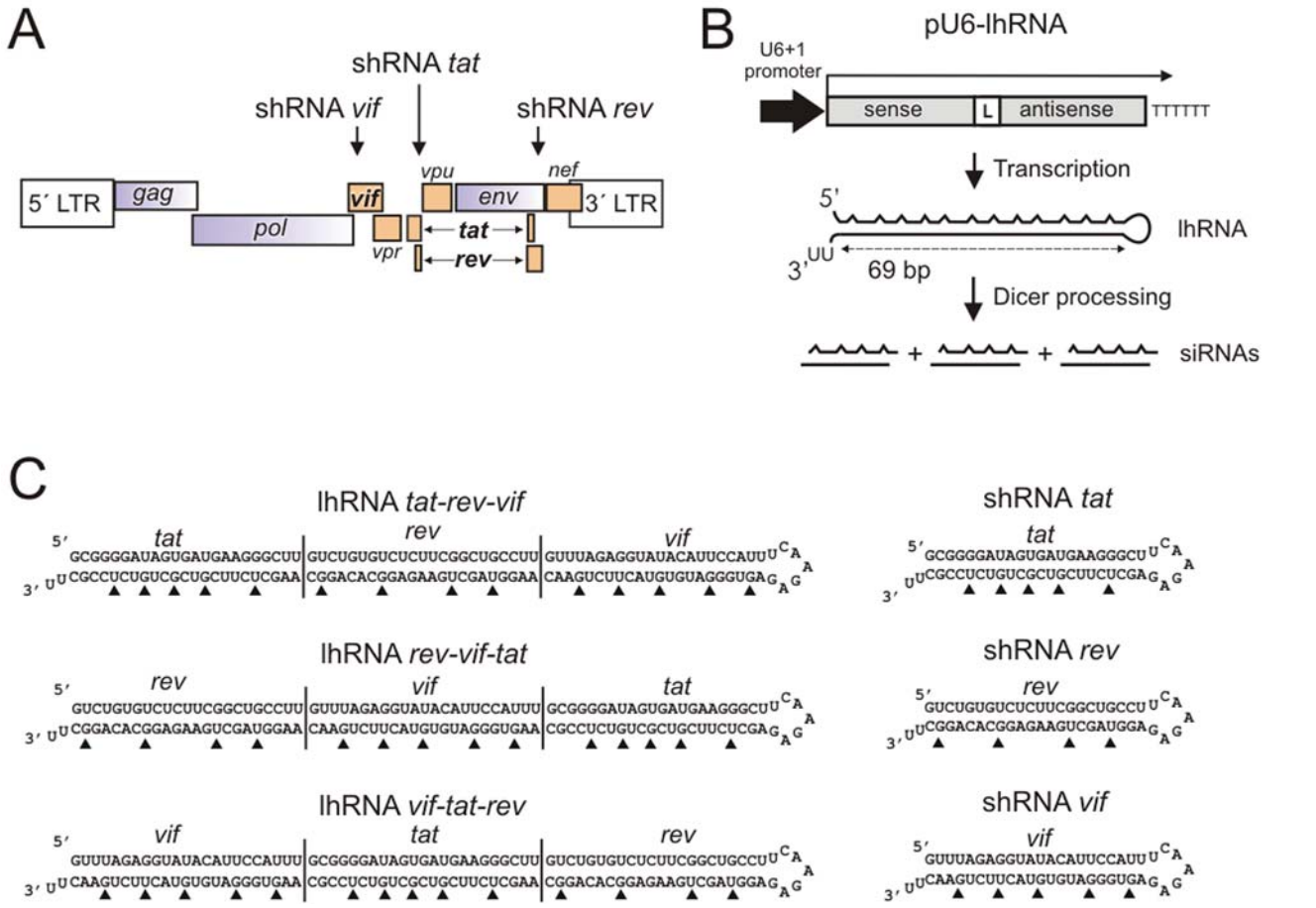


Figure 1. HIV-1 C subtype genome with sites targeted by lhRNA and shRNAs. A. Organization of HIV-1 subtype C genome indicating open reading frames (ORFs) together with the 5′ and 3′ long terminal repeats (LTRs). Arrows show the sites targeted by each of shRNA *vif*, shRNA *tat* and shRNA *rev*, as well as the lhRNAs. **B.** Schematic illustration of lhRNAs comprising 69 bp in the stem. G:U pairings are indicated as corrugated sense strand. A sequence of 2 U residues that are derived from the transcription termination signal is shown. The intended mechanism of transcription and processing of the lhRNAs to form 3 anti HIV-1 siRNAs is illustrated. **C.** Sequences and predicted structure of lhRNAs and shRNAs. The order of the siRNA-encoding sequences within the lhRNAs is indicated along the extent of the duplex. G:U and U:G pairings are indicated with an arrowhead.
doi:10.1371/journal.pone.0002602.g001

misalignment due to differential Dicer processing. To investigate this further, we used two 14-mer locked-nucleic acid (LNA) probes, *LNA-tat-1* and *LNA-tat-2*, which were partially or fully complementary to the tat siRNA respectively (Fig. 3B). *LNA-tat-2* was designed to fully complement any siRNAs generated by three Dicer processing reactions that are 21-bases apart. The results show that only the *tat* siRNA guide derived from the shRNA *tat* or lhRNA *tat-rev-vif* could be detected by both probes, which supports the theory that there is a considerable drop in siRNA concentration as Dicer processes along the lhRNA duplex.

Effect of spacing between siRNA-encoding sequences of the lhRNAs on silencing efficacy

To assess the effect of the spacing between siRNA-encoding sequences on silencing efficiency, the lhRNA *rev-vif-tat* encoding cassette was modified by insertion or deletion of 1–4 bases at each of the junctions of the siRNA-encoding sequences (Fig. 4A). When these modified lhRNA *rev-vif-tat*-derived expression cassettes were transfected into HEK293 cells together with psiCheck *tat-rev-vif* target, the silencing efficacy of each of the RNAi effector sequences was similar, and approximately 95% silencing was achieved (Fig. 4B). Slightly diminished efficacy was observed for lhRNA *rev-vif-tat* e. This sequence had a deletion of 2 bp at the *rev-vif* junction, which may influence processing and silencing efficacy of the siRNA originating from the stem base. Assessment of silencing of individual *tat*, *rev* and *vif* targets again showed that the silencing was greatest for each target cognate of the siRNA derived from the stem base of the lhRNA sequence (Fig. 4C). When using the psiCheck *rev* target, knockdown of approximately 90% was achieved by all of the hairpins except for lhRNA *rev-vif-tat* e, which again showed diminished efficacy. No rev siRNA guide was observed for lhRNA *rev-vif-tat* e by northern blot when probing for rev (Fig. 4D), explaining the lack of inhibitory activity for this lhRNA species when detecting effects at the first position (for *rev*, Fig 3C). Diminished knockdown of reporter gene activity was observed when the fused target corresponded to the second position siRNA within the lhRNA duplex. However, the different spacing arrangements resulted in significant variation in silencing efficiency at this position. Interestingly, lhRNA *rev-vif-tat* e was more effective than each of the other lhRNA *rev-vif-tat* cassettes against the *vif* target. The 2 bp deletion at the *rev-vif* junction of lhRNA *rev-vif-tat* e may be the most optimal spacing arrangement for the *vif* siRNA sequence, which is in the second position. Inhibition of the reporter-*tat* target (third position) was largely ineffective for all of the lhRNA variants. The results suggest that there exists considerable leeway in improving first and second

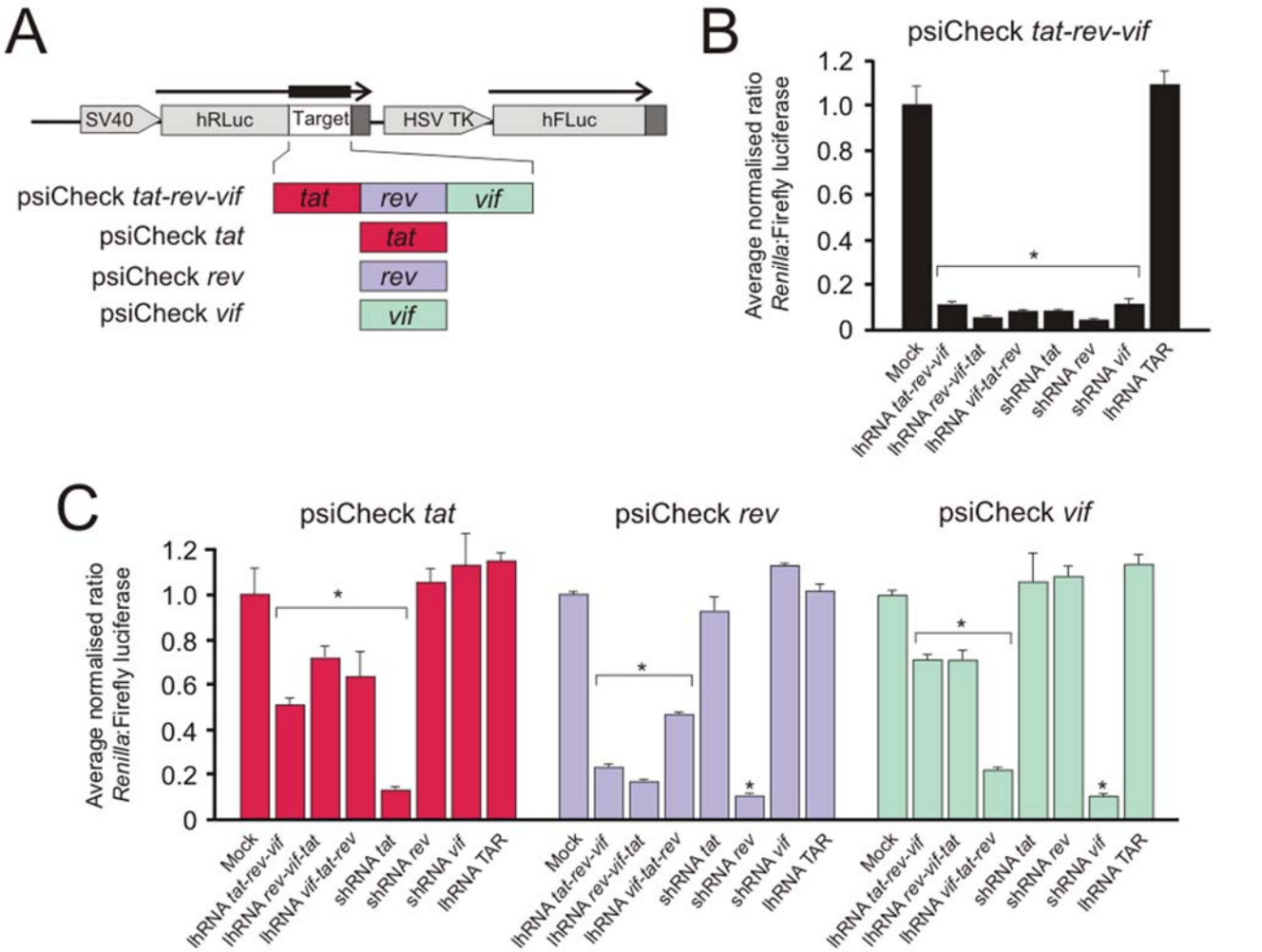


Figure 2. Knockdown of target-reporter fusion gene expression. A. psiCheck-derived vectors that include indicated HIV-1 target sequences inserted downstream of the *Renilla* luciferase ORF. The control Firefly luciferase cassette, present on the same plasmid, is also shown. Both cassettes are under control of constitutively active transcription regulatory elements: Herpes simplex virus thymidine kinase (HSV TK) and Simian virus 40 (SV40) promoters. **B.** Average normalized ratios of the *Renilla*:Firefly luciferase activity when cells were transfected with psiCheck *tat-rev-vif* dual luciferase reporter plasmid together with lhRNA- or shRNA-encoding plasmid vectors. **C.** Average normalized ratios of the *Renilla*:Firefly luciferase activity when cells were transfected with psiCheck *tat*, psiCheck *rev* or psiCheck *vif* dual luciferase reporter plasmids together with lhRNA- or shRNA-encoding plasmid vectors. The average values from three independent transfection experiments, with standard deviations, are given (*, $p<0.05$, *t*-test, relative to mock transfected control).
doi:10.1371/journal.pone.0002602.g002

position siRNA arrangements along an lhRNA duplex but that third position siRNAs are unlikely to be dramatically improved by these modifications.

Inhibition of HIV-1 replication in infected cells in culture

To assess the efficacy of lhRNA sequences in a culture model of HIV-1 infection, U87.CD_4.CCR5 cells were transfected with various lhRNA expression plasmids followed by viral challenge with a South African R5-tropic subtype C HIV-1 isolate, FV5 (accession: 05ZAFV5). Knockdown was assessed by determining p24 antigen levels and viral RNA genome equivalents (Fig. 5A) in the culture supernatant at day 6 post-infection. Of the lhRNA expression cassettes, lhRNA *tat-rev-vif* was most effective and achieved inhibition of markers of viral replication by 60–70%. shRNA *tat* was the most effective of the shRNA expression cassettes and effected inhibition of approximately 60%. Both lhRNA *rev-vif-tat* and shRNA *rev* were less effective whereas lhRNA *vif-tat-rev* and shRNA *vif* respectively had weak or no inhibitory effect on HIV-1 replication in this cell culture model. The efficacy of silencing was also observed longitudinally, again indicating the ineffective silencing by shRNA *vif* and lhRNA *vif-tat-rev* (Fig. 5B) . Silencing by the second and third position siRNAs from lhRNA *vif-tat-rev* are not contributing to the inhibition of HIV-1 replication, but the first position is the most significant for the other 2 lhRNAs. Analysis of the sequences of the targets from the FV5 isolate (Fig. 5C) reveals that the putative hairpin-derived *vif* guide is not perfectly complementary to its viral cognate and includes 3 G:U wobble mismatches. Although, shRNA vif was originally chosen as it proved to be effective at inhibiting viral progression and replication [34], it is possible that inhibiting *vif* may not immediately influence p24 output. To determine if the FV5 *vif* target sequence is refractory to silencing by shRNA *vif* and respective *vif*-containing lhRNAs, a psiCheck luciferase reporter vector was constructed containing HXB2 and FV5 *vif* shRNA target sequences. When compared to the inhibition of the HXB2 *vif* target, shRNA *vif* or lhRNA *vif-tat-rev* was unable to inhibit the

