Development of Replication-Defective Herpes Simplex Viral Vectors for Delivery of RNA Interference to Neurons of the Peripheral Nervous System

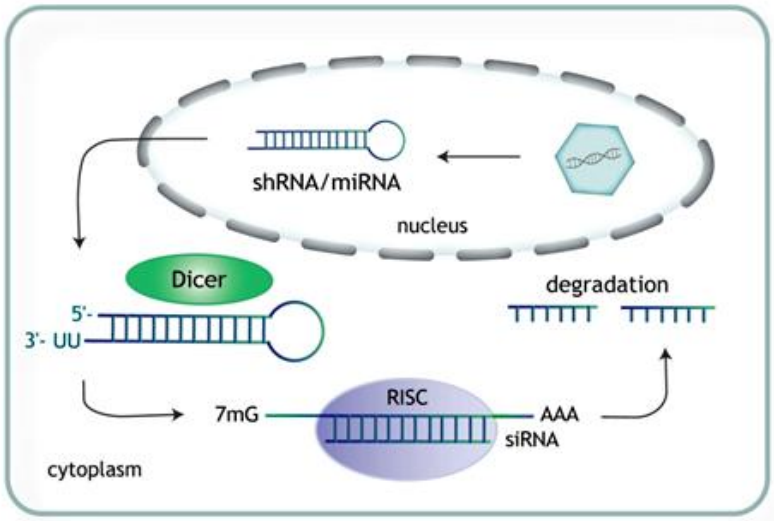
A thesis submitted to the University College London for the degree of Doctor of Philosophy

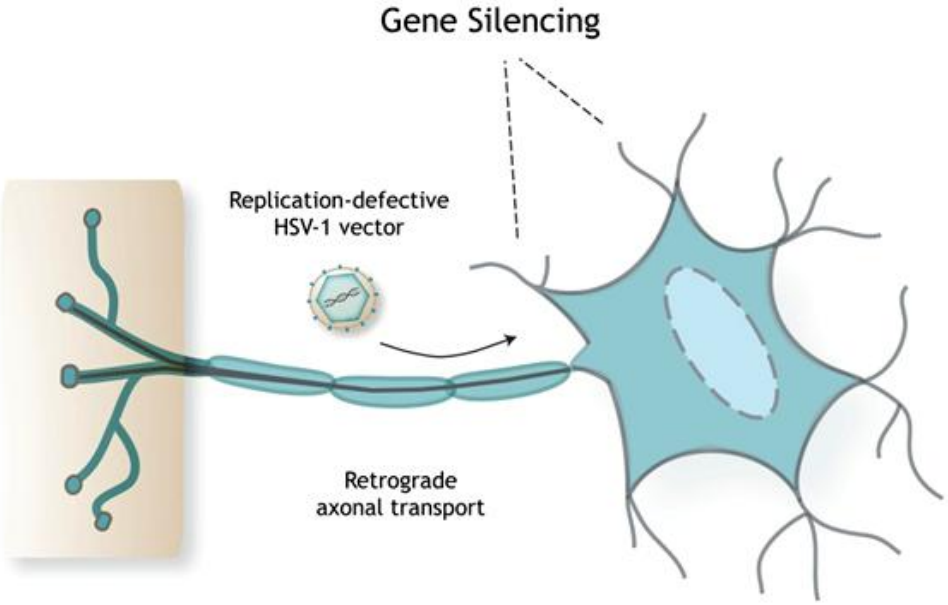
By

Anna-Maria Anesti

December 2009

Division of Infection & Immunity
The Windeyer Institute, 46 Cleveland St
London, W1T 4JF


shRNA/miRNA
nucleus
Dicer
degradation
5'-
3'- UU
7mG
RISC
AAA
siRNA
cytoplasm


Gene Silencing
Replication-defective
HSV-1 vector
Retrograde
axonal transport

ABSTRACT

Considerable interest has been focused on inducing RNA interference (RNAi) in neurons to study gene function and identify new targets for disease intervention. Although small interfering RNAs (siRNAs) have been used to silence genes in neurons, *in vivo* delivery of RNAi to the central and peripheral nervous system remains a major challenge limiting its applications.

This thesis describes the development of a highly efficient method for *in vivo* gene silencing in dorsal root ganglia (DRG) using replication-defective herpes simplex viral (HSV-1) vectors by identifying and evaluating various approaches to induce RNAi, i.e. expression of individual short-hairpin RNAs (shRNAs), artificial microRNAs (miRNAs) and multiple tandem miRNAs. Following the development of these systems, HSV-mediated delivery of shRNA or miRNA against reporter genes was shown to result in highly effective and specific silencing in neuronal and non-neuronal cells in culture and in the DRG of mice *in vivo*, including in a transgenic mouse model. Proof of concept was established by demonstrating *in vivo* silencing of the endogenous *trpv1* gene, thought to be involved in nociception, by assessing both mRNA and protein levels. These data are the first to show silencing in DRG neurons *in vivo* by vector-mediated delivery of shRNA and support the utility of HSV vectors for gene silencing in peripheral neurons and the potential application of this technology to the study of nociceptive processes and in pain gene target validation studies.

Moreover, a disabled HSV-1 vector targeting *p75*, *Lingo1* and *NgR2*, which are involved in myelin inhibition of axonal regeneration, was developed and evaluated for its ability to promote regeneration of sensory axons into the spinal cord, following injury of the dorsal roots. This is the first time such an appoach to silencing multiple genes has been employed. Although HSV-mediated delivery of multiple miRNAs resulted in highly effective silencing of these genes in dividing cells in culture, while highly effective silencing of *p75* was achieved, only modest silencing of *Lingo1* and *NgR2* was observed in

DRG neurons *in vivo*. Preliminary regeneration experiments, which were largely outside the scope of this thesis, were inconclusive and require more extensive study as a stand-alone project, if the *in vivo* potential of the approach developed for silencing multiple genes targeted at axonal regeneration is to be further explored.

ACKNOWLEDGEMENTS

I would like to thank my supervisor Dr. Robert Coffin for believing in me and giving me the opportunity to not only work on this project, but also make it my own. I would also like to thank my parents Lena and Ioannis for the love and endless support they have given me all these years and for teaching me that anything is possible. Special thanks to my colleagues Guy Simpson and Ziquin Han, Pieter Peeters and his team at Johnson & Johnson, and BioVex that generously sponsored this project. Finally, I would like to dedicate this thesis to my husband Stuart for supporting me in all my dreams and more importantly, for giving me the strength to make them happen.

DECLARATION

The work presented in this thesis is the work of Anna-Maria Anesti. Contributions by other researchers are gratefully acknowledged throughout this thesis and are summarised below:

- The quantitative RT-PCR experiments were performed by Dr. Ronald de Hoogt at Johnson & Johnson Pharmaceutical Research & Development in Belgium.

- The shRNA and miRNA sequences against *trpV1* were supplied by Dr. Ines Royaux at Johnson & Johnson Pharmaceutical Research & Development in Belgium.

- The dorsal root transections were performed by Prof. Patrick Anderson at UCL.

- The cortical culture in chapter 3 was prepared by Dr. Filitsa Groutsi at BioVex.

- The statistical analyses in chapter 3 and 4 were performed by Dr. Pieter Peeters at Johnson & Johnson Pharmaceutical Research & Development in Belgium.

PUBLICATIONS

- **Anesti A.M.**, Peeters P.J., Royaux I. and Coffin R.S. (2008) Efficient delivery of RNA interference to neurons *in vivo* using herpes simplex virus. *Nucleic Acids Research*, **36**(14): e86.

- Groutsi F., Mason M.R.J., Anderson P.N., Martins S., **Anesti M.**, Coffin R.S. and Campbell G. (2008) Retrograde viral transduction of cortical pyramidal neurons from the spinal cord. *Restorative Neurology and Neuroscience*, **26**: 509-520.

- **Anesti A.M.** and Coffin R.S. (2008) Herpes simplex virus vectors. *Concepts in Genetic Medicine*, Willey, Chapter 7: 85-102

- **Anesti A.M.**, Martins S. and Coffin RS. (2005) Herpes simplex virus vectors for neuronal gene delivery. Article featured in the American Biotechnology Laboratory.

- **Anesti A.M.** Delivery of RNAi to peripheral neurons *in vivo* using herpes simplex virus. *Methods in Molecular Biology: Pain and Analgesia*, Humana Press (in press).

- **Anesti A.M.** and Coffin R.S. Delivery of RNAi triggers to sensory neurons *in vivo* using herpes simplex virus. *Expert Opinion in Biological Therapy* (in press).

POSTER PRESENTATIONS

- **Anesti A.M.**, Thomas S., Peeters P.J., Rust M., Binlei L. and Coffin R.S. (2009) Highly efficient delivery to neurons *in vivo* using NeuroVex. *CHI's 7th Annual Discovery on Target Conference – RNAi for developing targeted therapeutics*, Boston MA.

- **Anesti A.M.**, Thomas S., Peeters P.J. and Coffin R.S. (2008) NeuroVex technology for gene silencing in neurons. *CHI's 6th Annual Discovery on Target Conference – RNAi for screening*, Boston MA.

- **Anesti A.M.**, Peeters P.J. and Coffin R.S. (2007) NeuroVex technology for gene silencing in neurons. *CHI's 5th Annual Discovery on Target Conference – RNAi for drug discovery and therapeutics*, Boston MA.

- **Anesti A.M.**, Peeters P.J. and Coffin R.S. (2007) NeuroVex technology for gene silencing in neurons. *4th Annual RNAi Europe*, Barcelona, Spain.

- **Anesti A.M.**, Campbell G., Groutsi F., Coffin R.S. (2006) NeuroVex technology for gene silencing in neurons. *CHI's 4th Annual Discovery on Target Conference – RNAi: From target discovery and validation to therapeutic development*, Boston MA.

- **Anesti A.M.**, Martins S., Campbell G., Groutsi F. and Coffin R.S. (2005) Delivery of RNA interference to the nervous system using recombinant herpes simplex virus 1 (HSV-1) vectors. *CHI's 3rd Annual Discovery on Target Conference – RNAi: Functional analysis and target validation*, Boston MA.

- **Anesti A.M.**, Martins S., Campbell G., Groutsi F. and Coffin R.S. (2005) Delivery of RNA interference to the nervous system using recombinant herpes simplex virus 1 (HSV-1) vectors. *CHI's 6th Annual Beyond Genome Conference*, San Francisco CA.

PRESENTATIONS

- **Anesti A.M.** Highly efficient delivery of RNAi to neurons *in vivo* using NeuroVex. (2009) *CHI's 7th Annual Discovery on Target Conference – RNAi for developing targeted therapeutics*, Boston MA.

ABBREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
AGO	Argonaute
ATP	Adenosine triphosphate
BGH	Bovine growth hormone
cDNA	Complementary DNA
CMV	Cytomegalovirus IE gene promoter
CMVenh	CMV IE gene enhancer
CNS	Central nervous system
CSPGs	Chondroitin sulfate proteoglycans
DNA	Deoxyribonucleic acid
DREZ	Dorsal root entry site
DRG	Dorsal root ganglion
dsRNA	Double-stranded RNA
EF1α	Elongation factor 1α
EmGFP	Emerald GFP
esiRNA	Endogenous siRNA
GFP	Green fluorescent protein
hcRNA	Heterochromatic-associated siRNA
HSV	Herpes simplex virus
ICP	Infected cell polypeptide
IE gene	Immediate early gene
IFN	Interferon
IR	Inverted repeat
IRES	Internal ribosome entry site
L4- L6	Lumbar dorsal root ganglia
lacZ	B-galactosidase
LAP	Latency active promoter
LAT	Latency associated transcript
Lingo1	LRR and Ig domain-containing, Nogo receptor–interacting protein
LV	Lentivirus
MAG	Myelin associated glycoprotein
miRNA	microRNA
MOI	Multiplicity of infection
mRNA	Messenger RNA
natsiRNA	Natural antisense siRNA
NgR	Nogo receptor
OMgp	Oligodendrocyte myelin glycoprotein
ORF	Open reading frame
NGR	Nerve growth factor
Pfu	Plaque forming units
piRNA	Piwi-interacting RNA
PKR	dsRNA-dependent protein kinase
PNS	Peripheral nervous system
Pol	RNA polymerase
PTGS	Post-transcriptional gene silencing
rasiRNA	Repeat-associated siRNA
RdRP	RNA-dependent RNA polymerase
RISC	RNAi-induced silencing complex
RITS	RNA-Induced transcriptional silencing
RNA	Ribonucleic acid
RNAi	RNA interference
SCI	Spinal cord injury
shRNA	Short-hairpin RNA
siRNA	Small-interfering RNA
tasiRNA	Trans-acting siRNA
TGS	Transcriptional gene silencing
tncRNA	Tiny-noncoding RNA
tRNA	Transfer RNA
trpV1	Transient receptor potential vanilloid
U_L U_S	Unique short and long regions
WCm	WHV mutated post-transcriptional regulatory element
WHV	Woodchuck hepatitis virus
x-gal	B-galactosidase stain

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION

1.1. RNA interference 18
1.1.1. Structure and biogenesis of siRNAs and miRNAs 18
1.1.2. Gene regulation by siRNAs and miRNAs 25
1.1.3. RISC complexes and Argonautes 25
1.1.4. Mechanisms of gene regulation by miRNAs 29
1.1.4.1. Translational repression 29
1.1.4.2. miRNA-induced mRNA degradation 35
1.1.5. Mechanisms of gene regulation by siRNAs 38
1.1.5.1. Transcriptional gene silencing 38
1.1.5.2. Secondary siRNAs 42
1.1.6. Silencing in mammalian cells 44
1.1.6.1. Silencing by synthetic siRNAs 46
1.1.6.2. Silencing by expression of shRNA/miRNA 56
1.1.6.3. Other nucleic acid-based gene silencing approaches 62

1.2. Viral vector-mediated delivery of RNAi 64
1.2.1. Adenoviral vectors 64
1.2.2. Adeno-associated viral vectors 65
1.2.3. Retroviral & lentiviral vectors 66

1.3. Herpes simplex virus 69
1.3.1. The biology of HSV-1 69
1.3.1.1. The structure of the virion 71
1.3.1.2. The HSV-1 genome 71
1.3.1.3. HSV-1 life cycle 71
1.3.1.4. Viral attachment and entry 72
1.3.1.5. Lytic infection 76
1.3.1.6. HSV-1 latency 83
1.3.2. Amplicon HSV-1 vectors 93
1.3.3. Replication-defective HSV-1 vectors 94
1.3.3.1. Minimizing toxicity 95
1.3.3.2. Long-term expression during latency 97

1.4. Delivery of RNAi to the nervous system 100

1.5. Aim of this thesis 106

CHAPTER 2: MATERIALS & METHODS

2.1. Materials 108
2.1.1. Suppliers 108
2.1.2. Bacterial strains 109
2.1.3. Plasmids 110
2.1.4. Cell lines 110
2.1.5. Primers 111
2.1.6. Double stranded DNA oligos 111
2.1.7. Antibodies 113
2.1.8. Animals 114
2.1.9. Standard solutions 114

2.2. Molecular biology techniques 115
2.2.1. Agarose gel electrophoresis 115
2.2.2. Restriction enzyme digestions 115
2.2.3. Blunt end reactions 115
2.2.4. Phosphatase treatment 116
2.2.5. Purification of DNA from gel bands 116
2.2.6. DNA ligation reactions 116
2.2.7. The Gateway technology 117
2.2.8. BP recombination reactions 118
2.2.9. LR recombination reactions 118
2.2.10. Bacterial cell culture 119
2.2.11. Preparation of competent cells 119
2.2.12. Transformation of bacteria 119
2.2.13. Small scale plasmid DNA purification (mini-prep) 120
2.2.14. High scale plasmid DNA purification (midi-prep) 120
2.2.15. DNA Sequencing 121
2.2.15. Polymerase chain reaction (PCR) 121
2.2.17. Quantitative reverse transcriptase (qRT)-PCR 122

2.3. Tissue culture techniques 124
2.3.1. Freezing and recovery of cells 124
2.3.2. Routine cell passage 124
2.3.3. Transfection of plasmids for transient expression 125
2.3.4. Construction of stable cell lines 125
2.3.5. Screening and purification of cell lines 127
2.3.6. Detection of ß-galactosidase expression 127
2.3.7. Detection of green fluorescent protein expression 127

2.4. Viral vector construction and propagation 128
2.4.1. Transfection for homologous recombination 128
2.4.2. Purification of viral recombinants 129
2.4.3. Production of high titre viral stock 129
2.4.4. Viral infectivity (plaque) assay 130
2.4.5. Viral DNA extraction 130

2.5. Primary neuronal cultures 131
2.5.1. Coating coverslips 131
2.5.2. Primary DRG neuronal cultures 131

2.6. *In vivo* gene delivery 132
2.6.1. Anaesthesia 132
2.6.2. Animal preparation 132
2.6.3. Sciatic nerve injection and conditioning lesion 133
2.6.4. Transection of the dorsal root (rhizotomy) 133
2.6.5. Transcardial perfusion 133
2.6.6. Sectioning of DRG and spinal cords 134
2.6.7. Detection of ß-galactosidase and GFP in whole DRG 134
2.6.8. Processing tissue for protein extraction 135
2.6.9. Processing tissue for RNA extraction 135

2.7. Protein isolation and analysis 136
2.7.1. Extraction of protein from DRG 136
2.7.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) 136
2.7.3. Western blot 137
2.7.4. Immunodetection of proteins on western blots 137
2.7.5. B-galactosidase activity assay 138
2.7.6. Immunofluorescence 138

Chapter 3: Development & Evaluation of Replication-Defective HSV-1 vectors Expressing shRNA using Different Approaches

3.1. Introduction 140
3.2. The 1764/4-/27+/RL1+ HSV-1 backbone 143
3.3. The complementing cell line 143
3.4. The pR19 expression cassette 144
3.5. Generation of plasmid vectors expressing shRNA 148
3.5.1. Expression of shRNA from a polymerase III promoter 148
3.5.2. Expression of shRNA from a hybrid pol II-pol III promoter 155
3.5.3. Expression of shRNA from a miRNA-like system 160
3.5.4. Expression of shRNA from a polymerase II promoter 166
3.6. Screening of shRNA sequences against GFP 174
3.7. Evaluation of plasmid vectors expressing shRNA 176
3.8. Choice of a disabled backbone for RNAi delivery to DRG 181
3.9. Generation of disabled HSV-1 vectors expressing shRNA 186
3.10. Construction of a cell line expressing ß-galactosidase 190
3.11. Evaluation of disabled vectors targeting lacZ in culture 194
3.12. HSV-mediated silencing in primary DRG neurons 195
3.13. Evaluation of disabled vectors targeting lacZ in DRG *in vivo* 199
3.14. HSV-mediated silencing of lacZ in a transgenic mouse model 204
3.15. Discussion 206

Chapter 4: HSV-Mediated Silencing of an Endogenous Gene Involved in Nociception – Silencing of TRPV1

4.1. Introduction 214
4.1.1. Nociception 214
4.1.2. Chronic pain, animal models and the role of TrpV1 215
4.1.3. Delivery of siRNAs to animal models of nociception 217
4.1.4. Rationale 218
4.2. Screening of shRNA / pre-miRNA sequences against trpV1 220
4.3. Generation of plasmid vectors targeting trpV1 220
4.4. Generation of disabled HSV-1 vectors targeting trpV1 226
4.5. Evaluation of disabled vectors targeting trpV1 in culture 228
4.6. HSV-mediated silencing of trpV1 in DRG neurons *in vivo* 230
4.6.1. Silencing of trpV1 – assessment of TrpV1 mRNA levels 230
4.6.2. Silencing of trpV1 – assessment of TrpV1 protein levels 231
4.7. Transduction efficacy of TrpV1-expressing neurons 235
4.8. Discussion 237

Chapter 5: HSV-Mediated Silencing to Promote Regeneration of Sensory Axons into the Spinal Cord

5.1. Introduction 242
5.1.1. Spinal cord injury 242
5.1.2. Degenerative processes following spinal cord injury 243
5.1.3. Inhibition of axonal regeneration 244
5.1.4. Myelin-associated inhibitors 245
5.1.5. Nogo, MAG and OMgp 235
5.1.6. NgR1 and NgR2 247

5.1.7. Lingo1, p75 and TROY 249
5.1.8. RhoA activation 250
5.1.9. Glial scar 251
5.1.10. Cell body responses to axotomy 253
5.1.11. Rationale 254
5.2. Screening of pre-miRNA sequences against p75, Lingo1 & NgR2 256
5.3. Assessment of silencing specificity 262
5.4. Chaining of pre-miRNAs against p75, Lingo1 & NgR2 264
5.5. Generation of a disabled HSV-1 vector against p75/Lingo1/NgR2 269
5.6. HSV-mediated silencing of p75/Lingo1/NgR2 in DRG *in vivo* 272
5.7. Labelling of sensory axons following sciatic nerve injection 275
5.8. Assessment of sensory axon regeneration into the spinal cord 279
5.9. Discussion 282

CHAPTER 6: GENERAL DISCUSSION 289

REFERENCES 294

PUBLICATION IN NUCLEIC ACIDS RESEARCH 359

FIGURES & TABLES

Figure 1.1: Endogenous siRNAs identified in mouse oocytes and D. melanogaster 20
Figure 1.2: Translational repression induced by miRNAs 34
Figure 1.3: Biogenesis and functions of mammalian miRNAs 37
Figure 1.4: Biogenesis and functions of tasiRNAs in plants 40
Figure 1.5: Biogenesis and functions of hcRNAs in S. pombe 41
Figure 1.6: Biogenesis and functions of siRNAs in plants and C. elegans 43
Figure 1.7: INF response induced in mammalian cells by long dsRNA 45
Figure 1.8: Off-target effects 49
Figure 1.9: Expression of shRNA from first and second generation vectors 60
Figure 1.10: Schematic representation of the HSV-1 virion 70
Figure 1.11: The life cycle of HSV-1 *in vivo* 73
Figure 1.12: HSV-1 attachment and entry 75
Figure 1.13: The VP16 induced complex 78
Figure 1.14: HSV gene expression and regulation 82
Figure 1.15: The HSV-1 LAT region 85
Figure 1.16: Proposed mechanisms that may define whether HSV enters latency or lytic infection 92
Figure 1.17: Reporter gene delivery to the nervous system using replication-defective HSV-1 vectors developed in our lab 99

Figure 3.1: Schematic representation of the disabled 1764 4-/27+/RL1+ HSV-1 genome and pR19 cassette 145
Figure 3.2: Expression of shRNA from the HSV-1 1764 4-/27+/RL1+ vector using different approaches 146
Figure 3.3: The pENTR-U6 vector from Invitrogen 147
Figure 3.4: Blast nucleotide sequence alignment between pENTR-U6shRNA and shLacZ or shGFP 152
Figure 3.5: Cloning of pR19U6shRNA plasmids 153
Figure 3.6: Cloning of the pR19CMVenhU6shLacZ plasmid 157
Figure 3.7: The pcDNA6.2/EmGFP-miR vector from Invitrogen 163
Figure 3.8: Cloning of pR19CMV/EmGFP-miR plasmids 164
Figure 3.9: The pSilencer 4.1-CMV vector from Ambion 168
Figure 3.10: Cloning of the pR19CMVshLacZ plasmid 169
Figure 3.11: Blast nucleotide sequence alignment between pR19CMVenhU6shLacZ, pcDNA6.2/EmGFP-miR-LacZ or pSilencer-shLacZ and shLacZ, CMV or U6 ... 171
Figure 3.12: Screening of shRNA sequences against GFP 175
Figure 3.13: Evaluation of plasmids expressing shRNA against β-galactosidase 178
Figure 3.14: Quantification of silencing in 293T transfected with pR19CMVLacZWCm and each of the plasmids expressing shLacZ..... 179
Figure 3.15: Assessment of silencing specificity 180
Figure 3.16: Evaluation of β-galactosidase expression in mouse DRG following sciatic nerve injection of HSV-1 1764 4-/27+/RL1+ virus expressing lacZ either from the CMV or the EF1α promoter 184
Figure 3.17: HSV-1 1764/4-/27+/RL1+ viruses are non-toxic to neurons in culture 185
Figure 3.18: PCR on viral DNA to confirm that the correct homologous recombination event had occurred 188
Figure 3.19: PCR on viral DNA to confirm that the correct homologous recombination event had occurred 189
Figure 3.20: Cloning of the pcDNA3-LacZ plasmid 192
Figure 3.21: The BHK-LacZ cell line 193
Figure 3.22: Evaluation of HSV vectors expressing shRNA against β-galactosidase 196
Figure 3.23: Quantification of silencing in BHK-LacZ cells transduced with disabled HSV vectors expressing shRNA against β-galactosidase 197

Figure 3.24: HSV-mediated silencing in primary DRG neuronal cultures 198
Figure 3.25: Evaluation of HSV-mediated silencing in DRG neurons *in vivo* 202
Figure 3.26: Quantification of silencing in sensory neurons *in vivo* 203
Figure 3.27: HSV-mediated silencing in a transgenic mouse model 205

Figure 4.1: Cloning of pR19U6shTRPV1 and pR19CMV/EmGFP-miR-TRPV1 223
Figure 4.2: Blast nucleotide sequence alignment of pR19U6shTRPV1 and pR19CMV/EmGFP-miR-TRPV1 against shTRPV1 and miR-TRPV1 224
Figure 4.3: PCR on viral DNA to confirm that the correct homologous recombination event had occurred 227
Figure 4.4: Silencing in 293T transfected with pSCREEN-iT/LacZ-trpV1 fused to ß-galactosidase and transduced with HSV-CMV/EmGFP-miR-TRPV1, HSV-U6shTRPV1, or negative control vectors 229
Figure 4.5: HSV-mediated silencing of endogenous TrpV1 - mRNA knockdown 232
Figure 4.6: HSV-mediated silencing of endogenous TrpV1 - protein knockdown 233
Figure 4.7: HSV-mediated silencing of endogenous TrpV1 - quantification of protein knockdown 234
Figure 4.8: TrpV1-positive sensory neurons transduced by the miRNA-like vector 236

Figure 5.1: Glial and myelin inhibition of axonal regeneration 252
Figure 5.2: Blast nucleotide alignments between pre-miRNA sequences and the p75, Lingo1 or NgR2 cDNA sequences 258
Figure 5.3: Construction of pSCREEN-iT/LacZ-p75, -Lingo1, and -NgR2 plasmids 260
Figure 5.4: Screening of pre-miRNA sequences against p75, Lingo1 and NgR2 261
Figure 5.5: Assessment of silencing specificity 263
Figure 5.6: Chaining of pre-miRNA sequences against p75, Lingo1 and NgR2 266
Figure 5.7: Blast nucleotide sequence alignment between pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 and pre-miR-p75, pre-miR-Lingo1 and pre-miR-NgR2 267
Figure 5.8: Evaluation of pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 268
Figure 5.9: PCR on viral DNA to confirm that the correct homologous recombination event had occurred 270
Figure 5.10: Evaluation of HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 in 293T cells 271
Figure 5.11: HSV-mediated silencing of p75, Lingo1 and NgR2 in DRG neurons *in vivo* ... 273
Figure 5.12: HSV-mediated silencing of p75, Lingo1 & NgR2 - quantification of protein knockdown 274
Figure 5.13: Schematic representation of a transverse section of spinal cord 276
Figure 5.14: Labelling of sensory axons projecting into the dorsal horn of the spinal following injection of HSV-CMV/EmGFP-miR-LacZ into the sciatic nerve ... 277
Figure 5.15: Labelling of sensory axons and motor neurons following injection of HSV-CMV/EmGFP-miR-LacZ into the sciatic nerve 278
Figure 5.16: A representative longitudinal section of spinal cord isolated 4 weeks after rhizotomy and injection with HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 281

Table 1.1: Classes of dsRNA molecules, their characteristics and functions 24
Table 1.2: *In vivo* delivery of synthetic siRNA 55
Table 1.3: Delivery of siRNA or shRNA to animal models of neurological disease 105

CHAPTER 1

INTRODUCTION

1.1 RNA INTERFERENCE

RNA interference (RNAi) is an evolutionarily conserved, sequence-specific, post-transcriptional gene silencing (PTGS) mechanism originally observed in C. elegans. Guo and Kemphues used antisense RNA to inhibit *par-1* mRNA expression, when they discovered that the sense RNA strand also repressed expression of *par-1* (Guo & Kemphues, 1995). Subsequently, Fire and Mello demonstrated in their Nobel Prize winning study that double-stranded RNA (dsRNA) was the trigger of gene silencing (Fire *et al.*, 1998). Thus, instead of antisense RNA passively initiating silencing by pairing with the target mRNA, the presence of low concentrations of both sense and antisense strands in the RNA preparation was proposed to result in small amounts of dsRNA. It is now clear that the RNAi pathway is one of many cellular responses to RNA induced by small dsRNA molecules, including small interfering RNAs (siRNAs) and microRNAs (miRNAs). Despite the field of RNAi being relatively young, it has already reshaped our understanding of gene regulation by revealing unsuspected layers of transcriptional and post-transcriptional gene regulatory mechanisms. The structure, biogenesis and functions of siRNAs and miRNAs and the application of RNAi as an effective gene silencing strategy are discussed in the following sections of this introduction.

1.1.1 Structure & biogenesis of small dsRNAs

Small dsRNAs (table 1.1) are generated through distinct biogenesis pathways. Although siRNAs and miRNAs were initially categorised in terms of their origin, exogenous or endogenous, since the discovery of endogenous siRNAs, it has become difficult to distinguish between them. siRNAs originate from endogenous or exogenous dsRNA that may be hundreds or thousands of base pairs long, and have been suggested to function in antiviral defence, silencing of mRNAs that are overproduced or translationally aborted, and guarding the genome from disruption by transgenes and transposons. Exogenous siRNAs are virally derived or experimentally introduced into the cytoplasm, whereas

endogenous siRNAs (esiRNAs) are derived from transcription of coding or non-coding genomic regions. Plant esiRNAs include natural antisense-siRNAs (natsiRNAs), trans-acting-siRNAs (tasiRNAs) and heterochromatic-associated siRNAs (hcRNAs). natsiRNAs originate from convergent partially overlapping transcripts (Borsani *et al.*, 2005). tasiRNAs are generated from specific non-coding genomic regions. Their biogenesis is initiated by miRNAs that cleave the single-stranded RNA transcript to produce fragments which serve as templates for dsRNA synthesis by the RNA-dependent RNA polymerase (RdRP) RDR6 (Allen *et al.*, 2005, Vazquez *et al.*, 2004). hcRNAs, which have also been identified in the yeast S. pombe, derive from long dsRNA precursors that are transcribed from genomic repeat regions. esiRNAs in C. elegans include tiny-noncoding RNAs (tncRNA), which are derived from non-coding, non-conserved sequences (Ambros *et al.*, 2003), and secondary siRNAs, which originate from unprimed RdRP synthesis of dsRNA (Pak & Fire, 2007, Sijen *et al.*, 2007) and have been also identified in plants.

Until recently, esiRNAs were not believed to exist in mammals and insects which lack RdRPs. Moreover, exposure of mammalian cells to long dsRNA results in the induction of the interferon (INF) response, which leads to the general inhibition of cellular translation and was widely perceived to preclude any roles for endogenous RNAi. However, recent studies have revealed diverse sources of esiRNAs in D. melanogaster (Chung *et al.*, 2008, Czech *et al.*, 2008, Ghildiyal *et al.*, 2008, Kawamura *et al.*, 2008, Okamura *et al.*, 2008) and mouse oocytes (Tam *et al.*, 2008, Watanabe *et al.*, 2008). These esiRNAs are derived from transposable elements (TE-siRNAs), overlapping RNAs formed by convergent or divergent transcription (cis-NAT-siRNAs), antisense transcribed pseudogenes pairing with homologous mRNAs (trans-NAT-siRNAs), and long inverted repeat transcripts (hpRNAs) (figure 1.1). Mammalian esiRNAs outside oocytes, where long dsRNA does induce the INF response, remain to be identified.

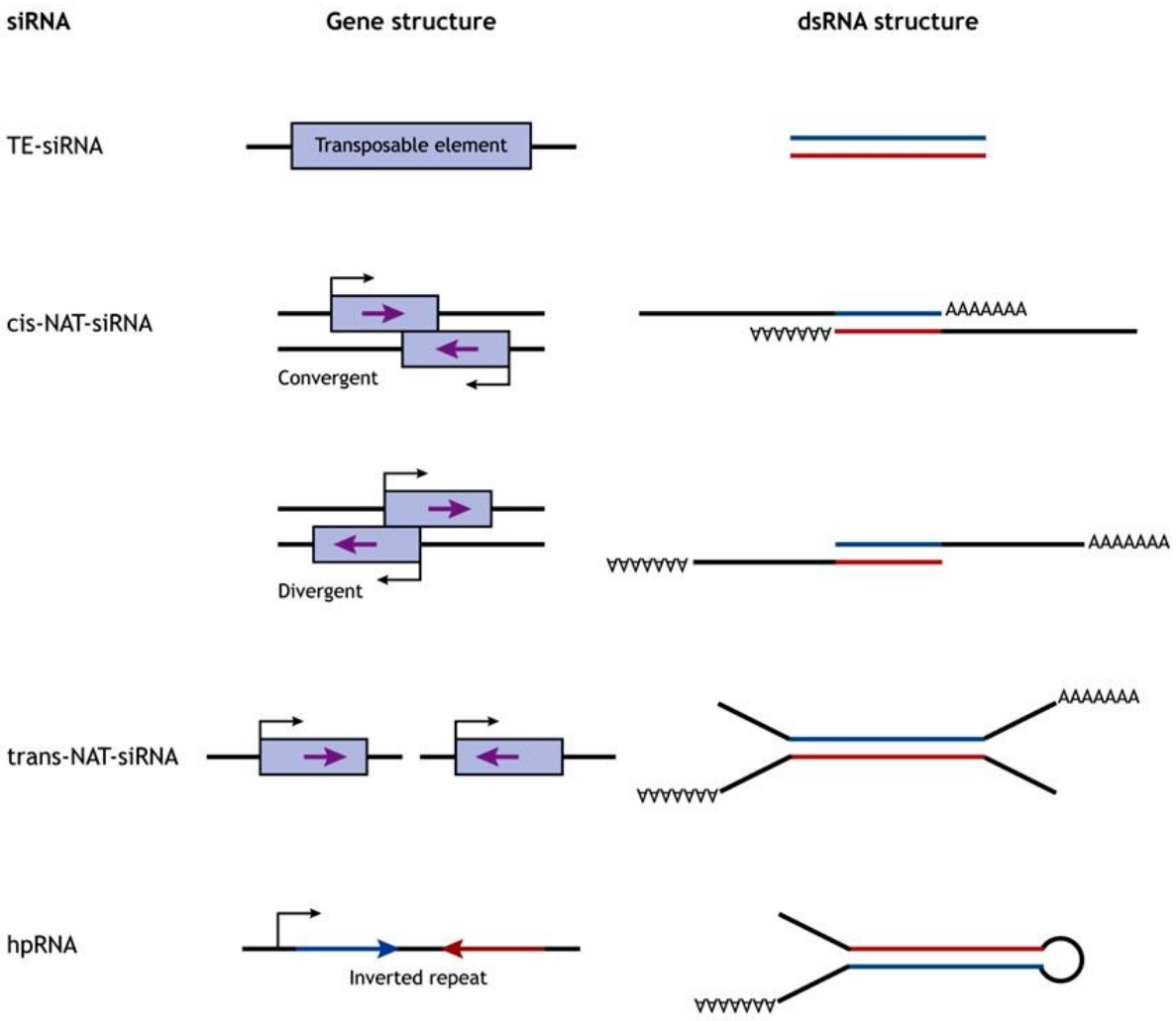


Figure 1.1 Endogenous siRNAs identified in mouse oocytes and D. melanogaster.

TE-siRNAs are derived from transposable elements.

cis-NAT-siRNAs are derived from overlapping RNAs formed by convergent or divergent transcription.

trans-NAT-siRNAs are derived from antisense transcribed pseudogenes pairing with homologous mRNAs.

hpRNAs are derived from long inverted repeat transcripts.

miRNAs are endogenous, regulatory non-coding RNA molecules involved in almost every developmental and cellular process investigated so far. The first miRNA, *lin-4*, was identified during a genetic screen in C. elegans (Lee *et al.*, 1993). Cloning of the locus revealed that *lin-4* produces a non-coding RNA that represses expression of *lin-14*, which is involved in post-embryonic development. Mammalian miRNAs are now predicted to regulate approximately 30% of all protein-coding genes (Lewis *et al.*, 2005). Over 500 miRNAs have been identified in humans (Landgraf *et al.*, 2007), and miRNAs have been recently implicated in the pathogenesis of human disease, including cancer (Esquela-Kerscher & Slack, 2006) and neurodegenerative disorders (Kim *et al.*, 2007, Lukiw *et al.*, 2008).

Almost half of all known mammalian miRNAs reside within the introns of protein-coding genes or within either the introns or exons of non-coding genes (Rodriguez *et al.*, 2004). Rarely, miRNAs lie within the exons of protein-coding mRNAs. These transcripts are thought to produce either miRNA or protein from a single mRNA molecule (Cullen, 2004). Some miRNAs form their own transcription units, whilst others are clustered and transcribed as polycistronic transcripts (Cai, 2004, He *et al.*, 2005). The majority of miRNAs are transcribed by RNA polymerase (pol) II; although a cluster of human miRNAs have been recently shown to utilize RNA pol III for their transcription (Cai, 2004). The long primary miRNA transcript (pri-miRNA) consists of a stem of approximately 33bp, a terminal loop and flanking regions. In collaboration with the dsRNA-binding protein Pasha in flies and DGCR8 in humans, the RNase III enzyme Drosha selectively cleaves the pri-miRNA at a position approximately one helical RNA turn into the stem to generate a 70-100nt hairpin precursor miRNA (pre-miRNA) (Denli *et al.*, 2004, Gregory *et al.*, 2004, Han *et al.*, 2004). Some spliced-out introns in C. elegans, D. melanogaster and mammals correspond precisely to pre-miRNAs (mirtrons), thus circumventing the requirement for Drosha-Pasha/DGCR8 (Okamura *et al.*, 2007, Ruby *et al.*, 2007). The discovery of mitrons suggests that any RNA, with a size comparable to a pre-miRNA and all the structural features of a

pre-miRNA, can be utilized by the miRNA processing machinery and potentially give rise to a functional miRNA. The pre-miRNA is subsequently exported to the cytoplasm by Exportin 5/Ran-GTP (Lund *et al.*, 2004). Silencing of Exportin 5 by siRNA results in a reduction in the levels of pre-miRNA not only in the cytoplasm but also in the nucleus, suggesting that Exportin 5 may also be important for stabilizing pre-miRNA in the nucleus (Yi *et al.*, 2003).

Both siRNAs and miRNAs are generated by Dicer, a cytoplasmic family of RNase III enzymes that cleave long dsRNA or pre-miRNA into 21-23nt dsRNA molecules with symmetric 2-3nt 3' overhangs (Bernstein *et al.*, 2001, Hutvagner *et al.*, 2001, Zhang *et al.*, 2002). In vertebrates, this reaction is carried out together with TRBP (transactivating region (Tar) RNA-binding protein) and PACT (interferon-inducible dsRNA-dependent protein kinase activator), which are both co-factors for strand selection (Chendrimada *et al.*, 2005, Lee *et al.*, 2006). In S. pombe, C. elegans and vertebrates, only a single Dicer gene has been identified (Bernstein *et al.*, 2001, Hutvagner *et al.*, 2001, Knight & Bass, 2001, Volpe *et al.*, 2002). In addition to two RNase III domains, mammalian Dicer has a PAZ domain and a C-terminal dsRNA binding domain, which recognize the 2nt 3'overhang generated by Drosha and long dsRNA, respectively (Zhang *et al.*, 2004a). Thus, the same Dicer that generates siRNA can also process pre-miRNA. Additional proteins may interact with Dicer to allow it to recognize different sources of dsRNA. In D. melanogaster, two Dicer paralogs have been identified. Dcr-1 partners with Loquacious and is required for miRNA processing, while Dcr-2 partners with R2D2 and is mainly involved in the processing of long dsRNA (Lee *et al.*, 2004). Interestingly, flies mutant in Loquacious, rather than R2D2, are highly depleted in esiRNAs, suggesting that a subpopulation of Dcr-2 that specifically recruits Loquacious may be devoted to the esiRNA pathway (Chung *et al.*, 2008, Czech *et al.*, 2008, Okamura *et al.*, 2008). It remains to be established whether processing of esiRNAs by Dicer takes place in the cytoplasm or nucleus, but it seems unlikely that the substrates for esiRNA production would be exported to the cytoplasm. In the plant A. thaliana, four Dicer paralogs have been identified. DCL1 processes miRNA

precursors (Park *et al.*, 2002, Reinhart *et al.*, 2002), DCL2 is required for production of natsiRNAs and siRNAs, DCL3 is involved in the production of hcRNAs, and DCL4 is required for the production of tasiRNAs (Xie *et al.*, 2005, Xie *et al.*, 2004b). DCL1, DCL3 and DCL4 are localised in the nucleus (Hiraguri *et al.*, 2005, Xie *et al.*, 2004a), and DCL1 is responsible for the processing of pri-miRNA to miRNA (Kurihara & Watanabe, 2004, Papp *et al.*, 2003). DCL2 is localised in both the cytoplasm and nucleus (Dorokhov *et al.*, 2006). Following processing by Dicer, the 3'end of plant miRNAs and siRNAs is modified by addition of a 2'-O-methyl group by the RNA methyltransferase HEN1 (Yang *et al.*, 2006).

Following unwinding of the siRNA or miRNA duplex, the strand with the thermodynamically less stable 5'end (Khvorova *et al.*, 2003, Schwarz *et al.*, 2003), termed the guide strand, is incorporated into related RNA-induced silencing complexes (RISCs), while the other strand, termed the passenger strand, is degraded. Naturally occurring miRNAs show a strong bias for accumulating only one strand into the RISC, and effective siRNA or miRNA duplexes show reduced thermodynamic stability at the 5' end of the antisense strand.

Class	Size (nt)	Precursor	Mechanism/ Function	3'end modifications	Organism
siRNA	20-23	dsRNA	mRNA cleavage	Unmodified	Mammals C. elegans
			Antiviral response Transposon defence	2'-O-methylated	Plants D. melanogaster
natsiRNA	21-22	dsRNA	mRNA cleavage	2'-O-methylated	Plants
			Regulation of gene expression		
tasiRNA	21-22	dsRNA	mRNA cleavage	2'-O-methylated	Plants
			Trans-acting cleavage of endogenous mRNAs		
hcRNA	24	dsRNA	Regulation of chromatin structure	2'-O-methylated	Plants
			Transcriptional gene silencing	Unknown	S. Pombe
tncRNA	22	dsRNA	Unknown	Unknown	C. elegans
			Unknown		
Secondary siRNAs	20-25	dsRNA	mRNA cleavage	Unmodified	C. elegans
			Enhancement of primary signal	2'-O-methylated	Plants
miRNA	20-23	Imperfect hairpin	Translational repression/mRNA cleavage	Unmodified	Mammals D. melanogaster C. elegans Viruses
			Regulation of gene expression	2'-O-methylated	Plants
piRNA	28-33	ssRNA	mRNA cleavage	2'-O-methylated	Mammals D. melanogaster
			Transposon defence in the germ line		
rasiRNA	23-28	ssRNA	Regulation of chromatin structure	2'-O-methylated	D. melanogaster
			Transcriptional gene silencing		

Table 1.1 Classes of small dsRNA molecules, their characteristics and functions.

1.1.2 Gene regulation by siRNAs and miRNAs

Whilst siRNAs and miRNAs differ in their biogenesis, they have the same regulatory potential, depending upon the degree of complementarity to their target mRNAs (Hutvagner & Zamore, 2002, Mourelatos *et al.*, 2002). Endonucleolytic cleavage of mRNA is favoured by perfect complementarity and although some mismatches can be tolerated, it requires base-pairing between bases 10 and 11 (Yekta *et al.*, 2004). Nevertheless, as explained in the following section, extensive base-pairing of a miRNA and its target mRNA is not always sufficient to induce mRNA degradation. siRNAs generally have perfect complementarity to their mRNA targets and thus, mediate silencing by mRNA degradation. They can, however, repress the translation or reduce the stability of mRNAs to which they anneal with imperfect complementarity. Moreover, esiRNAs have been shown to direct transcriptional gene silencing (TGS) through modulation of chromatin structure. Plant miRNAs generally base pair to their mRNA targets with nearly perfect complementarity and thus, trigger mRNA cleavage (Llave *et al.*, 2002, Tang *et al.*, 2003). Rarely, a similar mechanism is used by vertebrate and viral miRNAs (Davis *et al.*, 2005, Pfeffer *et al.*, 2004, Sullivan *et al.*, 2005, Yekta *et al.*, 2004). Unlike plants, animal miRNAs generally have imperfect complementarity to their target mRNAs, are present in multiple copies, bind to sites in the 3'UTR, and direct silencing by repressing translation or reducing mRNA stability. Finally, some plant miRNAs may act directly in promoting DNA methylation (Bao *et al.*, 2004), and recent studies describe a role for promoter-directed human miRNAs in facilitating repressive chromatin modifications and TGS (Gonzalez *et al.*, 2008, Kim *et al.*, 2008a). The main mechanisms of gene regulation by siRNAs and miRNAs are described in detail in sections 1.1.4 and 1.1.5.

1.1.3 RISC complexes and Argonautes

siRNAs and miRNAs function as components of RISCs (Hammond *et al.*, 2001) and ribonucleoprotein complexes (miRNPs) or miRISCs (Mourelatos *et al.*, 2002), respectively.

The key and best characterized components of the RISCs are Argonaute proteins. Other RISC-associated proteins include the VIG protein, the Tudor-SN protein, Fragile X-related protein and several RNA helicases, whose precise role in RNAi is still largely unknown. In humans, miRNPs reside in cytoplasmic foci, called processing bodies (P-bodies). Assembly of miRNPs is accomplished by a multiprotein complex termed the miRNA RISC Loading Complex (miRLC), whose core components are Argonaute and Dicer. The miRLC processes pre-miRNA into miRNA, unwinds the miRNA duplex and after loading of the mature miRNAs to Argonaute proteins, it disassembles resulting in the formation of the core miRNP (Maniataki & Mourelatos, 2005).

Argonautes is a diverse family of proteins that can be phylogenetically divided into AGO and PIWI based on similarities to Arabidopsis AGO1 and D. melanogaster PIWI proteins respectively, and WAGO (worm-specific Argonaute). There are eight Argonaute genes in humans: four AGO (AGO1-4) that are expressed in various adult tissues, including the nervous system, and four PIWI (PIWI1-4) that are mainly expressed in the testis (Sasaki *et al.*, 2003). AGO proteins interact with miRNAs/siRNAs, whereas PIWI bind a newly discovered class of small RNAs, known as piwi-interacting RNAs (piRNAs), which are found almost exclusively in the germline of mammals and insects. piRNAs, which include repeat-associated siRNAs (rasiRNAs) discovered in D. melanogaster, are 28-33nt long and 2'-O-methylated at their 3'end. piRNAs and rasiRNAs are believed to be processed from single-stranded RNA derived from defined genomic regions and retrotransposons or other repetitive elements, respectively, by a Dicer-independent mechanism that remains largely unknown (Aravin *et al.*, 2006, Aravin *et al.*, 2003, Girard *et al.*, 2006, Saito *et al.*, 2006, Vagin *et al.*, 2006). Mammalian piRNAs are believed to play a role in spermatogenesis and transposon regulation by mediating mRNA cleavage (Aravin *et al.*, 2007, Carmell *et al.*, 2007), whereas rasiRNAs have been suggested to regulate heterochromatin formation (Pal-Bhadra *et al.*, 2004, Yin & Lin, 2007).

Argonautes consist of a central PAZ domain and a C-terminal PIWI domain. The PAZ domain recognizes the single-stranded 2nt 3'overhangs characteristic of small RNAs processed by Dicer and binds to them with low affinity in a sequence-dependent manner (Lingel *et al.*, 2004, Ma *et al.*, 2004). The PIWI domain has an RNaseH-like fold and binds to the 5'end of small RNAs (Parker *et al.*, 2004, Song *et al.*, 2004a). Recent studies have revealed the presence of a middle domain similar to the cap-binding domain of the eukaryotic initiation factor eIF4E (Kiriakidou *et al.*, 2007), whose function is discussed in section 1.1.4.1.

Structural and biochemical studies have confirmed that Argonaute is the endonuclease of the RISC (Liu *et al.*, 2004, Rand *et al.*, 2004, Rivas *et al.*, 2005). In D. melanogaster and human cell lysates, Argonaute catalyses multiple rounds of mRNA cleavage, resulting in each siRNA directing the degradation of hundreds of target molecules (Haley & Zamore, 2004, Hutvagner & Zamore, 2002). Efficient release of the fragments resulting from mRNA cleavage requires adenosine triphosphate (ATP). Additional proteins are likely to be involved, as AGO2 alone can only direct a single round of mRNA cleavage (Rivas *et al.*, 2005). Following release of the fragments, the 3' fragment is degraded in the cytoplasm by 5'-3' exonuclease Xrn1, whilst the 5' fragment is degraded by the exosome via 3'-5' mRNA degradation. When miRNAs with perfect complementarity to their targets direct mRNA cleavage, a short poly(U) tail is added to the 3' end of the 5' cleavage fragment, suggesting decapping and 5'-3' mRNA degradation as an alternative route of degradation (Shen & Goodman, 2004). Certain Argonaute proteins, however, do not retain all the amino acids required for catalytic activity (Asp-Asp-Asp/Glu/His/Lys), which is referred to as slicer activity, and thus, are unable to cleave target mRNA. Interestingly, the presence of an intact PIWI domain catalytic centre only partially explains the cleavage activity of Argonaute proteins. The human AGO3, for instance, is incapable of mediating mRNA cleavage despite having an intact active site, which

indicates that additional cofactors may be required for catalytic activity (Meister *et al.*, 2004).

Most organisms examined to date contain multiple Argonaute proteins, with different Argonautes having specialized functions. In humans, AGO2 is the only Argonaute protein with a PIWI catalytic domain that can mediate cleavage of target mRNA (Meister *et al.*, 2004, Song *et al.*, 2004a). However, it has also been shown to direct translational repression in an engineered system (Pillai *et al.*, 2004). The function of AGO1 remains to be determined, but AGO3 and AGO4 are likely to mediate translational repression. In D. melanogaster, both Argonaute 1 and 2 are capable of cleavage (Okamura *et al.*, 2004), but Argonaute 1 is dedicated to the miRNA pathway, whilst Argonaute 2 mainly directs mRNA cleavage (Tolia & Joshua-Tor, 2007). In C. elegans, the Argonaute protein RDE-1 is required for mRNA cleavage, but plays little or no role in miRNA function (Tabara *et al.*, 2002), whereas ALG1 and 2 do not cleave mRNA, but are essential for miRNA function. Although the molecular basis for the selective interaction of certain Argonaute proteins to specific types of small dsRNAs remains to be elucidated, the biogenesis of small dsRNAs has been linked to effector programming (Förstemann *et al.*, 2007, Tomari *et al.*, 2007). In D. melanogaster, the structure of small dsRNAs allows sorting of miRNAs and siRNAs into Argonaute 1 and 2 complexes respectively, through Dicer-Argonaute interactions. However, although Argonaute 1 favours binding to small dsRNAs with central mismatches, a large proportion of miRNA duplexes with perfect complementarity in their central region still enter Argonaute 1-containing RISCs (Kawamura *et al.*, 2008). These studies indicate that a specific miRNA that preferentially interacts with a particular Argonaute may be unable to direct cleavage, even if it has perfect complementarity to its target mRNA. The elucidation of mammalian small dsRNA sorting rules may therefore have important implications for improving the efficacy of experimentally induced RNAi. Finally, different expression patterns and levels of Argonaute proteins may control the extent to which the different RNA silencing processes operate.

1.1.4 Mechanisms of gene regulation by miRNAs

Animal miRNAs, which generally have imperfect complementarity to their target mRNAs, bind to sites in the 3'UTR and direct silencing by repressing translation or reducing mRNA stability (figure 1.3). It is currently considered that the specificity of the miRNA for its target mRNA is primarily specified by the 5'end 'seed' region (nucleotides 2-8 of the guide strand). Due to the short length of this region, miRNAs are remarkably promiscuous and it has been suggested that some miRNAs can target hundreds of mRNAs (Lim *et al.*, 2005, Stark *et al.*, 2005). Strong base pairing within the 3'end of the miRNA and its target mRNA is not essential for repression, but may compensate for a weaker seed region or enhance repression (Brennecke *et al.*, 2005). Bulges or mismatches must be present in the central region of the miRNA-mRNA duplex to prevent Argonaute-induced cleavage. Furthermore, efficient translational repression often utilizes multiple miRNA-binding sites (Bartel & Chen, 2004) either because multiple RISCs act in an additive manner or to ensure at least one site will be occupied by RISC. Finally, sequences surrounding the miRNA-responsive elements contain certain features that could affect the effectiveness of silencing. AU-rich nucleotide composition and the relative position of the sites in the 3'UTR all have an impact on miRNA function (Grimson *et al.*, 2007).

1.1.4.1 Translational Repression

Although it is well documented that miRNAs mediate silencing by translational repression (Brennecke *et al.*, 2003, Chen, 2004, Lee *et al.*, 2003, Poy *et al.*, 2004, Wightman *et al.*, 1993), whether repression occurs at the initiation or post-initiation step remains a matter of intense debate.

There is substantial evidence to suggest that miRNAs repress translation initiation. It has been shown that the miRNA–RISC complex associates with the anti-translation initiation factor eIF6, which inhibits joining of the 60S to the 40S subunits (Chendrimada *et al.*, 2007). Depleting eIF6 in either human cells or C. elegans effectively abolishes

miRNA-mediated translational repression. However, in D. melanogaster cells, depletion of eIF6 did not relieve silencing, indicating that in a system where there is little redundancy of the miRNA repression machinery, eIF6 is not fully required (Eulalio *et al.*, 2008). In cap-dependent translation, which is the major mode of translation initiation, the 5' m7G cap is recognized by the cap-binding protein eIF4E. Several studies have concluded that the m7G cap is essential for translational repression and that miRNAs may cause an m7G cap-dependent impediment to the recruitment of the 80S ribosomes to mRNA (Humphreys *et al.*, 2005, Mathonnet *et al.*, 2007, Pillai *et al.*, 2005, Thermann & Hentze, 2007, Wakiyama *et al.*, 2007). Moreover, it has been demonstrated that mutations in the AGO2 central domain, which is similar to the cap-binding domain of eFI4E, abolishes the ability of AGO2 to repress translation when tethered to the mRNA 3'UTR (Kiriakidou *et al.*, 2007). It has been therefore proposed that AGO2 competes with eIF4E for m7G cap binding and thus, prevents translation of capped mRNAs, which accumulate in P-bodies (figure 1.2c). Finally, let-7 miRNA-mediated translational repression was recapitulated in two different cell-free systems established with extracts prepared from mouse cells (Mathonnet *et al.*, 2007) or human HEK293F cells over-expressing miRNA pathway components (Wakiyama *et al.*, 2007). In these systems, the polyA tail and 5'-cap were both required for translational repression, suggesting that let-7 represses translation by impairing the synergistic enhancement of translation by the 5'-cap and 3' polyA tail.

Other studies have suggested that translational repression occurs at the post-initiation step. In contrast to the study by Humphreys *et al.* (2005), which demonstrated that replacing the cap structure with an internal ribosome entry site (IRES) impairs miRNA-mediated repression, Petersen *et al.* demonstrated that cap-independent translation initiated through an IRES was repressed by miRNA (Petersen *et al.*, 2006). Moreover, sucrose gradient sedimentation analysis in C. elegans has revealed that lin-28 and lin-14 mRNAs are associated with polyribosomes capable of elongation *in vitro* (Olsen & Ambros, 1999, Seggerson *et al.*, 2002). Consistent with these observations, several recent studies

have reported similar findings that miRNAs and their targets co-sediment with polysomes engaged in active translation elongation based upon their sensitivity to puromycin treatment (Maroney *et al.*, 2006, Nottrott *et al.*, 2006, Petersen *et al.*, 2006). In the study by Nottrott *et al.* (2006), nascent polypeptides could not be detected by immunoprecipitation and the authors suggested the existence of a co-translational protein degradation mechanism (figure 1.2b). This would resemble the situation in yeast, in which many proteins undergo massive co-translational degradation without reaching a mature size (Turner & Varshavsky, 2000). However, targeting reporter proteins to the endoplasmic reticulum did not prevent repression mediated by either endogenous miRNAs or tethering of AGO2 to the mRNA 3′ UTR (Pillai *et al.*, 2005). If nascent proteins were degraded in the cytosol, co-translational insertion into the endoplasmic reticulum lumen would be expected to protect them, at least in part, from proteolysis. Moreover, proteasome inhibitors had no effect on miRNA-mediated repression and pulse-labelling experiments failed to detect either full-length or nascent polypeptides (Petersen *et al.*, 2006, Pillai *et al.*, 2005). Instead, Petersen *et al.* proposed a ribosome drop-off model, in which premature termination of translation accounts for the absence of protein production (figure 1.2a). Alternatively, the lack of nascent polypeptides may be due to repression at translation initiation. Finally, although some studies have assumed that the co-sedimentation of miRNA complexes with polysomes indicates that these complexes contain ribosomes, Thermann and Hentze (2007) have recently demonstrated that miRNA-mRNPs co-sedimented with polysomes are not necessarily being translated. These heavy RNP particles, termed pseudo-polysomes, were found to be insensitive to puromycin and thus, not actively elongating ribosomes.

Furthermore, recent studies have indicated that translational repression by miRNAs can be reversed in response to certain stimuli. When human hepatoma cells are subjected to amino acid starvation or other types of stress, CAT-1 mRNA that is repressed by miR-122 is released from P-bodies and recruited to polysomes through a mechanism that involves

binding of HuR (an AU-rich-element-binding embryonic lethal abnormal vision (ELAV) family protein) to the 3′ UTR of CAT-1 mRNA (Bhattacharyya *et al.*, 2006). Moreover, reporter genes controlled by let-7 miRNA and containing HuR-binding sites are upregulated in the same way in stressed HeLa cells. In cultured rat hippocampal neurons, miR-134, which is present at dendritic spines, negatively regulates translation of an mRNA encoding a protein kinase (Limk1) that is important for spine development (Schratt *et al.*, 2006). Treatment of cells with brain-derived neurotrophic factor (BDNF) stimulates Limk1 synthesis by relieving the miR-134 inhibition of Lim1 mRNA. The above data indicate that P bodies can also act as storage sites for mRNAs inhibited by miRNAs.

Finally, perhaps the most interesting development regarding miRNA-mediated translational regulation has been the finding that it oscillates between repression and activation in coordination with the cell cycle (Vasudevan & Steitz, 2008, Vasudevan *et al.*, 2007). Cell cycle arrest activates translation of mRNAs bearing AU-rich elements (AREs) in their 3′ UTR (Wilusz *et al.*, 2001). This activation was found to be mediated by the recruitment of AGO2 and the fragile X mental retardation-like protein 1 (FXR1P) to the AREs (Vasudevan & Steitz, 2007). Vasudevan and colleagues (2007) subsequently demonstrated that miRNA369-3, which contains two seed regions for the tumour necrosis factor-α (TNF-α) ARE, is specifically increased in cells following cell cycle arrest and recruits the AGO2-FXR1P complex to the TNF-α ARE to activate translation. Importantly, it was also demonstrated that let-7 induces translation upregulation of target mRNA on cell cycle arrest through miRNA target sites in the 3'UTR, while it represses translation in proliferating cells. The authors proposed that protein changes within the miRNP may result in this dual mode of regulation. Thus, the oscillation between activation and repression does not appear to be restricted to miRNAs targeting ARE sites. However, whether it applies to all miRNAs remains to be determined.

Although the precise mechanism by which miRNAs affect translation remains to be elucidated, it would appear from the available data that two distinct modes of

translational repression exist. It cannot be excluded that miRNAs modulate protein synthesis through many different routes, including repression at both the initiation and post-initiation steps of translation. The factors that determine the mechanisms by which miRNAs silence their targets remain unclear. The choice may depend on the specific miRNA, specific mRNA targets or specific tissue and cell types, as well as proteins that interact with miRNPs. Recently, Kong *et al*. have suggested that that the mode of translational repression may be dependent upon the promoter from which the target mRNA is transcribed (Kong *et al.*, 2008).

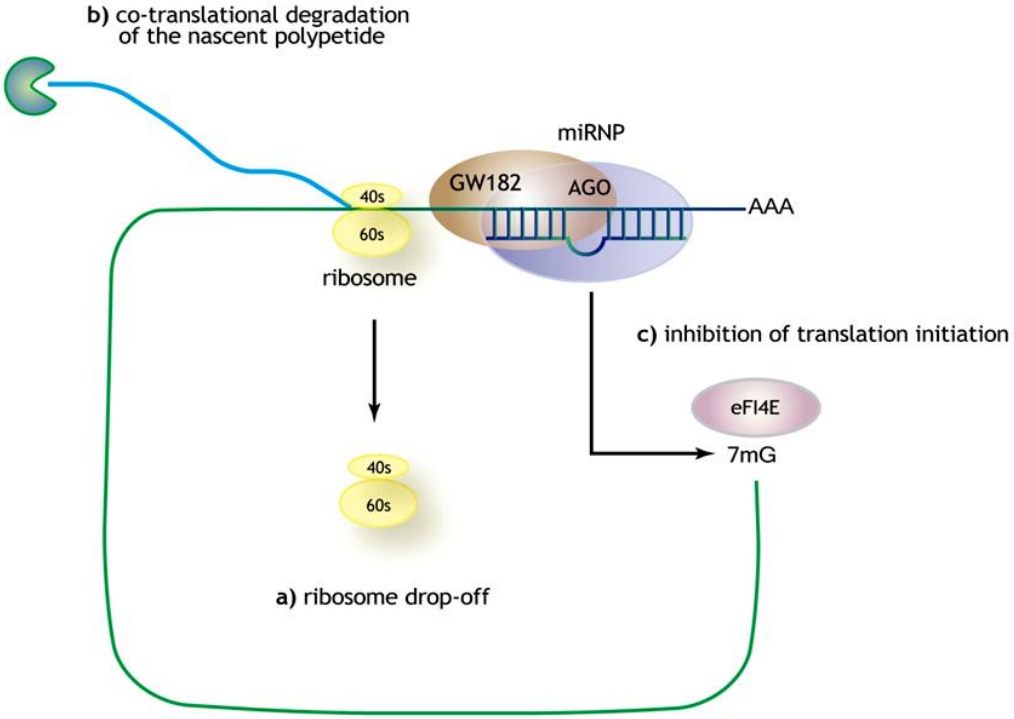


Figure 1.2 Translational repression induced by miRNAs

a) miRNAs have been suggested to repress translation at the post-initiation step via a ribosome drop-off mechanism, in which premature termination of translation occurs.

b) miRNAs have also been suggested to repress translation at the post-initiation step via a co-translational protein degradation mechanism.

c) miRNAs have been suggested to repress translation initiation. The middle domain of AGO2 may compete with eIF4E for m7G cap binding thus, preventing translation of capped mRNAs.

1.1.4.2 miRNA-induced mRNA degradation

Although early studies in C. elegans indicated that miRNAs reduce protein levels without affecting mRNA levels (Olsen & Ambros, 1999, Wightman *et al.*, 1993), it has since been demonstrated that miRNAs can also induce degradation of their mRNA targets (Bagga *et al.*, 2005, Behm-Ansmant *et al.*, 2006, Giraldez *et al.*, 2006, Jing *et al.*, 2005, Lim *et al.*, 2005, Wu *et al.*, 2006). Unlike mRNA degradation mediated by siRNAs or miRNAs with perfect complementarity to their target mRNAs, miRNA-induced mRNA degradation is not slicer-dependent. Most miRNA-mRNA sites required for endonucleolytic cleavage contain mismatches and none of the intermediates arising from slicer activity have been identified (Bagga *et al.*, 2005, Jing *et al.*, 2005)). Instead, it has been suggested that miRNAs induce mRNA degradation by promoting deadenylation, which is the gradual shortening of the polyA tail, followed by decapping, which is the removal of the mRNA cap structure.

Bagga and colleagues (2005) demonstrated in C. elegans that knockdown of Xrn1p, the enzyme required for mRNA degradation following decapping, resulted in attenuated silencing caused by let-7 and lin-4 miRNAs, and detected intermediates consistent with 5'-3' exonucleolytic mRNA degradation. Moreover, recent studies in D. melanogaster (Behm-Ansmant *et al.*, 2006), zebrafish embryos (Giraldez *et al.*, 2006) and human cells (Wu *et al.*, 2006) have revealed that mRNAs targeted by miRNAs for degradation undergo prior deadenylation. Importantly, mammalian Argonaute proteins, miRNAs and their mRNA targets co-localize to P-bodies, which are sites for mRNA degradation and contain amongst other components, the decapping DCP1:DCP2 complex, the CAF1:CCR4:NOT deadenylase complex and GW182 (Jakymiw *et al.*, 2005, Liu *et al.*, 2004, Meister *et al.*, 2004, Pillai *et al.*, 2005, Sen & Blau, 2005). This evidence has led to a model in which miRNAs sequester targets mRNAs to P-bodies and GW182 interacts with Argonautes to recruit deadenylases and decapping enzymes, leading to mRNA degradation. Nevertheless, P-bodies are not absolutely required for miRNA function, as depletion of Lsm1 or GW182 in human and D. melanogaster cells, which causes loss of P-bodies and disperses Argonaute proteins

throughout the cell, does not affect miRNA function (Chu & Rana, 2006, Eulalio *et al.*, 2008).

It would appear that miRNAs silence genes primarily by inhibiting protein synthesis and that mRNA degradation is a consequence of this primary event (Chendrimada *et al.*, 2007, Mathonnet *et al.*, 2007). However, mRNAs that cannot be translated are still subject to deadenylation and deadenylation still occurs in mRNAs when miRNA translational repression has been blocked (Wakiyama *et al.*, 2007, Wu *et al.*, 2006). These studies have suggested that deadenylation is a cause rather than a consequence of miRNA-mediated translational repression, particularly at the initiation step. Although deadenylation does not necessarily lead to mRNA decapping and degradation and may represent one mechanism to repress translation of target mRNAs, it does not fully account for translational repression (Behm-Ansmant *et al.*, 2006, Wu *et al.*, 2006). It has been suggested that when deadenylation is impaired, an alternative mechanism which may inhibit translation initiation, such as decapping, may be activated to bypass the requirement for deadenylation (Eulalio *et al.*, 2007). Thus, miRNAs may downregulate their targets by repressing translation, or inducing translation-dependent or independent mRNA degradation. The factors that determine the mechanism of miRNA silencing that occurs in individual cases remain to be determined.

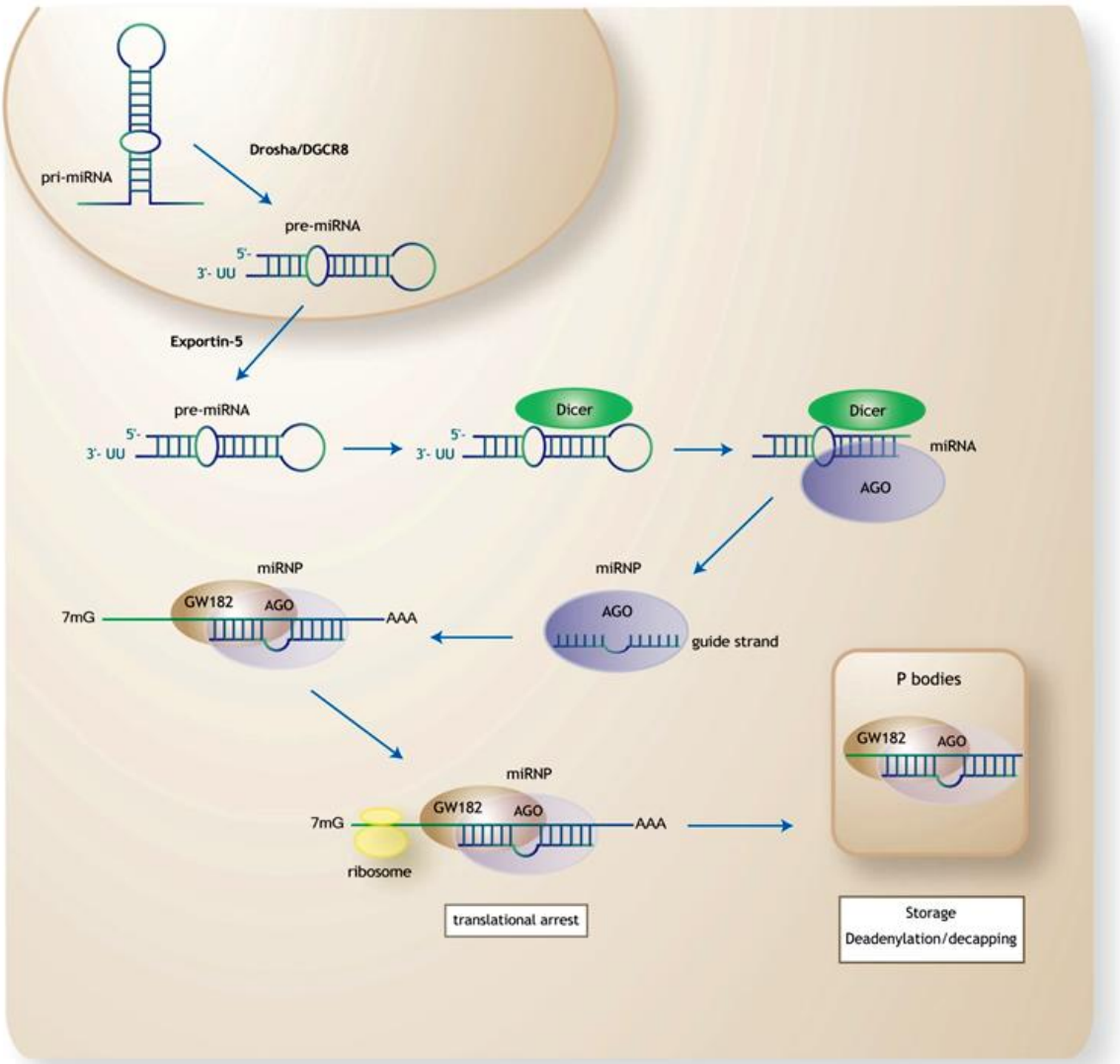


Figure 1.3 Biogenesis and functions of mammalian miRNAs

The pri-miRNA is processed in the nucleus by Drosha/DGCR8 into a pre-miRNA, which is transported to the cytoplasm by Exportin 5. The pre-miRNA is processed into a mature miRNA by the enzyme Dicer. The miRNA is loaded onto an Argonaute protein (AGO). Following unwinding of the miRNA duplex, the strand with the thermodynamically less stable 5' end, termed the guide strand, gets incorporated into a miRNP complex, while the other strand is degraded. The miRNP complex associates predominantly with GW182, which is required for P body integrity. Mammalian miRNAs generally have imperfect complementarity to their target mRNAs, are present in multiple copies, bind to sites in the 3'UTR, and direct silencing by repressing translation or reducing mRNA stability. mRNA degradation, which takes place in P bodies, is induced by promoting deadenylation and decapping. However, P bodies can also act as storage sites for mRNAs inhibited by miRNAs and translational repression can be reversed in response to certain stimuli.

1.1.5 Mechanisms of gene regulation by siRNAs

tasiRNAs (figure 1.4), natsiRNAs and most viral-derived siRNAs (figure 1.6) guide mRNA degradation, whereas hcRNAs play a role in heterochromatin regulation (figure 1.5). Genetic studies suggest that tasiRNAs may mediate cleavage of target mRNAs that are different from the sequences from which the tasiRNAs originate, playing a crucial role in plant development (Adenot *et al.*, 2006, Fahlgren *et al.*, 2006). The function of tncRNAs in C. elegans remains to be determined, whereas secondary siRNAs are believed to support the primary siRNA signal. Although the functions of animal esiRNAs remain largely unknown, they have been suggested to play a role analogous to piRNAs in suppressing the expression of mobile genetic elements in both germ and somatic cells (Chung *et al.*, 2008, Watanabe *et al.*, 2008). Moreover, they seem to regulate the expression of specific mRNAs and may also be involved in heterochromatin formation (Czech *et al.*, 2008, Okamura *et al.*, 2008, Tam *et al.*, 2008).

1.1.5.1 siRNA-mediated transcriptional gene silencing

Studies in S. pombe and plants have revealed that siRNAs can direct both transcriptional and post-transcriptional gene silencing. In S. pombe, hcRNAs are incorporated into RITS, a RISC-like complex that contains amongst other proteins Ago1 and the chromodomain protein Chp1 (figure 1.5). The RITS complex pairs with the nascent transcript repeat sequences and directs modification of histones to promote the formation of repressive heterochromatin on DNA by a mechanism that is largely unknown (Bühler *et al.*, 2006, Noma *et al.*, 2004, Verdel *et al.*, 2004, Volpe *et al.*, 2002). It has been demonstrated that DNA methylation in plants can be induced by miRNA with extensive complementarity exclusively to the corresponding spliced transcript, suggesting that RNA–DNA base-pairing is not required (Bao *et al.*, 2004). Plant hcRNAs, however, whose biogenesis requires RNA polymerase V, have been suggested to interact directly with the target enhancer region to induce RNA-directed DNA methylation (Daxinger *et al.*, 2009).

In S. pombe, hcRNAs correspond to repeat sequences that flank the centromeres, the sites of attachment of chromosomes to the spindle during cell division (Reinhart & Bartel, 2002). These sequences are normally embedded in heterochromatin that is important for centromere function. Mutations in components of the RNAi pathway disrupt this heterochromatin and lead to defects in chromosome segregation (Hall *et al.*, 2003, Volpe *et al.*, 2002). Chicken and mouse cells lacking Dicer also fail to assemble silent heterochromatin at their centromeres (Fukagawa *et al.*, 2004, Kanellopoulou *et al.*, 2005). Thus, esiRNAs may have a conserved role in the maintenance of pericentromeric hetero-chromatin. Moreover, they may be involved in the regulation of invasive genetic elements, such as retrotransposons, which are subject to RNAi-mediated transcriptional silencing in both fission yeast and plants (Hamilton *et al.*, 2002, Schramke & Allshire, 2003), and in transcriptional control of endogenous gene expression. A role for siRNAs in transcriptional gene regulation is supported by the discovery in plants that endogenous miRNAs direct methylation of a family of genes involved in leaf development (Bao *et al.*, 2004).

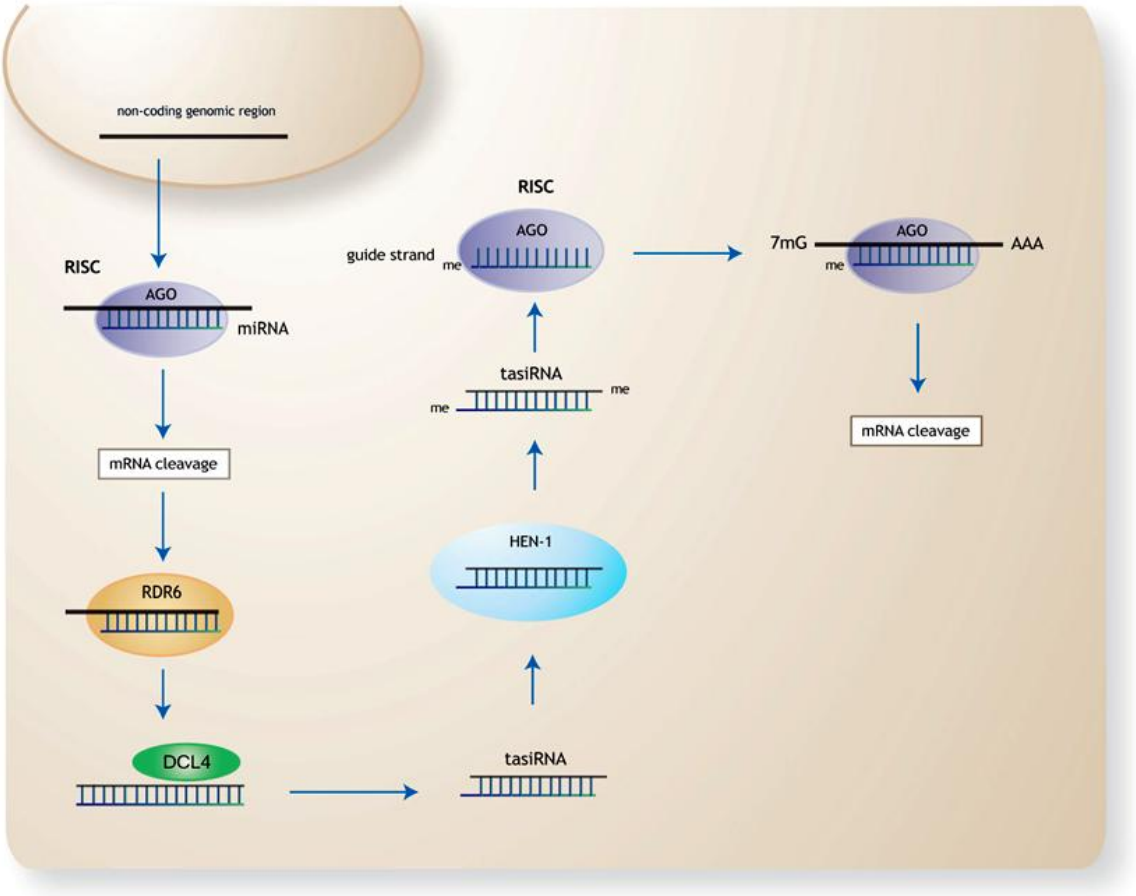


Figure 1.4 Biogenesis and functions of tasiRNAs in plants

tasiRNAs are generated from specific non-coding genomic regions. Their biogenesis is initiated by miRNAs, which in plants generally base pair to their mRNA targets with nearly perfect complementarity and thus, trigger mRNA cleavage. The miRNAs cleave the single-stranded RNA transcript to produce fragments which serve as templates for dsRNA synthesis by the RNA-dependent RNA polymerase RDR6. The long dsRNA is processed by DCL4 into tasiRNAs, which are 2'-O-methylated at the 3'ends by the RNA methyltransferase HEN-1. The guide strand of the tasiRNA is incorporated into a RISC which directs mRNA cleavage. It has been suggested that tasiRNAs mediate cleavage of target mRNAs that are different from the sequences from which the tasiRNAs originate, playing a crucial role in plant development

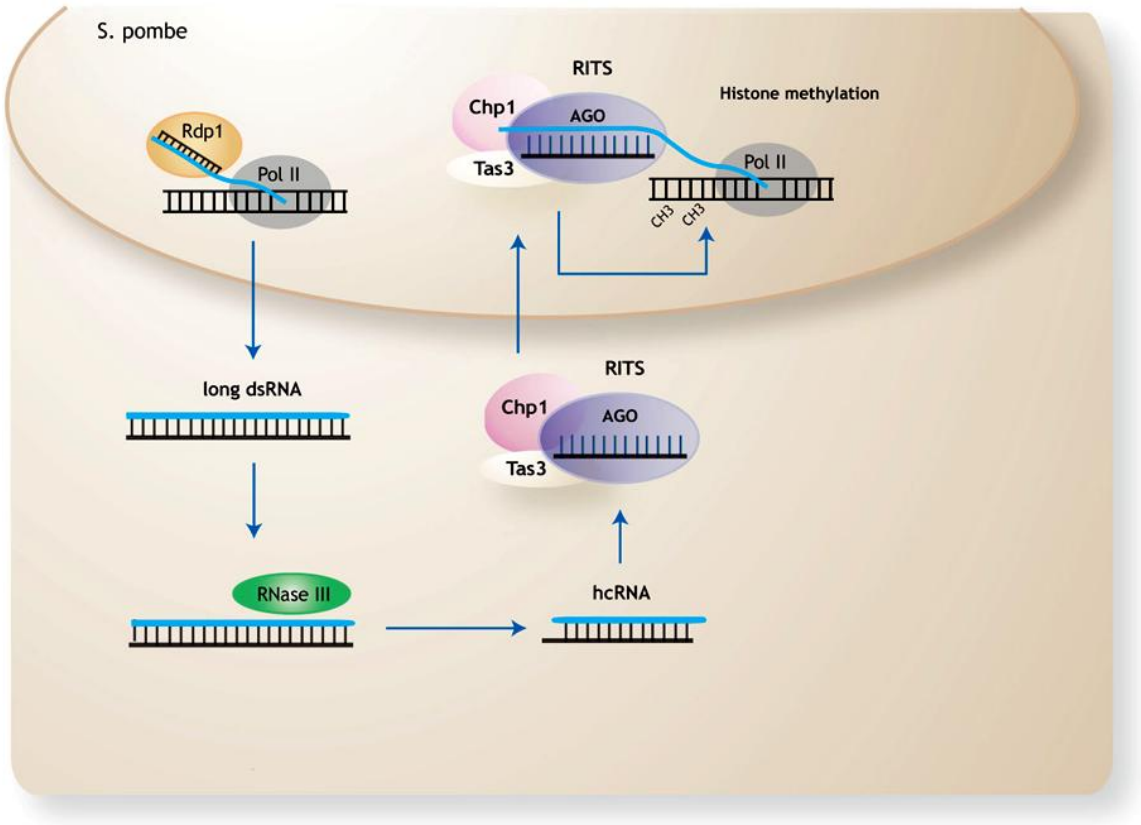


Figure 1.5 Biogenesis and functions of hcRNAs in S. pombe.

hcRNAs derive from long dsRNA precursors that are transcribed from genomic repeat regions. In S. pombe, hcRNAs correspond to repeat sequences that flank the centromeres. hcRNAs are incorporated into RITS, a RISC-like complex that contains amongst other proteins Ago1, the chromodomain protein Chp1 and targeting complex subunit 3 (Tas3). The RITS complex pairs with the nascent transcript repeat sequences and directs modification of histones to promote the formation of repressive heterochromatin on DNA.

1.1.5.2 Secondary siRNAs

Recent studies in C. elegans and plants indicate that experimentally induced RNAi by introducing either longer dsRNA (Pak & Fire, 2007) or a miRNA-like transgene (Sijen *et al.*, 2007) generates two distinct siRNA populations that form during primary and secondary phases (figure 1.6). Primary siRNAs arise directly from trigger RNA molecules, whereas secondary siRNAs require RdRPs. Although it remains to be determined how secondary siRNAs are formed, it is unlikely that RdRPs synthesizes them by priming off of primary siRNAs. Strong evidence comes from the fact that secondary siRNAs are perfectly complementary to the target mRNA even when given a mismatched primary siRNA as a trigger (Sijen *et al.*, 2007). The role of secondary siRNAs in endogenous regulation and exogenous RNAi-induced silencing remain to be elucidated. In plants, secondary siRNAs from exogenous siRNA or natsiRNA are believed to support the primary siRNA signal by inducing mRNA cleavage, whereas secondary siRNAs from hcRNA are believed to induce unidirectional spreading of DNA methylation (Daxinger *et al.*, 2009). In C. elegans, primary siRNAs interact with Argonaute RDE-1, whereas secondary siRNAs, which have a 5'-triphosphate, have been suggested to associate with SAGO-1 and SAGO-2 Argonautes, which are members of the WAGO family and lack residues essential for endonuclease activity (Pak & Fire, 2007, Yigit *et al.*, 2006). Secondary siRNAs may therefore induce indirect mRNA destabilization or transcriptional gene silencing, rather than direct target cleavage. It has been proposed that if RDE-1 was allowed to interact with secondary siRNAs, RdRP amplification of siRNA production could spread into non-targeted, essential genes resulting in unwanted off-target effects. Hence, specialization of Argonaute proteins may allow C. elegans to restrict a potent, amplified RNAi response to specific target loci.

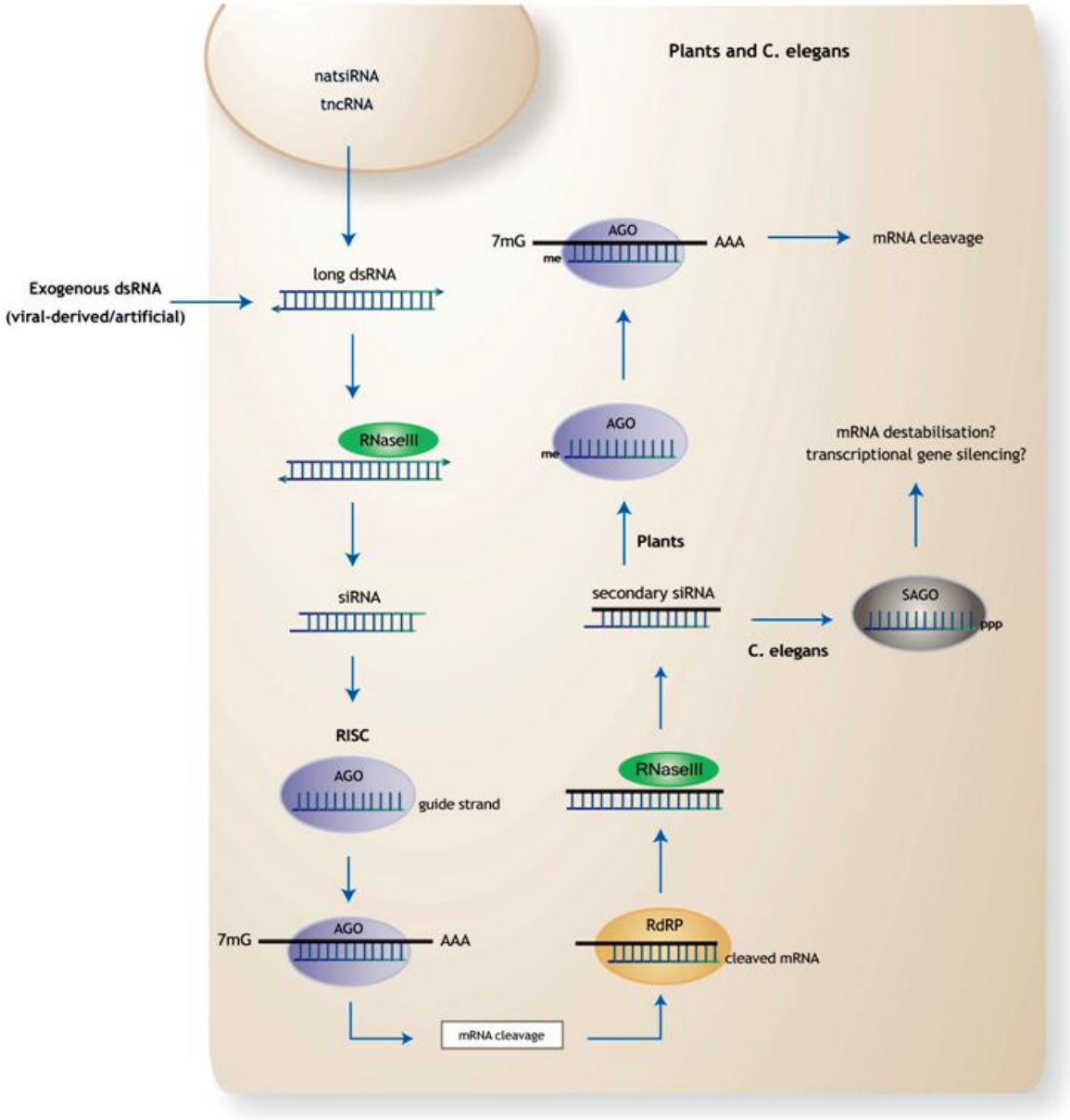


Figure 1.6 Biogenesis and functions of siRNAs in plants and C. elegans

Exogenous siRNAs are virally derived or experimentally introduced into the cytoplasm, whereas endogenous siRNAs are transcribed in the nucleus. natsiRNAs originate from convergent partially overlapping transcripts and tncRNAs are derived from non-coding, non-conserved sequences. Long dsRNA is processed into siRNA by an RNase III enzyme, Dicer in C. elegans and DCL2 in plants. The guide strand of the mature siRNA is incorporated into a RISC that contains an Argonaute protein (AGO) capable of directing target mRNA cleavage. In plants, the cleaved mRNA is converted into dsRNA by RdRP. The dsRNA is cleaved by DCL1 or DCL2 into a secondary siRNA, which induces further mRNA cleavage thus, amplifying the silencing effect. In C. elegans, secondary siRNAs have a 5' triphosphate and associate with SAGO Argonautes that lack endonuclease activity. Secondary siRNAs in C. elegans may therefore induce indirect mRNA destabilization or transcriptional gene silencing.

1.1.6 Silencing in mammalian cells

The discovery that RNAi operates in mammalian cells (Elbashir *et al.*, 2001) generated great excitement regarding potential applications in functional genomics and target validation, as well as harnessing RNAi as a therapeutic strategy to silence disease-causing genes. Whereas effective silencing in C. elegans and D. melanogaster can be achieved using long dsRNAs (Caplen *et al.*, 2000, Fire *et al.*, 1998), in mammalian systems, dsRNA of >30bp induces the INF response, which leads to non-specific translational inhibition and RNA degradation (figure 1.7). INF induces activation of RNA-dependent protein kinase (PKR) and 2', 5'-oligoadenylate synthetase, which regulate protein synthesis via phosphorylation of the α-subunit of the eukaryotic initiation factor 2α (eIF2α) and RNA degradation via activation of RNase L, respectively (Sledz & Williams, 2004, Stark *et al.*, 1998). Instead, RNAi in mammalian cells can be induced by the introduction of synthetic siRNAs and by plasmid or viral vector systems that express short-hairpin RNA (shRNA) or artificial miRNA. Target gene expression in culture can be reduced by as much as a ten-fold and the high degree of RNAi specificity has enabled the silencing of mutant genes with single nucleotide mutations, without affecting the expression of the wild-type alleles. However, two main challenges remain for the successful and safe application of RNAi *in vivo*; the design of siRNA/shRNA molecules so as to achieve optimal silencing with minimal off-target effects, and efficient delivery to target tissues.

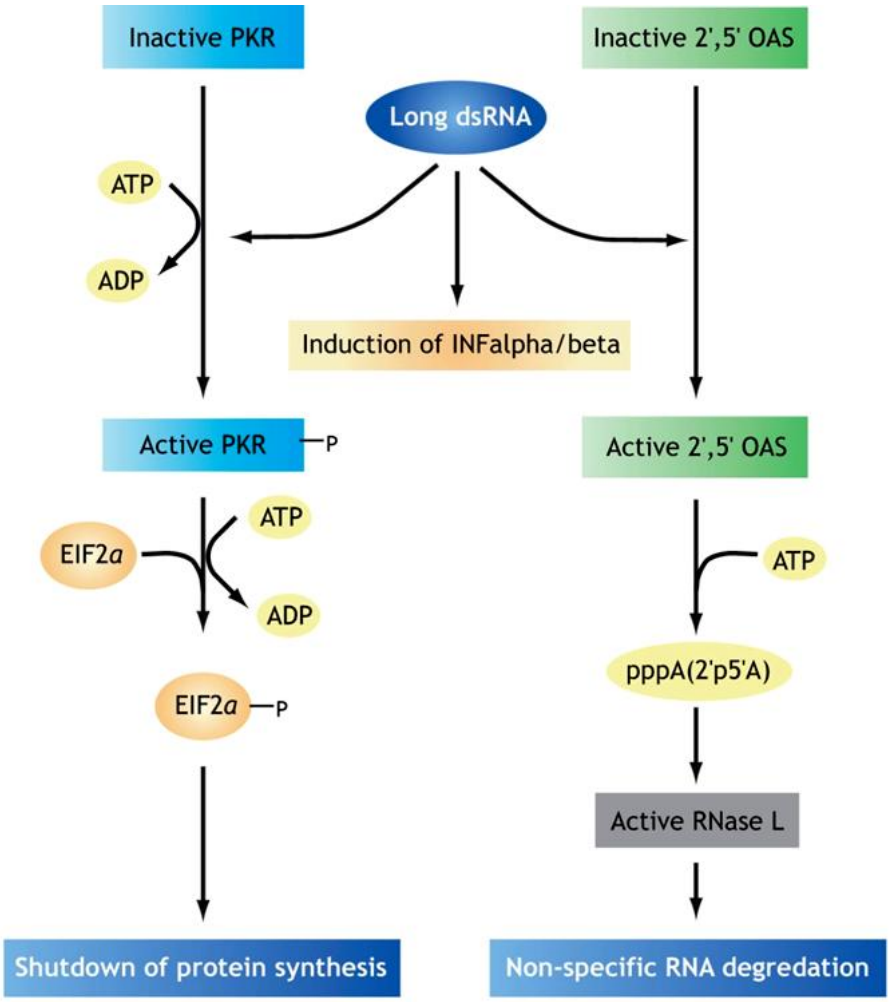


Figure 1.7 INF response induced in mammalian cells by long dsRNA

Long dsRNA induces the production of INFalpha and beta, which induce cell growth arrest and apoptosis. Activation of protein kinase PKR induces phosphorylation of the translation initiation factor EIF2a and results in inhibition of translation. Activation of 2',5' oligoadenylate synthetase (OAS) catalyses the conversion of ATP into long oligoadenylate chains (pppA(2'p5'A), which activates RNase L that degrades cellular RNA.

1.1.6.1 Silencing by synthetic siRNA

Ever since Tuschl and colleagues demonstrated that efficient gene silencing can be achieved by delivery of siRNAs to the cytoplasm of mammalian cells, chemically or enzymatically synthesised siRNAs have become a powerful tool for modulating gene expression. Enzymatically synthesised siRNAs are generated through processing of *in vitro* transcribed dsRNA into a pool of siRNAs (d-siRNA) by a recombinant Dicer (Myers and Ferrell, 2005, Myers & Ferrell, 2005). This approach eliminates the need to identify an individual effective siRNAs and is cost effective and relatively quick. However, unprocessed long dsRNA may activate the INF response and thus, gel purification of 21–23nt long siRNAs from unprocessed long dsRNAs and partially processed products is required. Moreover, competition from less effective siRNAs in a pool may reduce the overall silencing efficacy and it is more difficult to verify phenotypes arising from knockdown of target gene expression compared to when one optimal siRNA of known sequence is used.

Chemically synthesised siRNAs represent the gold standard for RNAi applications, but are considerably more expensive than enzymatically generated siRNAs. Provided some basic design rules are adhered to, synthetic siRNAs are well tolerated both *in vitro* and *in vivo*. Conventionally designed siRNAs are 21nt long with symmetric 2nt 3'overhangs (Elbashir *et al.*, 2001). However, 21-25nt duplexes with 2nt 3'overhangs (Caplen *et al.*, 2001, Elbashir *et al.*, 2002) and 21-29nt duplexes with blunt ends, symmetric or asymmetric 2nt overhangs have been shown to induce effective silencing (Kim *et al.*, 2005). Longer siRNAs are processed by Dicer that is believed to be important for efficient incorporation into the RISC, but may yield multiple 21nt long siRNAs with variable activity. Moreover, long siRNAs (>23nt) are more likely to induce the INF response, and different cell types have been shown to manifest drastically different cell viability and IFN responses to long siRNAs, suggesting that the dsRNA length threshold varies considerably amongst cell types (Reynolds *et al.*, 2006). Moreover, 5′-UGUGU-3′ motifs can induce the

INF response and the release of inflammatory cytokines through interaction with specific toll-like receptors (Judge *et al.*, 2005) and are therefore avoided, unless activation of an immune response together with siRNA-mediated gene silencing is desirable, such as in the treatment of viral infections and tumours. Modifications, such as locked nucleic acid (LNA) modifications, which add a methylene linkage between the 2′ and 4′ positions of the ribose, and 2′-O-methyl modifications into the sugar structure of selected nucleotides can abrogate the immunostimulatory activity of siRNAs (Hornung *et al.*, 2005, Judge *et al.*, 2005). However, depending on their extent and location, these modifications can also reduce or completely block the gene silencing activity of siRNAs and thus, extensive modifications are not desirable.

Different siRNAs against a target gene often manifest a spectrum of potency and may non-specifically target unrelated genes to which they anneal with partial complementary, termed off-target effects (Jackson *et al.*, 2003). Computational tools have been developed to increase the likelihood of selecting effective siRNAs and reduce potential off-target effects. Alternatively, validated siRNAs are commercially available and tested siRNA sequences can be obtained from the literature. Nevertheless, experimental validation is necessary to confirm the potency and specificity of the selected siRNAs. Reporter-based assays, which allow the target gene to be fused to a reporter gene and expressed from a plasmid vector, have been developed for rapid validation of siRNA sequences. Although downregulation of reporter activity correlates well with knockdown of target gene expression, it is necessary to test pre-validated siRNAs for their ability to silence endogenous gene expression.

Candidate siRNAs are often designed to target the coding sequence of the target gene that is generally better characterised than the 3’ or 5’ UTR. Moreover, siRNAs may be designed to target orthologs in more than one species or multiple splice variants of the target gene. Algorithms select candidate siRNA sequences based on the sequence and thermodynamic properties of functional siRNAs and in most cases, a genome-wide BLAST

search is automatically performed to identify potential similarities to other mRNAs that may be unintentionally targeted. Considerations in the design of siRNAs include strand bias (AU rich 5'end on the guide strand for sequence asymmetry), siRNA stability (low to medium GC content and low stability at positions 9-11 of the guide strand), absence of internal repeats, the accessibility of the mRNA target site, which requires prediction of mRNA secondary structure, and specificity (Chalk *et al.*, 2004, Khvorova *et al.*, 2003, Luo & Chang, 2004, Reynolds *et al.*, 2004). In addition to siRNA design, the success of RNAi-mediated gene silencing is also dependent on target gene expression and protein turnover.

Off-target effects may be caused by perfect complementarity between the central region of over half of the siRNA sequence and anywhere within the mRNA sequence, or by perfect complementarity between 6-7nt in the siRNA guide strand seed region and the 3'UTR of the mRNA (Birmingham *et al.*, 2006, Jackson *et al.*, 2003, Jackson *et al.*, 2006b, Lim *et al.*, 2005) (figure 1.8). In the latter case, siRNAs mimic miRNAs and mediate silencing by repressing translational and/or reducing mRNA stability. Although specificity can be enhanced by sequence asymmetry, introducing mismatches in the guide strand to the 3'UTR of undesired target mRNAs, 2'-O-methyl modifications in the seed region, and asymmetric 5'-O-methylation of siRNA duplexes, off-target effects cannot be completely eliminated (Holen *et al.*, 2005, Jackson *et al.*, 2003, Jackson *et al.*, 2006a, Jackson *et al.*, 2006b, Lin *et al.*, 2005). Delivery of a low dose of siRNA (≤20nM) and co-delivery of multiple siRNAs against a single target are effective strategies to minimise off-target effects (Semizarov *et al.*, 2003).

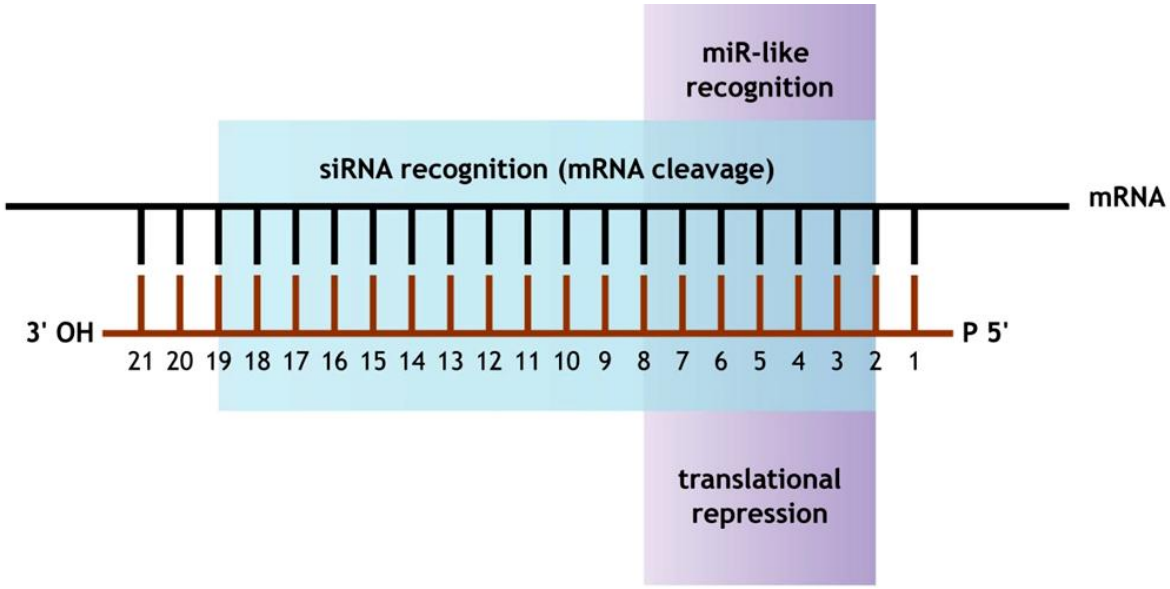


Figure 1.8 Off-target effects

Off-target activity can occur in two modes: By perfect complementarity between the central region of the siRNA sequence (blue region) and anywhere within the mRNA sequence, or by perfect complementarity between 6-7nt in the siRNA guide strand seed region (purple region) and the 3'UTR of the mRNA. In the latter case, the siRNA mimics a miRNA and is likely to mediate off-target effects by repressing translation.

Silencing by synthetic siRNAs is transient lasting for 3-7 days in dividing cells and up to several weeks in terminally differentiated cells, such as macrophages and neurons (Bartlett & Davis, 2006). Eventually siRNAs are diluted or degraded and thus, repeated administration is necessary to achieve a persistent effect. This is due to mammalian systems lacking RdRPs, which use the guide strand of the siRNA as a template to generate more siRNA duplexes, thus amplifying the silencing effect (Dillin, 2003). Moreover, siRNA duplexes are polyanionic macromolecules and their uptake by mammalian cells is generally poor. Transfection or electroporation of synthetic siRNAs is currently the most convenient method for silencing genes in mammalian cell cultures, where long-term inhibition of gene expression is not generally required. The most commonly used transfection reagents are cationic lipids, although cationic polymers and cationic cell penetrating peptides have also been used deliver siRNA in culture. A common property of these delivery agents, which aim to enhance cellular accumulation of siRNA molecules and facilitate release from endosomes, is their net positive charge that facilitates complex formation with the siRNA and interaction with the negatively charged cell membrane. However, it has been demonstrated that transfection reagents induce changes in gene expression, which may impact on siRNA activity and specificity (Hollins *et al.*, 2007). Toxicogenomic studies in A431 human epithelial cells revealed that the commonly used cationic lipids Lipofectin and Lipofectamine (Invitrogen) affect the expression of numerous genes involved in cell proliferation, differentiation and apoptosis thus, altering the expression profile of the cell and potentially interfering with the desired genotypes or phenotypes (Omidi *et al.*, 2003).

In vivo, the main challenge is efficient delivery of siRNAs to target tissues. Hydrodynamic delivery (high-pressure intravenous injection) is the most common method used for systemic siRNA delivery to organs such as liver and kidney, but is not appropriate for clinical applications (Song *et al.*, 2003). Delivery to localised regions, such as local tumours, eye, brain, spinal cord and mucus membranes, can be achieved by topical

delivery or direct injection of siRNAs. However, many tissues can only be reached by systemic administration of siRNAs in the bloodstream. Naked siRNAs have a very short half-life in blood (5-60min) due to rapid filtration by the kidney, uptake by phagocytes and degradation by serum nucleases. Moreover, egress from the bloodstream and across the vascular endothelium poses an additional challenge, as molecules larger than 5nm do not readily cross the endothelium. A variety of strategies have been employed to increase siRNA stability and facilitate siRNA delivery to specific cell types. Chemical modifications improve resistance to nucleases, coupling siRNAs to peptides or cationic polymers facilitate transport across the cell membrane, encasing them into nanoparticles or liposomes limit renal filtration, and linking them to cell surface receptor ligands target siRNAs to specific cell types.

Chemical modifications of the phosphorothioate linkage (backbone phosphate group O → S) or the boranophosphate linkage (backbone phosphate group O → BH_3) are considered a simple and effective method to increase the nuclease resistance of siRNAs (Amarzguioui *et al.*, 2003, Braasch *et al.*, 2004, Hall *et al.*, 2004). Modifications of the 2′-hydroxyl group of the pentone sugar, such as 2′-O-methyl, 2′-O-(2-methoxyethyl) and 2′-deoxy-2′floro, and LNA modifications, can also increase stability without affecting the silencing activity of siRNAs (Blidner, 2007, Dowler *et al.*, 2006, Elmén *et al.*, 2005, Prakash *et al.*, 2005). Strand bias can be influenced by chemical modifications or conjugation of siRNA and thus, either the 3′- or 5′-terminus of the sense strand is generally used.

Several small molecules and peptides have been linked to siRNAs. Most conjugates employ acid-cleavable and reducible bonds, such as ß-thio-propionate and disulfide linkages, which are cleaved in the acidic endosome compartments and the reductive cytosolic space, respectively, to facilitate release of intact siRNAs inside the cells. Cholesterol-conjugated siRNAs, which demonstrate improved stability compared to naked siRNAs, have been administered intravenously to silence apolipoprotein B (ApoB) in the mouse liver and consequently reduce cholesterol levels, without inducing an immune

response or any significant off-target effects (Soutschek *et al.*, 2004). Cholesterol-conjugated siRNAs linked to high-density lipoprotein (HDL) demonstrated five times more effective silencing of ApoB compared to unbound cholesterol-conjugated siRNAs through binding to the HDL receptor in the liver (Wolfrum *et al.*, 2007). Amongst cell penetrating peptides, the HIV-1 trans-activator protein (TAT), Penetratin and Transportan have been most commonly used to deliver siRNAs. Penetratin conjugated siRNAs were used to silence target genes, including superoxide dismutase, in primary neuronal cells without any signs of toxicity (Davidson *et al.*, 2004). However, a recent study has demonstrated that intrathecal delivery of siRNAs conjugated to Penetratin, unlike delivery of siRNAs conjugated to TAT or cholesterol, induces an immune response in mice and that siRNAs conjugated to cell penetrating peptides exhibit similar stability to naked siRNAs (Moschos *et al.*, 2007). Finally, cell-specific ligands, such as antibodies, aptamers, vitamins and hormones, have been linked to siRNAs and facilitate delivery to target tissues and cellular uptake by receptor-mediated endocytosis. Vitamin E-conjugated 27/29nt siRNAs with 2'-O-methyl and phosphorothioate linkage modifications reduced ApoB levels in the liver without inducing the INF response. Release of Vitamin E is facilitated through processing of the 27/29nt siRNA into 21nt siRNAs by Dicer (Nishina *et al.*, 2008).

Cationic peptides with an Arginine stretch can also serve as siRNA delivery vehicles. Peptide-siRNA complexes are formed through electrostatic interactions between the positively charged peptide and negatively charged siRNA. Delivery of siRNA against vascular endothelial growth actor (VEGF) using a cholesteryl oligoarginine peptide (Chol-9R) effectively inhibited tumour growth in colon adenocarcinoma (Kim *et al.*, 2006b). Moreover, the cationic polypeptide protamine, which was linked to the C-terminus of the antigen-binding region (Fab or scFv) of an antibody against the HIV-1 envelope protein gp160 or the hormone receptor ERBB2, has been used to target siRNAs to HIV-infected cells and breast cancer cells, respectively (Song *et al.*, 2005).

Cationic polymers bind to siRNAs and condense them into nanoparticles, which demonstrate reduced renal excretion and efficient delivery into cells. Amongst cationic polymers, cyclodextrin, polyethyleimine (PEI) and atelocollagen have been most commonly used to deliver siRNA (Ge *et al.*, 2004, Urban-Klein *et al.*, 2004). Intranasal administration of siRNAs complexed to PEI inhibited pulmonary influenza infection (Ge *et al.*, 2004) and atelocollagen has been used to deliver siRNAs against VEGF to various tumour models systemically and locally (Minakuchi *et al.*, 2004, Takeshita *et al.*, 2005). However, whilst cyclodextrin and atelocollagen are well tolerated (Heidel *et al.*, 2007), the use of PEI *in vivo* is hindered by its non-specific toxicity.

Polyethylene glycol (PEG) is a hydrophilic non-ionic polymer. PEG-siRNA conjugates can be complexed with cationic polymers or peptides to form colloidal nanoparticles (Kim *et al.*, 2006a). PEG-siRNA complexed with a cationic polymer forms a neutralised core surrounded by the PEG hydrophilic segments, which improve solubility. PEG-conjugation also increases siRNA stability, controls particle size, and prevents particle aggregation in the presence of serum. Moreover, PEG reduces the immunogenicity of the cationic polymer and uptake by phagocytes (Martina *et al.*, 2007). PEG-siRNAs complexed with PEI or a cationic fusogenic peptide (KALA) have successfully silenced VEGF in tumour cells. PEG-siRNA/PEI has also reduced tumour size following local or intravenous administration in mice (Kim *et al.*, 2008b, Lee *et al.*, 2007).

DOTAP (N-[1-(2,3-dioleoyloxy)]-N,N,N-trimethyl ammonium propane) and Oligofectamine (Invitrogen) were some of the first cationic lipid formulations to be used for *in vivo* delivery of siRNA and effective silencing of tumour necrosis factor receptor (TNF-R) and ß-catenin in mice (Sørensen *et al.*, 2003, Verma *et al.*, 2003). More recently, intra-vaginal delivery of siRNA against herpes simplex virus 2 (HSV-2) using Oligofectamine protected against lethal infection for up to 9 days (Palliser *et al.*, 2006). Liposomes consist of a lipid bilayer that forms a sphere with an aqueous core in which siRNAs are encased. Cationic liposomes (SNALPs) stabilised by PEG are a clinically approved delivery system

and have been successfully used to deliver siRNAs to mice and non-human primates (Morrissey *et al.*, 2005, Zimmermann *et al.*, 2006). Moreover, Geisbert and colleagues demonstrated that SNALP-mediated delivery of siRNA can protect guinea pigs against a lethal Ebola virus, whereas siRNAs delivered by PEI conferred partial protection from death (Geisbert *et al.*, 2006). However, cationic lipids have been shown to induce an immune response, in addition to potentially increasing off-target effects, as discussed earlier. Injection of siRNAs complexed with cationic lipids induced a potent cytokine response in mice that was debilitating to the organism, whilst injection of naked siRNAs or siRNAs conjugated to cholesterol had no significant effect on immune system activation (Heidel *et al.*, 2004, Hornung *et al.*, 2005, Judge *et al.*, 2005, Ma *et al.*, 2005, Soutschek *et al.*, 2004). Recently, PEG stabilised nanoparticles consisting of cationic lipids (DOTAP and cholesterol) and protamine, were used to deliver siRNAs without inducing an immune response. A ligand was attached to PEG to facilitate targeting to tumour cells (Li & Huang, 2006). Moreover, Sato and colleagues used Vitamin A-coupled liposomes to deliver siRNA against gp46 to hepatic cells and prolong survival of rats with lethal liver cirrhosis without inducing an immune response or any significant off-target effects (Sato *et al.*, 2008).

Material	Target	Target	Route	Animal	Ref
siRNA conjugates					
Cholesterol	Liver	ApoB	Intravenous	Mouse	Soutschek *et al.*, 2003
HDL-Chol	Liver	ApoB	Intravenous	Mouse	Wolfrum *et al.*, 2007
Vitamin E	Liver	ApoB	Intravenous	Mouse	Nishina *et al.*, 2008
Cell-penetrating peptides					
TAT	Lung	p38 MAP kinase	Intrathecal	Mouse	Moschos *et al.*, 2007
Penetratin	Lung	p38 MAP kinase	Intrathecal	Mouse	Moschos *et al.*, 2007
Cationic peptides					
Chol-9R	Tumour	VEGF	Intravenous	Mouse	Kim at el., 2006
Protamine-F105P	HIV- infected cells	gag	Intravenous	Mouse	Song *et al.*, 2005
Cationic polymers					
PEI	Lung	respiratory syncytial virus	Intranasal	Mouse	Ge *et al.*, 2004
PEI	Tumour	c-erbB2/neu	Intraperitoneal	Mouse	Urban-Klein *et al.*, 2005
Atelocollagen	Tumours	VEGF	Intravenous Intratumoural	Mouse	Takeshita *et al.*, 2005 Minakuchi *et al.*, 2004
PEGylated PEI	Tumours	VEGF	Intravenous Intratumoural	Mouse	Lee *et al.*, 2007; Kim *et al.*, 2008
Cationic lipids and Liposomes					
Oligofectamine	Vagina	HSV-2	Intravaginal	Mouse	Palliser *et al.*, 2006
SNALP	Ebola-infected cells	Polymerase L	Intravenous	Guinea pig	Geisbert *et al.*, 2006
SNALP	Liver	Hepatitis B virus	Intravenous	Mouse	Morrissey *et al.*, 2005
SNALP	Liver	ApoB	Intravenous	Monkey	Zimmermann *et al.*, 2006
LipoTrust	Liver	gp46	Intravenous	Rat	Sato *et al.*, 2008

Table 1.2 *In vivo* delivery of synthetic siRNA

Despite the problems associated with the *in vivo* delivery of synthetic siRNAs, several clinical trials are ongoing or planned for taking siRNA into the clinic. Advanced clinical trials for the treatment of age-related macular degeneration, which is a leading cause of blindness, and respiratory syncytial virus (RSV), which is a major cause of respiratory illness in children and infants, involve local delivery of naked siRNAs against VEGF and RSV nucleocapsid N gene to the eye and lung, respectively. These trials have reported that the siRNA doses used were well tolerated, with no adverse systemic effects. Phase I clinical trials for the treatment of hepatitis B and solid tumours involve systemic delivery of siRNAs using a cationic lipid and cyclodextrin decorated with a tumour specific ligand respectively, but results from these trials await publication. It has to be noted that a recent study has called into question the siRNA anti-angiogenic effect reported in the clinical trials for age-related macular degeneration by demonstrating that non-specific stimulation of Toll-like receptor 3 can reduce angiogenesis by downregulation of VEGF (Kleinman *et al.*, 2008).

1.1.6.2 Silencing by expression of shRNA/miRNA

In addition to introducing synthetic siRNA into the cytoplasm, silencing triggers can be expressed from vectors using either pol II or pol III promoters. The most commonly used approach involves transcription of sense and antisense 19-29bp long sequences connected by a loop of unpaired nucleotides (figure 1.9a). Expression under the control of a pol III promoter, such as the U6 snRNA or the H1 RNase P RNA promoter, results in the formation of a stable hairpin with a 3-4nt 3'overhang from the RNA pol III transcription termination that resembles an endogenous pre-miRNA (Lee *et al.*, 2002, Paul *et al.*, 2002, Yu *et al.*, 2002). Following expression in the nucleus, shRNAs are exported to the cytoplasm by Exportin 5 and are processed by Dicer to generate functional siRNAs. A variation on this theme uses a pol III-based tandem system to transcribe independently both sense and antisense strands, which following annealing of both strands, compose the siRNA duplex.

Nevertheless, it has been shown that tandem-type vectors present less silencing activity than hairpin-type vectors (Miyagishi *et al.*, 2004).

The conversion of active siRNA sequences into shRNAs can be problematic, as siRNAs do not require further processing and transport. As with the design of siRNAs, computational tools have been developed to increase the likelihood of selecting effective shRNA sequences and reduce potential off-target effects. Considerations in the design of shRNAs include, in addition to those discussed for siRNAs, no more than three consecutive U nucleotides anywhere within the hairpin or loop sequence as RNA pol III terminates at four or more Ts, five Ts at the 3′ end for efficient termination, and a G or A at position 1 of the hairpin for efficient RNA pol III transcription initiation. Another important factor is the size and sequence of the loop, which is important for successful export of pre-miRNA or shRNA from the nucleus (Brummelkamp *et al.*, 2002, Zeng & Cullen, 2003). A few artificial loops have been successfully tested, including the 5′-TTCAAGAGA-3’ loop (Pekarik *et al.*, 2003). Loops derived from naturally occurring miRNAs, such as the human miR-30 5′-CUGUGAAGCCACAGAUGGG-3′ and human miR-23 5′-CTTCCTGTCA-3′ loops, have been shown to further enhance the potency of shRNAs (Boden *et al.*, 2004, Xia *et al.*, 2004). Screening of multiple shRNA sequences is required in order to identify constructs that produce effective knockdown.

Pol II promoters, such as the cytomegalovirus immediate early (CMV IE) gene promoter, have also been used to express shRNA (Gou *et al.*, 2004, Xia *et al.*, 2004, Xia *et al.*, 2002). Some of the restrictions for expression of a functional shRNA include elimination of 5' overhang sequences and the use of a minimal polyA signal. There are several advantages to the use of pol II promoters, including tissue-specific and inducible transcription of shRNA. Although it is clear that functional siRNAs can be produced from pol II systems, given the requirement for a polyA signal at the 3' end of the transcriptional units, it remains unclear how the shRNAs are produced and which transport system is used to export them to the cytoplasm. More recently, Scherer and colleagues created tRNA-

shRNA chimeras that use the endogenous tRNA 3'-end processing endonuclease to release shRNAs that are subsequently processed into siRNAs. This system was shown to produce excellent strand selectivity (Scherer *et al.*, 2007).

As the understanding of miRNA biogenesis advanced, new generation RNAi triggers were developed (figure 1.9b). Artificial miRNAs, also known as shRNAmir, have been most commonly modelled on the well-characterised human miRNA, miR-30 (Boden *et al.*, 2004, Zeng & Cullen, 2003, Zeng *et al.*, 2002). However, vectors based on miR-155 and miR-26a, have also been described (Chung *et al.*, 2006, McManus *et al.*, 2002). The stem of the pri-miRNA, which is expressed using either pol III or pol II promoters, can be replaced with shRNA sequences against different target genes, without affecting normal miRNA maturation. The Drosha–DGCR8 complex excises the engineered stem–loops to generate intermediates that resemble endogenous pre-miRNAs, which are subsequently exported to the cytoplasm and processed by Dicer into functional miRNAs. These artificial miRNAs, which have perfect complementarity to their mRNA targets, have been demonstrated to mediate silencing by mRNA endonucleolytic cleavage rather than translational repression (Boden *et al.*, 2004, Chung *et al.*, 2006, Zeng *et al.*, 2002). The use of artificial miRNAs has become a very attractive alternative to the expression of shRNA (Boudreau *et al.*, 2008a, Silva *et al.*, 2005). Artificial miRNAs are amenable to pol II transcription and polycistronic strategies, which allow delivery of multiple shRNA sequences simultaneously and co-expression of a reporter gene or a biologically active protein together with the shRNA (Chung *et al.*, 2006). Expression of shRNA and artificial miRNA is discussed in more detail in chapter 3 of this thesis.

Several reversible and irreversible conditional systems have been developed for expression of shRNA from pol III or pol II promoters. Reversible inducible expression of shRNA has been most commonly achieved using deoxycycline-controlled cassettes (Chen *et al.*, 2003, Hosono *et al.*, 2004, Matsukura *et al.*, 2003, van de Wetering *et al.*, 2003). The E. coli Tet operator sequence (*tet*O), which is placed downstream of the TATA box, binds

to the tetracycline repressor (TetR) and represses shRNA expression. In the presence of deoxycycline, TetR no longer binds to *tet*O and expression of shRNA is initiated. However, insertion of the *tet*O sequence into the pol lll promoter results in weak expression of shRNA in the presence of deoxycycline, and this system does not guarantee complete repression of shRNA expression in the absence of the drug. To overcome these limitations, TetR has been fused to the Krüppel-associated box (KRAB) domain that is found in many zinc finger proteins and can silence promoters within 3kb of its binding site by inducing heterochromatin formation. This system, in which the *tet*O sequence was placed upstream of the H1 promoter, has allowed silencing of target genes with high efficiency in the presence of deoxycycline and suppression of shRNA expression in the presence of TetR-KRAB without any significant leakiness (Wiznerowicz & Trono, 2003). Conditional expression of artificial miRNA has also been achieved using a *tet*O-CMV pol II promoter (Dickins *et al.*, 2005). Irreversible inducible expression of shRNA has been demonstrated using Cre-mediated recombination. The U6-shRNA cassette has been inserted between two LoxP sequences. Following Cre-mediated recombination, the shRNA expression cassette is excised resulting in termination of knockdown. Alternatively, the TATA box of the U6 promoter has been replaced with a mutated LoxP-TATA site. The second LoxP-TATA site was inserted upstream of the shRNA. A sequence was inserted between the two LoxP sites to prevent transcription of shRNA. Following Cre-recombination, the inhibitory sequence is excised and the functional TATA box is restored (Tiscornia *et al.*, 2004, Ventura *et al.*, 2004).

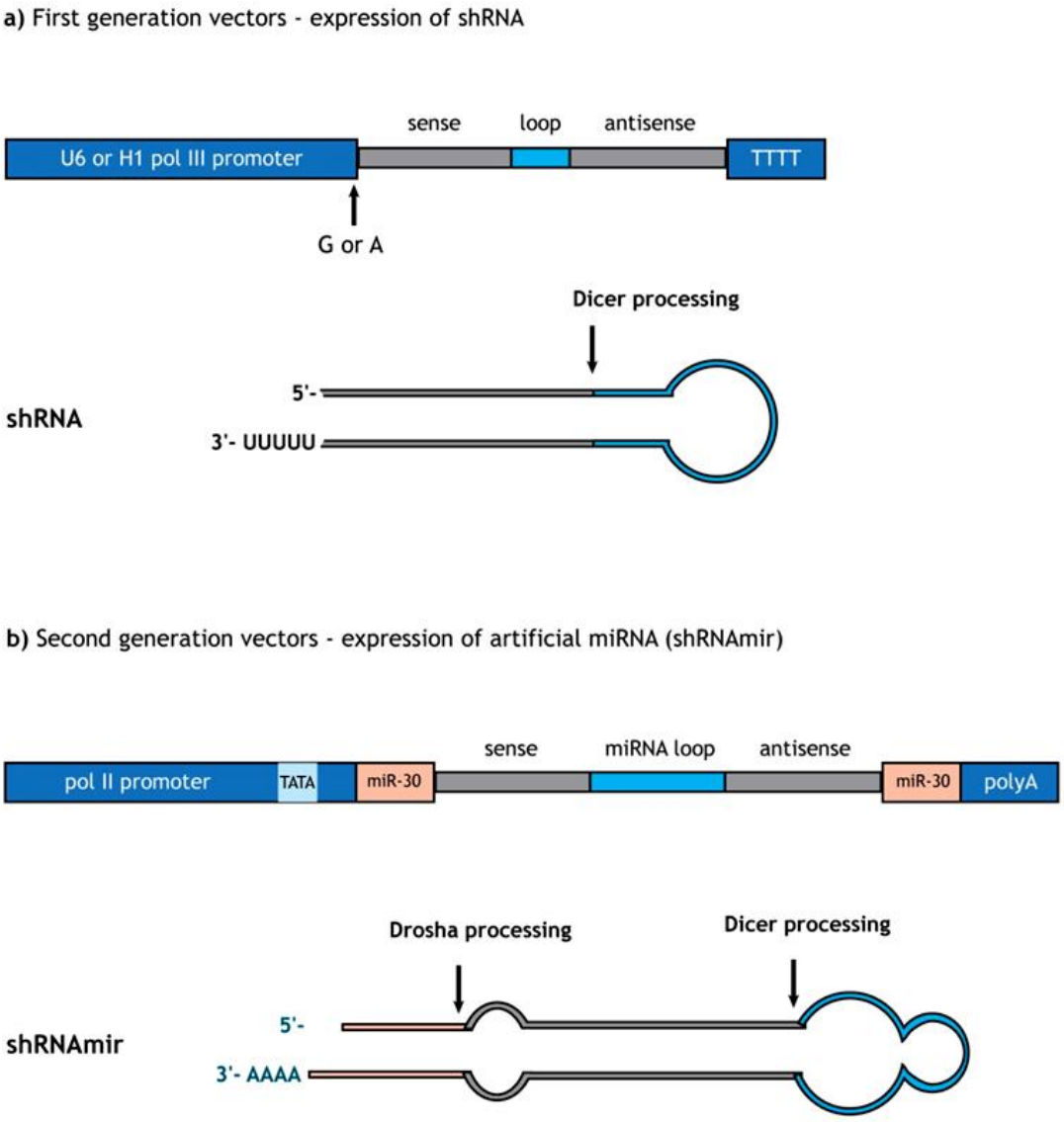


Figure 1.9 Expression of shRNA from first and second generation vectors

Expression of shRNA under the control of a pol III promoter, such as the U6 or H1 promoter, results in the formation of a stable hairpin with a 3-4nt 3'overhang from the RNA pol III transcription termination that resembles an endogenous pre-miRNA. For expression of artificial miRNA, the stem of the pri-miR-30 or pri-miR-155 transcript is replaced with the shRNA sequence, without affecting normal miRNA maturation.

Off-target effects have not been reported in systematic studies using shRNA or artificial miRNA. This is most likely due to shRNAs/miRNAs being dependent on endogenous processing by Drosha and Dicer, which are rate limiting steps in the generation of siRNAs and may therefore limit the concentration-dependent off-target effects observed with synthetic siRNAs. However, expression of shRNA has been shown to cause retraction of synapses and dendritic spines in primary hippocampal neurons by triggering the innate immune response (Alvarez *et al.*, 2006) and early embryonic lethality in zygotes that was associated with increased expression of the Oas1 interferon-induced gene (Cao *et al.*, 2005). Moreover, specific sequences capable of inducing an IFN response have been identified around the transcription start site in pol III driven shRNA expression systems (Pebernard & Iggo, 2004). Induction of the INF response gene Oas1 in primary cortical cultures by expression of shRNA was abolished by the introduction of the target sequence into a miR-30 backbone (Bauer *et al.*, 2008). Moreover, neurotoxicity in the mouse striatum caused by expression of shRNAs was significantly attenuated when these sequences were inserted in miR-30-based vectors (McBride *et al.*, 2008). This is most likely due to endogenous miRNAs having evolved to preclude the induction of dsRNA-triggered cellular immune response. Furthermore, high levels of shRNA expression, commonly achieved with pol III promoters, can lead to competition with endogenous miRNAs for limiting cellular factors, such as Exportin 5, Dicer and RISC (Castanotto *et al.*, 2007, Hutvágner, 2004, Yi *et al.*, 2005). This can result in oversaturation of the endogenous miRNA pathway, which has been shown to cause lethality in animals (Grimm *et al.*, 2006). Selection of potent shRNA sequences capable of effective silencing even when expressed at low levels and the use of promoters that mediate moderate levels of shRNA expression are an effective strategy to minimise toxicity. Unlike siRNAs and shRNAs, miRNAs, which are expressed in moderate levels and processed more efficiently than shRNAs, do not compete with the endogenous miRNA pathway and thus, have improved safety profiles (Boudreau *et al.*, 2008a, Castanotto *et al.*, 2007).

Hydrodynamic delivery of shRNA-expressing plasmids was shown to inhibit hepatitis C viral replication in the liver of mice (McCaffrey *et al.*, 2002, McCaffrey *et al.*, 2003). Moreover, transfection of a plasmid vector expressing shRNA into embryonic stem cells, followed by injection into blastocysts, has been used to generate transgenic mice (Kunath *et al.*, 2003). However, non-viral delivery of shRNA or artificial miRNA remains highly inefficient for many *in vitro* and most *in vivo* applications. Viral vectors allow highly efficient delivery of shRNA/miRNA to a wide range of mitotic and post-mitotic cells of different species origin, allow targeted delivery to specific cell types *in vivo*, and are versatile, as they allow transient transfection, stable integration, germline transmission and the creation of *in vivo* animal models. Delivery of shRNA/miRNA has been achieved using vectors based on adenovirus, adeno-associated virus, retrovirus/lentivirus and more recently, herpes simplex virus, which are discussed in detail in section 1.2 and 1.3.

1.1.6.3 Other nucleic acid-based gene silencing approaches

Interest in RNAi has rapidly overtaken interest in other nucleotide-based strategies for gene silencing, including those using antisense oligonucleotides and ribozymes. Antisense oligonucleotides are generally 20nt long and hybridize to pre-mRNA and mRNA to produce a substrate for ribonuclease H (RNase H), which specifically degrades the RNA strand of the RNA–DNA duplex. When modified in a way to prevent the action of RNase H, antisense oligonucleotides can inhibit translation of mRNA via steric hindrance, or splicing of pre-mRNAs (Kurreck, 2003). Ribozymes degrade target RNA by catalysing the hydrolysis of the phosphodiester backbone (Doudna & Cech, 2002). The hammerhead ribozyme, which is most commonly used, forms a unique secondary structure when hybridized to its target mRNA, as its catalytic region is flanked by sequences complementary to the target RNA sequences that flank the cleavage site.

Although a direct comparison between antisense oligonucleotides and siRNAs is difficult owing to their different mechanisms of action, numerous studies have concluded

that siRNAs are more potent than various types of antisense oligonucleotides (Bertrand *et al.*, 2002, Grunweller *et al.*, 2003, Kretschmer-Kazemi Far & Sczakiel, 2003, Miyagishi *et al.*, 2003). It has been estimated that antisense suppression of gene expression is approximatelly 100–1000-fold less effective than suppression by siRNAs. Moreover, antisense oligonucleotides can only be applied to downregulate the expression of certain genes. Although a systematic comparison of the gene silencing efficacy of ribozymes and siRNAs has not been performed, several experiments have indicated that siRNAs are also more effective than ribozymes (Yokota *et al.*, 2004).

1.2 VIRAL VECTORS FOR DELIVERY OF RNAi

The use of viral vectors to deliver genes and RNAi to the nervous system shows great promise for both basic research and therapeutic applications. Although many viral vectors are in common use for mainly pre-clinical studies, it is important to select the most suitable vector for each specific application. A number of factors must be considered when making this decision including the route of delivery, tropism, the duration of gene expression or silencing required, and the level of toxicity that can be tolerated.

1.2.1 Adenoviral vectors

Adenovirus (Ad) is a large (36kb), non-enveloped, double-stranded DNA virus that causes endemic and epidemic respiratory and intestinal infections in humans. Ad binds to the coxsackie adenovirus receptor and integrins on the plasma membrane and enters the cell by receptor-mediated endocytosis. Upon acidification of the endosome, the viral nucleocapsid is released into the cytoplasm and the linear DNA enters the nucleus through the nuclear pore, where it remains in an episomal form. Gene expression proceeds in a cascade fashion, with activation of early genes (E1-E4) followed by the onset of viral DNA replication and subsequently expression of late genes (L1-L5), leading to the production of infectious viral particles.

Recombinant adenoviruses infect a broad range of cell types, including neurons. Moreover, Ad vectors based on serotype 5 undergo retrograde transport from the nerve terminals of neurons to cell bodies *in vivo*. Deletion of essential E genes leads to replication-defective viruses, which can be propagated to high titres in producer cell lines that complement the deleted gene products *in trans*. First-generation replication-defective Ad vectors were constructed by deletion of the E1α and E1ß genes and deletions of varying sizes in the non-essential E3 region to allow the cloning of larger transgene expression cassettes (Akli *et al.*, 1993, Bajocchi *et al.*, 1993). These vectors have proven to have limited use in gene therapy applications due to cytopathic effects at high

multiplicities of infection (MOIs), which have been related to the expression of cellular transactivators with E1-like activity and a strong host immune response to viral antigens (Byrnes *et al.*, 1996, Yang *et al.*, 1994a). Deletion of E2α/ß in second-generation vectors and E4 in third-generation vectors resulted in decreased expression of viral proteins and a lower level of inflammatory response (Gao *et al.*, 1996, Yang *et al.*, 1994b). Retention of E3 also decreased vector antigenicity (Lee *et al.*, 1995). Gutless Ad vectors, in which all Ad genomic sequences have been deleted except for the non-coding inverted terminal repeats and packaging signal, have an increased transgene capacity (up to 30kb), diminished immune activation and the ability to confer long-term gene expression *in vivo* (Kumar-Singh & Farber, 1998, Thomas *et al.*, 2000). Moreover, the use of a cre-lox-based recombinase system allows propagation of Ad vectors to very high titres (up to 10^{13} infectious units per ml) without the need for a contaminating helper Ad virus (Hardy *et al.*, 1997). Although Ad vectors have distinctive advantages for efficient delivery to neurons both *in vivo* and *in vitro*, the high antigenicity of the virion and toxicity of the virion penton protein remain the most significant limitations for their use in long-term applications.

1.2.2 Adeno-associated viral vectors

Adeno-associated virus (AAV) is a naturally defective, small (4.7kb), single-stranded DNA virus that is known to be non-pathogenic in humans. Upon entry into the cell and release of the viral genome into the nucleus, AAV is propagated either by lytic infection in the presence of co-infecting Ad, or as a provirus by integration into the host genome. The linear AAV genome contains two open reading frames flanked by inverted terminal repeats (ITRs), which contain the minimal required *cis*-acting elements for AAV DNA replication, packaging, integration and rescue. In natural AAV infections, the virus encodes a set of replication (Rep) proteins that mediate replication of the viral DNA and facilitate site-specific integration into chromosome 19 of the host genome.

AAV infects both dividing and non-dividing cells, including neurons. Moreover, AAV-based vectors, in which all viral sequences are deleted except for the ITRs, are non-toxic, do not integrate into the host genome and facilitate long-term transgene expression (Hernandez *et al.*, 1999, McCown *et al.*, 1996). The combination of the ITRs and Rep proteins is particularly attractive, as it allows efficient, site-specific integration of inserted sequences (Balague *et al.*, 1997). The main limitations of recombinant AAV vectors are their small transgene capacity (4.5kb), which is not a critical limitation for RNAi, but may prevent expression of a biologically active protein together with shRNA, and the difficulty in propagating them to high titres. The replication and packaging of AAV-based vectors previously required the use of Ad helper virus, which was later heat-inactivated or separated by density gradient centrifugation (Ferrari *et al.*, 1996). Ad-free methods for producing high titre AAV stocks and new strategies for purification of AAV-based vectors have been described (Grimm *et al.*, 1998, Xiao *et al.*, 1998).

1.2.3 Retroviral & lentiviral vectors

Retroviruses and lentiviruses are enveloped RNA viruses. Following entry into the cell, the viral RNA is reverse transcribed into linear double-stranded DNA and transported to the nucleus, where it becomes integrated into the host genome. In retroviral/lentiviral vectors, essential viral genes are replaced by the gene of interest, which is flanked by the virus long terminal repeats (LTR) and packaging signal. Vector production utilises packaging cell lines that express the deleted viral genes from separate plasmids to minimise the possibility of generating replication-competent virus through recombination. Retroviral vectors derived from Moloney murine leukemia virus (MoMLV) have limited applications as delivery vectors to the nervous system, due to their inability to deliver genes to non-dividing cells and thus, post-mitotic neurons. They have, however, been extensively used for *ex vivo* transplantation strategies and delivery to neural precursors

and tumour cells, due to their high efficiency of transduction and long-term expression following stable integration in the host cell genome.

Lentiviral (LV) vectors, which are most commonly based on the human immuno-deficiency virus 1 (HIV-1), have a modest packaging capacity (8kb) and induce a minimal inflammatory response when introduced into the brain (Blomer *et al.*, 1997, Brooks *et al.*, 2002). The main advantage of LV vectors is their ability to produce long-term transgene expression by integration into the genome of both dividing and non-dividing cells, including neurons. Nevertheless, the consequence of stable delivery offered by LV vectors is the risk of insertional mutagenesis by activation of cellular proto-oncogenes. The risk of oncogenic transformation is less of a concern for neurons, but more for glial cells, which retain a mitotic competence. An important gain in viral safety could be achieved by directing integration to specific target sites through the use of viral integrase fused with a sequence-specific DNA-binding protein (Tan *et al.*, 2004b), or through the use of integration-defective LV vectors (Saenz *et al.*, 2004). The complex genome and replication cycle of lentiviruses have made the development of vectors and stable packaging cell lines difficult. Moreover, the restricted host range, low titres and pathogenic characteristics of HIV-1 have limited its use. Significant progress has, however, been made in generating safe and versatile vectors. HIV-based vectors pseudotyped with the vesicular stromatitus virus envelope G glycoprotein (VSV-G) show broad tropism. In addition, a three-plasmid expression system is used to minimize the possibility of generating replication-competent virus through recombination. This consists of a HIV-1 packaging plasmid, a vector plasmid containing viral integrase and promoter-driven transgenes and a plasmid expressing the surface VSV-G glycoprotein (Naldini *et al.*, 1996).

Most LV vectors incorporate the shRNA cassette between the two LTRs and also contain a pol II reporter or drug resistance gene cassette. However, LV vectors have also been developed by introducing the cassette into the 3'LTR of the viral genome, in which case following transcription and genomic integration, the proviral form contains two

sources of shRNA (Mangeot *et al.*, 2004, Tiscornia *et al.*, 2003). Knockdown mice expressing a shRNA transgene in selected tissues or in all tissues can be generated in a few months by transducing embryonic stem cells or embryos with shRNA expressing LV (Brummelkamp *et al.*, 2002, Rubinson *et al.*, 2003). This compares with several years often needed to generate knockout mice. The use of inducible promoters allows for the controlled expression of shRNA in response to an inducer, such as tetracycline (Wiznerowicz & Trono, 2003), and mice with graded degrees of gene knockdown can be produced to study subtle effects of gene expression (Hemann *et al.*, 2003). Furthermore, genome-wide libraries of retroviruses or LV expressing shRNA or artificial miRNA designed to target the complete human and mouse genomes have been generated to enable large scale RNAi screens (Berns *et al.*, 2004, Paddison *et al.*, 2004). These libraries have provided mammalian biologists for the first time with a genetic screening tool similar to that which has been used in more primitive organisms. Finally, a Phase I clinical trial that uses autologous bone marrow transplantation to treat AIDS-related lymphomas utilises a pol III promoter-shRNA cassette targeting the HIV tat and rev shared exons. The shRNA has been incorporated into an HIV-based LV vector, which in turn has been used to insert the shRNA along with two other RNA-based anti-HIV genes into blood stem cells that are subsequently infused into HIV-positive patients.

1.3 HERPES SIMPLEX VIRUS

Herpes simplex virus (HSV) has many unique features that support its development as a vector for the delivery of genes and RNAi to the nervous system. It is a highly infectious, naturally neurotrophic virus that is able to establish life-long latency in neurons following retrograde transport to the cell bodies. The HSV genome can be easily manipulated, has a high capacity to accept foreign DNA and does not integrate into the host chromosome, thus eliminating the possibility of insertional activation or inactivation of cellular genes. Whilst these aspects of the virus biology have been viewed as promising for some time, two main issues were required to be resolved for long-term application; these were the development of a vector genome which was safe and non-toxic and the development of promoters which were able to achieve long-term expression during latency. The biology of HSV-1 and the progress being made in the generation of vectors to solve these problems for neuronal gene delivery are discussed in the following sections.

1.3.1 The biology of HSV-1

HSV-1 is a member of the alphaherpesvirus subfamily and one of the most common human pathogens, infecting 40-80% of the adult population worldwide. HSV types 1 and 2 cause cold sores and genital herpes, respectively. Recurrent HSV-1 infection in the eye can result in corneal scarring and is one of the most common infectious causes of blindness in the developed world. Very rarely (approximately 1-2 cases per million per year) and primarily in immunocompromised individuals, HSV can spread to the central nervous system causing viral encephalitis.

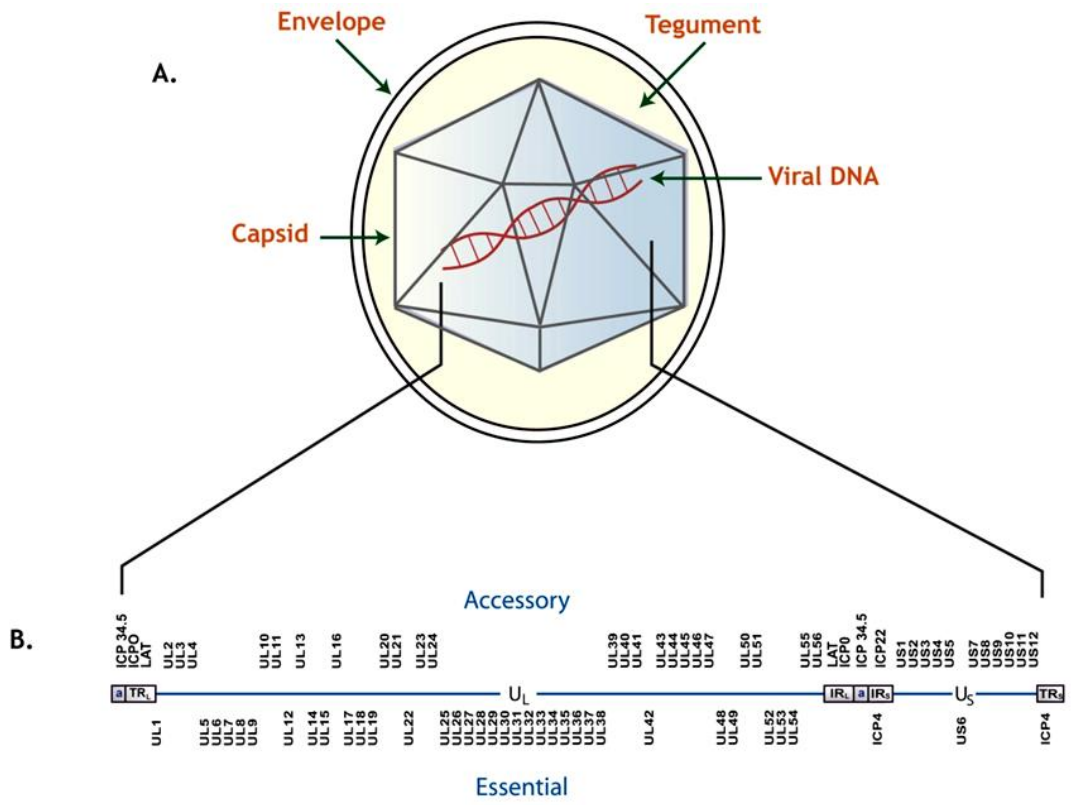


Figure 1.10 Schematic representation of the HSV-1 virion

A. The mature HSV-1 virion consists of a lipid envelope, the tegument proteins, an icosadeltahedral capsid and a core of linear, double-stranded DNA.

B. The 152kb long HSV-1 genome is arranged as unique long (UL) and short (US) regions flanked by terminal and inverted repeats (TR and IR respectively). The 'a' sequences are required for packaging of the genome into the capsid. The essential and non-essential genes are indicated.

1.3.1.1 Structure of the virion

The mature HSV-1 virion consists of a DNA core contained in an icosahedral capsid surrounded by a matrix of proteins, referred to as the tegument, and a trilaminar lipid envelope, in which are embedded at least 10 virally encoded glycoproteins (figure 1.10a). The envelope glycoproteins are involved in various aspects of the virus life cycle including attachment and entry, cell-to-cell spread, and immune evasion. The tegument proteins are responsible for initiating viral gene expression and for shutdown of host cell protein synthesis, and assist in virion maturation and egress.

1.3.1.2 The HSV-1 genome

The large HSV-1 genome consists of 152kb of linear double-stranded DNA (dsDNA) arranged as a unique long (U_L) and a unique short (U_S) region, each flanked by a pair of inverted repeats (IR) (figure 1.10b). Approximately half of the 84 viral genes expressed are essential for viral replication *in vitro*. The non-essential genes, which are involved in functions important for virus-host interactions *in vivo*, such as immune evasion and shutdown of host protein synthesis, can be deleted in the generation of vectors allowing insertion of large or multiple transgenes (approximately 30kb).

1.3.1.3 HSV-1 life cycle

The life cycle of HSV-1 *in vivo* begins in epithelial cells of the skin or mucous membrane. During primary infection, progeny virions enter sensory nerve terminals innervating the infection site, and the nucleocapsid and tegument proteins undergo retrograde axonal transport to the cell bodies in the sensory ganglia, where the viral genomes are retained in a latent state. Periodic reactivation from latency can occur spontaneously or in response to a variety of stimuli, including physical and emotional stress, hyperthermia, and UV irradiation (Sawtell & Thompson, 1992, Wagner *et al.*, 1975) and results in the production

of progeny virions that are anterogradely transported back to the nerve terminals (figure 1.11).

1.3.1.4 Viral attachment & entry

Entry of HSV-1 is a complex process involving several envelope glycoproteins and a number of different cellular receptors, and can be divided into two distinct phases: Attachment of the viral particle to the cell surface and entry of the nucleocapsid into the cell cytoplasm. Figure 1.12 summarises these processes.

Initial attachment of the viral particle to the host cell surface is mediated through binding of the envelope glycoproteins gC (Tal-Singer *et al.*, 1995) and/or gB (Herold *et al.*, 1994) to cell surface glycosaminoglycans, primarily heparan sulphate (Shieh *et al.*, 1992, WuDunn & Spear, 1989). These interactions significantly enhance infection efficiency, but are neither essential for infection, at least in culture, nor sufficient for viral entry. Deletion of gC reduces infection efficiency as much as 10-fold depending on HSV serotype and possibly on HSV strain and cell type (Cheshenko & Herold, 2002, Herold *et al.*, 1991). Deletion of both gB and gC severely reduces binding efficiency (Herold *et al.*, 1994) and abolishes infectivity partially due to the role of gB in viral entry, as discussed below. Cells deficient in heparan sulphate demonstrate significantly reduced susceptibility to HSV-1 infection, unless entry receptors are also absent, in which case susceptibility is abolished (Banfield *et al.*, 1995, Gruenheid *et al.*, 1993, Shieh *et al.*, 1992). In the absence of heparan sulphate, chondroitin sulphate may assist in viral attachment to the cell surface (Gruenheid *et al.*, 1993, Mardberg *et al.*, 2002).

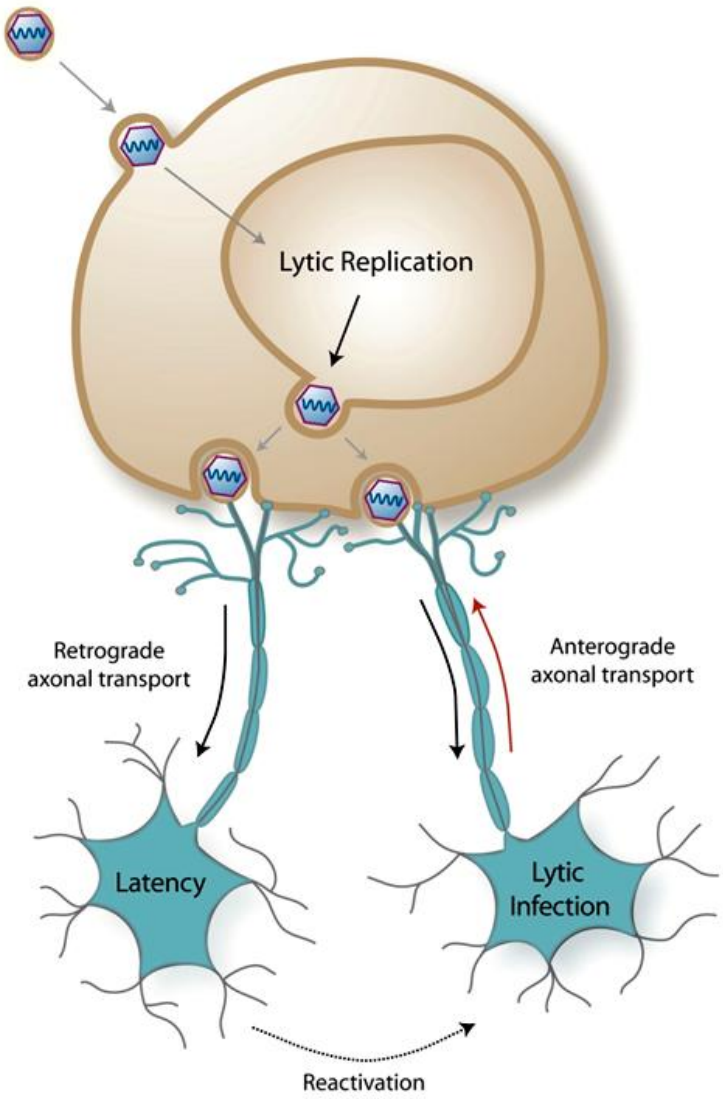


Figure 1.11 The Life Cycle of HSV-1 *in vivo*

Entry into epithelial cells is mediated through fusion of the viral envelope with the plasma membrane and release of the nucleocapsid and tegument proteins into the cytoplasm. The nucleocapsid is trasported to the nucleus in a microtubule-dependent manner and the genome is deposited into the nucleus. Following lytic replication in the infected epithelia, progeny virions are retrogradely transported to the cell bodies of sensory neurons innervating the infection site. Upon arrival to the cell body, the virus either initiates lytic expression or enters latency. Periodic reactivation from latency can occur spontaneously or in response to a variety of stimuli, including physical and emotional stress, hyperthermia, and UV irradiation, and results in the production of progeny virions that are anterogradely transported back to the nerve terminals.

Binding of the virus to the cell surface is followed by attachment of the envelope glycoprotein gD to an entry receptor. A number of such receptors have been identified including the herpes virus entry mediator A (HveA) and nectin-1 (HveC), which are members of the TNFα p75 receptor family (Montgomery *et al.*, 1996) and immunoglobulin superfamily (Geraghty *et al.*, 1998), respectively. These molecules are expressed in a variety of tissues and cell types, and account for the broad host range of the virus. HveC is expressed in epithelial cells and nearly all neurons in the peripheral and central nervous system (Haarr *et al.*, 2001). In the absence of any other entry receptors, resistant cells expressing heparan sulphate can be rendered susceptible to HSV-1 by expression of 3-O-sulphotransferases, which generate sites in heparan sulphate that can serve as receptors for gD (Shukla *et al.*, 1999).

Entry into the cell is mediated through pH-independent fusion of the viral envelope with the plasma membrane. At least four glycoproteins (gB, gD, gH and gL) are required for penetration (Cai *et al.*, 1988, Fuller & Spear, 1985, Roop *et al.*, 1993). Glycoprotein gL has been shown to form a heterodimer with gH (Hutchinson *et al.*, 1992, Peng *et al.*, 1998). A lipid-raft-associated receptor has been proposed to interact with gB (Bender *et al.*, 2005, Bender *et al.*, 2003), and integrin αVß3 has been shown to bind directly to gH-gL heterodimers (Parry *et al.*, 2005). Although the exact mechanism remains to be elucidated, it has been suggested that interaction of gD with cell surface receptors triggers the fusion machinery, and an activation signal is transmitted to gB and gH-gL resulting in conformational changes in these proteins that lead to membrane fusion (Fuller & Lee, 1992). Fusion at the plasma membrane is observed in the majority of cell types including sensory neurons. Recent studies, however, have demonstrated that HSV entry into cultured cells can also proceed via pH-independent or pH-dependent endocytosis (Milne *et al.*, 2005, Nicola *et al.*, 2005).

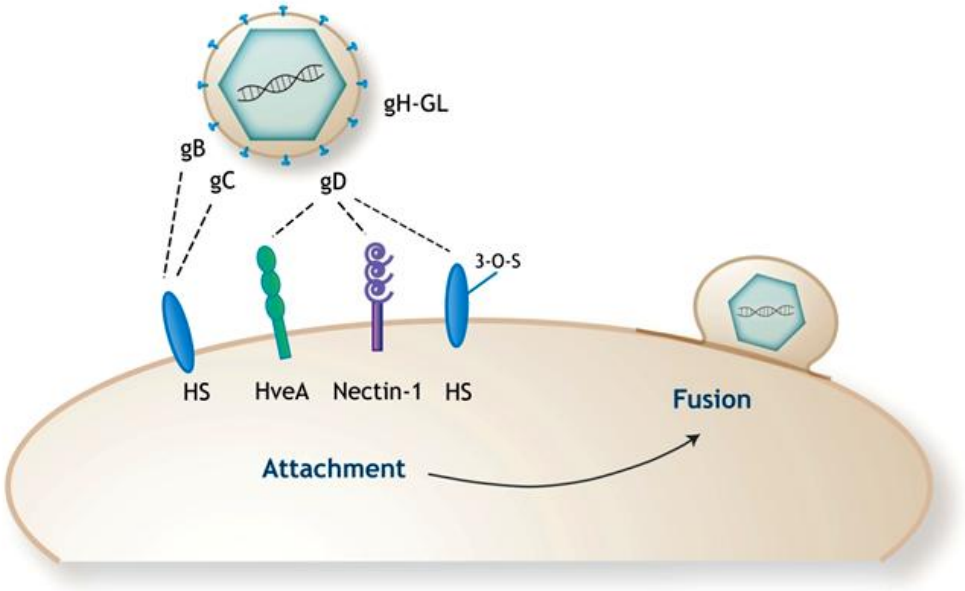


Figure 1.12 HSV-1 attachment and entry

Initial attachment of the viral particle to the host cell surface is mediated through binding of the envelope glycoproteins gC and/or gB to cell surface glycosaminoglycans, primarily heparan sulphate (HS). Binding of the virus to the cell surface is followed by attachment of the envelope glycoprotein gD to an entry receptor, including the herpes virus entry mediator A (HveA) and nectin-1 (HveC).). In the absence of any other entry receptors, resistant cells expressing heparan sulphate can be rendered susceptible to HSV-1 by expression of 3-O-sulphotransferases, which generate sites in heparan sulphate that can serve as receptors for gD. Entry into the cell is mediated through pH-independent fusion of the viral envelope with the plasma membrane. At least four glycoproteins (gB, gD, gH and gL) are required for penetration. It has been suggested that interaction of gD with cell surface receptors triggers the fusion machinery, and an activation signal is transmitted to gB and gH-gL resulting in conformational changes in these proteins that lead to membrane fusion

Following entry into the cell, the nucleocapsid and tegument proteins are released into the cytoplasm, where the tegument protein UL41 (vhs) remains and mediates shutdown of host cell protein synthesis through degradation of host and viral mRNA (Kwong & Frenkel, 1989). The nucleocapsid and some tegument proteins are transported to the nuclear pore and the genome is released into the nucleoplasm. Transport across the cytoplasm occurs in a microtubule-dependent manner. Nucleocapsids are retrogradely transported to the nucleus via a mechanism that involves interactions between the tegument protein UL34 and cellular dynein (Sodeik *et al.*, 1997, Ye *et al.*, 2000) and are anterogradely transported to the nerve terminals via a mechanism that involves interaction of US11 with kinesins (Diefenbach *et al.*, 2002).

1.3.1.5 Lytic Infection

Viral gene expression during lytic infection proceeds in a tightly regulated, interdependent cascade, in which three classes of viral genes are temporally expressed: immediate early (IE), early (E) and late (L) genes (Honess & Roizman, 1974). Expression of IE genes begins shortly after the genome arrives in the nucleus in the absence of de novo viral protein synthesis. This is followed by expression of E genes, which mainly encode proteins involved in viral DNA synthesis and replication. Expression of L genes, which mainly encode structural proteins, follows the onset of DNA replication.

Expression of IE genes is initiated by the tegument protein VP16 (also called Vmw65), which is synthesized late in infection. VP16 has a well-characterized acidic C-terminal activator domain and activates transcription by binding to the TAATGARAT elements present just upstream of the TATA box in all IE gene promoters (Sadowski *et al.*, 1988). VP16 forms a complex with the host cell factor (HCF) and localizes to the nucleus (La Boissière *et al.*, 1999), where it binds to the host transcription factor octamer binding protein 1 (Oct-1). The POU domain of Oct-1 binds to the TAAT region, and VP16 binds to the GARAT region of the TAATGARAT element (where R is a purine) (figure 1.13). This

enables the activator domain of VP16 to interact with the host transcription factor IID (TFIID) and assist in the recruitment of the host RNA pol II transcription machinery into the close proximity of IE promoters to initiate transcription of IE genes (Gerster & Roeder, 1988, Klemm *et al.*, 1995, O'Hare *et al.*, 1988). Other studies suggest that VP16 interacts with TFIIA (Kobayashi *et al.*, 1995, Kobayashi *et al.*, 1998) or TFIIB (Lin and Green, 19991; Choy and Green, 1993) to enhance formation of transcription pre-initiation complexes.

Whereas the transactivating function of VP16 is not essential for viral replication and assembly, certain mutations in VP16 prevent the production of infectious progeny virions, indicating that VP16 is required for virus assembly and egress (Ace *et al.*, 1989, Weinheimer *et al.*, 1992). Furthermore, there is evidence to suggest that VP16 binds to the vhs protein and downregulates its activity at intermediate and late times post-infection, thereby allowing the maintenance of viral protein synthesis (Lam *et al.*, 1996, Mossman *et al.*, 2000).

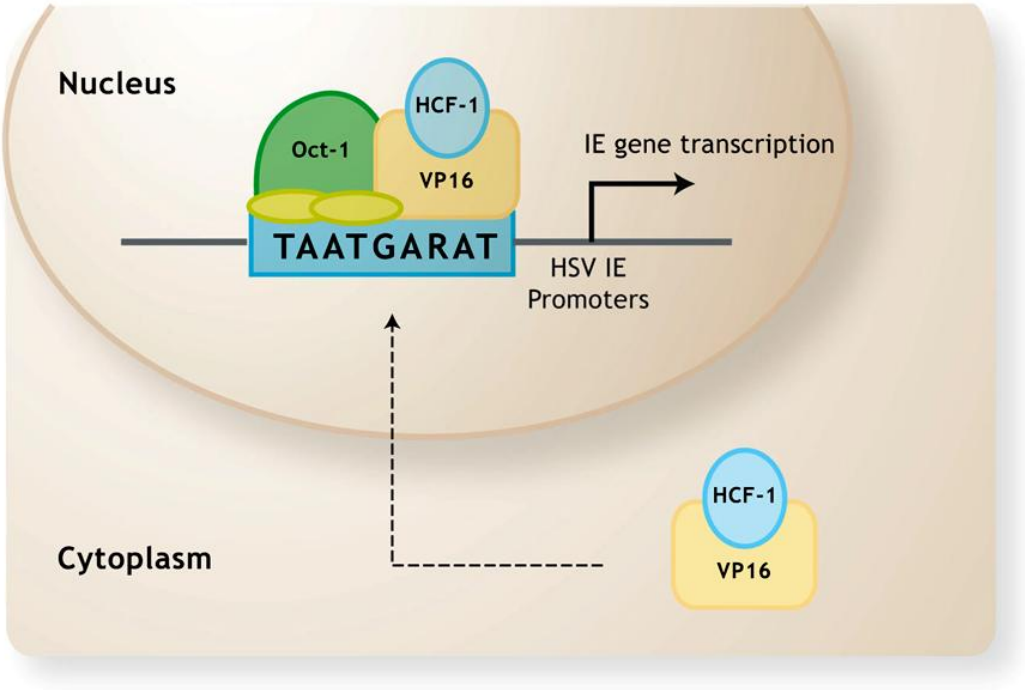


Figure 1.13 The VP16 induced complex

The tegument protein VP16 has a well-characterized acidic C-terminal activator domain and activates transcription by binding to the TAATGARAT elements present just upstream of the TATA box in all IE gene promoters. VP16 forms a complex with the host cell factor (HCF) and localizes to the nucleus, where it binds to the host transcription factor octamer binding protein 1 (Oct-1). The POU domain of Oct-1 binds to the TAAT region and VP16 binds to the GARAT region of the TAATGARAT element (where R is a purine). This enables the activator domain of VP16 to interact with the host transcription factor IID (TFIID) and assist in the recruitment of the host RNA pol II transcription machinery into the close proximity of IE promoters to initiate transcription of IE genes.

The virus encodes five IE proteins, designated infected cell polypeptides (ICP) 0, 4, 22, 27 and 47. With the exception of ICP47, these nuclear phosphoproteins are known to regulate the coordinated expression of the HSV genome. Only ICP4 and ICP27 are essential for virus replication (figure 1.14).

The RL2 gene encoding ICP0 (Vmw110) is located in the IR sequences flanking the U_L regions and is therefore present in two copies per genome. ICP0 is a promiscuous transactivator of all three classes of viral genes, non-viral genes in transient transfection assays (Everett, 1984, O'Hare & Hayward, 1985a), and a subset of cellular genes (Hobbs & DeLuca, 1999). ICP4 has been shown to interact with ICP0 to enhance this transactivation (Everett, 1984, Gelman & Silverstein, 1985). ICP0 enhances viral gene expression and growth both *in vitro* and *in vivo* (Cai & Schaffer, 1992, Chen & Silverstein, 1992) and thus, mutants lacking ICPO are replication competent, but grow very poorly at a low multiplicity of infection (Everett, 1989, Sacks & Schaffer, 1987, Stow & Stow, 1986). ICP0 does not bind DNA directly, but has been shown to interact with components of the translation (translation elongation factor EF-1δ (Kawaguchi *et al.*, 1997a)), transcription (transcription factor BML1 (Kawaguchi *et al.*, 2001)), cell cycle (cyclin D3 (Kawaguchi *et al.*, 1997b)) and proteolytic (ubiquitin-specific protease USP7 (Meredith *et al.*, 1995)) machinery. Newly synthesized ICP0 initially accumulates at nuclear structures known as ND10. ICP0 contains a short sequence similar to the N-terminus of CoREST, a co-repressor that forms complexes with the repressor REST and histone deacetylases (HDACs) 1 or 2 to repress cellular gene expression. It has been therefore proposed that co-localization of the virus genome and ICP0 at the ND10 structures serves to dissociate the HDAC1/2 from the CoREST/REST repressor complex, thereby blocking silencing of gene expression, and to disrupt ND10 structures and degrade its components, thereby blocking the IFN-mediated host response to infection (Gu *et al.*, 2005). Finally, ICP0 is required for efficient reactivation from latency, which will be discussed in more detail in the following section.

ICP4 is a major activator of E and L gene transcription (DeLuca and Schaffer, 1985), a repressor of ICP0 and its own transcription (O'Hare & Hayward, 1985b) and consequently necessary for the transition from the IE to the E phase of viral gene expression (Dixon & Schaffer, 1980). ICP4 exists as a homodimer (Metzler et al, 1985) and possesses two transactivation regions and a DNA-binding domain, which recognizes the degenerate RTCGTCNNYNYSG consensus sequence (where R is purine, Y is pyrimidine, S is C or G, and N is any base) (DiDonato *et al.*, 1991). While repression is mediated by high-affinity ICP4 binding sites when specifically positioned (Gu *et al.*, 1995, Kuddus *et al.*, 1995, Michael & Roizman, 1993), activation does not require any single site or collection of sites (Gu & DeLuca, 1994, Smiley *et al.*, 1992). ICP4 can form tripartite complexes on DNA with TFIIB and TFIID (Smith *et al.*, 1993). It has been suggested that the C-terminal region of ICP4 interacts with TFIID and TBP-associated factors to facilitate the binding of TFIID to the TATA box, which in turn facilitates the formation of transcription pre-initiation complexes (Carrozza & DeLuca, 1996, Grondin & DeLuca, 2000).

ICP27 functions primarily at the post-transcriptional level, but has also been proposed to have a role in translation initiation (Ellison *et al.*, 2005, Fontaine-Rodriguez *et al.*, 2004). ICP27 contributes to shutoff of host cell protein synthesis by inhibiting pre-mRNA splicing at early stages of infection (Hardy & Sandri-Goldin, 1994). Although the exact mechanism has not been fully defined, it has been suggested that ICP27 interacts with several splicing factors and affects their phosphorylation, resulting in inhibition of spliceosome assembly (Bryant *et al.*, 2001, Sciabica *et al.*, 2003). Later in infection, ICP27 begins to shuttle between the nucleus and cytoplasm and acts as a chaperone of viral mRNA across the nuclear membrane (Mears & Rice, 1998, Soliman *et al.*, 1997). It has been suggested that ICP27, which binds specifically to intronless mRNA (Sandri-Goldin, 1998), facilitates export of viral mRNA to the cytoplasm by interacting with the cellular adaptor protein Aly/REF and the cellular receptor TAP/NXF (Chen *et al.*, 2005). ICP27 is required for efficient DNA replication by promoting E gene expression and for the

transition from the E to the L phase of viral gene expression by activating L genes, whilst repressing IE and E gene expression (Rice & Lam, 1994, Uprichard & Knipe, 1996). It has also been suggested to modulate the activity of ICPO and ICP4, as well as the modification state of ICP4 (Sekulovich *et al.*, 1988, Su & Knipe, 1989).

ICP22 is not essential for growth in most cell types (Post & Roizman, 1981), but promotes efficient L gene expression in a cell-type specific manner (Sears *et al.*, 1985). ICP22 enables optimal expression of a subset of L genes (γ2), which depends on viral DNA synthesis for its expression, by mediating the stabilization of cyclin-dependent kinase cdc2 and the replacement of its cellular partner, cyclin B, with the viral DNA polymerase processivity factor U_L42 (Advani *et al.*, 2001). The cdc2-U_L42 complex recruits and activates topoisomerase IIα, which is one of the key enzymes required for viral DNA synthesis (Advani *et al.*, 2003). ICP22 is also involved in the production of a novel phosphorylated form of RNA polymerase II (Rice *et al.*, 1995) and regulates the stability and splicing of ICP0 mRNA (Carter & Roizman, 1996).

Finally, ICP47 blocks the presentation of antigenic peptides on the surface of infected cells to CD8+ T cells (cytotoxic T cells) and thus, helps the virus to escape immune surveillance (York *et al.*, 1994). ICP47 binds to and precludes transporters of antigen processing TAP1/TAP2 from transporting antigenic peptides into the endoplasmic reticulum, where they are loaded onto the major histocompatibility complex class I (MHC class I) for eventual presentation on the cell surface (Früh *et al.*, 1995, Hill *et al.*, 1995).

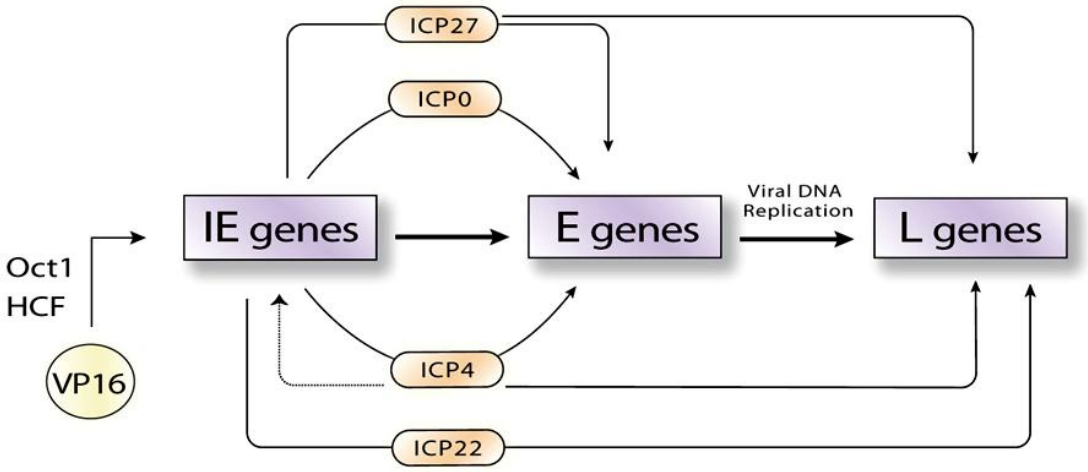


Figure 1.14 HSV-1 gene expression and regulation

The tegument protein VP16 interacts with Oct1 and HCF to transactivate IE gene promoters. Expression of IE genes is followed by expression of E genes, viral DNA replication and subsequently, expression of L genes. With the exception of ICP47, IE gene proteins regulate the coordinated expression of the HSV genome, but only ICP4 and ICP27 are essential for virus replication. ICP4 and ICP27 regulate expression of both E and L genes. ICP4 has been shown to interact with ICP0 to enhance this transactivation and is also a repressor of ICP0 and its own transcription. ICP27 contributes to shutoff of host cell protein synthesis by inhibiting pre-mRNA splicing at early stages of infection. Later in infection, ICP27 begins to shuttle between the nucleus and cytoplasm and acts as a chaperone of viral mRNA across the nuclear membrane. ICP0 and ICP22 regulate E and L gene expression, respectively. ICP0 is a promiscuous transactivator of viral and non-viral genes. ICP22 enables optimal expression of a subset of L genes by mediating the stabilization of cyclin-dependent kinase cdc2 and the replacement of its cellular partner, cyclin B, with the viral DNA polymerase processivity factor UL42.

According to the classical model of HSV-1 replication, circularization of viral genomes occurs before the onset of productive infection and thus, HSV-1 genomes replicate initially by a theta mechanism. This model has been recently challenged by Jackson and DeLuca (2003), who have demonstrated that circularization occurs during the establishment of latency, rather than early in productive infection. ICP0 inhibits the formation of circular genomes suggesting a mechanism for the establishment of latency (Jackson & DeLuca, 2003). Viral DNA replication proceeds via a rolling circle mechanism that leads to the production of HSV-1 genome concatemers (Jacob *et al.*, 1979). These are cleaved into genome-length units and packaged into the capsid through recognition of packaging sequences located in the IR. Nucleocapsids acquire tegument proteins and an envelope during budding through the inner nuclear membrane. Extracellular virions are produced by de-envelopment at the outer nuclear membrane followed by budding into the Golgi apparatus (Granzow *et al.*, 2001).

1.3.1.6 HSV-1 Latency

HSV latency is classically defined as the retention of a complete viral genome without the production of infectious virions. During latency, the viral genome is maintained in a stable episomal form and persists sometimes for the lifetime of the host in the absence of detectable infection.

The popular concept that HSV-1 latency is characterized by a complete lack of lytic gene expression has been called into question by several recent studies that have detected limited expression of ICP4, ICP8 and TK in mouse sensory ganglia (Feldman *et al.*, 2002, Kramer & Coen, 1995). It has been therefore suggested that the latent viral genome may exist at different stages of reactivation, but the factors that determine whether initiation of reactivation will lead to virion formation and emergence from latency are largely unknown.

During latency, whilst most viral gene expression is silenced, a single region within the long repeat remains transcriptionally active generating a population of RNA species designated latency-associated transcripts (LATs) (Stevens *et al.*, 1987) (Figure 1.15). The 8.3kb polyadenylated primary LAT transcribed from this region is spliced to give a non-polyadenylated 2kb LAT, which can be further spliced to produce smaller LATs (Wechsler *et al.*, 1988). The 2kb and 1.5kb LATs are abundant and accumulate in the nucleus as stable lariat introns (Farrell *et al.*, 1991). LAT expression is driven by two promoters, referred to as latency active promoters 1 (LAP1) and 2 (LAP2). LAP1 is located 660bp upstream of the 2kb LAT and contains a TATA box (Zwaagstra *et al.*, 1991). Although LAP1 is active in most cell types, it shows enhanced activity in neurons (Batchelor & O'Hare, 1992, Zwaagstra *et al.*, 1990), and activating transcription factor (ATF)/ cAMP response element (CRE) sites have been suggested to play a role in conferring neuronal specificity (Kenny *et al.*, 1994). LAP2 lies directly 3' to LAP1 and 257-58bp upstream of the 2kb LAT, and lacks a TATA box, but is a GC-rich promoter homologous to mammalian housekeeping gene promoters (Goins *et al.*, 1994). Deletion analysis suggests that LAP1 is primarily responsible for LAT expression during latency, whereas LAP2 is primarily responsible for LAT expression during lytic infection (Chen *et al.*, 1995). Although both promoters may be able to function independently, there is evidence to suggest that LAP2 contains elements required to drive long-term expression from LAP1 during latency (Lokensgard *et al.*, 1997).

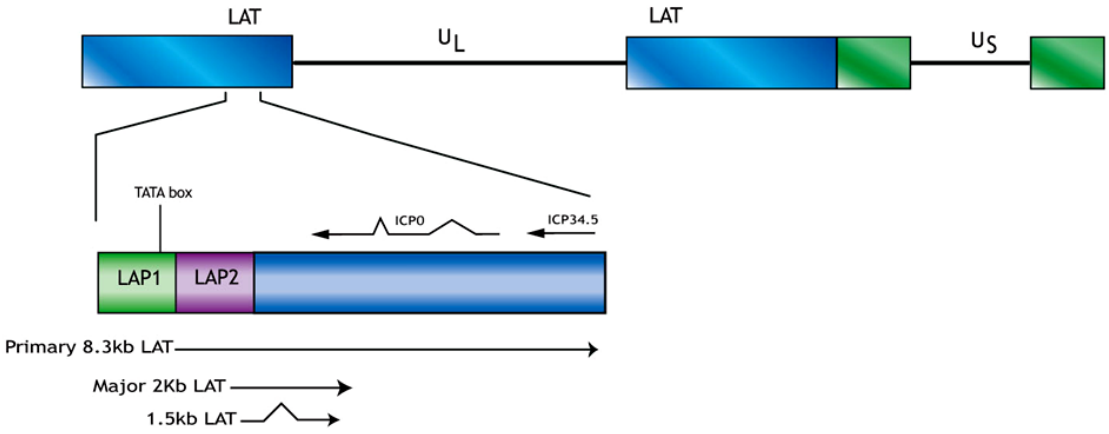


Figure 1.15 The HSV-1 LAT region

The LAT region is located within the long repeats of the HSV-1 genome and is the only region that remains transcriptionally active during latency generating a population of RNA species, termed latency associated transcripts (LATs). The 2kb and 1.5kb LATs are the most abundant. The last 723bp of the 2kb LAT are complementary to the 3' end of ICP0. LAT expression is driven by the LAP1 and LAP2 promoters. The LAP1 TATA box is located 736bp upstream of the 5' end of the 2kb LAT. LAP2 lies directly 3' to LAP1 and lacks a TATA box, but it is a GC-rich promoter homologous to mammalian housekeeping gene promoters. There is evidence to suggest that LAP2 contains elements required to drive long-term expression from LAP1 during latency.

Although the functions of the LATs remain largely unknown, it is clear that they are not essential for the establishment and maintenance of latency, or reactivation from latency (Ho & Mocarski, 1989, Javier *et al.*, 1988). Evidence suggests, however, that the LATs regulate the efficiency of each of these processes by aiding the prevention of apoptosis in infected neurons, regulating IE gene expression by an antisense mechanism, and by encoding a protein which may be important in the initiation of the reactivation from latency. A key problem for studies in each of these areas is the paucity of available animal models, none of which truly reflects the situation in humans. Thus, caution needs to be applied to the conclusions derived from any *in vivo* results, which are so far all generated in mice and rabbits.

Several studies of LAT-null mutants suggest that LATs are required for efficient reactivation from latency (Bloom *et al.*, 1996, Hill *et al.*, 1996, Perng *et al.*, 1994, Trousdale *et al.*, 1991). The region associated with decreased reactivation in these animal models has been mapped to a 385bp sequence in the 5'end of the 8.5kb LAT (Bloom *et al.*, 1996, Hill *et al.*, 1996). Thompson and Sawtell, however, demonstrated that LATs increase the percentage of neurons in which latency is established in mice, thereby increasing reactivation by increasing the pool of latently infected neurons available for reactivation, while no direct role in reactivation could be demonstrated (Thompson & Sawtell, 1997). Thus, LATs may function directly to promote reactivation and/or enhance the establishment of latency.

Since the primary LAT is transcribed antisense and complementary to ICP0, it has been suggested that LATs may aid the establishment and maintenance of latency by suppressing expression of ICP0. The 2kb LAT has been shown to inhibit transactivation of gene expression by ICP0 in transient transfection assays (Farrell *et al.*, 1991), and elevated levels of ICP0 mRNA were observed in cultures infected with mutants unable to produce 2kb LAT (Arthur *et al.*, 1998). Furthermore, LATs have been shown to suppress viral replication in a neuronal cell line by reducing the mRNA levels of ICP0 and to a

smaller extent, ICP4 and ICP27 mRNA levels, possibly due to reduced ICP0 expression (Mador *et al.*, 1998). Nevertheless, the 1.5kb region required for wild type levels of reactivation in rabbits does not overlap ICP0 (Perng *et al.*, 1996), and insertion of poly(A) at various sites within the 3'end of LAT, which terminates transcription, has no effect on reactivation (Bloom *et al.*, 1996). Thus, LATs appear to enhance reactivation, at least in the rabbit, by a mechanism that does not involve antisense regulation of ICP0.

Evidence from studies performed in our laboratory suggests that the LAT region encodes a protein product (Thomas *et al.*, 1999a, Thomas *et al.*, 2002). A large 2kb LAT-contained ORF, which is conserved between different strains of HSV-1, was shown to encode a protein that can profoundly enhance wild-type virus growth and compensate for deficiencies in ICP0 expression when expressed *in vitro*. Thus, whilst LAT expression may function to provide an antisense effect to ICP0 during the establishment and maintenance of latency, during reactivation and in response to as yet unidentified stimuli, translation of the LAT ORF may become possible, thereby relieving the ICP0 antisense effect of the LATs during latency and aiding the reactivation process.