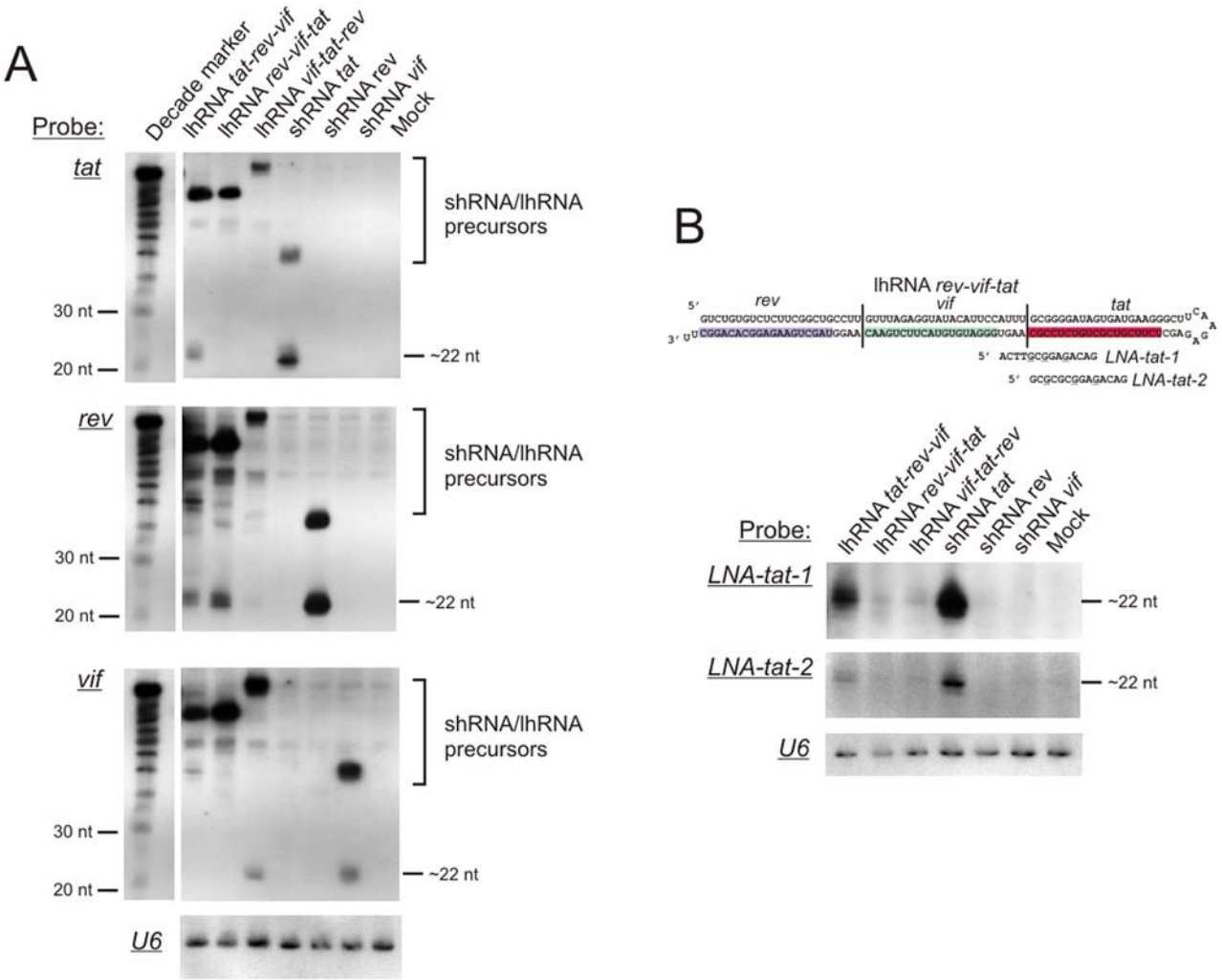


Figure 3. Northern blot analysis of RNA extracted from HEK293 cells that had been transfected with the indicated lhRNA and shRNA-expressing plasmids. A. A single blot was probed with an oligonucleotide that was complementary to putative *tat, rev* and *vif* guide sequences. **B.** The blot was probed with two 14-mer LNA oligonucleotides (LNA nucleotides underlined) which were complementary to the *rev* siRNA guide sequence and adjacent nucleotides as indicated in the illustration. Both blots (from **A** and **B**) were stripped and reprobed with an oligonucleotide complementary to U6 snRNA to control for equal RNA loading.
doi:10.1371/journal.pone.0002602.g003

FV5 target (Fig 5D). These data may explain why no viral inhibition was observed for shRNA *vif* and lhRNA *vif-tat-rev* (Figs 5A,B). The results of the challenge assay support the previous reporter knockdown data that lhRNA knockdown efficacy is primarily mediated by first position siRNA sequences located at the base of the duplex stem.

Discussion

The lhRNAs designed in this study were developed specifically to generate three separate functional siRNAs targeting known non-adjacent siRNA-susceptible regions of HIV-1. Such an approach is of tremendous value to efforts aimed at combinatorial RNAi strategies, where the targeting of highly mutagenic sequences, such as HIV-1, may help prevent the emergence of resistant viral variants [12,13]. Combinatorial RNAi approaches applied to date include the use of multiple RNA Pol III promoters to express shRNAs [15,35,36]. Various combinatorial shRNA-expression systems have been shown to delay effectively the emergence of shRNA-resistant HIV-1 in cell culture [15,36], proving in principle the efficacy of a multiple targeting RNAi strategy against a rapidly evolving target. However, there are reservations about the use of multiple RNA Pol III expression cassettes. Firstly, little is known about the long-term stability and efficacy of such a system: adjacent repeat sequences may recombine when delivered by viral vectors or when stably expressed in rapidly dividing cells [36]. Secondly, and more importantly, the use of multiple highly active RNA Pol III promoters can potentially flood the cell with shRNAs and abrogate the natural microRNA biogenesis pathway, leading to unwanted toxicities [37–39]. It is clear that the presence of several therapeutic hairpin species will require careful dosing in order to achieve the desired levels of silencing.

To date, lhRNAs expressed from RNA Pol III promoters have been used against a single contiguous target sequence in mammalian cells. Previous reports targeting HCV and HIV have suggested that lhRNAs (>50 bp) can adequately inhibit targets harboring mutations that abrogate the silencing efficiency of 21 bp shRNAs [24,30,33]. Yet, the efficacy of individually processed siRNAs generated from expressed lhRNAs has not been

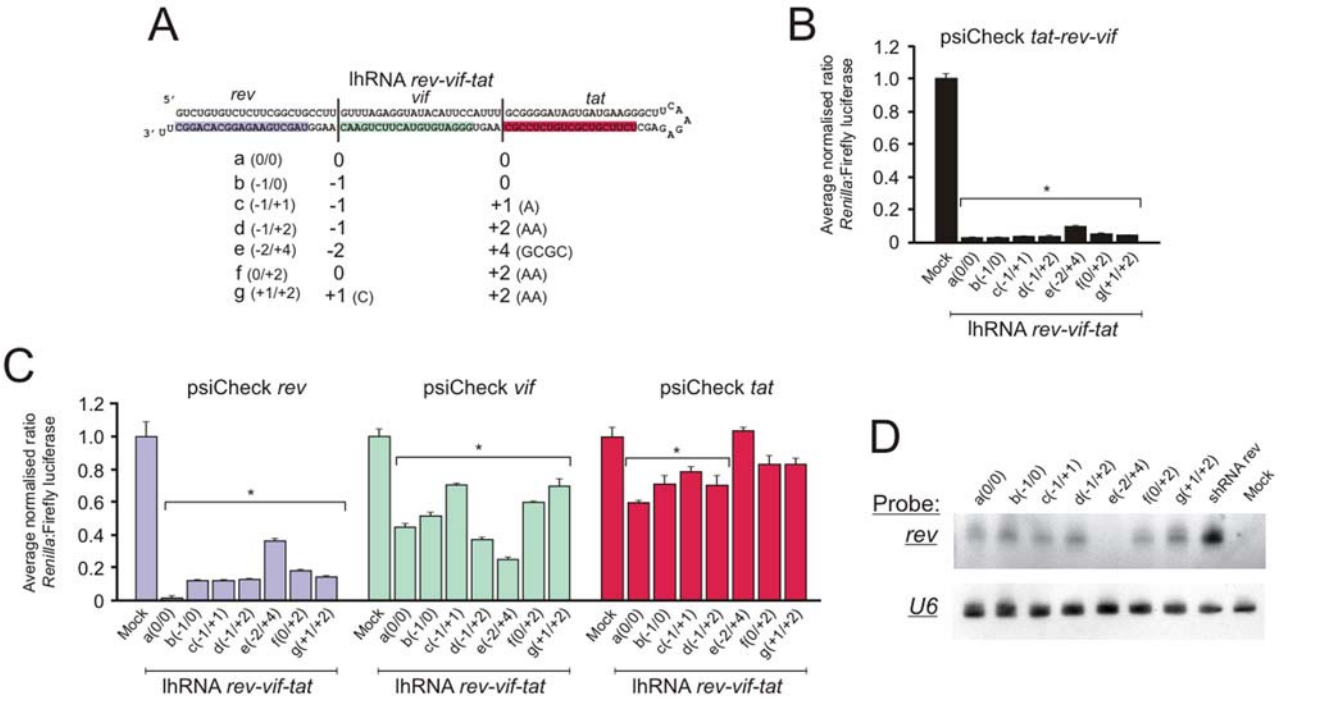


Figure 4. Effect of nucleotide spacing between *tat*, *rev* and *vif* siRNA-encoding sequences on silencing efficacy. A. Schematic illustration of hairpin sequences with boundaries between *tat*, *rev* and *vif* duplexes indicated. The sequences and numbers of bases inserted or deleted at the junctions of the RNAi effecter-encoding sequences are indicated for each of lhRNA *rev-vif-tat* a to lhRNA *rev-vif-tat* g. **B.** Average normalized *Renilla*:Firefly luciferase activity determined 48 hours after transfecting HEK293 cells with the psiCheck *tat-rev-vif* target together with each of lhRNA *rev-vif-tat* a to lhRNA *rev-vif-tat* g. **C.** Average normalized *Renilla*:Firefly luciferase activity determined 48 hours after transfecting HEK293 cells with the psiCheck *tat*, psiCheck *rev or* psiCheck *vif* target together with each of lhRNA *rev-vif-tat* a to lhRNA *rev-vif-tat* g. Results are given as the average values with standard deviations from three independent transfection experiments. (*, $p<0.05$, *t*-test, relative to mock transfected control). Mock transfected cells received the empty backbone U6+1 plasmid **D.** Northern blot analysis of RNA extracted from HEK293 cells that had been transfected with the indicated lhRNA and shRNA-expressing plasmids. The blot was probed with an oligonucleotide that was complementary to putative *rev* guide sequence. The blot was stripped and reprobed with an oligonucleotide complementary to U6 snRNA to control for equal RNA loading.
doi:10.1371/journal.pone.0002602.g004

adequately characterized, making it difficult for direct comparisons between lhRNAs and shRNA when targeted to the same sequence. We have previously observed that ~60 bp U6-expressed lhRNAs targeted to a contiguous sequence within HBV generated siRNAs more efficiently from the base of the hairpin stem, and this correlated with greater silencing efficacy [26]. However, we could not rule-out the possibility that second or third Dicer cleavage reactions generate siRNAs with ineffective guide sequences. We suspect that an increased variance of the siRNA pool and decreased siRNA concentration for second and third position cleavage products is likely to compromise the efficacy of siRNAs generated from lhRNAs that require more than two Dicer reactions. By placing three known effective siRNA sequences adjacent to each other along a 69 bp lhRNA duplex, some general principles concerning the efficacy of expressed lhRNAs for combinatorial RNAi have been deduced. By comparing different combinations of adjacent siRNA sequences within a lhRNA duplex, we show that sequences at the base of the hairpin stem are preferentially processed into effective siRNAs, and that the pattern of silencing appears to be independent of the siRNA sequence within the duplex. There was an exception, as construct lhRNA *rev-vif-tat* e (Fig 4) was not able to generate a guide strand for the first position. Some sequence differences do exist between lhRNA *rev-vif-tat* e and other lhRNA variants, and it may be possible that the sequence of the 2 nt 3′ overhang plays a role in siRNA recognition within RISC. The Paz domain of Dicer is known to have biased preference for different 3′ overhang sequences [40], and differential selection of processed siRNAs by the analogous Argonaut 2 Paz domain may occur similarly [41,42]. Overall, these data are in agreement with Dicer's preference for cleaving dsRNA duplex ends with 2-nt 3′-OH overhangs [40,43–45] but suggests that intracellular Dicer processivity is relatively inefficient. This perhaps underscores the function of human Dicer as a single-turnover enzyme specialized in generating mature miRNAs from a single cleavage reaction.

Initially, the three independent siRNA-encoding sequences were placed within the lhRNA duplex such that they were spaced at 23 bp intervals. Recently, Liu et al [32] showed that extended shRNAs with two independent siRNAs functioned optimally as independent siRNAs when spaced 4 bp apart. However, in our hands, such spacing arrangements were not necessarily optimal and it remains difficult to make gross generalizations regarding Dicer-processing positions along an expressed dsRNA duplex at this stage. Nevertheless, improvements in multiple targeting can be achieved by further investigating the addition or deletion of nucleotides at the siRNA junctions along the duplex. For one of the lhRNAs, lhRNA *rev-vif-tat*, efficient processing of the second siRNA was observed, albeit at reduced concentrations. If arranged correctly, augmented knockdown can be achieved for two independent siRNAs along a duplex, but this unlikely to be possible for three siRNAs. Thus, one can envisage that use of lhRNAs designed to efficiently inhibit at least two independent siRNA-susceptible regions may help to delay the onset of HIV-1 escape variants, especially when targeting only conserved

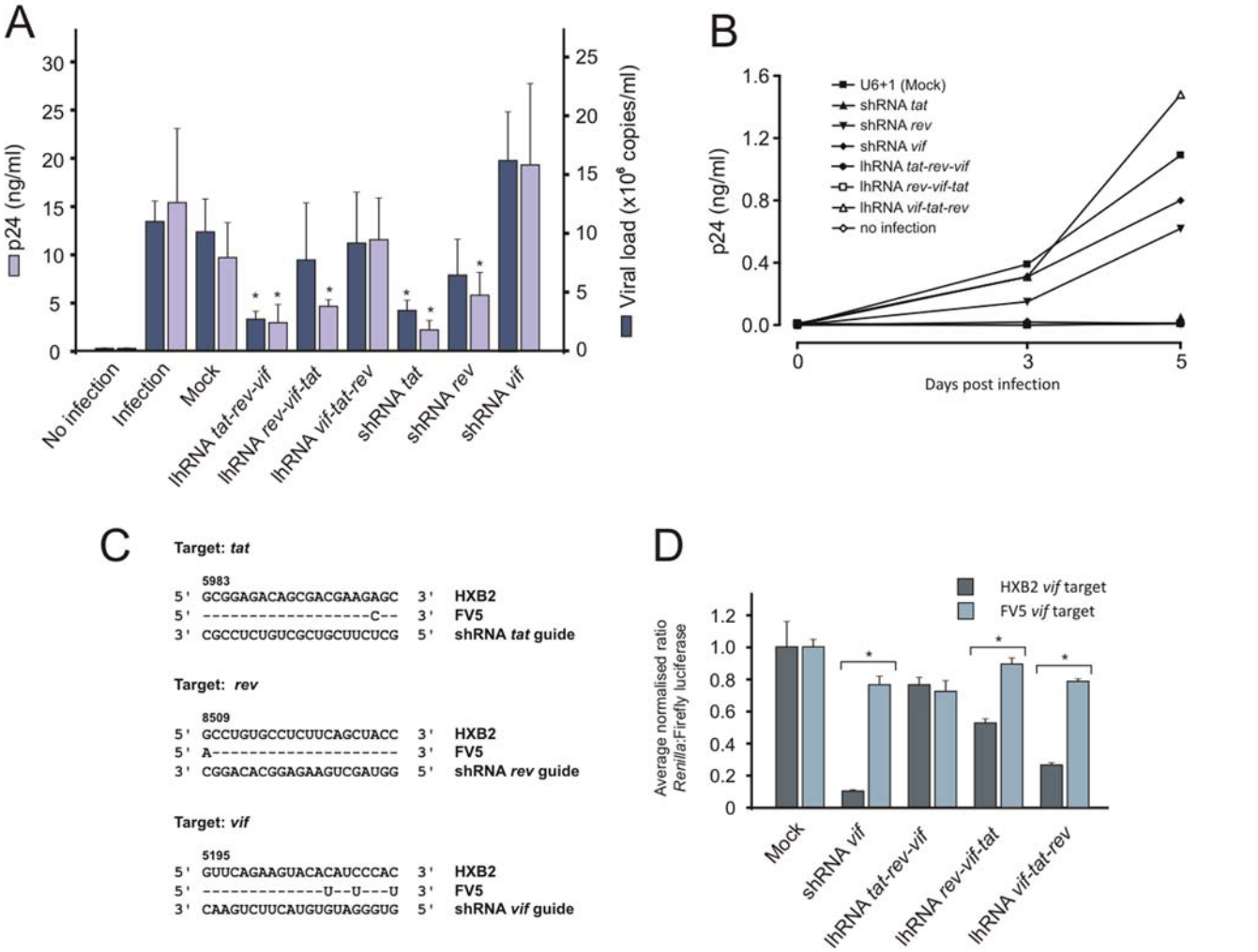


Figure 5. HIV-1 challenge assay. U87.CD_4.CCR5 cells were transfected with plasmids expressing the indicated hairpins and then subjected to infection with an equivalent of $TCID_{50}$ 1000 particles of the HIV-1 FV5 viral isolate. Concentrations of HIV-1 p24 in the culture supernatant and viral particle equivalents (**A**) were determined 6 days after infection. Results are expressed as the means with standard deviations of three independent experiments (*, $p<0.05$, *t*-test, relative to mock transfected control). **B.** Replication kinetics of a representative experiment included in (**A**). **C.** Sequence of lhRNA- and shRNA-derived *tat*, *rev* and *vif* guide sequences and complementary regions targeted within FV5 and HXB2 HIV-1 isolates. Mismatches between the putative guide and target sequences are shown. Sequence numbering is based on isolate HXB2, accession K03455. **D.** Knockdown of FV5 *vif* and HXB2 *vif* target-reporter fusion gene expression by shRNA *vif* and representative lhRNAs in HEK293 cells. Results are expressed as the means with standard deviations of three independent experiments (*; $p<0.05$, *t*-test, between annotated samples).
doi:10.1371/journal.pone.0002602.g005

sequences [46]. It is unlikely that a third siRNA produced by Dicer cleavage of an lhRNA will be present in sufficient concentration to produce three effective siRNAs. We therefore provide a note of caution for the use of lhRNAs containing more than two adjacent siRNA sequences aimed at effective combinatorial RNAi. Nevertheless, lhRNAs in combination with other multiple RNAi effector sequences, such as Pol II-expressed multiple miRNA precursors [14,18,19], are likely to provide an effective means of targeting rapidly evolving sequences such as HIV-1.