Several studies have concluded that LAT repress ICP4 expression (Chen *et al.*, 1997, Garber *et al.*, 1997, Mador *et al.*, 1998) and inhibit apoptosis (Branco & Fraser, 2005, Jin *et al.*, 2003, Perng *et al.*, 2000). It has been suggested that the LATs may function as non-coding RNAs, as recent studies predict that HSV-1 encodes eight miRNAs, three of which are located in the 8.3kb LAT region (Pfeffer *et al.*, 2004), and one miRNA is transcribed approximately 450bp upstream of the LAT transcription start site (Cui *et al.*, 2006). A miRNA encoded from exon 1 of the LAT gene was reported to accumulate in neuroblastoma and non-neuronal cells transfected with LAT or infected with wild-type HSV-1 (Gupta *et al.*, 2006). The authors suggested that this miRNA (miR-LAT) inhibits apoptosis by downregulation of transforming factor ß 1 (TGFß1). However, although it is clear that HSV-1 downregulates TGFß1, the study by Cupta et al. was retracted from Nature in 2008 (Gupta *et al.*, 2008), as further experiments from several other

laboratories failed to detect miR-LAT, but detected a neuroblastoma encoded miRNA instead. Finally, two small RNAs within the first 1.5kb of LAT have been recently detected, and although they are not mature miRNAs, they have the potential to base pare with ICP4 mRNA and may play role in latency reactivation by inhibiting apoptosis or reducing ICP4 expression (Peng et al., 2008). It has to be noted that no HSV-encoded miRNA has as yet been shown to be expressed during latency.

When human fibroblasts are infected with mutants defective in IE gene expression, the majority of viral genomes are retained in a quiescent state that resembles latency (Preston *et al.*, 1997, Samaniego *et al.*, 1998). Furthermore, VP16 mutants (in814) that are unable to form a complex with HCF-1 and Oct-1 establish latency in mice as efficiently as wild-type virus (Ace *et al.*, 1989, Steiner *et al.*, 1990, Valyi-Nagy *et al.*, 1991). These finding have lead to the hypothesis that the block to replication occurs before or at the level of IE gene expression and that establishment of latency in neurons may be caused by the absence of functional VP16-induced complex. Roizman and colleagues suggested that VP16 may not be capable of translocation to the cell body of sensory neurons. Expression of VP16 in neurons, however, failed to affect the ability of the virus to establish latency (Sears *et al.*, 1991). It has also been hypothesized that splice variants of Oct-2, which are unable to interact with VP16, repress IE gene expression by binding to TAATGARAT sites and blocking Oct-1 binding (Kemp *et al.*, 1990, Lillycrop *et al.*, 1991). Another study, however, failed to detect Oct-2 in sensory neurons, and overexpression of Oct-2 failed to suppress activation of an IE gene promoter (Hagmann *et al.*, 1995). It has also been suggested that Oct-1 may not be expressed in sensory neurons (Valyi-Nagy *et al.*, 1991). However, it was later demonstrated that Oct-1 is present at low levels (Hagmann *et al.*, 1995). Finally, it has been shown that HCF-1, which is localized to the nucleus in most cell types, accumulates in the cytoplasm of sensory neurons (Kristie *et al.*, 1999) and consequently, although VP16 translocates to the cell body, it may fail to localize to the nucleus.

Other proposed contributors to the establishment of latency are the partially overlapping ORF O and ORF P, which are located in the 3'end of the primary LAT, almost entirely antisense to ICP34.5. The transcription initiation site for both ORFs resides within a high-affinity ICP4 binding site. Hence, they are only expressed in the absence of functional ICP4 and their expression is completely repressed during productive infection (Lagunoff & Roizman, 1994, Lagunoff & Roizman, 1995, Randall *et al.*, 1997). Furthermore, ORF O and P interfere with the expression of multiple IE genes required for lytic gene expression and thus, appear to be ideal candidates for the regulation of latency. ORF P has been shown to inhibit expression of ICP0 and ICP22 by binding to splicing factors and interfering with mRNA splicing, and its transcription precludes the expression of ICP34.5 resulting in an attenuated phenotype (Bruni & Roizman, 1996, Randall & Roizman, 1997). ORF O interacts specifically with ICP4 and interferes *in vitro* with the binding of ICP4 to its cognate site (Randall *et al.*, 1997). Nevertheless, a recombinant virus expressing a truncated ORF P and mutated ORF O has been shown to reactivate from latency with similar efficiency to wild type virus (Lee & Schaffer, 1998). Moreover, another study, in which the start codon of both ORFs was replaced, has concluded that their expression is not essential for the establishment of latency (Randall *et al.*, 2000). It is however possible that ORF O and P play a role in the maintenance of latency, but such a role remains to be established.

Several studies have demonstrated that ICP0 is required for reactivation in latently infected human fibroblasts (Harris *et al.*, 1989, Hobbs *et al.*, 2001, Thompson & Sawtell, 2006). Investigation of latency in the mouse, however, has revealed that ICP0-null mutants reactivate less efficiently than wild-type virus, indicating that ICP0 has an important role in reactivation, but is not essential for reactivation (Halford & Schaffer, 2001, Thompson & Sawtell, 2006). As discussed above, the work of Thomas *et al* (1999, 2002) suggests that a protein encoded by the 2kb LAT may substitute for ICP0 at this time. It has also been suggested that although ICP0 is not required for the initiation of reactivation *in vivo* after

hypothermic treatment of mice, has an important role for later events in virus replication (Thompson & Sawtell, 2006). Furthermore, studies in latently infected trigeminal ganglion (TG) cell cultures and PC12 cells have demonstrated that expression of ICP0, ICP4 or VP16 is sufficient to induce reactivation, indicating that reactivation in neuronal cells can be induced in the absence of ICP0 (Halford *et al.*, 2001, Miller *et al.*, 2006). While VP16 is sufficient to induce reactivation in neuronal cells, it is unlikely that it contributes to the initiation of reactivation *in vivo*, since it is a L gene and its expression is dependent on IE and E gene expression. Moreover, VP16 is not necessary for efficient reactivation from TG explants (Steiner *et al.*, 1990). Collectively, the above findings suggest that IE genes play an important role in reactivation and thus, suppression of IE gene expression may be essential for the maintenance of latency.

Recent studies suggest that regulation of the chromatin structure on viral DNA determines whether HSV-1 enters latency or lytic infection (figure 1.16). Viral DNA is not associated with histones (Oh & Fraser, 2008) and upon arrival to the nucleus, host cell mechanisms attempt to silence viral gene expression by assembling chromatin. As latency is established, the LAT promoter and enhancer are associated with euchromatin and therefore exist in a transcriptionally permissive state (Kubat *et al.*, 2004), whereas lytic genes are progressively associated with heterochromatin and therefore exist in a transcriptionally non-permissive state (Wang *et al.*, 2005). Insulator elements that contain CCCTC sites bound by the CCCTC-binding factor have been identified upstream of the LAT promoter and in the LAT introns, and they are suggested to keep euchromatin activity within the LAT region (Amelio *et al.*, 2006b). Moreover, expression of LAT has been shown to promote heterochromatin and reduce euchromatin formation on lytic gene promoters by a mechanism that is yet to be defined (Wang *et al.*, 2005). Upon reactivation, the histones associated with LAT become deacetylated and lytic genes become associated with acetylated histones (Amelio *et al.*, 2006a). It has been hypothesized that in epithelial and other non-neuronal cells, VP16 promotes euchromatin formation on IE gene promoters.

Expression of ICP0 and ICP8 has also been suggested to promote euchromatin formation on lytic gene promoters, thereby further promoting lytic gene expression (Herrera & Triezenberg, 2004, Kent *et al.*, 2004). The mechanism by which these gene products affect chromatin structure on the viral genome is not entirely understood.

Finally, several studies have suggested a role for host immunity in suppressing viral gene expression in latently infected neurons. Leukocytes, including CD4+ and CD8+ T cells, infiltrate the TG during primary infection and remain for prolonged periods of time following the establishment of latency (Cantin *et al.*, 1995, Liu *et al.*, 2000). Moreover, the tightly regulated production of cytokines, such as gamma interferon (INF-γ), tumour necrosis factor alpha (TNF-α) and interleukin-6, in latently infected ganglia implies a persistent stimulation of the immune system in the absence of full reactivation (Halford *et al.*, 1997, Shimeld *et al.*, 1997). This concept is further supported by the recent observations that CD8+ T cells specific for gB are retained in latently infected sensory ganglia and are able to block full reactivation in TG cell cultures (Khanna *et al.*, 2003).

The precise mechanisms governing the establishment, maintenance of and reactivation from latency and the factors that induce reactivation have been the subject of intense investigation. Nevertheless, the molecular mechanisms controlling the repression of viral gene expression and reactivation from latency remain poorly understood, which as discussed above may relate to the delicate interactions between HSV and its natural human host which may not be fully reflected in other species.

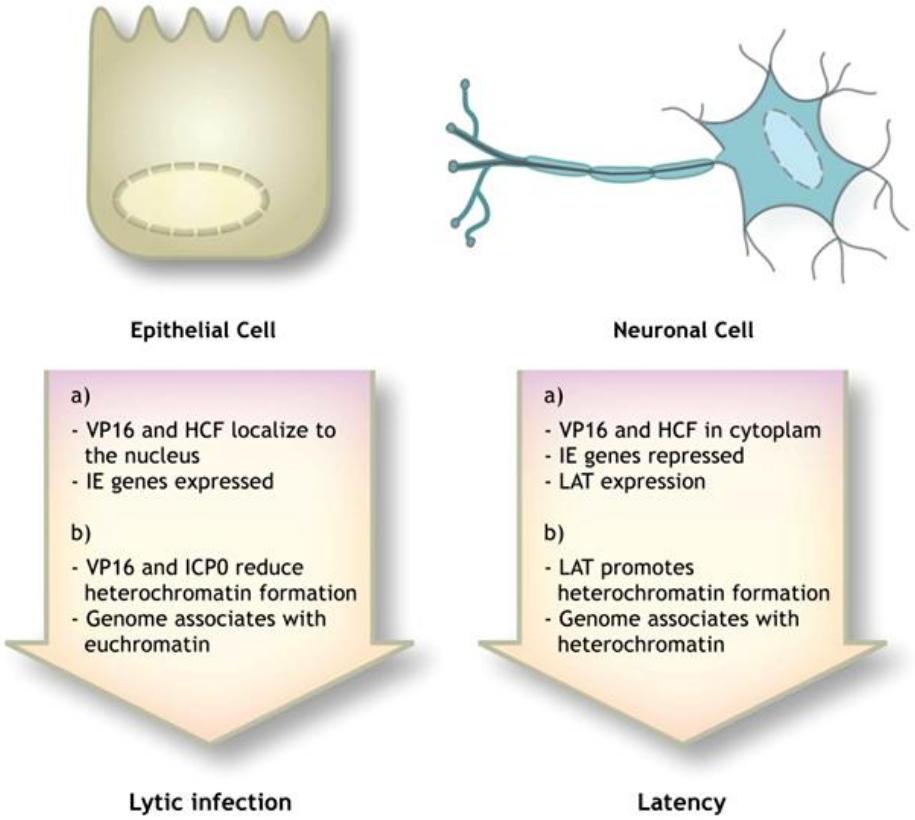


Figure 1.16 Proposed mechanisms that may determine whether HSV-1 enters latency or lytic infection

It has been suggested that in non-neuronal cells, VP16 promotes euchromatin formation on IE gene promoters. Subsequent expression of ICP0 and ICP8 has also been suggested to promote euchromatin formation on lytic gene promoters, thereby further promoting lytic gene expression. In neuronal cells, however, VP16 accumulates in the cytoplasm and the absence of functional VP16-induced complex promotes the establishment of latency. As latency is established, the LAT promoter and enhancer are associated with euchromatin and therefore exist in a transcriptionally permissive state, whereas lytic genes are progressively associated with heterochromatin and therefore exist in a transcriptionally non-permissive state. Expression of LATs has been shown to promote heterochromatin and reduce euchromatin formation on lytic gene promoters, thus contributing to the maintainance of latency. Upon reactivation, the histones associated with LAT become deacetylated and lytic genes become associated with acetylated histones.

1.3.2 Amplicon HSV-1 vectors

Amplicons are plasmids bearing the expression cassette of interest, the HSV-1 origin of replication and the HSV-1 packaging signal (Spaete & Frenkel, 1982). Amplicons are packaged as concatemers of up to one viral genome length into HSV virions in the presence of a helper virus. Helper viruses are traditionally replication-defective HSV-1 viruses containing mutations or deletions within an essential IE gene, such as ICP4 (Geller *et al.*, 1990). Replication of the defective helper virus and packaging of the amplicon are performed in a cell line capable of complementing the mutated or deleted gene products *in trans*. The main limitation of this system is that the final preparation contains a mixture of helper and amplicon viruses, which has not been possible to physically separate. Moreover, with each passage there is the possibility of recombination between the amplicon, helper virus genome and complementing cell line, which may result in production of replication-competent virus (During *et al.*, 1994). Recently, it has been possible to package amplicons free of helper virus by providing a packaging-deficient HSV genome via a bacterial artificial chromosome (BAC). The HSV BAC is deleted for ICP27 and the addition of ICP0 stuffer sequences render it too large to be packaged into the capsid. An ICP27-complementing plasmid is also co-transfected to allow the amplicon to be replicated and packaged (Saeki *et al.*, 2001).

Amplicons can be easily manipulated, contain multiple copies of the cassette of interest, have a large transgene capacity (potentially up to 150kb) and since no viral proteins are expressed, they are of low toxicity when packaged without contaminating helper virus. Moreover, they do not appear to enter latency and thus, the choice of promoter is not critical for long-term expression (discussed in the following section). However, it can be difficult to obtain reproducible data, as amplicons may contain different numbers of genome copies. Moreover, although gene expression is maintained for extended periods of time in both the central and peripheral nervous system (Federoff *et al.*, 1992, Kaplitt *et al.*, 1994, Lawrence *et al.*, 1996), amplicon vectors are eventually

degraded. To overcome this limitation, amplicons can be engineered to allow site-specific integration of the transgene into the cell genome, mediated by the rep protein of AAV (Cortes *et al.*, 2007). Although HSV-1 amplicon vectors have not been so far utilised to deliver shRNA to neurons, they have demonstrated effective silencing of tumour-related genes in glioma cells and human polyomavirus BK (BKV) T-antigen in BKV-transformed cells both *in vitro* and *in vivo* (Sabbioni *et al.*, 2006, Saydam *et al.*, 2005, Saydam *et al.*, 2007).

1.3.3 Replication-defective vectors

The alternative HSV vector system involves the introduction of the cassette of interest directly into the HSV-1 genome. This is achieved by insertion of the gene or shRNA into a plasmid that contains the expression cassette flanked by specific HSV sequences. Following co-transfection into complementing cells, the cassette is inserted into the HSV genome by homologous recombination. The viral vector genome contains a known copy number of the cassette and thus, the dose can be controlled.

Deletion of the essential IE genes ICP4 and/or ICP27 results in replication-defective viruses that can be directly propagated to high titres in appropriate cell lines which complement the deleted gene products *in trans*, without the need for a contaminating helper virus. Complementing cell lines for such deletions have been described, including E5 that complements ICP4 (DeLuca *et al.*, 1985), B130/2 that complements ICP27 (Howard *et al.*, 1998) and E26 that complements both ICP4 and ICP27 (Samaniego *et al.*, 1995). *In vivo,* the temporal cascade of viral gene expression is incapable of proceeding past the IE phase, resulting in recombinants that establish a persistent state very similar to latency. Furthermore, replication-defective vectors are unable to reactivate from latency and therefore persist for long periods of time in both neuronal cells and non-neuronal cells in culture.

1.3.3.1 Minimising toxicity

Although deletion of ICP4 is sufficient to create a replication-defective HSV-1 vector, the remaining IE gene products, which are highly toxic to cells, are abundantly expressed (DeLuca *et al.*, 1985) and thus, ICP4, ICP4/ICP22 or ICP4/ICP47 mutants are still toxic to neurons (Johnson *et al.*, 1992). To prevent cytotoxicity, vectors containing multiple IE deletions have been engineered. HSV-1 recombinants deleted for all IE genes appear to be non-toxic to cells (Samaniego *et al.*, 1997, Samaniego *et al.*, 1998). However, the efficient propagation of such viruses is problematic, because it requires that all IE gene products are provided *in trans* by a single complementing cell line, which is difficult to produce due to the toxicity of the IE gene products. Furthermore, the elimination of all IE genes significantly reduces transgene expression (Samaniego *et al.*, 1997). Retention of ICP0, which is a promiscuous transactivator of gene expression, allows efficient expression of transgenes and the virus to be propagated to high titres. Moreover, Johnson and colleagues demonstrated that ICP27 and ICP22 are more likely to contribute to the toxicity of ICP4 deleted viruses than ICP47 and ICP6 (Johnson *et al.*, 1994). Indeed, ICP4/ICP22/ICP27 mutants were found to be virtually non-toxic to primary cortical and DRG neurons and in the brain *in vivo* (Krisky *et al.*, 1998, Wu *et al.*, 1996). Nevertheless, deletion of ICP27 in combination with ICP4 results in large nuclear inclusions caused by ICP0 accumulating in the nucleus of infected cells (Zhu *et al.*, 1994, Zhu *et al.*, 1996).

VP16 cannot be deleted as it encodes an essential structural protein of the virion. However, mutations in VP16, which can be to some extent complemented by addition of hexamethylbisacetamide (HMBA) to the growth medium, reduce or abolish transactivation of IE genes and present an alternative approach to inactivating all IE genes (Ace *et al.*, 1989, Mossman & Smiley, 1999, Smiley & Duncan, 1997). A 12bp insertion into the VP16 transactivation domain, termed *in*1814, disrupts the interaction of VP16 with Oct-1 and HCF and significantly reduces activation of IE gene transcription, whilst VP16 remains capable of fulfilling its structural role (Ace *et al.*, 1988). At high MOIs, however, the virus

is still capable of replicating and mutations in VP16 need to therefore be accompanied by deletion of IE genes such as ICP4 and/or ICP27 (Johnson *et al.*, 1994, Lilley *et al.*, 2001, Palmer *et al.*, 2000, Preston *et al.*, 1997). In this case, HMBA is not sufficient for virus growth and a complementing cell line providing the equine herpesvirus 1 (EHV-1) homologue of VP16, which is not packaged into HSV, together with ICP4 and ICP27, has been developed in our laboratory to eliminate the possibility of recombinational repair of the VP16 mutation (Thomas *et al.*, 1999b).

A further block to replication in neurons is the deletion of non-essential genes, such as *tk* that encodes thymidine kinase, ICP6 that encodes the large subunit of ribonucleotide reductase, and ICP34.5, which are required for neurovirulence. Deletion of ICP34.5, which counteracts the INF-induced PKR-mediated inhibition of viral replication (He *et al.*, 1997), provides the greatest degree of neuroattenuation of any single non-essential gene and allows the virus to grow in several cell types *in vitro*, without the need for a complementing cell line (Coffin et al., 1996). HSV viruses only deleted for ICP34.5 transduce neurons of the peripheral and central nervous system with very low efficiency (Coffin *et al.*, 1996) and have been shown to cause severe inflammation in the brain (McMenamin *et al.*, 1998). They have shown, however, significant potential for the treatment of glioma and other cancers (Harrow *et al.*, 2004, Hu *et al.*, 2006, Walker *et al.*, 1999) and one such vector developed in our laboratory is now in a Phase III clinical trial in melanoma (Liu *et al.*, 2003).

Replication-defective HSV-1 vectors with deletions in ICP27, ICP4 and ICP34.5 and an inactivating mutation in VP16 (*in*1814) have been developed in our laboratory. These vectors are safe, non-toxic, can be produced to high titres, and allow efficient gene delivery to neurons in culture and both the peripheral and central nervous system (Lilley *et al.*, 2001, Palmer *et al.*, 2000) (figure 1.17).

1.3.3.2 Long-term expression during latency

The ability of HSV-1 to establish long-term latent infection in neurons has made it an attractive candidate as a vector for the delivery of genes to the nervous system. However, obtaining prolonged expression of a transgene in latently infected neurons proved challenging, due to transcriptional silencing of most HSV promoters, such as the *tk*, ICP0, ICP4, ICP6 and gC promoters (Ho & Mocarski, 1988, Palella *et al.*, 1988), as well as exogenous promoters introduced into the latent viral genome (Fink *et al.*, 1992, Lokensgard *et al.*, 1994). Thus, there was considerable interest in utilising the promoters driving expression of the LATs to drive expression of transgenes in neurons of the peripheral and central nervous system.

Insertion of a gene further downstream of the LAP1 TATA box resulted in 8 weeks of expression in the PNS (Ho & Mocarski, 1989) compared to 3 weeks when the gene was inserted immediately after the LAP1 TATA box (Dobson *et al.*, 1989, Margolis *et al.*, 1993). It therefore appeared that important elements in the sequences downstream of LAP1 were required for the promoter to function during latency. LAP2, but not LAP1, was able to drive very weak expression for at least 300 days in DRG when inserted at an ectopic locus within the HSV genome, such as gC (Goins *et al.*, 1994). When LAP1 linked to LAP2 was inserted into gC, efficient expression in the PNS was maintained for at least 28 days (Lokensgard *et al.*, 1997). Moreover, an approximately 800bp fragment of LAP1 without the TATA box linked to the MoMLV LTR and inserted into the gC locus was able to drive stable expression of reporter genes in the PNS for at least 42 days, although this was not possible when a number of other promoters were linked to LAP1 (Lokensgard *et al.*, 1994). Finally, insertion of an internal ribosome entry site (IRES) at a position 1.5 kb downstream of the 5' end of the primary 2kb LAT, which results in the maintenance of all cis-acting sequence elements for latent expression, allowed expression in the PNS for at least 307 days (Lachmann & Efstathiou, 1997, Marshall *et al.*, 2000).

The above findings suggested that although some of the elements necessary for expression during latency are present within LAP1, important sequences in LAP2 confer long-term expression on LAP1. The MoMLV LTR can substitute for LAP2 to achieve long-term expression in the PNS, further suggesting that the structure of LAP2 and its surrounding regions may be important for the LAT region to remain transcriptionally active during latency. Indeed, the LAT region has different dinucleotide content to the rest of the genome, which may reflect the chromatin structure of this region during latency (Coffin *et al.*, 1995). Based on this hypothesis, elements from the LAT region were utilised to confer long-term expression from heterologous promoters. A sequence 1.4kb downstream of the LAP1 TATA box (referred to as LAT P2), linked to a strong heterologous promoter, such as the cytomegalovirus (CMV) IE promoter, has been shown to drive efficient expression of reporter genes in DRG for at least 1 month (Palmer *et al.*, 2000). Importantly, genes could be expressed in the PNS from two promoters cloned in opposite orientations with the LAT P2 sequence centrally located and inserted into the vhs locus (UL43).

Obtaining long-term expression in the CNS has been even more challenging. This may be due to the different kinetics of gene expression from LAP, resulting in a gradual reduction in the number of transgene expressing neurons, or availability of different neuronal transcription factors. Nonetheless, when the CMV promoter was inserted after LAT P2, replication-defective vectors with ICP27, ICP4 and ICP35.4 deleted and an inactivating mutation in VP16 (*in*1814) were able to drive efficient expression of reporter genes in the CNS for at least 1 month (Lilley *et al.*, 2001, Perez *et al.*, 2004). These vectors were efficiently retrogradely transported, allowing expression not only around the injection site, but also at connected sites in the central nervous system (figure 1.17). These, and related vector backbones and promoter systems developed in our laboratory, have been used as the starting point for the studies reported in this thesis.

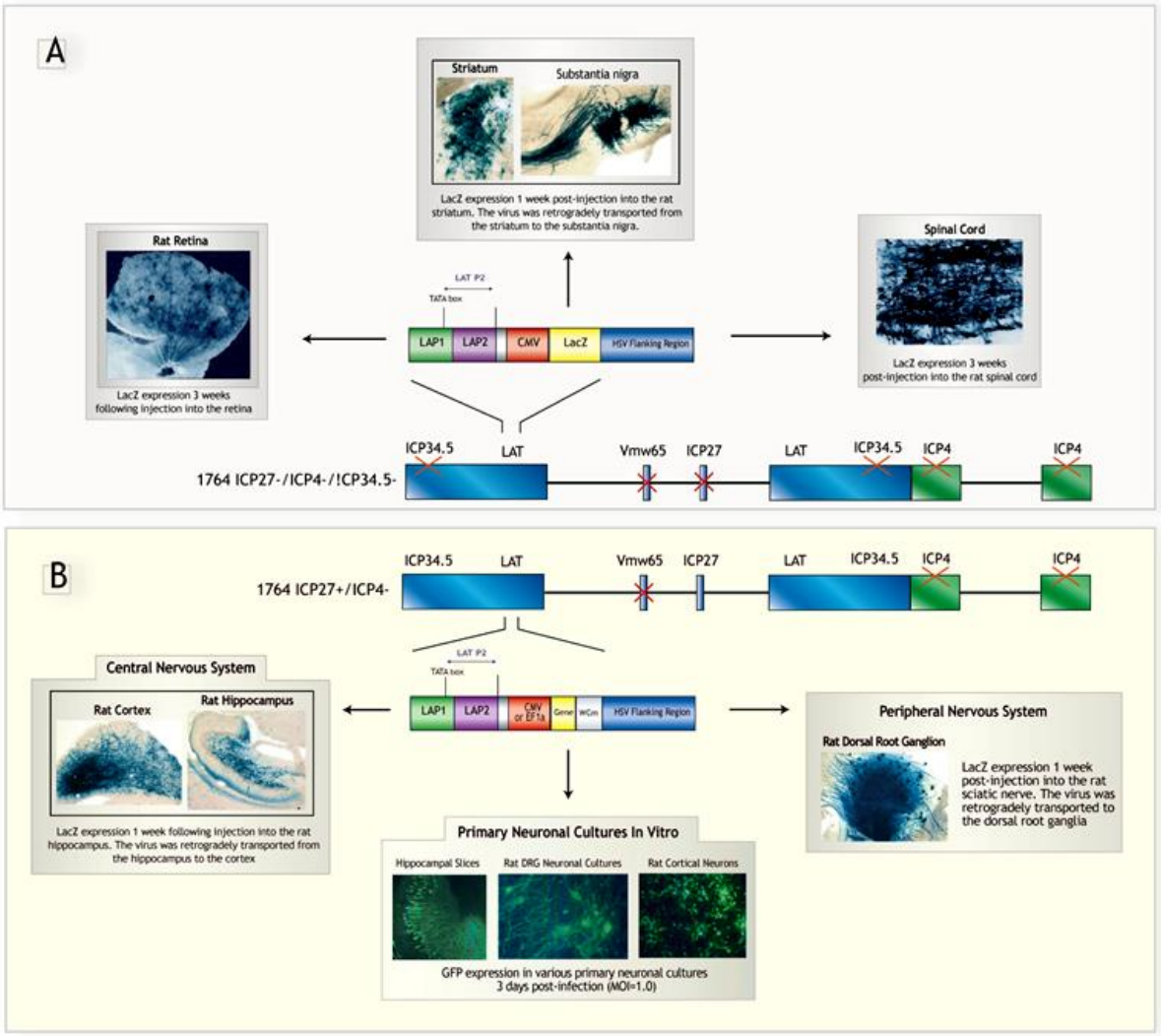


Figure 1.17 Delivery to the nervous system using replication-defective HSV-1 vectors developed in our lab

A. A multiply disabled (1764 ICP4-/ICP27-/ICP34.5-) HSV-1 vector expressing *lacZ* under the control of the CMV IE promoter was injected into the rat retina, striatum and spinal cord resulting in high levels of beta-galactosidase expression. The combination of this non-toxic vector backbone with the LATP2 promoter system allows trangene expression to persist for at least one month post-injection. Moreover, taking advantage of the retragrade transport capability of HSV-1, these vectors allow gene delivery to neurons distal from the site of injection (Lilley et al., 2001, Groutsi F., unpublished).

B. A series of less disabled (1764 ICP27+/ICP4-) replication-defective HSV-1 vectors have been constructed in our laboratory to efficiently deliver genes to neurons in culture and both the peripheral and central nervous system (unpublished data). Incorporation of the post-transcriptional regulatory element of the Woodchuck hepatitis virus (WCm) into the vector cassettes has increased expression levels even further. These vector backbones are also entirely non-toxic to neurons and combined with the LATP2 promoter system and either the CMV or EF1a promoters, they allow long-term expression in the central and peripheral nervous system.

1.4 DELIVERY OF RNAi TO NEURONS

Kosik and Krichevsky (2002) were the first to describe the induction of RNAi in primary neuronal cells. A growing number of publications have since demonstrated effective silencing in neurons both *in vitro* and *in vivo*. Although delivery of siRNA or shRNA has achieved silencing of molecular targets in various animal models of neurological disease, including pain and neurodegenerative disorders (summarised in table 1.3), efficient delivery to neurons remains a major hurdle for the successful application of RNAi as an experimental tool and for potential therapy.

For reasons that remain poorly understood, neurons are considerably more difficult to transfect than many other cell types. Moreover, whilst naked siRNA is taken up by endosomes in primary neuronal cells, insufficient amounts reach the cytoplasm (Lingor *et al.*, 2004). Cationic lipids, which are amongst the most commonly used siRNA transfection reagents, are highly toxic to primary neurons. Nevertheless, they have been used for the induction of RNAi when long-term survival of the neuronal culture is not necessary (Krichevsky & Kosik, 2002). Novel electroporation methods have emerged as a valuable alternative (Leclere *et al.*, 2005). Moreover, Penetratin and Transportan conjugated siRNAs have been used to silence genes in primary neuronal cultures with limited toxicity (Davidson *et al.*, 2004, Muratovska & Eccles, 2004). Finally, anionic liposomes, termed artificial virus-like particles (AVPs), and a cationic polypeptide (stearylated 8R) have demonstrated effective and non-toxic delivery of siRNAs to primary hippocampal neurons (Tonges *et al.*, 2006).

Delivery of siRNA to the brain parenchyma presents an even greater obstacle due to the presence of the blood-brain-barrier (BBB), which is only permeable to lipophilic molecules less than 400kDa in size. However, naked siRNA has induced effective silencing of target genes following intrathecal delivery to the spinal cord (Dorn *et al.*, 2004) and intra-ventricular infusion into the brain (Thakker *et al.*, 2005), which require continuous infusion and very high doses of siRNAs that may enhance potential off-target effects and

result in oversaturation of the endogenous miRNA pathway. Localised delivery of naked siRNAs, such as direct injection into the eye, requires lower doses of siRNAs and has been successfully used to protect axotomised retinal ganglion cells from death (Lingor *et al.*, 2005). Intravenous PEG stabilised immuno-liposome delivery has shown efficacy in delivering siRNAs against VEGF across the BBB and into glioma cells (Zhang *et al.*, 2004b). In this study, the monoclonal antibody which was conjugated to the liposome targeted a transferring receptor expressed in the BBB, and the other antibody targeted an insulin receptor expressed in brain cancer. More recently, Kumar et al. demonstrated that RVG (rabies virus glycoprotein)-9R complexed with siRNA against fatal Japanese encephalitis virus could efficiently cross the BBB following intravenous administration, without inducing an immune response (Kumar *et al.*, 2007). RVG interacts specifically with the nicotinic acetylcholine receptor expressed on the surface of neurons and enables efficient siRNA uptake into neuronal cells. Moreover, a single intrastriatal injection of cholesterol-conjugated siRNAs resulted in silencing of huntingtin and delayed disease progression in a mouse model of Huntington's disease (DiFiglia *et al.*, 2007). Finally, intracranial injection of siRNAs complexed with the cationic lipid i-FECT protected mice from fatal Japanese encephalitis virus and West Nile virus (Kumar *et al.*, 2006).

In a tissue as highly specialised as the CNS, targeted delivery of siRNA to specific cell types is mandatory for many applications. This can be achieved by viral-mediated delivery of shRNA or artificial miRNA. To bypass the BBB, viral vectors are stereotaxically injected directly into the brain, which results in localised delivery around the injection site, unless the virus is capable of retrograde and/or anterograde transport. Although direct injection into the brain is more invasive than systemic delivery, it requires considerably lower doses of RNAi triggers and a single administration is sufficient to induce long-term silencing. The first *in vivo* demonstration of viral-mediated delivery of shRNA to the nervous system made use of a recombinant Ad containing a DsRed expression cassette and a shRNA against GFP under the control of a modified version of the CMV pol II promoter (Xia *et al.*, 2002).

The authors reported highly effective silencing 5 days post-injection into the striatum of GFP transgenic mice.

Huntington's disease (HD) and spinocerebellar ataxia type 1 (SCA1) are dominantly inherited disorders caused by polyglutamine expansions (CAG codon) in the proteins huntingtin (htt) and ataxin-1, respectively (Gusella & MacDonald, 2000, Orr *et al.*, 1993). Xia and colleagues demonstrated that an AAV vector expressing shRNA against the CAG repeat region of mutant ataxin-1 under the control of the H1 promoter induced effective silencing in Purkinje cells, reduced intranuclear inclusions, and improved motor performance in a mouse model of SCA1 (Xia *et al.*, 2004). However, several injections in different sites were required to achieve efficient transduction of the cerebellum. The same group demonstrated improved behavioural and neuropathological abnormalities in a mouse model of HD, following striatal injection of an AAV vector expressing U6-shRNA against mutant htt (Harper *et al.*, 2005). Recently, Boudreau et al. demonstrated improved motor coordination and survival in a HD mouse model, following striatal injection of an AAV vector expressing artificial miRNA against both wild-type and mutant htt (Boudreau *et al.*, 2009).

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in humans. Dominantly inherited familial ALS is caused by mutations in the gene encoding Cu/Zn superoxide dismutase 1 (SOD1) (Bruijn *et al.*, 2004). Three studies have reported viral-mediated delivery of shRNA targeting a mutant form of SOD1 to mouse models of ALS. Raoul and colleagues injected a VSV-G pseudotyped LV vector, which expressed GFP and H1-shSOD1 from separate cassettes, directly into the lumbar spinal cord (Raoul *et al.*, 2005). Ralph and colleagues used a rabies-G envelope glycoprotein pseudotyped LV vector expressing H1-shSOD1 and ß-galactosidase from separate cassettes (Ralph *et al.*, 2005). Following multiple injections into the muscle, the LV vector was capable of delivery to the cell bodies of motor neurons in the spinal cord through retrograde transport mediated by the rabies G envelope glycoprotein. Both studies demonstrated delayed onset of ALS,

improvement of behavioural defects, and protection from neuronal degeneration in the spinal cord. Miller and colleagues demonstrated that intramuscular injection of an AAV serotype 2 vector expressing H1-shSOD1, which mainly transduces muscle fibres and to a lesser extent, enters the nerve terminals of motor neurons and undergoes retrograde transport to the spinal cord, reduced target gene expression in the spinal cord and delayed loss of hind-limp grip strength in a mouse model of ALS (Miller *et al.*, 2005).

Parkinson's disease, which is one of the most common neurodegenerative disorders, is characterised by selective loss of dopaminergic neurons from the substantia nigra pars compacta and accumulation of intracytoplasmic protein inclusions called Lewy bodies, which contain α-synuclein (Spillantini *et al.*, 1997). LV-mediated delivery of shRNA against a mutated form of human α-synuclein, which is associated with familial Parkinson's disease, demonstrated silencing in the human dopaminergic cell line SH-SY5Y *in vitro* and in the rat brain *in vivo*. However, expression of mutated human α-synuclein in the rat striatum was achieved experimentally from a separate LV vector (Sapru *et al.*, 2006). Fountaine and colleagues (2007) demonstrated that silencing of α-synuclein by transfection of siRNA into SH-SY5Y neuroblastoma dopaminergic cells was able to confer resistance to N-methyl-4-phenylpyridinium (MPP+), a potent and selective dopaminergic neurotoxin. Furthermore, viral-mediated RNAi has been used for the rapid generation of a Parkinson's disease model. Expression of U6-shRNA against the dopamine synthesis enzyme tyrosine hydroxylase, following injection of an AAV vector into the substantia nigra pars compacta of mice, resulted in localized silencing and behavioural changes, including a performance deficit (Hommel *et al.*, 2003).

Alzheimer's disease, which is the most common cause of dementia in the elderly, is characterised by neuritic plaques in the hippocampus and cortex and neurofibrillary tangles in the cell bodies of neurons in several regions of the brain. Alterations in the processing and clearance of the proteolytic products of the amyloid precursor protein (APP), such as C-terminal fragments (CTFs) and amyloid-ß peptides (Aß) that accumulate

in neuritic plaques, are believed to play a key role in the pathogenesis of Alzheimer disease (Selkoe & Schenk, 2003). CTFs and Aß peptides are generated by sequential cleavage of APP by ß-secretase (BACE1) and γ-secretase (Esler & Wolfe, 2001, Sinha *et al.*, 1999). LV–mediated expression of shRNA against BACE1 reduced Aß deposits in the hippocampus and attenuated cognitive deficits in two mouse models of Alzheimer's disease (Laird *et al.*, 2005, Singer *et al.*, 2005). Finally, a replication-defective HSV-1 vector expressing H1-shRNA against a mutant form of human APP, which was expressed from a co-injected LV, resulted in reduced Aß accumulation in the mouse hippocampus (Hong *et al.*, 2006). However, expression of mutant APP by co-injection of another vector is a somewhat artificial situation, and silencing of an endogenous gene using HSV vectors had not been demonstrated.

Induction of RNAi in sensory neurons, which are targeted with the vectors developed in this thesis, had only been achieved through delivery of synthetic siRNA. This is despite the fact silencing of genes in the cell bodies of these neurons in the dorsal root ganglia (DRG) is particularly attractive for both fundamental studies in nociception and neuronal regeneration and development, and for the development of potential therapeutic approaches. The efforts which have been made to silence genes in various animal models of nociception are reviewed in chapter 4.

RNAi trigger	Route of administration	Target gene	Disease model	Ref
naked siRNA	Intrathecal	P2X3	Pain	Dorn et al, 2004
naked siRNA	Injection in the eye	c-Jun, Bax, Apaf-1	Axotomy	Lingo et al., 2005
PEI siRNA	Intrathecal	NMDA	Pain	Tan et al., 2005
Cationic lipid siRNA	Intrathecal	DOR	Pain	Luo et al., 2005
Immunoliposome siRNA	Intravenous	VEGF	Glioblastoma	Zhang et al., 2004
RVG-9R-siRNA	Intravenous	JEV	Japanese Encephalitis virus	Kumar et al., 2007
Cholesterol-siRNA	Intrastriatal	htt	Huntington's disease	DiFiglia et al., 2007
i-FECT siRNA	Intracerebral	JEV, WNV	Japanese encephalitis virus and West Nile virus	Kumar et al., 2006
AAV-shRNA	Intrastriatal	htt	Huntington's disease	Harper et al., 2005
AAV-miRNA	Intrastriatal	htt	Huntington's disease	Boudreau et al., 2009
AAV-shRNA	Cerebellum injections	Ataxin-1	SCA1	Xia et al., 2004
LV-shRNA	Spinal cord	SOD1	ALS	Raoul et al., 2005
LV-shRNA	Intramuscular injections	SOD1	ALS	Ralph et al., 2005
LV-shRNA	Intramuscular	SOD1	ALS	Miller et al., 2005
LV-shRNA	Hippocampal injection	BACE1	Alzheimer's disease	Singer et al., 2005; Laird et al., 2005

Table 1.3 Delivery of siRNA or shRNA to animal models of neurological disease

THE AIM OF THIS THESIS

Delivery of synthetic siRNA to sensory neurons *in vivo* has proven problematic. Moreover, there have been no previous reports of delivery of shRNA to DRG, which are inaccessible by surgical techniques and thus, not amenable to direct injection with viral or plasmid vectors. The replication-defective HSV-1 vectors developed in our laboratory have allowed efficient delivery of reporter genes to sensory neurons, through retrograde transport from the injection site to the cell bodies in the DRG (Palmer *et al.*, 2000). Nevertheless, HSV had not been previously engineered to express shRNA. Thus, the aim of this thesis was to develop an efficient method for delivery of shRNA to sensory neurons *in vivo* to allow effective and specific RNAi-mediated silencing of targeted genes.

The primary aim of the work described in this thesis was to engineer replication-defective HSV-1 vectors expressing shRNA or artificial miRNA through the development of cassettes which would allow effective expression from within the HSV genome by taking advantage of regulatory elements derived from the LAT region. Effective expression from the HSV genome presents particular challenges, which whilst overcome for the expression of delivered genes (Lilley *et al.*, 2001, Palmer *et al.*, 2000), had not been previously addressed with respect to the expression of shRNA. Silencing from these vectors would be optimised by evaluating different approaches of shRNA expression both *in vitro* and in DRG neurons *in vivo*. Secondly, the work in this thesis aimed to establish proof of concept by demonstrating, for the first time, *in vivo* silencing of an endogenous gene expressed in sensory neurons. Finally, the work in this thesis aimed to present potential applications of this method in functional genomic studies and target validation by demonstrating silencing of a gene involved in nociception and simultaneous silencing of three genes believed to be involved in the inhibition of sensory axon regeneration following injury.

CHAPTER 2

MATERIALS & METHODS

2.1 MATERIALS

2.1.1 Suppliers

General laboratory chemicals were of analytical grade and obtained from Sigma-Aldrich Company Ltd. or Merck Chemicals Ltd. General disposable plasticware was supplied by Sterilin Ltd., Sarstedt Ltd, VWR International Ltd., Eppendorf Ltd. and CLP. All tissue culture plasticware was obtained from Nunc or Greiner Bio-One Ltd. The suppliers of all other materials are stated in the methods.

Supplier	Address
Alfa Aesar (Lancaster Synthesis)	Shore Road, Heysham, Lancashire, LA3 2XY, UK
Alpha Diagnostics International	6203 Woodlake Center, San Antonio, TX 78244 USA
Ambion (Applied Biosystems)	2130 Woodward St., TX 78744-1832, Austin, USA
Amersham (GE Healthcare)	The Grove Centre, Amersham, Bucks, HP7 9LL, UK
BD Biosciences	Edmund Halley Rd, Oxford Science Park, OX4 4DQ, UK
Bio-Rad Laboratories Ltd.	Maxted Road, Hemel Hempstead, Herts. HP2 7DX, UK
CLP (Neptune pipettor tips)	6190 Cornerstone Court East, San Diego, CA 92121, USA
Eppendorf UK Ltd	Endurance House, Histon, Cambridge, CB24 9ZR, UK
Gibco (Invitrogen)	3 Fountain Drive, Inchinnan Business Park, PA4 9RF, UK
Greiner Bio-One Ltd.	Brunel Way, Stonehouse, Gloustershire, GL10 3SX , UK
Hoffmann-La Roche Ltd	6 Falcon Way, Welwyn Garden City, Herts. AL7 1TW, UK
Insight Biotechnology Ltd.	Wembley, Middlesex, HA9 7YN, UK
Invitrogen Ltd.	3 Fountain Drive, Inchinnan Business Park, PA4 9RF, UK
Merck Ltd.	Boulevard Industrial Park, Nottingham NG9 2JR, UK

Millipore Ltd.	3-5 The Courtyards, Hatters Lane, Herts. WD18 8YH, UK
New England Biolabs Ltd.	75/77 Knowl Piece, Wilbury Way, Herts. SG4 0TY, UK
Nunc (Thermo Fischer Scientific)	Kamstrupvej 90, DK-4000, Roskilde, Denmark
Promega UK Ltd.	Southampton Science Park, Southampton, SO16 7NS, UK
Qiagen Ltd.	Fleming Way, Crawley, West Sussex, RH10 9NQ, UK
Santa Cruz Biotechnology Inc.	2145 Delaware Avenue, Santa Cruz, CA 95060, USA
Sarstedt Ltd.	68 Boston Road, Beaumont Leys, Leicester LE4 1AW, UK
Sartorius Ltd.	Longmead Business Centre, Epsom, Surrey, KT19 9QQ, UK
Sigma-Aldrich Company Ltd.	The Old Brickyard, Gillingham, Dorset, SP8 4XT, UK
Sterilin Ltd.	Angel Lane, Aberbargoed, Caerphilly, CF81 9FW, UK
Stratagene (Agilent Technologies)	Cheadle Royal Business Park, Stockport, SK8 3GR, UK
VWR International Ltd.	Hunter Boulevard, Leicestershire, LE17 4XN, UK
Whatman International Ltd.	Springfield Mill, Maidstone, ME14 2LE, UK

2.1.2 Bacterial Strains

Strain	Genotype	Source
OmniMAX™ 2-T1^{R}	F'(proAB^{+} lacI^{q} lacZΔM15 Tn10(Tet^{R}) Δ(ccdAB)} mcrA Δ(mrr-hsdRMS-mcrBC)φ80(lacZ)ΔM15 Δ(lacZA-argF) U169 endA1 recA1 supE44 thi-1 gyrA96 relA1 tonA panD	Invitrogen
DB3.1™ (CcdB resistant)	F^{-} gyr462 endA Δ(sr1-recA) mcrB mrr hsdS20 (r_B^-, m_B^-) supE44 ara14 galK2 lacY1 proA2 rpsL20(Str^{R}) xyl5 λ^{-} leu mtl1	Invitrogen
E. coli TOP10	F^{-} mcrA Δ(mrr-hsdRMS-mcrBC) φ80lacZΔM15 ΔlacZX74 recA1 araD139 Δ(ara-leu)7697 galU galK rpsL (Str^{R}) endA1 nupG	Invitrogen

2.1.3 Plasmids

Plasmid	Antibiotic Resistance	Source
pR19SYNeGFP	Ampicillin	BioVex
pR19CMVeGFP	Ampicillin	BioVex
pR19CMVLacZ	Ampicillin	BioVex
pR19CMVLacZWCm	Ampicillin	BioVex
pNOT3.5	Blasticidin	BioVex
pcDNA3	Ampicillin	Invitrogen
pENTR™/U6	Kanamycin	Invitrogen
pGEM-T Easy	Ampicillin	Promega
pSilencer™4.1-CMV	Ampicillin	Ambion
pcDNA™6.2-GW/EmGFP-miR	Spectinomycin	Invitrogen
pDONR™221	Kanamycin	Invitrogen
pSCREEN-iT™/LacZ-DEST	Ampicillin	Invitrogen
pSCREEN-iT™/LacZ-GW/CDK2	Ampicillin	Invitrogen

2.1.4 Cell Lines

Cell line	Origin	Culture Media	Reference/ Source
BHK 21 Clone 13	Baby Hamster Kidney cells	1 x Dulbecco's modified Eagle's media (DMEM) supplemented with 10%(v/v) foetal calf serum (FCS), 10%(v/v) tryptose phosphate broth (TPB) and containing 100U/ml penicillin and 100µg/ml streptomycin	(MacPherson & Stoker, 1962) ATCC CCL 10
27/12/M:4 (MAM49)	BHK cells that complement VP16, ICP4 and ICP27 deletions	As for BHK cells, but also containing 750µg/ml Zeocin and 800µg/ml G418 to maintain selection	(Thomas *et al.*, 1999b)

HEK 293T	Human Embryonic Kidney cells	As for BHK cells	(Graham *et al.*, 1977) ATCC CRL1573
9L/LacZ	Gliosarcoma cell line that stably expresses LacZ	As for BHK cells	ATCC CRL2200

ATCC is the registered trademark of the American Type Culture Collection

2.1.5 Primers

Primers were designed using VNTI and synthesized by Invitrogen:

Primer	Sequence 5'-3'
U6 Forward	GGACTATCATATGCTTACCG
M13 Reverse	CAGGAAACAGCTATGAC
AttR1 Forward	CACATTATACGAGCCGGAAGCAT
AttR2 Reverse	CAGTGTGCCGGTCTCCGTTATCG
pSilencer F	AGGCGATTAAGTTGGGTA
pSilencer R	CGGTAGGCGTGTACGGTG
LAT Forward	CTATGTTCCTGTTTCTGTTCTCC
LAT Reverse	TCCCCGAAAGCATCCTGCCAC
miR Forward	TCCCAAGCTGGCTAGTTAAG
miR Reverse	CTCTAGATCAACCACTTTGT
CMV Forward	TGACGTCAATGGGTGGACTA
GFP Forward	GTCCTGCTGGAGTTCGTGACC
WCm Reverse	GAGATCCGACTCGTCTGAGG
CMVenh Forward	GTTGACATTGATTATTGACTAG
CMVenh Reverse	GGCGAGCTCTGCCAAAACAAACTCCCATTG

2.1.6 Double Stranded DNA Oligos

The shRNA sequences were either supplied by Invitrogen or designed using Invitrogen's RNAi Designer tool (www.invitrogen.com/rnai). The two complementary single-stranded DNA oligonucleotides, one of which encoding the shRNA/miRNA of interest, were generated using Invitrogen's custom primer synthesis service and cloned into appropriate expression vectors.

Name	Sense Oligo Sequence (5'-3')	Target Gene
shLacZ	CACCGCTACACAAATCAGCGATTTCGAAAAATCGCT GATTTGTGTAG	ß-galactosidase (LacZ)
miR-LacZ	TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCA CTGACTGACGACTACACATCAGCGATTTCAGG	LacZ
shLacZ for pSilencer	AGCTTCTACACAAATCAGCGATTTTTCGAAATCGCT GATTTGTGTAGG	LacZ
shGFP1	CACCGCAAGCTGACCCTGAAGTTCGAAAACTTCAGG GTCAGCTTG	Green fluorescent protein (GFP)
shGFP2	CACCGCATCAAGGTGAACTTCAAGACGAATCTTGAA GTTCACCTTGATGC	GFP
shGFP3	CACCGCCACAACGTCTATATCATGGCGAACCATGAT ATAGACGTTGTGGC	GFP
miR-neg	TGCTGAAATGTACTGCGCGTGGAGACGTTTTGGCC ACTGACTGACGTCTCCACGCAGTACATTT	No known target
shTRPV1	GCGCATCTTCTACTTCAACTTCAAGAG AGTTGAAGTAGAAGATGCGC	Mouse (and rat) transient receptor potential vanilloid subtype 1(trpV1)
miR-TRPV1	TGCTGTGTAGTAGCTGTCTGTGTAGCGTTTTGGCC ACTGACTGACGCTACACACAGCTACTACA	Mouse (and rat) trpV1
miR-p75#1	TGCTGTAGACAGGAATGAGGTTGTCAGTTTTGGCC ACTGACTGACTGACAACCATTCCTGTCTA	Mouse nerve growth factor receptor (p75NTR nt884)
miR-p75#2	TGCTGAAAGGAGTCTATATGCTCCGGGTTTTGGCCA CTGACTGACCCGGAGCATAGACTCCTTT	Mouse p75NTR (nt1239)
miR-p75#3	TGCTGTGCACAGGCTCTCCACAATGTGTTTTGGCCA CTGACTGACACATTGTGGAGCCTGTGCA	Mouse p75NTR (nt1366)
miR-p75#4	TGCTGTAGACCTTGTGATCCATCGGCGTTTTGGCCA CTGACTGACGCCGATGGCACAAGGTCTA	Mouse p75NTR (nt718)
miR-p75#5	TGCTGAATATAGGCCACAAGGCCCACGTTTTGGCCA CTGACTGACGTGGGCCTTGGCCTATATT	Mouse (and rat) p75NTR (nt930)
miR-p75#6	TGCTGAATGTCAGCTCTCTGGATGCGGTTTTGGCCA CTGACTGACCGCATCCAGAGCTGACATT	Mouse (and rat) p75NTR (nt1350)

miR-Lingo1#1	TGCTGTAGTCTAGCAGGATGACGATCGTTTTGGCCA CTGACTGACGATCGTCACTGCTAGACTA	Mouse (and rat) leucine rich repeat and Ig domain containing 1 (Lingo1 nt855)
miR-Lingo1#2	TGCTGTGAAGTAGTTGGGTAGGAGTAGTTTTGGCC ACTGACTGACTACTCCTACAACTACTTCA	Mouse Lingo1 (nt1631)
miR-Lingo1#3	TGCTGATAATGAGCGTCTTGATGTCGGTTTTGGCCA CTGACTGACCGACATCAACGCTCATTAT	Mouse Lingo1 (nt2049)
miR-Lingo1#4	TGCTGTATAGGTCTTGGAACATGTAGGTTTTGGCCA CTGACTGACCTACATGTCAAGACCTATA	Mouse Lingo1 (nt873)
miR-Lingo1#5	TGCTGTACAAACACCTGCTGTGCCTTGTTTTGGCCA CTGACTGACAAGGCACAAGGTGTTTGTA	Mouse Lingo1 (nt1681)
miR-NgR2#1	TGCTGTACAGGTGTAGTGACTGCAGCGTTTTGGCC ACTGACTGACGCTGCAGTCTACACCTGTA	Mouse reticulon 4 receptor-like 2 (Rtn4rl2 or NgR2)
miR-NgR2#2	TGCTGTACTGTAGGCTGACCAAGCCTGTTTTGGCCA CTGACTGACAGGCTTGGAGCCTACAGTA	Mouse NgR2
miR-NgR2#3	TGCTGTGAACAGGTAGAGGATGGTGAGTTTTGGCC ACTGACTGACTCACCATCCTACCTGTTCA	Mouse NgR2
miR-NgR2#4	TGCTGAGGTACTGTAGGCTGACCAAGGTTTTGGCC ACTGACTGACCTTGGTCACTACAGTACCT	Mouse (and rat) NgR2
miR-NgR2#5	TGCTGAACAGGTAGAGGATGGTGAGGGTTTTGGCC ACTGACTGACCCTCACCACTCTACCTGTT	Mouse NgR2

2.1.7 Antibodies

Primary	Dilution/Assay	Secondary
Anti-Lingo1 rabbit polyclonal Abcam (ab23631)	1/500 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-p75 NGFR rabbit polyclonal Abcam (ab8874)	1/500 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-NgR2 rabbit affinity pure IgG Alpha Diagnostic (NGR21-A)	1/100 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-STAT1 rabbit polyclonal Abcam (ab31369)	1/500 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000

Anti-alpha tubulin rabbit polyclonal Abcam (ab4074)	1/500 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-TRPA1 rabbit polyclonal Abcam (ab31486)	1/1000 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-TRPV1 rabbit polyclonal Santa Cruz (sc-28759)	1/200 Western	Goat anti-rabbit IgG-HRP (Abcam) 1/3000
Anti-TRPV1 rabbit polyclonal Abcam (ab31895)	1/1000 Immunofluorescence	Goat anti-rabbit Alexa Fluor 594 (Invitrogen Molecular Probes) 1/200
Anti-GFP chicken polyclonal Abcam (ab13970)	1/1000 Immunofluorescence	Goat anti-chicken polyclonal IgY-FITC (Abcam) 1/250
Anti-GAPDH mouse monoclonal	1/800 Immunofluorescence	Goat anti-mouse IgG-FITC (Sigma) 1/100

2.1.8 Animals

The Balb/c mice used in this study were obtained from breeding colonies within the Department of Biological Services, UCL. Rosa26 transgenic mice (Gt(ROSA)26Sor), which are heterozygous for the ROSA26 retroviral insertion (Friedrich and Soriano, 1991) and express ß-galactosidase in most tissues of the adult mouse, were obtained from the Jackson Laboratory. All animal procedures were carried out in accordance to UK Home Office Regulations (Home Office project licence no: PPL70/4293).

2.1.9 Standard Solutions

The following standard solutions were used throughout:

TE: 10mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0

TAE: 400mM Tris, 200mM Sodium acetate, 20mM EDTA pH 8.3

TBE: 89mM Tris Base, 89mM Boric acid, 2mM EDTA pH 8.0

PBS: 137mM NaCl, 2.7mM KCl, 4.3mM $Na_2HPO_4 \cdot 7H_2O$, 1.4mM KH_2PO_4

SSC: 150mM NaCl, 15mM Sodium citrate

2.2 MOLECULAR BIOLOGY TECHNIQUES

2.2.1 Agarose Gel Electrophoresis

1% (w/v) agarose gels were prepared using 1xTAE. Ethidium bromide was added to a final concentration of 0.5µg/ml. Loading buffer (10x: 1xTAE, 50% (v/v) glycerol, 0.025% bromophenol blue) was added to DNA samples prior to loading at a 0.1 volume. 1kb plus DNA ladder (Gibco) was used in all cases as a marker for DNA size. The bands were visualized on a UV transilluminator.

2.2.2 Restriction Enzyme Digestions

Restriction enzyme digests were performed on plasmid DNA for clonal analysis or isolation of DNA fragments. Analytical digests were performed in a total volume of 20µl containing 5µl of mini-prep (approximately 50ng/µl) or 1µl of midi-prep DNA (approximately 1µg/µl), 1µl of each enzyme (10units/µl), 2µl of the appropriate restriction buffer (10x) as specified by the manufacturer (Promega) and ddH_20. Restriction digests for isolation of DNA fragments were performed in a total volume of 100µl containing 5µl of midi-prep DNA, 2µl of each enzyme, 10µl of the appropriate restriction buffer (10x) and ddH_20. The digests were incubated at the appropriate temperature for a minimum of 1 hour and then run on 1% agarose gel and visualized on a UV transilluminator.

2.2.3 Blunt End Reactions

To create compatible ends for cloning, restriction enzyme cleaved overhangs were converted to blunt double-stranded DNA ends. After restriction enzyme digestion as described before, 5µl of a dNTPs (dATP, dTTP, dCTP and dGTP) stock (25mM) and 2µl

(7.7units/µl) of T4 DNA polymerase (Promega) were added directly to DNA. The reaction was incubated at 37°C for 1 hour. The digests were heat-deactivated at 80°C for 20min and then cooled on ice for 20min before further restriction enzymes were added.

2.2.4 Phosphatase Treatment

To decrease the background associated with vector self-ligation, plasmid DNA was treated with calf intestinal alkaline phosphatase (CIAP, Promega) to remove 5' phosphates. Reactions were performed in a total volume of 40µl containing 4µl CIAP buffer (10x), 1pmol of 5' ends of DNA, 1µl of CIAP (1unit/µg DNA) and ddH_20. The reactions were incubated at 37°C for 15min. The CIAP was heat-deactivated at 65°C for 15min.

2.2.5 Purification of DNA from Gel Bands

DNA was purified from agarose using the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences) according to manufacturer's instructions. The maximum weight of gel slice that can be processed is 300mg. Briefly, DNA bands are excised using a clean scalpel and transferred to a pre-weighed 1.5ml microcentrifuge tube. The weight of the slice is determined and the appropriate volume of capture buffer is added to the gel, which is incubated at 60°C until completely melted. The sample is transferred to a GFX column and centrifuged. The column is washed and the DNA is eluted in 35µl of ddH_20. The concentration of purified DNA is determined using a nanodrop (ND-1000).

2.2.6 DNA Ligation Reactions

Ligations were performed in a total volume of 20µl containing vector and insert DNA at a molar ratio of 1:3 or 1:1, 1µl (3units/µl) of T4 DNA ligase (Promega), 2µl of ligation buffer (10x) and ddH20. Molar ratios were converted to mass ratios using the formula: [ng of

vector x kb size of insert/ kb size of vector] x molar ratio of insert/ vector = ng of insert. Blunt-end ligations were incubated at 16°C overnight, while sticky ends were ligated at room temperature for 3 hours or 4°C overnight.

2.2.7 The Gateway Technology

The Gateway® technology is a universal cloning method that takes advantage of the site-specific recombination properties of bacteriophage lambda (Landy, 1989) to provide a rapid and highly efficient way to move a gene of interest into multiple vector systems. To generate an entry clone, a BP recombination reaction is performed between a pDONR™ vector, in this study pDONR™221, and an attB expression clone. To generate the desired expression clone, an LR recombination reaction is performed between the entry clone and a Gateway® destination vector. The Gateway® technology is schematically represented below:

attB1-gene-attB2 (expression clone) x attP1-ccdB-attP2 (pDONR™) = attL1-gene-attL2 (entry clone) x attR1-ccdB-attR2 (destination vector)

CcdB is the F plasmid-encoded gene that inhibits growth in E. coli (Bernard & Couturier, 1992). The LR and BP recombination reactions were transformed into competent cells, which are not E. coli strains containing the F' episome that contain the ccdA gene and will prevent negative selection with the ccdB gene. To select, propagate and maintain the pDONR™ and Gateway® destination vectors, DB3.1™ competent cells were used that are resistant to ccdB.

2.2.8 BP Recombination Reactions

The attB x attP reaction is mediated by Gateway® BP Clonase™ II enzyme mix (Invitrogen), which contains the bacteriophage lambda recombination protein Integrase (Int), the E. coli-encoded protein Integration Host Factor (IHF), and reaction buffer. To perform the BP recombination reaction, 150ng of attB expression clone, 150ng of pDONR™ and TE buffer to 8µl are added to a 1.5ml microcentrifuge tube and mixed. The BP Clonase™ II enzyme mix (5x) is thawed on ice for 2min and 2µl are added to the reaction that is vortexed and incubated at 25°C for a minimum 1 hour or up to overnight to increase the yield of colonies. Following this incubation, 1µl of Proteinase K (Invitrogen) is added to the reaction, which is vortexed and incubated at 37°C for 10min. For BP reactions, the most efficient substrates are linear attB products and supercoiled attP donor vectors.

2.2.9 LR Recombination Reactions

The attL x attR reaction is mediated by Gateway® LR Clonase™ II enzyme mix (Invitrogen), which contains the bacteriophage lambda recombination proteins Int and Excisionase (Xis), the E. coli IHF, and reaction buffer. To perform the LR recombination reaction, 150ng of entry clone, 150ng of destination vector and TE buffer to 8µl are added to a 1.5ml microcentrifuge tube and mixed. The LR Clonase™ II enzyme mix (5x) is thawed on ice for 2min and 2µl are added to the reaction, which is vortexed and incubated at 25°C for a minimum 1 hour or up to overnight to increase the yield of colonies. Following this incubation, 1µl of Proteinase K is added to the reaction, which is vortexed and incubated at 37°C for 10min. For LR reactions, the most efficient substrates are supercoiled attL entry vectors and supercoiled attR destinations vectors. For entry clones <10kb the entry clone was linearised.

2.2.10 Bacterial Cell Culture

All bacteria were grown in LB media (1% w/v Bacto®-trypton, 1% w/v NaCl, 0.5 w/v Bacto®-yeast extract) or on plates containing LB and 2% Bacto®-Agar. LB was autoclaved at 120°C for 20min at 10lb/inch^2. Both media contained the appropriate antibiotic selection. Ampicillin was used at a final concentration of 100μg/ml. Chloramphenicol was used at a final concentration of 30μg/ml. Kanamycin and spectinomycin were used at a final concentration of 50μg/ml. LB cultures were incubated overnight at 37°C in an orbital incubator at 200rpm. Plates were incubated overnight at 37°C in a standard incubator.

2.2.11 Preparation of Competent cells

Competent cells were prepared using the standard $CaCl_2$ technique. A single colony was grown overnight in 10ml of LB containing no antibiotics. 100μl of this culture was used to inoculate 100ml of LB containing no antibiotics. The cells were allowed to grow for a minimum of 2 hours in the orbital incubator at 200rpm (until OD_{580} = approximately 0.5). Cells were pelleted by centrifugation at 2000rpm for 10min at 4°C, excess LB was discarded and the pellet was resuspended in 10ml of ice-cold $CaCl_2$ (100mM, 0.0218gr/ml). Cells were pelleted as before, resuspended in 4ml of ice-cold $CaCl_2$ and kept on ice until required. DB3.1™ (CcdB resistant) and OmniMAX™ 2-T1^R competent cells were purchased by Invitrogen.

2.2.12 Transformation of Bacteria

Competent cells were transformed by addition of plasmid DNA and subsequent incubation on ice for 30min. The cells were heat-shocked for 30sec at 42°C, incubated on ice for 2min and 250μl of LB containing no antibiotics were added. The cells were incubated for 1 hour in the orbital incubator at 200rpm. The transformed cells were plated on LB agar plates containing the appropriate antibiotic selection and colonies were allowed to grow

overnight at 37°C. Single colonies were isolated. Each colony was used to inoculate 3ml of LB containing antibiotics and incubated in the orbital shaker overnight.

2.2.13 Small Scale Plasmid DNA Purification (Mini-Prep)

Transformed cells from a 3ml LB overnight culture were pelleted by centrifugation at 7000rpm for 5min and resuspended in 100µl suspension buffer (50mM Tris-HCl pH 7.5, 10mM EDTA pH 8.0, and 100µg/ml RNase A). The cells were lysed by addition of 200µl of lysis buffer (0.2M NaOH, 1% Triton) and neutralized by addition of 150µl of neutralization buffer (3M potassium acetate, pH 4.8). The lysate was pelleted by centrifugation at 14,000rpm for 5min and the pelleted precipitate was removed with a bent hypothermic needle. The plasmid DNA was precipitated by addition of 500µl of isopropanol and centrifugation at 14,000rpm for 5min. The pellet was washed with 500µl of 70% ethanol and subsequent centrifugation at 14,000rpm for 5min. The supernatant was carefully discarded and the DNA pellet was allowed to air dry at room temperature for 20min. The purified plasmid DNA was resuspended in 50µl ddH20.

Extraction and purification of plasmid DNA from small scale cultures was also performed using the illustra plasmidPrep mini spin kit (GE Healthcare) according to manufacturer's instructions. The purified DNA was eluted in 50µl ddH20 and stored at -20°C. The concentration of purified DNA was determined using a nanodrop.

2.2.14 High Scale Plasmid DNA Purification (Midi-Prep)

Transformed cells from a 100ml overnight culture were pelleted by centrifugation at 3500rpm for 10min at 22°C. The plasmid DNA was extracted and purified using the PureLink HiPure Plasmid Midiprep kit (Invitrogen) according to the manufacturer's protocol. Briefly, the pellet was resuspended in 50mM Tris-HCl (pH 8.0), 10mM EDTA and

100µg/ml Rnase A. The cells were lysed in 0.2M NaOH and 1% (v/v) SDS and neutralized in 3.1M potassium acetate, pH 5.5. The lysate is passed through an ion-exchange column. The negatively charged phosphates on the backbone of the DNA interact with the positive charges on the surface of the resin. RNA, protein, carbohydrates and other impurities are washed away with 0.1M sodium acetate, pH 5.0 and 825mM NaCl. The purified DNA (a typical yield is 100µg) is eluted in 200µl of ddH20 and stored at -20^{o}C. The concentration of purified DNA was determined using a nanodrop.

2.2.15 DNA Sequencing

Plasmid DNA at a concentration of 50ng/µl was sequenced by the Sequencing Facility of the Department of Biochemistry at the University of Cambridge. Primers were synthesized by Invitrogen's custom primer synthesis service and were used at a concentration of 10pmole/µl.

2.2.16 Polymerase Chain Reaction (PCR)

PCR was performed on plasmid DNA to generate fragments for cloning or on viral DNA to confirm that the desired homologous recombination event had occurred. PCR amplification was achieved using the methodology described by Saiki and colleagues (Saiki *et al.*, 1985). For a standard 50µl reaction, 1µl viral DNA or 1µl plasmid DNA (1-10ng) was added to a 0.5ml PCR tube containing 5µl (10x) pfu buffer (100mM Tris-HCl pH 8.3, 500mM KCl, 20mM $MgCl_2$, Promega), 1µl of a 40mM dNTPs (dATP, dTTP, dCTP and dGTP) mixture, 100ng of each primer (10pmoles/µl), 0.5µl Taq DNA polymerase (5units/µl, Promega), and ddH_20. For cloning, proof reading DNA polymerase (pfu) was used (Promega). Filter pipettes were used to avoid contamination and all solutions were subjected to UV energy (999,900 uJ/cm^2) prior to their use to crosslink any contaminating DNA. The mixture was overlaid with 50µl of mineral oil to prevent evaporation and was put through different thermal

cycles: 1 cycle at 94°C for 2min, 30 cycles at 94°C for 30sec, at 50-60°C (depending on the Tm of the primers used) for 30sec and at 72°C for 1min (depending on the length of the PCR product; ~1min/kb), and 1 cycle at 72°C for 10min. PCR reactions were performed in an Eppendorf Mastercycler. The PCR products were visualized on 1% agarose gel.

2.2.17 Quantitative Reverse Transcriptase (qRT)-PCR

QRT-PCR experiments were performed by Ronald De Hoogt at Johnson & Johnson R&D in Belgium. Total RNA from cultured 293T cells was extracted and DNaseI treated using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Briefly, cell monolayers were lysed in RLT buffer containing guanidinium thiocyanate and ß-mercaptoethanol. The lysate was DNaseI treated and loaded onto a silica membrane. The RNA binds to the silica strongly, contaminants were washed away, and the RNA was eluted in water. 3µg of total RNA served as template for cDNA synthesis using Oligo dT primers and Superscript III RT (Invitrogen) in a volume of 20µl for 1 hour at 50°C. This was followed by inactivation of the enzyme at 70°C for 15min according to the manufacturer's instructions. Quantitative PCR was performed on an ABI Prism 7900-HT Sequence Detection System (Applied Biosystems) using a qPCR core kit without dUTP (Eurogentec). The thermal cycling conditions were 10min at 95°C, followed by 45 cycles of 15sec at 95°C and 1min at 60°C. Validated pre-designed Taqman Gene Expression Assays (Applied Biosystems) corresponding to the housekeeping genes ATP5b (Hs00969569_m1), B2m (Hs99999907_m1), GAPDH (Hs99999905_m1), GUSb (Hs99999908_m1) and an interferon response gene ISGF3G (Hs_00196051_m1) were used to generate standard curves on serial dilutions of cDNA. The relative standard curve method was used to calculate the expression values. Values were normalized against the values obtained for the B2m gene.

RNA from whole DRG was extracted using the Rneasy 96 kit (Qiagen) according to manufacturer's instructions. RNA was eluted in 100µl H_20 and vacuum dried to approximately 10µl. The RNA was subsequently amplified using a modified version of the two-cycle amplification kit from Affymetrix. The reaction was stopped after the first amplification step and a DNAseI digestion was performed. The RNA was cleaned in a 96-well plate, eluted twice in 50µl H_20 and vacuum dried to approximately 20µl. First strand cDNA synthesis was performed on 1µg of amplified RNA using random hexamer primers and Superscript II RT (Invitrogen). Quantitative PCR was performed using the TaqMan PCR kit (Applied Biosystems). Serial dilutions of cDNA were used to generate standard curves of threshold cycles versus the logarithms of concentration for Sart1 (endogenous control, mouse squamous cell carcinoma antigen recognised by T cells) and Trpv1 or GFP. For Sart1 mRNA levels, the TaqMan Gene Expression Assay (Applied Biosystems) was used. The Trpv1 primers and probe were designed manually:

Forward primer: 5'-AGAATTCAGTGCTGGAGGTGATC-3'

Reverse primer: 5'-GGCTCCACGAGAAGCATGTC-3'

Probe sequence: Carboxyfluorescein-5'-GAGCCAAGGGACCCACCCCATGGAGAGC-3'-tetramethylrhodamine (Eurogentec).

The GFP primers and probe were also designed manually:

Forward primer: 5'-AGCAAAGACCCCAACGAGAA-3'

Reverse primer: 5'-GGCGGCGGTCACGAA-3'

Probe sequence: Carboxyfluorescein-5'-CGCGATCACATGGTCCTGCTGG-3'-tetramethylrhodamine

2.3 TISSUE CULTURE TECHNIQUES

All tissue culture solutions, media and supplements were obtained from Gibco. All manipulations of cells were carried out under sterile conditions using standard aseptic techniques.

2.3.1 Freezing and Recovery of Cells

Cells were stored in liquid nitrogen suspended in 8% (v/v) dimethyl sulfoxide (DMSO), 30% (v/v) FCS and 68% (v/v) of the appropriate full growth medium (FGM) described in section 2.1.4. The cells from one 175cm^2 flask were trypsinized, resuspended in 1.8ml of this solution and aliquoted in freezing vials, which were slowly cooled to -70^{o}C and then immersed in liquid nitrogen. DMSO (Sigma) is toxic above 4ºC and thus when recovered, cells were rapidly thawed to minimize the toxic effects of the cryoprotectant, transferred to a 25cm^2 flask and slowly diluted in pre-warmed media. The media was changed the following day.

2.3.2 Routine Cell Passage

Cells were grown in 175cm^2 flasks in the appropriate media (see section 2.1.4). They were incubated at 37ºC/ 5% CO_2 and routinely passaged when 85-90% confluent. The medium was carefully removed and the cell monolayer was washed with HANKS balanced salt solution (HBSS) and incubated with trypsin: versene (1:10) until the cells were detached from the surface of the flask. The trypsinized cells were disaggregated, resuspended in a small volume of FGM to neutralize trypsin and seeded at a ratio of 1:10 (BHK and MAM49) or 1:3 (293T). When cells were grown in roller bottles, the roller bottles were gassed with $C0_2$ to a final concentration of 5% and placed in a rotary roller apparatus.

2.3.3 Transfection of Plasmids for Transient Expression

293T cells ($3x10^5$) grown in 24-well plates until 90% confluent were transiently transfected with supercoiled plasmid DNA using Lipofectamine (Invitrogen). For a single well transfection, the appropriate amount of plasmid DNA was diluted in 50µl DMEM and mixed gently. The appropriate amount of Lipofectamine was also diluted in 50µl DMEM, mixed gently and incubated for 5min at room temperature. The Lipofectamine and DNA were combined at a ratio 1:1 (v/w) and allowed to form complexes over 30min at room temperature. The cell media was replaced with 0.5ml DMEM, the Lipofectamine-DNA complexes (100µl) were added to the cells and the cells were incubated at 37°C for 4 hours. Following this incubation, the cells were overlaid with 0.5ml of FGM. The following day the media was replaced.

2.3.4 Construction of Stable Cell Lines

Stable cell lines based on BHK cells were generated by transfection of linearised plasmid DNA using a variation of the standard calcium phosphate transfection method (Stow & Wilkie, 1976).

HEBES Transfection Buffer:	140mM NaCl
	5mM KCl
Filter sterilise with a 0.2µm filter	0.7mM Na_2HPO_4
Store at 4°C	5.5mM D-glucose
	20mM Hepes
	pH 7.05 with NaOH

BHK cells were grown in a 6-well plate until 70% confluent.

The following tubes were set up:

Tube A:	31µl 2M CaCl2 10µg linearised plasmid DNA 20µg herring sperm DNA
Tube B:	400ml HEBES

The contents of tube A were gently mixed and added to tube B very slowly whilst the contents of tube B were continuously mixed. The mixture was incubated at room temperature for 40min to allow the DNA to precipitate. Following this incubation, the media was removed from the cells and the mixture was added to the cell monolayer in a dropwise manner. The cells were incubated for 40min at 37^{o}C and after 1ml of FGM was added, they were incubated at 37^{o}C for a further 4 hours. The media was removed and the cells were washed twice with 1ml of FGM. 1ml of ice cold 25% (v/v) DMSO in HEBES was added and left for 1.5min (no more than 2.5min). The DMSO solution was quickly removed, the cells were quickly washed twice with 2ml of FGM and 2ml of FGM without antibiotic selection were added. The following day, the media was replaced with fresh FGM containing the appropriate antibiotic selection at the concentration determined by a killing curve. In this study, the plasmids transfected for the generation of stable cell lines were based on pcDNA3 that expresses the neomycin gene. The concentration of G418 has been previously determined at 800µg/ml and no killing curve was therefore necessary. The transfection was split into petri dishes (1:10). The selective media was changed every 48-72 hours until individual colonies were clearly visible. Colonies were selected using an inverted microscope and transferred to 96-well plates.

2.3.5 Screening and Purification of Cell Lines

Colonies grown in 96-well plates were screened for either GFP or ß-galactosidase expression (see sections 2.3.6 and 2.3.7). The colonies exhibiting the highest expression levels were selected and purified under continual selection. Each of the selected clones was then grown in a well of a 24-well plate until 85% confluent and infected with disabled virus at a multiplicity of infection of 1.0 and 5.0 to ensure that the cell line allows high levels of transduction. The optimal clones were selected and amplified further under continual selection. The cells from one 175cm^2 flask were frozen and stored in liquid nitrogen. A new vial was defrosted every 4 weeks.

2.3.6 Detection of B-galactosidase Expression

For cells grown in 6-well plates, the media was removed and the cells were washed twice with 2ml 1xPBS and fixed with 1ml 0.05% glutaraldehyde in 1xPBS for 10min at room temperature. The cells were washed with 2ml 1xPBS and incubated at 37oC with 2ml of pre-warmed x-gal solution (1.4µg/ml potassium ferrocyanide [$K_3Fe(CN)_6$] and 1µg/ml potassium ferricyanide [$K_3Fe(CN)_6 \cdot 6H_20$] in 1xPBS, 1µl/ml 1M $MgCl_2$, and 2µl/ml 0.015gr X-gal [5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside] in 100µl DMSO) until stained. The x-gal stain was then removed and the cells were washed with 1xPBS. For screening cell colonies or purification of viral recombinants expressing ß-galactosidase, the cells were not fixed and the x-gal was removed as soon as the cells were stained to minimize toxicity.

2.3.7 Detection of Green Fluorescent Protein Expression

Cells expressing GFP or EmGFP were visualised directly using an inverted microscope at a wavelength of 500nm.

2.4 VIRAL VECTOR CONSTRUCTION AND PROPAGATION

The replication-incompetent virus used in this study was derived from HSV-1 strain $17syn^+$ and has ICP4 deleted and also an inactivating mutation (in814) in VP16. The virus was propagated on the MAM49 cell line, which complements deletions in both ICP4 and ICP27 and mutations in vwm65. The in814 mutation was also complemented by addition of 100mM hexamethyl bisacetamide (HMBA) to the growth medium.

2.4.1 Transfection for Homologous Recombination

Insertion of expression cassettes into the HSV-1 genome was achieved by homologous recombination. 80-85% confluent MAM49 cells were co-transfected with plasmid and viral DNA, using the standard calcium phosphate technique (Stow & Wilkie, 1976). 0.5-1µg of supercoiled or linearised plasmid DNA and 10-20µg of purified viral genomic DNA, together with 31µl of 2M $CaCl_2$ and 1µl of phenol-chloroform extracted herring sperm DNA (1µg/µl) were added to tube A and gently mixed. The contents of tube A were slowly added to tube B containing 400µl of room temperature Hebes transfection buffer (140mM NaCl, 5mM KCl, 0.7mM Na_2HPO_4, 5.5mM D-glucose, and 20mM Hepes), whilst tube B was continuously but gently mixed. The DNA was allowed to precipitate at room temperature for 30-40 minutes and the transfection mixture was added to the cells in a dropwise manner. The cells were incubated at 37°C for 30 minutes, and after 1ml of FGM was added to each well, they were incubated at 37°C for a further 5 hours. Cells were incubated with 1ml of 20% (v/v) DMSO in Hebes buffer for no more than 90 seconds and were immediately washed twice with 2ml FGM. 2ml FGM containing 3mM HMBA were added and the transfection was incubated at 37°C/ 5% CO_2 until cytopathic effect (CPE) had occurred. It was then harvested and freeze-thawed once to disrupt the cells and release the virus.

2.4.2 Purification of Viral Recombinants by Plaque Selection

Serial 10-fold dilutions of the original transfection were prepared in 100µl DMEM. 80% confluent MAM49 cells were infected in a total volume of 500µl/well and incubated at 37°C for 40-60 minutes to allow for the virus to be absorbed. Cells were overlaid with 2ml of a 1:2 (v/v) mixture of 1.6% (w/v) carboxymethyl cellulose (CMC): FGM containing antibiotic selection and HMBA, and incubated at 37°C for 48 hours. Recombinants plaques were picked using either GFP fluorescence microscopy or x-gal staining at a volume of 10µl using a P20 Gilson micropipette. Each plaque was resuspended in 100µl DMEM, freeze-thawed and used to infect two wells of a 6-well plate at 10µl and 90µl volumes. The plaque purification process was repeated until a pure population was obtained. Once pure, each plaque was used to infect a single well of a 6-well plate. When CPE was observed, the wells were harvested, freeze-thawed and half of the virus was used to generate a master viral stock, whereas the rest was kept at –80°C. Virus genome structures were confirmed by PCR.

2.4.3 Production of High Titre Viral Stock

MAM49 cells grown in ten 175cm^2 flasks until 80-90% confluent were seeded in ten 850cm^2 roller bottles, each containing 50ml FGM, gassed with 5% CO_2 and incubated at 37°C/ 5% CO_2 until 85-90% confluent. Roller bottles were infected with virus master stock at a multiplicity of infection (MOI) of 0.01 in a total volume of 500ml FGM supplemented with 3mM HMBA, and incubated at 37°C/ 5% CO_2 for 24 hours and then at 32°C/ 5% CO_2 until full CPE was observed. Cells were detached from the walls of the roller bottles by vigorous shaking and freeze-thawed. The cell suspension was transferred in 50ml tubes and centrifuged at 3,500rpm for 45 minutes at 4oC to remove the cell debris. The supernatant was filtered using a 5µm/0.45µm filter (SARTOBRAN 300 from sartorius) and centrifuged at 12,000rpm for 2 hours at 4°C to pellet the virus. The supernatant was discarded ant the

pelleted virus was resuspended in 500µl DMEM by gentle shaking overnight at 4°C. The virus was stored in liquid nitrogen in 25µl aliquots. One aliquot was freeze-thawed and used to determine the viral titre of the stock.

2.4.4 Viral Infectivity (Plaque) Assay

Serial 10-fold dilutions of the virus were prepared in DMEM (10^{-1}-10^{-10}) and plated onto 85% confluent MAM49 cells. 24-well plates were infected in a volume of 200µl DMEM per well. Cells were incubated at 37°C for 40-60 minutes to allow for the virus to be absorbed, overlaid with 1ml of CMC: FGM [1:2] containing 3mM HMBA and incubated at 37°C for a further 48 hours. Plaques were visualized by x-gal staining or GFP fluorescent microscopy. The viral titre was calculated as plaque forming units/ml (pfu/ml).

2.4.5 Viral DNA Extraction

Infected MAM49 cells were harvested from 175cm^2 flasks when full CPE was observed. The medium was removed from the flask and 7ml of DNAzol (genomic DNA isolation reagent, Helena Biosciences) were added to the monolayer. The lysate was transferred to an empty tube and overlaid with an equal volume of absolute ethanol to precipitate the viral DNA. The DNA was washed twice in 3ml of 75% ethanol, transferred to a clean tube and centrifuged at 5,000rpm for 1min. Excess ethanol was removed with a hypothermic needle and the pellet was allowed to air-dry at room temperature for 20min. The pelleted viral DNA was resuspended in 500µl of 8mM NaOH and incubated at 4°C overnight to allow for the viral DNA to dissolve. The following day, 57.5µl of 0.1M Hepes were added to the solution to neutralize the NaOH and the DNA was stored at –20°C until required. The integrity and approximate amount of the purified viral genomic DNA was determined by running 5µl on a 1% agarose gel.

2.5 PRIMARY NEURONAL CULTURES

2.5.1 Coating Coverslips

25mm sterile coverslips were placed into a 12-well plate and covered with poly-D-lysine (100µg/ml) overnight. The poly-D-lysine was removed and the coverslips were washed thoroughly with sterile H_20 and were allowed to dry completely (for approximately 2 hours) at room temperature.

2.5.2 Primary DRG Neuronal Cultures

A Lewis rat was terminally anesthetised. The head was removed and an incision was made along the length of the spinal column to expose the DRG. Each DRG was dissected, rinsed in 1xPBS, and the membrane and excess nerve were removed with fine forceps and scalpel. The ganglia were placed in 1.6ml DMEM. 400µl collagenase (Roche) and 40µl trypsin were added and the ganglia were incubated at 37^{o}C for 2 hours with agitation every 20min. The ganglia were washed twice in DMEM containing 10% horse serum to inactivate the collagenase and were dispersed using polished Pasteur pipettes with diminishing diameter. The DMEM was removed and 12ml of neurobasal medium supplemented with NSF-1, L-glutamine and penicillin-streptomycin was added to the cells that were plated on poly-D-lysine coated coverslips and incubated at 37^{o}C/ 5% CO_2. The following day, fresh pre-warmed media containing mitotic inhibitors (7.5µg/ml 5-fluoro-2'deoxyuridine and 17.5µg/ml Ara-C) were added to inhibit proliferation of Schwann cells. Neurons were allowed to mature for 9 days prior to being transduced. Half of the media was changed with fresh pre-warmed media every 3 days.

2.6 *IN VIVO* DELIVERY

All surgical instruments were obtained form Fine Science Tools (FST) and were sterilized in an autoclave for 20min at 120°C prior to each surgical procedure. Only one set of instruments was used per animal. Alternatively, the instruments and needle were autoclaved after each operation to avoid spreading infections between animals. A Zeiss operation microscope was used in performing surgical procedures. All animal procedures were carried out in accordance to UK Home Office Regulations.

2.6.1 Anaesthesia

Balb/c mice 6-8 weeks old were anesthetised by delivery of 1.5% oxygen, 3% nitrous oxide and 4% halothane in an anaesthetic chamber. Once sedated, the animal was prepared for surgery, transferred to the operating theatre and placed on a heated pad where anesthesia was delivered via a scavenging nose cone. Once the animal was deeply anesthetised, as indicated by the loss of toe-pinch reflex, the level was reduced to 1.5-2% to maintain anesthesia during surgery. The breathing pattern of the animal was monitored regularly and the anesthesia adjusted accordingly. Post-operatively the animals were kept in a clean cage and monitored every hour for the first six hours and then every day until stable.

2.6.2 Animal Preparation

Veterinary clippers were used to shave the appropriate areas prior to surgery. The skin was cleaned with 70% ethanol and the animal was covered with a sterile drape exposing only the areas of interest.

2.6.3 Sciatic Nerve Injection & Conditioning lesion

A skin incision, parallel and caudal to the femur, was made in the left thigh using a scalpel and the underlying muscles were exposed. The muscles (biceps femoris and semitendinosus muscles) were separated by blunt dissection and the left sciatic nerve was exposed. Fine forceps were used to separate the two main branches of the sciatic nerve. The tibial nerve, which is the larger branch, was injected with 5-7µl of virus over a period of 15min using a 10µl Hamilton blunt end syringe connected to a glass needle (Sigma; 50µl microcapillary pipettes) via fine tubing (SIMS Portex Ltd; Portex fine bore polythene tubing). The needle was carefully withdrawn, the nerve was loosely ligated with silk 5/O suture to prevent any outflow of virus and the skin was closed with Michel clips. Conditioning lesion of the sciatic nerve was performed following injection of the virus and ligation of the sciatic nerve by crushing both branches using fine forceps.

2.6.4 Transection of the dorsal root (Rhizotomy)

A left hemilaminectomy was performed at the L2 vertebra and the dura was opened. The L3 and L4 dorsal roots were identified by their large diameter and transected with microsurgical scissors. The cut ends were reanastomosed with a single 10/O suture. The dura was closed with 10/O sutures, the musculature was repaired with silk sutures and the skin was closed with Michel clips. This procedure was performed by Prof. Patrick Anderson.

2.6.5 Transcardial Perfusion

Animals were placed in a chamber and terminally anesthetised with $C0_2$. All animals were sacrificed in accordance to Schedule 2 Home Office regulations. The heart was exposed and after an incision of the pericardium was made, a blunt end needle was inserted into

the left vertical and 4% paraformaldehyde (PFA) in PBS was pumped through the heart using a peristaltic pump at a rate of 30rpm. When the animals were sufficiently perfused, the lumbar DRG (L1 to L6) ipsilateral to the inoculated side and/or the spinal cord were dissected and post-fixed in ice-cold 4% PFA for 1 hour on ice, after which time the tissue was washed twice with 1xPBS and sectioned.

2.6.6 Sectioning of DRG and Spinal Cords

Lumbar DRG and/or spinal cords were dissected from perfused animals and cryoprotected in 30% sucrose in 1xPBS at 4°C until no longer buoyant. The tissues were embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, N.C.) and immediately frozen. 10-20µm cryosections were cut on a Microm HM 500 M Cryostat and placed on Superfrost glass slides (WVR). The sections were stored at –80°C until either x-gal staining or immunofluorescence was performed. To assess GFP fluorescence, animals were not perfused and DRG sections were fixed with 70% ethanol instead of 4% PFA.

2.6.7 Detection of ß-galactosidase and GFP in whole DRG

Animals were not perfused. The lumbar DRG were dissected, post-fixed in 4% PFA overnight, washed twice with 1xPBS for 15min and stained with x-gal (5mM $[K_3Fe(CN)_6]$ and 5mM $[K_3Fe(CN)_6{\cdot}6H_20]$ in 1xPBS, 1mM $MgCl_2$, 0.02% sodium deoxycholate, 0.02% NP-40 and 40mg/ml x-gal in DMSO) for no more than 3 hours. To assess expression of both GFP and ß-galactosidase, GFP fluorescence was examined prior to fixation and x-gal staining, as this otherwise masks GFP fluorescence. Cell counts were performed in a blinded fashion in whole mount DRG preparations at a magnification of x40.

2.6.8 Processing Tissue for Protein Extraction

Animals were not perfused. The DRG were dissected on ice, rinsed with 1xPBS and immediately snap-frozen in liquid nitrogen. They were stored at –80^{o}C until homogenized and protein was extracted as described in section 2.7.1.

2.6.9 Processing Tissue for RNA Extraction

Animals were not perfused. The DRG were immediately dissected, rinsed with DEPC-treated 1xPBS and placed in sterile tubes containing RLT buffer (Qiagen) containing ß-mercaptoethanol. The DRG were immediately homogenized and the RNA was extracted as described in section 2.2.17.

2.7 PROTEIN ISOLATION & ANALYSIS

2.7.1 Extraction of Protein from DRG

The frozen DRG were transferred to a pestle and mortar which had been cooled on ice and were immediately homogenized in ice-cold RIPA (radio immuno precipitation assay) lysis buffer (150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 50mM Tris, pH 8.0) containing a cocktail of protease inhibitors (Roche). The homogenate was first passed through a 21 gauge needle 10 times and then a 25 gauge needle 10 times. It was then transferred to an ice-cold 1.5ml microcentrifuge tube and constant agitation was maintained for 2 hours at 4°C. The sample was centrifuged at 12,000rpm for 20min at 4°C and the supernatant was transferred to a clean ice-cold tube and kept on ice. The protein concentration was determined using the Bradford assay (Bio-Rad) according to the manufacturer's protocol and the concentration between samples was equalised in RIPA buffer so that 1-5mg/ml protein was loaded onto each gel. The samples were denatured in Laemmli standard loading buffer (5% β-mercaptoethanol, 50mM Tris-HCl pH 8.0, 6% (v/v) glycerol, 2% (w/v) SDS and 0.005% (w/v) bromophenol blue) at 70°C for 10min and were either loaded onto an SDS-polyacrylamide gel or stored at -20°C.

2.7.2 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The Laemmli system, which is the most widely used SDS-PAGE method for separating proteins (Laemmli, 1970), operates in a highly alkaline pH and may cause band distortion, loss of resolution, or artifact bands. For this reason, the NuPAGE® system (Invitrogen) was used, which operates in neutral pH 7.0 resulting in maximum stability of both proteins and gel matrix, thus providing better band resolution. 4-12% NuPAGE® Bis-Tris pre-cast gels were run in the XCell SureLock™ Mini-Cell vertical gel electrophoresis system (Invitrogen) in 1xNuPAGE® MES [2-(N-morpholino) ethane sulfonic acid] SDS Running Buffer. The upper buffer chamber (inner) was filled with running buffer above the level of the wells, the

wells were rinsed with running buffer, 10µl of denatured sample and 3µl of rainbow marker (Amersham) were loaded onto the gel and the lower buffer chamber (outer) was filled with 600ml of running buffer. The gels were run for 1 hour at 110/125 mA/gel (start) – 70/80 mA/gel (end).

2.7.3 Western Blotting

Proteins separated on NuPAGE® Bis-Tris gels were transferred to Hybond C nitrocellulose membranes (Amersham) using the wet transfer method (Towbin *et al.*, 1979). Briefly, the gel and nitrocellulose membrane were pre-soaked in 1xNuPAGE® Transfer Buffer (Invitrogen) and sandwiched between sheets of pre-soaked Whatman 3MM paper and Trans-Blot™ Cell (Bio-Rad). The transfer was performed at 200mA for 2 hours.

2.7.4 Immunodetection of Proteins on Western Blots

Nitrocellulose membranes were rinsed in 1xPBS with 0.1% Tween-20 (PBST) and blocked in 5% (w/v) skimmed milk in PBST overnight at 4°C with constant shaking. The blocked membranes were incubated with primary antibody diluted in 5% (w/v) skimmed milk in PBST overnight at 4°C with constant shaking (see section 2.1.7 for table of primary antibodies and dilutions) and washed three times at room temperature for 10min with PBST with constant shaking. They were then incubated with an HRP-conjugated secondary antibody diluted 5% (w/v) skimmed milk in PBST for 1 hour at room temperature with constant shaking (see section 2.1.7 for table of secondary antibodies and dilutions) and washed twice with PBST and once with PBS at room temperature for 10min with constant shaking. Immunodetection was performed using the ECL+ reagents (Amersham Biosciences). The membranes were exposed on x-ray film for 1sec to 10 minutes depending on the strength of the signal. The films were scanned and the pixel count and intensity of each band was quantified using the Scion Image software (Scion, Frederick,

MD). Signals were normalised against α-tubulin (loading control) and the result was expressed as a percentage of the negative control signal.

2.7.5 B-galactosidase Activity Assay

For determining ß-galactosidase protein levels, the High Sensitivity ß-Galactosidase Assay kit (Stratagene) was used according to the manufacturer's instructions. Cells were lysed and cell lysates were incubated with a reaction buffer and chlorophenol red-ß-D-galactopyranoside (CPRG) substrate. The activity of ß-galactosidase was quantified using a microplate reader.

2.7.6 Immunofluorescence

Sections were defrosted, fixed in 4% PFA for 15min, washed three times with 1xPBS and blocked in Image-iT FX Signal Enhancer (Molecular Probes) for 30min at room temperature. For Immunodetection of intracellular proteins, the cells were permeabilised by incubation with 1xPBS containing 0.25% Triton x-100 (PBST) for 10min at room temperature and washed three times in 1xPBS for 5min. The sections were then incubated with a mixture of primary antibodies in 1% bovine serum albumin (BSA) in PBST for 1 hour at room temperature in a humidified chamber, washed three times with 1xPBS for 5min and then treated with a mixture of secondary antibodies in 1% BSA for 1 hour at room temperature in a humidified chamber (see section 2.1.7). Coverslips were mounted with a drop of mounting medium, were sealed with nail polish to prevent drying and stored in the dark at -20^{o}C. All photographs were taken using an Axiovert 200M microscope equipped with an Axiocam HRc colour camera (Carl Zeiss, Jena, Germany).

CHAPTER 3

DEVELOPMENT AND EVALUATION OF REPLICATION-DEFECTIVE HSV-1 VECTORS EXPRESSING shRNA USING DIFFERENT APPROACHES

3.1 Introduction

The choice of promoter driving expression of shRNA is very important in the design of an efficient and versatile HSV-based method to deliver RNAi to neurons of the peripheral nervous system. Optimisation of shRNA expression will allow delivery of lower vector doses, thus reducing potential side effects, such as toxicity caused by the virus or off-target effects caused by shRNA. This chapter describes the development of a number of replication-defective HSV-1 vectors expressing shRNA against reporter genes. The aim of this chapter was to compare different shRNA expression systems described in the literature in the context of the HSV genome when combined with LAT-based expression systems, and evaluate their ability to silence target gene expression in DRG sensory neurons, with a view to developing an optimal system on which to base future work.

The pol III U6 and H1 promoters had been most commonly used to express shRNA from plasmid and viral vector systems (Abbas-Terki *et al.*, 2002, An *et al.*, 2003, Brummelkamp *et al.*, 2002, Huang *et al.*, 2004, Paul *et al.*, 2002, Sui *et al.*, 2002, Yu *et al.*, 2002) and thus, were an obvious choice with which to attempt to express shRNA from the disabled HSV-1 vectors used in this thesis. However, pol III promoters lack spatial and temporal control, and although inducible U6 and H1 promoters had been developed (Tiscornia *et al.*, 2004, van de Wetering *et al.*, 2003), *in vivo* application of such systems is challenging, as it requires co-delivery and expression of additional elements. Furthermore, previous work in our laboratory had demonstrated that the choice of promoter is important in achieving efficient and long-term HSV-mediated gene expression in neurons *in vivo* (Palmer *et al.*, 2000). As discussed in detail in the introduction of this thesis, expression from replication-defective HSV-1 vectors poses specific challenges, due to transcriptional silencing of most HSV promoters as well as exogenous promoters introduced into the latent viral genome. It has been suggested that the structure of LAP2 and its surrounding regions may be important for the LAT region to remain transcriptionally active during latency (Coffin *et al.*, 1995). In support of this hypothesis, a

sequence 1.4kb downstream of the LAP1 TATA box (referred to as LATP2) linked to a strong heterologous pol II promoter has been shown to drive highly efficient and sustained expression of reporter genes during latency in both the peripheral and central nervous system (Lilley *et al.*, 2001, Palmer *et al.*, 2000). Palmer *et al.* however, who investigated reporter gene expression in DRG from different promoters, demonstrated that the LATP2 region has the capability to allow long-term expression in a relatively non-promoter specific fashion. At the time the objectives of this chapter were conceived, HSV-mediated expression from pol III promoters had not been investigated and thus, there was no evidence to suggest that following insertion of the U6 or H1 promoter downstream of the LATP2 region, the LAT region would enable these promoters to remain transcriptionally active during latency.

In addition to pol III promoters, a modified pol II CMV promoter (Xia *et al.*, 2004, Xia *et al.*, 2002) and a hybrid CMV enhancer-U6 promoter (Xia *et al.*, 2003) had been utilised to express shRNA. The modified CMV promoter was found to be more efficient than the H1 promoter in PC3-6 neural cells, and the CMV enhancer element was shown to significantly improve silencing from the U6 promoter in HEK-293 cells. Moreover, an HIV-inducible pol II promoter was shown to efficiently express shRNA from a lentiviral vector and silence HIV-1 Rev protein in culture (Unwalla *et al.*, 2004). Finally, Zeng and colleagues demonstrated that shRNA could be efficiently expressed from the CMV promoter when embedded into endogenous miR-30 sequences and that these artificial miRNAs could induce specific degradation of target mRNAs similar to transfected siRNAs (Zeng & Cullen, 2003, Zeng *et al.*, 2002). Silencing of HIV-1 *tat* in 293 cells was found to be significantly improved when shRNA was expressed from a miR-30-based vector under the control of the U6 promoter compared to shRNA expressed from the same promoter (Boden *et al.*, 2004). Pol II promoters can be inducible or tissue-specific and the CMV promoter had been previously shown to allow efficient and long-term gene expression from the disabled HSV vectors developed in our lab (Lilley *et al.*, 2001, Palmer *et al.*, 2000). Moreover, it was

hypothesised that insertion of a hybrid CMV enhancer-U6 promoter downstream of the LATP2 region would be less likely to disturb the structure of the region surrounding LAP2, as the CMV promoter elements in the proximity of the LATP2 region would be retained.

At the time work in this chapter begun, silencing in the brain had been achieved through vector-mediated expression of shRNA from a lentivirus using the U6 promoter (Van den Haute *et al.*, 2003) and an adenovirus or AAV using a modified CMV promoter (Xia *et al.*, 2004, Xia *et al.*, 2002). However, expression of shRNA from an HSV-1 vector had not been demonstrated, and there were no reports of vector-mediated expression of shRNA in peripheral neurons *in vivo*. Moreover, *in vivo* expression of shRNA from a miRNA-like system or a hybrid CMV enhancer-U6 promoter had not been investigated. Thus, in order to identify the most effective system on which to base future work, expression of shRNA from the U6 promoter, a hybrid CMVenhancer-U6 promoter, a modified CMV promoter, or a miR-like system utilising the CMV promoter were evaluated, when linked to LAT-derived sequences, in dividing cells in culture and both *in vitro* and in DRG neurons *in vivo* following insertion of these cassettes into disabled HSV-1 vectors.

3.2 The 1764/4-/27+/RL1+ HSV-1 backbone

All virus backbones used in this thesis are 1764/4-/27+/RL1+. They were derived from HSV-1 strain 17syn+ (Brown *et al.*, 1973). The term 1764 is used to describe a virus with the in814 mutation in the gene encoding VP16 (Ace *et al.*, 1989) and with the genes encoding the neurovirulence factor ICP34.5 and ORF P completely deleted (McLean *et al.*, 1990). The *in814* mutation renders VP16 capable of fulfilling its structural role, yet incapable of transactivating IE gene expression. The term 4- refers to the deletion of ICP4, which is necessary for minimal toxicity. The 1764/4-/27- vectors initially developed in our lab had ICP27, ICP4 and ICP34.5 deleted and had the inactivating *in814* mutation in vmw65 (Lilley *et al.*, 2001, Palmer *et al.*, 2000). 27+ and RL1+ describe the re-insertion of ICP27 and ICP34.5 into the HSV genome (Palmer, unpublished). Re-insertion of ICP27 increases the ease of the production of the virus without significantly increasing toxicity, and re-insertion of ICP34.5 increases the expression levels of delivered genes in the context of this vector backbone (unpublished work). A schematic representation of the 1764/4-/27+/RL1+ vector backbone is shown in figure 3.1a.

3.3 The Complementing Cell Line

The complementing cell line 27/12/M:4 used for the propagation of the 1764/4-/27+/RL1+ viruses was previously constructed in our laboratory (Thomas *et al.*, 1999b). It expresses the equine herpesvirus 1 (EHV-1) homologue of vmw65, which has minimal similarity to the HSV gene to minimise the likelihood of the vmw65 mutation being repaired by homologous recombination. Moreover, although the EHV-1 gene 12 protein can functionally compensate for mutations in vmw65, it cannot structurally substitute for vmw65. Thus, the protein is not packaged in the virions and cannot transactivate IE gene expression when the virus produced is used to transduce non-complementing cells. The 27/12/M:4 cell line also expresses ICP27 from the ICP27 promoter and ICP4 from the MMTV promoter. Importantly, in the absence of viral infection, EHV-1 gene 12, ICP27 and ICP4 are only

weakly expressed, resulting in no observed toxicity and cells that can be stably propagated. Following viral infection and presumably transactivation of ICP0 by the EHV-1 gene 12 protein, these genes are abundantly expressed allowing efficient propagation of disabled viruses.

3.4 The pR19 Expression Cassette

All viruses in this thesis utilise the pR19 cassette, which has been previously described (Wagstaff *et al.*, 1998). To facilitate recombination into the LAT region, the pR19 cassette is flanked by sequences derived from the long repeat regions of the HSV-1 genome (nt 118,441-120,219 and nt 120,413-122,027). A strong heterologous promoter is inserted 1.4kb downstream of the LAP1 TATA box, referred to as the LAT P2 region, which allows long-term expression during latency in both the peripheral and central nervous system (Lilley *et al.*, 2001, Palmer *et al.*, 2000). The gene is placed under the control of a pol II promoter, such as the CMV IE gene, EF1α or synapsin promoters, and the bovine growth hormone polyadenylation sequence (BGH polyA). Expression from these vectors was further improved by insertion of a mutated form of the woodchuck hepatitis virus post-transcriptional regulatory element (WCm) between the gene and BGH polyA. WPRE is required for the accumulation of wild-type WHV RNAs (Donello *et al.*, 1998) and has been shown to enhance transgene expression in retroviral and lentiviral vector systems both *in vitro* and *in vivo* (Glover *et al.*, 2003, Klein *et al.*, 2002, Paterna *et al.*, 2000) possibly by enhancing mRNA nuclear export (Zufferey *et al.*, 1999). A schematic representation of the pR19 cassette is shown in figure 3.1b.

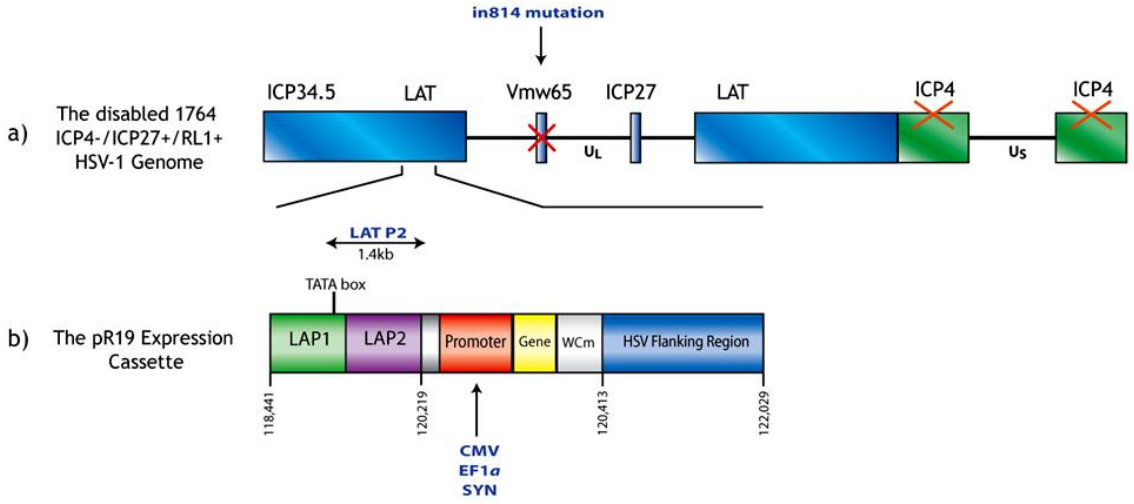


Figure 3.1 Schematic representation of the disabled 1764 4-/27+/RL1+ HSV-1 genome and pR19 cassette.

a) The virus is deleted for the essential IE gene ICP4 and also contains the *in1814* mutation in the Vmw65 gene encoding VP16, which abolishes transactivation of the remaining IE genes. These vectors are easily produced to high titres using a complementing cell line engineered to express ICP4 and the equine herpes virus homologue of VP16 (Thomas *et al.*, 1999)

b) The pR19 cassette contains flanking regions that facilitate recombination into the LAT region of the HSV genome. Following recombination with viral DNA, each cassette is present in two copies per genome. Insertion of a strong heterologous promoter, such as the CMV IE gene, EF1*a* or synapsin promoter, 1.4kb downstream of the LAP1 TATA box (after the LATP2 region) enables long-term expression of transgenes during latency in both the peripheral and central nervous system (Palmer *et al.*, 2000; Lilley *et al.*, 2001). Expression from these vectors was improved by addition of a mutated form of the WPRE element (WCm).

N.B. Not to scale

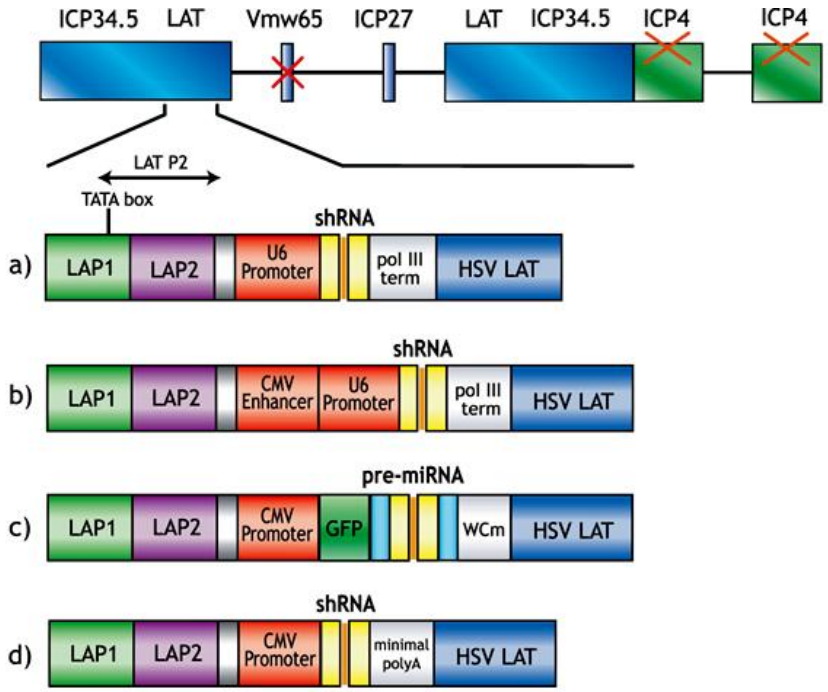


Figure 3.2 Expression of shRNA from the HSV-1 1764 4-/27+/RL1+ vector using different approaches

a) In the pR19U6shRNA cassette expression of shRNA is driven by the U6 pol III promoter that has been most commonly used to silence genes both *in vitro* and *in vivo*. Transcriptional termination by RNA pol III results in shRNA transcripts with defined ends that are recognised by Dicer.

b) In the pR19CMVenhU6shRNA cassette expression of shRNA is driven by the U6 pol III promoter and the enhancer element of the CMV pol II promoter, which has been shown to improve the activity of the U6 and H1 pol III promoters (Xia *et al.*, 2003; Ong *et al.*, 2005).

c) In the pR19CMV/EmGFP-miR cassette expression of pre-miRNA is driven by the CMV pol II promoter. To ensure that the miRNA is properly processed, the shRNA is inserted between 5' and 3' flanking regions derived from the endogenous miRNA-155 (Chung *et al.*, 2006). The reporter gene EmGFP is expressed from this system co-cistronically with the pre-miRNA to allow labelling of transduced cells.

d) In the pR19CMVshRNA cassette expression of shRNA is driven by the CMV pol II promoter. The hairpin is inserted almost immediately downstream of the CMV transcriptional start site and upstream of a synthetic minimal polyA (Xia *et al.*, 2002). This cassette results in the generation of functional siRNA molecules with minimal overhangs.

N.B. Not to scale

3.5 Generation of plasmid vectors expressing shRNA

In order to identify the most effective method of expressing shRNA from the 1764/4-/27+/RL1+ vector backbone, a number of different approaches, which are summarised in figure 3.2a-d, were evaluated. To compare silencing from these expression systems, plasmid vectors were generated to express a well-characterised hairpin sequence designed to target *lacZ*, the reporter gene encoding ß-galactosidase. Control plasmids were constructed to mediate expression of shRNA against green fluorescent protein (GFP) or a non-target shRNA sequence that is not predicted to target any known vertebrate gene (neg). Construction of these vectors is described in the following sections.

3.5.1 Expression of shRNA from a Polymerase III promoter

The pR19U6shRNA system (figure 3.2a) utilises the U6 pol III type promoter that has been widely used to successfully silence genes both *in vitro* and *in vivo*. The endogenous U6 promoter controls expression of the U6 RNA, a small nuclear RNA (snRNA) involved in splicing (Kunkel *et al.*, 1986), has been well characterised, and is active in most mammalian cell types. Transcriptional termination by RNA polymerase III provides a means of expressing shRNA transcripts with defined ends (Tazi *et al.*, 1993). Moreover, although most genes transcribed by RNA polymerase III require *cis*-acting regulatory elements within the transcribed regions, class III promoters such as U6 and H1, use exclusively non-transcribed promoter sequences (Reddy, 1988).

Inhibition of gene expression by a shRNA expressed from the U6 promoter has been shown to be more effective than inhibition by two siRNA strands expressed from separate U6 vectors (Yu *et al.*, 2002). Plasmid vectors allowing efficient expression of shRNA from the U6 promoter were commercially available and thus, the BLOCK-iT^{TM} U6 RNAi Entry Vector from Invitrogen (pENTR-U6) was used. The pENTR-U6 vector allows cloning of an approximately 50bp DNA oligonucleotide immediately downstream of the U6 pol III promoter and upstream the pol III termination sequence that consists of a cluster of 6

thymidine (T) residues (figure 3.3). The shRNA sequence consists of a 5'overhang (CACC), which allows directional cloning, the 1+-G of the U6 promoter, a 19nt sense sequence, a 4nt loop (CGAA), and a 19nt antisense sequence. Expression from this cassette results in the production of a shRNA, which enters the endogenous miRNA pathway, is transported to the cytoplasm and processed by Dicer into a 21nt siRNA. The siRNA guide strand becomes incorporated into a RISC, which targets mRNA with perfect complementarity to the siRNA for degradation resulting in effective knockdown of target gene expression.

The pENTR-U6 vector was supplied linearised with 4nt 5'overhangs on each strand to facilitate directional cloning of the double stranded DNA oligonucleotide. The validated sequence targeting ß-galactosidase (shLacZ) and the non-target shRNA sequence that is not predicted to target any known vertebrate gene (neg) were supplied by Invitrogen as annealed DNA oligonucleotides (sequences available in section 2.1.6) and were cloned into pENTR-U6 vectors (figure 3.3). The three sequences targeting GFP (shGFP1-3) were designed using Invitrogen's RNAi designer tool (sequences available in section 2.1.6). They were synthesized as single stranded DNA oligonucleotides, annealed and cloned into pENTR-U6 vectors. Each of the pENTR-U6shRNA vectors was sequenced using the U6 Forward and M13 Reverse primers (described in section 2.1.5) to ensure that the insert had been cloned in the correct orientation and more importantly, that the shRNA sequence had not been mutated (figure 3.4).

The pENTR-U6shRNA vectors allow transient expression of shRNA in cells that can be efficiently transfected with plasmid DNA and therefore enable rapid screening of shRNA sequences. Transfection of post-mitotic neurons is highly inefficient and often toxic. Thus, following initial screening, the U6shRNA cassette is transferred into an appropriate destination vector that serves as a shuttle plasmid and allows the cassette to be inserted into a disabled HSV vector.

The pR19-Gateway destination vector which was constructed contains flanking regions that allow recombination into the LAT region of the HSV genome (nt 118,441-

120,219 and nt 120,413-122,027) and the Gateway cassette (Invitrogen) that allows cloning using the recombination properties of bacteriophage lambda (figure 3.5e). The HSV flanking regions were derived from the pR19SYNeGFP vector (figure 3.5a), which drives expression of enhanced GFP from the synapsin promoter. To remove the BGH polyA sequence that is not essential for pol III expression, pR19SYNeGFP was digested with SacI-EcoNI. This step also removed the 5'end of the HSV nt 120,413-122,027 flanking region, which was excised as a BstXI-EcoNI fragment from pNot3.5 and cloned into pR19SYNeGFP (SacI-EcoNI) to generate the pR19SYNeGFP(no BGH polyA) vector (figure 3.5c). The pNot3.5 plasmid had been previously constructed in our laboratory (figure 3.5b). The LAT flanking regions were derived from the NotI region of the HSV genome (nt 118,439-122,025) and cloned into the unique NotI site of the pGEM5 cloning vector (Promega). The pR19SYNeGFP(no BGH polyA) vector was converted into a destination vector by cloning the Gateway cassette (figure 3.5d) between the ClaI-NsiI sites that had been blunted and treated with calf intestinal alkaline phosphatase (as described in sections 2.2.3 and 2.2.4). Finally, the U6shLacZ, U6-neg and U6shGFP2 (see section 3.6) cassettes, which were flanked by attL1-attL2 sequences, were inserted from pENTR (figure 3.5f) into pR19-Gateway by LR recombination (as described in section 2.2.9) to generate the pR19U6shLacZ, pR19U6-neg and pR19U6shGFP2 vectors, respectively. The cassettes were sequenced between the LAT flanking regions (sequences not shown). A map of pR19U6shRNA is shown in figure 3.5g.

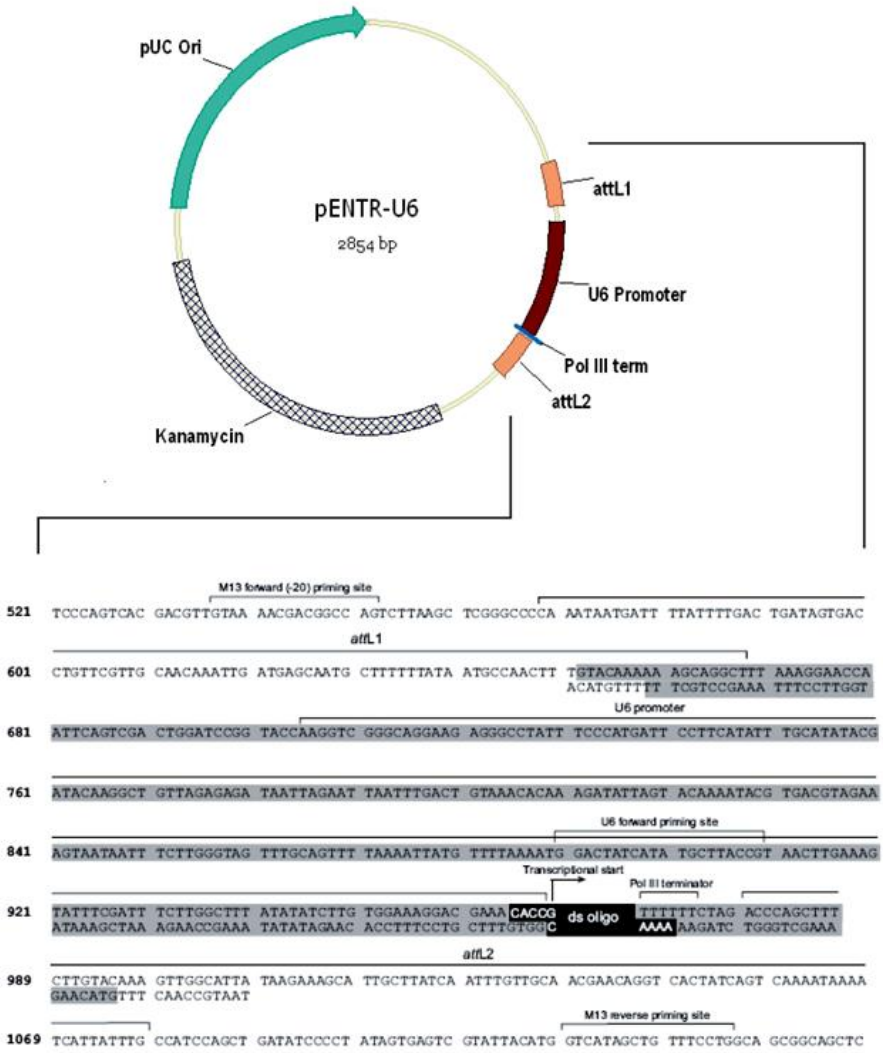


Figure 3.3 The pENTR-U6 vector from Invitrogen

The double stranded DNA oligonucleotide expressing the shRNA against the target gene is cloned immediately downstream of the U6 pol III promoter and upstream the pol III terminator that consists of a cluster of 6 thymidine (T) residues. The priming sites for the U6 Forward and M13 Reverse primers used to sequence the pENTR-U6shRNA plasmids are indicated in the vector sequence. The U6shRNA cassette is flanked by attL1-attL2 sequences that allow recombination into a destination vector (attR1-attR2).

a) pENTR-U6shLacZ sequencing

```
Sequence 1: lcl|1
Length = 47 (1 .. 47)  shLacZ

Sequence 2: lcl|65536
Length = 1358 (1 .. 1358)   pENTRU6shLacZ (U6 F primer)

Score = 91.1 bits (47), Expect = 2e-16
Identities = 47/47 (100%), Gaps = 0/47 (0%)
Strand=Plus/Plus

Query  1    CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTGTGTAG  47
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  37   CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTGTGTAG  83
```

The target sequence against ß-galactosidase in shLacZ:

```
gb|FJ440335.1|  Reporter cassette lacTeT, complete sequence
Length=5870

Score = 38.2 bits (19), Expect = 2.2
Identities = 19/19 (100%), Gaps = 0/19 (0%)
Strand=Plus/Minus

Query  29   AAATCGCTGATTTGTGTAG  47
            ||||||||||||||||||
Sbjct  745  AAATCGCTGATTTGTGTAG  727
```

b) pENTR-U6shGFP1-3 sequencing

```
Sequence 1: lcl|1
Length = 45 (1 .. 45) shGFP1

Sequence 2: lcl|65536
Length = 1434 (1 .. 1434) pENTRU6shGFP1 (M13 R primer)

Score = 87.2 bits (45), Expect = 3e-15
Identities = 45/45 (100%), Gaps = 0/45 (0%)
Strand=Plus/Minus

Query  1    CACCGCAAGCTGACCCTGAAGTTCGAAAACTTCAGGGTCAGCTTG  45
            |||||||||||||||||||||||||||||||||||||||||||||
Sbjct  174  CACCGCAAGCTGACCCTGAAGTTCGAAAACTTCAGGGTCAGCTTG  130
```

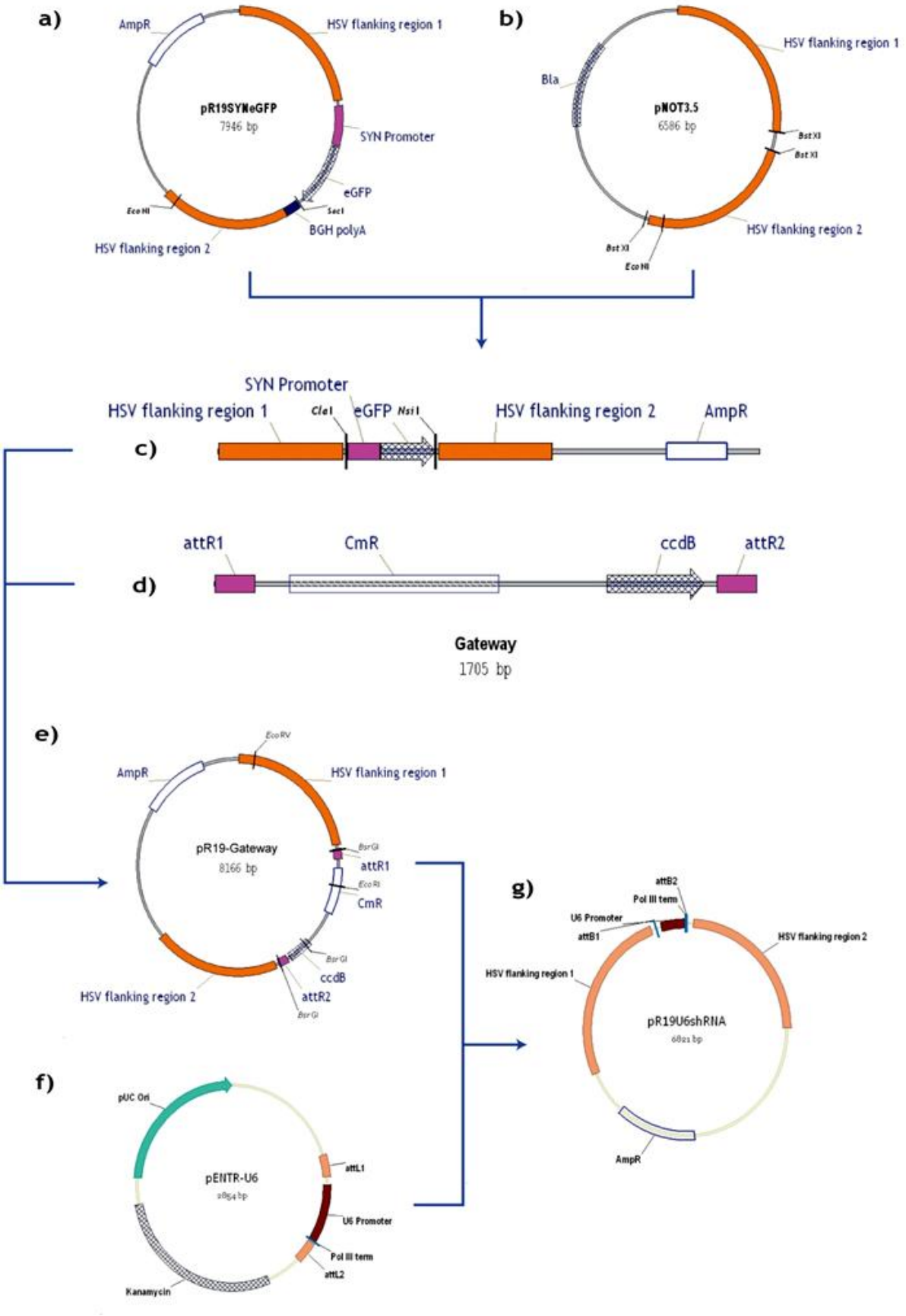
```
Sequence 1: lcl|1
Length = 50 (1 .. 50) shGFP2

Sequence 2: lcl|65536
Length = 1450 (1 .. 1450) pENTRU6shGFP2 (M13 R primer)

Score = 96.8 bits (50), Expect = 5e-18
Identities = 50/50 (100%), Gaps = 0/50 (0%)
Strand=Plus/Minus

Query  1    CACCGCATCAAGGTGAACTTCAAGACGAATCTTGAAGTTCACCTTGATGC  50
            ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  187  CACCGCATCAAGGTGAACTTCAAGACGAATCTTGAAGTTCACCTTGATGC  138
```

```
Sequence 1: lcl|1
Length = 50 (1 .. 50) shGFP3

Sequence 2: lcl|65536
Length = 1442 (1 .. 1442) pENTRU6shGFP3 (M13 R primer)

Score = 96.8 bits (50), Expect = 5e-18
Identities = 50/50 (100%), Gaps = 0/50 (0%)
Strand=Plus/Minus

Query  1   CACCGCCACAACGTCTATATCATGGCGAACCATGATATAGACGTTGTGGC  50
           ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 185 CACCGCCACAACGTCTATATCATGGCGAACCATGATATAGACGTTGTGGC  136
```

Figure 3.4 Blast nucleotide sequence alignment between pENTR-U6shRNA sequences obtained with the U6 Forward or M13 Reverse primers (described in section 2.1.5) and the oligonucleotide sequences expressing shRNA against a) ß-galactosidase (shLacZ) or b) enhanced GFP (shGFP). The target sequence against ß-galactosidase in shLacZ is indicated in the reporter cassette lacTeT sequence.

Figure 3.5 Cloning of pR19U6shRNA plasmids

The details of the cloning can be found in section 3.5.1. Briefly, pR19SYNeGFP was converted into the pR19-Gateway vector by removing the BGH polyA and cloning the Gateway cassette between ClaI-NsiI. The U6shLacZ, U6-neg and U6shGFP2 cassettes were inserted from pENTR (attL1-attL2) into pR19-Gateway vectors (attR1-attR2) by LR recombination to generate the pR19U6shLacZ, pR19U6-neg and pR19U6shGFP2 plasmids, respectively.


a)
AmpR
HSV flanking region 1
pR19SYNeGFP
7946 bp
SYN Promoter
eGFP
EcoNI
SacI
BGH polyA
HSV flanking region 2
b)
HSV flanking region 1
Bla
pNOT3.5
6586 bp
BstXI
BstXI
BstXI
EcoNI
HSV flanking region 2
SYN Promoter
HSV flanking region 1
ClaI
eGFP
NsiI
HSV flanking region 2
AmpR
c)
attR1
CmR
ccdB
attR2
d)
Gateway
1705 bp
e)
EcoRV
AmpR
HSV flanking region 1
pR19-Gateway
8166 bp
BsrGI
attR1
EcoRI
CmR
BsrGI
ccdB
attR2
BsrGI
HSV flanking region 2
f)
pUC Ori
attL1
pENTR-U6
2854 bp
U6 Promoter
Pol III term
attL2
Kanamycin
g)
attB2
Pol III term
U6 Promoter
attB1
HSV flanking region 2
HSV flanking region 1
pR19U6shRNA
6821 bp
AmpR

3.5.2 Expression of shRNA from a hybrid Pol II-Pol III promoter

Whilst some snRNAs are synthesized by RNA pol III, others are synthesized by pol II. These promoters have been shown to share enhancer elements (Carbon *et al.*, 1987, Kunkel & Peterson, 1988) and thus, a pol II enhancer may be able to enhance transcription from a pol III promoter. Indeed, various studies have demonstrated that the enhancer element of the CMV IE gene pol II promoter can improve the activity of the U6 or H1 pol III promoters (Ong *et al.*, 2005, Xia *et al.*, 2003). To test whether shRNA expression could be improved in this manner, pR19CMVenhU6shRNA was constructed, where expression of shRNA was placed under the control of a hybrid CMV enhancer-U6 promoter (figure 3.2b).

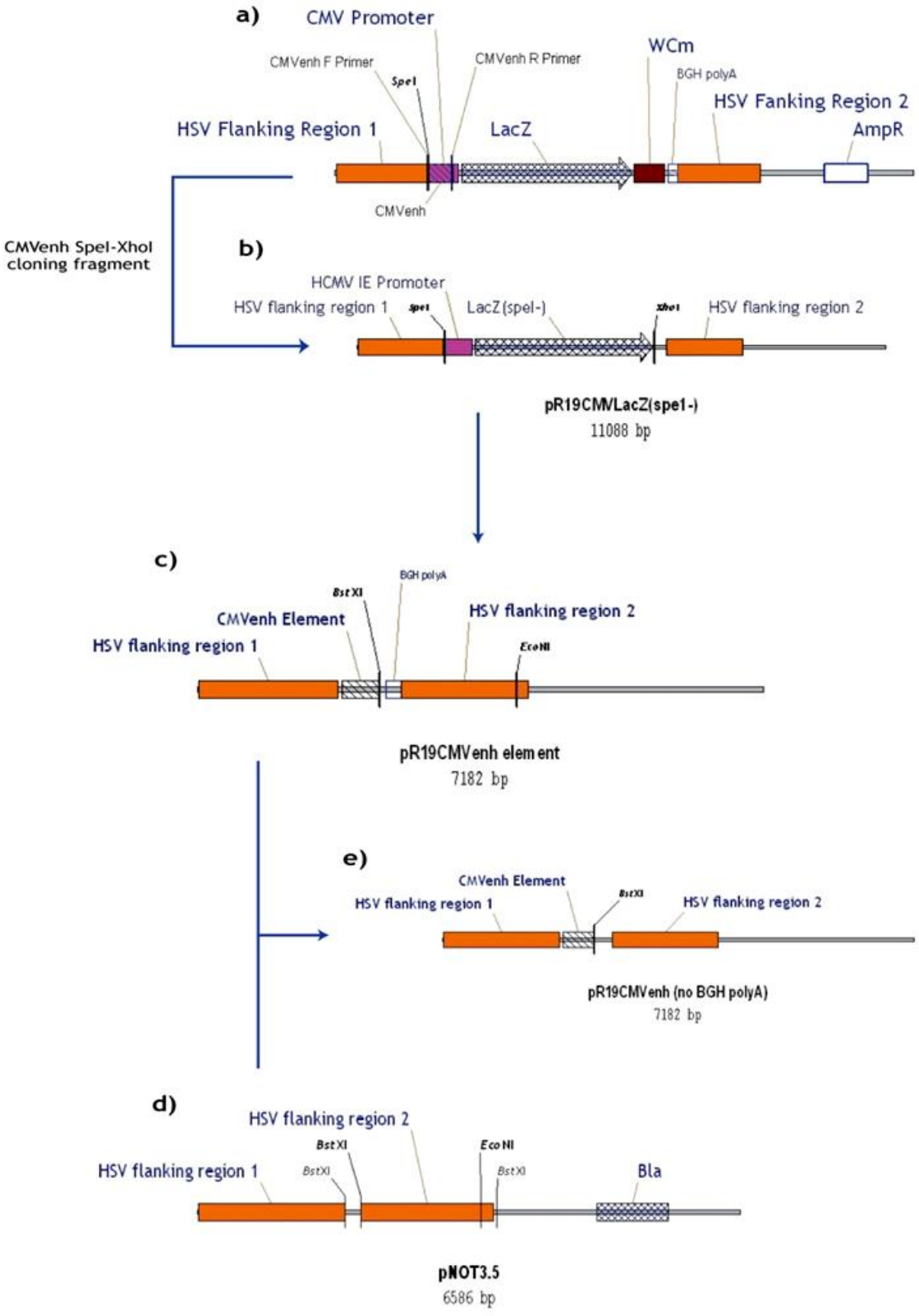
In the study by Xia *et al.* (2003), the authors evaluated a number of different constructs containing various combinations of U6 promoter and CMV enhancer. They demonstrated that the CMV enhancer element increased expression when placed either upstream or downstream of a full length U6 promoter in either forward or reverse orientation. In this study, a hybrid promoter consisting of the CMV enhancer element in a forward orientation inserted upstream of a full length U6 promoter was generated.

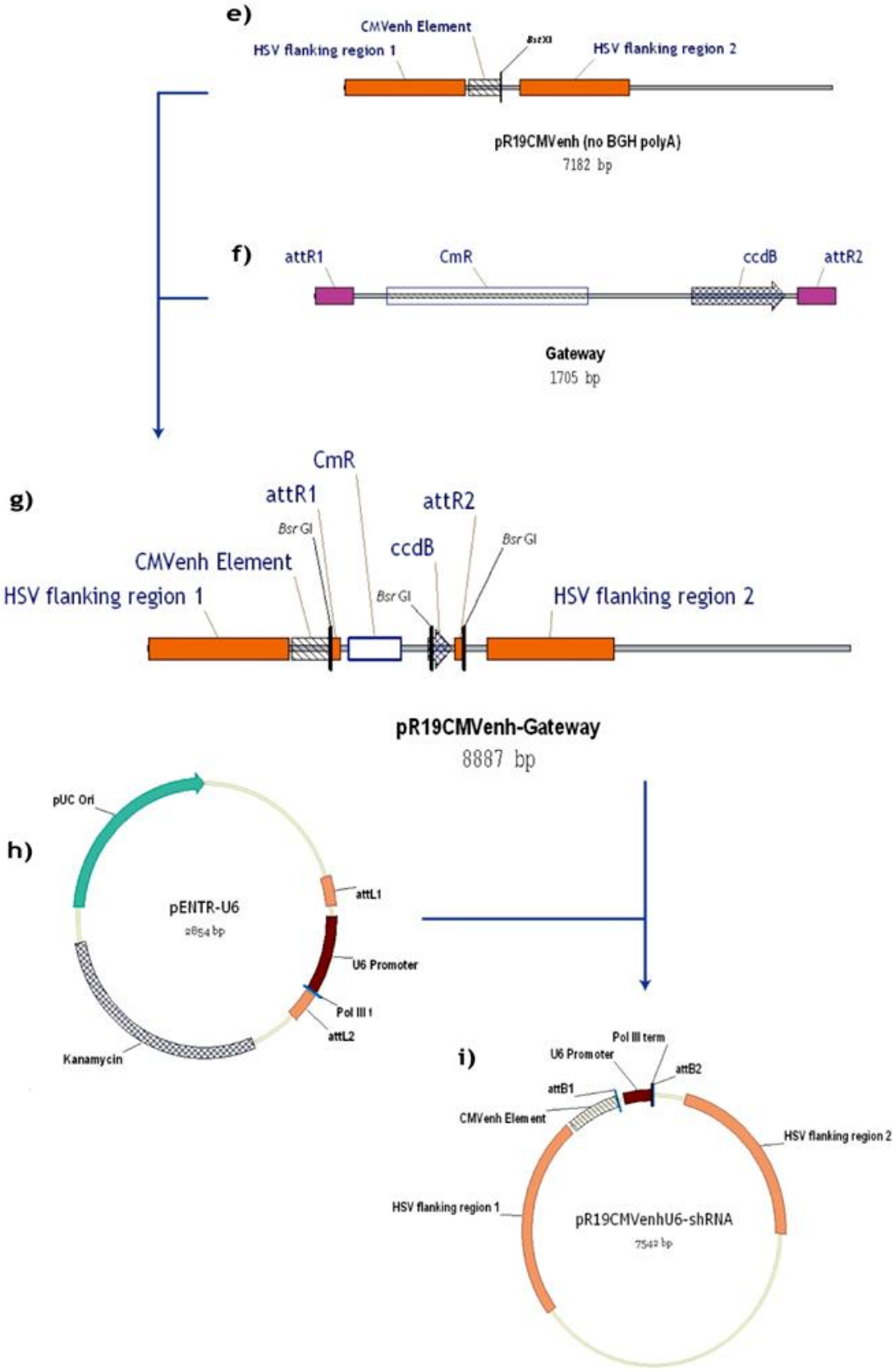
The CMV enhancer comprises nt 619-1024 of the human CMV IE gene promoter. A 486nt (nt 542-1027) CMV enhancer fragment was generated by PCR (as described in section 2.2.16) using the CMVenh F and CMVenh R primers (described in section 2.1.5) and the pR19CMVLacZWCm plasmid as a template (figure 3.6a). The CMVenh R primer was designed to add a XhoI restriction site to the 3'end of the PCR product to allow the CMV enhancer fragment to be cloned into pR19CMVLacZ between the SpeI-XhoI sites, in place of the CMV promoter and *lacZ* gene (figure 3.6b). To remove the BGH polyA sequence, the pR19CMVenh plasmid was digested with BstXI-EcoNI (figure 3.6c). This step also removed the 5'end of the HSV nt 120,413-122,027 flanking region, which was excised as a BstXI-EcoNI fragment from pNot3.5 (figure 3.6d) and cloned into pR19CMVenh (BstXI-EcoNI) to generate the pR19CMVenh(no BGH polyA) vector (figure 3.6e). The pR19CMVenh(no BGH polyA) plasmid was converted into the pR19CMVenh-Gateway destination vector (figure

3.6g) by cloning the Gateway cassette (figure 3.6f) into the BstXI site that had been blunted and treated with calf intestinal alkaline phosphatase. Finally, the U6shLacZ cassette (described in the previous section) was inserted from pENTR (figure 3.6h) into pR19CMVenh-Gateway by LR recombination. The pR19CMVenhU6shLaZ plasmid (figure 3.6i) was sequenced between the LAT flanking regions to ensure that the CMVenh-U6 hybrid promoter had been constructed correctly and that the shLacZ sequence had not been mutated (figure 3.11a).

Figure 3.6 Cloning of the pR19CMVenhU6shLacZ plasmid

The details of the cloning can be found in section 3.5.2. Briefly, the CMV enhancer was generated by PCR using the CMVenh F and CMVenh R primers (described in section 2.1.5) and the pR19CMVLacZWCm plasmid as a template. A XhoI restriction site was added to the 3'end of the fragment during PCR to allow the CMV enhancer to be cloned into pR19CMVLacZ between SpeI-XhoI. The BGH polyA was removed and the pR19CMVenh(no BGH polyA) plasmid was converted into the pR19CMVenh-Gateway destination vector by cloning the Gateway cassette into BstXI. The U6shLacZ cassette was inserted from pENTR into pR19CMVenh-Gateway by LR recombination to generate the pR19CMVenhU6shLacZ plasmid.


a)
CMV Promoter
CMVenh F Primer
CMVenh R Primer
WCm
BGH polyA
HSV Fanking Region 2
HSV Flanking Region 1
LacZ
AmpR
CMVenh
CMVenh SpeI-XhoI cloning fragment
b)
HCMV IE Promoter
HSV flanking region 1
LacZ(spel-)
HSV flanking region 2
pR19CMVLacZ(spe1-)
11088 bp
c)
BGH polyA
CMVenh Element
HSV flanking region 2
HSV flanking region 1
pR19CMVenh element
7182 bp
e)
CMVenh Element
HSV flanking region 1
HSV flanking region 2
pR19CMVenh (no BGH polyA)
7182 bp
d)
HSV flanking region 2
HSV flanking region 1
Bla
pNOT3.5
6586 bp


e)
CMVenh Element
HSV flanking region 1
HSV flanking region 2
pR19CMVenh (no BGH polyA)
7182 bp
f)
attR1
CmR
ccdB
attR2
Gateway
1705 bp
g)
CmR
attR1
attR2
Bsr GI
ccdB
Bsr GI
CMVenh Element
Bsr GI
HSV flanking region 1
HSV flanking region 2
pR19CMVenh-Gateway
8887 bp
h)
pUC Ori
attL1
pENTR-U6
2854 bp
U6 Promoter
Pol III t
attL2
Kanamycin
i)
Pol III term
U6 Promoter
attB2
attB1
CMVenh Element
HSV flanking region 2
HSV flanking region 1
pR19CMVenhU6-shRNA
7542 bp

3.5.3 Expression of shRNA from a miRNA-like system

Although pol III promoters have so far been most commonly used for expression of shRNA, there has been an interest in developing systems using pol II promoters. The miRNA-like expression system (pR19CMV/EmGFP-miR) constructed in this thesis takes advantage of the flexibility and variety of pol II promoters, which unlike pol III promoters, can be inducible and tissue-specific (figure 3.2c).

When inserted into pol II mRNA transcripts of irrelevant sequence, pre-miRNA sequences are readily excised (Zeng *et al.*, 2002). The sequence of the mature miRNA does not seem to be important for processing and can be altered to target the chosen gene (Zeng & Cullen, 2003). The BLOCK-iT^{TM} Pol II miR vector obtained from Invitrogen is based on pcDNA6.2 and allows directional cloning of a 68bp DNA oligonucleotide encoding an engineered pre-miRNA sequence downstream of the pol II CMV promoter (figure 3.7). The pre-miRNA sequence consists of a 5'overhang (TGCT), which is complementary to the vector and allows directional cloning, a 5'G followed by the 21nt antisense sequence, a 19nt spacer (GTTTTGGCCACTGACTGAC) that forms the terminal loop, and the sense sequence with 2nt (AA) removed to form an internal loop. To ensure that the miRNA is properly processed, the pre-miRNA sequence is inserted between 5' and 3' flanking regions derived from the endogenous miRNA-155 (Chung *et al.*, 2006, Tam & Dahlberg, 2006). Expression from this cassette results in the production of a pri-miRNA that resembles pri-miR-155. The pri-miRNA is processed in the nucleus by Drosha into a pre-miRNA that forms an intramolecular stem-loop structure similar to the structure of endogenous pre-miRNA. The pre-miRNA is subsequently transported to the cytoplasm by Exportin-5 and processed by Dicer into a 22nt mature miRNA, whose guide is incorporated into a miRNP. As described in detail in the introduction of this thesis, endogenous miRNAs can mediate silencing of target gene expression by mRNA endonucleolytic cleavage or translational repression and reducing mRNA stability, depending upon the degree of complementarity between the miRNA and its target mRNA. The engineered miRNAs

expressed from the miRNA-like cassette are designed to have perfect complementarity to their target mRNAs and have been shown to mediate silencing by mRNA cleavage in a similar manner to siRNAs (Chung *et al.*, 2006, Elbashir *et al.*, 2001, Zeng & Cullen, 2003). Extensive base-pairing of a miRNA and its target mRNA is not always sufficient to induce mRNA degradation, suggesting that the miRNA produced from the miRNA-like cassette preferentially interacts with a particular Argonaute that is able to direct cleavage. Finally, some miRNAs are located within the introns of protein coding genes and are co-expressed with mature mRNA, suggesting that pre-miRNAs can be excised from introns without disrupting production of mRNA (Baskerville & Bartel, 2005). A reporter gene encoding Emerald green fluorescent protein (EmGFP) is expressed from this system co-cistronically with the pre-miRNA and allows labelling of transduced cells to aid the monitoring of silencing efficiency.

The validated pre-miRNA sequence against ß-galactosidase (miR-LacZ) and the non-target pre-miRNA sequence (miR-neg), which is not predicted to target any known vertebrate gene, were supplied by Invitrogen as annealed DNA oligonucleotides (sequences available in section 2.1.6) and were cloned into pcDNA6.2/EmGFP vectors (figure 3.7). The 21nt target sequence against ß-galactosidase in pre-miR-LacZ (figure 3.11b) is identical to the target sequence in shLacZ (figure 3.4a) expressed from the U6 or CMVenh-U6 promoters (sections 3.5.1 and 3.5.2). The pcDNA6.2/EmGFP-miR-LacZ plasmid was sequenced using the GFP Forward primer (described in section 2.1.5) to ensure that the miRNA-LacZ sequence had not been mutated (figure 3.11b).

In pcDNA6.2/EmGFP-miR, the EmGFP-miR cassette is flanked by attB1-attB2 sequences that allow recombination into a suitable donor vector (attP1-attP2). The resulting entry clone (attL1-attL2) allows the cassette to be transferred into an appropriate destination vector (attR1-attR2) and ultimately into an HSV vector. The destination vector can be engineered to drive expression of the pre-miRNA from a constitutive, inducible or tissue specific pol II promoter. The EmGFP-miR cassette was

transferred from pcDNA6.2 (figure 3.8a) into pDONR-221 (figure 3.8b) by BP recombination reaction as described in section 2.2.8 (figure 3.8c). The pR19CMVLacZWCm plasmid (figure 3.8d) was converted into the pR19CMV-Gateway destination vector (figure 3.8f) by cloning the Gateway cassette (figure 3.8e) between the HindIII-XhoI sites that had been blunted and treated with calf intestinal alkaline phosphatase. Finally, the EmGFP-miR-LacZ and EmGFP-miR-neg cassettes were inserted from pDONR221 into pR19CMV-Gateway vectors by LR recombination. The pR19CMV/EmGFP-miR vectors were sequenced between the LAT flanking regions (sequences not shown) and a map is shown in figure 3.8g.

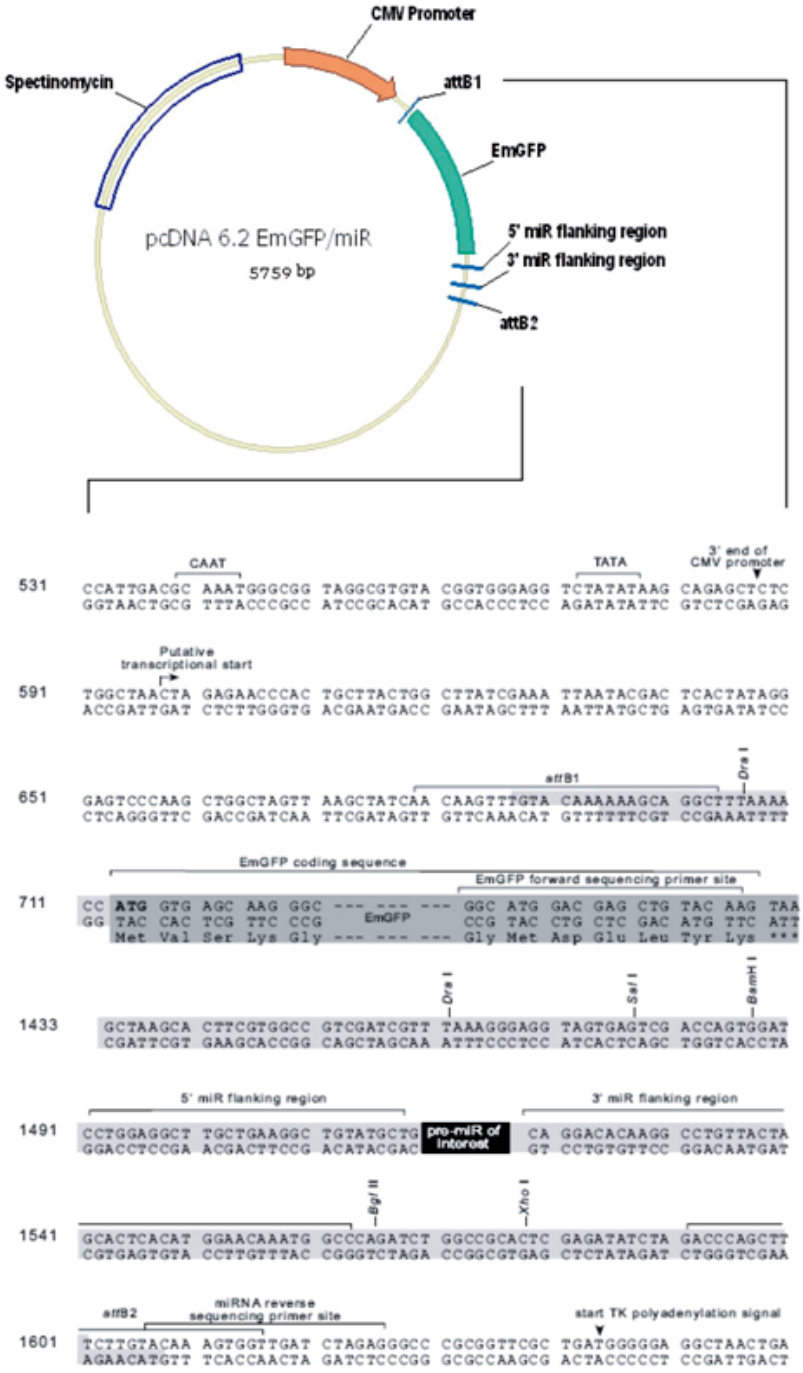
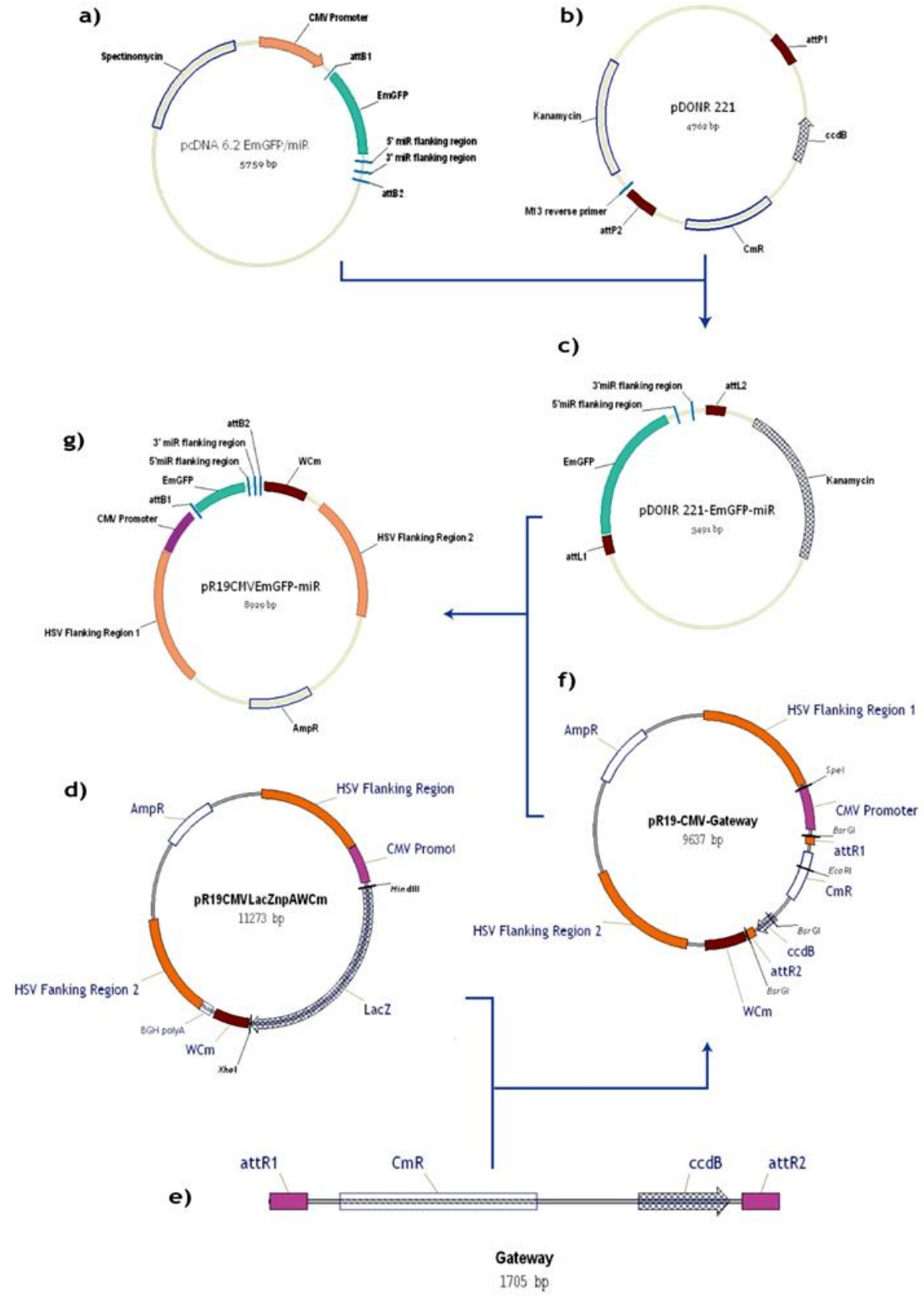


Figure 3.7 The pcDNA6.2/EmGFP-miR vector from Invitrogen

The 68bp DNA oligonucleotide encoding the engineered pre-miRNA sequence against the target gene is cloned downstream of the CMV pol II promoter and the EmGFP coding sequence. To ensure that the miRNA is properly processed, the pre-miRNA is inserted between 5' and 3' flanking regions derived from the endogenous miRNA-155 (Chung *et al.*, 2006).the EmGFP-miR cassette is flanked by attB1-attB2 sequences that allow recombination into pDONR-221 (attP1-attP2). The resulting entry clone (attL1-attL2) allows the cassette to be transferred into an appropriate destination vector (attR1-attR2).

Figure 3.8 Cloning of pR19CMV/EmGFP-miR plasmids

The details of the cloning can be found in section 3.5.3. Briefly, the EmGFP-miR cassette was transferred from pcDNA6.2 into pDONR-221 by BP recombination. The pR19CMVLacZWCm plasmid was converted into the pR19CMV-Gateway destination vector by cloning the Gateway cassette between HindIII-XhoI. The EmGFP-miR-LacZ and EmGFP-miR-neg cassettes were inserted from pDONR-221 into pR19CMV-Gateway vectors by LR recombination to generate the pR19CMV/EmGFP-miR plasmds.


a)
CMV Promoter
Spectinomycin
attB1
EmGFP
5' miR flanking region
3' miR flanking region
attB2
pcDNA 6.2 EmGFP/miR
5759 bp
b)
attP1
Kanamycin
pDONR 221
4762 bp
ccdB
M13 reverse primer
attP2
CmR
c)
3'miR flanking region
attL2
5'miR flanking region
EmGFP
Kanamycin
pDONR 221-EmGFP-miR
3491 bp
attL1
g)
attB2
3' miR flanking region
5'miR flanking region
WCm
EmGFP
attB1
CMV Promoter
HSV Flanking Region 2
pR19CMVEmGFP-miR
8929 bp
HSV Flanking Region 1
AmpR
f)
HSV Flanking Region 1
AmpR
SpeI
CMV Promoter
pR19-CMV-Gateway
9637 bp
BsrGI
attR1
EcoRI
CmR
HSV Flanking Region 2
BsrGI
ccdB
attR2
BsrGI
WCm
d)
HSV Flanking Region
AmpR
CMV Promot
HindIII
pR19CMVLacZnpAWCm
11273 bp
HSV Fanking Region 2
LacZ
BGH polyA
WCm
XhoI
attR1
CmR
ccdB
attR2
e)
Gateway
1705 bp

3.5.4 Expression of shRNA from a Polymerase II promoter

Expression of shRNA from the CMV pol II promoter results in a capped polyadenylated transcript in which the hairpin is extended on both 3' and 5' ends by vector sequences and polyA. A construct driving expression of shRNA from the CMV promoter and a full length polyA cassette failed to silence target gene expression in 293T cells (Paddison *et al.*, 2002, Xia *et al.*, 2002). To allow expression of functional shRNA from the CMV promoter, Xia and colleagues (2002) generated a construct in which the hairpin was inserted almost immediately downstream of the CMV transcriptional start site and upstream of a synthetic minimal polyA cassette. These authors concluded that the spacing of the hairpin immediate to the promoter is critically important and together with the minimal polyA, they result in the generation of functional siRNA molecules with minimal overhangs. To test whether shRNA could be expressed from a modified CMV promoter, the pR19CMVshRNA vector was generated (figure 3.2d).

The pSilencer 4.1-CMV vector from Ambion is based on the construct described by Xia *et al.* (2002) and allows cloning of a 55-60bp DNA oligonucleotide immediately downstream of the CMV transcriptional start site and upstream of a minimal SV40 polyA (figure 3.9). The vector was supplied linearised with BamHI and HindIII to facilitate directional cloning. The sequence against ß-galactosidase (available in section 2.1.6) was synthesized as two complementary oligonucleotides with single stranded overhangs corresponding to the BamHI and HindIII restriction sites. The target sequence against ß-galactosidase is identical to the target sequence in shLacZ (figure 3.4a) expressed from the U6 or CMVenh-U6 promoters (sections 3.5.1 and 3.5.2) and thus, identical to the target sequence in pre-miR-LacZ (figure 3.11b). The oligonucleotides were annealed and ligated into pSilencer, and the pSilencer-shLacZ vector was sequenced using the pSilencer Forward and pSilencer Reverse primers (described in section 2.1.5) to ensure that the shLacZ sequence had not been mutated (figure 3.11c).

The pSilencer-shLacZ vector (figure 3.10a) was digested with EcoRI-PvuI to remove the CMV-shLacZ-SV40 polyA cassette, which was double blunted and cloned into pR19SYNeGFP between the ClaI-NsiI sites (figure 3.10b). To remove the BGH polyA sequence, the resulting pR19CMVshLacZ plasmid was digested with SacI-EcoNI (figure 3.10c). This step also removed the 5'end of the HSV nt 120,413-122,027 flanking region, which was excised as a BstXI-EcoNI fragment from pNot3.5 (figure 3.10d) and cloned into pR19CMVshLacZ (SacI-EcoNI). The pR19CMVshLacZ vector was sequenced between the LAT flanking regions (sequences not shown) and a map is shown in figure 3.10e.

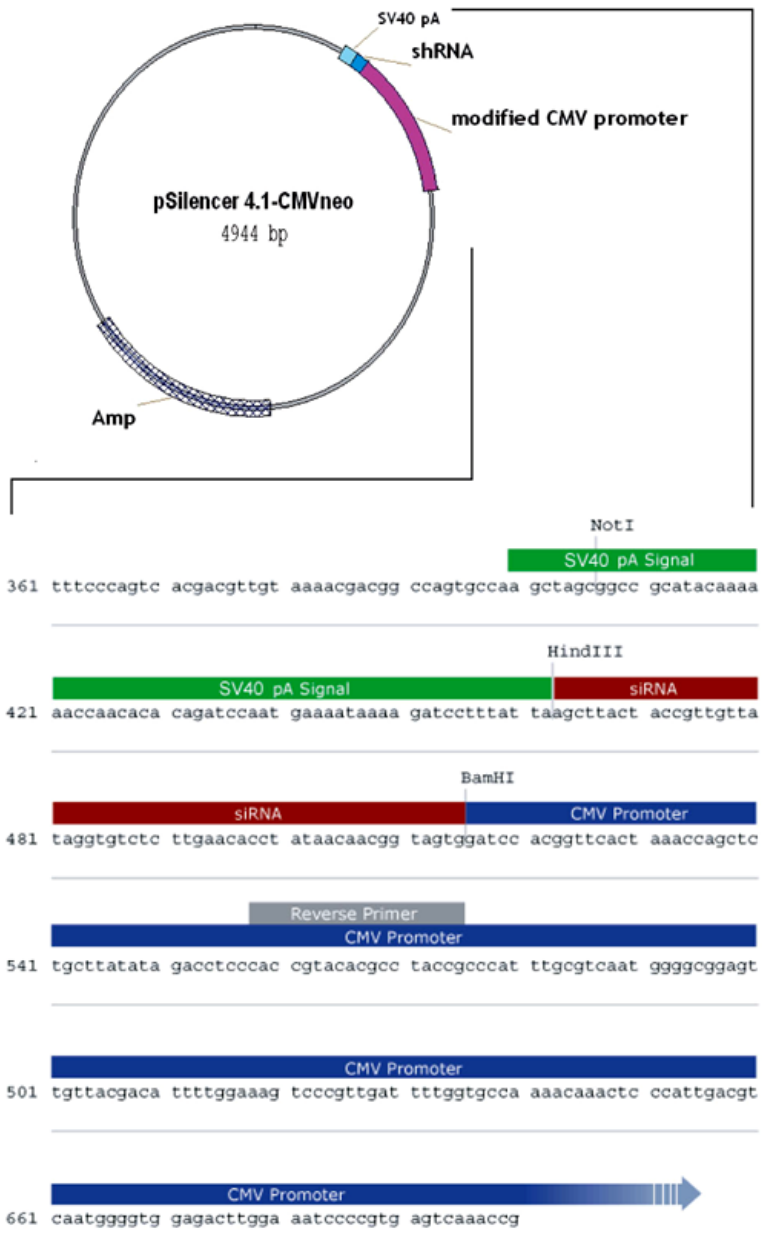
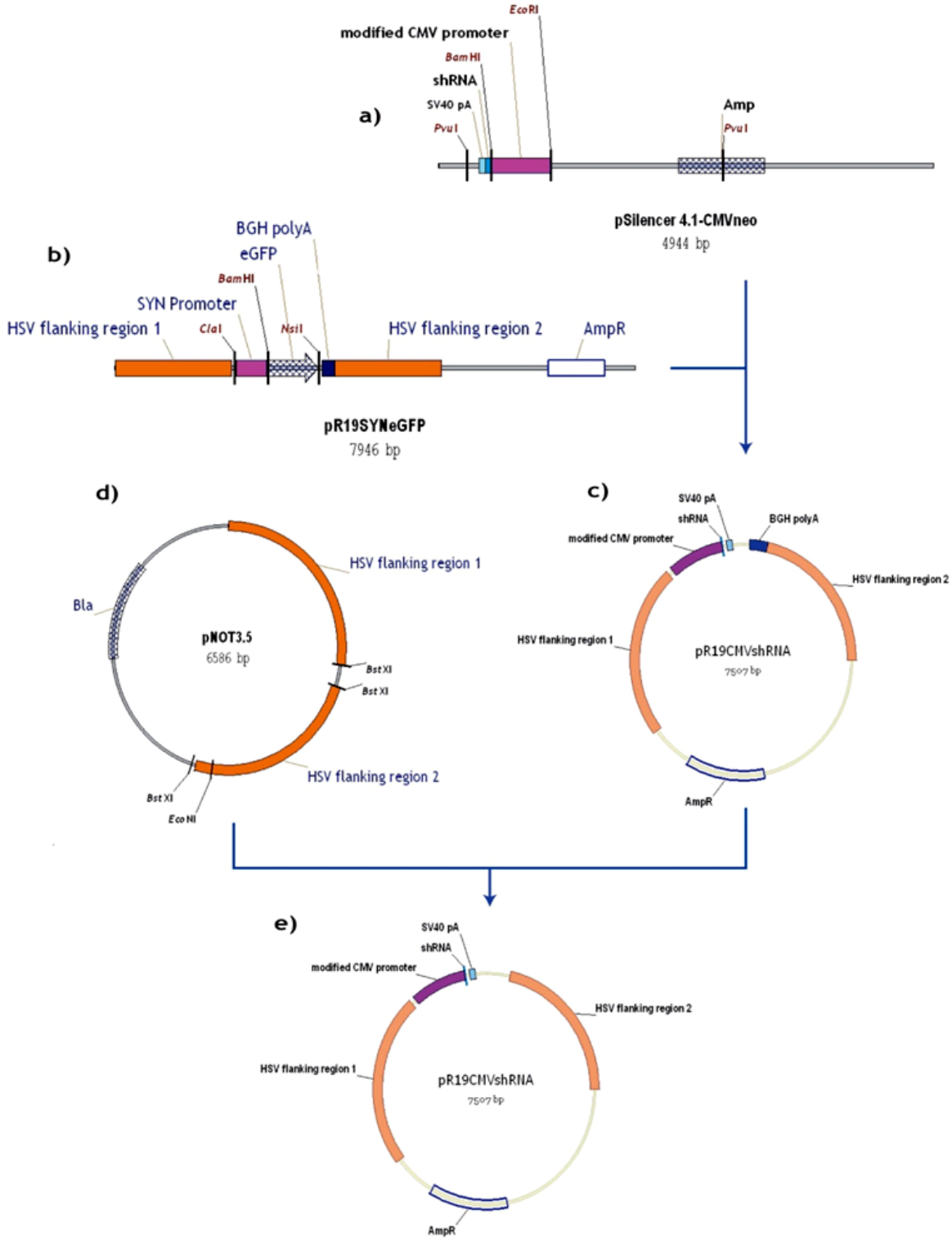


Figure 3.9 The pSilencer 4.1-CMV vector from Ambion

The pSilencer 4.1-CMV vector is based on the construct described by Xia *et al.* (2002) and allows cloning of a 55-60bp DNA oligonucleotide expressing the shRNA against the target gene immediately downstream of the CMV transcriptional start site and upstream of a minimal SV40 polyA. The shRNA is synthesized as two complementary oligonucleotides with single stranded overhangs encoding the BamHI and HindIII restriction sites to allow directional cloning into the vector.

Figure 3.10 Cloning of the pR19CMVshLacZ plasmid

The details of the cloning can be found in section 3.5.4. Briefly, pSilencer-shLacZ was digested with EcoRI-PvuI to remove the CMV-shLacZ-SV40 polyA cassette. The CMVshLacZ cassette was cloned into pR19SYNeGFP between ClaI-NsiI. Finally, the BGH polyA was removed to generate the pR19CMVshLacZ plasmid.


a)
modified CMV promoter
EcoRI
BamHI
shRNA
SV40 pA
PvuI
PvuI
Amp
pSilencer 4.1-CMVneo
4944 bp
b)
BGH polyA
eGFP
BamHI
SYN Promoter
HSV flanking region 1
ClaI
NsiI
HSV flanking region 2
AmpR
pR19SYNeGFP
7946 bp
d)
HSV flanking region 1
Bla
pNOT3.5
6586 bp
BstXI
BstXI
HSV flanking region 2
BstXI
EcoNI
c)
SV40 pA
shRNA
BGH polyA
modified CMV promoter
HSV flanking region 2
HSV flanking region 1
pR19CMVshRNA
7507 bp
AmpR
e)
SV40 pA
shRNA
modified CMV promoter
HSV flanking region 2
HSV flanking region 1
pR19CMVshRNA
7507 bp
AmpR

a) pR19CMVenhU6shLacZ sequencing

Sequence 1: lcl|1
Length = 47 (1 .. 47) shLacZ

Sequence 2: lcl|65536
Length = 1421 (1 .. 1421) **pR19CMVenhU6shLacZ (CMV Forward primer)**

Score = 91.1 bits (47), Expect = 2e-16
Identities = **47/47 (100%)**, Gaps = 0/47 (0%)
Strand=Plus/Plus

```
Query  1    CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTGTGTAG  47
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  608  CACCGCTACACAAATCAGCGATTTCGAAAAATCGCTGATTTGTGTAG  654
```

pR19CMVenhU6shLacZ sequenced using the LAT Forward primer:

emb|X03922.1|HEHCMVP1 Human cytomegalovirus (HCMV) IE1 gene promoter region
Length=1848

Score = 898 bits (486), Expect = 0.0
Identities = 486/486 (100%), Gaps = 0/486 (0%)
Strand=Plus/Plus

```
Query  50    TTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAG 109
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  542   TTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAG  601

Query  110   CCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCC  169
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  602   CCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCC  661

Query  170   CAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGG  229
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  662   CAACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCAATAGG  721

Query  230   GACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA  289
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  722   GACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCACTTGGCAGTACA  781

Query  290   TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGC  349
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  782   TCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGC  841

Query  350   CTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGT  409
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  842   CTGGCATTATGCCCAGTACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGT  901

Query  410   ATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATA  469
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  902   ATTAGTCATCGCTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATA  961

Query  470   GCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTT  529
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  962   GCGGTTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTTGTT  1021

Query  530   TTGGCA  535
             ||||||
Sbjct  1022  TTGGCA  1027
```

pR19CMVenhU6shLacZ sequenced using the CMV Forward primer:

```
gb|AY623053.1|  Homo sapiens clone phU6A U6 small nuclear RNA, promoter region
Length=485

Score =  492 bits (266),  Expect = 2e-135
Identities = 266/266 (100%), Gaps = 0/266 (0%)
Strand=Plus/Plus

Query  346  CCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGAT  405
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  220  CCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTCATATTTGCATATACGAT  279

Query  406  ACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTAC  465
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  280  ACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACTGTAAACACAAAGATATTAGTAC  339

Query  466  AAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTT  525
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  340  AAAATACGTGACGTAGAAAGTAATAATTTCTTGGGTAGTTTGCAGTTTTAAAATTATGTT  399

Query  526  TTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT  585
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  400  TTAAAATGGACTATCATATGCTTACCGTAACTTGAAAGTATTTCGATTTCTTGGCTTTAT  459

Query  586  ATATCTTGTGGAAAGGACGAAACACC  611
            |||||||||||||||||||||||||
Sbjct  460  ATATCTTGTGGAAAGGACGAAACACC  485
```

b) pcDNA6.2/EmGFP-miR-LacZ sequencing

Sequence 1: lcl|1
Length = 68 (1 .. 68) pre-miR-LacZ

Sequence 2: lcl|65536
Length = 1340 (1 .. 1340) **pcDNA6.2/EmGFP-miR-LacZ (GFP Forward primer)**

```
Score =  131 bits (68),  Expect = 3e-28
Identities = 68/68 (100%), Gaps = 0/68 (0%)
Strand=Plus/Plus

Query  1    TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCACTGACTGACGACTACACATCAGCG  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 105   TGCTGAAATCGCTGATTTGTGTAGTCGTTTTGGCCACTGACTGACGACTACACATCAGCG 164

Query  61   ATTTCAGG  68
            ||||||||
Sbjct  165  ATTTCAGG  172
```

The target sequence against ß-galactosidase in pre-miR-LacZ:

```
gb|FJ440335.1|  Reporter cassette lacTeT, complete sequence
Length=5870

Score = 41.0 bits (44),  Expect = 0.40
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

Query  4    AAATCGCTGATTTGTGTAGTC  26
            |||||||||||||||||||||
Sbjct  745  AAATCGCTGATTTGTGTAGTC  725
```

c) pSilencer-shLacZ sequencing

Sequence 1: lcl|1
Length = 48 (1 .. 48) **shLacZ for pSilencer**

Sequence 2: lcl|65536
Length = 1336 (1 .. 1336) **pSilencer-shLacZ (pSilencer Reverse primer)**

Score = 93.0 bits (48), Expect = 7e-17
Identities = **48/48 (100%)**, Gaps = 0/48 (0%)
Strand=Plus/Minus

```
Query  1    AGCTTCTACACAAATCAGCGATTTTTCGAAATCGCTGATTTGTGTAGG  48
            ||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  72   AGCTTCTACACAAATCAGCGATTTTTCGAAATCGCTGATTTGTGTAGG  25
```

Figure 3.11 Blast nucleotide alignments between a) pR19CMVenhU6shLacZ, b) pcDNA6.2/EmGFP-miR-LacZ or c) pSilencer-shLacZ sequences obtained using appropriate sequencing primers and the oligonucleotide sequences against ß-galactosidase. The pR19CMVenhU6shLacZ sequences obtained with the LAT Forward and CMV Reverse primers (described in section 2.1.5) were aligned against the CMV IE gene and U6 promoter sequences to ensure that the CMVenh-U6 hybrid promoter had been successfully constructed. The target sequence against ß-galactosidase in pre-miR-LacZ is indicated in the reporter cassette lacTeT sequence.

3.6 Screening of shRNA sequences against GFP

To identify the most potent shRNA sequence against GFP, 293T cells were co-transfected with pR19CMVeGFPWCm (100ng) that expresses enhanced green fluorescent protein and either pENTRU6shLacZ, pENTRU6shGFP1, pENTRU6shGFP2, or pENTRU6shGFP3 (1µg) constructed in section 3.5.1. Transfection was performed using Lipofectamine as described in section 2.3.3. Fluorescent microscopy revealed highly effective knockdown of GFP expression as early as 12hrs post-transfection when either shGFP2 or shGPF3 were transfected (data not shown). Figure 3.12 demonstrates *gfp* silencing at 48 hrs post-transfection. The shGFP1 sequence, which was converted into shRNA from a validated siRNA sequence supplied by Ambion, failed to induce any silencing (figure 3.12b). This result is in agreement with numerous other studies that have concluded that a validated siRNA sequence is not necessarily effective when converted into a shRNA. Transfection with the pENTRU6shLacZ had no effect on GFP levels indicating that silencing is specific (figure 3.12a). The U6shGFP2 cassette that induced near complete knockdown of GFP expression (figure 3.12c) was selected and inserted into pR19-Gateway as described in section 3.5.1.

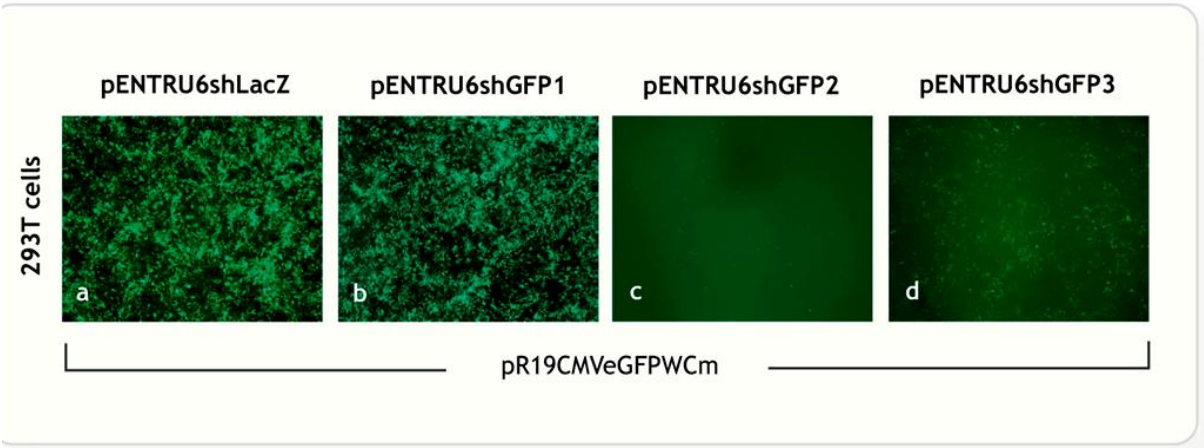


Figure 3.12 Screening of shRNA sequences against GFP

293T cells were co-transfected with pR19CMVeGFPWCm (100ng) and either: a) pENTRU6shLacZ negative control, b) pENTRU6shGFP1, c) pENTRU6shGFP2, or d) pENTRU6shGFP3 (1:10)

Fluorescent microscopy for GFP revealed highly effective silencing at 72hrs post-transfection when either shGFP2 or shGPF3 were transfected (objective x10). The shGFP1 sequence, which was converted into shRNA from a validated siRNA sequence supplied by Ambion, failed to induce any silencing. Transfection with the pENTRU6shLacZ had no effect on GFP levels indicating that silencing is specific. The shGFP2 sequence was selected and subcloned into pR19.

3.7 Evaluation of plasmid vectors expressing shRNA

The plasmid vectors constructed in section 3.5 were tested for their ability to express shRNA against ß-galactosidase. 293T cells were co-transfected with pR19CMVLacZWCm (100ng) which expresses ß-galactosidase and either pR19U6-neg, pR19U6shLacZ, pR19CMVenhU6shLacZ, or pR19CMVshLacZ (1µg) using Lipofectamine. In each of these conditions, the pR19CMVeGFPWCm plasmid (100ng) that expresses enhanced GFP was also co-transfected to evaluate the transfection efficiency. Similarly, 293T cells were co-transfected with pR19CMVLacZWCm (100ng) and either pR19CMV/EmGFP-miR-neg or pR19CMV/EmGFP-miR-LacZ (1µg). Transfection efficiency was monitored by the presence of the EmGFP signal expressed from these constructs.

X-gal staining (as described in section 2.3.6) at 72 hrs post-transfection demonstrated that each of the plasmids targeting *lacZ* substantially reduced ß-galactosidase levels (figure 3.13b, c, and f) except for pR19CMVshLacZ that failed to induce any silencing (figure 3.13d). GFP levels assessed by fluorescent microscopy prior to x-gal staining indicated that the transfection efficiency between different conditions had been normalised. Importantly, transfection with the negative controls had no effect on ß-galactosidase levels indicating that silencing is specific (figure 3.13a and d).

Analysis of ß-galactosidase expression by enzyme activity assay (as described in section 2.7.5) revealed that the pR19U6shLacZ construct silenced *lacZ* by 89.0 ± 1.5% (n=3, mean ± s.d.), the pR19CMVenhU6shLacZ construct by 89.4 ± 4.6% and the pR19CMVGFP-miR-LacZ construct by 93.0 ± 1.7% (figure 3.14). Although these results were highly encouraging, the level of sensitivity achieved with this system is greater than that achieved with endogenous target gene knockdown, because the constructs expressing *lacZ* and shRNA/pre-miRNA are co-transfected simultaneously and thus, delivery to all cells is not required in order to achieve an RNAi response.

To investigate RNAi specificity further, the mRNA levels of four endogenous genes were assessed in 293T cells transfected with each of the above constructs. Induction of

the INF response by shRNA/miRNA or any other sequences in the expression cassettes would result in non-specific mRNA degradation and significantly reduced mRNA levels of housekeeping genes. Quantitative RT-PCR (described in section 2.217) for ATP5b, GAPDH and GUSb revealed that there is no significant reduction in the expression levels of any of these housekeeping genes 72 hrs following introduction of shRNA or pre-miRNA (figure 3.15). Moreover, quantitative RT-PCR for ISGF3G, the interferon-stimulated transcription factor 3 gamma, confirmed that silencing is not caused by a non-specific effect associated with induction of the interferon (INF) response (figure 3.15). The qRT-PCR experiment was performed by Ronald De Hoogt at Johnson & Johnson Research and Development in Belgium.