In conclusion, we show that RNA Pol III-expressed lhRNAs are capable of producing independent siRNAs that induce significant knockdown of non-contiguous siRNA-susceptible regions of HIV-1. Importantly, the position and arrangement of the siRNA-encoding sequences along the lhRNA duplex plays an important role in determining the overall efficacy of the lhRNA in target suppression. Nonetheless, by optimizing the particular arrangement of siRNA-encoding sequences along the lhRNA duplex, effective multiple targeting is possible for up to two Dicer processing reactions. We therefore provide a useful framework for investigating the use of RNA Pol III-expressed lhRNAs aimed at effective combinatorial RNAi in mammalian cells.

Materials and Methods

Target plasmids

The psiCheck target plasmids were prepared by directed insertion of the *Xho*I-*Not*I digested HIV-1 PCR fragments into the plasmid psiCheck2 (Promega, WI, USA) such that the target sequences were within the 3′ UTR of *Renilla* Luciferase. The individual shRNA target sequences were amplified by PCR from pNL4-3 template [47] using the following primers: sh *tat* target F 5′- GAT CTC GAG AGT GTT GCT TTC ATT GCC AA-3′ (29 nt), sh *tat* target R 5′-GAT CGC GGC CGC GCA TTA CAT GTA CTA CTT ACT GCT T-3′ (37 nt). sh *rev* target F 5′-GAT CTC GAG AAG GTG GAG AGA GAG ACA GA-3′ (29 nt), sh *rev* target R 5′-GAT CGC GGC CGC CAC CAA TAT TTG

AGG GCT TC-3′ (32 nt). sh *vif* target F 5′-GAT CTC GAG ATT TCA AGG AAA GCT AAG GA-3′ (29 nt), sh vif target R 5′-GAT CGC GGC CGC AAT GCC AGT CTC TTT CTC CT-3′ (32 nt). To generate a product consisting of all three target sites adjacent to one another, complementary oligonucleotides were treated with polynucleotide kinase (Promega, WI, USA), annealed, and cloned directly into the XhoI-NotI sites of psiCheck2. To facilitate screening, an EcoRV site was inserted within each annealed dsDNA insert. The oligonucleotides used include: target tat-rev-vif (+) 5′- GAT CTC GAG GCG GAG ACA GCG ACG AAG AGC TTG CCT GTG CCT CTT CAG CTA CC-3′ (53 nt) and target tat-rev-vif (−) 5′-GAT CGC GGC CGC GTG GGA TGT GTA CTT CTG AAC AAG GTA GCT GAA GAG GCA CAG GC-3′ (58 nt). Similarly, psiCheck clones were constructed for a HXB2 and FV5 vif target: target FV5-vif (+) 5′-TCG AGA TAT CGT TCA GAA GTA CAT ATT CCA TGC -3′ and target FV5-vif (−) 5′-GGC CGC ATG GAA TAT GTA CTT CTG AAC GAT ATC -3′; target HXB2-vif (+) 5′-TCG AGA TAT CGT TCA GAA GTA CAC ATC CCA CGC -3′ and target HXB2-vif (−) 5′-GGC CGC GTG GGA TGT GTA CTT CTG AAC GAT ATC -3′.

Long hairpin RNA and short hairpin RNA expression plasmids

The procedure for generating Pol III U6 shRNA cassettes has been previously described [4,48]. A similar 2 step PCR approach was used to produce the lhRNA and shRNA vectors complementary to the HIV-1 *vif*, *tat*, and *rev* genes. The first amplification was carried out with a universal U6 forward primer and first lhRNA or shRNA reverse primer with U6 promoter plasmid DNA as template. The amplified product was used as template for a PCR step with a second lhRNA or shRNA reverse primer and again the universal U6 forward primer. The sequence of the U6 universal forward primer was 5′- CTA ACT AGT GGC GCG CCA AGG TCG GGC AGG AAG AGG G-3′. Sequences of the reverse primers for the amplifications were as follows: lhRNA *tat-rev-vif* R1 5′- CTT GAA ATG GAA TGT ATA CCT CTA AAC AAG GCA GCC GAA GAG ACA CAG ACA AGC CCT TCA TCA CTA TCC CCG CGG TGT TTC GTC CTT TCC ACA A -3′ (94 nt), lhRNA *tat-rev-vif* R2 5′- AAA AAA GCG GAG ACA GCG ACG AAG AGC TTG CCT GTG CCT CTT CAG CTA CCT TGT TCA GAA GTA CAC ATC CCA CTC TCT TGA AAT GGA ATG TAT A -3′ (94 nt). lhRNA *rev-vif-tat* R1 5′- CTT GAA GCC CTT CAT CAC TAT CCC CGC AAA TGG AAT GTA TAC CTC TAA ACA AGG CAG CCG AAG AGA CAC AGA CGG TGT TTC GTC CTT TCC ACA A -3′ (94 nt), lhRNA *rev-vif-tat* R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TTG TTC AGA AGT ACA CAT CCC ACT TGC GGA GAC AGC GAC GAA GAG CTC TCT TGA AGC CCT TCA TCA C -3′ (94 nt). lhRNA *vif-tat-rev* R1 5′- CTT GAA GGC AGC CGA AGA GAC ACA GAC AAG CCC TTC ATC ACT ATC CCC GCA AAT GGA ATG TAT ACC TCT AAA CGG TGT TTC GTC CTT TCC ACA A -3′ (94 nt), lhRNA *vif-tat-rev* R2 5′- AAA AAA GTT CAG AAG TAC ACA TCC CAC TTG CGG AGA CAG CGA CGA AGA GCT TGC CTG TGC CTC TTC AGC TAC CTC TCT TGA AGG CAG CCG AAG A -3′ (94 nt). shRNA *tat* R1 5′- CTC TTG AAG CCC TTC ATC ACT ATC CCC GCG GTG TTT CGT CCT TTC CAC AA -3′ (50 nt), shRNA *tat* R2 5′- AAA AAA GCG GAG ACA GCG ACG AAG AGC TCT CTT GAA GCC CTT CAT CAC -3′ (48 nt). shRNA *rev* R1 5′- CTC TTG AAG GCA GCC GAA GAG ACA CAG ACG GTG TTT CGT CCT TTC CAC AA -3′ (50 nt), shRNA *rev* R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TCT CTT GAA GGC AGC CGA AGA -3′ (48 nt). shRNA *vif* R1 5′- CTC TTG AAA TGG AAT TGT ATA CCT CTA AAC GGT GTT TCG TCC TTT CCA CAA -3′ (50 nt), shRNA *vif* R2 5′- AAA AAA GTT CAG AAG TAC ACA TCC CAC TCT CTT GAA ATG GAA TGT ATA -3′ (48 nt). The sequences for variants of lhRNA *rev-vif-tat* were: lhRNA *rev-vif-tat* b R1 5′- CTC TTG AAG CCC TTC ATC ACT ATC CCC GCA AAT GGA ATG TAT ACC TCT AAA CAG GCA GCC GAA GAG ACA CAG ACG GTG TTT CGT CCT TTC CAC AA-3′ (95 nt), lhRNA *rev-vif-tat* b R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TGT TCA GAA GTA CAC ATC CCA CTT GCG GAG ACA GCG ACG AAG AGC TCT CTT GAA GCC CTT CAT C-3′ (91 nt). lhRNA *rev-vif-tat* c R1 5′- CTC TTG AAG CCC TTC ATC ACT ATC CCC GCT AAA TGG AAT GTA TAC CTC TAA ACA GGC AGC CGA AGA GAC ACA GAC GGT GTT TCG TCC TTT CCA CAA -3′ (96 nt), lhRNA *rev-vif-tat* c R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TGT TCA GAA GTA CAC ATC CCA CTT AGC GGA GAC AGC GAC GAA GAG CTC TCT TGA AGC CCT TCA TCA -3′ (93 nt). lhRNA *rev-vif-tat* d R1 5′- CTC TTG AAG CCC TTC ATC ACT ATC CCC GCT TAA ATG GAA TGT ATA CCT CTA AAC AGG CAG CCG AAG AGA CAC AGA CGG TGT TTC GTC CTT TCC ACA A -3′ (97 nt), lhRNA *rev-vif-tat* d R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TGT TCA GAA GTA CAC ATC CCA CTT AAG CGG AGA CAG CGA CGA AGA GCT CTC TTG AAG CCC TTC ATC A -3′ (94 nt). lhRNA *rev-vif-tat* e R1 5′- CTT GAA GCC CTT CAT CAC TAT CCC CGC GCG CAA ATG GAA TGT ATA CCT CTA AAC GGC AGC CGA AGA GAC ACA GAC GGT GTT TCG TCC TTT CCA CAA -3′ (96 nt), lhRNA *rev-vif-tat* e R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC GTT CAG AAG TAC ACA TCC CAC TTG CGC GCG GAG ACA GCG ACG AAG AGC TCT CTT GAA GCC CTT CAT CAC -3′ (96 nt). lhRNA *rev-vif-tat* f R1 5′- CTT GAA GCC CTT CAT CAC TAT CCC CGC TTA AAT GGA ATG TAT ACC TCT AAA CAA GGC AGC CGA AGA GAC ACA GAC GGT GTT TCG TCC TTT CCA CAA -3′ (96 nt), lhRNA *rev-vif-tat* f R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TTG TTC AGA AGT ACA CAT CCC ACT TAA GCG GAG ACA GCG ACG AAG AGC TCT CTT GAA GCC CTT CAT CAC -3′ (96 nt). lhRNA *rev-vif-tat* g R1 5′- CTT GAA GCC CTT CAT CAC TAT CCC CGC TTA AAT GGA ATG TAT ACC TCT AAA CGA AGG CAG CCG AAG AGA CAC AGA CGG TGT TTC GTC CTT TCC ACA A -3′ (97 nt), lhRNA *rev-vif-tat* g R2 5′- AAA AAA GCC TGT GCC TCT TCA GCT ACC TTC GTT CAG AAG TAC ACA TCC CAC TTA AGC GGA GAC AGC GAC GAA GAG CTC TCT TGA AGC CCT TCA TCA C -3′ (97 nt). The 63 bp lhRNA control plasmid, lhRNA TAR, which was designed to target an irrelevant site which included the HIV-1 TAR stem-loop (complementary to positions 454–512, numbering according to HIV-1 HXB2 sequence, accession K03455), has been previously described [26]. For all lhRNA constructs, each pair of primers had an overlapping sequence of 19 bases that enabled extension of the PCR product to generate a U6 promoter lhRNA cassette with a RNA Pol III transcription termination signal [48]. Amplified PCR products were ligated to a T/A cloning vector (pTZ57R/T, Fermentas, WI, USA) to generate pTZ-U6 lhRNA and shRNA plasmids. Sequences were confirmed by standard procedures.

Cell culture

The human embryonic kidney cell line, HEK293, was maintained in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker, MD, USA) supplemented with 10% heat inactivated fetal calf serum (FCS, Delta Bioproducts, Johannesburg, SA) at

37°C and 5% CO_2. The human astrocyte glioblastoma cell line, U87.CD_4.CCR5 (NIH AIDS Research and Reference Reagent Program), was maintained in DMEM supplemented with 15% heat inactivated FCS, 50 IU/mL Penicillin/50 μg/mL Streptomycin mix (Gibco, BRL, UK), 1 μg/mL Puromycin (Merck, London, UK), 300 μg/mL G418 (Sigma, MO, USA) and 1% L-glutamine (Sigma, MO, USA) at 37°C and 5% CO_2.

Transfections

Transfections were carried out using a ratio of 1 μL Lipofectamine2000 (Invitrogen, CA, USA) to 1 μg total DNA per well according to the manufacturer's instructions. Media was changed 24 hours post transfection, and analysis of cells was carried out 24 hours thereafter. Equivalent transfection efficiencies were verified by fluorescence microscopy by cotransfecting a plasmid that constitutively produces enhanced green fluorescent protein (pCI-eGFP) [49].

To evaluate the effects of the lhRNA and shRNA encoding plasmids on a reporter target, HEK293 cells were seeded 24 hours prior to transfection at 120 000 cells per well in 24 well culture dishes. HEK293 cells were transfected with 150 ng of target plasmid, 750 ng of lhRNA or shRNA encoding plasmid and 100 ng of pCI-eGFP.

To determine the induction of IFN response-related genes, HEK293 cells were seeded as described above and transfected with 900 ng of lhRNA or shRNA encoding plasmid and 100 ng pCI-eGFP per well. Control double stranded RNA, poly (I:C) (Sigma, MO, USA), was transfected at equivalent amounts to the hairpin encoding plasmids.

For Northern blot analysis HEK293 cells were seeded at 80% confluency in 10 cm culture dishes 24 hours prior to transfection. Cells were transfected using Lipofectamine with 16 μg of lhRNA or shRNA encoding plasmid, 3 μg of target plasmid, and 1 μg pCI-eGFP.

To assess the effects of the lhRNA encoding plasmids on a subtype C HIV-1 primary isolate in an infection challenge assay, U87.CD_4.CCR5 cells (NIH HIV/AIDS Reagent and Reference Program) were washed with 1×PBS, treated for 5 minutes with 1×trypsin, counted as described above and seeded 24 hours prior to transfection at 100 000 cells per well in 12 well culture dishes using DMEM supplemented with 15% heat inactivated FCS only. The following day, cells were co-transfected with 900 ng of lhRNA encoding plasmid and 100 ng of pCI-GFP per well as described above.

Dual luciferase assay

These were carried out according to the manufacturer's instructions (Promega, WI, USA) using a Veritas dual-injection luminometer (Turner Biosystems, C A, USA). Target-specific *Renilla* luciferase expression was normalized to background firefly luciferase expression. Average expression ratios for a control plasmid containing the U6 promoter was set to 100%, and relative expression levels for other samples calculated accordingly. Two independent experiments in triplicate were performed and the data are expressed as the mean±standard deviation.