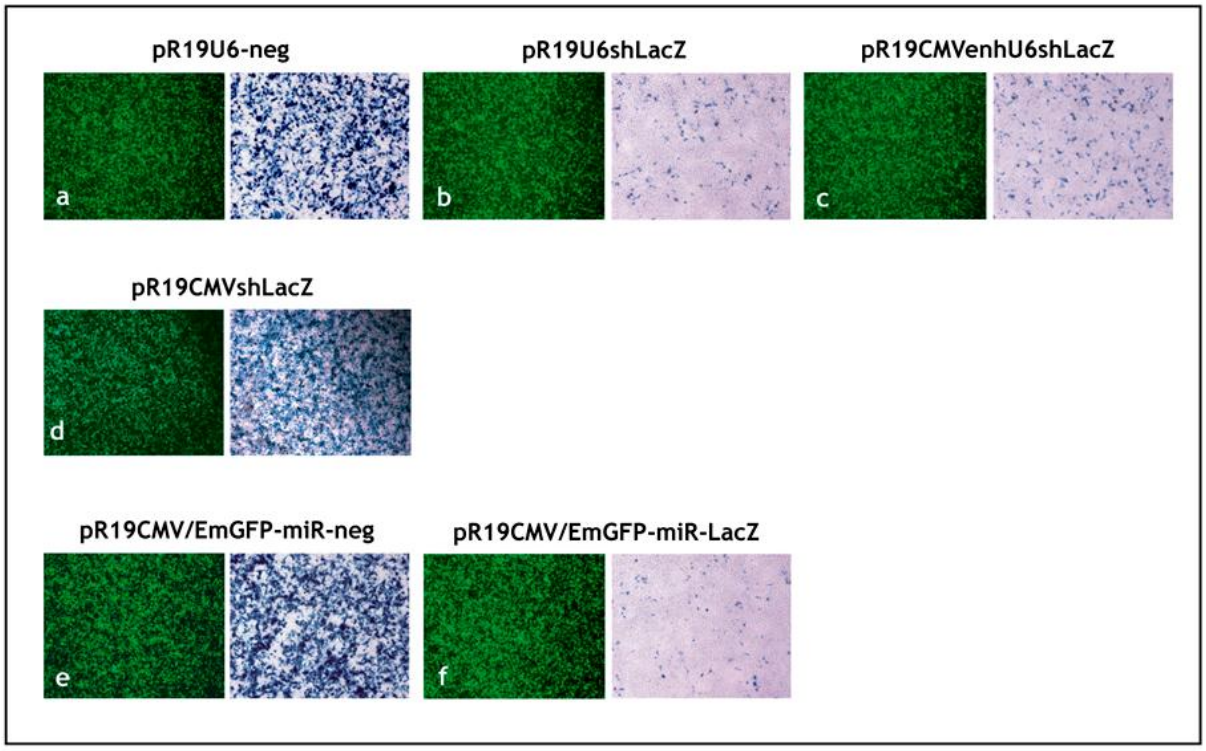


Figure 3.13 Evaluation of plasmids expressing shRNA against beta-galactosidase

293T cells were co-transfected with pR19CMVLacZWCm (100ng) and either: a) pR19U6-neg, b) pR19U6shLacZ, c) pR19CMVenhU6shLacZ, or d) pR19CMVshLacZ (1:10). In each of these conditions, the pR19CMVeGFPWCm plasmid (100ng) was also co-transfected to normalise transfection efficiency.

Similarly, 293T cells were co-transfected with pR19CMVLacZWCm (100ng) and either: e) pR19CMV/EmGFP-miR-neg or f) pR19CMV/EmGFP-miR-LacZ (1:10). Transfection efficiency was monitored by the presence of the EmGFP signal expressed from these constructs.

X-gal staining at 72 hrs post-transfection demonstrated that each of the plasmids targeting lacZ substantially reduced beta-galactosidase levels except for pR19CMVshLacZ that failed to induce any silencing. GFP levels assessed by fluorescent microscopy prior to x-gal staining indicated that the transfection efficiency between different conditions had been normalised. Transfection with the negative controls had no effect on beta-galactosidase levels indicating that silencing is specific (objective x10).

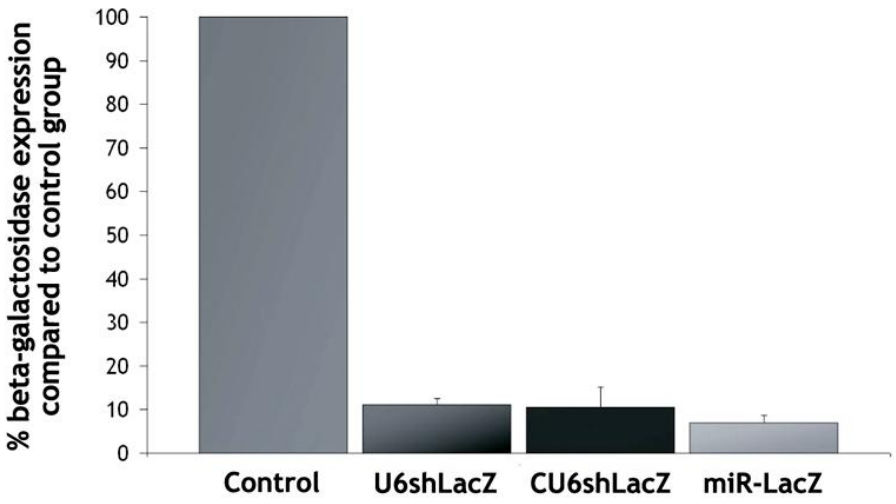


Figure 3.14 Quantification of silencing in 293T cells transfected with pR19CMVLacZWCm expressing beta-galactosidase and each of the plasmids expressing shRNA against beta-galactosidase.

Analysis of beta-galactosidase expression by enzyme activity assay at 72 hrs post-transfection revealed that the pR19U6shLacZ construct silenced lacZ by 89.0 ± 1.5%, the pR19CMVenhU6shLacZ construct silenced lacZ by 89.4 ± 4.6%, and the pR19CMVGFP-miR-LacZ construct silenced lacZ by 93.0 ± 1.7% (n=3, mean ± s.d.). Beta-galactosidase expression was calculated as a percentage of the expression in cells transfected with negative controls (pR19U6-neg for the pR19U6shLacZ and pR19CMVenhU6shLaZ plasmids and pR19CMV/EmGFP-miR-neg for the pR19CMV/EmGFP-miR-LacZ plasmid).

X-gal staining images of the conditions quantified here are shown in figure 3.12.

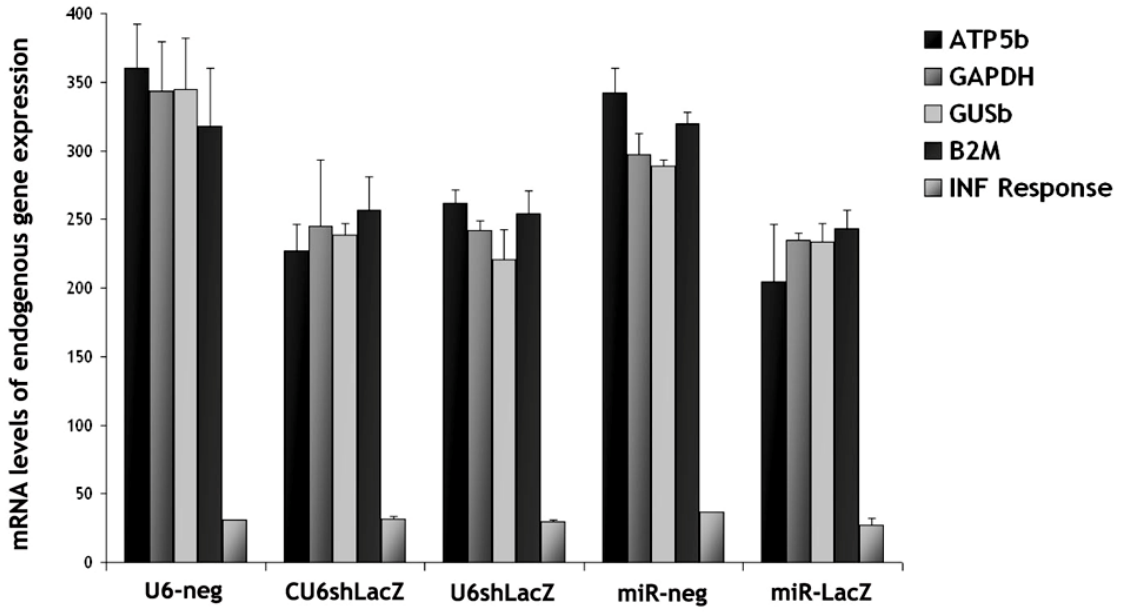


Figure 3.15 Assessment of silencing specificity

Quantitative RT-PCR was performed in 293T cells transfected with plasmids expressing shRNA (details of the transfection can be found in section 3.7) to investigate whether the mRNA levels of unrelated genes were non-specifically reduced by expression of shRNA.

qRT-PCR for ATP5b, GAPDH and GUSb demonstrated no significant reduction in the expression levels of any of these housekeeping genes 72 hrs following introduction of shRNA or pre-miRNA into 293T cells. Moreover, qRT-PCR for ISGF3G, the interferon-stimulated transcription factor 3 gamma, confirmed that silencing is not caused by a non-specific effect associated with induction of the INF response.

3.8 Choice of disabled backbone for RNAi delivery to DRG

Prior to generating viral vectors expressing shRNA, it was important to identify a disabled HSV-1 backbone that would make a suitable vector for delivery of RNAi to mouse sensory neurons. The 1764/4-/27+/RL1+ and 1764/4-/27- backbones described in section 3.2, which have different levels of disablement, were both available at the time this project began. Previous work in our laboratory had already concluded that expression in the brain (Palmer, unpublished) and spinal cord (Groutsi, unpublished) was considerably improved by re-insertion of ICP34.5 and ICP27 into the HSV genome. These studies also demonstrated that despite its level of disablement, the 1764/4-/27+/RL1+ vector is not appreciably more toxic or immunogenic than the more disabled 1764/4-/27- backbone. Although the less disabled 1764/4-/27+/RL1+ backbone was the obvious choice upon which to base all the shRNA-expressing viruses constructed in this thesis, its potential to deliver high levels of relatively long-term expression to DRG sensory neurons had not been properly evaluated.

The highly disabled 1764/4-/27- viruses had already been tested for gene delivery to the rat DRG after footpad inoculation and direct sciatic nerve injection with somewhat disappointing results (Palmer *et al.*, 2000). At higher titres ($5x10^8$ pfu/ml), gene delivery was considerably improved following injection into the sciatic nerve, but not following footpad inoculation. These results suggested that some level of replication competence might be necessary for efficient gene delivery to DRG, especially for the virus to penetrate various cell types in the footpad and enter the sciatic nerve. Thus, replication-defective viruses allow efficient gene delivery to DRG at high titres when injected directly into the sciatic nerve. Consequently, injection into the sciatic nerve was chosen as the route of administration to be used throughout this thesis (described in section 2.6.3).

Based on the above observations, the less disabled 1764/4-/27+/RL1+ backbone might be expected to allow more efficient gene delivery to the DRG than the 1764/4-/27- backbone (Palmer *et al.*, 2000). To confirm this and also compare expression from

different promoters, the 1764/4-/27+/RL1+ pR19CMVLacZWCm or pR19EF1αLacZWCm vectors were injected into the sciatic nerve of mice ($5x10^5$ pfu per animal) and the lumbar DRG (L3 and L4) were isolated at 3 and 21 days post-injection. X-gal staining in whole mount DRG preparations (as described in section 2.6.7) revealed that injection of the virus into the tibial branch of the sciatic nerve results in delivery predominantly to the L4 DRG. At 3 days post-injection, the 1764/4-/27+/RL1+ backbone allowed highly efficient gene delivery to mouse sensory neurons following injection into the sciatic nerve, with the CMV promoter driving considerably higher levels of expression in these neurons than the EF1α promoter (figure 3.15a).

Whilst expression in DRG is maintained for at least 21 days, it is considerably reduced compared to that at early times post-injection (figure 3.15b). Previous work in our lab has shown that in DRG the pR20.9 cassette (which allows expression of two transgenes from a MMLV LTR and a LAP1 promoter inserted on either side of the LAT P2 element), when inserted into UL43 of a 1764 virus, maintained gene expression during latency as efficiently as the endogenous LAT promoters maintained expression of the LATs (Palmer *et al.*, 2000). The same was suggested for the pR19 cassette inserted into a 1764/4-/27- virus. It was therefore suggested that reduction in gene expression over time is not due to the promoter being transcriptionally inactivated during latency, but may due to the virus being cleared by the host immune response. Other work in our laboratory has shown that following injection of 1764/4-/27- and 1764/4-/27+/RL1+ pR19CMVLacZ viruses into the rat cervical spinal cord, the inflammatory response appears to be the same for both viruses (Groutsi, unpublished). This could be due to ICP34.5 being re-inserted into the genome which may have a function in protecting against such endogenous responses; ICP34.5 has a role in immune evasion by suppressing INFα/ß and MHC Class I and II production from infected cells and the less disabled vectors may therefore suppress the local immune response. Moreover, the 1764 4-/27+/RL1+ pR19CMVeGFPWCm and pR19CMVLacZWcm vectors were found to be non-toxic when used to transduce rat DRG

(figure 3.16a) and rat embryonic E14 cortical neuronal cultures (figure 3.16b) respectively, at an MOI=5.0. Although this data is sufficient for the purpose of using the vectors as target validation tools, a more detailed investigation of the IE gene expression pattern of this backbone would be required for the vectors developed in this thesis to be utilized for potential long-term therapy.

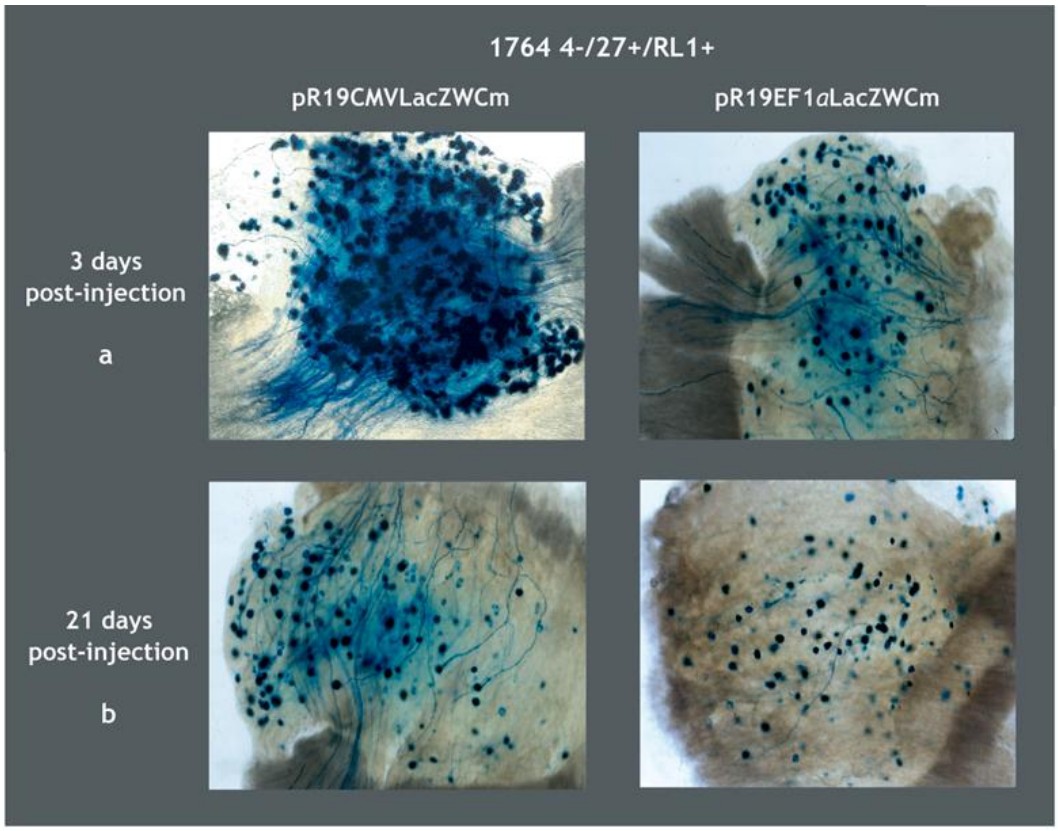


Figure 3.16 Evaluation of beta-galactosidase expression in mouse DRG following sciatic nerve injection of HSV-1 1764 4-/27+/RL1+ virus expressing lacZ either from the CMV or the EF1*a* promoter.

X-gal staining on whole mount L4 DRG preparations at:

a) 3 days post-injection demonstrated that the 1764/4-/27+/RL1+ backbone allows highly efficient gene delivery to mouse sensory neurons following injection into the sciatic nerve, with the CMV promoter driving considerably higher levels of expression in these neurons than the EF1*a* promoter.

b) 21 days post-injection demonstrated that expression is maintained for long periods of time, but it is considerably reduced compared to that at early times post-injection.

(objective x5)

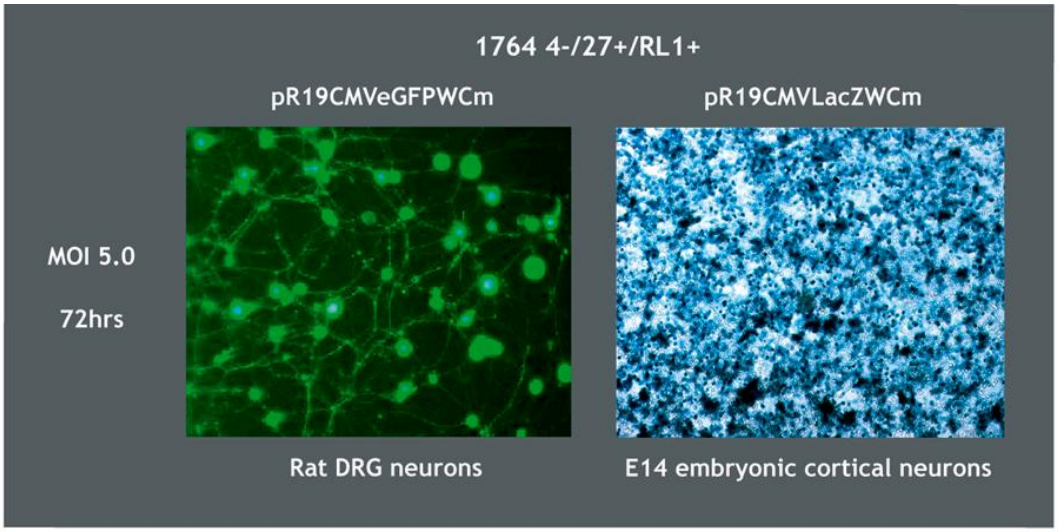


Figure 3.17 HSV-1 1764/4-/27+/RL1+ viruses are non-toxic to neurons in culture.

1764 4-/27+/RL1+ pR19CMVeGFPWCm and pR19CMVLacZWcm vectors were used to transduce rat DRG neurons (prepared as described in section 2.5.2) and rat embryonic E14 cortical neurons (prepared and transduced by F. Groutsi), respectively, at an MOI=5.0. X-gal staining or fluorescent microscopy at 72 hrs post-transduction revealed that neurons remain healthy following tranduction with the virus and show no obvious signs of toxicity caused by the virus (objective x10).

3.9 Generation of disabled HSV-1 vectors expressing shRNA

Following evaluation of shRNA expression from the plasmid vectors constructed in section 2.5 and selection of the disabled backbone that would make a suitable vector for delivery of RNAi to mouse DRG (section 3.8), the cassettes that successfully reduced ß-galactosidase levels (section 3.7) were inserted into 1764 4-/27+/RL1+ HSV-1 by homologous recombination. The pR19CMVshLacZ system that failed to induce any silencing was abandoned. The pR19 cassette is flanked by sequences that allow recombination into the LAT region of the HSV genome and thus, following recombination with viral DNA, each cassette was inserted immediately 3' of the LAT P2 region and was present in two copies per genome.

The pR19U6shLacZ, pR19U6-neg and pR19CMVenhU6shLacZ plasmids constructed in sections 3.5.1 and 3.5.2 were each co-transfected with 1764 4-/27+/RL1+ pR19CMVeGFP WCm viral DNA (prepared as described in section 2.4.5) into complementing MAM49 cells as described in section 2.4.1. Viral recombinants were identified by their inability to express GFP (white plaques) using fluorescent microscopy and were plaque purified as described in section 2.4.2. The pR19U6shGFP2 plasmid constructed in section 3.5.1 was co-transfected with 1764 4-/27+/RL1+ pR19CMVLacZWCm viral DNA. White plaques were identified following x-gal staining for ß-galactosidase. White plaques may arise from a mutation in the reporter gene or the promoter and thus, during negative selection, i.e. when white plaques are selected from a coloured background, there is an increased risk of selecting recombinants that may not be of the desired structure. It was therefore particularly important to confirm that the cassette expressing GFP or LacZ had been replaced by the cassette expressing shRNA and that the shRNA sequence had not been mutated. This was achieved by performing PCR on viral DNA extracted from each plaque (figure 3.18) followed by sequencing of the PCR products. One plaque from each recombinant virus was selected and grown to a high titre as described in section 2.4.3.

The pR19CMV/EmGFP-miR-LacZ and pR19CMV/EmGFP-miR-neg plasmids described in section 3.5.3 were linearised using a unique XmnI site in the plasmid backbone. 1764 4-/27+/RL1+ pR19CMVLacZWCm viral DNA was co-transfected with linearised pR19CMV/EmGFP-miR-neg. The pR19CMV/EmGFP-miR-LacZ plasmid was not transfected with viral DNA expressing ß-galactosidase, as the *lacZ* would be silenced by the virus expressing miR-LacZ making it impossible to select the correct recombinants. Instead, pR19CMV/EmGFP-miR-LacZ was co-transfected with 1764 4-/27+/RL1+ pR19U6shLacZ viral DNA. Viral recombinants were identified by their ability to express GFP (green plaques) using fluorescent microscopy. Two recombinant populations arose from these transfections represented by faint green and bright green plaques. As two copies of the LAT region are present in the genome, they were believed to be a consequence of the miR cassette having been inserted in the genome once and then twice, respectively. The bright green plaques were preferentially selected and purified. When a pure green population was obtained, the purity of the pR19CMV/EmGFP-miR-neg plaques was confirmed further by x-gal staining. Expression of GFP from the pR19CMV/EmGFP-miR-LacZ and pR19CMV/EmGFP-miR-neg recombinants indicated that the desired homologous recombination event had occurred. However, to confirm that the pre-miRNA sequence had not been mutated, PCR was performed on viral DNA extracted from each plaque using the GFP Forward and WCm Reverse primers described in section 2.1.5 (figure 3.19) and the PCR products were sequenced. One plaque from each recombinant virus was selected and grown to a high titre.

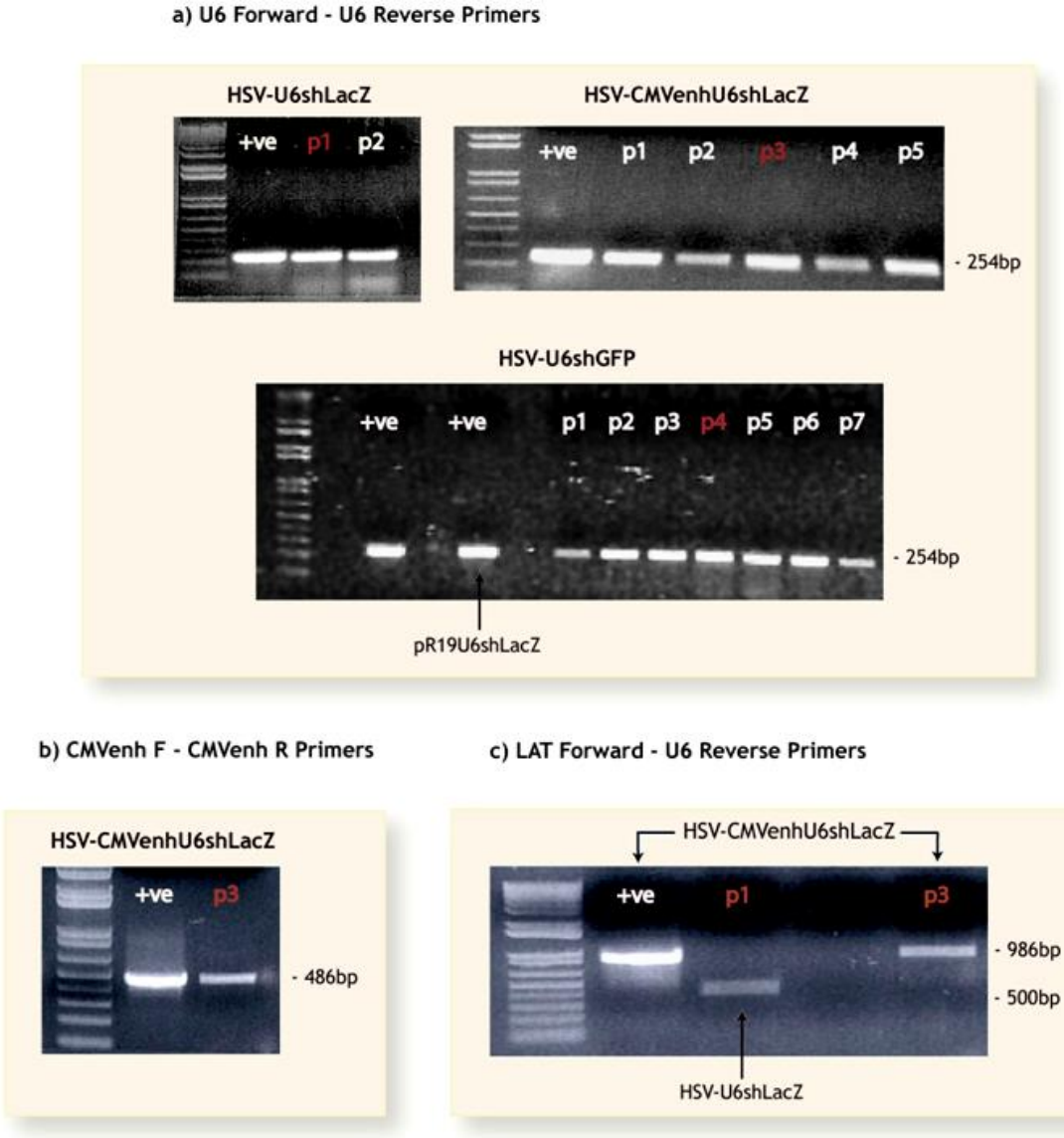


Figure 3.18 PCR on viral DNA to confirm that the correct homologous recombination event had occured. Unless stated otherwise, the positive control used in each experiment is the plasmid DNA used to generate the plaques tested. The letter "p" refers to plaque. Plaques in red were selected and grown to a high titre.

a) PCR on viral DNA extracted from HSV-U6shLacZ, HSV-CMVenhU6shLacZ and HSV-U6shGFP plaques using the U6 Forward and U6 Reverse primers produced a band of 254bp thus confirming the presence of the U6 promoter.

b) PCR on viral DNA extracted from HSV-CMVenhU6shLacZ plaque 3 using the CMVenh Forward and CMVenh Reverse primers produced a 486bp product thus confirming the presence of the CMV enhancer upstream of U6.

c) PCR on viral DNA extracted from HSV-U6shLacZ plaque 1 and HSV-CMVenhU6shLacZ plaque 3 using the LAT Forward and U6 Reverse primers confirmed that the cassette is inserted within the LAT region of HSV.

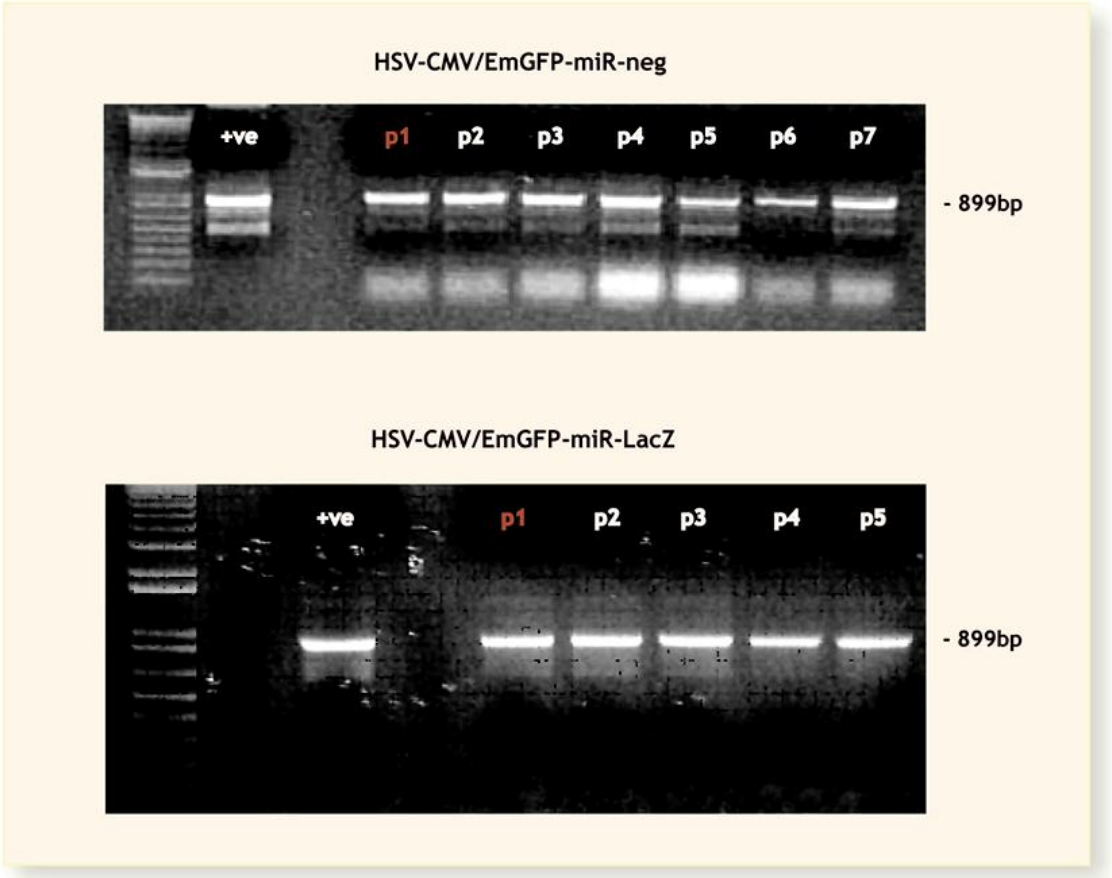


Figure 3.19 PCR on viral DNA to confirm that the correct homologous recombination event had occured.

The positive control used in each experiment is the plasmid DNA used to generate the plaques tested. The letter "p" refers to plaque. Plaques in red were selected and grown to a high titre.

PCR on viral DNA extracted from HSV-CMV/EmGFP-miR-neg and HSV-CMV/EmGFP-miR-LacZ plaques using the GFP Forward and WCm Reverse primers produced a band of 899bp thus confirming the presence of the pre-miRNA cassette.

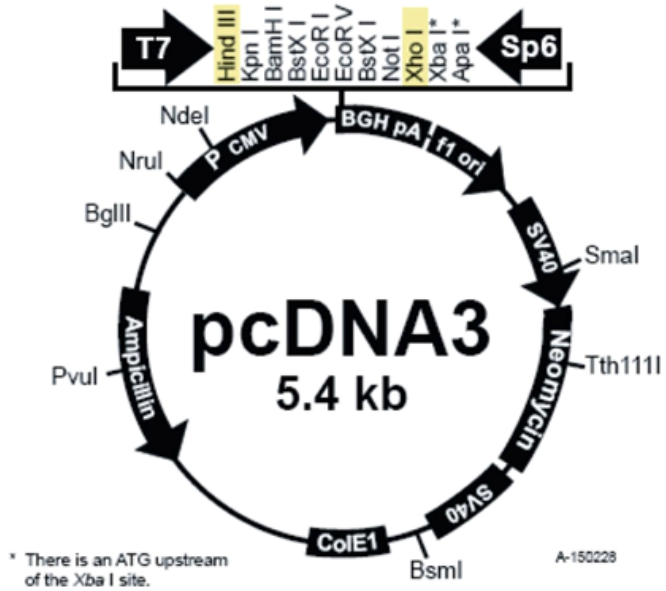
3.10 Construction of a cell line expressing ß-galactosidase

Evaluation of plasmid vectors expressing shRNA against ß-galactosidase revealed that the systems which were effective induced approximately 90% silencing of *lacZ* when the reporter gene and shRNA were co-delivered simultaneously (section 3.7). The level of sensitivity achieved with this system is greater than that achieved with endogenous gene silencing, because delivery to all cells is not required in order to achieve an RNAi response. To evaluate the efficiency of HSV-mediated silencing *in vitro*, a cell line that expresses ß-galactosidase would be more appropriate, as it would alleviate the need to deliver *lacZ* together with the RNAi viruses and would allow assessment of silencing in a system closer to endogenous gene expression.

The only available cell line expressing ß-galactosidase was the 9L/LacZ rat gliosarcoma cell line. 9L cells have been reported to be highly susceptible to infection with replication-defective ICP4- HSV-1 mutants (Krisky *et al.*, 1998, Moriuchi *et al.*, 2000). However, when 9L/LacZ cells were transduced with 1764 4-/27+/RL1+ pR19CMVeGFPWCm (MOI=10.0), the number of GFP positive cells at 72 hrs post-transduction was very low (data not shown). Thus, 9L/LacZ cells do not allow efficient gene expression from the disabled vectors used in this thesis and are not an appropriate cell line to evaluate HSV-mediated silencing. BHK cells are routinely used to assess expression levels from the vectors developed in our lab and therefore, a BHK cell line that constitutively expresses high levels of ß-galactosidase was constructed for the purpose of evaluating the vectors generated in section 3.10.

The ß-galactosidase coding sequence without the polyadenylation signal was excised from pR19CMVLacZWCm as a HindIII-XhoI restriction fragment and subcloned into the pcDNA3 multiple cloning site between HindIII-XhoI (figure 3.20). The pcDNA3-LacZ plasmid was linearised with SpeI and transfected into BHK cells as described in section 2.3.4. 100 neomycin (G418) resistant colonies were selected and screened for their ability to express ß-galactosidase. The colony expressing the highest levels was selected and the BHK-LacZ

the cell line was generated as detailed in section 2.3.5. Finally, BHK-LacZ cells were transduced with 1764 4-/27+/RL1+ pR19CMVeGFPWCm at an MOI=5.0. Fluorescent microscopy for GFP followed by x-gal staining at 48 hrs post-transduction demonstrated that the BHK-LacZ cell line uniformly expresses high levels of ß-galactosidase and allows high levels of HSV-mediated expression (figure 3.21).



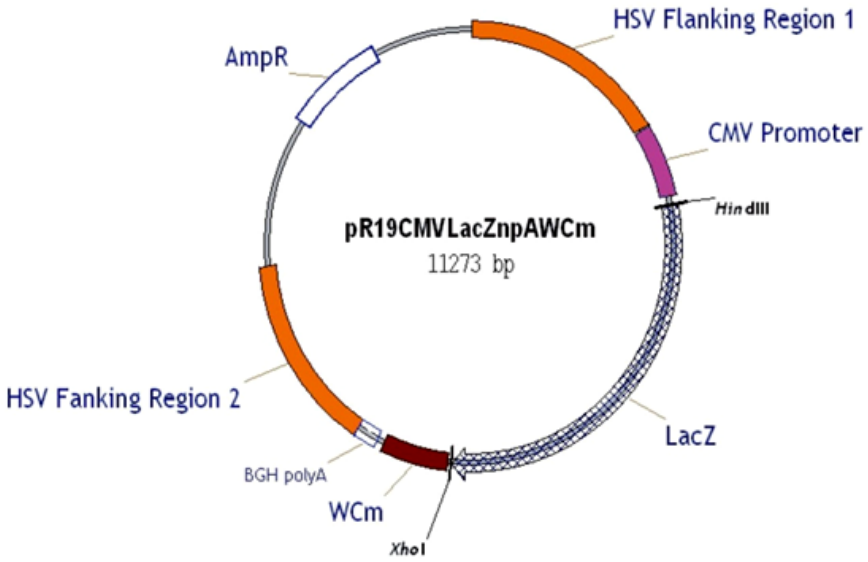


Figure 3.20 Cloning of the pcDNA3-LacZ plasmid

The lacZ gene was excised from pR19CMVLacZWCm as a HindIII-XhoI restriction fragment and subcloned into the pcDNA3 multiple cloning site between HindIII-XhoI (Invitrogen). Details of how the BHK-LacZ cell line was contructed can be found in section 3.10.

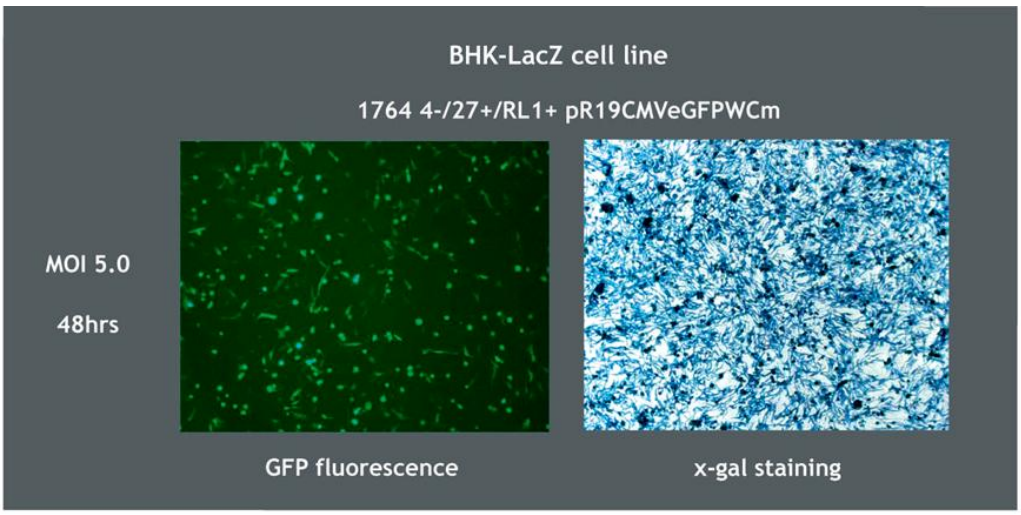


Figure 3.21 The BHK-LacZ cell line

Details of how the BHK-LacZ cell line was constructed can be found in section 3.10. BHK-LacZ cells were transduced with 1764 4-/27+/RL1+ pR19CMVeGFPWCm (MOI=5.0). Fluorescent microscopy for GFP followed by x-gal staining at 48 hrs post-transduction demonstrated that the BHK-LacZ cell line uniformly expresses high levels of beta-galactosidase and allows high levels of HSV-mediated expression (objective x10).

3.11 Evaluation of disabled vectors targeting lacZ in culture

To compare silencing from the HSV vectors expressing shRNA using different approaches (constructed in section 3.9), BHK-LacZ cells were transduced with each vector targeting *lacZ* (HSV-U6shLacZ, HSV-CMVenhU6shLacZ or HSV-CMV/EmGFP-miR-LacZ) or the negative control vectors (HSV-U6shGFP or HSV-CMV/EmGFP-miR-neg) at an MOI=10.0 (figure 3.22a). The expression of functional shRNA from the HSV-U6shGFP negative control (MOI=5.0) was confirmed by the reduction of GFP levels in BHK cells co-transduced with the 1764 4-/27+/RL1+ pR19CMVeGFPWCm virus expressing enhanced GFP (MOI=1.0) (figure 3.22b). Expression of miRNA from the HSV-CMV/EmGFP-miR-neg control was confirmed by the presence of the EmGFP signal (figure 3.22a-iv). Silencing was assessed by x-gal staining at 72hrs post-transduction. Transduction with the HSV-U6shGFP (figure 3.22a-i) or HSV-CMV/EmGFP-miR-neg (figure 3.22a-iv) control vectors had no effect on ß-galactosidase expression levels. Transduction with HSV-U6shLacZ (figure 3.22-ii), HSV-CMVenhU6shLacZ (figure 3.22a-iii) or HSV-CMV/EmGFP-miR-LacZ (figure 3.22a-v) resulted in a significant reduction in ß-galactosidase levels. Importantly, GFP expression in BHK-LacZ cells transduced with HSV-CMV/EmGFP-miR vectors was not affected by expression of miR-LacZ as evaluated by fluorescent microscopy (figure 3.22a-iv and v). It can be therefore concluded that silencing is not caused by a non-specific effect mediated by the HSV vector backbone. Unlike off-target effects, this would most likely be a non-sequence specific effect caused by induction of the INF response or toxicity from the virus and thus affect the expression levels of both *lacZ* and *gfp*.

B-galactosidase activity assay performed in BHK-LacZ cells at various times post-transduction with HSV-U6shLacZ, HSV-CMVenhU6shLacZ or HSV-CMV/EmGFP-miR-LacZ revealed reduced protein levels by up to 84.0 ± 8.0% (n = 6, mean ± s.d.), 91.0 ± 7.0% and 86.0 ± 7.0%, respectively (figure 3.23a). B-galactosidase levels were calculated as a percentage of the expression in the cells transduced with the negative controls (HSV-U6shGFP or HSV-CMV/EmGFP-miR-neg). HSV-CMV/EmGFP-miR-LacZ is most effective at 48

hrs, HSV-CMVenhU6shLacZ at 72 hrs and HSV-U6shLacZ at 4 days post-transduction, reflecting the different kinetics of expression from the promoters used. B-galactosidase activity assay performed at 72 hrs post-transduction at MOI=1.0, 5.0 and 10.0 revealed that silencing is dose-dependent (figure 3.23b). Transduction of BHK-LacZ cells with these vectors at an MOI higher that 10.0 resulted in no further improvement of silencing, whilst cell viability was considerably reduced, indicating that the endogenous miRNA pathway may be saturated (data not shown).

3.12 HSV-mediated silencing in primary DRG neurons

To determine whether HSV-mediated delivery of shRNA could induce silencing in primary neuronal cells, rat DRG neuronal cultures (prepared as described in section 2.5.2) were co-transduced with 1764 4-/27+/RL1+ pR19CMVeGFPWCm (MOI=1.0), and either HSV-U6shLacZ or HSV-U6shGFP (MOI=5.0). High transduction efficiencies were achieved with over 90% of neurons being positive for GFP. Fluorescent microscopy at 48 hrs post-transduction revealed that transduction with HSV-U6shLacZ had no effect on GFP expression levels, whereas transduction with HSV-U6shGFP resulted in near complete inhibition of GFP expression (figure 3.24). This data indicates highly effective silencing in neurons *in vitro* induced by HSV-mediated RNAi.

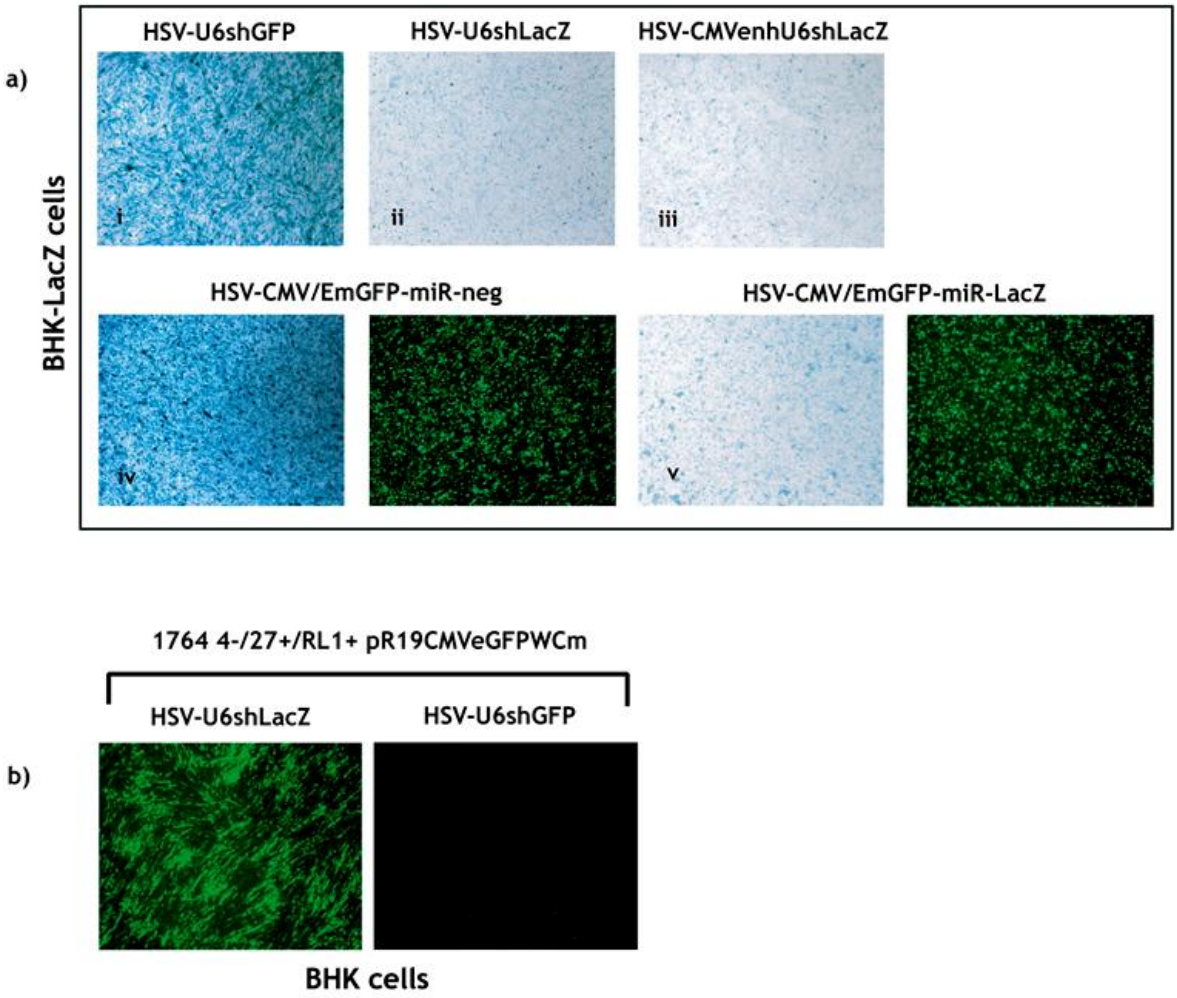


Figure 3.22 Evaluation of HSV vectors expressing shRNA against beta-galactosidase

a) BHK-LacZ cells were transduced with i) HSV-U6shGFP negative control, ii) HSV-U6shLacZ, iii) HSV-CMVenhU6shLacZ, iv) HSV-CMV/EmGFP-miR-neg negative control or v) HSV-CMV/EmGFP-miR-LacZ (MOI=10.0). Silencing was assessed by x-gal staining at 72hrs post-transduction. Transduction with the negative control vectors had no effect on beta-galactosidase expression levels (i and iv). Transduction with HSV-U6shLacZ, HSV-CMVenhU6shLacZ or HSV-CMV/EmGFP-miR-LacZ resulted in a significant reduction in beta-galactosidase levels (ii, iii and v).

b) BHK cells were co-transduced with the 1764 4-/27+/RL1+ pR19CMVeGFPWCm virus and either HSV-U6shLacZ or HSV-U6shGFP (1:10). Fluorescent microscopy revealed that transduction with HSV-U6shGFP resulted in a dramatic reduction of GFP expression levels.

(objective x10)

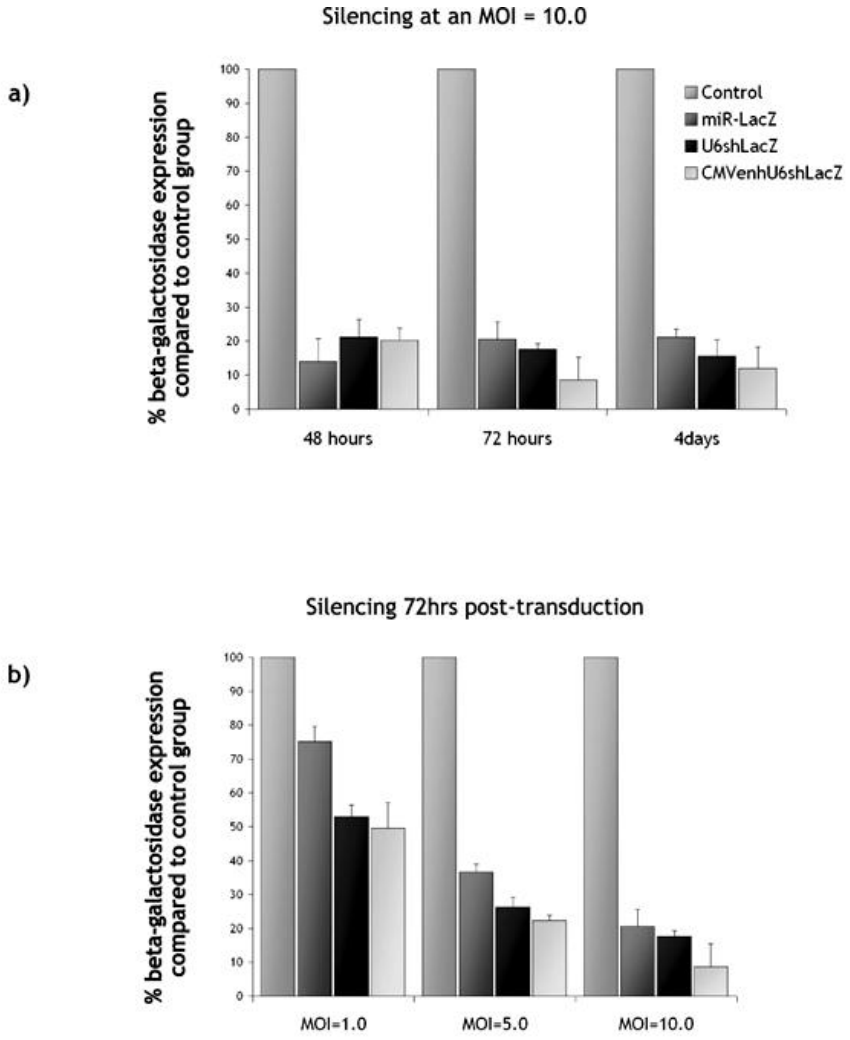


Figure 3.23 Quantification of silencing in BHK-LacZ cells transduced with disabled HSV vectors expressing shRNA against beta-galactosidase.

a) B-galactosidase activity assay at various times post-transduction demonstrated reduced protein levels by up to with 84.0 ± 8.0% HSV-U6shLacZ, 91.0 ± 7.0% with HSV-CMVenhU6shLacZ and 86.0 ± 7.0% with HSV-CMV/EmGFP-miR-LacZ (n = 6, mean ± s.d.). HSV-CMV/EmGFP-miR-LacZ is most effective at 48 hrs, HSV-CMVenhU6shLacZ at 72 hrs and HSV-U6shLacZ at 4 days post-transduction.

b) B-galactosidase activity assay performed at 72 hrs post-transduction at MOI=1.0, 5.0 and 10.0 revealed that silencing is dose-dependent.

Beta-galactosidase expression was calculated as a percentage of the expression in cells transfected with negative controls (HSV-U6shGFP for the HSV-U6shLacZ and HSV-CMVenhU6shLaZ, and HSV-CMV/EmGFP-miR-neg for pR19CMV/EmGFP-miR-LacZ).

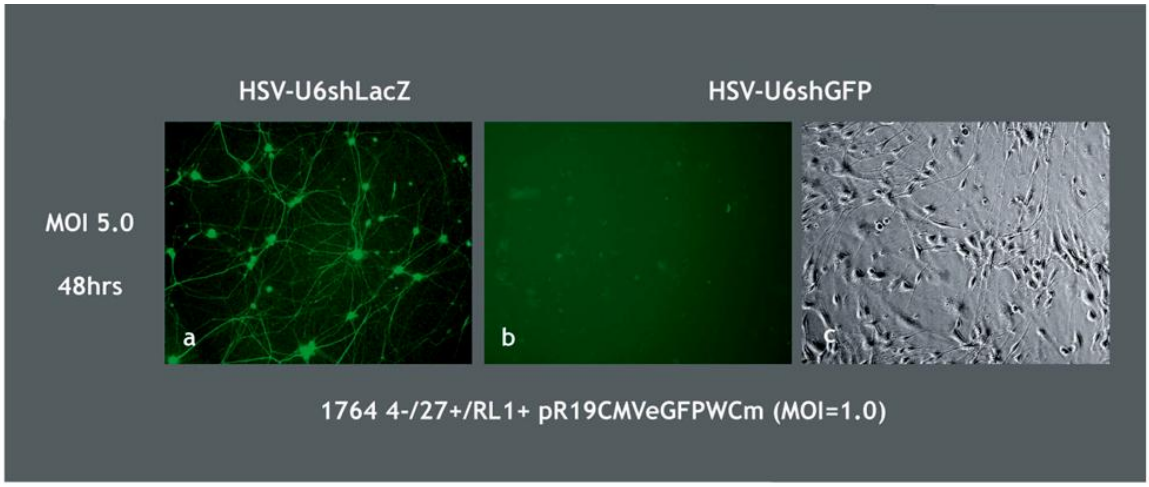


Figure 3.24 HSV-mediated silencing in primary DRG neuronal cultures

Rat DRG neuronal cultures (prepared as described in section 2.5.2) were co-transduced with 1764 4-/27+/RL1+ pR19CMVeGFPWCm (MOI=1.0), and either a) HSV-U6shLacZ or b) HSV-U6shGFP (MOI=5.0). High transduction efficiencies were achieved with over 90% of neurons being positive for GFP. Fluorescent microscopy at 48 hrs post-transduction demonstrated that whilst HSV-U6shLacZ has no effect on the levels of GFP, HSV-U6shGFP results in near complete inhibition of GFP expression. Image c shows a phase contrast photomicrograph of neurons transduced HSV-U6shGFP (objective x10).

3.13 Evaluation of disabled vectors targeting lacZ in DRG *in vivo*

To compare the efficiency of HSV-mediated silencing in sensory neurons *in vivo*, ß-galactosidase was overexpressed in the DRG of BALB/c mice using a disabled HSV-1 vector. A single injection into the sciatic nerve was performed as described in section 2.6.3 to co-deliver 1764 4-/27+RL1+ pR19CMVLacZWCm expressing ß-galactosidase and each of the HSV-U6shLacZ, HSV-CMVenhU6shLacZ and HSV-CMV/EmGFP-miR-LacZ vectors targeting *lacZ* or each of the HSV-U6-neg and HSV-CMV/EmGFP-miR-neg negative controls, at a ratio 1:5. In animals injected with HSV-U6-neg, HSV-U6shLacZ or HSV-CMVenhU6shLacZ, the 1764 4-/27+/RL1+ pR19CMVeGFPWCm vector was also co-injected to normalise transduction efficiency (figure 3.25a). In animals injected with HSV-CMV/EmGFP-miR vectors, transduction efficiency was monitored by the presence of the EmGFP signal expressed from these vectors (figure 3.25b).

7 days post-injection, the lumbar DRG (L4/L5) from the injected side of the animals were isolated and analysed as described in section 2.6.7. High transduction efficiencies were achieved in all injections (n=15) mainly to the L4 DRG, with the majority of neurons being positive for GFP as assessed by fluorescent microscopy. Figures 3.25a and b demonstrate representative L4 DRG from these animals. X-gal staining in whole mount DRG preparations revealed a dramatic reduction in ß-galactosidase levels in animals injected with HSV-U6shLacZ (figure 3.25a-ii), HSV-CMVenhU6shLacZ (figure 3.25a-iii) or HSV-CMV/EmGFP-miR-LacZ (figure 3.25b-ii) compared to control groups injected with either HSV-U6-neg (figure 3.25a-i) or HSV-CM/EmGFP-miR-neg (figure 3.25b-i).

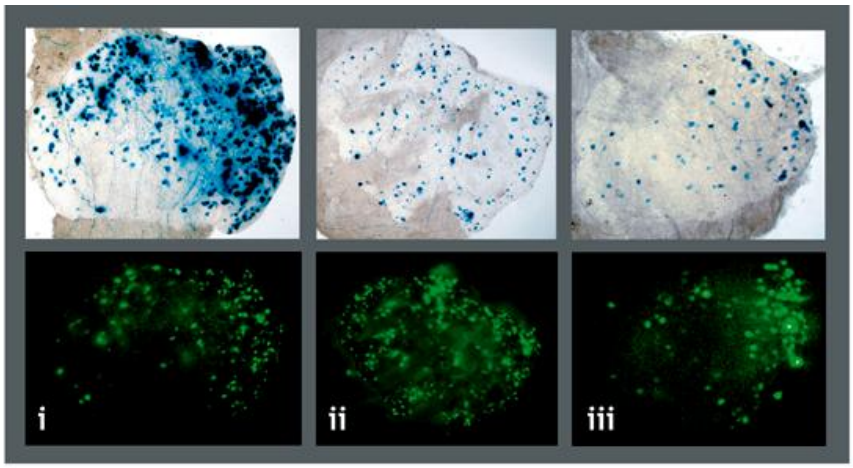
In the experiments described in this section, silencing can only be achieved in neurons that have been transduced with both ß-galactosidase- and shRNA-expressing vectors. In animals also injected with the GFP-expressing vector (figure 3.25a), expression of GFP allows monitoring of transduction efficiency, but not direct labelling of neurons expressing shRNA. Previous work in our lab has demonstrated that co-injection of viruses expressing different reporter genes from the same backbone results in highly overlapping

transduction patterns in DRG (S.Martins, unpublished data). This is also demonstrated by the high degree of ß-galactosidase and GFP co-localisation evident in figure 3.25a-i. Nevertheless, the high levels of ß-galactosidase displayed in some neurons when the ß-galactosidase-expressing and HSV-U6shLacZ or HSV-CMVenhU6shLacZ vectors were co-injected may be due to incomplete transduction overlap (figure 3.25a-ii and iii). In the DRG of animals injected with HSV-CMV/EmGFP-miR-LacZ (figure 3.5b-ii), EmGFP expression allows identification of transduced neurons and >90% of GFP-positive neurons display markedly reduced or abolished ß-galactosidase expression.

Silencing was quantified by directly counting the number of LacZ-positive versus GFP-positive neurons in whole mount DRG preparations. Thus, transduction efficacy was normalised between different conditions and silencing was assessed in relation to the transduced population of neurons rather than the total number of neurons in the DRG. We found that HSV-U6shLacZ reduces ß-galactosidase levels by 78.0 ± 6.8% (p = 0.001) (n = 3, mean ± s.d.), HSV-CMVenhU6shLacZ by 62.1 ± 20.9% (p = 0.02) and HSV-GFP-miR-LacZ by 92.0 ± 3.0% (p = 0.0002) (figure 3.26). B-galactosidase levels were calculated as a percentage of the expression in DRG injected with the negative controls. Whereas the number of GFP-positive neurons remained essentially stable at 170 ± 53 cells (n = 15, mean ± s.d.) in each of the different conditions, the level of lacZ-positive neurons dropped from 464 ± 68 cells (n = 6, mean ± s.d.) in DRG injected with the negative controls to 122 ± 68, 148 ± 60 and 63 ± 15 cells (n = 3, mean ± s.d.) in DRG injected with HSV-U6shLacZ, HSV-CMVenhU6shLacZ or HSV-GFP-miR-LacZ, respectively. The number of GFP-positive neurons was found to be much lower than the number of LacZ-positive neurons in DRG injected with the negative controls. This is mainly due to the fact that x-gal staining used to visualise LacZ-positive cells is more sensitive than fluorescence microscopy used to visualise GFP-positive cells. Moreover, in the HSV-miRNA vectors, EmGFP is co-cistronically expressed with the pre-miRNA and this may result in inefficient GFP mRNA processing.

The above data confirm robust silencing in peripheral neurons *in vivo* induced by HSV-mediated RNAi. Importantly, injection with the negative controls had no effect on ß-galactosidase levels and injection with the vectors targeting *lacZ* does not seem to have an effect on the levels of GFP indicating silencing specificity.

a)



b)

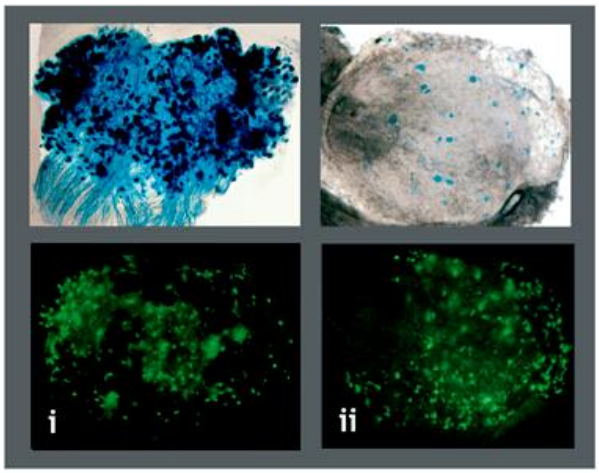


Figure 3.25 Evaluation of HSV-mediated silencing in DRG neurons *in vivo*.

a) BALB/c mice (3 animals/group) were injected once directly into the sciatic nerve with 1x10^6 plaque forming units (pfu) of 1764 4-/27+/RL1+ pR19CMVLacZWCm vector and 5x10^6 pfu of: (i) the HSV-U6-neg control, (ii) HSV-U6shLacZ, or (iii) HSV-CMVenhU6shLacZ. The 1764 4-/27+/RL1+ pR19CMVeGFPWCm vector was also co-injected (1x10^6 pfu) to normalise transduction efficiency.

b) BALB/c mice (3 animals/group) were injected once directly into the sciatic nerve with 1x10^6 pfu of 1764 4-/27+/RL1+ pR19CMVLacZCm vector and 5x10^6 pfu of: (i) the HSV-CMV/EmGFP-miR-neg control or (ii) HSV-CMV/EmGFP-miR-LacZ. EmGFP expression from this system allows direct labelling of transduced cells.

GFP levels were assessed by fluorescent microscopy prior to x-gal staining. Silencing was assessed by x-gal staining in whole mount L4 DRG preparations at 7days post-injection (objective x5).

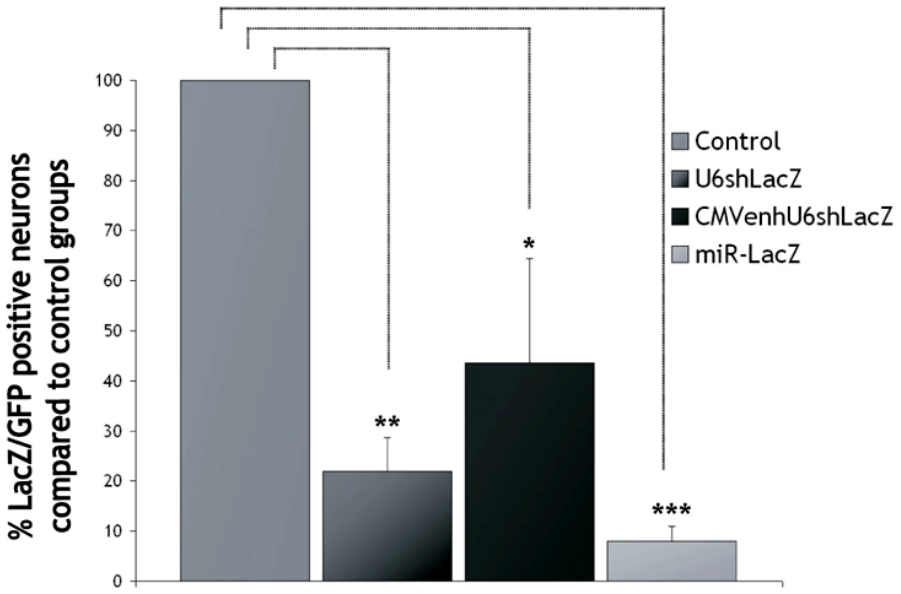


Figure 3.26 Quantification of silencing in sensory neurons *in vivo*

Silencing in sensory neurons *in vivo* was quantified by directly counting the number of LacZ-positive versus GFP-positive neurons in whole mount L4 DRG preparations (at a magnification x40) isolated from animals injected as described in section 3.13.

HSV-U6shLacZ reduces beta-galactosidase protein levels by 78.0 ± 6.8% (**p = 0.001), HSV-CMVenhU6shLacZ by 62.1 ± 20.9% (*p = 0.02) and HSV-CMV/EmGFP-miR-LacZ by 92.0 ± 3.0% (***p = 0.0002) (n = 3, mean ± s.d., results analysed for significance using t-test). B-galactosidase levels were calculated as a percentage of the expression in DRG injected with the HSV-U6-neg and HSV-CMV/EmGFP-miR-neg negative controls. Whereas the number of GFP-positive neurons remained essentially stable at 170 ± 53 cells (n = 15, mean ± s.d.) in each of the different conditions, the level of lacZ-positive neurons dropped from 464 ± 68 cells (n = 6, mean ± s.d.) in DRG injected with the negative controls to 122 ± 68, 148 ± 60 and 63 ± 15 cells (n = 3, mean ± s.d.) in DRG injected with HSV-U6shLacZ, HSV-CMVenhU6shLacZ or HSV-GFP-miR-LacZ respectively.

3.14 HSV-mediated silencing of lacZ in a transgenic mouse model

The results described in the previous section demonstrate silencing in a system where a marker gene has been delivered coincidentally with silencing. It was therefore important to determine whether *lacZ* could be effectively silenced in a transgenic mouse model, where ß-galactosidase expression resembles endogenous gene expression. The HSV-CMV/EmGFP-miR-LacZ vector, which was shown in the previous section to be the most effective at silencing *in vivo*, was selected for further testing. Moreover, co-expression of EmGFP from these vectors allows direct labelling of transduced neurons.

Rosa26 transgenic mice (supplied by Johnson & Johnson) were injected into the sciatic nerve with HSV-CMV/EmGFP-miR-neg or HSV-CMV/EmGFP-miR-LacZ (as described in section 2.6.3). 7 days post-injection, the lumbar DRG from the injected side of the animals were isolated and sectioned (as described in section 2.6.6). Rosa26 mice express high levels of ß-galactosidase in the majority of their tissues, including neuronal and non-neuronal cells of the DRG. Quantification of ß-galactosidase expression in the whole DRG would measure silencing not only in sensory neurons, which is the target cell population, but also in non-neuronal cells not transduced by the vector. The DRG were, therefore, sectioned to clearly demonstrate silencing in individual neurons transduced by the virus.

Fluorescence microscopy for GFP demonstrated high transduction efficiencies with the vectors throughout all sections taken from injected animals. Figure 3.27 shows representative sections from L4 DRG. In all 4 independent experiments, neurons transduced with HSV-CMV/EmGFP-miR-LacZ (figure 3.27-ii), whilst also GFP-positive, displayed significantly reduced x-gal staining compared to animals injected with the negative control (figure 3.27-i). B-galactosidase expression in these sections was also markedly reduced compared to sections taken from the DRG of the non-injected side of the same animals (data not shown).

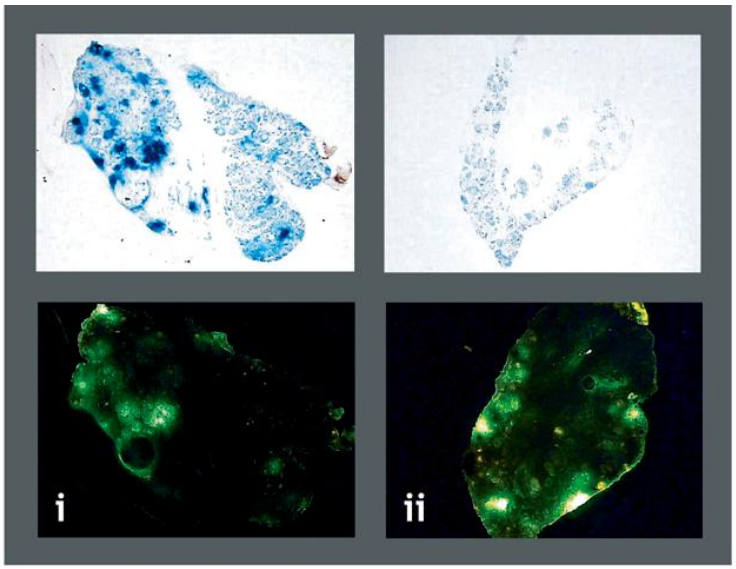


Figure 3.27 HSV-mediated silencing in a transgenic mouse model

Rosa26 transgenic mice were injected into the sciatic nerve with 5x10^6 pfu of: (i) HSV-CMV/EmGFP-miR-neg or (ii) HSV-CMV/EmGFP-miR-LacZ. 7days post-injection, the L4 DRG were isolated and sectioned. GFP expressed from these vectors allows simultaneous localisation of transduced neurons and monitoring of silencing efficacy. Neurons transduced with HSV-CMV/EmGFP-miR-LacZ are GFP positive and display significantly reduced x-gal staining compared to DRG sections taken from animals injected with the negative control (objective x10).

3.15 Discussion

RNAi has become a powerful tool for modulating gene expression. Delivery to neurons however poses specific challenges. Whereas delivery of siRNAs to primary neuronal cultures has been achieved with relatively high efficiency (Davidson *et al.*, 2004), *in vivo* delivery of RNAi to neurons and DRG neurons in particular, has been problematic. The development of an efficient method for *in vivo* delivery of RNAi to peripheral neurons would allow a better understanding of gene function, enable the validation of novel gene targets in drug discovery, and potentially allow the development of RNAi-based treatments.

The replication-defective HSV-1 vectors developed in our laboratory allow highly efficient gene delivery to neurons of the central and peripheral nervous system both *in vitro* and *in vivo* (Lilley *et al.*, 2001, Palmer *et al.*, 2000) and are particularly efficient at targeting DRG neurons in both mice and rats through retrograde transport following injection into the sciatic nerve. Whilst work in our laboratory had previously identified regulatory elements in the HSV genome that allow efficient expression of exogenous genes from heterologous promoters in a relatively non-promoter specific fashion, expression of shRNA from these vectors had not been previously investigated.

The work described in this chapter demonstrates that replication-defective HSV-1 vectors can express shRNA and induce highly effective and specific silencing in dividing cells in culture, primary neuronal cells and DRG neurons *in vivo*. Silencing in sensory neurons *in vivo* was assessed in an overexpression system and more importantly in a transgenic mouse model. Moreover, it was established that expression of shRNA from the disabled 1764/4-/27+/RL1+ HSV-1 vector backbone can be achieved using either pol II or pol III promoters linked to the LATP2 region.

With the exception of the modified CMV promoter that failed to induce any silencing, the shRNA expression systems tested in this thesis demonstrated similar levels of highly effective silencing in dividing cells in culture. However, when these promoter systems

were tested in DRG neurons *in vivo*, the miRNA-like system was found to be considerably more effective. These results are in agreement with the more recent findings by Hassani *et al.*, who have demonstrated that when plasmid vectors expressing shRNA from the H1 promoter or a hybrid CMV enhancer-H1 promoter were tested *in vitro* on two different cell lines, silencing efficacy was independent of the promoter used. However, when these vectors were tested *in vivo* in either the developing or the adult mouse brain, the efficiency of the shRNA depended markedly on the promoter used (Hassani *et al.*, 2007). There is now general consensus that the choice of promoter for expression of shRNA varies in different *in vitro* and *in vivo* situations and thus, the conclusions drawn from the evaluation performed in this thesis apply only to the cell types and conditions tested.

The reasons behind the disappointing results obtained with the modified CMV promoter were not investigated further. The highly promising data obtained with the other shRNA expression systems evaluated in this chapter was sufficient for this work to progress to the *in vivo* evaluation phase, which was the main focus of this thesis. Nevertheless, the failure to produce any obvious reduction in the levels of ß-galactosidase can only be attributed to the lack of functional shRNA being expressed from this system, as the target sequence against ß-galactosidase was identical in all the cassettes tested, which were also fully sequenced to confirm that the shRNA sequence had not been mutated. This result was highly surprising especially since silencing was investigated following transfection into 293T cells and identical shRNA expression cassettes have been shown to induce effective silencing of *gfp* or *ataxin-1* following transfection into HEK-293 cells. These cassettes also induced effective silencing of *ß-glucuronidase* and *ataxin-1* in the mouse brain, when inserted into adenoviral and AAV vectors, respectively (Xia *et al.*, 2004, Xia *et al.*, 2002). Moreover, two independent studies have since confirmed that efficient expression of shRNA can be achieved from tissue-specific pol II promoters using cassettes similar to the one described by Xia and colleagues (Giering *et al.*, 2008, Gou *et al.*, 2004).

The enhancer element of the CMV promoter failed to improve silencing from the U6 promoter when transfected into 293T cells, it demonstrated marginally improved silencing in BHK-LacZ cells when expressed from an HSV vector, and this system was identified as the least effective at silencing *in vivo*. These findings contradict a previous study that demonstrated approximately 50% enhanced silencing in 293T cells when the same system was expressed from a plasmid (Xia *et al.*, 2003). However, in the study by Xia *et al.* silencing achieved from the U6 promoter was relatively modest. It has been suggested that expression from the U6 promoter may be regulated by a mechanism that aims to prevent accumulation of U6 RNAs (Noonberg *et al.*, 1996). Thus, improving silencing from the U6 promoter may only be feasible in situations where the activity of the U6 promoter is relatively weak. Studies in the mouse brain have demonstrated that although the U6 promoter is more effective that the H1 promoter when inserted into a lentivirus (Mäkinen *et al.*, 2006), the CMV enhancer significantly improves silencing from the H1 promoter (Hassani *et al.*, 2007). It remains to be established whether a hybrid CMV enhancer-H1 promoter is more efficient than the CMV enhancer-U6.

Although the miRNA-based vector demonstrated the most effective silencing in DRG neurons *in vivo*, a more systematic comparison is required in order to identify whether expressing shRNA from a miRNA-like system is superior to expressing shRNA from pol III promoters. Boden *et al.* demonstrated that shRNA embedded into miR-30 sequences and expressed from the U6 promoter was 80% more effective in reducing HIV-1 p24 antigen production in 293 cells than shRNA expressed from the same promoter (Boden *et al.*, 2004). Northern blot analysis revealed that shRNA expressed from a miRNA-like system is more efficiently processed into siRNA. Similar results were obtained by Silva *et al.*, who demonstrated that HEK-293 cells transfected with miR-30-based plasmids contained approximately 12 times more siRNA than cells transfected with plasmids expressing shRNA (Silva *et al.*, 2005). Expression of shRNA from miRNA-like plasmids resulted in more effective and consistent knockdown of target gene expression. In contrast to the above

studies in which shRNAs with 21nt stem / 9nt loop and 29nt stem / 4nt loop were evaluated respectively, Li *et al*. demonstrated that shRNAs with a 19nt stem and 9nt loop expressed from the U6 promoter were considerably more effective than synthetic miRNAs expressed from miR-30-based vectors under the control of either the CMV or U6 promoter (Li *et al*., 2007). Boudreau *et al*. argued that the shRNAs tested by Boden *et al*. and Silva *et al*. had sub-optimal 5'overhangs (5' XbaI site and a 27nt leader sequence respectively), which are important substrates for Exportin 5 and Dicer, and that none of the previous comparison studies had assessed strand biasing, i.e. which strand of the siRNA duplex enters the RISC and mediates gene silencing (Boudreau *et al*., 2008b). The authors demonstrated that when variability between shRNA and miRNA-like vectors is minimised by taking into account the transcribed product, processing sites and strand bias, shRNAs expressed from the U6 promoter are more effective that synthetic miRNAs expressed from the same promoter both *in vitro* and in mice *in vivo*. In the experiments described in this thesis, the shRNA sequences were designed to have 3′ overhangs resulting from pol III termination, which provide optimal substrates for Exportin 5 and Dicer. Moreover, they were selected to account for the +1-G nucleotide of the U6 promoter and to contain AU-rich 3′ ends, both of which promote loading of the antisense strand. However, although the target sequence against ß-galactosidase was identical in pre-miRNA and shRNA sequences, the stem length and loop structure were different. Moreover, expression of shRNA and synthetic miRNA was driven by different promoters and thus, it can be argued that a fair comparison of shRNA and miRNA-like vectors was not provided.

Nevertheless, expression of a marker gene together with miRNA from a single transcription unit and the potential to use inducible or tissue-specific pol II promoters hold clear advantages for the use of miRNA-like expression vectors. The latter may be a requirement in the design of safe vectors, as there is evidence to suggest that shRNAs compete with endogenous miRNAs for limiting cellular factors required for processing of small RNAs, such as Exportin 5 and RISC (Castanotto *et al*., 2007, Hutvágner, 2004, Yi *et*

al., 2005). This can result in oversaturation of the endogenous miRNA pathway, downregulation of endogenous miRNA production, toxicity and even death (Grimm *et al.*, 2006). The risk of oversaturation can be minimised by optimising the shRNA dose and sequence, as well as expressing shRNA from inducible or tissue-specific promoters. However, expression of shRNA from miRNA-based vectors requires the additional step of excision from the longer pri-miRNA sequence by the Drosha-DGCR8 complex (Han *et al.*, 2006). This additional step may add another point of competition with endogenous miRNAs, thereby increasing the risk of saturation. Nevertheless, it has been demonstrated that unlike siRNAs and shRNAs, synthetic miRNAs do not compete with the endogenous miRNAs pathway (Castanotto *et al.*, 2007). Moreover, synthetic miRNAs were shown to have improved safety profiles compared to shRNAs both *in vitro* and the mouse cerebella *in vivo* when expressed from an AAV vector (Boudreau *et al.*, 2008a). Although synthetic miRNAs were found to be less potent than shRNAs, shRNAs generate an abundance of unprocessed precursors, whereas synthetic miRNAs are processed more efficiently (Boudreau *et al.*, 2008b). It was subsequently revealed that shRNAs disrupt miRNA biogenesis and function, whereas synthetic miRNAs have negligible effects even when dosed to silence as effectively as shRNAs. Moreover, shRNAs caused Purkinje cell neurotoxicity, whereas synthetic miRNA expression was well tolerated and sufficient to silence *ataxin-1* in SCA1 mice. Finally, whilst shRNA has been shown to induce an INF response in neurons (Alvarez *et al.*, 2006, Cao *et al.*, 2005), neurotoxicity in cortical cultures and the mouse striatum was attenuated when shRNA sequences were inserted in miRNA-based lentiviral and AAV vectors, respectively (Bauer *et al.*, 2008, McBride *et al.*, 2008). Collectively, the above findings suggest that miRNA-based vectors are better suited for silencing in the mammalian nervous system.

Additionally, pol III promoters can only allow expression of a single shRNA and thus, inhibition of multiple genes requires expression from multiple promoters or different vectors, resulting in variable levels of shRNA expression. The miRNA-like system allows

expression of multiple miRNA cassettes from a single transcript and it is therefore possible to simultaneously silence multiple gene targets (Chung *et al.*, 2006, Zeng & Cullen, 2003). Alternatively, delivery of RNAi can be combined with expression of a biologically active protein in place of the reporter gene. These properties of the miRNA-like vectors may prove particularly useful in the study or treatment of complex conditions that are likely to be caused by a combination of factors (an example is described in Chapter 5 of this thesis). Finally, a single miRNA cassette may be multimerised in tandem, up to at least eight copies, resulting in increased inhibition of target gene expression (Chung *et al.*, 2006). It has to be noted that vectors based on miR-30 expressing multiple copies of miRNA in tandem have yielded conflicting results. Zhou *et al.* reported reduced silencing from a plasmid vector expressing two copies of miRNA in tandem compared to a plasmid expressing a single copy (Zhou *et al.*, 2005), whilst Sun *et al.* reported improved silencing efficacy in 293 cells when two copies of the same miRNA were expressed in tandem from a lentiviral vector, but addition of a third copy yielded a disproportionally smaller increase (Sun *et al.*, 2006). Factors such as copy number and positioning, and intervening spacer length and composition are all likely to affect miRNA processing. Although, during the course of this thesis, another study was published to describe expression of shRNA from a replication-defective HSV-1 (Hong *et al.*, 2006), this is the only demonstration of HSV-mediated expression of artificial miRNA.

Specificity has become a major concern in the use of RNAi. Non-specific silencing may result from a non-sequence-specific effect caused by the virus, sequence-specific off-target effects, or induction of the INF response. Throughout this study, negative control vectors had no effect on target gene expression. Moreover, the levels of GFP were not affected by expression of shRNA or miRNA targeting *lacZ*, and vice versa, thus excluding any potential non-specific effects induced by the HSV vector backbone. Furthermore, expression of shRNA/miRNA against ß-galactosidase in 293T cells had no effect on the expression levels of four unrelated endogenous genes, including a gene involved in the

induction of an INF response, indicating that silencing is not caused by a general non-specific shutdown of protein synthesis. Nevertheless, a microarray analysis is required in order to identify any potential off-target effects caused by the hairpin sequence against *lacZ*. Moreover, although expression of shRNA from a pol III-based system or a miRNA-based vector resulted in no obvious toxicity in primary neurons *in vitro* or DRG neurons *in vivo*, a more detailed investigation is required to exclude neurotoxicity caused by induction of an INF response or competition with the endogenous miRNA pathway.

In conclusion, the present chapter has developed replication-defective HSV-1 vectors that can be used to efficiently deliver RNAi to peripheral neurons both *in vitro* and *in vivo*. These highly promising results provided the basis for the work described in the following two chapters of this thesis, which aimed to provide proof of concept that endogenous genes can be silenced in sensory neurons *in vivo* using the vectors developed in this chapter and also demonstrate potential applications of this method in target validation and functional studies.

CHAPTER 4

HSV-MEDIATED SILENCING OF AN ENDOGENOUS GENE INVOLVED IN NOCICEPTION – SILENCING OF TRPV1

4.1 Introduction

The recent discovery that RNAi operates in mammalian neurons has generated great excitement, not only with respect to potential applications in functional genomic studies and target validation, but also harnessing RNAi as a therapeutic strategy to silence disease-causing genes. Although delivery of synthetic siRNAs to the nervous system has achieved silencing of molecular targets in various models of neurological disease including pain (summarised in section 4.1.3), silencing requires frequent administration and relatively high doses of siRNAs. As a more efficient alternative, targeted delivery of RNAi to neurons can be achieved using viral vectors. At the time the objectives of this chapter were conceived, lentiviruses, adenoviruses and AAV had been engineered to deliver shRNA to the central nervous system (Harper *et al.*, 2005, Ralph *et al.*, 2005, Raoul *et al.*, 2005, Singer *et al.*, 2005, Xia *et al.*, 2004). There were no reports, however, of vector-mediated delivery of shRNA to DRG neurons *in vivo*, the main target site for studying nociceptive processes and for the development of new analgesics.

This chapter investigates the potential of the disabled HSV vectors developed in the previous chapter to silence the endogenous *trpv1* gene, which is thought to be involved in nociceptive processes, in sensory neurons *in vivo*. The following introductory sections provide a brief overview of the neuroanatomy of the structures involved in nociception, chronic pain and the role of TrpV1, and review the efforts being made to validate pain-related genes using synthetic siRNAs.

4.1.1 Nociception

Nociception is the term commonly used to refer to the perception of pain. Acute nociceptive pain serves a crucial physiological function, warning the body of impending or actual tissue damage, preventing further damage, and aiding the healing process. The peripheral terminals of primary afferent fibres respond to a variety of stimuli and translate this information into the dorsal horn of the spinal cord (grey matter), where the central

ends of these fibres terminate. This information is conveyed by spinal projection neurons to higher centres in the brain, including the reticular formation, thalamus and ultimately the cerebral cortex, where non-noxious and noxious signals can be perceived. Sensory fibres arise from cell bodies in the trigeminal and dorsal root ganglia, and can be categorised into Aß-fibres, Aδ-fibres, and C-fibres. Aß-fibres are large in diameter, are highly myelinated and detect innocuous stimuli applied to skin, muscle and joints. Aδ-fibres are smaller in diameter, are thinly myelinated and respond to both thermal and mechanical stimuli. C-fibres are the smallest type of primary afferents, are unmyelinated and detect selectively nociceptive stimuli. Collectively, Aδ- and C-fibres are termed nociceptors and respond to noxious stimuli. Efforts to determine how these neurons detect pain-producing stimuli of a thermal, mechanical or chemical nature have revealed new signalling mechanisms and improved our understanding of the molecular events that facilitate transitions from acute to persistent pain.

4.1.2 Chronic pain, animal models & the role of TrpV1

Chronic pain is a major socio-economic burden with more than 320 million sufferers worldwide. Despite great progress being made in elucidating the molecular mechanisms underlying nociception, pain remains poorly treated due to the analgesic drugs currently available resulting in only partial and transient relief and severe side effects. There are two types of chronic pain, inflammatory and neuropathic. Inflammatory pain is associated with tissue damage and the resulting inflammatory process. Neuropathic pain is caused by damage to the neurons mainly in the peripheral, but also central nervous system, and is generally associated with hyperalgesia (hypersensitivity to noxious stimuli) and allodynia (hypersensitivity to normally non-noxious stimuli).

Mechanistic and molecular-based understanding of chronic pain has relied heavily on animal studies. There are three widely used animal models of neuropathic pain: the chronic constriction injury model (Bennett & Xie, 1988), partial ligation of the sciatic

nerve (Seltzer *et al.*, 1990), and spinal nerve ligation (Kim & Chung, 1992). One common feature of these models is that only a proportion of sciatic nerve fibres are damaged and the undamaged axons still innervating the hind paw allow assessment of altered pain responses after nerve injury. In the capsaicin model, intradermal administration of capsaicin induces hypersensitivity to mechanical and thermal stimulation, as well as spontaneous pain (Gilchrist *et al.*, 1996). Other experimental animal models, such as injection of formalin, Complete Freund's Adjuvant or Carrageenan, mimic inflammatory pain and have been widely used in assessing the efficacy of analgesic compounds.

Hyperexcitability in peripheral sensory nerves acts as an important driving mechanism for neuropathic pain and can account for the initiation and maintenance of central hyperexcitability. Amongst the proposed molecular drivers of peripheral hyperexcitability is the transient receptor potential vanilloid subtype 1 (TrpV1) receptor, which is predominantly expressed in nociceptive afferent fibres of the DRG, trigeminal ganglia and nodose ganglia. TrpV1 is a ligand-gated cation channel activated by capsaicin, noxious heat (>45^{o}C) and protons (Caterina *et al.*, 1997, Tominaga *et al.*, 1998) and regulated by a variety of intracellular pathways and molecules, including inflammatory mediators and neuroregulators (Cortright & Szallasi, 2004, Zhang *et al.*, 2005). TrpV1 knockout mice exhibit diminished sensitivity to heat and lack of capsaicin-induced pain behaviour, but show normal responses to noxious mechanical stimuli after partial nerve ligation (Caterina *et al.*, 2000, Davis *et al.*, 2000). In contrast, selective TrpV1 antagonists, which aim to inhibit peripheral nerve fibre activity by blocking TrpV1 transduction, have shown attenuated mechanical hyperalgesia and allodynia in various rat models of neuropathic pain (Christoph *et al.*, 2006a, Honore *et al.*, 2005, Kanai Y, 2005, Pomonis *et al.*, 2003). Thus, TrpV1 appears to be a central molecular integrator of noxious stimuli and although it is believed to play a key role in neural sensitization caused by inflammation and nerve injury, its specific role in neuropathic pain has been controversial.

4.1.3 Delivery of siRNAs to animal models of nociception

The pain-related RNAi approaches published to date involve delivery of synthetic siRNAs by repeated injections or continuous infusion. Silencing of several pain-related genes has been achieved in various animal models of nociception using this experimental design. However, knockdown is transient lasting only up to several days, requires high doses of siRNAs and lacks specificity in the sense that uptake of siRNAs cannot be restricted to neurons or a specific subset of neurons.

Dorn *et al.* achieved silencing of the P2X3 receptor, whose role in inflammatory and neuropathic pain had already been established (Honore *et al.*, 2002, Jarvis *et al.*, 2002), by intrathecal delivery of naked siRNAs infused via a mini pump (Dorn *et al.*, 2004). Silencing of P2X3 in the DRG and dorsal horn of the spinal cord inhibited hyperalgesia and allodynia and provided pain relief in a rat model of neuropathic pain. Luo *et al.* achieved silencing of the delta opioid receptor (DOR) by daily intrathecal injections of siRNAs mixed with a cationic lipid (i-Fect^{TM}) (Luo *et al.*, 2005). Transfection resulted in more efficient delivery of siRNAs to the lumbar DRG and spinal cord and thus, a lower dose was required to induce effective silencing of DOR, which inhibited antinociception induced by a DOR selective agonist. Tan *et al.* achieved silencing of the NR2B subunit of the N-methyl-D-aspartate (NMDA) receptor that had been suggested to play a role in chronic pain (Coderre & Melzack, 1992) by intrathecal injection of siRNAs complexed with polyethyleimine (Tan *et al.*, 2004a). Reduced mRNA and protein levels of NR2B in the spinal cord abolished formalin-induced pain behaviours in rats. Furthermore, Guo *et al.* demonstrated that injection of siRNAs against TrkB, the receptor for BDNF, into the rostral ventromedial medulla, followed by electroporesion, prevented the upregulation of TrkB expression induced by inflammation caused by injection of Freund's adjuvant (Guo *et al.*, 2006). More recently, silencing of the P2X7 receptor in satellite cells of the lumbar DRG by intrathecal delivery of siRNAs elicited up-regulation of the P2X3 receptor, increased the activity of

sensory neurons responding to painful stimuli and evoked abnormal nociceptive behaviours in rats (Chen *et al.*, 2008).