Viral propagation and challenge assay

FV5 is a primary HIV-1 CCR5-utilizing subtype C virus that was isolated from a drug-naïve HIV-positive AIDS patient admitted to the Johannesburg Hospital AIDS clinic, and propagated by standard PBMC co-culture techniques. The co-receptor tropism of FV5 was established genotypically by automated sequencing of the V3 loop of the viral *env* gene (accession 05ZAFV5), and confirmed phenotypically by MT-2 fusion assay. Twenty four hours post-transfection, U87.CD_4.CCR5 cells were infected with FV5 using a $TCID_{50}$ 1000. Twenty four hours post infection cells were washed three times using 1×PBS and fresh media was added. At days 0 (day of washing), 3, 5 and 6, 100 μL of supernatant was collected per well and analysed by ELISA (Murex Biotech LTD, Dartford, UK) for p24 antigen production as a marker of viral replication. Viral RNA was extracted from 300 μL of day 6 supernatant using the COBAS Ampliprep instrument (Roche, Germany), followed by a viral load assay with the COBAS Amplicor (Roche, Germany) according to manufacturer's specifications. Day 0 p24 data was completed and in all cases no viral p24 protein or RNA was detected indicating that all residual infecting virus had been removed from the cultures.

Northern blot analysis

Total RNA was extracted from HEK293 cells using TriReagent™ (Sigma, MO, USA) according the manufacturer's instructions 48 hours post-transfection. Twenty-five micrograms of RNA was resolved on urea denaturing 15% polyacrylamide gels and blotted onto nylon membranes. RNA molecular weight markers, which were radioactively labeled as described below, were run alongside the cellular RNA. Blots were hybridized to three DNA oligonucleotides (probes *tat*, *rev* and *vif*) to detect products of hairpin processing. These were complimentary to regions spanning the antisense sequence of the long hairpin. Probes were labeled at their 5′ ends with [γ-^{32} P] ATP and T4 polynucleotide kinase. After purification using standard procedures, they were hybridized to immobilized RNA, exposed to X-ray film and then stripped and reprobed. An oligonucleotide sequence complementary to U6 small nuclear RNA was used as a control to verify equal loading of the cellular RNA. Probe oligonucleotide sequences were as follows: probe *tat*: 5′-GCG GAG ACA GCG ACG AAG AGC TT-3′; probe *rev*: 5′-GCC TGT GCC TCT TCA GCT ACC TT-3′; probe *vif*: 5′-GTT CAG AAG TAC ACA TCC CAC TT-3′; and U6 small nuclear RNA probe: 5′-TAG TAT ATG TGC TGC CGA AGC GAG CA-3′. The LNA probe sequences were as follows: probe *LNA-tat-1*: 5′-ACT TGC GGA GAC AG-3′; probe *LNA-tat-2*: 5′-GCG CGC GGA GAC AG-3′. The LNA nucleotides are underlined.

Statistics

Statistical calculations were determined using the GraphPad Prism software package (GraphPad, Software, Inc., CA, USA). Statistical difference was considered significant when $p<0.05$ and was determined using either an unpaired Student's *t*-test or by ANOVA.

Acknowledgments

We would like to acknowledge Lynn Morris for her initial assistance, Maria Papathanasopoulos for help with the FV5 subtype C HIV-1 isolate and Naazneen Moolla for assisting with the viral load assays.

Author Contributions

Conceived and designed the experiments: MW SS. Performed the experiments: MW SS SB. Analyzed the data: KM MW SS AC PA. Contributed reagents/materials/analysis tools: KM MW AC. Wrote the paper: KM MW PA.

References

1. Fire A, Xu S, Montgomery MK, Kostas SA, Driver SE, et al. (1998) Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391: 806–811.
2. Elbashir SM, Lendeckel W, Tuschl T (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes and Development 15: 188–200.
3. Brummelkamp TR, Bernards R, Agami R (2002) A system for stable expression of short interfering RNAs in mammalian cells. Science 296: 550–553.
4. Lee NS, Dohjima T, Bauer G, Li H, Li MJ, et al. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat Biotechnol 20: 500–505.
5. Unwalla HJ, Li MJ, Kim JD, Li HT, Ehsani A, et al. (2004) Negative feedback inhibition of HIV-1 by TAT-inducible expression of siRNA. Nat Biotechnol 22: 1573–1578.
6. Zhou H, Xia XG, Xu Z (2005) An RNA polymerase II construct synthesizes short-hairpin RNA with a quantitative indicator and mediates highly efficient RNAi. Nucleic Acids Res 33: e62.
7. Lee Y, Jeon K, Lee JT, Kim S, Kim VN (2002) MicroRNA maturation: stepwise processing and subcellular localization. Embo J 21: 4663–4670.
8. Boden D, Pusch O, Silbermann R, Lee F, Tucker L, et al. (2004) Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. Nucleic Acids Res 32: 1154–1158.
9. Boden D, Pusch O, Lee F, Tucker L, Ramratnam B (2003) Human Immunodeficiency Virus Type 1 Escape from RNA Interference. Journal of Virology 77: 11531–11535.
10. Das AT, Brummelkamp TR, Westerhout EM, Vink M, Madiredjo M, Bernards R, Berkhout B (2004) Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol 78: 2601–2605.
11. Westerhout EM, Ooms M, Vink M, Das AT, Berkhout B (2005) HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Res 33: 796–804.
12. Grimm D, Kay MA (2007) Combinatorial RNAi: A Winning Strategy for the Race Against Evolving Targets? Mol Ther 15: 878–888.
13. Scherer L, Rossi JJ, Weinberg MS (2007) Progress and prospects: RNA-based therapies for treatment of HIV infection. Gene Ther 14: 1057–1064.
14. Gonzalez S, Castanotto D, Li H, Olivares S, Jensen MC, et al. (2005) Amplification of RNAi–targeting HLA mRNAs. Mol Ther 11: 811–818.
15. ter Brake O, Konstantinova P, Ceylan M, Berkhout B (2006) Silencing of HIV-1 with RNA interference: a multiple shRNA approach. Mol Ther 14: 883–892.
16. Henry SD, van der Wegen P, Metselaar HJ, Tilanus HW, Scholte BJ, et al. (2006) Simultaneous targeting of HCV replication and viral binding with a single lentiviral vector containing multiple RNA interference expression cassettes. Mol Ther 14: 485–493.
17. Xia XG, Zhou H, Xu Z (2006) Multiple shRNAs expressed by an inducible pol II promoter can knock down the expression of multiple target genes. Biotechniques 41: 64–68.
18. Sun D, Melegari M, Sridhar S, Rogler CE, Zhu L (2006) Multi-miRNA hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. Biotechniques 41: 59–63.
19. Chung KH, Hart CC, Al-Bassam S, Avery A, Taylor J, et al. (2006) Polycistronic RNA polymerase II expression vectors for RNA interference based on BIC/miR-155. Nucleic Acids Res 34: e53.
20. Manche L, Green SR, Schmedt C, Mathews MB (1992) Interactions between double-stranded RNA regulators and the protein kinase DAI. Mol Cell Biol 12: 5238–5248.
21. Diallo M, Arenz C, Schmitz K, Sandhoff K, Schepers U (2003) Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures. Oligonucleotides 13: 381–392.
22. Park WS, Miyano-Kurosaki N, Hayafune M, Nakajima E, et al. (2002) Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Res 30: 4830–4835.
23. Tran N, Raponi M, Dawes IW, Arndt GM (2004) Control of specific gene expression in mammalian cells by co-expression of long complementary RNAs. FEBS Lett 573: 127–134.
24. Akashi H, Miyagishi M, Yokota T, Watanabe T, Hino T, et al. (2005) Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. Mol Biosyst 1: 382–390.
25. Strat A, Gao L, Utsuki T, Cheng B, Nuthalapaty S, Mathis JM, et al. (2006) Specific and nontoxic silencing in mammalian cells with expressed long dsRNAs. Nucleic Acids Res 34: 3803–3810.
26. Weinberg MS, Ely A, Barichievy S, Mufamadi S, Carmona S, et al. (2007) Specific inhibition of HBV replication *in vitro* and *in vivo* with expressed long hairpin RNA. Molecular Therapy 15: 534–541.
27. Marques JT, Devosse T, Wang D, Zamanian-Daryoush M, et al. (2006) A structural basis for discriminating between self and nonself double-stranded RNAs in mammalian cells. Nature Biotechnology 24: 559–565.
28. Robbins MA, Li M, Leung I, Li H, Boyer DV, et al. (2006) Stable expression of shRNAs in human CD34+ progenitor cells can avoid induction of interferon responses to siRNAs *in vitro*. Nature Biotechnology 24: 566–571.
29. Watanabe T, Sudoh M, Miyagishi M, Akashi H, Arai M, et al. (2006) Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther 13: 883–892.
30. Nishitsuji H, Kohara M, Kannagi M, Masuda T (2006) Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. J Virol 80: 7658–7666.
31. Barichievy S, Saayman S, von Eije KJ, Morris KV, et al. (2007) The inhibitory efficacy of RNA POL III-expressed long hairpin RNAs targeted to untranslated regions of the HIV-1 5′ long terminal repeat. Oligonucleotides 17: 419–431.
32. Liu YP, Haasnoot J, Berkhout B (2007) Design of extended short hairpin RNAs for HIV-1 inhibition. Nucleic Acids Res 35: 5683–5693.
33. Sano M, Li H, Nakanishi M, Rossi JJ (2008) Expression of long anti-HIV-1 hairpin RNAs for the generation of multiple siRNAs: advantages and limitations. Mol Ther 16: 170–177.
34. Lee SK (2005) Lentiviral delivery of short hairpin RNAs protects CD4 T cells from multiple clades and primary isolates of HIV. Blood 106: 818–826.
35. Chang LJ, Liu X, He J (2005) Lentiviral siRNAs targeting multiple highly conserved RNA sequences of human immunodeficiency virus type 1. Gene Ther 12: 1133–1144.
36. Brake OT, Hooft K, Liu YP, Centlivre M, Jasmijn von Eije K, Berkhout B (2008) Lentiviral vector design for multiple shRNA expression and durable HIV-1 inhibition. Mol Ther 16: 557–564.
37. An DS, Qin FX, Auyeung VC, Mao SH, Kung SK, et al. (2006) Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. Mol Ther 14: 494–504.
38. Grimm D, Streetz KL, Jopling CL, Storm TA, Pandey K, et al. (2006) Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature 441: 537–541.
39. Castanotto D, Sakurai K, Lingeman R, Li H, Shively L, et al. (2007) Combinatorial delivery of small interfering RNAs reduces RNAi efficacy by selective incorporation into RISC. Nucleic Acids Res 35: 5154–5164.
40. Vermeulen A, Behlen L, Reynolds A, Wolfson A, Marshall WS, et al. (2005) The contributions of dsRNA structure to Dicer specificity and efficiency. Rna 11: 674–682.
41. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song JJ, Hammond SM, Joshua-Tor L, Hannon GJ (2004) Argonaute2 is the catalytic engine of mammalian RNAi. Science 305: 1437–1441.
42. Song JJ, Smith SK, Hannon GJ, Joshua-Tor L (2004) Crystal structure of Argonaute and its implications for RISC slicer activity. Science 305: 1434–1437.
43. Provost P, Dishart D, Doucet J, Frendewey D, Samuelsson B, et al. (2002) Ribonuclease activity and RNA binding of recombinant human Dicer. Embo J 21: 5864–5874.
44. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W (2002) Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. Embo J 21: 5875–5885.
45. Macrae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, et al. (2006) Structural basis for double-stranded RNA processing by Dicer. Science 311: 195–198.
46. von Eije KJ, ter Brake O, Berkhout B (2008) Human immunodeficiency virus type 1 escape is restricted when conserved genome sequences are targeted by RNA interference. J Virol 82: 2895–2903.
47. Adachi A, Gendelman HE, Koenig S, Folks T, Willey R, et al. (1986) Production of acquired immunodeficiency syndrome-associated retrovirus in human and nonhuman cells transfected with an infectious molecular clone. J Virol 59: 284–291.
48. Castanotto D, Li H, Rossi JJ (2002) Functional siRNA expression from transfected PCR products. RNA 8: 1454–1460.
49. Passman M, Weinberg M, Kew M, Arbuthnot P (2000) In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. Biochem Biophys Res Commun 268: 728–733.

OLIGONUCLEOTIDES 17:419–431 (2007)

DOI: 10.1089/oli.2007.0095

The Inhibitory Efficacy of RNA POL III-Expressed Long Hairpin RNAs Targeted to Untranslated Regions of the HIV-1 5′ Long Terminal Repeat

SAMANTHA BARICHIEVY,[1] SHEENA SAAYMAN,[1] KARIN J. VON EIJE,[2] KEVIN V. MORRIS,[3] PATRICK ARBUTHNOT,[1] and MARC S. WEINBERG[1]

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) is a lentivirus that causes persistent infection resulting in the demise of immune regulatory cells, and ensuing diseases associated with acquired immune deficiency syndrome (AIDS). Although current therapeutic modalities have had a significant impact on mortality rates, novel therapies are constantly needed to prevent the emergence of resistant viral variants that escape the effects of antivirals. RNA Interference (RNAi) is a promising therapeutic modality for the inhibition of HIV-1 RNAs. Traditionally, RNAi effector sequences include expressed short hairpin RNAs (shRNAs) or short interfering RNAs (siRNAs). Recently, expressed long hairpin RNAs (lhRNAs) have been used with the aim of generating multiple independent siRNAs, which simultaneously target different susceptible sites on HIV-1. Here, modified lhRNAs expressed from U6 RNA Pol III promoters were targeted to sites within the first transcribed sequences of the HIV-1 5′ long terminal repeat (LTR) region. Both Tat-dependent and independent suppressive efficacy was demonstrated against subtype B and C reporter sequences; however, lhRNAs complementary to the TAR stem–loop were refractory to silencing. None of the lhRNAs induced an unwanted interferon response as measured by interferon beta levels. Silencing by the lhRNAs was not equal across the extent of its cognate sequence, with the greatest efficacy observed for sequences located at the base of the stem. Nevertheless, direct antireplicative activity was seen when targeting lhRNAs to a subtype B HIV clone pNL4-3 Luc and a subtype C wild-type HIV-1 strain, FV5. These data highlight distinct target loci within the 5′ LTR of HIV-1 that are susceptible to lhRNA targeting, and may prove to have an important advantage over other RNAi target sites within HIV-1. Although lhRNAs themselves require further manipulation to improve their overall efficacy in generating multiple functioning siRNAs, they may prove useful in any combinatorial-based approach to treating HIV-1 infection.

INTRODUCTION

INFECTION with the human immunodeficiency virus type 1 (HIV-1) causes demise of CD4+ immune regulatory cells. When untreated, this leads inexorably to acquired immunodeficiency syndrome (AIDS) (Barre-Sinoussi et al., 1983; Gallo et al., 1984; UNAIDS, 2005). Clearance of HIV-1 by the immune system is inefficient,

[1]Antiviral Gene Therapy Unit, Department of Molecular Medicine and Haematology, University of Witwatersrand, Johannesburg, South Africa.

[2]Laboratory of Experimental Virology, Department of Medical Microbiology, University of Amsterdam, Amsterdam, The Netherlands.

[3]Department of Molecular and Experimental Medicine, The Scripps Research Institute, La Jolla, California.

as integration of proviral DNA into the genome of host cells acts as a permanent template for the production of replicating virus. This is compounded by high viral mutation rates that lead to variants that differ significantly from the original viral lineage (Bhattacharya et al., 2007).

Current therapies aimed at inhibiting HIV-1 replication include drugs that target reverse transcriptase (RT), protease (PR), and more recently, viral entry or fusion inhibitors. These agents, particularly in combinations of two or more, have had an important impact on the morbidity and mortality of HIV-related illness (Palella et al., 1998). However, serious concerns regarding drug toxicity and drug resistance has prompted the search for novel therapeutic approaches. Additionally, the efficacy of current antiretroviral therapy is seriously threatened by the persistence of latent viral reservoirs (Lassen et al., 2004).

Resistance to antiviral agents can be managed by varying existing treatment strategies and by developing novel therapeutic agents (Daar and Richman, 2005). The emergence of posttranscriptional knockdown approaches that exploit the RNA Interference (RNAi) pathway has proven to be a consistently potent mechanism of inhibiting HIV-1 infection and replication in infected cell cultures. Already there are promising RNAi-based candidate therapeutic agents that are likely to be tested in future human clinical trials (Rossi, 2006).

Initial experiments involving RNAi as an anti-HIV strategy have successfully targeted genomic and subgenomic RNAs of HIV-1, thereby inhibiting both early and late stages of the viral life cycle (Coburn and Cullen, 2002; Jacque et al., 2002; Lee et al., 2002; Novina et al., 2002; Surabhi and Gaynor, 2002; Morris and Rossi, 2004). Effective viral suppression has also been achieved by targeting host-derived viral accessory proteins that are necessary for viral replication (Novina et al., 2002; Chiu et al., 2004; Ping et al., 2004; Ye et al., 2004; Anderson and Akkina, 2005).

RNAi-based strategies against HIV-1 mostly involve the use of RNA Polymerase (Pol) III-derived expression cassettes that produce short hairpin RNAs (shRNAs). These are often targeted to HIV-1-susceptible cells by lentiviral vector delivery (Boden et al., 2004; Nishitsuji et al., 2004; Li and Rossi, 2005). However, one major drawback of using shRNAs has been the emergence of shRNA viral escape mutants (Boden, 2003; Das et al., 2004; Westerhout et al., 2005). Consequently, it has been proposed that an effective RNAi-based approach to treating HIV-1 infection requires simultaneous targeting of multiple sites (Berkhout, 2004; Grimm and Kay, 2007).

A recent innovation to multiple targeting has been to adapt long double-stranded RNAs (dsRNA) (Park et al., 2002; Diallo et al., 2003; Tran et al., 2004) into safe RNAi-inducing agents in mammalian cells (Akashi et al., 2005; Konstantinova et al., 2006). These constructs provide both potent and specific gene-silencing capabilities, and appear to be processed into multiple short interfering RNAs (siRNAs) by the intracellular RNAi machinery in mammalian cells (Akashi et al., 2005; Nishitsuji et al., 2006; Strat et al., 2006; Watanabe et al., 2006; Konstantinova et al., 2007; Weinberg et al., 2007). Most importantly, intracellularly expressed long dsRNAs do not elicit the type 1 interferon (IFN) response *in vitro* or *in vivo* (Akashi et al., 2005; Weinberg et al., 2007).

Long dsRNA has been used to suppress HIV-1 when targeted to *nef* (Yamamoto et al., 2002), and various long hairpin RNA expression constructs (lhRNAs) have been used successfully to suppress HIV-1 replication when targeted to wild-type *integrase* (*int*) transcripts as well as anti-*int* shRNA escape mutants (Nishitsuji et al., 2006). HIV-1 *tat*, *rev*, and *nef* transcripts have also been successfully targeted without the induction of an IFN response (Konstantinova et al., 2006, 2007).

Importantly, most RNAi approaches to date have focused on targeting laboratory strains of HIV-1 subtype B sequences, with little attention given to inhibiting primary subtype C sequences, which constitute the majority (56%) of HIV-1 infections worldwide (UNAIDS, 2005). Taking into consideration the importance of a multiple targeted approach and the issue of viral latency, the first 180 nucleotides of the subtype C genomic RNA were chosen for targeting using three 60-base pair (bp) lhRNAs expressed from U6 RNA Pol III cassettes. The targeted region is present within genomic and subgenomic viral RNA species and includes conserved *cis*-regulatory sequences within the R and U5 regions of the 5′ long terminal repeat (LTR) of HIV-1 (Fig. 1). Importantly, the latent HIV-1 provirus only generates nonprocessive mRNA transcripts due to the absence of the transcriptional transactivator protein Tat. During active transcription involving Tat, viral transcripts are processed into complete mRNAs. In the current study, expressed lhRNAs were investigated for their ability to suppress viral replication and to inhibit Tat-mediated processive and Tat-independent nonprocessive transcription from both subtype B and C LTR promoters as an indicator of their ability to target active as well as latent forms of the virus.

MATERIALS AND METHODS

Target plasmids

The subtype B LTR-Luc vector, pLTR-Luc, has been described previously (Jeeninga et al., 2000) (NIH AIDS Research and Reference Reagent Program), and contains an LTR promoter sequence derived from the HIV-1 LAI molecular clone (Peden et al., 1991). To generate a subtype C LTR–luc construct, a two-step subcloning processes was used. First, the LTR sequence from proviral

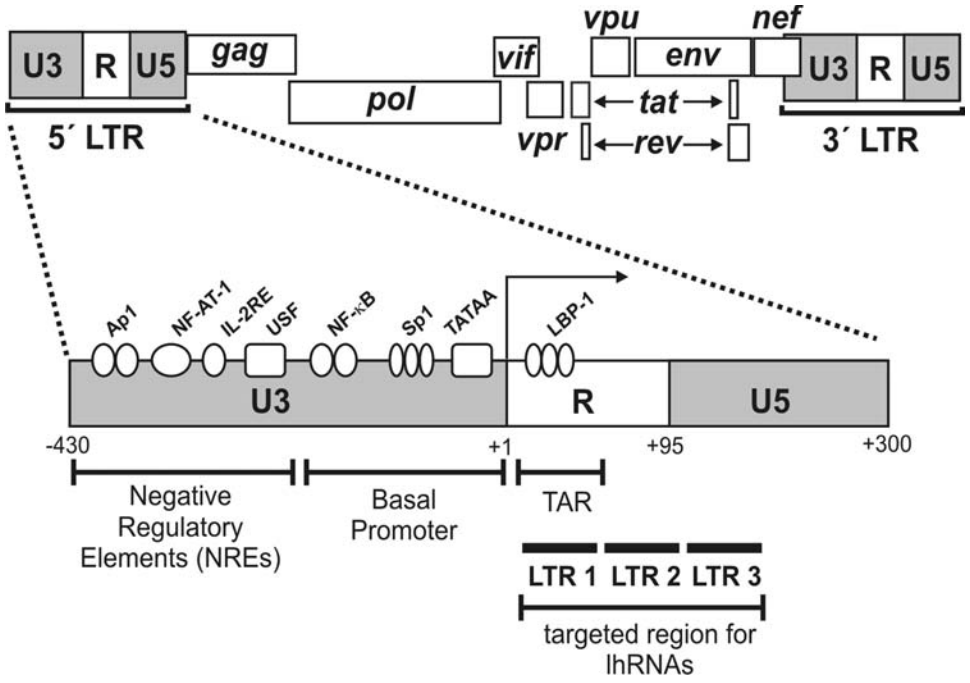


FIG. 1. Diagrammatic representation of the HIV-1 proviral genome depicting all viral open reading frames flanked by 5′ and 3′ long terminal repeat (LTR) sequences. A more detailed map of the 5′ LTR promoter is shown, highlighting the important *cis* and *trans* regulatory elements. The 60-bp sites targeted by the expressed lhRNAs are shown. LTR 1 spans the TAR loop completely with LTR 2 and 3 adjacent downstream. The complete lhRNA target region spans conserved sequences within the R and U5 untranslated transcript.

samples of the HIV-1 isolate Du151 (Williamson et al., 2003) were amplified with the following primers: Du151 LTR F 5′-GATCGTCGACGATATCAGATCTGGAAGGGTTAATTTACTCTAAG-3′ containing *Sal*I, *Eco*RV, and *Bgl*III linkers, and Du151 LTR R 5′-ATCGAATTCAAGCTTGTTCGGGCGCCACTGCTAGAGATTTTCCA-3′ with *Hind*III, *Eco*RI linkers. Amplified PCR products were cloned into the T/A vector, pTZ57R/T (Fermentas, Glen Burnie, MD) to generate pTZ–Du151–LTR. Second, the Luc–SV40poly(A) sequence derived from the Firefly luciferase cassette of plasmid pGL3 (Promega, Madison, WI) was amplified by PCR using the following primers: Luc–SV40pA F 5′-ATTAGAATTCATGGAAGACGCCAAAAA-3′ Luc–SV40pA R 5′-ATTAGGGCCCACTAGTAAGCTTACCACATTTGTAGAGGTTTTAC-3′ containing *Apa*I, *Spe*I, and *Hind*III linkers. PCR products were cloned into the T/A vector pCR2.1 (Invitrogen, Carlsbad, CA) to generate pCR2.1–Luc–SV40pA. For both constructed plasmids, positive orientation clones were selected and verified by sequencing. The *Bam*HI–*Pst*I digested fragment of pCR2.1–Luc–SV40pA was directionally cloned into the same sites in pTZ–Du151–LTR to generate the expression plasmid pLTR^c–Luc–SV40pA. The expression plasmid pLTR^c–Luc–SV40pA–puro was constructed by amplifying the LTRc–Luc–SV40pA region using primers Du151 LTR F and Luc–SV40pA R and inserting the PCR product in the T/A vector psiLentGene-puro (Invitrogen) according to the manufacturer's instructions. pLTR^c–Luc–SV40pA–puro was used to generate a stable HEK293 cell line under Puromycin selection (refer to Transfections). The psiCheck–LTR 2 target plasmid was prepared by directed insertion of the *Xho*I–*Not*I digested PCR fragment into the plasmid psiCheck2 (Promega) such that the LTR sequences are within the 3′ UTR of *Renilla* Luciferase. The lhRNA LTR 2 target sequence was amplified by PCR amplification using LTR lhRNA 2 F (5′-GATCTCGAGGAACCCACTGCTTAAGCCTC-3′) and LTR lhRNA 2 R (5′-GATCGCGGCCGCTTTCCACACTAACACAAAGG-3′) primer combinations. To generate multiple short targets that "tile" the entire LTR lhRNA 2 target, complementary oligonucleotides were treated with polynucleotide kinase (Promega), annealed, and cloned directly into the *Xho*I–*Not*I sites of psiCheck2. To facilitate screening, an *Eco*RV site was inserted within each annealed dsDNA insert. The complementary oligonucleotide sequences used were: LTR 2 Target A F 5′-TCGAGATATCGAACCCACTGCTTAAGCCTCAAGC-3′ and LTR 2 Target A R 5′-GGCCGCTTGAGGCTTAAGCAGTGGGTTCGATATC-3′; LTR 2 Target B F5′-TCGAGATATCTTAAGCCTCAATAAAGCTTGC-

CGC-3′ and LTR 2 Target B R 5′-GGCCGCGGCAAGCTTTATTGAGGCTTAAGATATC-3′; LTR 2 Target C F 5′-TCGAGATATCCAATAAAGCTTGCCTTGAGTGCGC-3′ and LTR 2 Target C R 5′-GGCCGCGCACTCAATTCAAGCTTTATTGGATATC-3′; LTR 2 Target D F 5′-TCGAGATATCTGCCTTGAGTGCTCTAAGTAGTGC-3′ and LTR 2 Target D R 5′-GGCCGCACTACTTAGAGCACTCAAGGCAGATATC-3′; LTR 2 Target E F 5′-TCGAGATATCGTGCTCTAAGTAGTGTGTGCCCGC-3′ and LTR 2 Target E R 5′-GGCCGCGGGCACACACTACTTAGAGCACGATATC-3′.

Long hairpin RNA expression plasmids. The generation of the Pol III U6 shRNA II cassette has been previously described (Lee et al., 2002). A similar two-step PCR approach was used to produce the lhRNA vectors complementary to HIV-1 coordinates 454–512 (lhRNA LTR 1), 507–565 (HIV lhRNA 2), and 566–624 (HIV lhRNA 3) (numbering according to HXB2 sequence, accession K03455). The first amplification was carried out with a universal U6 forward primer and first lhRNA reverse primer with U6 promoter plasmid DNA as a template. The amplified product was used as a template for a PCR step with a second lhRNA reverse primer and again the universal U6 forward primer. The sequence of the U6 universal forward primer was 5′-CTAACTAGTGGCGCGCCAAGGTCGGGCAGGAAGAGGG-3′. Sequences of the reverse primers for the amplifications were as follows: lhRNA LTR 1-R1 5′-CCTCTCTTGAAGAGTCCCCTAAATAACCAGAGAACCCCCGGACTCAGATCCGGTCCACCCAGAAAGAACCGGTGTTTCGTCCTTTCCACAA-3′ (91 nt), lhRNA LTR 1-R2 5′-GATCTCTAGAAAAAAGGGTCTCTCTAGGTAGACCAGATCTGAGCCCGGGAGCTCTCTGGCTATCTAGGGAACCCTCTCTTGAAGAGTCCCC-3′ (91 nt). lhRNA LTR 2-R1 5′-TGCCCTCTCTTGAAAGACACACACCACT-CAGAACACCCAAGACAAACTTCATTAAGGCCTAAACAGTAGGCTCGGTGTTTCGTCCTTTCCACAA-3′ (94 nt), lhRNA LTR 2-R2 5′-AAAAAAGAACCCACTGCTTAAGCCTCAAT-AAAGCTTGCCTTGAGTGCTCTAAGTAGTGTGTGCCCTCTCTTGAAAGAC-3′ (79 nt), lhRNA LTR 3-R1 5′-GGAAATCTCTTGAACTCCCACACCAACACAAAGAGTCTAAGAGACCTCCAGTCACCAGAATCACACAACAAACGGTGTTTCGTCCTTTCCACAA-3′ (94 nt), lhRNA LTR 3-R2 5′-AAAAAAGTCTGTTGTATGACTCTGGTAACTAGAGATCCCTCAGACCCTTTGTGTTAGTGTGGAAATCTCTTGAACTCC-3′ (79 nt). The lhRNA control plasmid targeted to an irrelevant site (HBV), lhRNA HBV, was constructed according to previously described methods (Weinberg et al., 2007). Each pair of primers had an overlapping sequence of 19 bases that enabled extension of the PCR product to generate a U6 promoter lhRNA cassette with a transcription termination signal (Castanotto et al., 2002). Amplified PCR products were ligated to a T/A cloning vector (pTZ57R/T, Fermentas) to generate pTZ–U6 lhRNA plasmids (pU6 lhRNA LTR 1, pU6 lhRNA LTR 2, and pU6 lhRNA LTR 3). The sequences were confirmed by standard procedures.

Cell culture. The human embryonic kidney cell line, HEK293, were maintained in Dulbecco's Modified Eagle's Medium (DMEM, BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal calf serum (FCS, Delta Bioproducts, Johannesburg, SA) at 37°C and 5% CO_2 and transfected using the same medium. The human astrocyte glioblastoma cell line, U87.CD_4.CCR5 (NIH AIDS Research and Reference Reagent Program), was maintained in DMEM supplemented with 15% heat-inactivated FCS, 50 IU/mL Penicillin/50 μg/mL Streptomycin mix (Gibco, BRL, UK), 1 μg/mL Puromycin (Merck, London, UK), 300 μg/mL G418 (Sigma, St. Louis, MO), and 1% L-glutamine (Sigma) at 37°C and 5% CO_2.

Transfections. HEK293 cells were seeded 24 hours prior to transfection at 120,000 cells per well in 24-well culture dishes. Transfections were carried out using 1 μL Lipofectamine 2000 to 1 μg DNA per well as per the manufacturer's instructions (Invitrogen) with OptiMem (Gibco, BRL). Media was changed 24 hours posttransfection, and cells were assayed 24 hours thereafter.

To generate a stable cell line that constitutively expresses Firefly luciferase from a HIV-1 subtype C 5′LTR promoter, HEK293 cells were seeded 24 hours prior to transfection at 2.5 million cells per 10-cm dish and transfected with 10 μg pLTR^c–Luc–SV40pA–puro using Lipofectamine 2000 as described above. Media was changed 24 hours posttransfection and supplemented with 1 μg/mL Puromycin for selection of stable clones. Single colonies were picked roughly 4 weeks following transfection and maintained in DMEM supplemented with antibiotic until a functional assay had been performed. This included assessment of basal and Tat-transactivated Firefly luciferase expression levels as described below. Thereafter stable cells were maintained as described above.