Finally, inhibition of TrpV1 expression has been achieved by delivery of antisense oligonucleotides (Christoph *et al.*, 2007) or siRNAs (Christoph *et al.*, 2006b). Intrathecal injection of naked siRNAs against *trpV1* reduced cold allodynia by more than 50% over a time period of approximately 5 days in rats with chronic constriction injury and diminished spontaneous visceral pain behaviour induced by capsaicin in mice. Moreover, Christoph and colleagues have recently published the generation of a transgenic mouse expressing shRNA against *trpV1*, which develops mechanical allodynia and hyperalgesia, thus providing further evidence for the relevance of TrpV1 in neuropathic pain (Christoph *et al.*, 2008).

4.1.4 Rationale

The primary aim of this chapter was to provide proof of concept that an endogenous neuronally expressed gene could be silenced using the replication-defective HSV-1 vectors developed in the previous chapter. Trpv1 is an ideal target for testing the efficacy of HSV-mediated silencing *in vivo*, as it is predominantly expressed in DRG sensory neurons that can be efficiently targeted following injection of the virus into the sciatic nerve. Furthermore, TrpV1 has been shown to play a key role in inflammatory and neuropathic pain and is considered to be an attractive target for new analgesic drugs. Thus, effective and specific silencing of *trpV1* in DRG neurons *in vivo* would demonstrate the potential of the vectors developed in this thesis as a valuable tool for the study of nociceptive processes and the validation of potential novel targets for pain therapy.

The work described in this chapter was performed in parallel to the second half of the work described in chapter 3. It followed evaluation of different shRNA expression systems in BHK-LacZ cells, which revealed that similar levels of specific silencing could be achieved with all three vectors tested (section 3.11). Thus, two vectors were constructed

to express shRNA against *trpV1* from the U6 pol III promoter or pre-miRNA against *trpV1* from the CMV pol II promoter. Work in this chapter was performed in collaboration with Johnson & Johnson Research and Development in Belgium.

4.2 Screening of shRNA / pre-miRNA sequences against trpV1

In preliminary experiments performed by Ines Royaux at Johnson & Johnson Research and Development in Belgium, several plasmid vectors expressing shRNA sequences under the control of the U6 promoter or pre-miRNA sequences under the control of the CMV promoter were evaluated for their ability to knockdown mouse *trpv1* by at least 80% following transfection into 293T cells (data not shown). Screening of sequences against TrpV1 was performed by cloning the mouse *trpV1* cDNA sequence (GI:49616384) into the pSCREEN-iT/LacZ-DEST vector (Invitrogen), which allows generation of a screening construct containing the target gene fused to the *lacZ* reporter gene. The pSCREEN-iT/LacZ-trpV1 plasmid was co-transfected into 293T cells with each of the plasmids expressing shTRPV1 or pre-miR-TRPV1 using Lipofectamine. Target gene knockdown was assessed by measuring ß-galactosidase levels. This system allows rapid screening of target genes without the need for an antibody or prior knowledge of the knockdown phenotype and is particularly useful when screening sequences targeting neuronally expressed genes, as transfection of neuronal cell lines with plasmid DNA is highly inefficient.

4.3 Generation of plasmid vectors targeting trpV1

The most effective shRNA sequence against TrpV1, which targeted the region containing nucleotides 1296-1316 of the mouse *trpv1* gene, was used to generate the pR19U6shTRPV1 vector (figure 4.1a). The shTRPV1 sequence (available in section 2.1.6) was synthesized as single stranded DNA oligonucleotides, annealed and cloned into pENTR-U6 (described in figure 3.3). The U6shTRPV1 cassette was then inserted from pENTR-U6 into pR19-Gateway (constructed in section 3.5.1) by LR recombination. The pR19U6shTRPV1 plasmid was sequenced between the LAT flanking regions to ensure that the shTRPV1 sequence had not been mutated (figure 4.2a). Figure 4.2a also demonstrates that the shTRPV1 sequence is predicted to target the rat and human *trpV1* gene, in addition to the mouse gene.

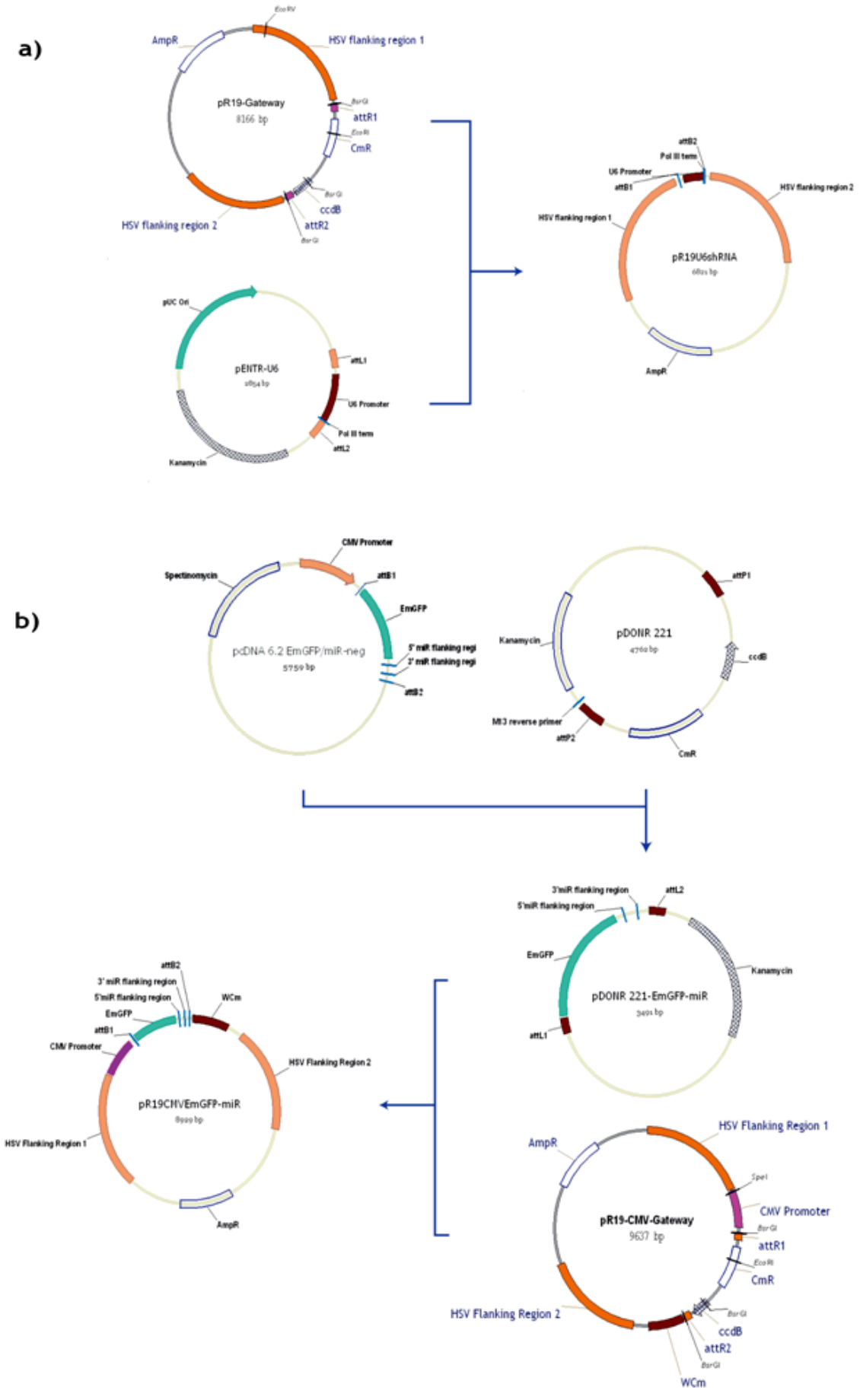
The most effective pre-miRNA sequence against TrpV1, which targeted the region containing nucleotides 581-601 of the mouse *trpv1* gene, was used to generate the pR19CMV/EmGFP-miR-TRPV1 vector (figure 4.1b). The pre-miR-TRPV1 sequence (available in section 2.1.6) was synthesized as single stranded DNA oligonucleotides, annealed and cloned into pcDNA6.2/EmGFP-miR (described in figure 3.7). The EmGFP-miR-TRPV1 cassette was inserted into pDONR-221 by BP recombination and then into pR19CMV-Gateway (constructed in section 3.5.3) by LR recombination. The pR19CMV/EmGFP-miR-TRPV1 plasmid was sequenced between the LAT flanking regions to ensure that the pre-miR-TRPV1 sequence had not been mutated (figure 4.2b). Figure 4.2b also demonstrates that the pre-miR-TRPV1 sequence is predicted to target the rat *trpV1* gene, in addition to the mouse gene.

Figure 4.1 Cloning of pR19U6shTRPV1 and pR19CMV/EmGFP-miR-TRPV1

a) The U6shTRPV1 cassette was inserted from pENTR-U6 into pR19-Gateway by LR recombination.

b) The EmGFP-miR-TRPV1 cassette was inserted from pcDNA6.2 into pDONR-221 by BP recombination and then into pR19CMV-Gateway by LR recombination.

The details of the cloning can be found in section 4.3. The details of the cloning of the pR19-Gateway plasmid can be found in section 3.5.1 and the details of the cloning of the pR19CMV-Gateway can be found in section 3.5.3. The LR and BP recombination reactions are described in section 2.2.9 of the materials and methods.


a)
AmpR
HSV flanking region 1
pR19-Gateway
8166 bp
attR1
CmR
ccdB
attR2
HSV flanking region 2
pUC Ori
pENTR-U6
attL1
U6 Promoter
Pol III term
attL2
Kanamycin
attB2
Pol III term
U6 Promoter
attB1
HSV flanking region 2
HSV flanking region 1
pR19U6shRNA
AmpR
b)
CMV Promoter
Spectinomycin
attB1
EmGFP
pcDNA 6.2 EmGFP/miR-neg
5759 bp
attB2
Kanamycin
attP1
pDONR 221
ccdB
M13 reverse primer
attP2
CmR
3'miR flanking region
attL2
5'miR flanking region
EmGFP
Kanamycin
pDONR 221-EmGFP-miR
attL1
attB2
3' miR flanking region
5'miR flanking region
WCm
EmGFP
attB1
CMV Promoter
HSV Flanking Region 2
pR19CMVEmGFP-miR
HSV Flanking Region 1
AmpR
HSV Flanking Region 1
AmpR
CMV Promoter
pR19-CMV-Gateway
9637 bp
attR1
CmR
HSV Flanking Region 2
ccdB
attR2
WCm

a) pR19U6shTRPV1 sequencing

Sequence 1: lcl|1
Length = 47 (1 .. 47) shTRPV1

Sequence 2: lcl|65536
Length = 1205 (1 .. 1205) **pR19U6shTRPV1 (U6 Forward primer)**

Score = 91.1 bits (47), Expect = 2e-16
Identities = 47/47 (100%), Gaps = 0/47 (0%)
Strand=Plus/Plus

```
Query  1    GCGCATCTTCTACTTCAACTTCAAGAGAGTTGAAGTAGAAGATGCGC  47
            |||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  223  GCGCATCTTCTACTTCAACTTCAAGAGAGTTGAAGTAGAAGATGCGC  269
```

The target sequence against mouse, human and rat TrpV1 in shTRPV1:

gb|AY445519.1| Mus musculus transient receptor potential cation channel V1 (Trpv1) mRNA, complete cds
Length=2520

Score = 44.1 bits (22), Expect = 0.036
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Plus

```
Query  1     GCGCATCTTCTACTTCAACTTC  22
             ||||||||||||||||||||||
Sbjct  1296  GCGCATCTTCTACTTCAACTTC  1317
```

gb|BC136633.1| Homo sapiens transient receptor potential cation channel, subfamily V, member 1, mRNA, complete cds
Length=4021

Score = 44.1 bits (22), Expect = 0.036
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Plus

```
Query  1     GCGCATCTTCTACTTCAACTTC  22
             ||||||||||||||||||||||
Sbjct  1775  GCGCATCTTCTACTTCAACTTC  1796
```

gb|AF029310.1|AF029310 Rattus norvegicus transient receptor potential cation channel, subfamily V, member 1 (Trpv1), mRNA
Length=2847

Score = 44.1 bits (22), Expect = 0.036
Identities = 22/22 (100%), Gaps = 0/22 (0%)
Strand=Plus/Plus

```
Query  1     GCGCATCTTCTACTTCAACTTC  22
             ||||||||||||||||||||||
Sbjct  1373  GCGCATCTTCTACTTCAACTTC  1394
```

b) pR19CMV/EmGFP-miR-TRPV1 sequencing

Sequence 1: lcl|1
Length = 64 (1 .. 64) **pre-miR-TRPV1**

Sequence 2: lcl|65536
Length = 1397 (1 .. 1397) **pR19CMV/EmGFP-miR-TRPV1 (GFP Forward primer)**

Score = 123 bits (64), Expect = 6e-26
Identities = **64/64 (100%)**, Gaps = 0/64 (0%)
Strand=Plus/Plus

```
Query  1    TGCTGTGTAGTAGCTGTCTGTGTAGCGTTTTGGCCACTGACTGACGCTACACACAGCTAC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct 122   TGCTGTGTAGTAGCTGTCTGTGTAGCGTTTTGGCCACTGACTGACGCTACACACAGCTAC  181

Query  61   TACA  64
            ||||
Sbjct  182  TACA  185
```

The target sequence against mouse and rat TrpV1 in pre-miRNA-TRPV1:

gb|AY445519.1| Mus musculus transient receptor potential cation channel V1 (Trpv1) mRNA, complete cds
Length=2520

Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  6    TGTAGTAGCTGTCTGTGTAGC  26
            |||||||||||||||||||||
Sbjct  601  TGTAGTAGCTGTCTGTGTAGC  581
```

gb|AF029310.1|AF029310 Rattus norvegicus transient receptor potential cation channel, subfamily V, member 1 (Trpv1), mRNA
Length=2847

Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21 (0%)
Strand=Plus/Minus

```
Query  6    TGTAGTAGCTGTCTGTGTAGC  26
            |||||||||||||||||||||
Sbjct  678  TGTAGTAGCTGTCTGTGTAGC  658
```

Figure 4.2 Blast nucleotide sequence alignment of a) the pR19U6shTRPV1 sequence obtained with the U6 Forward primer and b) the pR19CMV/EmGFP-miR-TRPV1 sequence obtained with the GFP Forward primer against the oligonucleotide sequences expressing shRNA and pre-miRNA against TrpV1, respectively. The primer sequences are described in section 2.1.5. The target sequence in shTRPV1 is indicated in the mouse, rat and human *trpV1* gene sequences. The target sequence in pre-miR-TRPV1 is indicated in the mouse and rat *trpV1* gene sequences

4.4 Generation of disabled HSV-1 vectors targeting trpV1

The U6shTRPV1 and EmGFP/miR-TRPV1 cassettes were inserted into the LAT region of the replication-defective 1764/4-/27+/RL1+ HSV-1 genome immediately 3' to the LATP2 region, which allows long term expression during latency.

The pR19U6shTRPV1 plasmid was co-transfected with 1764 4-/27+/RL1+ pR19CMVeGFPWCm viral DNA (prepared as described in section 2.4.5) into complementing MAM49 cells as described in section 2.4.1. Viral recombinants were identified by their inability to express GFP (white plaques) using fluorescent microscopy and were plaque purified as described in section 2.4.2. To confirm that the cassette expressing GFP had been replaced by the cassette expressing shRNA and that the shTRPV1 sequence had not been mutated, PCR was performed on viral DNA extracted from each plaque using the U6 Forward and LAT Reverse primers described in section 2.1.5 (figure 4.3a). This was followed by sequencing of the PCR products. One recombinant plaque was selected and grown to a high titre as described in section 2.4.3.

The pR19CMV/EmGFP-miR-TRPV1 plasmid was linearised using a unique XmnI site in the plasmid backbone and was co-transfected with 1764 4-/27+/RL1+ pR19CMVLacZWCm viral DNA. Viral recombinants were identified by their ability to express GFP (green plaques) using fluorescent microscopy. Bright green plaques were selected and purified. When a pure green population was obtained, the purity of the plaques was confirmed further by x-gal staining. Expression of GFP from these recombinants indicated that the desired homologous recombination event had occurred. However, to confirm that the pre-miR-TRPV1 sequence had not been mutated, PCR was performed on viral DNA extracted from each plaque using the GFP Forward and WCm Reverse primers described in section 2.1.5 (figure 4.3b). The PCR products were sequenced and one recombinant plaque was selected and grown to a high titre.

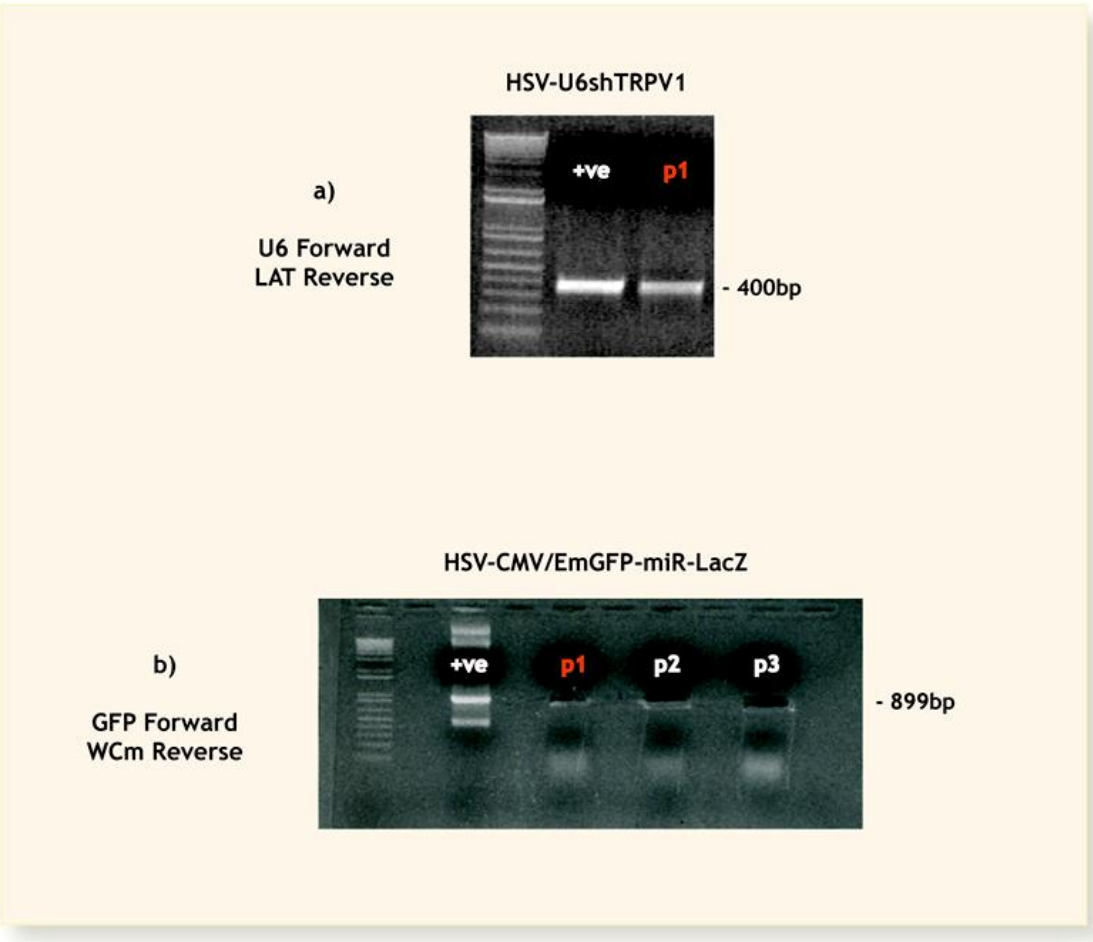


Figure 4.3 PCR on viral DNA to confirm that the correct homologous recombination event had occured.

The positive control used in each experiment is the plasmid DNA used to generate the plaques tested. The letter "p" refers to plaque. Plaques in red were selected and grown to a high titre.

a) PCR on viral DNA extracted from the HSV-U6shTRPV1 plaque using the U6 Forward and LAT Reverse primers produced a band of 400bp thus confirming that the shTRPV1 sequence was inserted into the LAT region downstream of the U6 promoter.

b) PCR on viral DNA extracted from HSV-CMV/EmGFP-miR-TRPV1 plaques using the GFP Forward and WCm Reverse primers produced a band of 899bp thus confirming the presence of the pre-miRNA cassette. N.B. That the PCR bands were excised prior to the gel being photographed.

4.5 Evaluation of disabled vectors targeting trpV1 in culture

Prior to testing the HSV-U6shTRPV1 and HSV-CMV/EmGFP-miR-TRPV1 vectors in DRG neurons *in vivo*, the viruses were evaluated for their ability to silence *trpV1* in 293T cells transfected with the pSCREEN-iT/LacZ-trpV1 plasmid (supplied by Ines Royaux). The HSV-U6shGFP and HSV-CMV/EMGFP-miR-neg vectors constructed in section 3.9 were used as negative controls.

293T cells were transfected with 100ng pSCREEN-iT/LacZ-trpV1 using Lipofectamine (as described in section 2.3.3) and were allowed to recover overnight prior to being transduced with viruses at an MOI = 5.0. Β-galactosidase activity assay at 48 hrs post-transduction revealed highly effective knockdown of TrpV1 fused to β-galactosidase, when either HSV-U6shTRPV1 or HSV-CMV/EmGFP-miR-TRPV1 were transduced compared to the negative controls. The HSV-miR-TRPV1 vector induced 88.5 ± 6.8% silencing (n=3, mean ± s.d.) and was found to be more effective than the HSV-U6shTRPV1, which resulted in a 74.5 ± 5.0% in silencing (figure 4.4). This result is in agreement with the data obtained in BHK-LacZ cells transduced with vectors targeting β-galactosidase from different promoters, which revealed marginally improved silencing of *lacZ* at 48 hrs with the vector expressing pre-miR-LacZ compared to the vectors expressing shLacZ. However, bearing in mind that the pre-miR-TRPV1 and shTRPV1 sequences target different regions of the *trpV1* gene (figure 4.2), this may also be due to the pre-miR-TRPV1 sequence being more effective than the shTRPV1 sequence.

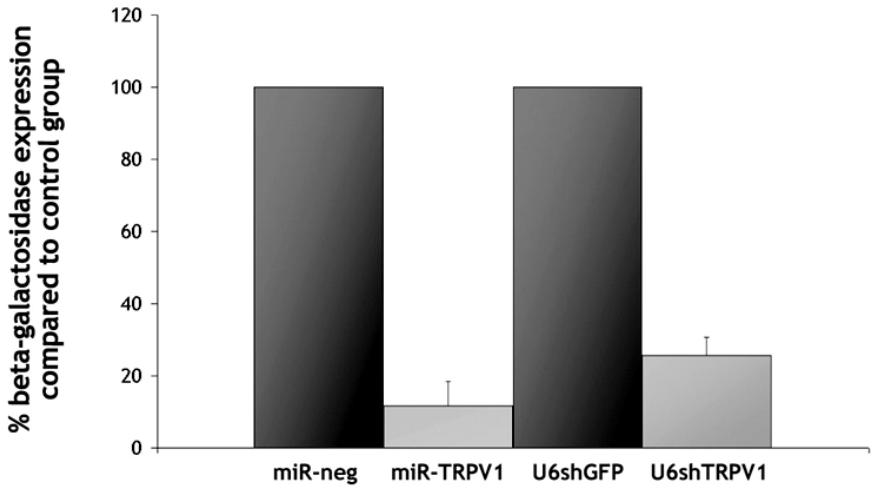


Figure 4.4 Silencing in 293T cells transfected with pSCREEN-iT/LacZ-trpV1 expressing mouse TrpV1 fused to beta-galactosidase and transduced with HSV-CMV/EmGFP-miR-TRPV1, HSV-U6shTRPV1, or negative control vectors.

Analysis of beta-galactosidase expression by enzyme activity assay at 48 hrs post-transduction revealed that HSV-CMV/EmGFP-miR-TRPV1 silenced trpV1 by 88.5 ± 6.8% and HSV-U6shTRPV1 silenced trpV1 by 75.5 ± 5.0% (n=3, mean ± s.d.). Beta-galactosidase expression was calculated as a percentage of the expression in cells transduced with the HSV-CMV/EmGFP-miR-neg and HSV-U6shGFP negative controls, respectively.

4.6 HSV-mediated silencing of trpV1 in DRG neurons *in vivo*

This section describes silencing of *trpV1* in DRG neurons *in vivo* following injection of HSV-U6shTRPV1 or HSV-CMV/EMGFP-miR-TRPV1 into the sciatic nerve of mice. Silencing was assessed at the mRNA and protein levels. As explained in section 4.1.4, following injection of the HSV-U6shTRPV1 vector and assessment of TrpV1 mRNA levels, evaluation of disabled vectors expressing shRNA using different approaches (described in section 3.13) revealed that expression of shRNA from a miRNA-like system was more effective at silencing *lacZ* in DRG neurons *in vivo* than expression of shRNA from the U6 promoter. Thus, the HSV-U6shTRPV1 vector was abandoned and silencing was further assessed at the protein level using the HSV-CMV/EmGFP-miR-TRPV1 vector, which had also been found to be more effective at silencing *trpV1* in 293T cells transfected with pSCREEN-iT/LacZ-trpV1 and allows direct labelling of transduced cells through expression of EmGFP together with the pre-miRNA.

4.6.1 Silencing of trpV1 – assessment of TrpV1 mRNA levels

The HSV-U6shTRPV1 vector was injected into the sciatic nerve of BALB/c mice (n=4). Mice injected with HSV-U6shLacZ were used as negative controls (n=2). The 1764 4-/27+/RL1+ pR19CMVeGFPWCm vector expressing GFP was also co-injected to monitor transduction efficiency. At day 8, the left DRG (injected side) and right DRG (non-injected side) were isolated and analysed for GFP and Trpv1 levels. GFP expression assessed by quantitative RT-PCR confirmed delivery to all injected animals, predominantly to the L4 DRG (data not shown). Quantitative RT-PCR for Trpv1 revealed markedly reduced mRNA levels in all animals injected with HSV-U6shTRPV1 compared to animals injected with the negative control (figure 4.5). Trpv1 mRNA in the left DRG isolated from these animals was reduced by 53.6 ± 11.2% (one-way *t*-test, p=0.01) (n = 4, mean ± s.d.) compared to the DRG isolated from the non-injected side of the same animals. Silencing was assessed between the L4 DRG of the same animals to avoid variation in TrpV1 expression between different

animals. Importantly, HSV-U6shLacZ does not seem to have an effect on the levels of the endogenous *trpV1* gene further indicating the specificity of this method. The qRT-PCR experiment was performed by Johnson & Johnson Research and Development in Belgium as described in section 2.2.17.

4.6.2 Silencing of trpV1 – assessment of TrpV1 protein levels

In the previous chapter, it was demonstrated that the HSV-CMV/EmGFP-miR vector is more effective at silencing *in vivo* than the HSV-U6shRNA system. Subsequent experiments were therefore performed using the vector expressing pre-miRNA against *trpV1*. Detection of mRNA degradation by RT-PCR is often used to assess RNAi effects. However, it has been reported that in some cases the RISC could merely inhibit mRNA translation without mRNA degradation by a mechanism similar to the miRNA translation inhibition. To evaluate silencing at the protein level, BALB/c mice were injected directly into the sciatic nerve with HSV-CMV/EmGFP-miR-neg (n=10) or HSV-CMV/EmGFP-miR-TRPV1 (n=10). 8 days post-injection the DRG from injected animals were isolated as described in section 2.6.8. Delivery was assessed by fluorescent microscopy for GFP, which confirmed delivery to all injected animals mainly to the L4 DRG. Western blot analysis (as described in sections 2.7.2-2.7.4) for TrpV1, TrpA1 and Stat1 (antibodies described in section 2.17) was performed on protein extracted from the L4 DRG isolated from each group (as described in section 2.7.1) (figure 4.6). Quantification of band density using the Scion Image software revealed an average 59% reduction in the levels of TrpV1 protein compared to the negative control (figure 4.7). The levels of Stat1 remained essentially unchanged indicating that silencing is specific and not caused by induction of the INF response. However, protein extracted from the DRG of non-injected animals should have been included as a negative control to exclude the possibility that the miR-neg induces the INF response. Finally, although TrpA1 was not downregulated, this is not enough data to conclude that the miR-TRPV1 sequence does not cause any off-target effects.

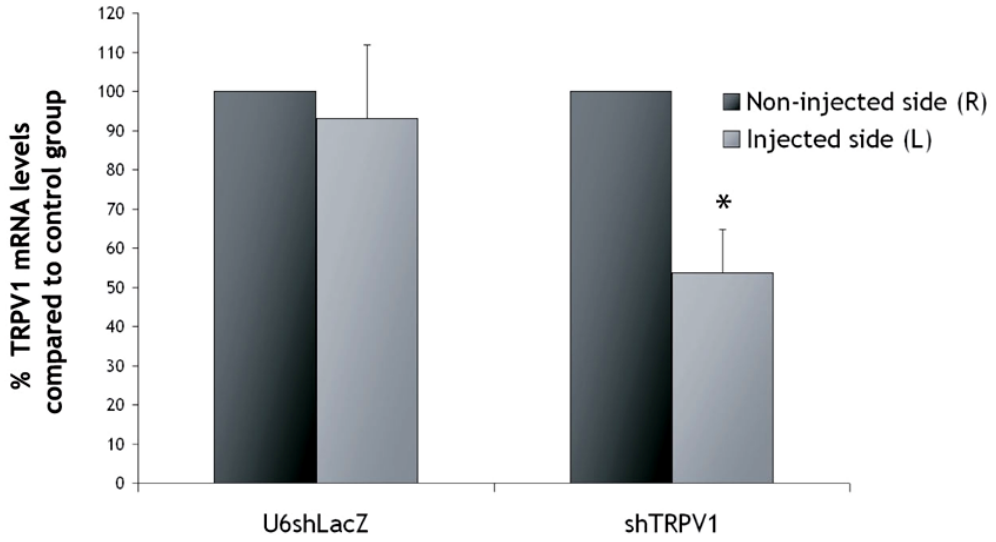


Figure 4.5 HSV-mediated silencing of endogenous TrpV1 - mRNA knockdown

BALB/c mice were injected once directly into the sciatic nerve with 2.5x10^6 pfu of either HSV-U6shTRPV1 (n = 4) or HSV-U6shLacZ (n = 2). A GFP-expressing vector was also co-injected to assess transduction efficiency (1x10^6 pfu). At day 8, both the left (injected) and right (non-injected) L4 DRG were isolated. Quantitative RT-PCR revealed that TrpV1 mRNA levels in the DRG of animals injected with HSV-U6shTRPV1 were reduced by 53.6 ± 11.2% (mean ± s.d.) compared to the right DRG. In DRG injected with the HSV-U6shLacZ vector, TrpV1 mRNA levels were essentially unchanged compared to the right DRG.

Statistical differences between the two injected groups were determined by one-way t-test, *p = 0.01.

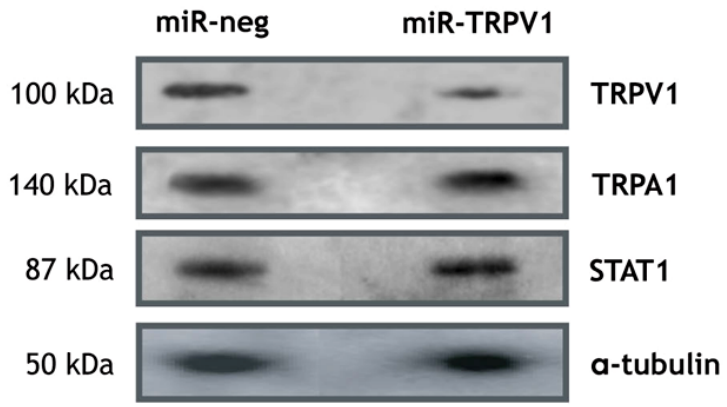


Figure 4.6 HSV-mediated silencing of endogenous TrpV1 - protein knockdown

BALB/c mice were injected once directly into the sciatic nerve with 5x10^6 pfu of either HSV-CMV/EmGFP-miR-TRPV1 (n = 10) or HSV-CMV/EmGFP-miR-neg (n = 10). At day 8, the L4 DRG isolated from each group were pooled, protein was extracted and western blots were performed for TrpV1, TrpA1 and Stat1. The protein levels of *α*-tubulin were essentially the same between the two groups, indicating that an equal amount of protein was loaded. Whilst the levels of TrpA1 and Stat1 remained the same, the levels of TrpV1 were significantly lower in animals injected with HSV-CMV/EmGFP-miR-TRPV1 compared to animals injected with the negative control.

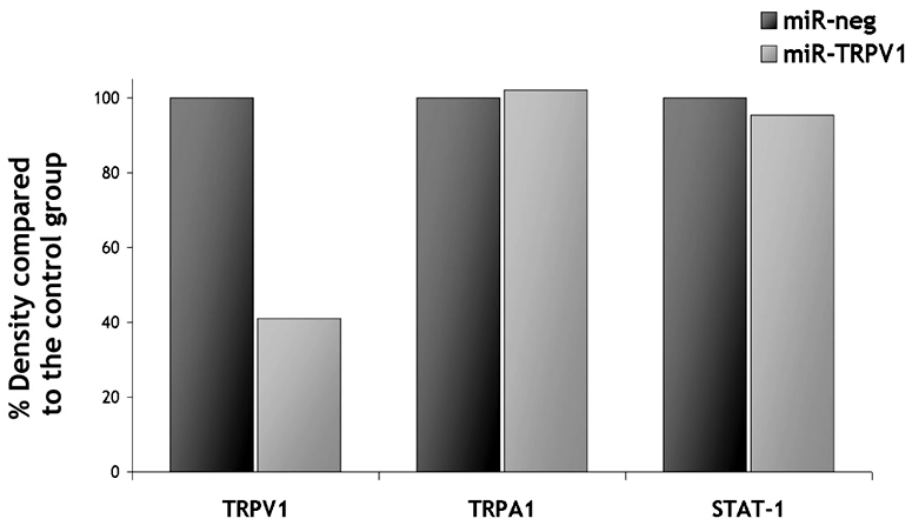


Figure 4.7 HSV-mediated silencing of endogenous TrpV1 - quantification of protein knockdown

Following western blot analysis (shown in figure 4.6) on protein extracted from the DRG of injected animals (described in detail in section 4.6.2), the density of the bands was quantified using the Scion Image software. Quantification of band density revealed an average 59% knockdown of TrpV1 protein in animals injected with HSV-CMV/EmGFP-miR-TRPV1 (n=10) compared to animals injected with the HSV-CMV/EmGFP-miR-neg control (n=10). The levels of TrpA1 and Stat1 were essentially the same between the two groups. Values were normalized against *a*-tubulin (loading control).

4.7 Transduction efficacy of TrpV1-expressing neurons

The *in vivo* data obtained with both vectors targeting *trpV1* is particularly promising as it represents overall mRNA and protein levels in both transduced and non-transduced sensory neurons expressing TrpV1. It should be noted that the 92% knockdown of ß-galactosidase expression described in section 3.13 was calculated by counting LacZ-positive vs. GFP-positive neurons and therefore reflects the proportion of transduced neurons in which lacZ was completely silenced.

The DRG is comprised of a heterologous population of neurons and TrpV1 is almost exclusively expressed in small- and medium- diameter sensory fibres. Thus, to provide an explanation as to why complete *trpV1* silencing was not achieved with the vectors constructed in this chapter, the efficiency TrpV1-expressing neurons are transduced by the vector following injection into the sciatic nerve was investigated. BALB/c mice (n=2) were injected with HSV-CMV/EmGFP-miR-neg and immunofluorescence (as described in section 2.7.6) was performed on L4 DRG sections isolated from these animals to investigate the degree of co-localization between EmGFP expressed by the vector and TrpV1 (antibodies described in section 2.17). Figure 4.8 shows representative sections from DRG isolated 4 days post-injection. Immunofluorescence confirmed that the vector transduces a heterologous population of neurons, including an estimated 80% of neurons that express TrpV1 (neurons not counted). However, 20% of such neurons were not transduced. This provides one explanation for the 53.6% reduction in TrpV1 mRNA using the U6 vector expressing shTRPV1 (figure 4.5) and 59% reduction of TrpV1 protein using the miRNA version (figure 4.6).

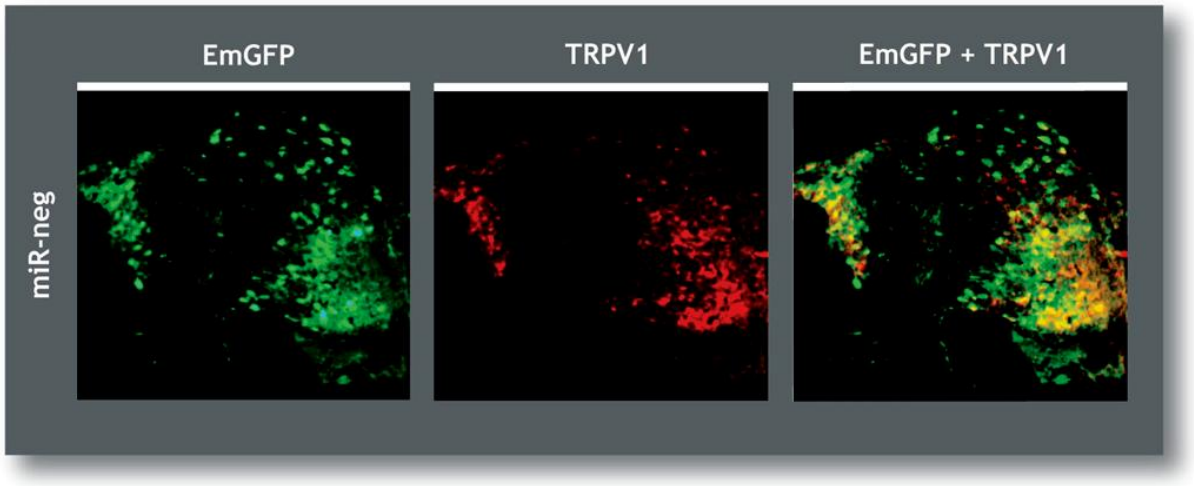


Figure 4.8 TrpV1-positive sensory neurons transduced by the miRNA-like vector

BALB/c mice were injected into the sciatic nerve with 5x10^6 pfu of HSV-CMV/EmGFP-miR-neg. 4 days post-injection the L4 DRG were isolated, sectioned and immunofluorescence for EmGFP and TrpV1 was performed to reveal the number of TrpV1-expressing neurons transduced by the vector. An estimated 80% of nociceptive TrpV1-positive neurons, which are predicted to be mainly C-fibres, are transduced by the virus (yellow) following injection into the sciatic nerve (objective x5).

4.8 Discussion

Although replication-defective HSV-1 vectors have been extensively used to deliver genes to peripheral neurons, including in animal models of neuropathic and inflammatory pain (Goss *et al.*, 2001, Hao *et al.*, 2003a, Hao *et al.*, 2003b), HSV vectors had not been previously developed to provide silencing of an endogenously expressed gene in sensory neurons *in vivo*. The previous chapter of this thesis demonstrated that disabled HSV-1 vectors can induce highly effective and specific silencing of reporter genes in dividing cells in culture, primary neuronal cells and DRG neurons *in vivo*. This chapter demonstrates that in addition to the silencing of transgenically expressed reporter genes, HSV-mediated expression of shRNA or artificial miRNA to DRG neurons *in vivo* results in effective and specific silencing of the endogenous *trpv1* gene, which is involved in nociceptive processing and therefore is a potential target for therapeutic pain relief.

Both vectors constructed in this chapter were effective at silencing *trpV1* in DRG following injection into the sciatic nerve. Trpv1 mRNA levels were reduced by 54% in sensory neurons from mice injected with the vector expressing shTRPV1. This downregulation is highly promising as intrathecal delivery of siRNAs against other pain-related genes has achieved a maximum 40% mRNA reduction in DRG neurons *in vivo* (Dorn *et al.*, 2004, Luo *et al.*, 2005). Previous studies have shown that partial injury of the sciatic nerve results in downregulation of TrpV1 in the L4 and L5 DRG (Hudson *et al.*, 2001, Michael & Priestley, 1999). Quantitative RT-PCR revealed no significant difference in TrpV1 mRNA levels between L4 DRG taken from the injected and non-injected side of the same animals. This result demonstrates that the disabled HSV backbone and/or introduction of shRNA do not induce the INF response, but also suggests that the procedure used to inject the virus into the sciatic nerve does not alter TrpV1 expression levels by injuring the nerve fibres. In addition to reduced mRNA levels, TrpV1 protein levels were reduced by 59% in sensory neurons from mice injected with the vector expressing miR-TRPV1. Protein levels were assessed by western blot, because using

immunocytochemistry to detect a partial reduction of TrpV1 expression against the large TrpV1 pool existing prior to delivery of shRNA would be challenging. To investigate silencing specificity further, western blot analysis demonstrated that the pre-miRNA sequence against *trpV1* had no effect on the expression levels of Stat-1, which would be significantly upregulated in the event of an induction of the INF response. Furthermore, the pre-miRNA sequence against *trpV1* had no effect on the expression levels of TrpA1, although it has to be noted, that *trpV1* has limited homology to *trpA1*.

Incomplete knockdown of TrpV1 may be due to residual protein in DRG neurons expressed prior to expression of shRNA. Thus, silencing will depend on the half-life of the protein expressed from the target gene. Moreover, the RNAi efficacy of the method described in this thesis will critically depend upon the efficiency at which the vector transduces the specific subset of neurons expressing the target gene. TrpV1 is almost exclusively expressed by unmyelinated small- and medium-diameter DRG neurons, mainly C-fibers (Michael & Priestley, 1999). Further investigation into the reasons complete silencing of *trpV1* was not achieved revealed that injection of the vector into the sciatic nerve results in incomplete transduction of the subset of DRG neurons expressing TrpV1. Additional experiments are required to determine the subpopulation of DRG neurons transduced with disabled HSV following injection of the sciatic nerve and thus, predict the potency of this approach against future targets.

TrpV1, whose role in inflammatory and neuropathic pain has already been established (Christoph *et al.*, 2006, 2007 and 2008), has been used here as a proof of concept that an endogenous gene can be specifically silenced in DRG using the vector system developed. Since the primary aim was to evaluate a means of effective and specific RNAi delivery to DRG neurons *in vivo* rather that to validate the role of *trpV1* itself, the assessment of any behavioural effects was beyond the scope of this thesis, although Johnson & Johnson are separately undertaking this work. Previous studies suggest that only a small number of TrpV1-expressing neurons have to be reached by siRNA

treatment to evoke an analgesic response and that knockdown of TrpV1 protein by antisense oligonucleotides, despite being too subtle to detect in the DRG or spinal cord, was sufficient to reduce neuropathic pain behaviour (Christoph *et al.*, 2006 and 2007). Thus, 59% knockdown of TrpV1 protein demonstrated here would be expected to confer a biological effect, which it is hoped will be confirmed in a capsaicin mouse model by Johnson & Johnson in the coming months.

In conclusion, the results described in this chapter highlight the potential of the disabled HSV-1 vectors developed in this thesis, which can theoretically be engineered to target any gene, as a valuable tool for the study of nociceptive processes and the development of new analgesic drugs. Furthermore, these vectors could ultimately be applicable to the development of an HSV vector-based approach to pain control. Such an approach may be particularly relevant in the case of TrpV1 silencing. Current analgesic strategies are aimed at developing TrpV1 agonists or antagonists to attenuate excitability in sensory fibres, and several antagonists are in various stages of clinical development for the treatment of pain. Although TrpV1 is most strongly expressed in sensory neurons, it is functionally expressed in many other tissues throughout the body, including the central nervous system (Szallasi *et al.*, 2007), where it has been suggested to play an important role in hippocampal synaptic plasticity (Gibson *et al.*, 2008). Thus, systemic TrpV1 antagonists or non-targeted delivery of siRNAs against *trpV1* may interfere with processes such as learning and memory, and adversely affect cognitive function. Administration of the vectors developed in this thesis, via the relatively non-invasive route of sciatic nerve injection, allows targeted delivery of shRNA to the cell bodies of sensory neurons in the DRG and α-motor neurons in the ventral horn of the spinal cord though retrograde transport. However, disabled HSV vectors are incapable of trans-synaptic transport and thus, unable to spread to the central nervous system to induce any adverse side effects that might result from knockdown of TrpV1 at that site.

Shortly after the work described in this chapter was completed, a replication-defective HSV-1 vector was shown to express shRNA in the mouse brain to suppress mutant APP exogenously expressed from a co-injected LV vector (Hong *et al.*, 2006). Furthermore, HSV amplicon vectors have demonstrated silencing of genes in tumour cells *in vitro* and *in vivo* (Sabbioni *et al.*, 2006, Saydam *et al.*, 2005, Saydam *et al.*, 2007). Nevertheless, the work described in chapters 3 and 4 of this thesis represents the first and so far only demonstration of endogenous gene silencing in neurons *in vivo* using HSV and of gene silencing in sensory neurons *in vivo* by vector-mediated delivery of shRNA or artificial miRNA and (Anesti *et al.*, 2008).

CHAPTER 5

HSV-MEDIATED SILENCING TO PROMOTE REGENERATION OF SENSORY AXONS INTO THE SPINAL CORD

5.1 Introduction

The regenerative capacity of the injured adult mammalian CNS is extremely limited. There is now widespread agreement that the failure of axonal regeneration is multifactorial. A combination of inhibitory molecules, attenuated cell body responses to axotomy and inadequate neurotrophic support are all believed to play some part in preventing CNS axonal regeneration. Effective regeneration will therefore depend on strategies directed at blocking multiple inhibitors simultaneously, whilst boosting the intrinsic capacity of CNS neurons to regenerate. To effectively restore function, such approaches should be combined with strategies aimed at directing regenerating axons to reconnect with their target neurons and reconstituting neuronal circuits.

This chapter investigated the potential of a disabled HSV-1 vector to induce regeneration of sensory axons into the spinal cord by silencing a combination of genes suggested to be involved in the inhibition of axonal regeneration. The following introductory sections provide a brief overview of the degenerative processes following CNS injury, cell body responses to axotomy and inhibitory molecules that contribute to the failure of axonal regeneration, with special focus on the inhibitors that are targeted by the vector constructed in this chapter.

5.1.1 Spinal cord injury

An estimated 2.5 million people worldwide live with spinal cord injury (SCI), with more than 130,000 new injuries reported each year. Injury to the spinal cord can result in dramatic disability. Although many patients show some spontaneous recovery, most patients with significant damage to the spinal cord have permanent symptoms, such as loss of voluntary movement and chronic pain. SCI cannot yet be repaired by any therapy. During the past 50 years, however, improvements in medical and surgical care, the advent of rehabilitation programmes and new devices have drastically changed the prognosis of

serious SCI from utter hopelessness, great suffering and early death to the expectation of long-term survival and a significantly improved quality of life.

5.1.2 Degenerative processes following spinal cord injury

Traumatic injury to the spinal cord causes immediate death of cells in the vicinity of the injury site, including neurons, oligodendrocytes, astrocytes and endothelial cells, and although injury can directly damage the cell bodies of neurons with typically modest functional consequences, this often results in substantial axonal damage. Following axotomy, the axon proximal segment survives, but unlike axotomised neurons of the peripheral or embryonic nervous system, the damaged fails to regenerate beyond the lesion site. The distal axon segment retracts from post-synaptic neurons and undergoes progressive degeneration, a process known as Wallerian degeneration. Myelin sheaths are wrapped around axons to ensure rapid propagation of action potentials. Peripheral myelin is produced by Schwann cells, whereas central myelin is produced by oligodendrocytes. Axonal loss leads to the damage of myelin, death of oligodendrocytes and the accumulation of cellular debris rich in degenerating myelin. In the PNS, myelin debris is phagocytosed by Schwann cells and macrophages. In the CNS, Wallerian degeneration is relatively slow and degenerating myelin can persist for years due to delayed and inefficient macrophage responses (George & Griffin, 1994). Moreover, resident and invading inflammatory cells, such as microglia and macrophages, can mediate various destructive processes, such as further cell death, membrane damage and apoptosis. Finally, within hours of injury, recruitment of inflammatory cells and reactive astrocytes leads to the formation of a glial scar often accompanied by a fluid-filled cyst, which act as an additional barrier to axonal growth.

5.1.3 Inhibitors of axonal regeneration

Despite early claims that adult CNS axons are incapable of regeneration (Ie Gross Clark, 1943), sprouting within the lesion site (Guth *et al.*, 1985, Liu & Chambers, 1958) and even short distance sprouting from surviving axons have been observed in several different experimental models (Freund *et al.*, 2006, Li & Raisman, 1994, Taylor *et al.*, 2006). Ramon and Cajal (1928), who were the first to describe the anatomical responses to nervous system injury, reported that the ends of lesioned axons become swollen into dystrophic endballs. Recent studies have revealed that these dystrophic growth cones are highly active structures stalled in an inhibitory environment (Kerschensteiner *et al.*, 2005, Tom *et al.*, 2004). Moreover, DRG neurons that have axons in both the CNS and PNS can only regenerate their peripheral processes, and some injured CNS axons can regenerate over long distances in the permissive environment of a peripheral nerve graft (David & Aguayo, 1981, Richardson *et al.*, 1980). These observations suggested that adult CNS axons have an intrinsic capacity for regeneration, which is severely limited by the inhibitory and nonpermissive nature of the injured mammalian CNS.

An increasing number of CNS inhibitory cues have been identified, including the prototypic myelin inhibitors Nogo-A, myelin-associated glycoprotein (MAG), and oligodendrocyte myelin glycoprotein (OMgp), as well as several types of chondroitin sulfate proteoglycans (CSPGs), which are upregulated following injury particularly near the site of the glial scar formation (discussed in section 5.1.8). In addition, recent evidence suggests that developmental axon guidance molecules belonging to the semaphorin, ephrin and netrin families, which are also upregulated following injury, may repel regenerating axons and inhibit axonal regrowth (Yiu & He, 2006). Although interfering with signaling from axon guidance molecules has been suggested to be as effective in promoting functional recovery following SCI as interfering with myelin-associated inhibitors (Bolsover *et al.*, 2008), this is beyond the scope of this thesis. The

following sections focus on myelin-associated inhibitors and their role in the failure of axonal regeneration.

5.1.4 Myelin-associated inhibitors

In the late 1980s, pioneering work in the lab of Martin Schwab demonstrated that oligodendrocytes and myelin membranes are major inhibitors of axonal growth within the CNS (Schwab & Caroni, 1988). Recent evidence suggests that myelin inhibition is complex, involving multiple and distinct receptor systems that may operate in a redundant manner. Furthermore, the relative contribution of different ligand-receptor systems to myelin inhibition may vary amongst different neuronal cell types. Whilst great progress has been made in identifying myelin-associated inhibitors, defining their mechanism of action and their relative contribution to the failure of axonal regeneration is of fundamental importance for the rational design of therapies to promote functional recovery following CNS injury.

5.1.5 Nogo, MAG and OMgp

Nogo is a member of the reticulon (RTN) family of membrane-associated proteins. At least three isoforms (Nogo-A, -B and -C) are generated by alternative splicing and promoter usage. Amongst them, Nogo-A is highly expressed in developing and mature oligodendrocytes and in some subpopulations of neurons, but is absent from peripheral myelin (Huber *et al.*, 2002). Nogo-A contains two distinct inhibitory domains that have both been shown to inhibit neurite outgrowth and growth cone collapse (Caroni & Schwab, 1988, GrandPre *et al.*, 2000, Oertle *et al.*, 2003). The unique N-terminal domain (amino-Nogo) is not shared by Nogo-B and Nogo-C, whereas the 66 amino acid hydrophilic loop (Nogo-66), which is flanked by two transmembrane domains and is part of the C-terminal RTN homology domain, is common to all three isoforms. The topology of Nogo-A remains controversial. Although initial evidence places Nogo-66 on the cell surface and amino-Nogo

in the cytoplasm, amino-Nogo can also be detected on the cell surface (Oertle *et al.*, 2003). It has been hypothesized that a large part of Nogo-A may be exposed following oligodendrocyte membrane damage, further contributing to growth inhibition after injury. A monoclonal antibody against amino-Nogo (IN-1) has been shown to promote axonal regeneration and functional recovery following SCI (Bregman *et al.*, 1995, Brosamle *et al.*, 2000, Liebscher, 2005, Schnell & Schwab, 1990). However, ascending sensory fibres do not exhibit enhanced regeneration into the rat spinal cord when treated with an antibody against amino-Nogo (NI-35/250), suggesting that the relative contribution of Nogo-A to the failure of regeneration varies between different fibre systems (Oudega *et al.*, 2000). Furthermore, studies with Nogo null mice have failed to demonstrate any significant regeneration of corticospinal fibers following dorsal hemisection (Simonen *et al.*, 2003, Zheng *et al.*, 2003) and only one study has reported robust regeneration (Kim *et al.*, 2003), raising questions about whether blocking Nogo alone is sufficient to promoter axonal regeneration following SCI. Due to the lack of reproducibility of these transgenic studies, the role of Nogo in axonal regeneration remains controversial.

MAG is comprised of five extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain and a cytoplasmic domain. It is expressed in both oligodendrocytes in the CNS and Schwann cells in the PNS, and has been implicated in myelin formation and maintenance (Schachner, 2000). Interestingly, MAG promotes embryonic and neonatal neurite outgrowth (Hasegawa *et al.*, 2004, Johnson *et al.*, 1989). The molecular basis underlying the transition from promotion to inhibition of growth is not fully understood and depending on the neuronal cell type, it occurs at different developmental stages. Following postnatal day 6, however, all neuronal cell types examined to date, including DRG neurons, are strongly inhibited by MAG (Cai *et al.*, 2001, Mukhopadhyay *et al.*, 1994). Whilst MAG has been shown to be a potent inhibitor of axonal growth *in vitro* (Wong *et al.*, 2003), MAG null mice do not demonstrate improved axonal growth following SCI (Bartsch *et al.*, 1995). Moreover, MAG null mice exhibit defects in

myelin stability (Li *et al.*, 1994, Montag *et al.*, 1994), late-onset progressive PNS axonal atrophy, increased Wallerian regeneration, and CNS axon degeneration (Loers *et al.*, 2004, Pan *et al.*, 2005).

Finally, OMgp is a GPI-anchored protein that contains a leucine-rich repeat (LRR) domain (Mikol *et al.*, 1990). Similar to Nogo-A, OMgp is expressed by neurons and oligodendrocytes in the developing and adult CNS (Habib *et al.*, 1998). Recent evidence suggests that it is present at Nodes of Ranvier and may act to prevent collateral sprouting (Huang *et al.*, 2005). It remains to be determined whether blocking OMgp function *in vivo* leads to enhanced axonal regeneration after SCI.

5.1.6 NgR1 and NgR2

The Nogo receptor gene family consists of three members NgR1, NgR2 and NgR3. Nogo receptors are GPI-anchored proteins containing a LRR cluster flanked by cysteine–rich N-terminal and C-terminal LRR capping domains. NgR1 is expressed in many types of CNS neurons, including a subpopulation of adult DRG neurons (Hunt *et al.*, 2002). The NgR1 LRR cluster can bind with high affinity to the Nogo-66 domain of Nogo-A (Fournier *et al.*, 2001), as well as MAG (Domeniconi *et al.*, 2002) and OMgp (Wang *et al.*, 2002) that are structurally different to Nogo. The finding that all three myelin inhibitors, which seem to be redundant for axonal growth, signal through a single receptor, made NgR1 an attractive candidate to block in order to promote axonal regeneration.

Administration of a function-blocking NgR1 ectodomain resulted in sprouting of corticospinal and raphespinal fibres, which correlated with improved spinal cord electrical conduction and improved locomotion (Li *et al.*, 2004a). Moreover, some of the most robust regeneration following SCI has been observed in animals treated with the NgR1 antagonistic peptide NEP1-40 (Li & Strittmatter, 2003). This study, however, could not be reproduced by Steward and colleagues (Steward *et al.*, 2008). Furthermore, NgR1 null mice failed to show enhanced regeneration of corticospinal fibres (Kim *et al.*, 2004, Zheng

et al., 2005), although some regeneration of raphespinal and rubrospinal descending fibres was observed after complete transection, suggesting that the effect of blocking NgR1 may vary between different populations of spinal cord fibres (Kim *et al.*, 2004). Moreover, although DRG neurons isolated from wild-type mice demonstrated a significant increase in growth cone collapse in response to soluble myelin inhibitors compared to DRG neurons from NgR1 null mice (Kim *et al.*, 2004), it was later shown that neurite outgrowth inhibition in DRG neurons grown on substrate bound Nogo-66, MAG and OMgp is NgR1 independent (Chivatakarn *et al.*, 2007, Zheng *et al.*, 2005). These results suggest that growth cone collapse and growth inhibition may be separate activities of myelin inhibitors. Finally, RNAi-mediated knockdown of NgR1 further confirmed that blocking NgR1 expression does not result in enhanced neurite outgrowth on membrane bound MAG (Chivatakarn *et al.*, 2007). Collectively, recent studies have challenged the oversimplified scenario that all three inhibitors converge on NgR1 to mediate their effects and imply the existence of redundancy at the receptor level. Thus, great interest has been focused on defining the mechanism of action and relative contribution of NgR2 to myelin inhibition.

NgR2 is predominantly expressed in neurons of the embryonic and adult central and peripheral nervous systems, including a subpopulation of DRG neurons, and has a distinct though largely overlapping expression pattern to NgR1 (Barrette *et al.*, 2007, Funahashi *et al.*, 2008, Hunt *et al.*, 2002). NgR2 binds MAG with an approximately five-fold greater affinity than NgR1, but does not support binding of Nogo-66 or OMgp (Venkatesh *et al.*, 2005) and thus, NgR2 has been suggested to substitute for NgR1 in mediating MAG inhibition. NgR1 and NgR2 double mutant mice are not yet available and therefore, the relative contribution of NgR1 and NgR2 to myelin inhibition remains to be established.

Finally, Nogo receptor independent mechanisms to MAG inhibition may also exist, as neurons treated with an enzyme that cleaves all GPI-linked proteins can still respond to MAG (Niederost *et al.*, 2002). Some evidence has implicated ganglioside GT1b in MAG inhibition (Vyas *et al.*, 2002). Combined pharmacological inhibition of gangliosides

biosynthesis and NgR1 resulted in increased neuronal growth of DRG neurons cultured on substrate bound MAG than either treatment alone, further suggesting that combined blockage of multiple binding molecules may be necessary to overcome myelin inhibition (Mehta *et al.*, 2007).

5.1.7 Lingo, p75 and TROY

NgR1 lacks an intracellular domain and has been shown to interact with p75 or TROY, which are members of the tumour necrosis factor receptor family, and Lingo1, which is a nervous system specific type-1 membrane protein, to mediate transduction across the cell membrane. Early studies suggested that neurite outgrowth inhibition by Nogo-66, MAG and OMgp is mediated by an NgR1/p75/Lingo1 receptor complex. Lingo1 and NgR1 show distinct but largely overlapping neuronal expression patterns (Barrette *et al.*, 2007, Llorens *et al.*, 2008). Lingo1 is an essential component of the NgR1 complex in culture (Mi *et al.*, 2004) and a Lingo1 antagonist has been shown to promote axonal sprouting and functional recovery following SCI (Ji *et al.*, 2006). Moreover, neurons from p75 null mice showed dramatically reduced responses to Nogo-66, MAG and OMgp (Yamashita *et al.*, 2002). Subsequently, p75 that was originally identified as a neurotrophin receptor was shown to associate with NgR1 (Wang *et al.*, 2002, Wong *et al.*, 2002) and undergo intramembrane proteolysis by α- and γ-secretase, which is important for RhoA activation and subsequent inhibition of neurite outgrowth (Domeniconi *et al.*, 2005). RNAi-mediated knockdown of p75 in cultured DRG neurons resulted in enhanced neurite outgrowth in the presence of MAG and CNS myelin (Ahmed *et al.*, 2005, Higuchi *et al.*, 2003). However, similar to studies with Nogo-A knockout mice, p75 null mice failed to regenerate corticospinal fibres following SCI injury (Song *et al.*, 2004b). Moreover, p75 is not expressed in most populations of mature neurons and thus, many types of CNS neurons that are inhibited by myelin do not express p75.

NgR1 was subsequently found to interact with TROY with eight-fold higher affinity than with p75. Functional studies demonstrated that ectopic expression of dominant negative TROY in DRG neurons attenuates Nogo-66 inhibition (Park *et al.*, 2005). Moreover, DRG neurons isolated from TROY null mice are significantly more resistant to Nogo-66, OMgp and CNS myelin inhibition of neurite outgrowth *in vitro* (Shao *et al.*, 2005). Thus, TROY has been suggested to substitute for p75 in neurons that do not express p75. However, whilst TROY has been reported to be expressed in DRG neurons amongst other neurons (Park *et al.*, 2005), these findings have not been confirmed by subsequent studies (Hisaoka *et al.*, 2006), suggesting that additional signal-transducing co-receptors exist.

5.1.8 RhoA activation

Expression of NgR1, p75 or TROY, and Lingo1 is sufficient to confer responsiveness of non-neuronal cells to myelin inhibition by activating the downstream signal RhoA and its effector RhoA-associated kinase (ROCK) (Park *et al.*, 2005, Shao *et al.*, 2005). RhoA belongs to the Rho family of small intercellular GTPases that signal modulation of polymerization of the actin cytoskeleton. RhoA is directly activated in response to myelin-associated inhibitors and this activation correlates with signals that induce growth cone collapse (Winton *et al.*, 2002). Inhibition of RhoA using C3 transferase or dominant negative RhoA and inhibition of ROCK resulted in enhanced neuronal outgrowth on inhibitory substrates (Fournier *et al.*, 2003, Lehmann *et al.*, 1999, Niederost *et al.*, 2002). Furthermore, silencing of RhoA in DRG cultures resulted in more enhanced neurite outgrowth in the presence of CNS myelin than when p75 or NgR1 were silenced (Ahmed *et al.*, 2005). More importantly, inhibition of RhoA promoted sprouting of corticospinal fibres following dorsal hemisection, and inhibition of ROCK promoted significant regeneration of corticospinal fibres and functional recovery (Dergham *et al.*, 2002, Fournier *et al.*, 2003, Lehmann *et al.*, 1999). Ephrins, semaphorins and CSPGs have also been suggested to converge on the RhoA pathway (Hunt *et al.*, 2002, Sandvig *et al.*, 2004). Thus, the RhoA-

ROCK pathway is an attractive candidate to block in order to promote axonal regeneration.

5.1.9 Glial scar

In addition to degenerating myelin, another important source of inhibition is the glial scar, which is composed predominantly of reactive astrocytes that extend their hypertrophied processes to form both a physical and chemical barrier (Berry *et al.*, 1983, Fitch & Silver, 1997). The glial scar has traditionally been regarded inhibitory to axonal regeneration mainly due to indirect evidence, such as labeled axons stopping at the region of astrogliosis (Davies *et al.*, 1997) and detection of inhibitory CSPGs (Davies *et al.*, 1999, Levine, 1994). Breakdown of CSPGs using the enzyme chodroitinase ABC (ChABC) has been shown to promote regeneration of sensory and corticospinal axons, and improve functional recovery after SCI (Barritt *et al.*, 2006, Bradbury *et al.*, 2002, Caggiano *et al.*, 2005). Studies with transgenic animals, however, have clearly demonstrated that although the glial scar may be a source of inhibitory molecules, its formation plays a critical role in limiting the extent of the injury (Faulkner *et al.*, 2004, Okada *et al.*, 2006). Moreover, a transgenic mouse that overexpresses ChABC demonstrated sensory axon regeneration and behavioural recovery following dorsal rhizotomy, and regeneration of corticospinal axons but no significant recovery after dorsal hemisection (Cafferty *et al.*, 2007). Thus, neutralizing specific components of the glial scar that inhibit axon regeneration without significantly altering its wound healing properties in combination to blocking myelin-associated inhibitors may be a promising strategy to promote functional recovery following CNS injury.

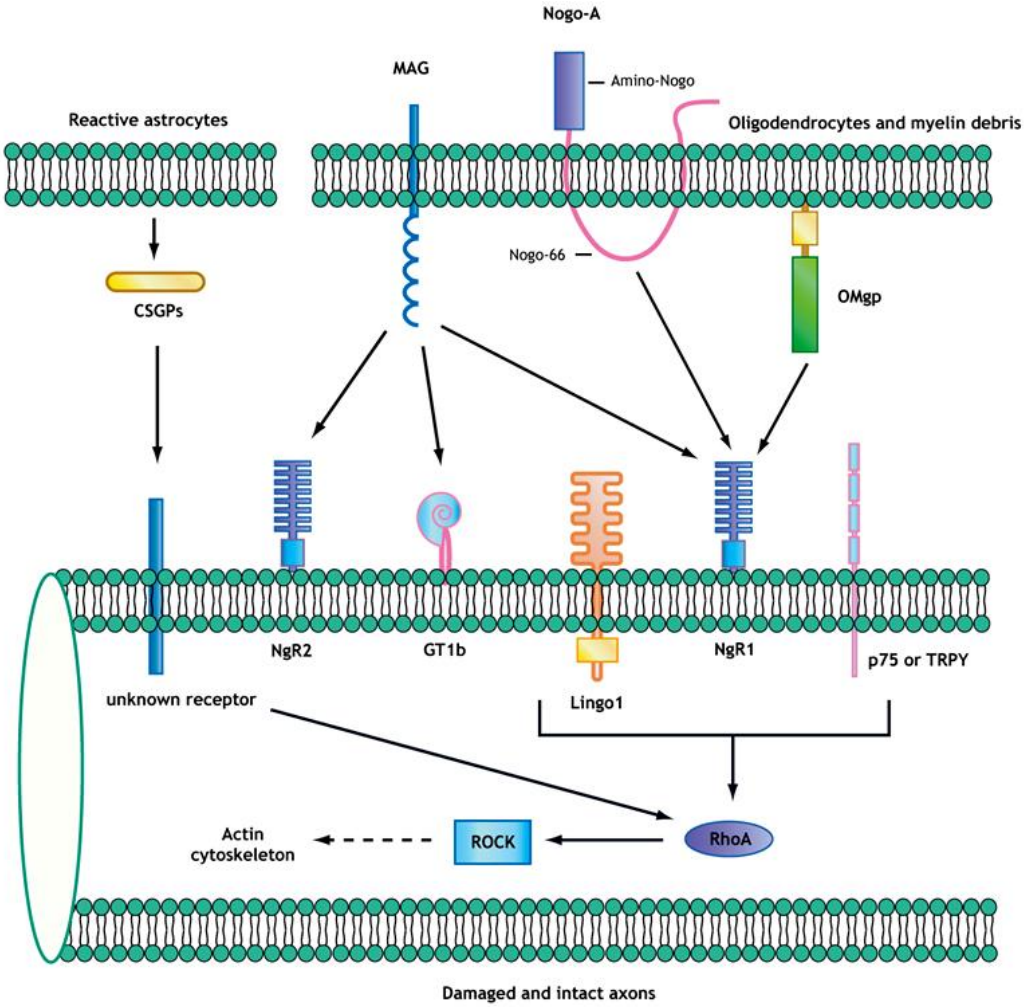


Figure 5.1 Glial and myelin inhibition of axonal regeneration

The myelin inhibitors Nogo-A, MAG and OMgp have been shown to interact with NgR1. NgR2 may substitute for NgR1 in mediating MAG-specific inhibitory effects. Some evidence has implicated GT1b in MAG inhibition. NgR1 has been shown to associate with Lingo and p75 or TROY, although other co-receptors may exist, to mediate transduction across the cell membrane. Myelin inhibitors, as well as CSGPs that are expressed by activated astrocytes at the glial scar, activate RhoA and its downstream effector ROCK, which modulate the polymerization of the actin cytoskeleton.

5.1.10 Cell body responses to axotomy

The central axons of DRG neurons have been shown to regenerate into peripheral nerve transplants inserted into the dorsal column of the spinal cord, when their peripheral axons have been previously transected (Richardson & Issa, 1984). More recently, Neumann and Woolf observed sprouting of central DRG axons across the non-permissive spinal cord injury site, when DRG neurons were conditioned by sciatic nerve transection (Neumann & Woolf, 1999). Conditioning lesion of the peripheral DRG axon triggers upregulated expression of a number of genes, collectively referred to as regeneration-associated genes or RAGs (Broude *et al.*, 1997, Schreyer & Skene, 1993), which include genes expressing transcription factors, cytoskeletal proteins, cell adhesion molecules, axonal guidance molecules and neurotrophic factors (Befort *et al.*, 2003, Boeshore *et al.*, 2004, Tanabe *et al.*, 2003).

Upregulation of RAGs in the CNS is weaker and more transient than in the PNS, and in some systems, altogether absent (Mason *et al.*, 2003, Tetzlaff *et al.*, 1991). Thus, although some transected axons may sprout, grow collaterals and even new functional connections, RAGs are downregulated soon after injury and injured axons eventually form dystrophic growth cones. Moreover, although axotomy induces the expression of RAGs in both central and peripheral neurons, adult CNS neurons may lack the downstream effectors, such as ATF3/c-jun (Hunt *et al.*, 2004, Mason *et al.*, 2003), to translate these signals into long-distance regeneration. Thus, enhancing the expression of developmental genes associated with axonal growth in combination to blocking myelin-associated inhibitors may be necessary to promote meaningful axonal regeneration.

cAMP has been identified as an important intrinsic determinant of axonal growth (Neumann *et al.*, 2002, Qiu *et al.*, 2002) and has been suggested to mediate some of its effects by regulating cAMP-responsive binding protein (CREB) and CRE-mediated transcription of genes required for regeneration (Gao *et al.*, 2004). The endogenous levels of cAMP decline dramatically with development and this spontaneous decline coincides

with the switch to the inhibition of regeneration by MAG (Cai *et al.*, 2001). Moreover, it has been suggested that priming neurons with particular neurotrophins elevates cAMP and prevents subsequent inhibition of regeneration by MAG through activation of PKA (Cai *et al.*, 1999). Indeed, *in vivo* treatment with nerve growth factor (NGF), neurotrophin-3 (NT3) or glial-derived neurotrophic factor (GDNF) promoted regeneration of sensory neurons across the dorsal root entry zone and into the spinal cord and demonstrated functional recovery in rats with injured dorsal roots (Ramer *et al.*, 2000).

5.1.11 Rationale

Genetic deletions of individual myelin components or individual components of the NgR1 complex have produced conflicting results. It is clear from the studies described in this introduction that no single component is solely responsible for the failure of regeneration in the adult mammalian CNS. A reasonable next step would therefore be to use approaches to simultaneously target multiple and potentially compensatory inhibitory pathways. Moreover, treatments to enhance the intrinsic regenerative capacity of injured neurons may also be required for successful regeneration.

As described in chapter 3, the miRNA-like disabled HSV-1 vector developed in this thesis has the potential to allow silencing of multiple genes simultaneously through expression of multiple miRNA cassettes from a single transcript. The primary aim of this chapter was to investigate whether silencing of multiple genes could be achieved cells in culture and sensory neurons *in vivo* using the miRNA-like vector system. Furthermore, preliminary experiments were performed to test the novel hypothesis that silencing *p75*, *Lingo1* and *NgR2* in combination to a conditioning lesion of the sciatic nerve can promote regeneration of sensory axons into the spinal cord following injury of the dorsal roots (rhizotomy). This hypothesis is substantiated by previous studies suggesting that 1) p75, which is highly expressed in sensory neurons, is important for Nogo-66, MAG and OMgp neurite outgrowth inhibition in these neurons (Yamashita *et al.*, 2002), 2) Lingo1 is an

essential component of the NgR1 complex (Mi *et al.*, 2004), 3) Nogo-A is redundant for outgrowth inhibition in sensory neurons and thus, MAG inhibition may play an important role (Oudega *et al.*, 2000), and 4) NgR2, which does not signal through p75 or Lingo and is highly expressed in sensory neurons, may substitute for NgR1 to mediate MAG-specific inhibitory effects (Venkatesh *et al.*, 2005). At the time the objectives of this chapter were conceived, there were no reports of silencing multiple genes simultaneously using vector-mediated delivery of shRNA, or silencing receptors for myelin-associated inhibitors *in vivo*. Work in this chapter was performed in collaboration with Prof. Patrick Anderson at the Department of Anatomy, UCL.

5.2 Screening of pre-miRNA sequences against p75, Lingo1 & NgR2

Six pre-miRNA sequences against mouse *p75* (GI:23468246), five pre-miRNA sequences against mouse *Lingo1* (GI:41351214) and five pre-miRNA sequences against mouse *NgR2* (GI:124376089) were designed using Invitrogen's RNAi designer tool (sequences available in section 2.1.6). Figure 5.2 shows the 21-22nt regions within each gene that are targeted by these sequences. The sixteen pre-miRNAs were synthesized as single stranded DNA oligonucleotides, annealed and cloned into pcDNA6.2/EmGFP vectors (shown in figure 3.7). The pcDNA6.2/EmGFP-miR plasmids were sequenced using the GFP Forward primer (described in section 2.1.5) to ensure that the pre-miRNA sequences had not been mutated (data not shown).

To screen the pre-miRNA sequences in 293T cells, the mouse p75 (IMAGE:5367638), Lingo1 (IMAGE:5685897) and NgR2 (IMAGE:40130800) cDNA sequences were cloned into pSCREEN-iT/LacZ-DEST vectors (Invitrogen), which allow generation of screening constructs containing each target gene fused to the *lacZ* reporter gene (figure 5.3a). The mouse p75 cDNA sequence (Geneservice) was supplied cloned into the pCMVSPORT6 vector between the SalI-NotI sites and was subcloned into pENTR4 (Invitrogen) between SalI-NotI. The mouse Lingo1 cDNA sequence (Geneservice) was supplied cloned into the pYX-Asc vector between the EcoR1-NotI sites and was subcloned into pENTR4 between EcoRI-NotI. A map of pENTR4 is shown in figure 5.3b. The mouse NgR2 cDNA sequence, which was a gift to Prof. Anderson, was supplied cloned into pFLAG-CMV-1 between the HindIII-XbaI sites (figure 5.3c) and was subcloned into pENTR-U6shLacZ between BamHI-XbaI in place of the U6 promoter, shLacZ and pol III termination signal (figure 5.3d). The resulting entry clones (attL1-attL2) allowed the p75, Lingo1 and NgR2 cDNA sequences to be transferred into pSCREEN-iT/LacZ-DEST destination vectors (attR1-attR2).

The pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1 and pSCREEN-iT/LacZ-NgR2 vectors (100ng) were then co-transfected into 293T cells with each of the plasmids expressing pre-miR-p75(1-6), pre-miR-Lingo1(1-5) and pre-miR-NgR2(1-5) (1µg)

respectively, using Lipofectamine (as described in section 2.3.3). The pcDNA6.2/EmGFP-miR-neg plasmid constructed in section 3.5.3 was used as a negative control. Target gene knockdown was assessed by measuring ß-galactosidase levels (as described in section 2.7.5) at 48 hrs post-transfection. EmGFP levels expressed from the pre-miRNA constructs were assessed by fluorescent microscopy prior to assessing ß-galactosidase levels, to ensure that differences in knockdown were not due to variable transfection efficiency between the different conditions (data not shown). Ideally, sequences would be selected for their ability to knockdown target gene expression by over 80%. However, the most effective pre-miRNA sequences against p75, Lingo1 and NgR2 were found to induce silencing at somewhat lower levels of up to 65% (figure 5.4). Nevertheless, as the pre-miRNA sequences tested were predicted by Invitrogen's RNAi designer tool to be the most potent, no additional sequences were screened. The pre-miR-p75-1, pre-miR-Lingo1-1 and pre-miR-NgR2-1 sequences, found to silence *p75* by 63±4.0%, *Lingo1* by 65±9.0% and *NgR2* by 57±2.0% respectively (n=4, mean ± s.d.), were selected for further testing.

pcDNA6.2/EmGFP-miR-p75 sequences

gb|BC038365.1| Mus musculus nerve growth factor receptor (TNFR superfamily, member 16), mRNA (cDNA clone MGC:35588 IMAGE:5367638), complete cds, Length=3441

The target sequence in pre-miR-p75-1:

```
Score = 41.0 bits (44), Expect = 0.40
Identities = 22/22 (100%), Gaps = 0/22
(0%) Strand=Plus/Minus

Query  5    GAAATCGCTGATTTGTGTAGTC  26
            ||||||||||||||||||||||
Sbjct 746   GAAATCGCTGATTTGTGTAGTC 725
```

The target sequence in pre-miR-p75-2:

```
Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21
(0%) Strand=Plus/Minus

Query  6     AAAGGAGTCTATATGCTCCGG  26
             |||||||||||||||||||||
Sbjct  1259  AAAGGAGTCTATATGCTCCGG  1239
```

The target sequence in pre-miR-p75-3:

```
Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21
(0%)Strand=Plus/Minus

Query  6     TGCACAGGCTCTCCACAATGT  26
             |||||||||||||||||||||
Sbjct  1386  TGCACAGGCTCTCCACAATGT  1366
```

The target sequence in pre-miR-p75-4:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%) Strand=Plus/Minus

Query  5    GTAGACCTTGTGATCCATCGGC  26
            ||||||||||||||||||||||
Sbjct  739  GTAGACCTTGTGATCCATCGGC  718
```

The target sequence in pre-miR-p75-5:

```
Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21
(0%)Strand=Plus/Minus

Query  6    AATATAGGCCACAAGGCCCAC  26
            |||||||||||||||||||||
Sbjct  950  AATATAGGCCACAAGGCCCAC  930
```

The target sequence in pre-miR-p75-6:

```
Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21
(0%)Strand=Plus/Minus

Query  6     AATGTCAGCTCTCTGGATGCG  26
             |||||||||||||||||||||
Sbjct  1370  AATGTCAGCTCTCTGGATGCG  1350
```

pcDNA6.2/EmGFP-miR-Lingo sequences

gb|BC052384.1| Mus musculus leucine rich repeat and Ig domain containing 1, mRNA (cDNA clone MGC:57943 IMAGE:5703857), complete cds Length=3321

The target sequence in pre-miR-Lingo1-1:

```
Score = 42.8 bits (46), Expect = 0.11
Identities = 23/23 (100%), Gaps = 0/23
(0%) Strand=Plus/Minus

Query  4    TGTAGTCTAGCAGGATGACGATC  26
            |||||||||||||||||||||||
Sbjct  932  TGTAGTCTAGCAGGATGACGATC  910
```

The target sequence in pre-miR-Lingo1-2:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  5     GTGAAGTAGTTGGGTAGGAGTA  26
             ||||||||||||||||||||||
Sbjct  1707  GTGAAGTAGTTGGGTAGGAGTA  1686
```

The target sequence in pre-miR-Lingo1-3:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  5     GATAATGAGCGTCTTGATGTCG  26
             ||||||||||||||||||||||
Sbjct  2125  GATAATGAGCGTCTTGATGTCG  2104
```

The target sequence in pre-miR-Lingo1-4:

```
Score = 42.8 bits (46), Expect = 0.11
Identities = 23/23 (100%), Gaps = 0/23
(0%)Strand=Plus/Minus

Query  4    TGTATAGGTCTTGGAACATGTAG  26
            |||||||||||||||||||||||
Sbjct  950  TGTATAGGTCTTGGAACATGTAG  928
```

The target sequence in pre-miR-Lingo1-5:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  6     TACAAACACCTGCTGTGCCTTG  27
             ||||||||||||||||||||||
Sbjct  1756  TACAAACACCTGCTGTGCCTTG  1735
```

---

pcDNA6.2/EmGFP-miR-NgR2 sequences

gb|BC138154.1| Mus musculus reticulon 4 receptor-like 2, mRNA (cDNA clone MGC:169778, IMAGE:8861173), complete cds Length=1401

The target sequence in pre-miR-NgR2-1:

```
Score = 39.2 bits (42), Expect = 1.4
Identities = 21/21 (100%), Gaps = 0/21
(0%)Strand=Plus/Minus

Query  6    TACAGGTGTAGTGACTGCAGC  26
            |||||||||||||||||||||
Sbjct  480  TACAGGTGTAGTGACTGCAGC  460
```

The target sequence in pre-miR-NgR2-2:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  5    GTACTGTAGGCTGACCAAGCCT  26
            ||||||||||||||||||||||
Sbjct  541  GTACTGTAGGCTGACCAAGCCT  520
```

The target sequence in pre-miR-NgR2-3:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  6    TGAACAGGTAGAGGATGGTGAG  27
            ||||||||||||||||||||||
Sbjct  770  TGAACAGGTAGAGGATGGTGAG  749
```

The target sequence in pre-miR-NgR2-4:

```
Score = 41.0 bits (44), Expect = 0.39
Identities = 22/22 (100%), Gaps = 0/22
(0%)Strand=Plus/Minus

Query  5    GAGGTACTGTAGGCTGACCAAG  26
            ||||||||||||||||||||||
Sbjct  544  GAGGTACTGTAGGCTGACCAAG  523
```

The target sequence in pre-miR-NgR2-5:

```
Score = 39.2 bits (42), Expect = 1.3
Identities = 21/21 (100%), Gaps = 0/21
(0%)Strand=Plus/Minus