To determine the effects of the lhRNA encoding plasmids on a reporter target, HEK293 cells were transfected with 90 ng of target plasmid, 900 ng of lhRNA encoding plasmid, and 10 ng of a plasmid vector that constitutively produces enhanced green fluorescent protein (pCI–eGFP) per well (Passman et al., 2000) . Where necessary, 90 ng of pCMV–Tat (a gift from J. van Harmelen) or pCI neo (Promega) control were included per well. Equivalent transfection efficiencies were verified by fluorescence microscopy. Where target plasmids only con-

tained a Firefly luciferase expression cassette, 5 ng of Pol II CMV driven *Renilla* luciferase encoding plasmid (pRSV–RLuc, a gift from J.J. Rossi) was included per sample.

To evaluate the lhRNA constructs on targeting and downregulation of a subtype B-based HIV-1 molecular clone, HEK293 cells were seeded as described above and cotransfected with 40 ng pNL4-3.Luc.E^-R^-, 20 ng pRSV–Ren (*Renilla* luciferase encoding plasmid), 80 ng pBluescript SK (Strategene, La Jolla, CA) (carrier) and 40 ng of the lhRNA construct of interest using Lipofectamine 2000 as described above. pNL4-3.Luc.E^-R^- is a luciferase reporter HIV-1 clone provided by the NIH AIDS Research and Reference Reagent Program that consists of pNL4-3 with the gene for the firefly luciferase reporter fused to the 5′-end of the *nef* coding region using *Not*I and *Xho*I sites. Two frame shifts render this clone Env− and Vpr−, and therefore competent for a single round of replication (Connor et al., 1995; He et al., 1995).

To assess the effects of the lhRNA encoding plasmids on a subtype C-based WT HIV-1 strain in a challenge assay, U87.CD_4.CCR5 cells were washed with 1 × PBS, treated for 5 minutes with 1 × trypsin, counted as described above, and seeded 24 hours prior to transfection at 100,000 cells per well in 12-well culture dishes using DMEM supplemented with 15% heat-inactivated FCS only. The following day, cells were cotransfected with 900 ng of lhRNA encoding plasmid and 100 ng of pCI–GFP per well as described above.

Dual luciferase assay. These were carried out according to the manufacturer's instructions (Promega). The samples were assayed in a Veritas dual-injection luminometer (Turner Biosystems, Sunnyvale, C A). Target-specific Firefly luciferase expression was normalized to background *Renilla* luciferase expression. Average expression ratios for control lhRNA HBV was set to 100%, and relative expression levels for other samples calculated accordingly. Two independent experiments in triplicate were performed, and the data are expressed as the mean ± standard error of the mean (SEM). Statistical difference was considered significant for $p < 0.05$. A Dunnett's multiple comparison was calculated with the GraphPad Prism software package (GraphPad Software Inc., San Diego, CA).

Quantitative real-time RT-PCR. To measure concentrations of IFN response-related genes, total RNA from lhRNA or control plasmid transfected HEK293 cells was extracted using TriReagent™ (Sigma) according to the manufacturer's instructions. RNA pellets were resuspended in 10 μL of nuclease free water, DNAse treated for 60 minutes at 37°C, and reverse transcribed using Sensiscript (Qiagen, GmbH, Germany) and oligo-dT primer (10 μM) according to the manufacturer's instructions. The following primer sets (IDT, Coralville, IA) were used to amplify *IFN-β* and *GAPDH* mRNA: IFN-β Forward: 5′-TCC AAA TTG CTC TCC TGT TGT GCT-3′, *IFN-β* Reverse: 5′-CCA CAG GAG CTT CTG ACA CTG AAA A-3′, *GAPDH* Forward: 5′-AGG GGT CAT TGA TGG CAA CAA TAT CCA-3′ and GAPDH Reverse: 5′-TTT ACC AGA GTT AAA AGC AGC CCT GGT G-3′. All real-time PCRs were carried out using the Roche Lightcycler V.2. Controls included water blanks and RNA extracts that were not subjected to reverse transcription. Taq Readymix with SYBR green (Sigma) was used to amplify and detect DNA during the reaction. Thermal cycling parameters consisted of a hot start for 30 seconds at 95°C followed by 50 cycles of 95°C for 10 seconds, 58°C for 10 seconds, and 72°C for 10 seconds. Specificity of the PCR products was verified by melting curve analysis.

Viral propagation and challenge assay. FV5 is a primary HIV-1 CCR5-utilizing subtype C virus that was isolated from a drug-naive HIV-positive AIDS patient admitted to the Johannesburg Hospital AIDS clinic, and propagated by standard PBMC coculture techniques. The coreceptor tropism of FV5 was established genotypically by automated sequencing of the V3 loop of the viral *env* gene (Accession 05ZAFV5), and confirmed phenotypically by MT-2 fusion assay. Twenty four hours posttransfection, U87.CD_4.CCR5 cells were infected with FV5 using a $TCID_{50}$ 1000. Twenty-four hours postinfection cells were washed three times using 1 × PBS and fresh media was added. At days 0 (day of washing) and 4, 100 μL of supernatant was collected per well and analyzed by ELISA (Murex Biotech LTD, Dartford, UK) for p24 antigen production as a marker of viral replication. Day 0 p24 data was completed, and in all cases no viral p24 protein was detected, indicating that all residual infecting virus had been removed from the cultures (data not shown).

RESULTS

Because lhRNAs can be processed by Dicer to generate multiple siRNAs, such constructs have the distinct advantage of limiting the possibility for viral escape induced by base changes to sites targeted by single siRNA sequences. In this study we generated three U6 snRNA RNA Pol III promoter-driven cassettes (LTR lhRNA 1–3) that encode lhRNAs with ~60-bp stems targeted to conserved adjacent sites within the R/U5 (untranslated region) of the HIV-1 subtype C 5′ LTR promoter (Fig. 1). The lhRNAs were designed to include G:U mismatches (approximately every 4–8 nt) within the sense strand to facilitate cloning and to prevent the possible in-

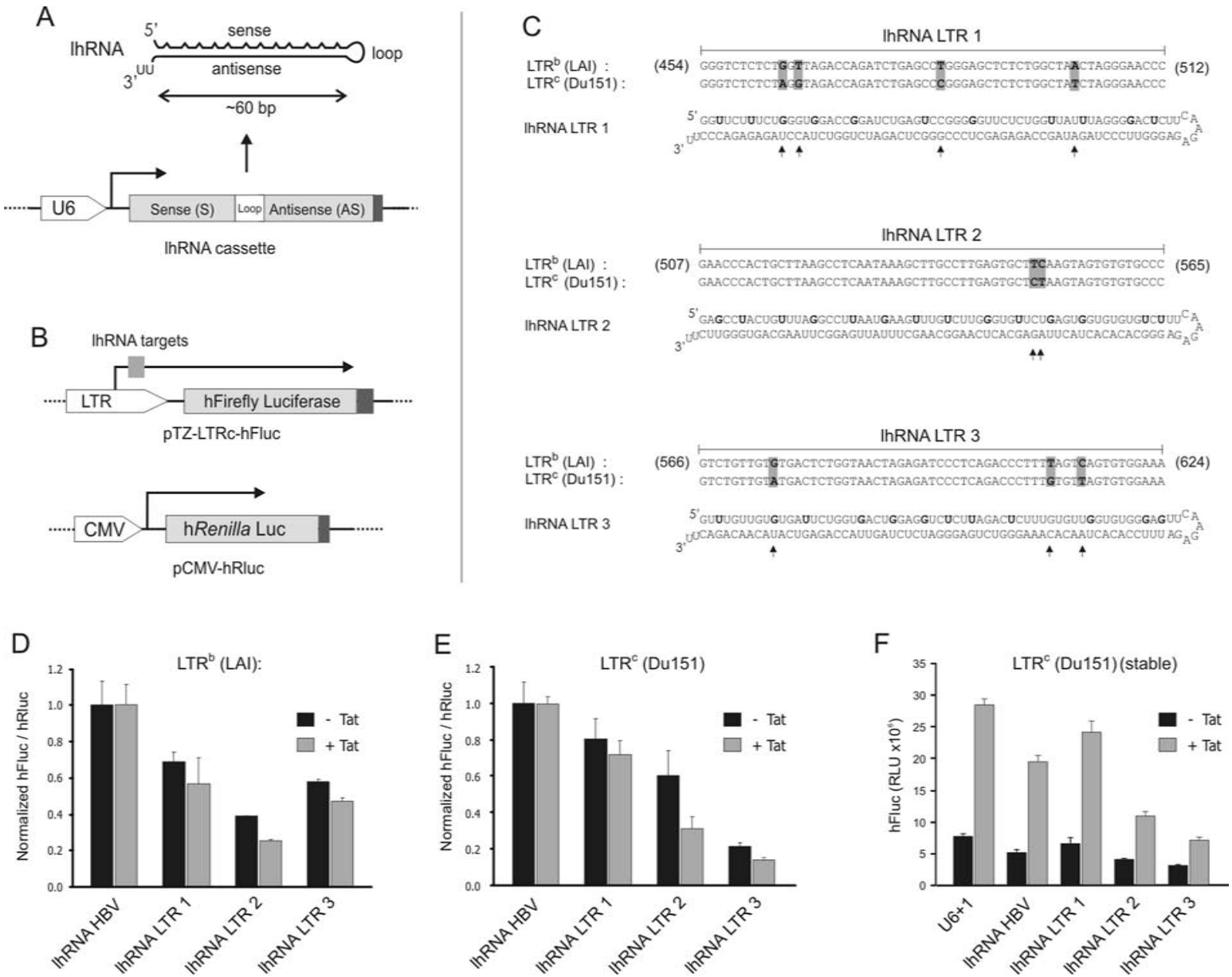

A
lhRNA
5'
sense
loop
3'UU
antisense
~60 bp
U6
Sense (S)
Loop
Antisense (AS)
lhRNA cassette
B
lhRNA targets
LTR
hFirefly Luciferase
pTZ-LTRc-hFluc
CMV
hRenilla Luc
pCMV-hRluc
C
lhRNA LTR 1
LTRb (LAI) :
LTRc (Du151) :
(454)
(512)
lhRNA LTR 1
lhRNA LTR 2
(507)
(565)
lhRNA LTR 2
lhRNA LTR 3
(566)
(624)
lhRNA LTR 3
D
LTRb (LAI):
Normalized hFluc / hRluc
- Tat
+ Tat
lhRNA HBV
lhRNA LTR 1
lhRNA LTR 2
lhRNA LTR 3
E
LTRc (Du151)
F
LTRc (Du151) (stable)
hFluc (RLU x10⁶)
U6+1

FIG. 2. Long hairpin RNAs mediate targeted suppression of HIV-1 subtype B and C LTR-driven Firefly luciferase reporter constructs. (**A**) LhRNAs were designed to be expressed as 60-bp transcripts from a U6 Pol III cassette containing G:U wobble mismatches within the sense strand of the duplex. (**B**) Two separate Firefly reporter constructs were generated by cloning the subtype C (strain Du151) or subtype B (strain NL4-3) LTR promoter of HIV-1 upstream of a GL3 Firefly luciferase open reading frame. A CMV Pol II cassette expressing *Renilla* luciferase was used as a background control in cotransfections. (**C**) Alignment and comparison between LTR^b and LTR^c sequences derived from isolates LAI and Du151, respectively. Long hairpins are shown with respect to their cognate target sequence, and G:U wobbles are in bold text. Numbering is according to the molecular clone HXB2. (**D** and **E**) Three lhRNAs and controls were cotransfected with target reporter constructs in the presence and absence of exogenous Tat in HEK293 cells. Data is expressed as a ratio of firefly to *Renilla* luciferase normalized to a nonspecific lhRNA HBV control determined 48 hours posttransfection ($n = 3$, $\pm$SEM). (**F**) Three lhRNAs and controls were transfected in the presence of exogenous Tat in HEK293 cells stably expressing subtype C LTR-driven Firefly luciferase. Data is expressed as an average Firefly luciferase value normalized to an empty vector control determined 48 hours posttransfection.

duction of the IFN response by dsRNA-dependent protein kinase R (PKR) (Akashi et al., 2005) (Fig. 2A and C). Initially, the inhibitory effects of the three lhRNAs were determined by transient cotransfection with subtype B (LTR^b) and subtype C (LTR^c)-derived LTR-driven luciferase reporter constructs. The LTR^c sequence used in this study, Du151, was derived from a local South African isolate (Williamson et al., 2003), while the LTR^b sequence corresponds to the LAI molecular clone (Jeeninga et al., 2000; Peden et al., 1991) (Fig. 2B and C).

Transient cotransfections in cell culture showed that two of the three lhRNAs efficiently reduced LTR-driven luciferase activity in the presence or absence of exogenously supplied Tat (Fig. 2D and E). However, lhRNA LTR 1, which targets the region containing the stable TAR loop, proved to be ineffective in the context of both subtype B and C LTR sequences. These data are in line with previously observed studies which have shown that the TAR loop is refractory to siRNA-mediated inhibition (Yoshinari et al., 2004). For lhRNA LTR 2 and 3, there were differences in the degree of inhibition when targeted to either LTR^b or LTR^c sequences. Interestingly, lhRNA LTR 2 was particularly effective against LTR^b sequences, while conversely, lhRNA LTR 3 was more effective against LTR^c sequences. LhRNA LTR 2 inhibited LTR^b and LTR^c-driven luciferase activity by 61% and 39%, respectively, in the presence of Tat; and by 75% and 69%, respectively, in the absence of Tat. Similarly, lhRNA LTR 3 inhibited LTR^b and LTR^c-driven luciferase activity by 42% and 78%, respectively, in the presence of Tat; and by 53% and 86%, respectively, in the absence of Tat. Minor sequence variations exist between LTR^b and LTR^c targets, and these may be responsible for the differences in efficacy for both lhRNAs LTR 2 and LTR 3 (Fig. 2C). This is especially the case for lhRNA LTR 3, where the target LTR^b has a G (compared to an A for LTR^c) at position 575, 10 nucleotides from the base of the stem (Fig. 2C). Because siRNAs have been shown previously to be generated predominantly from the stem region of a lhRNA duplex (Weinberg et al., 2007), sequence changes in this regions may have a dramatic effect on the efficacy of a given lhRNA.

Following infection of HIV-1 permissive cells, the virus integrates into the host chromosome forming a proviral copy of its genome and establishing a latent infection. To test the efficacy of lhRNAs at suppressing their cognate mRNAs when expressed from an integrated (as opposed to an episomal) target, transfections where performed in an LTR^c-driven Firefly luciferase stable cell line (Fig. 2F). The pattern of inhibition following transient transfection of lhRNAs in the presence or absence of Tat was largely the same, with a slightly better inhibitory effect seen in the presence of Tat. Moreover, as shown previously, lhRNA LTR 3 proved to be the most effective lhRNA against a stably-expressed LTR^c target. Interestingly, a nonspecific lhRNA targeted to an unrelated hepatitis B virus sequence (lhRNA HBV) also showed minor suppressive activity, which may be due to some nonspecific off-target effects (Fig. 2F). Nevertheless, these data suggest that minor sequence variations may be tolerated by lhRNAs, suggesting that these sequences may provide important advantages for their use against multiple HIV-1 strains, viral escape mutants, and possibly latently infected virus. This is further demonstrated by the ability of the lhRNAs to inhibit both nonprocessive (Tat-dependent) and processive (Tat-independent) transcripts, in principle showing inhibition of early and late events in the life-cycle of HIV-1.