Query  5    AACAGGTAGAGGATGGTGAGG  25
            |||||||||||||||||||||
Sbjct  768  AACAGGTAGAGGATGGTGAGG  748
```

Figure 5.2 Blast nucleotide alignments between the oligonucleotides expressing pre-miRNA sequences and the p75, Lingo1 and NgR2 cDNA sequences.

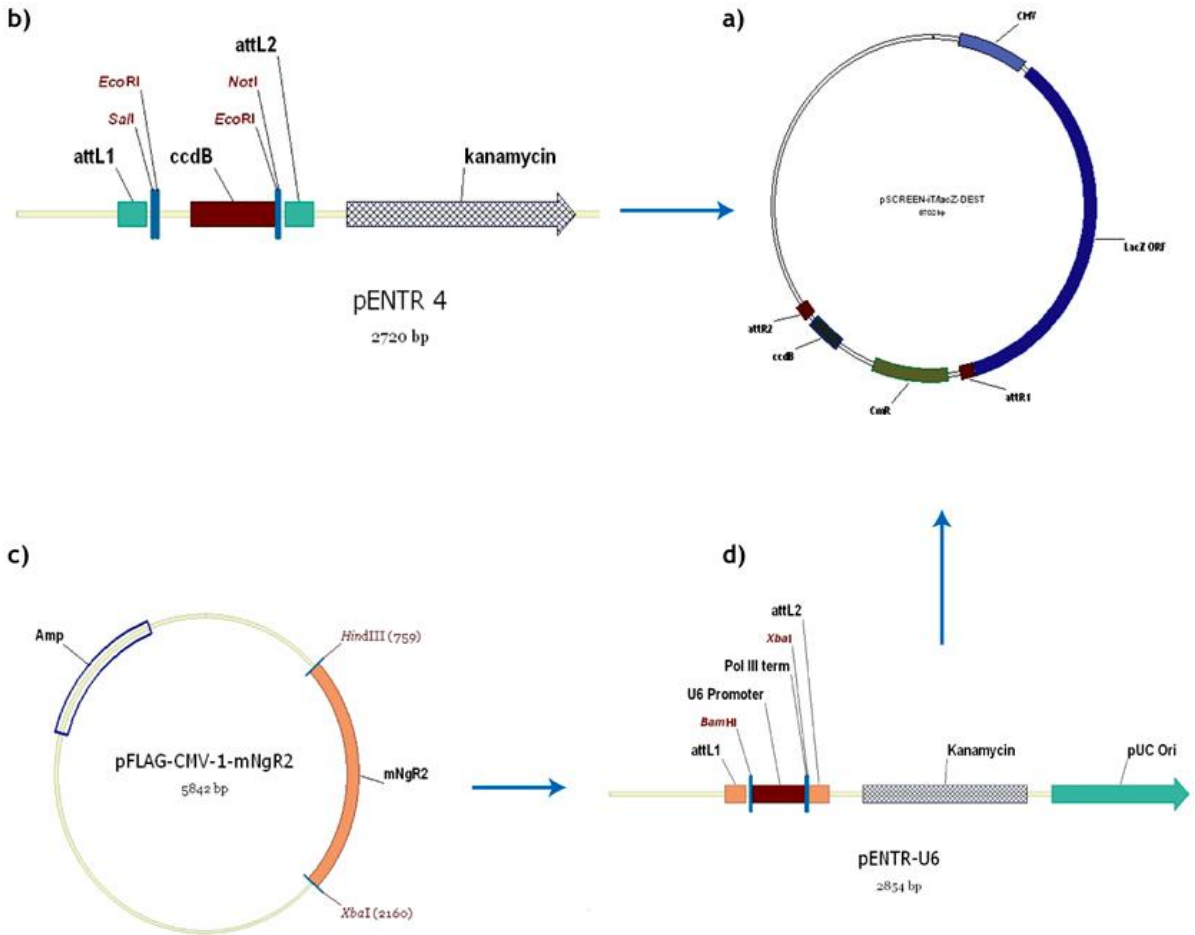


Figure 5.3 Construction of pSCREEN-iT/LacZ-p75, -Lingo1, and -NgR2 plasmids

The mouse p75 cDNA was cloned into pENTR4 between SalI-NotI. The mouse Lingo1 cDNA was cloned into pENTR4 between EcoRI-NotI. The mouse NgR2 cDNA sequence was supplied cloned into pFLAG-CMV-1 between the HindIII-XbaI sites and was subcloned into pENTR-U6shLacZ between BamHI-XbaI in place of the U6 promoter, shLacZ and pol III termination signal. The resulting entry clones (attL1-attL2) allowed the p75, Lingo1 and NgR2 cDNA sequences to be transferred into pSCREEN-iT/LacZ-DEST destination vectors (attR1-attR2).

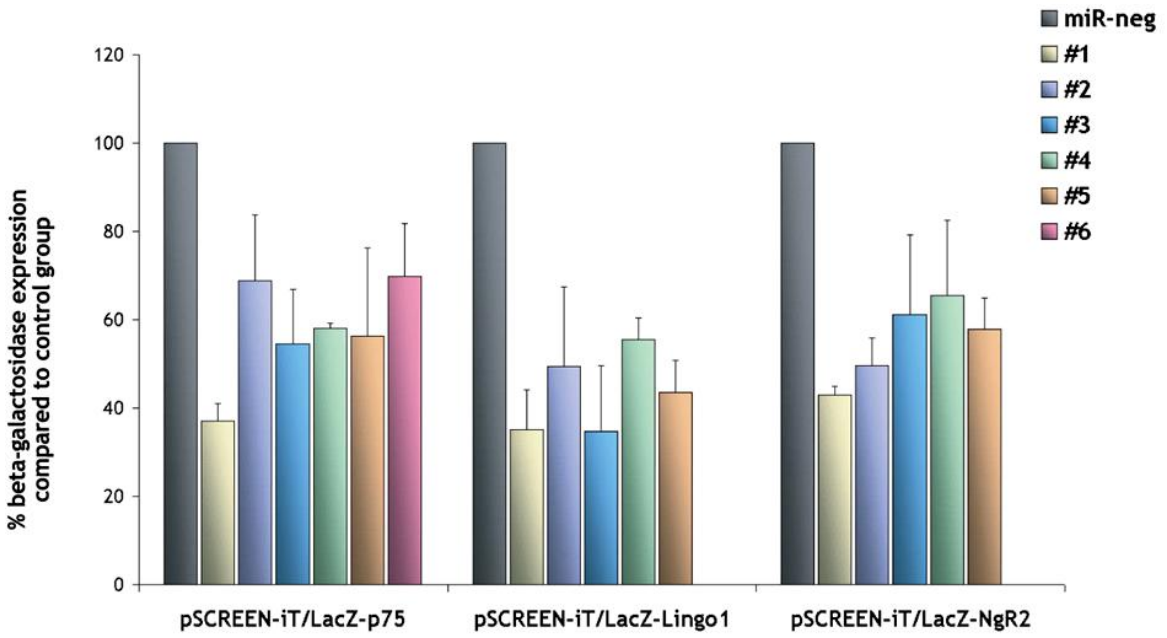


Figure 5.4 Screening of pre-miRNA sequences against p75, Lingo1 and NgR2

The pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1 and pSCREEN-iT/LacZ-NgR2 vectors were co-transfected into 293T cells with each of the plasmids expressing pre-miR-p75, pre-miR-Lingo1 and pre-miR-NgR2 respectively. The plasmid expressing pre-miR-neg was used as a negative control. Transfection efficiency was monitored by the EmGFP signal expressed from these constructs. Silencing was assessed by measuring beta-galactosidase levels at 48 hrs post-transfection. The pre-miR-p75-1, pre-miR-Lingo1-1 and pre-miR-NgR2-1 sequences that were found to silence p75 by 63±4.0%, Lingo1 by 65±9.0% and NgR2 by 57±2.0% respectively (n=4, mean ± s.d.), were selected.

5.3 Assessment of silencing specificity

There is no significant sequence similarity between *p75*, *Lingo1* and *NgR2*. Nevertheless, prior to expressing the three pre-miRNA sequences from a single vector, it was necessary to exclude any potential off-target effects arising from a miRNA against one gene non-specifically silencing any of the genes that it is not predicted to target. 293T cells were therefore co-transfected with pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1, or pSCREEN-iT/LacZ-NgR2 (100ng) and each of the plasmids expressing pre-miR-neg, pre-miR-p75-1, pre-miR-Lingo1-1, or pre-miR-NgR2-1 (1μg) using Lipofectamine. Transfection efficiency was monitored by the presence of the EmGFP signal expressed from the pre-miRNA constructs. B-galactosidase activity assay at 48 hrs post-transfection confirmed that each miRNA silences its intended target gene only with comparable potency to that demonstrated in the previous section, indicating that silencing is specific. However, p75, Lingo and NgR2 expression levels were found to be lower in cells transfected with the pcDNA6.2/EmGFP-miR-neg plasmid than in cells co-transfected with the plasmids expressing pre-miR-p75, pre-miR-Lingo1 or pre-miR-NgR2 and the genes that they are not predicted to target (figure 5.5). The downregulation of Lingo1 by the negative control miRNA appears to be statistically significant. Thus, miR-neg represses Lingo1 expression by a non-specific effect, which may be due to an off-target effect or induction of the INF response. The quantitative RT-PCR performed in chapter 3 demonstrated that miR-neg does not induce the INF response in 293T cells (section 3.7). Moreover, miR-neg is not predicted to target any known vertebrate gene. The reasons behind this result were not further investigated, but as a result miR-neg was not used as a negative control in any further experiments performed in this chapter.

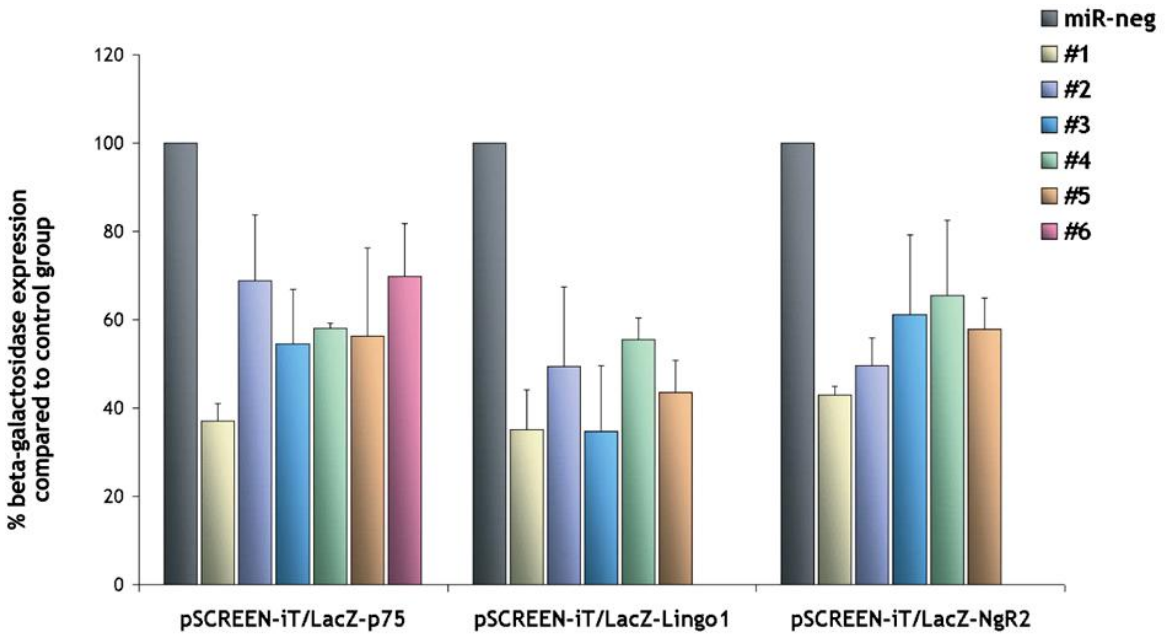


Figure 5.4 Screening of pre-miRNA sequences against p75, Lingo1 and NgR2

The pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1 and pSCREEN-iT/LacZ-NgR2 vectors were co-transfected into 293T cells with each of the plasmids expressing pre-miR-p75, pre-miR-Lingo1 and pre-miR-NgR2 respectively. The plasmid expressing pre-miR-neg was used as a negative control. Transfection efficiency was monitored by the EmGFP signal expressed from these constructs. Silencing was assessed by measuring beta-galactosidase levels at 48 hrs post-transfection. The pre-miR-p75-1, pre-miR-Lingo1-1 and pre-miR-NgR2-1 sequences that were found to silence p75 by 63±4.0%, Lingo1 by 65±9.0% and NgR2 by 57±2.0% respectively (n=4, mean ± s.d.), were selected.

5.4 Tandem expression of pre-miRNAs against p75, Lingo1 & NgR2

Endogenous miRNAs are often expressed in clusters by RNA pol II (He *et al.*, 2005, Suh *et al.*, 2004). Cloning of pre-miRNA sequences in tandem may potentially allow co-cistronic expression of multiple pre-miRNAs and thus, silencing of multiple genes from a single transcript. The most potent pre-miRNA sequences against *p75*, *Lingo1* and *NgR2* were cloned in tandem and transferred into a pR19 vector, which allows the cassette expressing all three pre-miRNAs in series to be inserted into a disabled HSV vector.

The pcDNA6.2/EmGFP-miR-Lingo1 plasmid was digested with BamHI-XhoI to release the pre-miR-Lingo1 sequence together with the miR-155 flanking regions. This fragment was inserted into pcDNA6.2/EmGFP-miR-p75 between BglII-XhoI. The resulting pcDNA6.2/EmGFP-miR-p75/Lingo1 plasmid was digested with BglII-XhoI and was used as a backbone to insert the pre-miR-NgR2 and miR-155 flanking regions, which were excised from pcDNA6.2/EmGFP-miR-NgR2 using BamHI-XhoI (figure 5.6). The resulting pcDNA6.2/EmGFP-miR-p75/Lingo1/NgR2 plasmid was sequenced using the GFP Forward primer (described in section 2.1.5) to ensure that the three pre-miRNA sequences had been chained correctly. The pre-miR-p75/ Lingo1/NgR2 cassette was inserted into pDONR-221 by BP recombination and then into pR19CMV-Gateway (constructed in section 3.5.3) by LR recombination. The pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 plasmid was sequenced between the LAT flanking regions (figure 5.7).

To investigate whether tandem expression of pre-miRNA sequences targeting different genes results in knockdown of each individual gene, 293T cells were co-transfected with pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1, or pSCREEN-iT/LacZ-NgR2 (100ng) and either pR19CMV/EmGFP-miR-TRPV1 or pR19CMV/EmGFP-miR-p75/ Lingo1/NgR2 (1µg) using Lipofectamine. The pR19CMV/EmGFP-miR-TRPV1 plasmid (constructed in section 4.3) was used as negative control to avoid using the plasmid expressing pre-miR-neg or the pR19CMV/EmGFP-miR-LacZ plasmid (constructed in section 3.5.3), which would silence the *lacZ* gene that is fused to the target genes. Transfection

efficiency was monitored by the presence of the EmGFP signal expressed from the pre-miRNA constructs. B-galactosidase activity assay at 48 hrs post-transfection revealed that pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 silences *p75* by 74±14%, *Lingo1* by 75±11% and *NgR2* by 63±10% (n=4, mean ± s.d.), suggesting that tandem expression of pre-miRNA sequences has no effect on the processing of individual pre-miRNAs into functional miRNAs (figure 5.8). Silencing from these miRNAs seems to be considerably improved indicating that the miR-TRPV1, which is used as a negative control, does not reduce expression of the targeted genes.

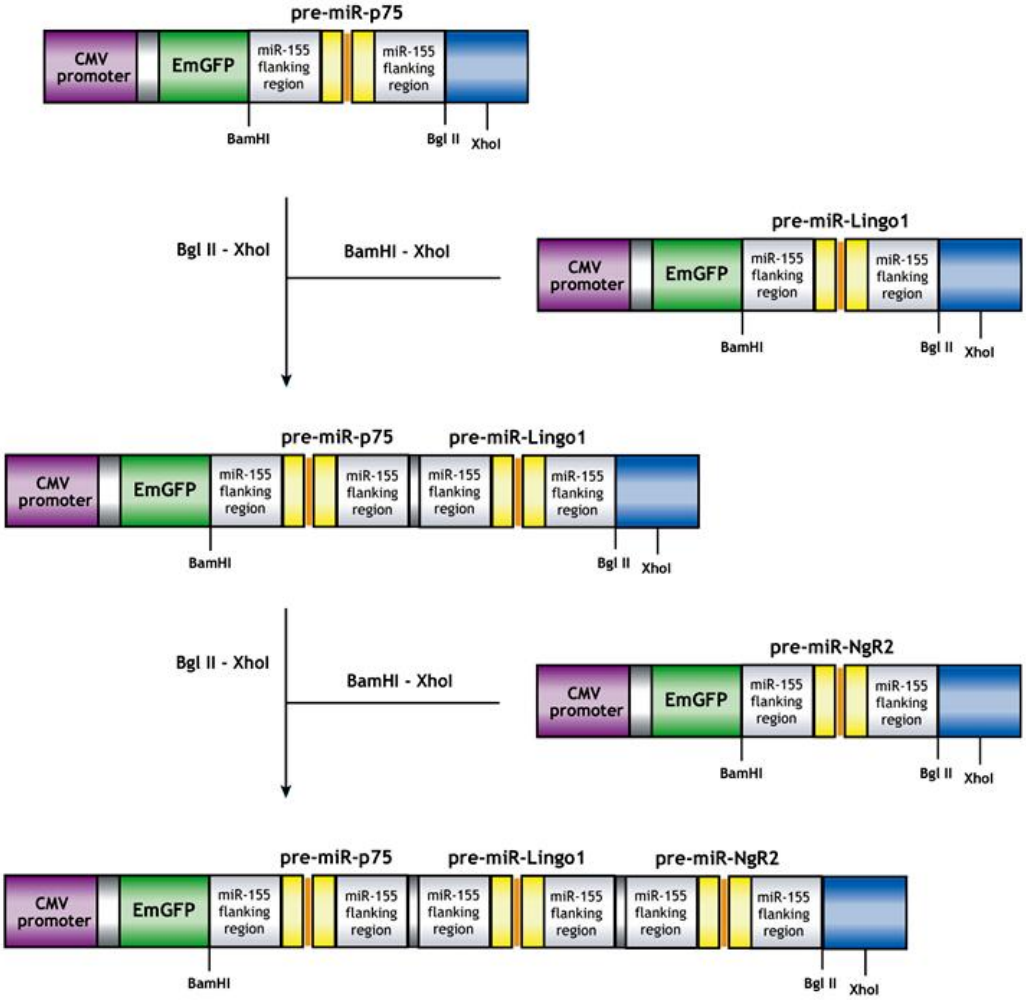


Figure 5.6 Chaining of pre-miRNA sequences against p75, Lingo1 and NgR2

The pcDNA6.2/EmGFP-miR-Lingo1 plasmid was digested with BamHI-XhoI to release the pre-miR-Lingo1 sequence together with the miR-155 flanking regions. This fragment was inserted into pcDNA6.2/EmGFP-miR-p75 between BglII-XhoI. The resulting pcDNA6.2/EmGFP-miR-p75/Lingo1 plasmid was digested with BglII-XhoI and was used as a backbone to insert the pre-miR-NgR2 and miR-155 flanking regions, which were excised from pcDNA6.2/EmGFP-miR-NgR2 using BamHI-XhoI.

N.B. Not to scale

Sequence 1: lcl|1
Length = 1431 (1 .. 1431) **pre-miR-p75-1**

Sequence 2: lcl|65536
Length = 64 (1 .. 64) **pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 (GFP F Primer)**

Score = 123 bits (64), Expect = 2e-24
Identities = **64/64 (100%)**, Gaps = 0/64 (0%)
Strand=Plus/Plus

```
Query  145  TGCTGTAGACAGGAATGAGGTTGTCAGTTTTGGCCACTGACTGACTGACAACCATTCCTG  204
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCTGTAGACAGGAATGAGGTTGTCAGTTTTGGCCACTGACTGACTGACAACCATTCCTG  60

Query  205  TCTA  208
            ||||
Sbjct  61   TCTA  64
```

Sequence 1: lcl|1
Length = 1431 (1 .. 1431) **pre-miR-Lingo1-1**

Sequence 2: lcl|65536
Length = 64 (1 .. 64) **pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 (GFP F Primer)**

Score = 123 bits (64), Expect = 2e-24
Identities = 64/64 (100%), Gaps = 0/64 (0%)
Strand=Plus/Plus

```
Query  283  TGCTGTAGTCTAGCAGGATGACGATCGTTTTGGCCACTGACTGACGATCGTCACTGCTAG  342
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCTGTAGTCTAGCAGGATGACGATCGTTTTGGCCACTGACTGACGATCGTCACTGCTAG  60

Query  343  ACTA  346
            ||||
Sbjct  61   ACTA  64
```

Sequence 1: lcl|1
Length = 1431 (1 .. 1431) **pre-miR-NgR2-1**

Sequence 2: lcl|65536
Length = 64 (1 .. 64) **pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 (GFP F Primer)**

Score = 123 bits (64), Expect = 2e-24
Identities = 64/64 (100%), Gaps = 0/64 (0%)
Strand=Plus/Plus

```
Query  421  TGCTGTACAGGTGTAGTGACTGCAGCGTTTTGGCCACTGACTGACGCTGCAGTCTACACC  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  1    TGCTGTACAGGTGTAGTGACTGCAGCGTTTTGGCCACTGACTGACGCTGCAGTCTACACC  60