Although efficient RNAi can be induced by lhRNAs expressed from intracellular gene vectors, differentiated mammalian cells can react to these constructs by activating a global IFN response. At worst, this results in nonspecific mRNA degradation, translational suppression, and cell death via apoptosis. The IFN response mediates antiviral or antigrowth activities, and is elicited by interaction of dsRNA with cellular proteins such as PKR, myxovirus A (MxA), and 2′,5′-oligoadenylate synthase 1 (OAS1) genes, among others. The use of dsRNA < 30 bp, and specifically the use of 21-bp short-interfering RNAs (siRNAs), was initially shown to evade stimulat-

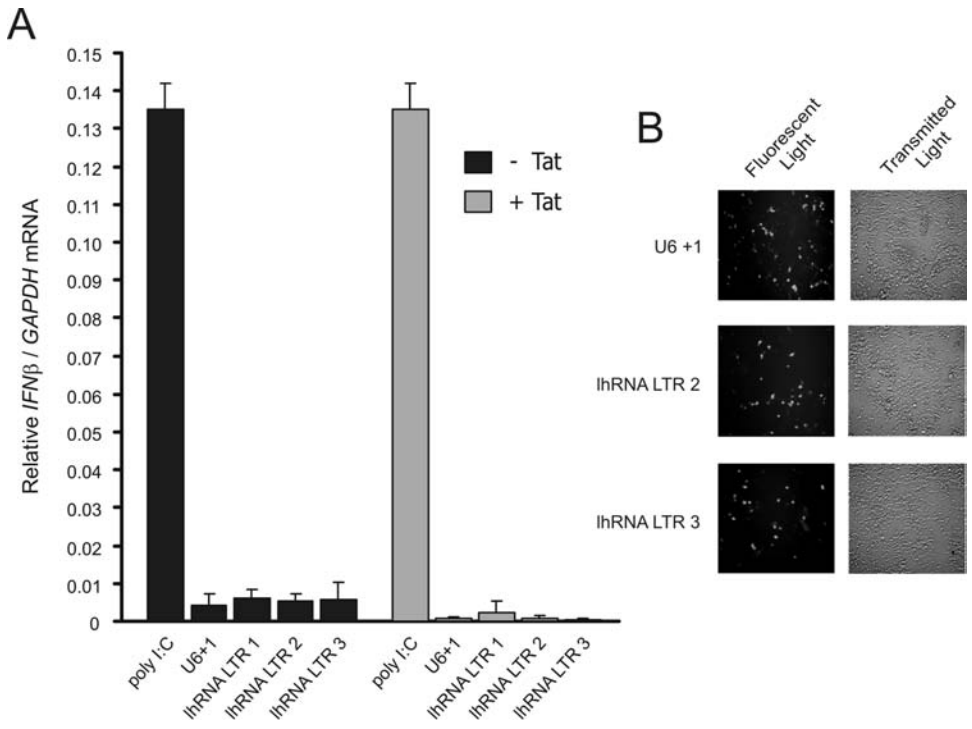


FIG. 3. Long hairpin RNAs targeted to the LTR of HIV-1 subtype C do not elicit a nonspecific interferon response. (**A**) As an index of the activation of interferon induction, quantitative real-time RT PCR was used to determine the expression of *IFN-β* relative to *GAPDH* expression 48 hours posttreatment in treated cell cultures. The dsRNA complex Poly I:C was used as a positive control ($n = 3$, ± SEM). (**B**) HEK293 cells were cotransfected with the lhRNA expressing constructs and pCI–eGFP, a GFP expressing plasmid, to verify equivalent transfection efficiencies between samples 48 hours posttransfection. Data is represented at 100× original magnification.

ing the type 1 IFN response (Elbashir, 2001; Sledz et al., 2003). As an index of activation of an interferon response by the lhRNAs used in this study, we assayed interferon beta (*IFN-β*) expression in the presence and absence of Tat by quantitative real-time RT-PCR. None of the constructs elicited *IFN-β* expression compared to a dsRNA control (Fig. 3A), and moreover, cell viability and transfection efficiencies were conserved in control and lhRNA-treated cultures (Fig. 3B). These data suggest that the lhRNAs are capable of inhibiting HIV-1 gene expression without inducing an unwanted toxic cellular response.

The lhRNAs used here were generated to target ~60 bp of cognate HIV-1 sequence. To characterize Dicer-mediated processing of the lhRNAs, a knockdown assay was performed on "tiled" segments of the lhRNA LTR 2 target sequence. Briefly, a set of target luciferase reporter vectors were constructed that span either the complete LTR 2 sequence or shorter overlapping segments that together cover the entire LTR 2 target site (Fig. 4A). These sequences were placed in the 3′ UTR of a *Renilla* luciferase reporter construct and Firefly luciferase was used as an internal control (Fig. 4B). Following cotransfection with either the complete lhRNAs or their sense or antisense counterparts alone, suppression was only observed when the entire LTR 2 Target site or Target A site was present (Fig. 4C). Target A corresponds to the first 22 bp of the complete sequence, suggesting that the majority of siRNAs generated from the lhRNAs by Dicer occurs from the 5′ end of the lhRNA stem. These observations are in accordance with our previously published results that indicate that siRNAs are preferentially produced from the stem region of a U6-generated lhRNA (Weinberg et al., 2007).

The ability of the various lhRNAs to suppress HIV-1 viral replication was determined using two different assays. First, LTR lhRNAs were cotransfected with a HIV-1 subtype B molecular clone (pNL4-3 Luc), and luciferase activity was measured. These results revealed a similar pattern of suppression, with LTR lhRNA 2 again proving to be the most effective lhRNA construct when targeted to a subtype B LTR sequence (79% knockdown). However, the knockdown efficiency was lower than that observed for shRNA II, a previously described shRNA that targets a conserved region of the Rev open reading frame (Lee et al., 2002) (Fig. 5A). Second, LTR lhRNAs were transfected into U87.CD_4.CCR5 cells that were subsequently challenged with a South African R5-tropic subtype C HIV-1 isolate, FV5. Knockdown was assessed by determining p24 antigen levels at day 4 postinfection. Both LTR lhRNA 2 and 3 inhibited HIV-1

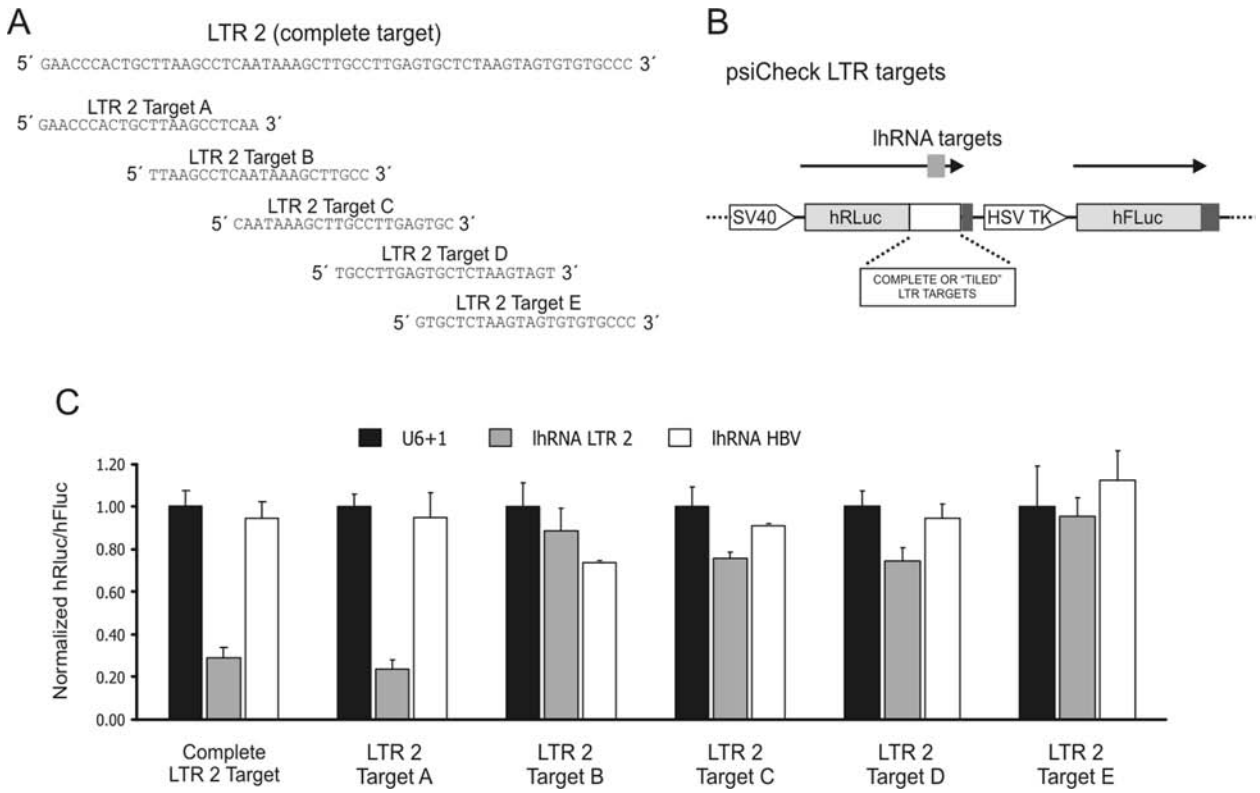


FIG. 4. Silencing efficiency across the region spanning the lhRNA LTR 2 and 3 target sites. (**A** and **B**) The complete LTR2/3 target as well as 22-bp individual sequences (LTR 2 Targets A–E) that overlap to span only the LTR 2 sequence were inserted downstream of the *Renilla* luciferase open reading frame within the pSiCheck vector. The tiled target sites (A–E) are depicted relative to the complete sequence (LTR). (**C**) Suppression efficiency of lhRNA 2, and controls were determined at 48 hours post-transfection against the complete target and individual tile targets. Data is expressed as a ratio of Firefly to *Renilla* luciferase normalized to an empty vector control ($n = 3$, $\pm$SEM).

replication by ~50% relative to a nonspecific lhRNA control (Fig. 5B). Surprisingly, lhRNA LTR 1 also showed some suppression, which may indicate that in the context of a replicating virus, RNAi-refractory secondary structures may become temporarily accessible for cleavage. Yoshinari et al. (2004) has previously shown that if secondary structural elements in the TAR stem–loop are relaxed, this region is sensitive to siRNA-mediated suppression. These results are an encouraging proof of concept because while the knockdown efficacy of the LTR-specific lhRNAs is not as marked when compared to the U6-expressed anti-Tat/Rev shRNA, these data were generated from a transfection assay. We plan to improve knockdown by transducing cells with a lentiviral vector carrying a LTR-specific lhRNA payload.

DISCUSSION

The lhRNAs generated in this study were specifically designed to target pregenomic and viral mRNAs species that include the first 180 nucleotides of transcribed sequences within the R/U5 5′ UTR region of the HIV-1 LTR in an effort to block both early and late stages of the viral life cycle. Although two out of the three lhRNAs were capable of suppressing viral RNAs *in vitro* and *in vivo*, there was a notable lack of suppression by LTR lhRNA 1. This was probably due to the target site and not the RNAi effector itself, as the LTR 1 sequence overlaps completely with the TAR loop found in all HIV-1 transcripts and the extensive secondary structure of this site may cause this region to be refractory to RNAi mediated inhibition (Yoshinari et al., 2004).

The use of shRNAs in concert involves the introduction of multiple expression cassettes into a cell, each of which requires RNAi machinery to generate functional siRNAs from the DNA based vector. This scenario may lead to promoter occlusion either through competition for transcription factors, or competitive inhibition of siRNA precursors for RNAi processing machinery, or a combination of these two factors. Therefore, an advantage of using long hairpin RNAs is that a single RNA Pol III promoter expresses a single transcript that can be processed by cellular Di into siRNAs that target multiple sites.

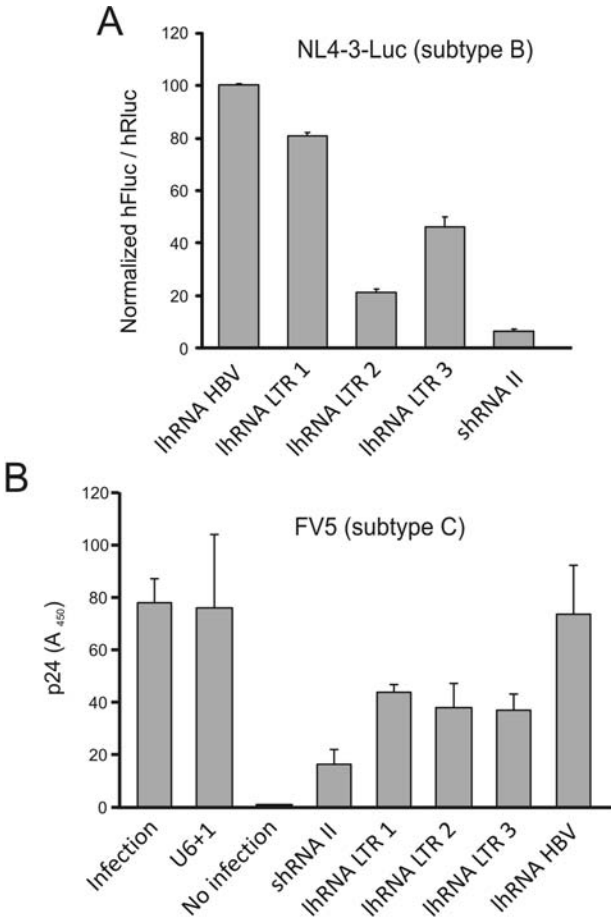


FIG. 5. Long hairpin RNA silencing of HIV-1 gene expression. (**A**) The three lhRNAs, control lhRNA HBV and a shRNA control targeted to Tat were cotransfected with HIV-1 subtype B clone pNL3-4 Luc in HEK293 cells and relative Firefly luciferase expression determined 48 hours later. Data is expressed as a ratio of Firefly to *Renilla* luciferase normalized to a nonspecific lhRNA HBV control ($n = 3$, ± SEM). (**B**) Challenge assay testing all three lhRNAs against HIV-1 subtype C FV5 strain at a $TCID_{50}$ 1000. Viral p24 antigen was assayed 4 days postinfection by ELISA. Data is expressed as a percentage knockdown relative to a nonspecific lhRNA HBV control ($n = 3$, ±SEM).

Another advantage of using lhRNAs that can be spliced into multiple siRNAs is their potential limitation of the evolution of viral escape mutants. Certainly the *in vitro* data presented here argues for some tolerance of sequence variation by the lhRNAs that may prove useful when targeting various strains or subtypes of HIV-1. However, a larger number of variant strains would need to be tested to confirm these findings. Expression of both HIV-1 subtype B and C LTR sequences were effectively suppressed by the lhRNAs used here, although minor sequence variations in the targets, such as that seen in lhRNA LTR 3 at position 575 (Fig. 2C), may have resulted in the observed differences in inhibition.

At present, it remains unclear whether long dsRNAs actively generate effective multiple siRNAs. We have previously shown that RNA Pol III expressed lhRNAs (between 50 and 100 bp) targeted to the HBV X open reading frame are preferentially processed into siRNAs at the duplex stem base, with processing and knockdown efficacy tapering-off across the full length of the duplex (Weinberg et al., 2007). These results are in accordance with our LTR 2 "tiling" array studies (Fig. 4), which suggest that multiple siRNAs may not be processed efficiently from a single lhRNA template. These effects may be due to inefficiencies in the processing of multiple rounds by intracellular Dicer. Alternatively, siRNAs that are processed along the duplex may not be functionally active, because the incorrect strand could be selected into RISC.

The two lhRNAs that showed the best suppressive effects exhibited knockdown in the presence and absence of Tat when targeted to an episomal site. Furthermore, a similar pattern was observed in the presence of an integrated target. This may have important implications for the treatment of latently infected pools of cells (Morris and Rossi, 2006). Following integration of the viral genome into host chromosome, initial HIV-1 transcripts do not elongate (nonprocessive transcription) unless Tat is present to aid RNA Pol II. Current antiretrovirals are only active against replicating forms of HIV-1, and thus, any potential therapeutic that targets latent virus will be a useful addition to existing antiretroviral regimens.

Progress on the use of RNAi as a single anti-HIV therapeutic or in combination with other treatment strategies has been slowed by concerns regarding the delivery of large nucleic acid complexes to CD4+ lymphocytes and with the emergence of drug-resistant viral mutants (Morris, 2006). These problems have been exacerbated by the recent observation that manipulation of the natural RNAi pathway within mammalian cells can lead to saturation of cellular RNAi components and serious off-target effects, leading in some cases to death in animal studies (An et al., 2006; Grimm et al., 2006). Consequently, the applicability of RNAi as an adjunctive anti-HIV therapy will require a careful understanding of specific dosage requirements. Nevertheless, the data presented here suggest that off-target effects, as characterized by the IFN response, is not being elicited in the presence of lhRNAs, although further *in vivo* studies remain necessary.

Recently, a conserved HIV-1 *int* region was successfully targeted with a U6-expressed 50-bp lhRNA. This study showed that lhRNAs were capable of suppressing viral replication in a variant resistant to an anti-*int* shRNA (Nishitsuji et al., 2006). However, it remains unclear whether these lhRNA constructs are capable of delaying the onset of viral escape. In addition, for some longer hairpin duplexes (>100 bp and up to 1000 bp), the silencing efficacy appears to be highly variable in a

mammalian cell context. Recently, a replication-competent *nef*-deleted HIV-1 variant with a 300-bp lhRNA targeted to *nef* showed significant inhibition of HIV-1 in *trans,* although, intriguingly through a non-RNAi-mediated mechanism (Konstantinova et al., 2007). Unfortunately, longer hairpin duplexes often result in lower lentiviral titres due in part to the instability of hairpin RNAs in the packaging RNA (Konstantinova et al., 2007). These complications may be resolved in future by packaging lentiviruses in cells defective in one or more RNAi component, or by introducing mismatches such as G:U wobbles within the long hairpin duplex.

In conclusion, lhRNAs might prove efficacious in suppressing HIV-1 replication while avoiding the emergence of viral variants. The lhRNAs described in this study are functional in suppressing LTR-driven luciferase activity across differing subtypes of HIV-1 that including subtype B and C sequences. Moreover, we have shown that lhRNAs are active against processive and nonprocessive transcripts, and do not induce a detectable IFN response. Importantly, these anti-LTR lhRNAs showed knockdown against a primary isolate of HIV-1. Future plans include the incorporation of these RNAi effectors into lentiviral vectors. These will then be assessed for their efficacy against viral isolates following transduction into HIV-1 permissive lymphocytes and followed longitudinally to determine the ability of the lhRNAs at preventing or delaying the emergence of escape mutants.

ACKNOWLEDGMENTS

The authors would like to thank Lynn Morris, John J. Rossi, and Lisa Scherer for practical assistance, and Maria Papathanasopoulos, Alexio Capovilla, and Naazneen Moolla for help with the FV5 subtype C HIV-1 isolate. The cell line U87.CD_4.CCR5 and the plasmid pNL4-3.Luc.R^-E^- was obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH. This work was supported by the South African National Research Foundation (NRF), Medical Research Council (MRC), Poliomyelitis Research Foundation (PRF), and the German Academic Exchange (DAAD).

REFERENCES

AKASHI, H., MIYAGISHI, M., YOKOTA, T., WATANABE, T., HINO, T., NISHINA, K., KOHARA, M., and TAIRA, K. (2005). Escape from the interferon response associated with RNA interference using vectors that encode long modified hairpin-RNA. Mol. Biosyst. **1,** 382–390.

AN, D.S., QIN, F.X., AUYEUNG, V.C., MAO, S.H., KUNG, S.K., BALTIMORE, D., and CHEN, I.S. (2006). Optimization and functional effects of stable short hairpin RNA expression in primary human lymphocytes via lentiviral vectors. Mol. Ther. **14,** 494–504.

ANDERSON, J., and AKKINA, R. (2005). HIV-1 resistance conferred by siRNA cosupression of CXCR4 and CCR5 coreceptors by a specific lentiviral vector. AIDS Res. Ther. **2,** 1–12.

BARRE-SINOUSSI, F., CHERMAN, J.-C., REY, R., NUGEYRE, M.T., CHAMARET, S., GRUEST, J., DAUGUET, C., AXLER-BLIN, C., VEZINET-BRUN, F., ROUZIOUX, C., ROZENBAUM, W., and MONTAGNIER, L. (1983). Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220,** 868–871.

BERKHOUT, B. (2004). RNA interference as an antiviral approach: targeting HIV-1. Curr. Opin. Mol. Ther. **6,** 141–145.

BHATTACHARYA, T., DANIELS, M., HECKERMAN, D., FOLEY, B., FRAHM, N., KADIE, C., CARLSON, J., YUSIM, K., MCMAHON, B., GASCHEN, B., MALLAL, S., MULLINS, J.I., NICKLE, D.C., HERBECK, J., ROUSSEAU, C., LEARN, G.H., MIURA, T., BRANDER, C., WALKER, B., and KORBER, B. (2007). Founder effects in the assessment of HIV polymorphisms and HLA allele associations. Science **315,** 1583–1586.

BODEN, D., PUSCH, O., LEE, F., TUCKER, L., and RAMRATNAM, B. (2003). Human immunodeficiency virus type 1 escape from RNA interference. J. Virol. **77,** 11531–11535.

BODEN, D., PUSCH, O., LEE, F., TUCKER, L., and RAMRATNAM, B. (2004). Efficient gene transfer of HIV-1-specific short hairpin RNA into human lymphocytic cells using recombinant adeno-associated virus vectors. Mol. Ther. **9,** 396–402.

CASTANOTTO, D., LI, H., and ROSSI, J.J. (2002). Functional siRNA expression from transfected PCR products. RNA **8,** 1454–1460.

CHIU, Y.L., CAO, H., JACQUE, J.M., STEVENSON, M., and RANA, T.M. (2004). Inhibition of human immunodeficiency virus type 1 replication by RNA interference directed against human transcription elongation factor P-TEFb (CDK9/CyclinT1). J. Virol. **78,** 2517–2529.

COBURN, G.A., and CULLEN, B.R. (2002). Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. **76,** 9225–9231.

CONNOR, R.I., CHEN, B., CHOE, S., and LANDAU, N.R. (1995). Vpr is required for efficient replication of human immunodeficiency virus type 1 in mononuclear phagocytes. Virology **206,** 935–944.

DAAR, E.S., and RICHMAN, D.D. (2005). Confronting the emergence of drug-resistant HIV type 1: impact of antiretroviral therapy on individual and population resistance. AIDS Res. Hum. Retroviruses **21,** 343–357.

DAS, A.T., BRUMMELKAMP, T.R., WESTERHOUT, E.M., VINK, M., MADIREDJO, M., BERNARDS, R., and BERKHOUT, B. (2004). Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J. Virol. **78,** 2601–2605.

DIALLO, M., ARENZ, C., SCHMITZ, K., SANDHOFF, K., and SCHEPERS, U. (2003). Long endogenous dsRNAs can induce complete gene silencing in mammalian cells and primary cultures. Oligonucleotides **13,** 381–392.

ELBASHIR, S.M., LENDECKEL, W., and TUSCHL, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev. **15,** 188–200.

GALLO, R.C., SALAHUDDIN, S.Z., POPOVIC, M., SHEARER, G.M., KAPLAN, M., HAYNES, B.F., PALKER, T.J., REDFIELD, R., OLESKE, J., SAFAI, B., et al. (1984). Frequent detection and isolation of cytopathic retrovirus (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224,** 500–503.

GRIMM, D., and KAY, M.A. (2007). Combinatorial RNAi: a winning strategy for the race against evolving targets? Mol. Ther. **15,** 878–888.

GRIMM, D., STREETZ, K.L., JOPLING, C.L., STORM, T.A., PANDEY, K., DAVIS, C.R., MARION, P., SALAZAR, F., and KAY, M.A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. Nature **441,** 537–541.

HE, J., CHOE, S., WALKER, R., DI MARZIO, P., MORGAN, D.O., and LANDAU, N.R. (1995). Human immunodeficiency virus type 1 Viral Protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. **69,** 6705–6711.

JACQUE, J., TRIQUES, K., and STEVENSON, M. (2002). Modulation of HIV-1 replication by RNA interference. Nature **418,** 435–438.

JEENINGA, R.E., HOOGENKAMP, M., ARMAND-UGON, M., DE BAAR, M., VERHOEF, K., and BERKHOUT, B. (2000). Functional differences between the long terminal repeat transcriptional promoters of human immunodeficiency virus type 1 subtypes A through G. J. Virol. **74,** 3740–3751.

KONSTANTINOVA, P., DE VRIES, W., HAASNOOT, J., TER BRAKE, O., DE HAAN, P., and BERKHOUT, B. (2006). Inhibition of human immunodeficiency virus type 1 by RNA interference using long-hairpin RNA. Gene Ther. **13,** 1403–1413.

KONSTANTINOVA, P., TER BRAKE, O., HAASNOOT, J., DE HAAN, P., and BERKHOUT, B. (2007). Trans-inhibition of HIV-1 by a long hairpin RNA expressed within the viral genome. Retrovirology **4,** 15.

LASSEN, K., HAN, Y., ZHOU, Y., SILICIANO, J., and SILICIANO, R.F. (2004). The multifactorial nature of HIV-1 latency. Trends Mol. Med. **10,** 525–531.

LEE, N.S., DOHJIMA, T., BAUER, G., LI, H. T., LI, M., EHSANI, A., SALVATERRA, P., and ROSSI, J.J. (2002). Expression of small interfering RNAs targeted against HIV-1 *rev* transcripts in human cells. Nat. Biotechnol. **19,** 500–505.

LI, M., and ROSSI, J.J. (2005). Lentiviral vector delivery of siRNA and shRNA encoding genes into cultured and primary hematopoietic cells. Methods Mol. Biol. **309,** 261–272.

MORRIS, K.V. (2006). Therapeutic potential of siRNA-mediated transcriptional gene silencing. Biotechniques **Suppl.,** 7–13.

MORRIS, K.V., and ROSSI, J.J. (2004). Anti-HIV-1 gene expressing lentiviral vectors as an adjunctive therapy for HIV-1 infection. Curr. HIV Res. **2,** 185–191.

MORRIS, K.V., and ROSSI, J.J. (2006). Lentiviral-mediated delivery of siRNAs for antiviral therapy. Gene Ther. **13,** 553–558.

NISHITSUJI, H., IKEDA, T., MIYOSHI, H., OHASHI, T., KANNAGI, M., and MASUDA, T. (2004). Expression of small hairpin RNA by lentivirus-based vector confers efficient and stable gene-suppression of HIV-1 on human cells including primary non-dividing cells. Microbes Infect. **6,** 76–85.

NISHITSUJI, H., KOHARA, M., KANNAGI, M., and MASUDA, T. (2006). Effective suppression of human immunodeficiency virus type 1 through a combination of short- or long-hairpin RNAs targeting essential sequences for retroviral integration. J. Virol. **80,** 7658–7666.

NOVINA, C.D., MURRAY, M.F., DYKXHOORN, D.M., BERESFORD, P.J., RIESS, J., LEE, S., COLLMAN, R.G., LIEBERMAN, J., SHANKAR, P., and SHARP, P.A. (2002). siRNA-directed inhibition of HIV-1 infection. Nat. Med. **8,** 681–686.

PALELLA, F.J., JR., DELANEY, K.M., MOORMAN, A.C., LOVELESS, M.O., FUHRER, J., SATTEN, G.A., ASCHMAN, D.J., and HOLMBERG, S.D. (1998). Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. N. Engl. J. Med. **338,** 853–860.

PARK, W.S., MIYANO-KUROSAKI, N., HAYAFUNE, M., NAKAJIMA, E., MATSUZAKI, T., SHIMADA, F., and TAKAKU, H. (2002). Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Res. **30,** 4830–4835.

PASSMAN, M., WEINBERG, M., KEW, M., and ARBUTHNOT, P. (2000). In situ demonstration of inhibitory effects of hammerhead ribozymes that are targeted to the hepatitis Bx sequence in cultured cells. Biochem. Biophys. Res. Commun. **268,** 728–733.

PEDEN, K., EMERMAN, M., and MONTAGNIER, L. (1991). Changes in growth properties on passage in tissue culture of viruses derived from infectious molecular clones of HIV-1LAI, HIV-1MAL, and HIV-1ELI. Virology **185,** 661–672.

PING, Y.H., CHU, C., CAO, H., JACQUE, J.M., STEVENSON, M. and RANA, T.M., (2004). Modulating HIV-1 replication by RNA interference directed against human transcription elongation factor SPT5. Retrovirology **1,** 46.

ROSSI, J.J. (2006). RNAi as a treatment for HIV-1 infection. Biotechniques **Suppl,** 25–29.

SLEDZ, C.A., HOLKO, M., DE VEER, M.J., SILVERMAN, R.H., and WILLIAMS, B.R. (2003). Activation of the interferon system by short-interfering RNAs. Nat. Cell Biol. **5,** 834–839.

STRAT, A., GAO, L., UTSUKI, T., CHENG, B., NUTHALAPATY, S., MATHIS, J.M., ODAKA, Y., and GIORDANO, T. (2006). Specific and nontoxic silencing in mammalian cells with expressed long dsRNAs. Nucleic Acids Res. **34,** 3803–3810.

SURABHI, R.M., and GAYNOR, R.B. (2002). RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J. Virol. **76,** 12963–12973.

TRAN, N., RAPONI, M., DAWES, I.W., and ARNDT, G.M. (2004). Control of specific gene expression in mammalian cells by co-expression of long complementary RNAs. FEBS Lett. **573,** 127–134.

UNAIDS. (2005). AIDS Epidemic Update December 2005. pp. 1–98.

WATANABE, T., SUDOH, M., MIYAGISHI, M., AKASHI, H., ARAI, M., INOUE, K., TAIRA, K., YOSHIBA, M., and KOHARA, M. (2006). Intracellular-diced dsRNA has enhanced efficacy for silencing HCV RNA and overcomes variation in the viral genotype. Gene Ther. **13,** 883–892.

WEINBERG, M.S., ELY, A., BARICHIEVY, S., MUFAMADI, S., CARMONA, S., and ARBUTHNOT, P. (2007). Specific inhibition of HBV replication *in vitro* and *in vivo* with expressed long hairpin RNA. Mol. Ther. **15,** 534–541.

WESTERHOUT, E.M., OOMS, M., VINK, M., DAS, A.T., and BERKHOUT, B. (2005). HIV-1 can escape from RNA interference by evolving an alternative structure in its RNA genome. Nucleic Acids Res. **33,** 796–804.

WILLIAMSON, C., MORRIS, L., MAUGHAN, M.F., PING, L.H., DRYGA, S.A., THOMAS, R., REAP, E.A., CILLIERS, T., VAN HARMELEN, J., PASCUAL, A., RAMJEE, G., GRAY, G., JOHNSTON, R., KARIM, S.A., and SWANSTROM, R. (2003). Characterization and selection of HIV-1 subtype C isolates for use in vaccine development. AIDS Res. Hum. Retroviruses **19,** 133–144.

YAMAMOTO, T., OMOTO, S., MIZUGUCHI, M., MIZUKAMI, H., OKUYAMA, H., OKADA, N., SAKSENA, N.K., BRISIBE, E.A., OTAKE, K., and FUJI, Y.R. (2002). Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. Microbiol. Immunol. **46,** 809–817.

YE, Y., DE LEON, J., YOKOYAMA, N., NAIDU, Y., and CAMERINI D. (2005); DBR1 siRNA inhibition of HIV-1 replication. Retrovirology **2,** 63.

YOSHINARI, K., MIYAGISHI, M., and TAIRA, K. (2004). Effects on RNAi of the tight structure, sequence and position of the targeted region. Nucleic Acids Res. **32,** 691–699.

Address reprint requests to:
Marc S. Weinberg
Department of Molecular Medicine and Haematology
University of Witwatersrand
Johannesburg, South Africa

E-mail: marc.weinberg@wits.ac.za

Received June 3, 2007; accepted in revised form July 6, 2007