Query  481  TGTA  484
            ||||
Sbjct  61   TGTA  64
```

Figure 5.7 Blast nucleotide sequence alignment between the pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 sequence obtained with the GFP Forward primer and the oligonucleotide sequences expressing pre-miRNA against *p75*, *Lingo1* and *NgR2*. The pre-miRNA sequences were cloned in the right order and were not mutated.

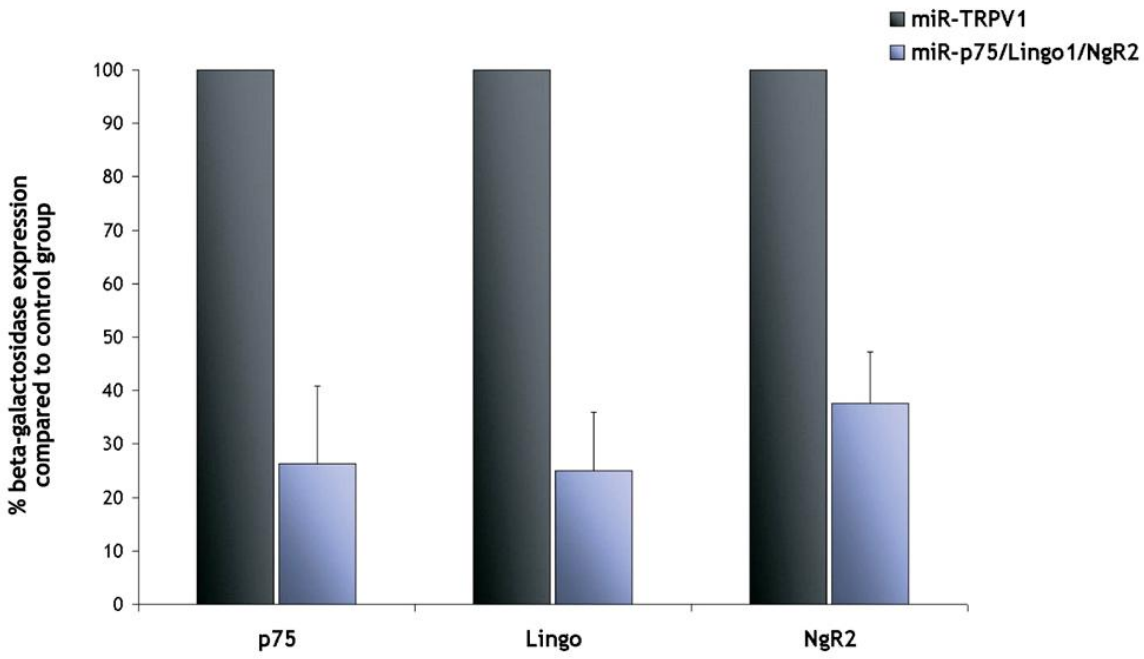


Figure 5.8 Evaluation of pR19CMV/EmGFP-miR-p75/Lingo1/NgR2

293T cells were co-transfected with pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1 or pSCREEN-iT/LacZ-NgR2 and either pR19CMV/EmGFP-miR-TRPV1 or pR19CMV/EmGFP-miR-p75/ Lingo1/NgR2. B-galactosidase activity assay at 48 hrs post-transfection revealed that pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 silences p75 by 74±14%, Lingo1 by 75±11% and NgR2 by 63±10% (n=4, mean ± s.d.), indicating that chaining different pre-miRNAs has no effect on the expression and processing of functional miRNAs

5.5 Generation of a disabled HSV-1 vector against p75/Lingo1/NgR2

The EmGFP/miR-p75/Lingo1/NgR2 cassette was inserted into the LAT region of the replication-defective 1764/4-/27+/RL1+ HSV-1 genome immediately 3' to the LATP2 region, which allows long term expression during latency. The pR19CMV/ EmGFP-miR-p75/Lingo1/NgR2 plasmid was linearised using a unique XmnI site in the plasmid backbone and was co-transfected into MAM49 complementing cells with 1764 4-/27+/RL1+ pR19CMVLacZWCm viral DNA (as described in section 2.4.2). Viral recombinants were identified by their ability to express GFP (green plaques) using fluorescent microscopy. Bright green plaques were selected and purified. When a pure green population was obtained, the purity of the plaques was confirmed further by x-gal staining. Expression of GFP from these recombinants indicated that the desired homologous recombination event had occurred. However, to confirm that the pre-miR-p75/Lingo1/NgR2 sequence had not been mutated, PCR was performed on viral DNA extracted from each plaque using the GFP Forward and WCm Reverse primers described in section 2.1.5 (figure 5.9). The PCR products were sequenced (data not shown) and one recombinant plaque was selected and grown to a high titre as described is section 2.4.3.

293T cells were transfected with pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1, or pSCREEN-iT/LacZ-NgR2 (100ng) and were allowed to recover for 24hrs prior to being transduced with HSV-CMV/EmGFP-miR-TRPV1 (negative control) or HSV-CMV/EmGFP-miR-p75/ Lingo1/NgR2 at an MOI=10.0. B-galactosidase activity assay at 72 hrs post-transfection (figure 5.10) revealed that HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 silences *p75* by 94±0.9%, *Lingo1* by 94±0.8% and *NgR2* by 96±0.5% (n=3, mean ± s.d.). Thus, knockdown was significantly improved when the pre-miRNA cassette was expressed from a disabled HSV vector as compared to when it was expressed from a plasmid vector (figure 5.8), presumably due to the more efficient delivery by the viral rather than plasmid vector.

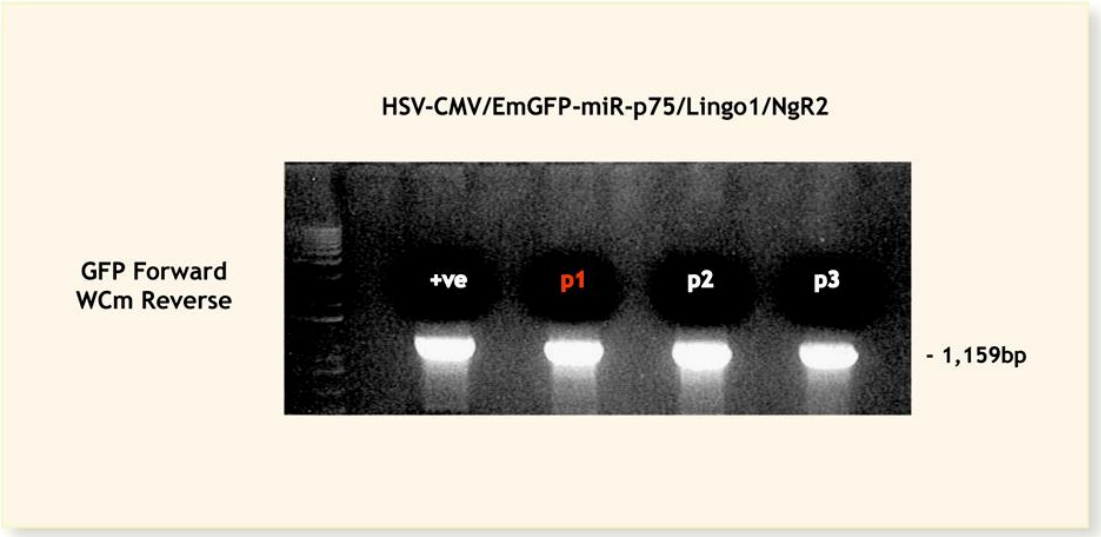


Figure 5.9 PCR on viral DNA to confirm that the correct homologous recombination event had occurred

PCR on viral DNA extracted from HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 plaques using the GFP Forward and WCm Reverse primers produced a band of 899bp thus confirming the presence of the pre-miRNA cassette. The positive control is the pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 used to generate the plaques tested. The letter "p" refers to plaque. Plaques in red were selected and grown to a high titre.

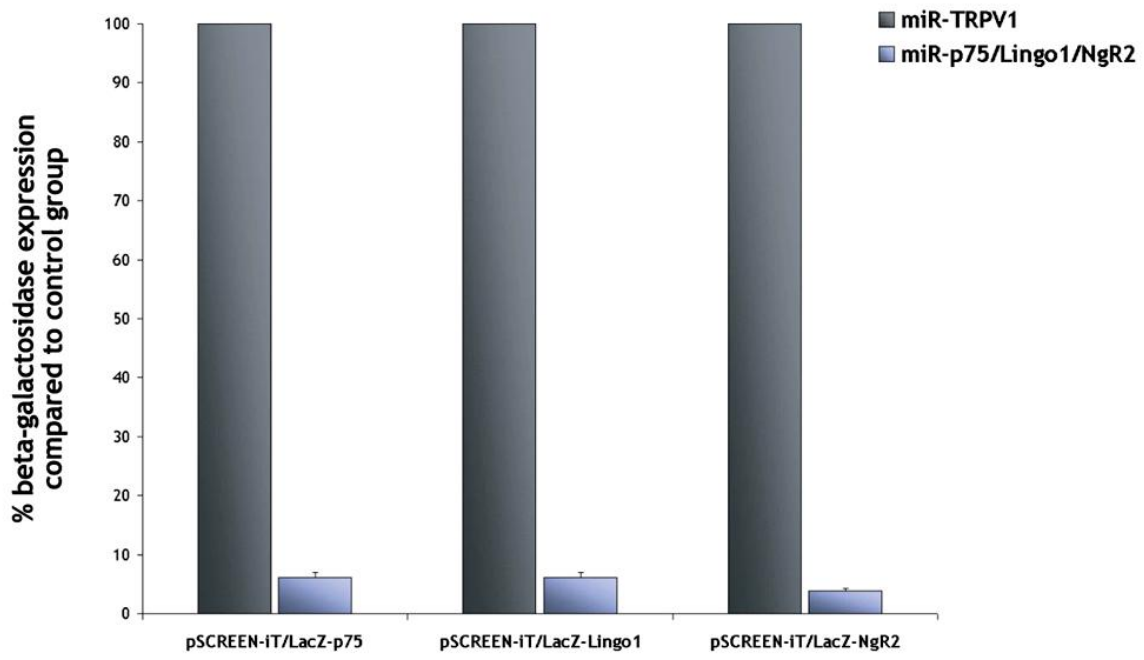


Figure 5.10 Evaluation of HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 in 293T cells

293T cells were transfected with pSCREEN-iT/LacZ-p75, pSCREEN-iT/LacZ-Lingo1 or pSCREEN-iT/LacZ-NgR2 (100ng) and were allowed to recover for 24hrs prior to being transduced with HSV-CMV/EmGFP-miR-TRPV1 or HSV-CMV/EmGFP-miR-p75/ Lingo1/NgR2 at an MOI=10.0. B-galactosidase activity assay at 72 hrs post-transfection revealed that HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 silences p75 by 94±0.9%, Lingo1 by 94±0.8% and NgR2 by 96±0.5% (n=3, mean ± s.d.).

5.6 HSV-mediated silencing of p75/Lingo1/NgR2 in DRG *in vivo*

To evaluate HSV-mediated silencing of *p75*, *Lingo1* and *NgR2* in DRG neurons *in vivo*, BALB/c mice were injected directly into the sciatic nerve with HSV-CMV/EmGFP-miR-LacZ (n=10) or HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 (n=10). 7 days post-injection the DRG were isolated as described in section 2.6.8. Fluorescent microscopy for EmGFP confirmed high levels of delivery to all injected animals, mainly to the L4 DRG. Western blot analysis for p75, Lingo1 and NgR2 (antibodies described in section 2.17) was performed on protein extracted from the L4 DRG isolated from each group as described in sections 2.7.1-2.7.4 (figure 5.11). Quantification of band density using the Scion Image software revealed an average 85% reduction in the levels of p75, 40% reduction in the levels of Lingo1, but only 20% reduction in the levels of NgR2 compared to the negative control (figure 5.12). Protein levels were normalized against α-tubulin.

The experiment described in the previous section that investigated the efficiency at which the tandem pre-miRNAs are expressed and processed when inserted into a single HSV vector, revealed highly effective silencing of all target genes in 293T cells (section 5.5). However, HSV-mediated delivery of these pre-miRNAs expressed from a single transcript resulted in highly effective silencing of *p75*, modest silencing of *Lingo1* and poor silencing of *NgR2* in DRG neurons *in vivo*. In the DRG, p75, Lingo1 and NgR2 proteins have distinct though largely overlapping expression patterns. HSV-1 has been shown to transduce a heterologous population of DRG neurons following injection into the sciatic nerve (section 4.7; figure 4.8). Thus, although the potency of the pre-miRNA sequence against each target gene is important, the relative efficiency at which the virus transduces the specific subpopulations of DRG neurons expressing p75, Lingo1 and NgR2 may be a major limiting factor for silencing all three individual target genes in target tissue *in vivo*.

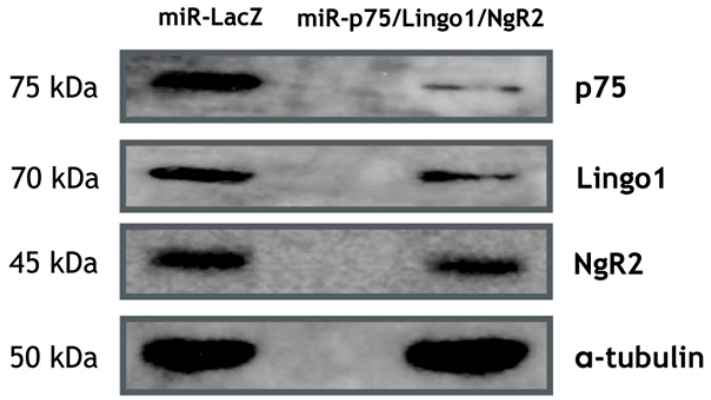


Figure 5.11 HSV-mediated silencing of p75, Lingo1 and NgR2 in DRG neurons *in vivo*

BALB/c mice were injected directly into the sciatic nerve with 5x10^6 pfu of either HSV-CMV/EmGFP-miR-LacZ (n=10) or HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 (n=10). Western blot analysis for p75, Lingo1 and NgR2 was performed on protein extracted from the L4 DRG isolated from each group at 7 days post-injection. The protein levels of *a*-tubulin remained essentially the same between the two groups, indicating that a similar amount of protein was loaded. The levels of p75 were dramatically reduced in animals injected with HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 compared to the animals injected with the negative control, whilst the levels of Lingo1 were reduced considerably less and the levels of NgR2 were reduced only slightly.

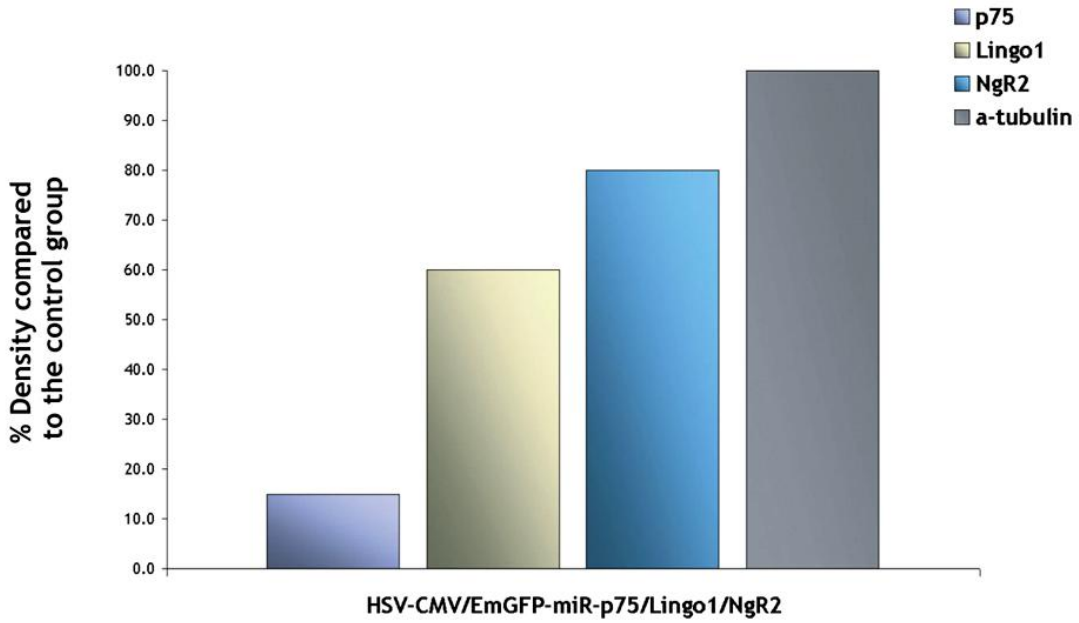


Figure 5.12 HSV-mediated silencing of p75, Lingo1 & NgR2 - quantification of protein knockdown

Following western blot analysis (shown in figure 5.11) on protein extracted from the DRG of injected animals (described in detail in section 5.6), the density of the bands was quantified using the Scion Image software. Quantification of band density revealed an average 85% reduction in the levels of p75, 40% reduction in the levels of Lingo1 and only 20% reduction in the levels of NgR2 compared to the negative control. Values were normalized against *a*-tubulin (loading control).

5.7 Labelling of sensory axons following sciatic nerve injection

Sensory axons enter the spinal cord from the dorsal root and terminate at distinct laminae in the spinal cord grey matter (figure 5.13). Different subtypes of sensory axons show prominent lamina-specific connectivity. Calcitonin gene-related peptide (CGRP)-positive and substance P (SP)-positive nociceptors, terminate in lamina I and the outer aspect of lamina II (IIo), isolectin B4 (IB4)-positive nociceptors terminate in the inner aspect of lamina II (IIi), and TrkC-positive proprioceptors terminate in Clark's column and the ventral horn. C-fibres are CGRP-positive, SP-positive, or bind to IB4 and thus terminate in laminae I and II.

To identify which types of sensory axons projecting into the spinal cord are most efficiently transduced by the virus, BALB/c mice were injected directly into the sciatic nerve with HSV-CMV/EmGFP-miR-LacZ (n=3). 3 days post-injection, the lumbar spinal cords were removed and sectioned (as described in section 2.6.6). Immunofluorescence for EmGFP (antibodies described in section 2.17) performed as described in section 2.7.6 revealed strong labelling of sensory axons terminating in laminae I and II of the dorsal horn (figure 5.14 and figure 5.15b). Thus, following injection into the sciatic nerve, the virus preferentially enters the peripheral axons of nociceptive afferents, which are mainly C-fibers, and gets retrogradely transported along the dorsal root to the DRG, where it expresses EmGFP together with pre-miRNA (figure 5.13). The EmGFP expressed in the cell bodies of these neurons diffuses along the axons to label laminae I and II of the dorsal horn, where their central axons terminate. Moreover, following sciatic nerve injection, the virus enters the axons of motor neurons and gets retrogradely transported along the ventral root resulting in highly efficient delivery to the cell bodies of motor neurons, which are located in the ventral horn of the spinal cord grey matter (figure 5.13), as shown in figure 5.15a.

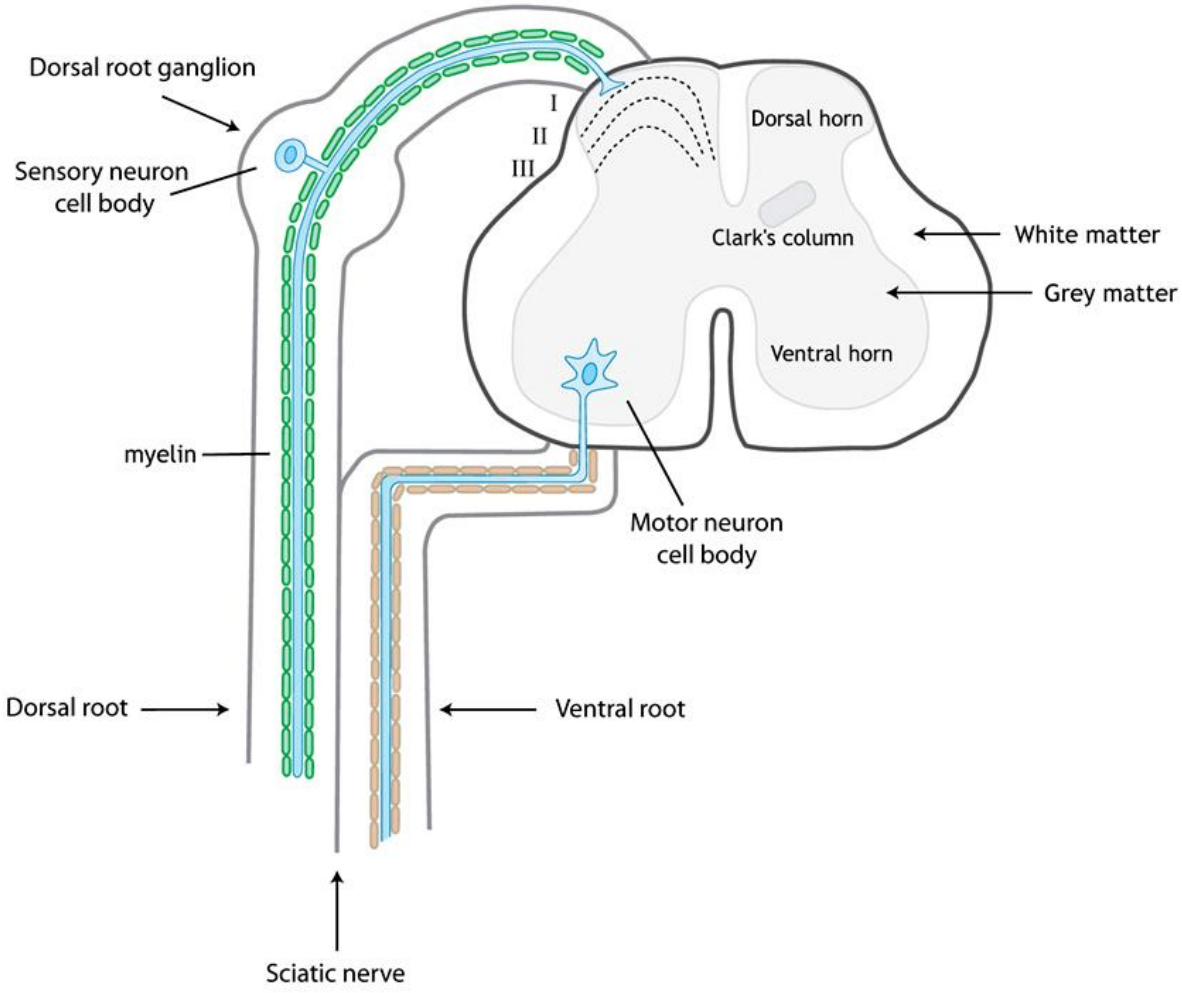


Figure 5.13 Schematic representation of a transverse section of spinal cord showing the grey and white matter, as well as the dorsal and ventral roots along which afferent and efferent fibres of the peripheral nervous system enter and exit the spinal cord, respectively. Sensory axons enter the spinal cord from the dorsal root and terminate at distinct laminas in the spinal cord grey matter. CGRP-positive and SP-positive nociceptors, which are mainly C-fibres, terminate in lamina I and the outer aspect of lamina II (IIo), isolectin B4-binding nociceptors terminate in the inner aspect of lamina II (IIi), and TrkC-positive proprioceptors terminate in Clark's column and the ventral horn.

Following injection into the sciatic nerve, the virus enters the peripheral axons of sensory neurons and the axons of motor neurons and gets retrogradely transported along the dorsal and ventral roots to their cell bodies in the DRG and ventral horn, respectively.

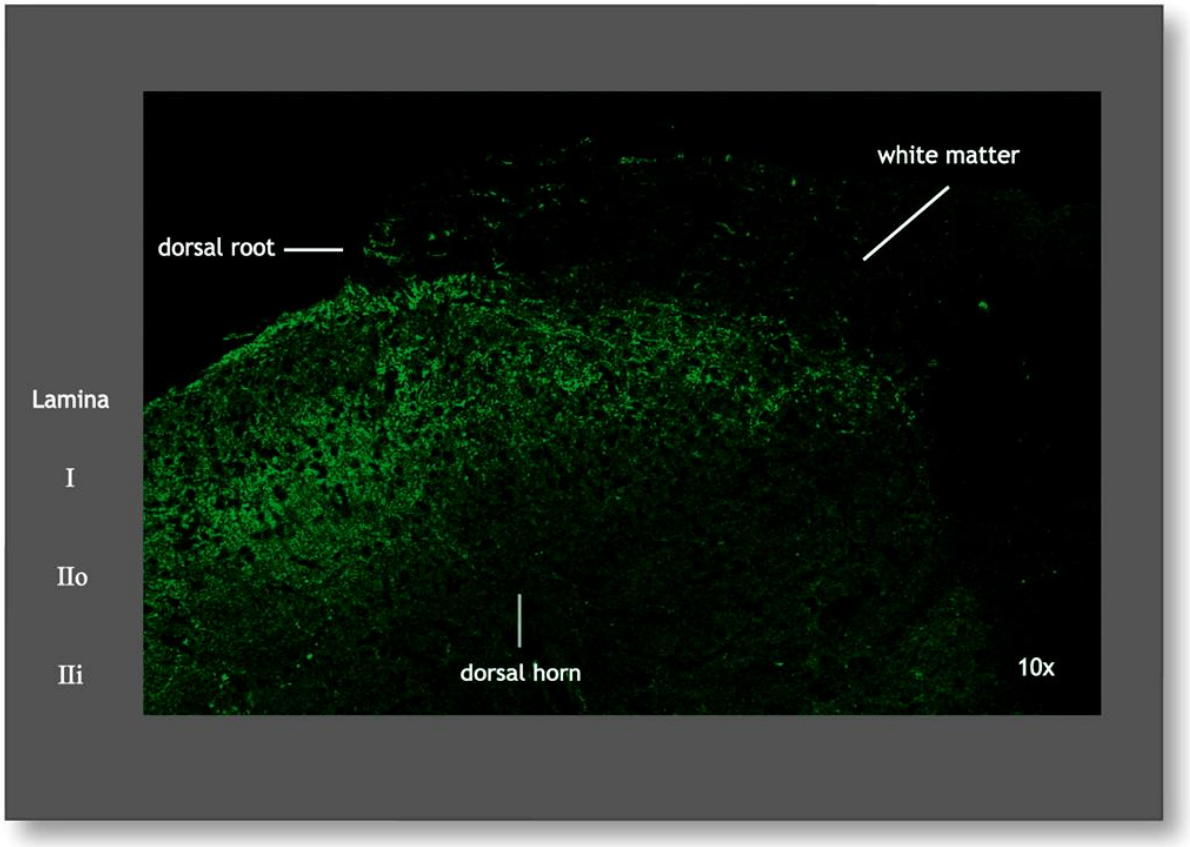


Figure 5.14 Labelling of sensory axons projecting into the dorsal horn of the spinal following injection of 5x10^6 pfu of HSV-CMV/EmGFP-miR-LacZ into the sciatic nerve of Balb/c mice. Immunocytochemistry for EmGFP preformed on transverse sections of lumbar spinal cord revealed that the virus transduces mainly C-fibers, whose central axons terminate in laminas I and II of the grey matter.

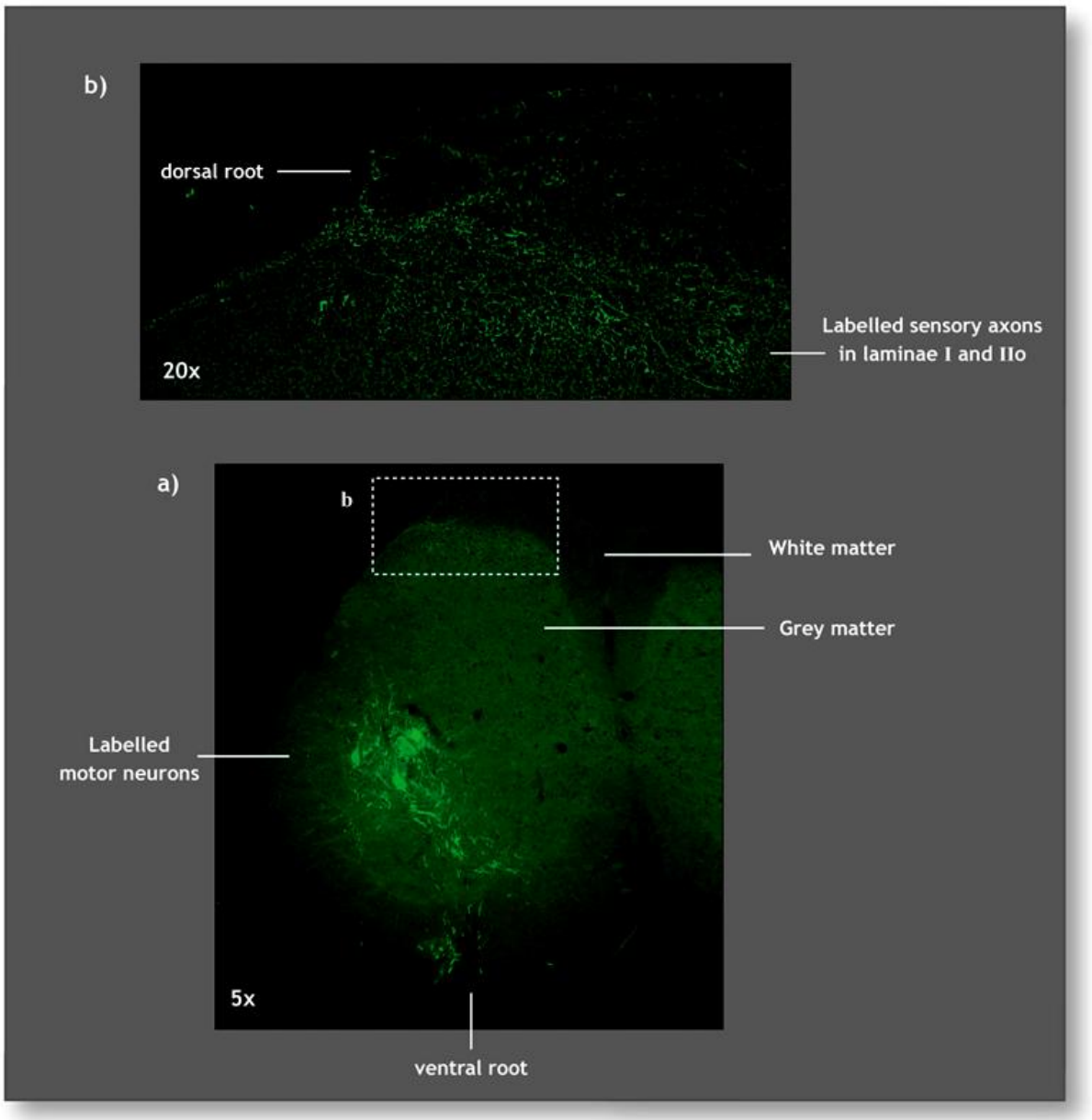


Figure 5.15 Labelling of sensory axons and motor neurons following injection of 5x10^6 pfu of HSV-CMV/EmGFP-miR-LacZ into the sciatic nerve of Balb/c mice. Immunocytochemistry for EmGFP preformed on transverse sections of lumbar spinal cord revealed that:

a) The virus enters the axons of motor neurons and gets retrogradely transported along the ventral root to the cell bodies in the ventral horn of the spinal cord. Boxed area b is shown at a higher magnification.

b) The virus enters the peripheral axons of nociceptive afferents, which are mainly C-fibers, and gets retrogradely transported to the DRG. The EmGFP expressed in the DRG diffuses along the central axons and labels laminae I and IIo of the dorsal horn, where these axons terminate.

5.8 Assessment of sensory axon regeneration into the spinal cord

The regenerative capacity of the central axons of DRG neurons is considerably weaker than their peripheral axons. Although sensory axons can regenerate through the dorsal root after dorsal root injury (rhizotomy), they are unable to cross the dorsal root entry zone (DREZ) to reconnect with their target neurons in the dorsal horn of the spinal cord. The DREZ, which is a distinct interface between peripheral and central nerve tissues, is permissive to the regeneration of adult sensory axons under normal circumstances, but becomes inhibitory after dorsal root axons have been injured (McPhail *et al.*, 2005). Following injury, the DREZ is believed to form a physical and chemical barrier to regenerating sensory axons by reactive astrocytes and factors produced by them, including various proteoglycans (Beggah *et al.*, 2005). Astrogliosis that begins within days after dorsal rhizotomy is followed by Wallerian degeneration, which does not begin until 1 week later and results in the breakdown and accumulation of inhibitory myelin (Li Liu, 1998, Ramer *et al.*, 2001). Thus, although rhizotomy is a suitable and simplified model to study axonal regeneration, there is no evidence to suggest that blocking myelin-associated proteins or their receptors is sufficient to overcome inhibition by the DREZ, which spatially and temporally precedes myelin inhibition. Moreover, even when the regenerative capacity of DRG axons is enhanced by a conditioning lesion of the sciatic nerve, vigorously regenerating axons usually fail to penetrate the DREZ (Chong *et al.*, 1999). The preliminary experiments performed in this section were aimed at investigating whether HSV-mediated silencing of *p75*, *Lingo1* and *NgR2* in combination to a conditioning lesion of the sciatic nerve are sufficient to promote regeneration of sensory axons across the DREZ, overcome myelin inhibition in the white matter and allow regenerating axons to reconnect with their targets in the dorsal horn.

BALB/c mice (n=6) received complete transection of the left L4 and L5 dorsal roots as described in section 2.6.4. 3 days after rhizotomy, these mice were injected directly into the sciatic nerve with either HSV-CMV/EmGFP-miR-LacZ (n=3) or HSV-CMV/EmGFP-

miR-p75/Lingo1/NgR2 (n=3) and also received a conditioning lesion of the sciatic nerve (as described in section 2.6.3). 4 weeks after rhizotomy, the lumbar spinal cords, dorsal roots and DRG were removed and sectioned. Immunofluorescence for EmGFP was performed to identify axons transduced by the virus. Figure 5.16 shows a representative longitudinal (horizontal) section from animals injected with HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2. The dorsal column (DC) and dorsal roots (DR) are shown, and dashed lines outline the borders of the DREZ and dorsal roots. Although many axons in the dorsal roots are strongly labelled, EmGFP could not be detected in the DRG (not shown), indicating that the virus has ceased expression in the cell bodies 4 weeks post-injection. Labelled axons in the dorsal root, which was accidentally folded, extend beyond the DREZ and into the dorsal horn of the spinal cord (figure 5.16a, indicated by arrows). This is most likely due to incomplete transection of the dorsal root. Strongly labelled sensory axons from injured dorsal roots regenerate through the dorsal root injury site and towards the DREZ. When they reach the DREZ, however, they exhibit typical behaviour associated with DREZ inhibition (figure 5.16b, indicated by arrows with stars). Axons inhibited by astrocytes form endbulb structures (Carlstedt, 1985) or are re-directed around the DREZ (Zhang *et al.*, 2001), and axons encountering repulsive cues at or near the DREZ are repelled and turn back into the dorsal roots (Reier *et al.*, 1983). Some axons, however, seem to penetrate the DREZ (figure 5.16b, indicated by arrows). This may be due to effective silencing of *p75*, *Lingo1* and *NgR2*, the conditioning lesion, incomplete transection of the dorsal root, or a combination of the above. Nevertheless, these axons fail to extend beyond the DREZ and into the dorsal horn (figure 5.16a).

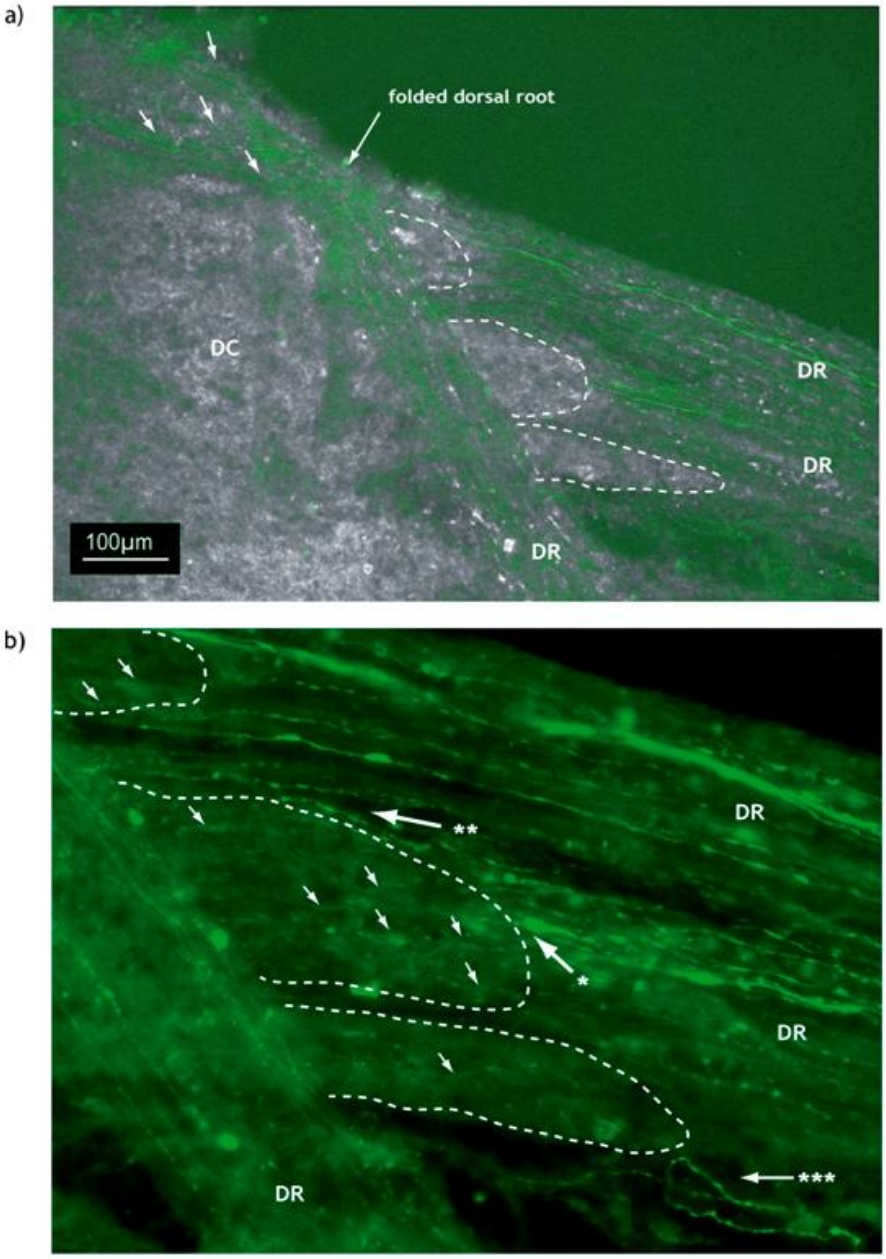


Figure 5.16 A representative longitudinal section of spinal cord isolated 4 weeks after rhizotomy from an animal injected with 5x10^6 pfu of HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 and stained for EmGFP is shown at a) 10x magnification and b) 20x magnification. The dorsal column (DC) and dorsal roots (DR) are indicated, and dashed lines outline the borders of the DREZ and dorsal roots.

a) The dorsal root that was accidentally folded over the borders of the DREZ and dorsal column is indicated. Axons from this dorsal root extend beyond the DREZ and into the dorsal horn of the spinal cord (indicated by arrows). No labelled injured axons seem to regenerate across the DREZ and into the dorsal horn.

b) Labelled axons that penetrate the DREZ are indicated by small arrows. Arrows with stars indicate axons inhibited by the DREZ: (*) axons form endbulbs, (**) axons are re-directed around the DREZ, or (***) axons are repelled and turn back into the dorsal root.

5.9 Discussion

The weak regenerative capacity of the injured adult mammalian CNS is partially attributed to various inhibitory components present in CNS myelin. As discussed in the introduction of this chapter, studies with transgenic mice have revealed that blocking the expression of individual myelin-associated inhibitors and receptors has limited effects on axonal regeneration *in vivo*. Moreover, different types of CNS neurons possess very different regenerative capacities and respond differently to experimental treatment strategies *in vivo*. Thus, depending on the fibre tract injured, different combinations of myelin inhibitors may need to be targeted in order to promote meaningful regeneration. The miR-like disabled HSV-1 vector developed in this thesis has the potential to silence multiple genes simultaneously through expression of multiple miRNA cassettes from a single transcript and can be engineered to theoretically target any combination of genes appropriate to the fibre tract injured. This was clearly demonstrated *in vitro* where very high level vector-mediated silencing of three genes in parallel was demonstrated. Furthermore, the use of transgenic animals with germline mutations, which often lead to compensatory processes and/or developmental defects, has been a major drawback in the field of regeneration. HSV-mediated delivery of RNAi to validate genes believed to be involved in the failure of axonal regeneration offers control over the duration of knockdown and may overcome these limitations.

In addition to numerous inhibitory molecules present in the environment of injured axons, the failure of axonal regeneration is also attributed to the poor intrinsic capacity of CNS axons to regenerate following injury. Although many experimental approaches have been developed to overcome these obstacles individually, there is now a general consensus that combinatorial approaches will be needed to elicit sustained and effective regenerative responses. The miR-like disabled HSV-1 vector developed in this thesis allows expression of a biologically active protein, in place of EmGFP, together with the pre-miRNA. Thus, it may be possible, in combination to silencing multiple inhibitors of axonal

regeneration, to overexpress developmental axonal guidance molecules in an attempt to boost the intrinsic capacity of CNS neurons to regenerate. Although such an approach was not investigated in this thesis, it represents a highly attractive strategy to promote axonal regeneration following SCI injury. In the present study, a conditioning lesion of the sciatic nerve was performed in order to increase cAMP levels and enhance the regenerative capacity of the central axons of DRG neurons. In studies demonstrating regeneration of DRG axons into the spinal cord, cAMP was administered to DRG either 2 days (Neumann *et al.*, 2002) or 7 days (Qiu *et al.*, 2002) before the spinal cord injury was induced. The conditioning lesion in the experiments described in this chapter was performed 3 days following injury. Within the context of translating any positive results into a clinically relevant strategy for spinal cord repair, the beneficial effects of manipulating cAMP levels would have to be demonstrated after injury. However, there is no evidence to suggest that a conditioning lesion performed after injury has the same positive effect in promoting regeneration as when it is performed before injury.

In the present study, a disabled HSV-1 vector was engineered to silence *p75*, *Lingo1* and *NgR2* in an attempt to target distinct and potentially compensatory pathways of myelin inhibition. Prior to investigating the potential of this vector to promote regeneration of sensory axons into the spinal cord, its ability to simultaneously silence these genes in DRG neurons *in vivo* was investigated. Western blot analysis revealed highly effective silencing of *p75*, modest silencing of *Lingo1* and inefficient silencing of *NgR2*, when the virus was injected into the sciatic nerve. This result raised numerous concerns. The 85% silencing of *p75* is not predicted on its own to be sufficient in blocking myelin inhibition through NgR1, as TROY or other unidentified co-receptors are likely to substitute for p75. The 40% silencing of *Lingo1* on its own or in combination to the silencing of *p75* may be sufficient to block inhibition by Nogo-A and OMgp in a subset of neurons. However, NgR2 that is only silenced by 20% may substitute for NgR1 to mediate MAG-specific inhibitory effects. Nogo-A has been shown to be redundant for outgrowth

inhibition in sensory neurons and thus, blocking inhibition by MAG may be essential to promote regeneration of sensory axons (Oudega *et al.*, 2000). Nevertheless, the majority of neurons expressing p75 have been shown to also express NgR2 and Lingo1 (Venkatesh *et al.*, 2005). Thus, it cannot be excluded that in a subset of DRG neurons transduced by the vector, all target genes may be sufficiently silenced. This remains to be established using immunocytochemistry on DRG sections taken from animals injected with the vector, rather than western blot that measures total protein levels in the whole DRG. Thus while proof of principle that the expression of multiple genes can be simultaneously repressed in DRGs *in vivo* was achieved, further work, outside of the scope of this thesis, is required.

Immunocytochemistry for EmGFP, which is expressed co-cistronically with the pre-miRNA, demonstrated axonal labelling of nociceptive neurons, when the disabled virus expressing pre-miR-LacZ was injected into the sciatic nerve. This is in agreement with the results obtained in the previous chapter, which demonstrated that the virus expressing pre-miR-neg transduces a heterologous population of DRG neurons, including an estimated 80% of TrpV1-positive neurons, which are mainly C-fibers (section 4.7). Approximately half of all sensory neurons of different sizes express high levels of p75 (Schecterson & Bothwell, 1992, Wright & Snider, 1995). Moreover, NgR2 has been shown to be expressed in a heterologous population of DRG neurons, the majority of which also express p75 and Lingo1 (Venkatesh *et al.*, 2005). Thus, the 20% silencing of NgR2 in DRG neurons *in vivo* cannot be entirely attributed to the efficiency at which NgR2-positive neurons are transduced by the virus. It is possible that effective silencing of *p75* may have triggered a compensatory response resulting in upregulation of the levels of NgR2. Moreover, it cannot be excluded that processing of tandem pre-miRNAs into functional miRNAs may not be as efficient in neurons as in 293T cells. This could be investigated by transducing primary DRG neurons and assessing expression of pre-miRNAs or miRNAs against each target using northern blots, or assessing silencing of target genes when the pre-miRNAs are chained in a different sequence.

Despite the incomplete silencing observed by western blot analysis in DRG *in vivo*, the disabled vector targeting *p75*, *Lingo1* and *NgR2* was in any case evaluated for its ability to promote regeneration of sensory axons into the spinal cord following dorsal rhizotomy in combination with a conditioning lesion of the sciatic nerve. This was thought worthwhile not least because a subset of cells may well have had all three target genes effectively silenced, which it was not possible to determine by western blot. Dorsal rhizotomy disconnects primary sensory neurons from their spinal targets and DRG neurons fail to regenerate their central axons across the DREZ, often even when their regenerative capacity is enhanced by a conditional lesion of the sciatic nerve. Clinically, lack of primary afferent regeneration across the DREZ results in loss of sensation and the development of severe pain. Numerous strategies have been employed to promote regeneration of sensory axons across the DREZ, including viral vector-mediated delivery of polysialic acid, which acts as a permissive agent for cell migration (Zhang *et al.*, 2007), viral vector-mediated delivery or intrathecal infusion of neurotrophins (Ramer *et al.*, 2002, Zhang *et al.*, 1998), transplantation of olfactory ensheathing cells (Li *et al.*, 2004b), and degradation of proteoglycans (Steinmetz *et al.*, 2005). These strategies have produced some level of regeneration across the DREZ. However, both astrocytic and myelin-derived inhibitors are present following dorsal rhizotomy. Regenerating DRG axons first come into contact with the DREZ and then with degenerating myelin. Whilst axons treated with NT-3 have been shown to cross the DREZ (Ramer *et al.*, 2002, Zhang *et al.*, 1998), following the onset of myelin degeneration, they fail to elongate and remain within the CNS compartment of the dorsal root. Although, degenerating myelin represents a substantial barrier, blocking myelin-associated inhibitors or their receptors to promote regeneration of sensory axons into the spinal cord had not been previously investigated.

Following injection of the HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 vector into the sciatic nerve of mice that had received complete transection of the dorsal roots, the majority of labelled axons either stopped at the DREZ or were re-directed around the

DREZ, whilst some axons were repelled back into the dorsal root. A few labelled axons seemed to have penetrated the DREZ, but failed to regenerate into the dorsal horn. Thus, despite a conditioning lesion of the sciatic nerve and possibly some degree of resistance to myelin inhibition achieved by the vector, axons failed to regenerate across the DREZ and into the dorsal horn of the spinal cord. There is no evidence to suggest that overcoming myelin inhibition is sufficient to overcome inhibition by the DREZ, which spatially and temporally precedes myelin inhibition. Thus, it is not possible to formally conclude that the levels of p75, Lingo1 and NgR2 were sufficiently reduced by vector-mediated RNAi to confer resistance of injured neurons to myelin inhibition, or that the failure of regeneration across the DREZ was due to insufficiently effective silencing of *p75*, *Lingo1* and *NgR2*. Further work may contemplate HSV-mediated delivery of NT3 or a cocktail of neurotrophic factors to the spinal cord, as has otherwise been previously achieved in our laboratory (unpublished), to attempt to overcome inhibition by the DREZ prior to silencing *p75*, *Lingo1* and *NgR2*. Moreover, the conditioning lesion may have been more effective when received prior to dorsal rhizotomy. Finally, dorsal rhizotomy may not be a suitable model to investigate the potential of the HSV-CMV/EmGFP-miR-p75/Lingo1/NgR2 vector to promote axonal regeneration following injury. Disabled HSV-1 vectors have been shown to efficiently transduce corticospinal neurons following direct injection into the spinal cord (Groutsi *et al.*, 2008). Future experiments may therefore be directed at investigating the potential of this vector to promote regeneration of descending fibres following SCI. NgR2 and Lingo1 are strongly expressed in several neuronal populations of the adult mouse brain projecting into the spinal cord, including corticospinal, rubrospinal, reticulospinal, and raphespinal tracts. Expression of p75 is restricted to neuronal descending pathways from the brainstem that also express NgR2 and Lingo1 (Barrette *et al.*, 2007). Thus, silencing *p75*, *Lingo1* and *NgR2* in these fibre systems may be a promising strategy to promote axonal regeneration following SCI, indeed potentially more so than in the model preliminary tested here.

In conclusion, while multiple genes can be clearly silenced simultaneously *in vitro* and *in vivo* using the vectors developed, *in vivo* the situation is more complex and likely dependent upon the genes targeted, their expression patterns as compared to the cells which are transduced by the vector, and the largely unknown functional consequences which may result from silencing. The *in vitro* experiments performed in this chapter provide the first demonstration of silencing of multiple genes using HSV-mediated RNAi, and indeed while complete silencing was not achieved for all genes in parallel *in vivo*, even partial silencing of multiple genes in DRGs has not been previously achieved. Unfortunately, however, due to severe time constraints, the preliminary regeneration experiments presented here could not be further expanded such that conclusive phenotypic effects were not observed and thus, the potential of miR-based HSV-1 vectors for unequivocally promoting regeneration remained unproven. This could of course be a consequence of the combination of gene targets chosen as, as yet, it is not known knockdown of which if any gene combination may result in a regenerative effect. It remains to be established, therefore, whether simultaneous silencing of *p75*, *Lingo1* and *NgR2* is sufficient to promote axonal regeneration following PNS injury, future experiments in models of CNS injury also being justified.

Another important question that remains to be answered is whether and to what extent the RNAi machinery remains functional in axotomised neurons. Protein synthesis in neurons occurs in the cell body, as well as in axons and nerve terminals (Brittis *et al.*, 2002, Zheng *et al.*, 2001). The axons of adult and developing DRG neurons have been shown to contain components of RISCs/miRNPs, including Argonaute proteins, which assemble and function independently of the cell body (Hengst *et al.*, 2006, Murashov *et al.*, 2007). The mechanism by which miRNAs, which are expressed in the cell body, are transported along the axons to regulate the expression of axonally localised transcripts remains unknown. Thus, disruption of the RNAi machinery in axons or shRNA/miRNA transport along axons, following axotomy, may prevent effective silencing of axonally

localised mRNAs, such as RhoA (Wu *et al.*, 2005). Considerable further work is therefore clearly required in the field of using miRNAs to suppress gene expression in neurons, particularly of proteins which are usually present in axons or axonal terminals, the work in this chapter providing a potential starting point from which such work using HSV vectors might proceed.

CHAPTER 6

GENERAL DISCUSSION

RNA interference is a fascinating cellular gene regulation machinery that has rapidly emerged as a powerful experimental and target validation tool. Moreover, RNAi holds considerable promise as a therapeutic strategy to silence disease-causing genes, particularly those that encode non-druggable targets, i.e. those which are not amenable to conventional therapeutics such as small molecules, proteins, or monoclonal antibodies. Despite great progress being made in elucidating the cellular processes involved in the RNAi/miRNA pathway, delivery and specificity have remained the main limitations for the effective use of RNAi *in vivo*. Advances in our understanding of esiRNA and miRNA biogenesis and functions are expected to result in the improved design and specificity of silencing triggers. In recent years, considerable effort has also been focused on developing methods to improve delivery of RNAi to target tissues *in vivo*, which is the main focus of this thesis.

This thesis aimed to develop an efficient method for delivery of RNAi to specific target tissues, sensory neurons, *in vivo*. Delivery to sensory neurons has been problematic using non-viral systems, and had not been feasible using the vector systems previously tested. It was anticipated that efficient expression of shRNA or artificial miRNA in sensory neurons would improve the understanding of sensory neuronal pathways, allow the generation of transgenic knockdown animals, enable the validation of novel gene targets in drug discovery, and may assist in the development of RNAi-based therapeutics for peripheral neuropathies, such as chronic pain. The results obtained during the course of this thesis, recently published in Nucleic Acids Research (Anesti et al., 2008), demonstrate that the novel replication-defective HSV-1 vectors developed in the thesis can express shRNA, artificial miRNA and multiple miRNAs in tandem, and induce effective and specific silencing of targeted genes in dividing cells in culture, in primary neurons *in vitro* and DRG neurons *in vivo*.

Chapter 3 of this thesis evaluated various approaches to RNAi expression from within the HSV genome by utilising regulatory elements derived from the LAT region.

Optimisation of RNAi strategies remains essential due to potential side effects caused by siRNAs or shRNAs competing with the endogenous miRNA machinery, stimulating cellular responses to dsRNA, or silencing unintended target mRNAs due to partial complementarity. These side effects can result in severe cellular toxicity and even death. Improved gene silencing strategies would be expected to allow the use of lower doses of the silencing trigger, thus reducing the likelihood of RNAi-induced side effects. With the exception of the modified CMV promoter, which failed to induce any silencing for reasons that were not elucidated, the RNAi expression systems evaluated in this thesis each demonstrated similar levels of highly effective silencing in dividing cells in culture. However, the miRNA-based system was found to clearly be the most effective at providing silencing in DRG neurons *in vivo*. At the time the disabled HSV-1 vectors described in this thesis were developed, it was unclear in the field as to whether expression of artificial miRNA was superior to expressing shRNA, and so both were tested. Recent publications have clearly demonstrated that expression of shRNA embedded into endogenous miRNA sequences does not saturate the endogenous miRNA pathway and prevents induction of the INF response. Thus, miRNA-based vectors are currently considered more suitable than all other available systems for gene silencing in the mammalian nervous system. The miRNA-based vector developed in this thesis represents the first and so far only demonstration of HSV-mediated silencing by expression of artificial miRNA and currently is the only available method allowing expression of artificial miRNA in sensory neurons *in vivo*.

Chapter 4 of this thesis provided proof of concept that an endogenous gene can be effectively and specifically silenced in DRG using the vectors developed. Assessment of both protein and mRNA levels revealed that HSV-mediated expression of shRNA or artificial miRNA reduces the levels of the neuronally expressed TrpV1 gene by half, and thus demonstrated the potential of this technology as a gene target validation tool for genes involved in nociception. Indeed, the vector expressing miRNA against *trpV1* is currently under evaluation by a number of biotechnology and pharmaceutical companies with a view

to utilizing this technology as a routine gene target validation tool in the development of novel analgesics.

Chapter 5 of this thesis demonstrated that it is possible to simultaneously silence multiple gene targets through expression of multiple artificial miRNAs in tandem using the miRNA-based vector system developed in this thesis. Whilst expression of multiple miRNAs in culture resulted in highly effective silencing of each targeted gene, silencing in DRG neurons *in vivo* was found to decrease in potency the further away the miRNA in question was inserted from the promoter. The reasons behind this result were not clear. Furthermore, it remains to be determined whether simultaneous silencing of *p75*, *Lingo1* and *NgR2*, the genes targeted, is sufficient to promote regeneration of sensory axons into the spinal cord following rhizotomy. Nevertheless, the experiments performed in this thesis provide the first demonstration of silencing of multiple genes using HSV, and the first example of silencing multiple genes in peripheral neurons *in vivo*, even though this silencing was not complete. Although the potential of the vector expressing artificial miRNA against *p75*, *Lingo1* and *NgR2* to promote axonal regeneration following PNS injury was not fully investigated, this vector is under evaluation by others at UCL for its ability to promote regeneration of corticospinal fibres following SCI.

Silencing with the disabled HSV vectors developed in this thesis was sustained for at least 1 week in DRG. Furthermore, EmGFP could be detected in the axons and nerve terminals of transduced DRG neurons at 4 weeks post-injection of the 1764 4-/27+/RL1+ pR19CMV/EmGFP-miR-p75/Lingo1/NgR2 vector. However, expression of miRNA in the cell bodies of these neurons, assessed by expression of EmGFP in the DRG, was reduced to below detectable levels by this time. Thus, this vector is potentially ideal for targeting inhibitors of axonal regeneration, as induction of RNAi is only required for sufficiently long periods of time to promote regeneration, and expression of these genes should be eventually allowed to return to normal levels. Furthermore, although complete silencing of targeted genes was not achieved, complete silencing of targeted genes is neither

necessary nor desirable for many applications. TrpV1, for example, plays an important role in nociception and thus, complete silencing of this gene may result in undesirable side effects. Furthermore, a reduction in TrpV1 expression by half is predicted to be sufficient in alleviating pain. This is currently being assessed by a pharmaceutical company who are performing behavioural studies in animal models of pain.

Finally, although this thesis has focused on delivery of RNAi to peripheral neurons, it has formed the basis of a project funded by the Michael J. Fox Foundation (which the thesis author is conducting and which is currently underway) to investigate the potential of the miRNA-based system developed to simultaneously silence α-synuclein and Lingo-1 in the brain of Parkinson's disease animal models. A vector produced on this project has already demonstrated highly efficient silencing of both α-synuclein and Lingo-1 in 293T cells transfected with plasmids expressing each target gene. Furthermore, preliminary experiments in primary ventral midbrain neurons indicate that silencing of α-synuclein and Lingo-1 is sufficient to protect neurons against toxicity caused by MTPT treatment. This result has also provided evidence that processing of tandem pre-miRNAs into functional miRNAs can be efficiently achieved in neurons. Evaluation of silencing in the brain, following injection of the virus into the striatum and retrograde transport to the substantia nigra, is currently underway. These results will demonstrate the general utility of the technology developed during the course of the thesis and particularly that knockdown of genes in central as well as peripheral neurons *in vivo* is feasible using a replication-defective HSV-1 vector.

REFERENCES

Abbas-Terki, T., Blanco-Bose, W., Dã©Glon, N., Pralong, W. & Aebischer, P. (2002) Lentiviral-mediated rna interference. *Human Gene Therapy,* 13(18), 2197-2201.

Ace, C. I., Dalrymple, M. A., Ramsay, F. H., Preston, V. G. & Preston, C. M. (1988) Mutational analysis of the herpes simplex virus type 1 trans-inducing factor vmw65. *J Gen Virol,* 69(10), 2595-2605.

Ace, C. I., Mckee, T. A., Ryan, J. M., Cameron, J. M. & Preston, C. M. (1989) Construction and characterization of a herpes simplex virus type 1 mutant unable to transinduce immediate-early gene expression. *J. Virol.,* 63(5), 2260-2269.

Adenot, X., Elmayan, T., Lauressergues, D., Boutet, S., Bouché, N., Gasciolli, V. & Vaucheret, H. (2006) Drb4-dependent tas3 trans-acting sirnas control leaf morphology through ago7. 16(9), 927-932.

Advani, S. J., Weichselbaum, R. R. & Roizman, B. (2001) Cdc2 cyclin-dependent kinase binds and phosphorylates herpes simplex virus 1 ul42 DNA synthesis processivity factor. *J. Virol.,* 75(21), 10326-10333.

Advani, S. J., Weichselbaum, R. R. & Roizman, B. (2003) Herpes simplex virus 1 activates cdc2 to recruit topoisomerase iiî± for post-DNA synthesis expression of late genes. *Proceedings of the National Academy of Sciences of the United States of America,* 100(8), 4825-4830.

Ahmed, Z., Dent, R. G., Suggate, E. L., Barrett, L. B., Seabright, R. J., Berry, M. & Logan, A. (2005) Disinhibition of neurotrophin-induced dorsal root ganglion cell neurite outgrowth on cns myelin by sirna-mediated knockdown of ngr, p75ntr and rho-a. *Molecular and Cellular Neuroscience,* 28(3), 509-523.

Akli, S., Caillaud, C., Vigne, E., Stratford-Perricaudet, L. D., Poenaru, L., Perricaudet, M., Kahn, A. & Peschanski, M. R. (1993) Transfer of a foreign gene into the brain using adenovirus vectors. *Nat Genet,* 3(3), 224-228.

Allen, E., Xie, Z., Gustafson, A. M. & Carrington, J. C. (2005) Microrna-directed phasing during trans-acting sirna biogenesis in plants. *Cell,* 121(2), 207-221.

Alvarez, V. A., Ridenour, D. A. & Sabatini, B. L. (2006) Retraction of synapses and dendritic spines induced by off-target effects of rna interference. *J. Neurosci.,* 26(30), 7820-7825.

Amarzguioui, M., Holen, T., Babaie, E. & Prydz, H. (2003) Tolerance for mutations and chemical modifications in a sirna. *Nucl. Acids Res.,* 31(2), 589-595.

Ambros, V., Lee, R. C., Lavanway, A., Williams, P. T. & Jewell, D. (2003) Micrornas and other tiny endogenous rnas in c. Elegans. *Current Biology,* 13(10), 807-818.

Amelio, A. L., Giordani, N. V., Kubat, N. J., O'neil, J. E. & Bloom, D. C. (2006a) Deacetylation of the herpes simplex virus type 1 latency-associated transcript (lat) enhancer and a decrease in lat abundance precede an increase in icp0 transcriptional permissiveness at early times postexplant. *J. Virol.,* 80(4), 2063-2068.

Amelio, A. L., Mcanany, P. K. & Bloom, D. C. (2006b) A chromatin insulator-like element in the herpes simplex virus type 1 latency-associated transcript region binds ccctc-binding factor and displays enhancer-blocking and silencing activities. *J. Virol.,* 80(5), 2358-2368.

An, D. S., Xie, Y., Mao, S. H., Morizono, K., Kung, S. K. P. & Chen, I. S. Y. (2003) Efficient lentiviral vectors for short hairpin rna delivery into human cells. *Human Gene Therapy,* 14(12), 1207-1212.

Anesti, A.-M., Peeters, P. J., Royaux, I. & Coffin, R. S. (2008) Efficient delivery of rna interference to peripheral neurons in vivo using herpes simplex virus. *Nucl. Acids Res.,* 36(14), e86-.

Aravin, A., Gaidatzis, D., Pfeffer, S. B., Lagos-Quintana, M., Landgraf, P., Iovino, N., Morris, P., Brownstein, M. J., Kuramochi-Miyagawa, S., Nakano, T., Chien, M., Russo, J. J., Ju, J., Sheridan, R., Sander, C., Zavolan, M. & Tuschl, T. (2006) A novel class of small rnas bind to mili protein in mouse testes. *Nature,* 442(7099), 203-207.

Aravin, A. A., Lagos-Quintana, M., Yalcin, A., Zavolan, M., Marks, D., Snyder, B., Gaasterland, T., Meyer, J. & Tuschl, T. (2003) The small rna profile during drosophila melanogaster development. *Developmental Cell,* 5(2), 337-350.

Aravin, A. A., Sachidanandam, R., Girard, A., Fejes-Toth, K. & Hannon, G. J. (2007) Developmentally regulated pirna clusters implicate mili in transposon control. *Science,* 316(5825), 744-747.

Arthur, J., Everett, R., Brierley, I. & Efstathiou, S. (1998) Disruption of the 5' and 3' splice sites flanking the major latency- associated transcripts of herpes simplex virus type 1: Evidence for alternate splicing in lytic and latent infections. *J Gen Virol,* 79(1), 107-116.

Bagga, S., Bracht, J., Hunter, S., Massirer, K., Holtz, J., Eachus, R. & Pasquinelli, A. E. (2005) Regulation by let-7 and lin-4 mirnas results in target mrna degradation. *Cell,* 122(4), 553-563.

Bajocchi, G., Feldman, S. H., Crystal, R. G. & Mastrangeli, A. (1993) Direct in vivo gene transfer to ependymal cells in the central nervous system using recombinant adenovirus vectors. *Nat Genet,* 3(3), 229-234.

Balague, C., Kalla, M. & Zhang, W. (1997) Adeno-associated virus rep78 protein and terminal repeats enhance integration of DNA sequences into the cellular genome. *J. Virol.,* 71(4), 3299-3306.

Banfield, B., Leduc, Y., Esford, L., Schubert, K. & Tufaro, F. (1995) Sequential isolation of proteoglycan synthesis mutants by using herpes simplex virus as a selective agent: Evidence for a proteoglycan- independent virus entry pathway. *J. Virol.,* 69(6), 3290-3298.

Bao, N., Lye, K.-W. & Barton, M. K. (2004) Microrna binding sites in arabidopsis class iii hd-zip mrnas are required for methylation of the template chromosome. *Developmental Cell,* 7(5), 653-662.

Barrette, B., Vallières, N., Dubé, M. & Lacroix, S. (2007) Expression profile of receptors for myelin-associated inhibitors of axonal regeneration in the intact and injured mouse central nervous system. *Molecular and Cellular Neuroscience,* 34(4), 519-538.

Barritt, A. W., Davies, M., Marchand, F., Hartley, R., Grist, J., Yip, P., Mcmahon, S. B. & Bradbury, E. J. (2006) Chondroitinase abc promotes sprouting of intact and injured spinal systems after spinal cord injury. *J. Neurosci.,* 26(42), 10856-10867.

Bartel, D. P. & Chen, C.-Z. (2004) Micromanagers of gene expression: The potentially widespread influence of metazoan microrna. *Nat Rev Genet,* 5(5), 396-400.

Bartlett, D. W. & Davis, M. E. (2006) Insights into the kinetics of sirna-mediated gene silencing from live-cell and live-animal bioluminescent imaging. *Nucl. Acids Res.,* 34(1), 322-333.

Bartsch, U., Bandtlow, C., Schnell, L., Bartsch, S., Spillmann, A., Rubin, B., Hillenbrand, R., Montag, D., Schwab, M. & Schachner, M. (1995) Lack of evidence that myelin-associated glycoprotein is a major inhibitor of axonal regeneration in the cns. *Neuron,* 15(6), 1375-81.

Baskerville, S. & Bartel, D. (2005) Microarray profiling of microrna reveals frequent coexpression with neighboring mirnas and host genes. *RNA,* 11(3), 241-247.

Batchelor, A. H. & O'hare, P. (1992) Localization of cis-acting sequence requirements in the promoter of the latency-associated transcript of herpes simplex virus type 1 required for cell-type-specific activity. *J. Virol.,* 66(6), 3573-3582.

Bauer, M., Kinkl, N., Meixner, A., Kremmer, E., Riemenschneider, M., Forstl, H., Gasser, T. & Ueffing, M. (2008) Prevention of interferon-stimulated gene expression using microrna-designed hairpins. *Gene Ther,* 16(1), 142-147.

Befort, K., Laurie Karchewski, Christopher Lanoue & Woolf, C. J. (2003) Selective up-regulation of the growth arrest DNA damage-inducible gene gadd45 alpha in sensory and motor neurons after peripheral nerve injury. *European Journal of Neuroscience,* 18(4), 911-922.

Beggah, A. T., Dours-Zimmermann, M. T., Barras, F. M., Brosius, A., Zimmermann, D. R. & Zurn, A. D. (2005) Lesion-induced differential expression and cell association of neurocan, brevican, versican v1 and v2 in the mouse dorsal root entry zone. *Neuroscience,* 133(3), 749-762.

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P. & Izaurralde, E. (2006) Mrna degradation by mirnas and gw182 requires both ccr4:Not deadenylase and dcp1:Dcp2 decapping complexes. *Genes & Development,* 20(14), 1885-1898.

Bender, F. C., Whitbeck, J. C., Lou, H., Cohen, G. H. & Eisenberg, R. J. (2005) Herpes simplex virus glycoprotein b binds to cell surfaces independently of heparan sulfate and blocks virus entry. *J. Virol.,* 79(18), 11588-11597.

Bender, F. C., Whitbeck, J. C., Ponce De Leon, M., Lou, H., Eisenberg, R. J. & Cohen, G. H. (2003) Specific association of glycoprotein b with lipid rafts during herpes simplex virus entry. *J. Virol.,* 77(17), 9542-9552.

Bennett, G. & Xie, Y. (1988) A peripheral mononeuropathy in rat that produces disorders of pain sensation like those seen in man. *Pain,* 33(1), 87-107.

Bernard, P. & Couturier, M. (1992) Cell killing by the f plasmid ccdb protein involves poisoning of DNA-topoisomerase ii complexes. *J Mol Biol,* 226(3), 735-45.

Berns, K., Hijmans, E. M., Mullenders, J., Brummelkamp, T. R., Velds, A., Heimerikx, M., Kerkhoven, R. M., Madiredjo, M., Nijkamp, W., Weigelt, B., Agami, R., Ge, W., Cavet, G., Linsley, P. S., Beijersbergen, R. L. & Bernards, R. (2004) A large-scale rnai screen in human cells identifies new components of the p53 pathway. *Nature,* 428(6981), 431-437.

Bernstein, E., Caudy, A. A., Hammond, S. M. & Hannon, G. J. (2001) Role for a bidentate ribonuclease in the initiation step of rna interference. *Nature,* 409(6818), 363-366.

Berry, M., Maxwell, W., Logan, A., Mathewson, A., Mcconnell, P., Ashhurst, D. & Thomas, G. (1983) Deposition of scar tissue in the central nervous system. *Acta Neurochir Suppl (Wien),* 32(31-53.

Bertrand, J., Pottier, M., Vekris, A., Opolon, P., Maksimenko, A. & Claude, M. (2002) Comparison of antisense oligonucleotides and sirnas in cell culture and in vivo. *Biochem. Biophys. Res. Commun.,* 296(4), 1000-4.

Bhattacharyya, S. N., Habermacher, R., Martine, U., Closs, E. I. & Filipowicz, W. (2006) Relief of microrna-mediated translational repression in human cells subjected to stress. *Cell,* 125(6), 1111-1124.

Birmingham, A., Anderson, E. M., Reynolds, A., Ilsley-Tyree, D., Leake, D., Fedorov, Y., Baskerville, S., Maksimova, E., Robinson, K., Karpilow, J., Marshall, W. S. & Khvorova, A. (2006) 3[prime] utr seed matches, but not overall identity, are associated with rnai off-targets. *Nat Meth,* 3(3), 199-204.

Blidner, R., Robert P. Hammer, Mandi J. Lopez, Sandra O. Robinson, W. Todd Monroe, (2007) Fully 2'-deoxy-2'-fluoro substituted nucleic acids induce rna interference in mammalian cell culture. *Chemical Biology & Drug Design,* 70(2), 113-122.

Blomer, U., Naldini, L., Kafri, T., Trono, D., Verma, I. & Gage, F. (1997) Highly efficient and sustained gene transfer in adult neurons with a lentivirus vector. *J. Virol.,* 71(9), 6641-6649.

Bloom, D., Hill, J., Devi-Rao, G., Wagner, E., Feldman, L. & Stevens, J. (1996) A 348-base-pair region in the latency-associated transcript facilitates herpes simplex virus type 1 reactivation. *J. Virol.,* 70(4), 2449-2459.

Boden, D., Pusch, O., Silbermann, R., Lee, F., Tucker, L. & Ramratnam, B. (2004) Enhanced gene silencing of hiv-1 specific sirna using microrna designed hairpins. *Nucl. Acids Res.,* 32(3), 1154-1158.

Boeshore, K., Rebecca C. Schreiber, Stacey A. Vaccariello, Hilary Hyatt Sachs, Rafael Salazar, Junghee Lee, Rajiv R. Ratan, Patrick Leahy & Zigmond, R. E. (2004) Novel changes in gene expression following axotomy of a sympathetic ganglion: A microarray analysis. *Journal of Neurobiology,* 59(2), 216-235.

Bolsover, S., Fabes, J. & Anderson, P. N. (2008) Axonal guidance molecules and the failure of axonal regeneration in the adult mammalian spinal cord. *Restorative Neurology and Neuroscience,* 26(2), 117-130.

Borsani, O., Zhu, J., Verslues, P. E., Sunkar, R. & Zhu, J.-K. (2005) Endogenous sirnas derived from a pair of natural cis-antisense transcripts regulate salt tolerance in arabidopsis. *Cell,* 123(7), 1279-1291.

Boudreau, R. L., Martins, I. & Davidson, B. L. (2008a) Artificial micrornas as sirna shuttles: Improved safety as compared to shrnas in vitro and in vivo. *Mol Ther,* 17(1), 169-175.

Boudreau, R. L., Mcbride, J. L., Martins, I., Shen, S., Xing, Y., Carter, B. J. & Davidson, B. L. (2009) Nonallele-specific silencing of mutant and wild-type huntingtin demonstrates therapeutic efficacy in huntington's disease mice. *Mol Ther,* 17(6), 1053-1063.

Boudreau, R. L., Monteys, A. M. & Davidson, B. L. (2008b) Minimizing variables among hairpin-based rnai vectors reveals the potency of shrnas. *RNA,* 14(9), 1834-1844.

Braasch, D. A., Paroo, Z., Constantinescu, A., Ren, G., Öz, O. K., Mason, R. P. & Corey, D. R. (2004) Biodistribution of phosphodiester and phosphorothioate sirna. *Bioorganic & Medicinal Chemistry Letters,* 14(5), 1139-1143.

Bradbury, E. J., Moon, L. D. F., Popat, R. J., King, V. R., Bennett, G. S., Patel, P. N., Fawcett, J. W. & Mcmahon, S. B. (2002) Chondroitinase abc promotes functional recovery after spinal cord injury. *Nature,* 416(6881), 636-640.

Branco, F. J. & Fraser, N. W. (2005) Herpes simplex virus type 1 latency-associated transcript expression protects trigeminal ganglion neurons from apoptosis. *J. Virol.,* 79(14), 9019-9025.

Bregman, B., Kunkel-Bagden, E., Schnell, L., Dai, H., Gao, D. & Schwab, M. (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature,* 378(498-501.

Brennecke, J., Hipfner, D. R., Stark, A., Russell, R. B. & Cohen, S. M. (2003) Bantam encodes a developmentally regulated microrna that controls cell proliferation and regulates the proapoptotic gene hid in drosophila. *Cell,* 113(1), 25-36.

Brennecke, J., Stark, A., Russell, R. B. & Cohen, S. M. (2005) Principles of microrna–target recognition. *PLoS Biology,* 3(3), e85.

Brittis, P. A., Lu, Q. & Flanagan, J. G. (2002) Axonal protein synthesis provides a mechanism for localized regulation at an intermediate target. 110(2), 223-235.

Brooks, A. I., Stein, C. S., Hughes, S. M., Heth, J., Mccray, P. M., Sauter, S. L., Johnston, J. C., Cory-Slechta, D. A., Federoff, H. J. & Davidson, B. L. (2002) Functional correction of established central nervous system deficits in an animal model of lysosomal storage disease with feline immunodeficiency virus-based vectors. *Proceedings of the National Academy of Sciences of the United States of America,* 99(9), 6216-6221.

Brosamle, C., Huber, A. B., Fiedler, M., Skerra, A. & Schwab, M. E. (2000) Regeneration of lesioned corticospinal tract fibers in the adult rat induced by a recombinant, humanized in-1 antibody fragment. *J. Neurosci.,* 20(21), 8061-8068.

Broude, E., Mcatee, M., Kelley, M. S. & Bregman, B. S. (1997) C-jun expression in adult rat dorsal root ganglion neurons: Differential response after central or peripheral axotomy. *Experimental Neurology,* 148(1), 367-377.

Brown, S. M., Ritchie, D. A. & Subak-Sharpe, J. H. (1973) Genetic studies with herpes simplex virus type 1. The isolation of temperature-sensitive mutants, their arrangement into complementation groups and recombination analysis leading to a linkage map. *J Gen Virol,* 18(3), 329-346.

Bruijn, L., Miller, T. & Cleveland, D. (2004) Unraveling the mechanisms involved in motor neuron degeneration in als. *Annu Rev Neurosci,* 27(723-49.

Brummelkamp, T. R., Bernards, R. & Agami, R. (2002) A system for stable expression of short interfering rnas in mammalian cells. *Science,* 296(5567), 550-553.

Bruni, R. & Roizman, B. (1996) Open reading frame p--a herpes simplex virus gene repressed during productive infection encodes a protein that binds a splicing factor and reduces synthesis of viral proteins made from spliced mrna. *Proceedings of the National Academy of Sciences of the United States of America,* 93(19), 10423-10427.

Bryant, H. E., Wadd, S. E., Lamond, A. I., Silverstein, S. J. & Clements, J. B. (2001) Herpes simplex virus ie63 (icp27) protein interacts with spliceosome-associated protein 145 and inhibits splicing prior to the first catalytic step. *J. Virol.,* 75(9), 4376-4385.

Bühler, M., Verdel, A. & Moazed, D. (2006) Tethering rits to a nascent transcript initiates rnai- and heterochromatin-dependent gene silencing. *Cell,* 125(5), 873-86.

Byrnes, A. P., Maclaren, R. E. & Charlton, H. M. (1996) Immunological instability of persistent adenovirus vectors in the brain: Peripheral exposure to vector leads to renewed inflammation, reduced gene expression, and demyelination. *J. Neurosci.,* 16(9), 3045-3055.

Cafferty, W. B. J., Yang, S.-H., Duffy, P. J., Li, S. & Strittmatter, S. M. (2007) Functional axonal regeneration through astrocytic scar genetically modified to digest chondroitin sulfate proteoglycans. *J. Neurosci.,* 27(9), 2176-2185.

Caggiano, A. O., Zimber, M. P., Ganguly, A., Blight, A. R. & Gruskin, E. A. (2005) Chondroitinase abci improves locomotion and bladder function following contusion injury of the rat spinal cord. *Journal of Neurotrauma,* 22(2), 226-239.

Cai (2004) Human microrna s are processed from capped, polyadenylated transcripts that can also function as mrnas. *RNA,* 10(12), 1957-1966.

Cai, D., Qiu, J., Cao, Z., Mcatee, M., Bregman, B. S. & Filbin, M. T. (2001) Neuronal cyclic amp controls the developmental loss in ability of axons to regenerate. *J. Neurosci.,* 21(13), 4731-4739.

Cai, D., Shen, Y., De Bellard, M., Tang, S. & Filbin, M. T. (1999) Prior exposure to neurotrophins blocks inhibition of axonal regeneration by mag and myelin via a camp-dependent mechanism. 22(1), 89-101.

Cai, W., Person, S., Debroy, C. & Gu, B. (1988) Functional regions and structural features of the gb glycoprotein of herpes simplex virus type 1. An analysis of linker insertion mutants. *J Mol Biol,* 201(3), 575-88.

Cai, W. & Schaffer, P. A. (1992) Herpes simplex virus type 1 icp0 regulates expression of immediate-early, early, and late genes in productively infected cells. *J. Virol.,* 66(5), 2904-2915.

Cantin, E., Hinton, D., Chen, J. & Openshaw, H. (1995) Gamma interferon expression during acute and latent nervous system infection by herpes simplex virus type 1. *J. Virol.,* 69(8), 4898-4905.

Cao, W., Hunter, R., Strnatka, D., Mcqueen, C. & Erickson, R. (2005) DNA constructs designed to produce short hairpin, interfering rnas in transgenic mice sometimes show early lethality and an interferon response. *Journal of Applied Genetics,* 46(2), 217-225.

Caplen, N. J., Fleenor, J., Fire, A. & Morgan, R. A. (2000) Dsrna-mediated gene silencing in cultured drosophila cells: A tissue culture model for the analysis of rna interference. *Gene,* 252(1-2), 95-105.

Caplen, N. J., Parrish, S., Imani, F., Fire, A. & Morgan, R. A. (2001) Specific inhibition of gene expression by small double-stranded rnas in invertebrate and vertebrate systems. *Proceedings of the National Academy of Sciences of the United States of America,* 98(17), 9742-9747.

Carbon, P., Murgo, S., Ebel, J.-P., Krol, A., Tebb, G. & Mattaj, I. W. (1987) A common octamer motif binding protein is involved in the transcription of u6 snrna by rna polymerase iii and u2 snrna by rna polymerase ii. 51(1), 71-79.

Carlstedt, T. (1985) Regenerating axons form nerve terminals at astrocytes. *Brain Research,* 347(1), 188-191.

Carmell, M. A., Girard, A., Van De Kant, H. J. G., Bourc'his, D., Bestor, T. H., De Rooij, D. G. & Hannon, G. J. (2007) Miwi2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Developmental Cell,* 12(4), 503-514.

Caroni, P. & Schwab, M. (1988) Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.,* 106(4), 1281-1288.

Carrozza, M. & Deluca, N. (1996) Interaction of the viral activator protein icp4 with tfiid through taf250. *Mol. Cell. Biol.,* 16(6), 3085-3093.

Carter, K. & Roizman, B. (1996) Alternatively spliced mrnas predicted to yield frame-shift proteins and stable intron 1 rnas of the herpes simplex virus 1 regulatory gene alpha 0 accumulate in the cytoplasm of infected cells. *Proc Natl Acad Sci,* 93(22), 12535-40.

Castanotto, D., Sakurai, K., Lingeman, R., Li, H., Shively, L., Aagaard, L., Soifer, H., Gatignol, A., Riggs, A. & Rossi, J. J. (2007) Combinatorial delivery of small interfering rnas reduces rnai efficacy by selective incorporation into risc. *Nucl. Acids Res.,* 35(15), 5154-5164.

Caterina, M. J., Leffler, A., Malmberg, A. B., Martin, W. J., Trafton, J., Petersen-Zeitz, K. R., Koltzenburg, M., Basbaum, A. I. & Julius, D. (2000) Impaired nociception and pain sensation in mice lacking the capsaicin receptor. *Science,* 288(5464), 306-313.

Caterina, M. J., Schumacher, M. A., Tominaga, M., Rosen, T. A., Levine, J. D. & Julius, D. (1997) The capsaicin receptor: A heat-activated ion channel in the pain pathway. *Nature,* 389(6653), 816-824.

Chalk, A. M., Wahlestedt, C. & Sonnhammer, E. L. L. (2004) Improved and automated prediction of effective sirna. *Biochemical and Biophysical Research Communications,* 319(1), 264-274.

Chen, I.-H. B., Li, L., Silva, L. & Sandri-Goldin, R. M. (2005) Icp27 recruits aly/ref but not tap/nxf1 to herpes simplex virus type 1 transcription sites although tap/nxf1 is required for icp27 export. *J. Virol.,* 79(7), 3949-3961.

Chen, J. & Silverstein, S. (1992) Herpes simplex viruses with mutations in the gene encoding icp0 are defective in gene expression. *J. Virol.,* 66(5), 2916-2927.

Chen, S., Kramer, M., Schaffer, P. & Coen, D. (1997) A viral function represses accumulation of transcripts from productive- cycle genes in mouse ganglia latently infected with herpes simplex virus. *J. Virol.,* 71(8), 5878-5884.

Chen, X. (2004) A microrna as a translational repressor of apetala2 in arabidopsis flower development. *Science,* 303(5666), 2022-2025.

Chen, X., Schmidt, M., Goins, W. & Glorioso, J. (1995) Two herpes simplex virus type 1 latency-active promoters differ in their contributions to latency-associated transcript expression during lytic and latent infections. *J. Virol.,* 69(12), 7899-7908.

Chen, Y., Stamatoyannopoulos, G. & Song, C.-Z. (2003) Down-regulation of cxcr4 by inducible small interfering rna inhibits breast cancer cell invasion in vitro. *Cancer Res,* 63(16), 4801-4804.

Chen, Y., Zhang, X., Wang, C., Li, G., Gu, Y. & Huang, L.-Y. M. (2008) Activation of p2x7 receptors in glial satellite cells reduces pain through downregulation of p2x3 receptors in nociceptive neurons. *Proceedings of the National Academy of Sciences,* 105(43), 16773-16778.

Chendrimada, T. P., Finn, K. J., Ji, X., Baillat, D., Gregory, R. I., Liebhaber, S. A., Pasquinelli, A. E. & Shiekhattar, R. (2007) Microrna silencing through risc recruitment of eif6. *Nature,* 447(7146), 823-828.

Chendrimada, T. P., Gregory, R. I., Kumaraswamy, E., Norman, J., Cooch, N., Nishikura, K. & Shiekhattar, R. (2005) Trbp recruits the dicer complex to ago2 for microrna processing and gene silencing. *Nature,* 436(7051), 740-744.

Cheshenko, N. & Herold, B. C. (2002) Glycoprotein b plays a predominant role in mediating herpes simplex virus type 2 attachment and is required for entry and cell-to-cell spread. *J Gen Virol,* 83(9), 2247-2255.

Chivatakarn, O., Kaneko, S., He, Z., Tessier-Lavigne, M. & Giger, R. J. (2007) The nogo-66 receptor ngr1 is required only for the acute growth cone-collapsing but not the chronic growth-inhibitory actions of myelin inhibitors. *J. Neurosci.,* 27(27), 7117-7124.

Chong, M., C.J. Woolf, N.S.K. Haque & Anderson, P. N. (1999) Axonal regeneration from injured dorsal roots into the spinal cord of adult rats. *The Journal of Comparative Neurology,* 410(1), 42-54.

Christoph, T., Bahrenberg, G., De Vry, J., Englberger, W., Erdmann, V., Frech, M., Kögel, B., Röhl, T., Schiene, K., Schröder, W., Seibler, J. & Kurreck, J. (2008) Investigation of trpv1 loss-of-function phenotypes in transgenic shrna expressing and knockout mice. *Mol Cell Neurosci,* 37(3), 579-89.

Christoph, T., Gillen, C., Mika, J., Grünweller, A., Schäfer, M., Schiene, K., Frank, R., Jostock, R., Bahrenberg, G., Weihe, E., Erdmann, V. & Kurreck, J. (2007) Antinociceptive effect of antisense oligonucleotides against the vanilloid receptor vr1/trpv1. *Neurochem Int,* 50(1), 281-90.

Christoph, T., Grünweller, A., Mika, J., Schäfer, M., Wade, E., Weihe, E., Erdmann, V., Frank, R., Gillen, C. & Kurreck, J. (2006a) Silencing of vanilloid receptor trpv1 by rnai reduces neuropathic and visceral pain in vivo. *Biochem. Biophys. Res. Commun.,* 350(1), 238-43.

Christoph, T., Grünweller, A., Mika, J., Schäfer, M. K. H., Wade, E. J., Weihe, E., Erdmann, V. A., Frank, R., Gillen, C. & Kurreck, J. (2006b) Silencing of vanilloid receptor trpv1 by rnai reduces neuropathic and visceral pain in vivo. *Biochemical and Biophysical Research Communications,* 350(1), 238-243.

Chu, C.-Y. & Rana, T. M. (2006) Translation repression in human cells by microrna-induced gene silencing requires rck/p54. *PLoS Biology,* 4(7), e210.

Chung, K.-H., Hart, C. C., Al-Bassam, S., Avery, A., Taylor, J., Patel, P. D., Vojtek, A. B. & Turner, D. L. (2006) Polycistronic rna polymerase ii expression vectors for rna interference based on bic/mir-155. *Nucl. Acids Res.,* 34(7), e53-.

Chung, W.-J., Okamura, K., Martin, R. & Lai, E. C. (2008) Endogenous rna interference provides a somatic defense against drosophila transposons. *Current Biology,* 18(11), 795-802.

Coderre, T. & Melzack, R. (1992) The role of nmda receptor-operated calcium channels in persistent nociception after formalin-induced tissue injury. *J. Neurosci.,* 12(9), 3671-3675.

Coffin, R., Maclean, A., Latchman, D. & Brown, S. (1996) Gene delivery to the central and peripheral nervous systems of mice using hsv1 icp34.5 deletion mutant vectors. *Gene Ther,* 3(10), 886-91.

Coffin, R. S., Howard, M. K. & Latchman, D. S. (1995) Altered dinucleotide content within the latently transcribed regions of the DNA of alpha herpes viruses--implications for latent rna expression and DNA structure. *Virology,* 209(2), 358-365.

Cortes, M. L., Oehmig, A., Saydam, O., Sanford, J. D., Perry, K. F., Fraefel, C. & Breakefield, X. O. (2007) Targeted integration of functional human atm cdna into genome mediated by hsv/aav hybrid amplicon vector. *Mol Ther,* 16(1), 81-88.

Cortright, D. N. & Szallasi, A. (2004) Biochemical pharmacology of the vanilloid receptor trpv1. *European Journal of Biochemistry,* 271(10), 1814-1819.

Cui, C., Griffiths, A., Li, G., Silva, L. M., Kramer, M. F., Gaasterland, T., Wang, X.-J. & Coen, D. M. (2006) Prediction and identification of herpes simplex virus 1-encoded microrna. *J. Virol.,* 80(11), 5499-5508.

Cullen, B. R. (2004) Transcription and processing of human microrna precursors. *Molecular Cell,* 16(6), 861-865.

Czech, B., Malone, C. D., Zhou, R., Stark, A., Schlingeheyde, C., Dus, M., Perrimon, N., Kellis, M., Wohlschlegel, J. A., Sachidanandam, R., Hannon, G. J. & Brennecke, J. (2008) An endogenous small interfering rna pathway in drosophila. *Nature,* 453(7196), 798-802.

David, S. & Aguayo, A. (1981) Axonal elongation into peripheral nervous system "Bridges" After central nervous system injury in adult rats. *Science,* 214(931-3.

Davidson, T. J., Harel, S., Arboleda, V. A., Prunell, G. F., Shelanski, M. L., Greene, L. A. & Troy, C. M. (2004) Highly efficient small interfering rna delivery to primary mammalian neurons induces microrna-like effects before mrna degradation. *J. Neurosci.,* 24(45), 10040-10046.

Davies, S. J. A., Fitch, M. T., Memberg, S. P., Hall, A. K., Raisman, G. & Silver, J. (1997) Regeneration of adult axons in white matter tracts of the central nervous system. *Nature,* 390(6661), 680-683.

Davies, S. J. A., Goucher, D. R., Doller, C. & Silver, J. (1999) Robust regeneration of adult sensory axons in degenerating white matter of the adult rat spinal cord. *J. Neurosci.,* 19(14), 5810-5822.

Davis, E., Caiment, F., Tordoir, X., Cavaillé, J., Ferguson-Smith, A., Cockett, N., Georges, M. & Charlier, C. (2005) Rnai-mediated allelic trans-interaction at the imprinted rtl1/peg11 locus. *Current Biology,* 15(8), 743-749.

Davis, J. B., Gray, J., Gunthorpe, M. J., Hatcher, J. P., Davey, P. T., Overend, P., Harries, M. H., Latcham, J., Clapham, C., Atkinson, K., Hughes, S. A., Rance, K., Grau, E., Harper, A. J., Pugh, P. L., Rogers, D. C., Bingham, S., Randall, A. & Sheardown, S. A. (2000) Vanilloid receptor-1 is essential for inflammatory thermal hyperalgesia. *Nature,* 405(6783), 183-187.

Daxinger, L., Kanno, T., Bucher, E., Van Der Winden, J., Naumann, U., Matzke, A. J. M. & Matzke, M. (2009) A stepwise pathway for biogenesis of 24-nt secondary sirnas and spreading of DNA methylation. *EMBO J,* 28(1), 48-57.

Deluca, N. A., Mccarthy, A. M. & Schaffer, P. A. (1985) Isolation and characterization of deletion mutants of herpes simplex virus type 1 in the gene encoding immediate-early regulatory protein icp4. *J. Virol.,* 56(2), 558-570.

Denli, A. M., Tops, B. B. J., Plasterk, R. H. A., Ketting, R. F. & Hannon, G. J. (2004) Processing of primary micrornas by the microprocessor complex. *Nature,* 432(7014), 231-235.

Dergham, P., Ellezam, B., Essagian, C., Avedissian, H., Lubell, W. D. & Mckerracher, L. (2002) Rho signaling pathway targeted to promote spinal cord repair. *J. Neurosci.,* 22(15), 6570-6577.

Dickins, R. A., Hemann, M. T., Zilfou, J. T., Simpson, D. R., Ibarra, I., Hannon, G. J. & Lowe, S. W. (2005) Probing tumor phenotypes using stable and regulated synthetic microrna precursors. *Nat Genet,* 37(11), 1289-1295.

Didonato, J., Spitzner, J. & Muller, M. (1991) A predictive model for DNA recognition by the herpes simplex virus protein icp4. *J Mol Biol,* 219(3), 451-70.

Diefenbach, R. J., Miranda-Saksena, M., Diefenbach, E., Holland, D. J., Boadle, R. A., Armati, P. J. & Cunningham, A. L. (2002) Herpes simplex virus tegument protein us11 interacts with conventional kinesin heavy chain. *J. Virol.,* 76(7), 3282-3291.

Difiglia, M., Sena-Esteves, M., Chase, K., Sapp, E., Pfister, E., Sass, M., Yoder, J., Reeves, P., Pandey, R. K., Rajeev, K. G., Manoharan, M., Sah, D. W. Y., Zamore, P. D. & Aronin, N. (2007) Therapeutic silencing of mutant huntingtin with sirna attenuates striatal and cortical neuropathology and behavioral deficits. *Proceedings of the National Academy of Sciences,* 104(43), 17204-17209.

Dillin, A. (2003) The specifics of small interfering rna specificity. *Proceedings of the National Academy of Sciences of the United States of America,* 100(11), 6289-6291.

Dixon, R. A. & Schaffer, P. A. (1980) Fine-structure mapping and functional analysis of temperature-sensitive mutants in the gene encoding the herpes simplex virus type 1 immediate early protein vp175. *J. Virol.,* 36(1), 189-203.

Dobson, A. T., Sederati, F., Devi-Rao, G., Flanagan, W. M., Farrell, M. J., Stevens, J. G., Wagner, E. K. & Feldman, L. T. (1989) Identification of the latency-associated transcript promoter by expression of rabbit beta-globin mrna in mouse sensory nerve ganglia latently infected with a recombinant herpes simplex virus. *J. Virol.,* 63(9), 3844-3851.

Domeniconi, M., Cao, Z., Spencer, T., Sivasankaran, R., Wang, K. C., Nikulina, E., Kimura, N., Cai, H., Deng, K., Gao, Y., He, Z. & Filbin, M. T. (2002) Myelin-associated glycoprotein interacts with the nogo66 receptor to inhibit neurite outgrowth. 35(2), 283-290.

Domeniconi, M., Zampieri, N., Spencer, T., Hilaire, M., Mellado, W., Chao, M. V. & Filbin, M. T. (2005) Mag induces regulated intramembrane proteolysis of the p75 neurotrophin receptor to inhibit neurite outgrowth. 46(6), 849-855.

Donello, J. E., Loeb, J. E. & Hope, T. J. (1998) Woodchuck hepatitis virus contains a tripartite posttranscriptional regulatory element. *J. Virol.,* 72(6), 5085-5092.

Dorn, G., Patel, S., Wotherspoon, G., Hemmings-Mieszczak, M., Barclay, J., Natt, F. J. C., Martin, P., Bevan, S., Fox, A., Ganju, P., Wishart, W. & Hall, J. (2004) Sirna relieves chronic neuropathic pain. *Nucl. Acids Res.,* 32(5), e49-.

Dorokhov, Y., Frolova, O. Y., Skurat, E. V., Ivanov, P. A., Gasanova, T. V., Sheveleva, A. A., Ravin, N. V., Mäkinen, K. M., Klimyuk, V. I., Skryabin, K. G., Gleba, Y. Y. & Atabekov, J. G. (2006) A novel function for a ubiquitous plant enzyme pectin methylesterase: The enhancer of rna silencing. *FEBS Lett,* 580(16), 3872-3878.

Doudna, J. A. & Cech, T. R. (2002) The chemical repertoire of natural ribozymes. *Nature,* 418(6894), 222-228.

Dowler, T., Bergeron, D., Tedeschi, A.-L., Paquet, L., Ferrari, N. & Damha, M. J. (2006) Improvements in sirna properties mediated by 2'-deoxy-2'-fluoro-{beta}-d-arabinonucleic acid (fana). *Nucl. Acids Res.,* 34(6), 1669-1675.

During, M. J., Naegele, J. R., O'malley, K. L. & Geller, A. I. (1994) Long-term behavioral recovery in parkinsonian rats by an hsv vector expressing tyrosine hydroxylase. *Science,* 266(5189), 1399-1403.

Elbashir, S. M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. & Tuschl, T. (2001) Duplexes of 21-nucleotide rnas mediate rna interference in cultured mammalian cells. *Nature,* 411(6836), 494-498.

Elbashir, S. M., Harborth, J., Weber, K. & Tuschl, T. (2002) Analysis of gene function in somatic mammalian cells using small interfering rnas. *Methods,* 26(2), 199-213.

Ellison, K. S., Maranchuk, R. A., Mottet, K. L. & Smiley, J. R. (2005) Control of vp16 translation by the herpes simplex virus type 1 immediate-early protein icp27. *J. Virol.,* 79(7), 4120-4131.

Elmén, J., Thonberg, H., Ljungberg, K., Frieden, M., Westergaard, M., Xu, Y., Wahren, B., Liang, Z., Ørum, H., Koch, T. & Wahlestedt, C. (2005) Locked nucleic acid (lna) mediated improvements in sirna stability and functionality. *Nucl. Acids Res.,* 33(1), 439-447.

Esler, W. & Wolfe, M. (2001) A portrait of alzheimer secretases--new features and familiar faces. *Science,* 293(5534), 1449-54.

Esquela-Kerscher, A. & Slack, F. J. (2006) Oncomirs [mdash] microrna s with a role in cancer. *Nat Rev Cancer,* 6(4), 259-269.

Eulalio, A., Behm-Ansmant, I., Schweizer, D. & Izaurralde, E. (2007) P-body formation is a consequence, not the cause, of rna-mediated gene silencing. *Mol. Cell. Biol.,* 27(11), 3970-3981.

Eulalio, A., Huntzinger, E. & Izaurralde, E. (2008) Gw182 interaction with argonaute is essential for mirna-mediated translational repression and mrna decay. *Nat Struct Mol Biol,* 15(4), 346-353.

Everett, R. (1984) Trans activation of transcription by herpes virus products: Requirement for two hsv-1 immediate-early polypeptides for maximum activity. *EMBO J.,* 3(13), 3135-41.

Everett, R. D. (1989) Construction and characterization of herpes simplex virus type 1 mutants with defined lesions in immediate early gene 1. *J Gen Virol,* 70(5), 1185-1202.

Fahlgren, N., Montgomery, T. A., Howell, M. D., Allen, E., Dvorak, S. K., Alexander, A. L. & Carrington, J. C. (2006) Regulation of auxin response factor3 by tas3 ta-sirna affects developmental timing and patterning in arabidopsis. 16(9), 939-944.

Farrell, M. J., Dobson, A. T. & Feldman, L. T. (1991) Herpes simplex virus latency-associated transcript is a stable intron. *Proceedings of the National Academy of Sciences of the United States of America,* 88(3), 790-794.

Faulkner, J. R., Herrmann, J. E., Woo, M. J., Tansey, K. E., Doan, N. B. & Sofroniew, M. V. (2004) Reactive astrocytes protect tissue and preserve function after spinal cord injury. *J. Neurosci.,* 24(9), 2143-2155.

Federoff, H. J., Geschwind, M. D., Geller, A. I. & Kessler, J. A. (1992) Expression of nerve growth factor in vivo from a defective herpes simplex virus 1 vector prevents effects of axotomy on sympathetic ganglia. *Proceedings of the National Academy of Sciences of the United States of America,* 89(5), 1636-1640.

Feldman, L. T., Ellison, A. R., Voytek, C. C., Yang, L., Krause, P. & Margolis, T. P. (2002) Spontaneous molecular reactivation of herpes simplex virus type 1 latency in mice. *Proceedings of the National Academy of Sciences of the United States of America,* 99(2), 978-983.

Ferrari, F., Samulski, T., Shenk, T. & Samulski, R. (1996) Second-strand synthesis is a rate-limiting step for efficient transduction by recombinant adeno-associated virus vectors. *J. Virol.,* 70(5), 3227-3234.

Fink, D. J., Sternberg, L. R., Weber, P. C., Mata, M., Goins, W. F. & Glorioso, J. C. (1992) In vivo expression of î²-galactosidase in hippocampal neurons by hsv-mediated gene transfer. *Human Gene Therapy,* 3(1), 11-19.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. & Mello, C. C. (1998) Potent and specific genetic interference by double-stranded rna in caenorhabditis elegans. *Nature,* 391(6669), 806-811.

Fitch, M. T. & Silver, J. (1997) Activated macrophages and the blood-brain barrier: Inflammation after cns injury leads to increases in putative inhibitory molecules. *Experimental Neurology,* 148(2), 587-603.

Fontaine-Rodriguez, E., Taylor, T., Olesky, M. & Knipe, D. (2004) Proteomics of herpes simplex virus infected cell protein 27: Association with translation initiation factors. *Virology,* 330(2), 487-92.

Förstemann, K., Horwich, M. D., Wee, L., Tomari, Y. & Zamore, P. D. (2007) Drosophila microrna s are sorted into functionally distinct argonaute complexes after production by dicer-1. *Cell,* 130(2), 287-297.

Fournier, A. E., Grandpre, T. & Strittmatter, S. M. (2001) Identification of a receptor mediating nogo-66 inhibition of axonal regeneration. *Nature,* 409(6818), 341-346.

Fournier, A. E., Takizawa, B. T. & Strittmatter, S. M. (2003) Rho kinase inhibition enhances axonal regeneration in the injured cns. *J. Neurosci.,* 23(4), 1416-1423.

Freund, P., Schmidlin, E., Wannier, T., Bloch, J., Mir, A., Schwab, M. E. & Rouiller, E. M. (2006) Nogo-a-specific antibody treatment enhances sprouting and functional recovery after cervical lesion in adult primates. *Nat Med,* 12(7), 790-792.

Früh, K., Ahn, K., Djaballah, H., Sempé, P., Van Endert, P., Tampé, R., Peterson, P. & Yang, Y. (1995) A viral inhibitor of peptide transporters for antigen presentation. *Nature,* 375(6530), 415-8.

Fukagawa, T., Nogami, M., Yoshikawa, M., Ikeno, M., Okazaki, T., Takami, Y., Nakayama, T. & Oshimura, M. (2004) Dicer is essential for formation of the heterochromatin structure in vertebrate cells. *Nat Cell Biol,* 6(8), 784-791.

Fuller, A. O. & Lee, W. C. (1992) Herpes simplex virus type 1 entry through a cascade of virus-cell interactions requires different roles of gd and gh in penetration. *J. Virol.,* 66(8), 5002-5012.

Fuller, A. O. & Spear, P. G. (1985) Specificities of monoclonal and polyclonal antibodies that inhibit adsorption of herpes simplex virus to cells and lack of inhibition by potent neutralizing antibodies. *J. Virol.,* 55(2), 475-482.

Funahashi, S., Tomohiko Hasegawa, Akira Nagano & Kohji Sato (2008) Differential expression patterns of messenger rnas encoding nogo receptors and their ligands in the rat central nervous system. *The Journal of Comparative Neurology,* 506(1), 141-160.

Gao, G., Yang, Y. & Wilson, J. (1996) Biology of adenovirus vectors with e1 and e4 deletions for liver- directed gene therapy. *J. Virol.,* 70(12), 8934-8943.

Gao, Y., Deng, K., Hou, J., Bryson, J. B., Barco, A., Nikulina, E., Spencer, T., Mellado, W., Kandel, E. R. & Filbin, M. T. (2004) Activated creb is sufficient to overcome inhibitors in myelin and promote spinal axon regeneration in vivo. 44(4), 609-621.

Garber, D., Schaffer, P. & Knipe, D. (1997) A lat-associated function reduces productive-cycle gene expression during acute infection of murine sensory neurons with herpes simplex virus type 1. *J. Virol.,* 71(8), 5885-5893.

Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H. N. & Chen, J. (2004) Inhibition of influenza virus production in virus-infected mice by rna interference. *Proceedings of the National Academy of Sciences of the United States of America,* 101(23), 8676-8681.

Geisbert, Thomasâ w., Hensley, Lisaâ e., Kagan, E., Yu, Erikâ z., Geisbert, Joanâ b., Daddarioâ€¤Dicaprio, K., Fritz, Elizabethâ a., Jahrling, Peterâ b., Mcclintock, K., Phelps, Janetâ r., Lee, Amyâ c. Â h., Judge, A., Jeffs, Lloydâ b. & Maclachlan, I. (2006) Postexposure protection of guinea pigs against a lethal ebola virus challenge is conferred by rna interference. *The Journal of Infectious Diseases,* 193(12), 1650-1657.

Geller, A. I., Keyomarsi, K., Bryan, J. & Pardee, A. B. (1990) An efficient deletion mutant packaging system for defective herpes simplex virus vectors: Potential applications to human gene therapy and neuronal physiology. *Proceedings of the National Academy of Sciences of the United States of America,* 87(22), 8950-8954.

Gelman, I. & Silverstein, S. (1985) Identification of immediate early genes from herpes simplex virus that transactivate the virus thymidine kinase gene. *Proc Natl Acad Sci,* 82(16), 5265-9.

George, R. & Griffin, J. (1994) The proximo-distal spread of axonal degeneration in the dorsal columns of the rat. *J Neurocytol,* 23(11), 657-67.

Geraghty, R. J., Krummenacher, C., Cohen, G. H., Eisenberg, R. J. & Spear, P. G. (1998) Entry of alphaherpesviruses mediated by poliovirus receptor-related protein 1 and poliovirus receptor. *Science,* 280(5369), 1618-1620.

Gerster, T. & Roeder, R. (1988) A herpesvirus trans-activating protein interacts with transcription factor otf-1 and other cellular proteins. *Proc Natl Acad Sci,* 85(17), 6347-51.

Ghildiyal, M., Seitz, H., Horwich, M. D., Li, C., Du, T., Lee, S., Xu, J., Kittler, E. L. W., Zapp, M. L., Weng, Z. & Zamore, P. D. (2008) Endogenous sirnas derived from transposons and mrnas in drosophila somatic cells. *Science,* 320(5879), 1077-1081.

Gibson, H. E., Edwards, J. G., Page, R. S., Van Hook, M. J. & Kauer, J. A. (2008) Trpv1 channels mediate long-term depression at synapses on hippocampal interneurons. 57(5), 746-759.

Giering, J. C., Grimm, D., Storm, T. A. & Kay, M. A. (2008) Expression of shrna from a tissue-specific pol ii promoter is an effective and safe rnai therapeutic. *Mol Ther,* 16(9), 1630-1636.

Gilchrist, H., Allard, B. & Simone, D. (1996) Enhanced withdrawal responses to heat and mechanical stimuli following intraplantar injection of capsaicin in rats. *Pain,* 67(1), 178-88.

Giraldez, A. J., Mishima, Y., Rihel, J., Grocock, R. J., Van Dongen, S., Inoue, K., Enright, A. J. & Schier, A. F. (2006) Zebrafish mir-430 promotes deadenylation and clearance of maternal mrnas. *Science,* 312(5770), 75-79.

Girard, A. L., Sachidanandam, R., Hannon, G. J. & Carmell, M. A. (2006) A germline-specific class of small rnas binds mammalian piwi proteins. *Nature,* 442(7099), 199-202.

Glover, C., Alison S. Bienemann, Margaret Hopton, Thomas C. Harding, James N. Kew & Uney, J. B. (2003) Long-term transgene expression can be mediated in the brain by adenoviral vectors when powerful neuron-specific promoters are used. *The Journal of Gene Medicine,* 5(7), 554-559.

Goins, W. F., Sternberg, L. R., Croen, K. D., Krause, P. R., Hendricks, R. L., Fink, D. J., Straus, S. E., Levine, M. & Glorioso, J. C. (1994) A novel latency-active promoter is contained within the herpes simplex virus type 1 ul flanking repeats. *J. Virol.,* 68(4), 2239-2252.

Gonzalez, S., Pisano, D. & Serrano, M. (2008) Mechanistic principles of chromatin remodeling guided by sirnas and mirnas. *Cell cycle,* 7(16), 2601 - 2608

Goss, J., Mata, M., Goins, W., Wu, H., Glorioso, J. & Fink, D. (2001) Antinociceptive effect of a genomic herpes simplex virus-based vector expressing human proenkephalin in rat dorsal root ganglion. *8,* 7(551-6).

Gou, D., Narasaraju, T., Chintagari, N. R., Jin, N., Wang, P. & Liu, L. (2004) Gene silencing in alveolar type ii cells using cell-specific promoter in vitro and in vivo. *Nucl. Acids Res.,* 32(17), e134-.

Graham, F. L., Smiley, J., Russell, W. C. & Nairn, R. (1977) Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol,* 36(1), 59-72.

Grandpre, T., Nakamura, F., Vartanian, T. & Strittmatter, S. M. (2000) Identification of the nogo inhibitor of axon regeneration as a reticulon protein. *Nature,* 403(6768), 439-444.

Granzow, H., Klupp, B. G., Fuchs, W., Veits, J., Osterrieder, N. & Mettenleiter, T. C. (2001) Egress of alphaherpesviruses: Comparative ultrastructural study. *J. Virol.,* 75(8), 3675-3684.

Gregory, R. I., Yan, K.-P., Amuthan, G., Chendrimada, T., Doratotaj, B., Cooch, N. & Shiekhattar, R. (2004) The microprocessor complex mediates the genesis of micrornas. *Nature,* 432(7014), 235-240.

Grimm, D., Kern, A., Rittner, K. & Kleinschmidt, J. R. A. (1998) Novel tools for production and purification of recombinant adenoassociated virus vectors. *Human Gene Therapy,* 9(18), 2745-2760.

Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., Marion, P., Salazar, F. & Kay, M. A. (2006) Fatality in mice due to oversaturation of cellular microrna/short hairpin rna pathways. *Nature,* 441(7092), 537-541.

Grimson, A., Farh, K. K.-H., Johnston, W. K., Garrett-Engele, P., Lim, L. P. & Bartel, D. P. (2007) Microrna targeting specificity in mammals: Determinants beyond seed pairing. *Molecular Cell,* 27(1), 91-105.

Grondin, B. & Deluca, N. (2000) Herpes simplex virus type 1 icp4 promotes transcription preinitiation complex formation by enhancing the binding of tfiid to DNA. *J. Virol.,* 74(24), 11504-11510.

Groutsi, F., Mason, M. R. J., Anderson, P. N., Martins, S., Anesti, M., Coffin, R. S. & Campbell, G. (2008) Retrograde viral transduction of cortical pyramidal neurons from the spinal cord. *Restorative Neurology and Neuroscience,* 26(6), 509-520.

Gruenheid, S., Gatzke, L., Meadows, H. & Tufaro, F. (1993) Herpes simplex virus infection and propagation in a mouse l cell mutant lacking heparan sulfate proteoglycans. *J. Virol.,* 67(1), 93-100.

Grunweller, A., Wyszko, E., Bieber, B., Jahnel, R., Erdmann, V. A. & Kurreck, J. (2003) Comparison of different antisense strategies in mammalian cells using locked nucleic acids, 2'-o-methyl rna, phosphorothioates and small interfering rna. *Nucl. Acids Res.,* 31(12), 3185-3193.

Gu, B. & Deluca, N. (1994) Requirements for activation of the herpes simplex virus glycoprotein c promoter in vitro by the viral regulatory protein icp4. *J. Virol.,* 68(12), 7953-7965.

Gu, B., Kuddus, R. & Deluca, N. (1995) Repression of activator-mediated transcription by herpes simplex virus icp4 via a mechanism involving interactions with the basal transcription factors tata-binding protein and tfiib. *Mol. Cell. Biol.,* 15(7), 3618-3626.

Gu, H., Liang, Y., Mandel, G. & Roizman, B. (2005) Components of the rest/corest/histone deacetylase repressor complex are disrupted, modified, and translocated in hsv-1-infected cells. *Proceedings of the National Academy of Sciences of the United States of America,* 102(21), 7571-7576.

Guo, S. & Kemphues, K. J. (1995) Par-1, a gene required for establishing polarity in c. Elegans embryos, encodes a putative ser/thr kinase that is asymmetrically distributed. *Cell,* 81(4), 611-620.

Guo, W., Robbins, M. T., Wei, F., Zou, S., Dubner, R. & Ren, K. (2006) Supraspinal brain-derived neurotrophic factor signaling: A novel mechanism for descending pain facilitation. *J. Neurosci.,* 26(1), 126-137.

Gupta, A., Gartner, J. J., Sethupathy, P., Hatzigeorgiou, A. G. & Fraser, N. W. (2006) Anti-apoptotic function of a microrna encoded by the hsv-1 latency-associated transcript. *Nature,* 442(7098), 82-85.

Gupta, A., Gartner, J. J., Sethupathy, P., Hatzigeorgiou, A. G. & Fraser, N. W. (2008) Anti-apoptotic function of a microrna encoded by the hsv-1 latency-associated transcript. *Nature,* 451(7178), 600-600.

Gusella, J. & Macdonald, M. (2000) Molecular genetics: Unmasking polyglutamine triggers in neurodegenerative disease. *Nat Rev Neurosci,* 1(2), 109-15.

Guth, L., Barrett, C., Donati, E., Anderson, F., Smith, M. & Lifson, M. (1985) Essentiality of a specific cellular terrain for growth of axons into a spinal cord lesion. *Exp Neurol,* 88(1), 1-12.

Haarr, L., Shukla, D., Rødahl, E., Dal Canto, M. & Spear, P. (2001) Transcription from the gene encoding the herpesvirus entry receptor nectin-1 (hvec) in nervous tissue of adult mouse. *Virology,* 287(2), 301-9.

Habib, A., Linda S. Marton, Brenda Allwardt, Jeffrey R. Gulcher, Daniel D. Mikol, Thorbergur Högnason, Naibedya Chattopadhyay & Kári Stefansson (1998) Expression of the oligodendrocyte-myelin glycoprotein by neurons in the mouse central nervous system. *Journal of Neurochemistry,* 70(4), 1704-1711.

Hagmann, M., Georgiev, O., Schaffner, W. & Douville, P. (1995) Transcription factors interacting with herpes simplex virus {alpha} gene promoters in sensory neurons. *Nucl. Acids Res.,* 23(24), 4978-4985.

Haley, B. & Zamore, P. D. (2004) Kinetic analysis of the rnai enzyme complex. *Nat Struct Mol Biol,* 11(7), 599-606.

Halford, W. P., Kemp, C. D., Isler, J. A., Davido, D. J. & Schaffer, P. A. (2001) Icp0, icp4, or vp16 expressed from adenovirus vectors induces reactivation of latent herpes simplex virus type 1 in primary cultures of latently infected trigeminal ganglion cells. *J. Virol.,* 75(13), 6143-6153.

Halford, W. P. & Schaffer, P. A. (2001) Icp0 is required for efficient reactivation of herpes simplex virus type 1 from neuronal latency. *J. Virol.,* 75(7), 3240-3249.

Halford, W. P., Veress, L. A., Gebhardt, B. M. & Carr, D. J. J. (1997) Innate and acquired immunity to herpes simplex virus type 1. *Virology,* 236(2), 328-337.

Hall, A. H. S., Wan, J., Shaughnessy, E. E., Ramsay Shaw, B. & Alexander, K. A. (2004) Rna interference using boranophosphate sirnas: Structure-activity relationships. *Nucl. Acids Res.,* 32(20), 5991-6000.

Hall, I. M., Noma, K.-I. & Grewal, S. I. S. (2003) Rna interference machinery regulates chromosome dynamics during mitosis and meiosis in fission yeast. *Proceedings of the National Academy of Sciences of the United States of America,* 100(1), 193-198.

Hamilton, A., Voinnet, O., Chappell, L. & Baulcombe, D. (2002) Two classes of short interfering rna in rna silencing. *EMBO J.,* 21(17), 4671-9.

Hammond, S. M., Boettcher, S., Caudy, A. A., Kobayashi, R. & Hannon, G. J. (2001) Argonaute2, a link between genetic and biochemical analyses of rnai. *Science,* 293(5532), 1146-1150.

Han, J., Lee, Y., Yeom, K.-H., Kim, Y.-K., Jin, H. & Kim, V. N. (2004) The drosha-dgcr8 complex in primary microrna processing. *Genes & Development,* 18(24), 3016-3027.

Han, J., Lee, Y., Yeom, K.-H., Nam, J.-W., Heo, I., Rhee, J.-K., Sohn, S. Y., Cho, Y., Zhang, B.-T. & Kim, V. N. (2006) Molecular basis for the recognition of primary micrornas by the drosha-dgcr8 complex. 125(5), 887-901.

Hao, S., Mata, M., Goins, W., Glorioso, J. & Fink, D. (2003a) Transgene-mediated enkephalin release enhances the effect of morphine and evades tolerance to produce a sustained antiallodynic effect in neuropathic pain. *Pain,* 102(1-2), 135-42.

Hao, S., Mata, M., Wolfe, D., Huang, S., Glorioso, J. & Fink, D. (2003b) Hsv-mediated gene transfer of the glial cell-derived neurotrophic factor provides an antiallodynic effect on neuropathic pain. *Mol Ther,* 8(3), 367-75.

Hardy, S., Kitamura, M., Harris-Stansil, T., Dai, Y. & Phipps, M. (1997) Construction of adenovirus vectors through cre-lox recombination. *J. Virol.,* 71(3), 1842-1849.

Hardy, W. R. & Sandri-Goldin, R. M. (1994) Herpes simplex virus inhibits host cell splicing, and regulatory protein icp27 is required for this effect. *J. Virol.,* 68(12), 7790-7799.

Harper, S. Q., Staber, P. D., He, X., Eliason, S. L., Martins, I. S. H., Mao, Q., Yang, L., Kotin, R. M., Paulson, H. L. & Davidson, B. L. (2005) Rna interference improves motor and neuropathological abnormalities in a huntington's disease mouse model. *Proceedings of the National Academy of Sciences of the United States of America,* 102(16), 5820-5825.

Harris, R. A., Everett, R. D., Zhu, X. X., Silverstein, S. & Preston, C. M. (1989) Herpes simplex virus type 1 immediate-early protein vmw110 reactivates latent herpes simplex virus type 2 in an in vitro latency system. *J. Virol.,* 63(8), 3513-3515.

Harrow, S., Papanastassiou, V., Harland, J., Mabbs, R., Petty, R., Fraser, M., Hadley, D., Patterson, J., Brown, S. M. & Rampling, R. (2004) Hsv1716 injection into the brain adjacent to tumour following surgical resection of high-grade glioma: Safety data and long-term survival. *Gene Ther,* 11(22), 1648-1658.

Hasegawa, Y., Fujitani, M., Hata, K., Tohyama, M., Yamagishi, S. & Yamashita, T. (2004) Promotion of axon regeneration by myelin-associated glycoprotein and nogo through divergent signals downstream of gi/g. *J. Neurosci.,* 24(30), 6826-6832.

Hassani, Z., Francois, J.-C., Alfama, G., Dubois, G. M., Paris, M., Giovannangeli, C. & Demeneix, B. A. (2007) A hybrid cmv-h1 construct improves efficiency of pei-delivered shrna in the mouse brain. *Nucl. Acids Res.,* 35(9), e65-.

He, B., Chou, J., Brandimarti, R., Mohr, I., Gluzman, Y. & Roizman, B. (1997) Suppression of the phenotype of gamma(1)34.5- herpes simplex virus 1: Failure of activated rna-dependent protein kinase to shut off protein synthesis is associated with a deletion in the domain of the alpha47 gene. *J. Virol.,* 71(8), 6049-6054.

He, L., Thomson, J. M., Hemann, M. T., Hernando-Monge, E., Mu, D., Goodson, S., Powers, S., Cordon-Cardo, C., Lowe, S. W., Hannon, G. J. & Hammond, S. M. (2005) A microrna polycistron as a potential human oncogene. *Nature,* 435(7043), 828-833.

Heidel, J. D., Hu, S., Liu, X. F., Triche, T. J. & Davis, M. E. (2004) Lack of interferon response in animals to naked sirnas. *Nat Biotech,* 22(12), 1579-1582.

Heidel, J. D., Yu, Z., Liu, J. Y.-C., Rele, S. M., Liang, Y., Zeidan, R. K., Kornbrust, D. J. & Davis, M. E. (2007) Administration in non-human primates of escalating intravenous doses of targeted nanoparticles containing ribonucleotide reductase subunit m2 sirna. *Proceedings of the National Academy of Sciences,* 104(14), 5715-5721.

Hemann, M. T., Fridman, J. S., Zilfou, J. T., Hernando, E., Paddison, P. J., Cordon-Cardo, C., Hannon, G. J. & Lowe, S. W. (2003) An epi-allelic series of p53 hypomorphs created by stable rnai produces distinct tumor phenotypes in vivo. *Nat Genet,* 33(3), 396-400.

Hengst, U., Cox, L. J., Macosko, E. Z. & Jaffrey, S. R. (2006) Functional and selective rna interference in developing axons and growth cones. *J. Neurosci.,* 26(21), 5727-5732.

Hernandez, Y. J., Wang, J., Kearns, W. G., Loiler, S., Poirier, A. & Flotte, T. R. (1999) Latent adeno-associated virus infection elicits humoral but not cell-mediated immune responses in a nonhuman primate model. *J. Virol.,* 73(10), 8549-8558.

Herold, B. C., Visalli, R. J., Susmarski, N., Brandt, C. R. & Spear, P. G. (1994) Glycoprotein c-independent binding of herpes simplex virus to cells requires cell surface heparan sulphate and glycoprotein b. *J Gen Virol,* 75(6), 1211-1222.

Herold, B. C., Wudunn, D., Soltys, N. & Spear, P. G. (1991) Glycoprotein c of herpes simplex virus type 1 plays a principal role in the adsorption of virus to cells and in infectivity. *J. Virol.,* 65(3), 1090-1098.

Herrera, F. J. & Triezenberg, S. J. (2004) Vp16-dependent association of chromatin-modifying coactivators and underrepresentation of histones at immediate-early gene promoters during herpes simplex virus infection. *J. Virol.,* 78(18), 9689-9696.

Higuchi, H., Yamashita, T., Yoshikawa, H. & Tohyama, M. (2003) Functional inhibition of the p75 receptor using a small interfering rna. *Biochemical and Biophysical Research Communications,* 301(3), 804-809.

Hill, A., Jugovic, P., York, I., Russ, G., Bennink, J., Yewdell, J., Ploegh, H. & Johnson, D. (1995) Herpes simplex virus turns off the tap to evade host immunity. *Nature,* 375(6539), 411-5.

Hill, J., Gebhardt, B., Wen, R., Bouterie, A., Thompson, H., O'callaghan, R., Halford, W. & Kaufman, H. (1996) Quantitation of herpes simplex virus type 1 DNA and latency-associated transcripts in rabbit trigeminal ganglia demonstrates a stable reservoir of viral nucleic acids during latency. *J. Virol.,* 70(5), 3137-3141.

Hiraguri, A., Itoh, R., Kondo, N., Nomura, Y., Aizawa, D., Murai, Y., Koiwa, H., Seki, M., Shinozaki, K. & Fukuhara, T. (2005) Specific interactions between dicer-like proteins and hyl1/drb-family dsrna-binding proteins in arabidopsis thaliana. *Plant Molecular Biology,* 57(2), 173-188.

Hisaoka, T., Yoshihiro Morikawa, Tadasuke Komori, Takuya Sugiyama, Toshio Kitamura & Emiko Senba (2006) Characterization of troy-expressing cells in the developing and postnatal cns: The possible role in neuronal and glial cell development. *European Journal of Neuroscience,* 23(12), 3149-3160.

Ho, D. Y. & Mocarski, E. S. (1988) [beta]-galactosidase as a marker in the peripheral and neural tissues of the herpes simplex virus-infected mouse. *Virology,* 167(1), 279-283.

Ho, D. Y. & Mocarski, E. S. (1989) Herpes simplex virus latent rna (lat) is not required for latent infection in the mouse. *Proceedings of the National Academy of Sciences of the United States of America,* 86(19), 7596-7600.

Hobbs, W. E., Brough, D. E., Kovesdi, I. & Deluca, N. A. (2001) Efficient activation of viral genomes by levels of herpes simplex virus icp0 insufficient to affect cellular gene expression or cell survival. *J. Virol.,* 75(7), 3391-3403.

Hobbs, W. E., Ii & Deluca, N. A. (1999) Perturbation of cell cycle progression and cellular gene expression as a function of herpes simplex virus icp0. *J. Virol.,* 73(10), 8245-8255.

Holen, T., Moe, S. E., Sorbo, J. G., Meza, T. J., Ottersen, O. P. & Klungland, A. (2005) Tolerated wobble mutations in sirnas decrease specificity, but can enhance activity in vivo. *Nucl. Acids Res.,* 33(15), 4704-4710.

Hollins, A. J., Omidi, Y., Benter, I. F. & Akhtar, S. (2007) Toxicogenomics of drug delivery systems: Exploiting delivery system-induced changes in target gene expression to enhance sirna activity. *Journal of Drug Targeting,* 15(1), 83-88.

Hommel, J. D., Sears, R. M., Georgescu, D., Simmons, D. L. & Dileone, R. J. (2003) Local gene knockdown in the brain using viral-mediated rna interference. *Nat Med,* 9(12), 1539-1544.

Honess, R. W. & Roizman, B. (1974) Regulation of herpesvirus macromolecular synthesis i. Cascade regulation of the synthesis of three groups of viral proteins. *J. Virol.,* 14(1), 8-19.

Hong, C. S., Goins, W. F., Goss, J. R., Burton, E. A. & Glorioso, J. C. (2006) Herpes simplex virus rnai and neprilysin gene transfer vectors reduce accumulation of alzheimer's disease-related amyloid-[beta] peptide in vivo. *Gene Ther,* 13(14), 1068-1079.

Honore, P., Kage, K., Mikusa, J., Watt, A., Johnston, J., Wyatt, J., Faltynek, C., Jarvis, M. & Lynch, K. (2002) Analgesic profile of intrathecal p2x(3) antisense oligonucleotide treatment in chronic inflammatory and neuropathic pain states in rats. *Pain,* 99(1-2), 11-9.

Honore, P., Wismer, C. T., Mikusa, J., Zhu, C. Z., Zhong, C., Gauvin, D. M., Gomtsyan, A., El Kouhen, R., Lee, C.-H., Marsh, K., Sullivan, J. P., Faltynek, C. R. & Jarvis, M. F. (2005) A-425619 [1-isoquinolin-5-yl-3-(4-trifluoromethyl-benzyl)-urea], a novel transient receptor potential type v1 receptor antagonist, relieves pathophysiological pain associated with inflammation and tissue injury in rats. *J Pharmacol Exp Ther,* 314(1), 410-421.

Hornung, V., Guenthner-Biller, M., Bourquin, C., Ablasser, A., Schlee, M., Uematsu, S., Noronha, A., Manoharan, M., Akira, S., De Fougerolles, A., Endres, S. & Hartmann, G. (2005) Sequence-specific potent induction of ifn-[alpha] by short interfering rna in plasmacytoid dendritic cells through tlr7. *Nat Med,* 11(3), 263-270.

Hosono, T., Mizuguchi, H., Katayama, K., Xu, Z.-L., Sakurai, F., Ishii-Watabe, A., Kawabata, K., Yamaguchi, T., Nakagawa, S., Mayumi, T. & Hayakawa, T. (2004) Adenovirus vector-mediated doxycycline-inducible rna interference. *Human Gene Therapy,* 15(8), 813-819.

Howard, M., Kershaw, T., Gibb, B., Storey, N., Maclean, A., Zeng, B., Tel, B., Jenner, P., Brown, S., Woolf, C., Anderson, P., Coffin, R. & Latchman, D. (1998) High efficiency gene transfer to the central nervous system of rodents and primates using herpes virus vectors lacking functional icp27 and icp34.5. *Gene Ther,* 5(8), 1137-47.

Hu, J. C. C., Coffin, R. S., Davis, C. J., Graham, N. J., Groves, N., Guest, P. J., Harrington, K. J., James, N. D., Love, C. A., Mcneish, I., Medley, L. C., Michael, A., Nutting, C. M., Pandha, H. S., Shorrock, C. A., Simpson, J., Steiner, J., Steven, N. M., Wright, D. & Coombes, R. C. (2006) A phase i study of oncovexgm-csf, a second-generation oncolytic herpes simplex virus expressing granulocyte macrophage colony-stimulating factor. *Clin Cancer Res,* 12(22), 6737-6747.

Huang, A., Chen, Y., Wang, X., Zhao, S., Su, N. & White, D. (2004) Functional silencing of hepatic microsomal glucose-6-phosphatase gene expression in vivo by adenovirus-mediated delivery of short hairpin rna. *FEBS Lett,* 558(1-3), 69-73.

Huang, J. K., Phillips, G. R., Roth, A. D., Pedraza, L., Shan, W., Belkaid, W., Mi, S., Fex-Svenningsen, A., Florens, L., Yates, J. R., Iii & Colman, D. R. (2005) Glial membranes at the node of ranvier prevent neurite outgrowth. *Science,* 310(5755), 1813-1817.

Huber, A. B., Weinmann, O., Brosamle, C., Oertle, T. & Schwab, M. E. (2002) Patterns of nogo mrna and protein expression in the developing and adult rat and after cns lesions. *J. Neurosci.,* 22(9), 3553-3567.

Hudson, L., S. Bevan, G. Wotherspoon, C. Gentry, A. Fox & J. Winter (2001) Vr1 protein expression increases in undamaged drg neurons after partial nerve injury. *European Journal of Neuroscience,* 13(11), 2105-2114.

Humphreys, D. T., Westman, B. J., Martin, D. I. K. & Preiss, T. (2005) Micrornas control translation initiation by inhibiting eukaryotic initiation factor 4e/cap and poly(a) tail function. *Proceedings of the National Academy of Sciences of the United States of America,* 102(47), 16961-16966.

Hunt, D., Hossain-Ibrahim, K., Mason, M., Coffin, R., Lieberman, A., Winterbottom, J. & Anderson, P. (2004) Atf3 upregulation in glia during wallerian degeneration: Differential expression in peripheral nerves and cns white matter. *BMC Neuroscience,* 5(1), 9.

Hunt, D., Mason, M., Campbell, G., Coffin, R. & Anderson, P. (2002) Nogo receptor mrna expression in intact and regenerating cns neurons. *Mol Cell Neurosci,* 20(4), 537-52.

Hutchinson, L., Browne, H., Wargent, V., Davis-Poynter, N., Primorac, S., Goldsmith, K., Minson, A. C. & Johnson, D. C. (1992) A novel herpes simplex virus glycoprotein, gl, forms a complex

with glycoprotein h (gh) and affects normal folding and surface expression of gh. *J. Virol.,* 66(4), 2240-2250.

Hutvagner, G., Mclachlan, J., Pasquinelli, A. E., Balint, E., Tuschl, T. & Zamore, P. D. (2001) A cellular function for the rna-interference enzyme dicer in the maturation of the let-7 small temporal rna. *Science,* 293(5531), 834-838.

Hutvágner, G., Simard Mj, Mello Cc, Zamore Pd (2004) Sequence-specific inhibition of small rna function. *PLoS Biology,* 2(4), e98.

Hutvagner, G. & Zamore, P. D. (2002) A microrna in a multiple-turnover rnai enzyme complex. *Science,* 297(5589), 2056-2060.

Ie Gross Clark, W. (1943) The problem of neuronal regeneration in the central nervous system: Ii. The insertion of peripheral nerve stumps into the brain. *J Anat,* 77(251-9.

Jackson, A. L., Bartz, S. R., Schelter, J., Kobayashi, S. V., Burchard, J., Mao, M., Li, B., Cavet, G. & Linsley, P. S. (2003) Expression profiling reveals off-target gene regulation by rnai. *Nat Biotech,* 21(6), 635-637.

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. (2006a) Position-specific chemical modification of sirnas reduces â€œoff-targetâ€¤ transcript silencing. *RNA,* 12(7), 1197-1205.

Jackson, A. L., Burchard, J., Schelter, J., Chau, B. N., Cleary, M., Lim, L. & Linsley, P. S. (2006b) Widespread sirna â€œoff-targetâ€¤ transcript silencing mediated by seed region sequence complementarity. *RNA,* 12(7), 1179-1187.

Jackson, S. A. & Deluca, N. A. (2003) Relationship of herpes simplex virus genome configuration to productive and persistent infections. *Proceedings of the National Academy of Sciences of the United States of America,* 100(13), 7871-7876.

Jacob, R. J., Morse, L. S. & Roizman, B. (1979) Anatomy of herpes simplex virus DNA xii. Accumulation of head-to-tail concatemers in nuclei of infected cells and their role in the generation of the four isomeric arrangements of viral DNA. *J. Virol.,* 29(2), 448-457.

Jakymiw, A., Lian, S., Eystathioy, T., Li, S., Satoh, M., Hamel, J., Fritzler, M. & Chan, E. (2005) Disruption of gw bodies impairs mammalian rna interference. *Nature Cell Biology* 7(1167 - 1174

Jarvis, M. F., Burgard, E. C., Mcgaraughty, S., Honore, P., Lynch, K., Brennan, T. J., Subieta, A., Van Biesen, T., Cartmell, J., Bianchi, B., Niforatos, W., Kage, K., Yu, H., Mikusa, J.,

Wismer, C. T., Zhu, C. Z., Chu, K., Lee, C.-H., Stewart, A. O., Polakowski, J., Cox, B. F., Kowaluk, E., Williams, M., Sullivan, J. & Faltynek, C. (2002) A-317491, a novel potent and selective non-nucleotide antagonist of p2x3 and p2x2/3 receptors, reduces chronic inflammatory and neuropathic pain in the rat. *Proceedings of the National Academy of Sciences of the United States of America,* 99(26), 17179-17184.

Javier, R. T., Stevens, J. G., Dissette, V. B. & Wagner, E. K. (1988) A herpes simplex virus transcript abundant in latently infected neurons is dispensable for for establishment of the latent state. *Virology,* 166(1), 254-257.

Ji, B., Li, M., Wu, W.-T., Yick, L.-W., Lee, X., Shao, Z., Wang, J., So, K.-F., Mccoy, J. M., Blake Pepinsky, R., Mi, S. & Relton, J. K. (2006) Lingo-1 antagonist promotes functional recovery and axonal sprouting after spinal cord injury. *Molecular and Cellular Neuroscience,* 33(3), 311-320.

Jin, L., Peng, W., Perng, G.-C., Brick, D. J., Nesburn, A. B., Jones, C. & Wechsler, S. L. (2003) Identification of herpes simplex virus type 1 latency-associated transcript sequences that both inhibit apoptosis and enhance the spontaneous reactivation phenotype. *J. Virol.,* 77(11), 6556-6561.

Jing, Q., Huang, S., Guth, S., Zarubin, T., Motoyama, A., Chen, J., Di Padova, F., Lin, S.-C., Gram, H. & Han, J. (2005) Involvement of microrna in au-rich element-mediated mrna instability. *Cell,* 120(5), 623-634.

Johnson, P., Abramow-Newerly, W., Seilheimer, B., Sadoul, R., Tropak, M., Arquint, M., Dunn, R., Schachner, M. & Roder, J. (1989) Recombinant myelin-associated glycoprotein confers neural adhesion and neurite outgrowth function. *Neuron,* 3(377-85).

Johnson, P. A., Miyanohara, A., Levine, F., Cahill, T. & Friedmann, T. (1992) Cytotoxicity of a replication-defective mutant of herpes simplex virus type 1. *J. Virol.,* 66(5), 2952-2965.

Johnson, P. A., Wang, M. J. & Friedmann, T. (1994) Improved cell survival by the reduction of immediate-early gene expression in replication-defective mutants of herpes simplex virus type 1 but not by mutation of the virion host shutoff function. *J. Virol.,* 68(10), 6347-6362.

Judge, A. D., Sood, V., Shaw, J. R., Fang, D., Mcclintock, K. & Maclachlan, I. (2005) Sequence-dependent stimulation of the mammalian innate immune response by synthetic sirna. *Nat Biotech,* 23(4), 457-462.

Kanai Y, N. E., Fujiuchi a, Hara T, Imai A. (2005) Involvement of an increased spinal trpv1 sensitization through its up-regulation in mechanical allodynia of cci rats. *Neuropharmacology,* 49(7), 977-84.

Kanellopoulou, C., Muljo, S. A., Kung, A. L., Ganesan, S., Drapkin, R., Jenuwein, T., Livingston, D. M. & Rajewsky, K. (2005) Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. *Genes & Development,* 19(4), 489-501.

Kaplitt, M. G., Kwong, A. D., Kleopoulos, S. P., Mobbs, C. V., Rabkin, S. D. & Pfaff, D. W. (1994) Preproenkephalin promoter yields region-specific and long-term expression in adult brain after direct in vivo gene transfer via a defective herpes simplex viral vector. *Proceedings of the National Academy of Sciences of the United States of America,* 91(19), 8979-8983.

Kawaguchi, Y., Bruni, R. & Roizman, B. (1997a) Interaction of herpes simplex virus 1 alpha regulatory protein icp0 with elongation factor 1delta: Icp0 affects translational machinery. *J. Virol.,* 71(2), 1019-1024.

Kawaguchi, Y., Tanaka, M., Yokoymama, A., Matsuda, G., Kato, K., Kagawa, H., Hirai, K. & Roizman, B. (2001) Herpes simplex virus 1 î± regulatory protein icp0 functionally interacts with cellular transcription factor bmal1. *Proceedings of the National Academy of Sciences of the United States of America,* 98(4), 1877-1882.

Kawaguchi, Y., Van Sant, C. & Roizman, B. (1997b) Herpes simplex virus 1 alpha regulatory protein icp0 interacts with and stabilizes the cell cycle regulator cyclin d3. *J. Virol.,* 71(10), 7328-7336.

Kawamura, Y., Saito, K., Kin, T., Ono, Y., Asai, K., Sunohara, T., Okada, T. N., Siomi, M. C. & Siomi, H. (2008) Drosophila endogenous small rnas bind to argonaute[thinsp]2 in somatic cells. *Nature,* 453(7196), 793-797.

Kemp, L. M., Dent, C. L. & Latchman, D. S. (1990) Octamer motif mediates transcriptional repression of hsv immediate-early genes and octamer-containing cellular promoters in neuronal cells. 4(2), 215-222.

Kenny, J. J., Krebs, F. C., Hartle, H. T., Gartner, A. E., Chatton, B., Leiden, J. M., Hoeffler, J. P., Weber, P. C. & Wigdahl, B. (1994) Identification of a second atf/creb-like element in the herpes simplex virus type 1 (hsv-1) latency-associated transcript (lat) promoter. *Virology,* 200(1), 220-235.

Kent, J. R., Zeng, P.-Y., Atanasiu, D., Gardner, J., Fraser, N. W. & Berger, S. L. (2004) During lytic infection herpes simplex virus type 1 is associated with histones bearing modifications that correlate with active transcription. *J. Virol.,* 78(18), 10178-10186.

Kerschensteiner, M., Schwab, M. E., Lichtman, J. W. & Misgeld, T. (2005) In vivo imaging of axonal degeneration and regeneration in the injured spinal cord. *Nat Med,* 11(5), 572-577.

Khanna, K. M., Bonneau, R. H., Kinchington, P. R. & Hendricks, R. L. (2003) Herpes simplex virus-specific memory cd8+ t cells are selectively activated and retained in latently infected sensory ganglia. 18(5), 593-603.

Khvorova, A., Reynolds, A. & Jayasena, S. D. (2003) Functional sirnas and mirnas exhibit strand bias. *Cell,* 115(2), 209-216.

Kim, D.-H., Behlke, M. A., Rose, S. D., Chang, M.-S., Choi, S. & Rossi, J. J. (2005) Synthetic dsrna dicer substrates enhance rnai potency and efficacy. *Nat Biotech,* 23(2), 222-226.

Kim, D. H., Sã¦Trom, P. L., Snã¸Ve, O. & Rossi, J. J. (2008a) Microrna-directed transcriptional gene silencing in mammalian cells. *Proceedings of the National Academy of Sciences,* 105(42), 16230-16235.

Kim, J.-E., Li, S., Grandpré, T., Qiu, D. & Strittmatter, S. M. (2003) Axon regeneration in young adult mice lacking nogo-a/b. 38(2), 187-199.

Kim, J.-E., Liu, B. P., Park, J. H. & Strittmatter, S. M. (2004) Nogo-66 receptor prevents raphespinal and rubrospinal axon regeneration and limits functional recovery from spinal cord injury. 44(3), 439-451.

Kim, J., Inoue, K., Ishii, J., Vanti, W. B., Voronov, S. V., Murchison, E., Hannon, G. & Abeliovich, A. (2007) A microrna feedback circuit in midbrain dopamine neurons. *Science,* 317(5842), 1220-1224.

Kim, S. & Chung, J. (1992) An experimental model for peripheral neuropathy produced by segmental spinal nerve ligation in the rat. *Pain,* 50(3), 355-63.

Kim, S. H., Jeong, J. H., Lee, S. H., Kim, S. W. & Park, T. G. (2006a) Peg conjugated vegf sirna for anti-angiogenic gene therapy. *Journal of Controlled Release,* 116(2), 123-129.

Kim, S. H., Jeong, J. H., Lee, S. H., Kim, S. W. & Park, T. G. (2008b) Local and systemic delivery of vegf sirna using polyelectrolyte complex micelles for effective treatment of cancer. *Journal of Controlled Release,* 129(2), 107-116.

Kim, W. J., Christensen, L. V., Jo, S., Yockman, J. W., Jeong, J. H., Kim, Y.-H. & Kim, S. W. (2006b) Cholesteryl oligoarginine delivering vascular endothelial growth factor sirna effectively inhibits tumor growth in colon adenocarcinoma. *Mol Ther,* 14(3), 343-350.

Kiriakidou, M., Tan, G. S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P. T. & Mourelatos, Z. (2007) An mrna m7g cap binding-like motif within human ago2 represses translation. *Cell,* 129(6), 1141-1151.

Klein, R., Hamby, M., Sonntag, C., Millard, W., King, M. & Meyer, E. (2002) Measurements of vector-derived neurotrophic factor and green fluorescent protein levels in the brain. *Methods,* 28(2), 286-92.

Kleinman, M. E., Yamada, K., Takeda, A., Chandrasekaran, V., Nozaki, M., Baffi, J. Z., Albuquerque, R. J. C., Yamasaki, S., Itaya, M., Pan, Y., Appukuttan, B., Gibbs, D., Yang, Z., Kariko, K., Ambati, B. K., Wilgus, T. A., Dipietro, L. A., Sakurai, E., Zhang, K., Smith, J. R., Taylor, E. W. & Ambati, J. (2008) Sequence- and target-independent angiogenesis suppression by sirna via tlr3. *Nature,* 452(7187), 591-597.

Klemm, R., Goodrich, J., Zhou, S. & Tjian, R. (1995) Molecular cloning and expression of the 32-kda subunit of human tfiid reveals interactions with vp16 and tfiib that mediate transcriptional activation. *Proc Natl Acad Sci,* 92(13), 5788-92.

Knight, S. W. & Bass, B. L. (2001) A role for the rnase iii enzyme dcr-1 in rna interference and germ line development in caenorhabditis elegans. *Science,* 293(5538), 2269-2271.

Kobayashi, N., Boyer, T. & Berk, A. (1995) A class of activation domains interacts directly with tfiia and stimulates tfiia-tfiid-promoter complex assembly. *Mol Cell Biol.,* 15(11), 6465-73.

Kobayashi, N., Horn, P., Sullivan, S., Triezenberg, S., Boyer, T. & Berk, A. (1998) Da-complex assembly activity required for vp16c transcriptional activation. *Mol Cell Biol.,* 18(7), 4023-31.

Kong, Y. W., Cannell, I. G., De Moor, C. H., Hill, K., Garside, P. G., Hamilton, T. L., Meijer, H. A., Dobbyn, H. C., Stoneley, M., Spriggs, K. A., Willis, A. E. & Bushell, M. (2008) The mechanism of micro-rna-mediated translation repression is determined by the promoter of the target gene. *Proceedings of the National Academy of Sciences,* 105(26), 8866-8871.

Kramer, M. & Coen, D. (1995) Quantification of transcripts from the icp4 and thymidine kinase genes in mouse ganglia latently infected with herpes simplex virus. *J. Virol.,* 69(3), 1389-1399.

Kretschmer-Kazemi Far, R. & Sczakiel, G. (2003) The activity of sirna in mammalian cells is related to structural target accessibility: A comparison with antisense oligonucleotides. *Nucl. Acids Res.,* 31(15), 4417-4424.

Krichevsky, A. M. & Kosik, K. S. (2002) Rnai functions in cultured mammalian neurons. *Proceedings of the National Academy of Sciences of the United States of America,* 99(18), 11926-11929.

Krisky, D., Wolfe, D., Goins, W., Marconi, P., Ramakrishnan, R., Mata, M., Rouse, R., Fink, D. & Glorioso, J. (1998) Deletion of multiple immediate-early genes from herpes simplex virus

reduces cytotoxicity and permits long-term gene expression in neurons. *Gene Ther,* 5(12), 1593-603.

Kristie, T. M., Vogel, J. L. & Sears, A. E. (1999) Nuclear localization of the c1 factor (host cell factor) in sensory neurons correlates with reactivation of herpes simplex virus from latency. *Proceedings of the National Academy of Sciences of the United States of America,* 96(4), 1229-1233.

Kubat, N. J., Amelio, A. L., Giordani, N. V. & Bloom, D. C. (2004) The herpes simplex virus type 1 latency-associated transcript (lat) enhancer/rcr is hyperacetylated during latency independently of lat transcription. *J. Virol.,* 78(22), 12508-12518.

Kuddus, R., Gu, B. & Deluca, N. (1995) Relationship between tata-binding protein and herpes simplex virus type 1 icp4 DNA-binding sites in complex formation and repression of transcription. *J. Virol.,* 69(9), 5568-5575.

Kumar-Singh, R. & Farber, D. (1998) Encapsidated adenovirus mini-chromosome-mediated delivery of genes to the retina: Application to the rescue of photoreceptor degeneration. *Hum. Mol. Genet.,* 7(12), 1893-1900.

Kumar, P., Lee, S. K., Shankar, P. & Manjunath, N. (2006) A single sirna suppresses fatal encephalitis induced by two different flaviviruses. *PLoS Med,* 3(4), e96.

Kumar, P., Wu, H., Mcbride, J. L., Jung, K.-E., Hee Kim, M., Davidson, B. L., Kyung Lee, S., Shankar, P. & Manjunath, N. (2007) Transvascular delivery of small interfering rna to the central nervous system. *Nature,* 448(7149), 39-43.

Kunath, T., Gish, G., Lickert, H., Jones, N., Pawson, T. & Rossant, J. (2003) Transgenic rna interference in es cell-derived embryos recapitulates a genetic null phenotype. *Nat Biotech,* 21(5), 559-561.

Kunkel, G. & Peterson, T. (1988) Upstream elements required for efficient transcription of a human u6 rna gene resemble those of u1 and u2 genes even though a different polymerase is used. *Genes & Development,* 2(2), 196-204.

Kunkel, G. R., Maser, R. L., Calvet, J. P. & Pederson, T. (1986) U6 small nuclear rna is transcribed by rna polymerase iii. *Proceedings of the National Academy of Sciences of the United States of America,* 83(22), 8575-8579.

Kurihara, Y. & Watanabe, Y. (2004) Arabidopsis micro-rna biogenesis through dicer-like 1 protein functions. *Proceedings of the National Academy of Sciences of the United States of America,* 101(34), 12753-12758.

Kurreck, J. (2003) Antisense technologies. *European Journal of Biochemistry,* 270(8), 1628-1644.

Kwong, A. D. & Frenkel, N. (1989) The herpes simplex virus virion host shutoff function. *J. Virol.,* 63(11), 4834-4839.

La Boissière, S., Hughes, T. & O'hare, P. (1999) Hcf-dependent nuclear import of vp16. *EMBO J.,* 18(2), 480-9.

Lachmann, R. & Efstathiou, S. (1997) Utilization of the herpes simplex virus type 1 latency-associated regulatory region to drive stable reporter gene expression in the nervous system. *J. Virol.,* 71(4), 3197-3207.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage t4. *Nature,* 227(5259), 680-685.

Lagunoff, M. & Roizman, B. (1994) Expression of a herpes simplex virus 1 open reading frame antisense to the gamma(1)34.5 gene and transcribed by an rna 3' coterminal with the unspliced latency-associated transcript. *J. Virol.,* 68(9), 6021-6028.

Lagunoff, M. & Roizman, B. (1995) The regulation of synthesis and properties of the protein product of open reading frame p of the herpes simplex virus 1 genome. *J. Virol.,* 69(6), 3615-3623.

Laird, F. M., Cai, H., Savonenko, A. V., Farah, M. H., He, K., Melnikova, T., Wen, H., Chiang, H.-C., Xu, G., Koliatsos, V. E., Borchelt, D. R., Price, D. L., Lee, H.-K. & Wong, P. C. (2005) Bace1, a major determinant of selective vulnerability of the brain to amyloid-{beta} amyloidogenesis, is essential for cognitive, emotional, and synaptic functions. *J. Neurosci.,* 25(50), 11693-11709.

Lam, Q., Smibert, C., Koop, K., Lavery, C., Capone, J., Weinheimer, S. & Smiley, J. (1996) Herpes simplex virus vp16 rescues viral mrna from destruction by the virion host shutoff function. *EMBO J.,* 15(10), 2575-81.

Landgraf, P., Rusu, M., Sheridan, R., Sewer, A., Iovino, N., Aravin, A., Pfeffer, S., Rice, A., Kamphorst, A. O., Landthaler, M., Lin, C., Socci, N. D., Hermida, L., Fulci, V., Chiaretti, S., Foà, R., Schliwka, J., Fuchs, U., Novosel, A., Müller, R.-U., Schermer, B., Bissels, U., Inman, J., Phan, Q., Chien, M., Weir, D. B., Choksi, R., De Vita, G., Frezzetti, D., Trompeter, H.-I., Hornung, V., Teng, G., Hartmann, G., Palkovits, M., Di Lauro, R., Wernet, P., Macino, G., Rogler, C. E., Nagle, J. W., Ju, J., Papavasiliou, F. N., Benzing, T., Lichter, P., Tam, W., Brownstein, M. J., Bosio, A., Borkhardt, A., Russo, J. J., Sander, C., Zavolan, M. & Tuschl, T. (2007) A mammalian microrna expression atlas based on small rna library sequencing. *Cell,* 129(7), 1401-1414.

Landy, A. (1989) Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annual Review of Biochemistry,* 58(1), 913-941.

Lawrence, M., Ho, D., Sun, G., Steinberg, G. & Sapolsky, R. (1996) Overexpression of bcl-2 with herpes simplex virus vectors protects cns neurons against neurological insults in vitro and in vivo. *J. Neurosci.,* 16(2), 486-496.

Leclere, P., Panjwani, A., Docherty, R., Berry, M., Pizzey, J. & Tonge, D. (2005) Effective gene delivery to adult neurons by a modified form of electroporation. *J Neurosci Methods,* 142(1), 137-43.

Lee, L. Y. & Schaffer, P. A. (1998) A virus with a mutation in the icp4-binding site in the l/st promoter of herpes simplex virus type 1, but not a virus with a mutation in open reading frame p, exhibits cell-type-specific expression of gamma 134.5 transcripts and latency-associated transcripts. *J. Virol.,* 72(5), 4250-4264.

Lee, M., Abina, M., Haddada, H. & Perricaudet, M. (1995) The constitutive expression of the immunomodulatory gp19k protein in e1-, e3- adenoviral vectors strongly reduces the host cytotoxic t cell response against the vector. *Gene Ther,* 2(4), 256-62.

Lee, R. C., Feinbaum, R. L. & Ambros, V. (1993) The c. Elegans heterochronic gene lin-4 encodes small rnas with antisense complementarity to lin-14. *Cell,* 75(5), 843-854.

Lee, S. H., Kim, S. H. & Park, T. G. (2007) Intracellular sirna delivery system using polyelectrolyte complex micelles prepared from vegf sirna-peg conjugate and cationic fusogenic peptide. *Biochemical and Biophysical Research Communications,* 357(2), 511-516.

Lee, Y., Ahn, C., Han, J., Choi, H., Kim, J., Yim, J., Lee, J., Provost, P., Radmark, O., Kim, S. & Kim, V. N. (2003) The nuclear rnase iii drosha initiates microrna processing. *Nature,* 425(6956), 415-419.

Lee, Y., Hur, I., Park, S.-Y., Kim, Y.-K., Suh, M. S. & Kim, V. N. (2006) The role of pact in the rna silencing pathway. *EMBO J.*

Lee, Y., Jeon, K., Lee, J., Kim, S. & Kim, V. (2002) Microrna maturation: Stepwise processing and subcellular localization. *EMBO J.,* 21(17), 4663-70.

Lee, Y. S., Nakahara, K., Pham, J. W., Kim, K., He, Z., Sontheimer, E. J. & Carthew, R. W. (2004) Distinct roles for drosophila dicer-1 and dicer-2 in the sirna/mirna silencing pathways. *Cell,* 117(1), 69-81.

Lehmann, M., Fournier, A., Selles-Navarro, I., Dergham, P., Sebok, A., Leclerc, N., Tigyi, G. & Mckerracher, L. (1999) Inactivation of rho signaling pathway promotes cns axon regeneration. *J. Neurosci.,* 19(17), 7537-7547.

Levine, J. (1994) Increased expression of the ng2 chondroitin-sulfate proteoglycan after brain injury. *J. Neurosci.,* 14(8), 4716-4730.

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

Li, C., Tropak, M., Gerlai, R., Clapoff, S., Abramow-Newerly, W., Trapp, B., Peterson, A. & Roder, J. (1994) Myelination in the absence of myelin-associated glycoprotein. *Nature,* 369(747-50.

Li, L., Lin, X., Khvorova, A., Fesik, S. W. & Shen, Y. (2007) Defining the optimal parameters for hairpin-based knockdown constructs. *RNA,* 13(10), 1765-1774.

Li Liu, J. K. E. P., Mikael Svensson, Håkan Aldskogius, (1998) Glial cell responses, complement, and clusterin in the central nervous system following dorsal root transection. *Glia,* 23(3), 221-238.

Li, S.-D. & Huang, L. (2006) Surface-modified lpd nanoparticles for tumor targeting. *Annals of the New York Academy of Sciences.*

Li, S., Liu, B. P., Budel, S., Li, M., Ji, B., Walus, L., Li, W., Jirik, A., Rabacchi, S., Choi, E., Worley, D., Sah, D. W. Y., Pepinsky, B., Lee, D., Relton, J. & Strittmatter, S. M. (2004a) Blockade of nogo-66, myelin-associated glycoprotein, and oligodendrocyte myelin glycoprotein by soluble nogo-66 receptor promotes axonal sprouting and recovery after spinal injury. *J. Neurosci.,* 24(46), 10511-10520.

Li, S. & Strittmatter, S. M. (2003) Delayed systemic nogo-66 receptor antagonist promotes recovery from spinal cord injury. *J. Neurosci.,* 23(10), 4219-4227.

Li, Y., Carlstedt, T., Berthold, C.-H. & Raisman, G. (2004b) Interaction of transplanted olfactory-ensheathing cells and host astrocytic processes provides a bridge for axons to regenerate across the dorsal root entry zone. *Experimental Neurology,* 188(2), 300-308.

Li, Y. & Raisman, G. (1994) Schwann cells induce sprouting in motor and sensory axons in the adult rat spinal cord. *J. Neurosci.,* 14(7), 4050-4063.

Liebscher, T., Lisa Schnell, Dina Schnell, Jeannette Scholl, Regula Schneider, Mirjam Gullo, Karim Fouad, Anis Mir, Martin Rausch, Diana Kindler, Frank P. T. Hamers, Martin E. Schwab, (2005)

Nogo-a antibody improves regeneration and locomotion of spinal cord-injured rats. *Annals of Neurology,* 58(5), 706-719.

Lilley, C. E., Groutsi, F., Han, Z., Palmer, J. A., Anderson, P. N., Latchman, D. S. & Coffin, R. S. (2001) Multiple immediate-early gene-deficient herpes simplex virus vectors allowing efficient gene delivery to neurons in culture and widespread gene delivery to the central nervous system in vivo. *J. Virol.,* 75(9), 4343-4356.

Lillycrop, K. A., Dent, C. L., Wheatley, S. C., Beech, M. N., Ninkina, N. N., Wood, J. N. & Latchman, D. S. (1991) The octamer-binding protein oct-2 represses hsv immediate-early genes in cell lines derived from latently infectable sensory neurons. 7(3), 381-390.

Lim, L. P., Lau, N. C., Garrett-Engele, P., Grimson, A., Schelter, J. M., Castle, J., Bartel, D. P., Linsley, P. S. & Johnson, J. M. (2005) Microarray analysis shows that some micrornas downregulate large numbers of target mrnas. *Nature,* 433(7027), 769-773.

Lin, X., Ruan, X., Anderson, M. G., Mcdowell, J. A., Kroeger, P. E., Fesik, S. W. & Shen, Y. (2005) Sirna-mediated off-target gene silencing triggered by a 7 nt complementation. *Nucl. Acids Res.,* 33(14), 4527-4535.

Lingel, A., Simon, B., Izaurralde, E. & Sattler, M. (2004) Nucleic acid 3[prime]-end recognition by the argonaute2 paz domain. *Nat Struct Mol Biol,* 11(6), 576-577.

Lingor, P., Koeberle, P., Kugler, S. & Bahr, M. (2005) Down-regulation of apoptosis mediators by rnai inhibits axotomy-induced retinal ganglion cell death in vivo. *Brain,* 128(3), 550-558.

Lingor, P., Michel, U., Schöll, U., Bähr, M. & Kügler, S. (2004) Transfection of "Naked" Sirna results in endosomal uptake and metabolic impairment in cultured neurons. *Biochemical and Biophysical Research Communications,* 315(4), 1126-1133.

Liu, B., Robinson, M., Han, Z., Branston, R., Englis, H. C., Reay, P., Mcgrath, Y., Thomas, S., Thornton, M., Bullock, P., Love, C. & Coffin, R. (2003) Icp34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. *Gene Ther,* 10(4), 292-303.

Liu, C. & Chambers, W. (1958) Intraspinal sprouting of dorsal root axons; development of new collaterals and preterminals following partial denervation of the spinal cord in the cat. *AMA Arch Neurol Psychiatry,* 79(1), 46-61.

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. (2004) Argonaute2 is the catalytic engine of mammalian rnai. *Science,* 305(5689), 1437-1441.

Liu, T., Khanna, K. M., Chen, X., Fink, D. J. & Hendricks, R. L. (2000) Cd8+ t cells can block herpes simplex virus type 1 (hsv-1) reactivation from latency in sensory neurons. *J. Exp. Med.,* 191(9), 1459-1466.

Llave, C., Xie, Z., Kasschau, K. D. & Carrington, J. C. (2002) Cleavage of scarecrow-like mrna targets directed by a class of arabidopsis mirna. *Science,* 297(5589), 2053-2056.

Llorens, F., Vanesa Gil, Susana Iraola, Laura Carim-Todd, Eulàlia Martí, Xavier Estivill, Eduardo Soriano, José Antonio Del Rio & Sumoy, L. (2008) Developmental analysis of lingo-1/lern1 protein expression in the mouse brain: Interaction of its intracellular domain with myt1l. *Developmental Neurobiology,* 68(4), 521-541.

Loers, G., Aboul-Enein, F., Bartsch, U., Lassmann, H. & Schachner, M. (2004) Comparison of myelin, axon, lipid, and immunopathology in the central nervous system of differentially myelin-compromised mutant mice: A morphological and biochemical study. *Mol Cell Neurosci,* 27(2), 175-89.

Lokensgard, J., Berthomme, H. & Feldman, L. (1997) The latency-associated promoter of herpes simplex virus type 1 requires a region downstream of the transcription start site for long-term expression during latency. *J. Virol.,* 71(9), 6714-6719.

Lokensgard, J. R., Bloom, D. C., Dobson, A. T. & Feldman, L. T. (1994) Long-term promoter activity during herpes simplex virus latency. *J. Virol.,* 68(11), 7148-7158.

Lukiw, W. J., Zhao, Y. & Cui, J. G. (2008) An nf-{kappa}b-sensitive micro rna-146a-mediated inflammatory circuit in alzheimer disease and in stressed human brain cells. *J. Biol. Chem.,* 283(46), 31315-31322.

Lund, E., Guttinger, S., Calado, A., Dahlberg, J. E. & Kutay, U. (2004) Nuclear export of microrna precursors. *Science,* 303(5654), 95-98.

Luo, K. Q. & Chang, D. C. (2004) The gene-silencing efficiency of sirna is strongly dependent on the local structure of mrna at the targeted region. *Biochemical and Biophysical Research Communications,* 318(1), 303-310.

Luo, M.-C., Zhang, D.-Q., Ma, S.-W., Huang, Y.-Y., Shuster, S., Porreca, F. & Lai, J. (2005) An efficient intrathecal delivery of small interfering rna to the spinal cord and peripheral neurons. *Molecular Pain,* 1(1), 29.

Ma, J.-B., Ye, K. & Patel, D. J. (2004) Structural basis for overhang-specific small interfering rna recognition by the paz domain. *Nature,* 429(6989), 318-322.

Ma, Z., Li, J., He, F., Wilson, A., Pitt, B. & Li, S. (2005) Cationic lipids enhance sirna-mediated interferon response in mice. *Biochemical and Biophysical Research Communications,* 330(3), 755-759.

Macpherson, I. & Stoker, M. (1962) Polyoma transformation of hamster cell clones--an investigation of genetic factors affecting cell competence. *Virology,* 16(147-51.

Mador, N., Goldenberg, D., Cohen, O., Panet, A. & Steiner, I. (1998) Herpes simplex virus type 1 latency-associated transcripts suppress viral replication and reduce immediate-early gene mrna levels in a neuronal cell line. *J. Virol.,* 72(6), 5067-5075.

Mangeot, P.-E., Cosset, F.-L., Colas, P. & Mikaelian, I. (2004) A universal transgene silencing method based on rna interference. *Nucl. Acids Res.,* 32(12), e102-.

Maniataki, E. & Mourelatos, Z. (2005) A human, atp-independent, risc assembly machine fueled by pre-mirna. *Genes & Development,* 19(24), 2979-2990.

Mardberg, K., Trybala, E., Tufaro, F. & Bergstrom, T. (2002) Herpes simplex virus type 1 glycoprotein c is necessary for efficient infection of chondroitin sulfate-expressing gro2c cells. *J Gen Virol,* 83(2), 291-300.

Margolis, T. P., Bloom, D. C., Dobson, A. T., Feldman, L. T. & Stevens, J. G. (1993) Decreased reporter gene expression during latent infection with hsv lat promoter constructs. *Virology,* 197(2), 585-592.

Maroney, P. A., Yu, Y., Fisher, J. & Nilsen, T. W. (2006) Evidence that microrna s are associated with translating messenger rnas in human cells. *Nat Struct Mol Biol,* 13(12), 1102-1107.

Marshall, K. R., Lachmann, R. H., Efstathiou, S., Rinaldi, A. & Preston, C. M. (2000) Long-term transgene expression in mice infected with a herpes simplex virus type 1 mutant severely impaired for immediate-early gene expression. *J. Virol.,* 74(2), 956-964.

Martina, M.-S., Nicolas, V., Wilhelm, C., Ménager, C., Barratt, G. & Lesieur, S. (2007) The in vitro kinetics of the interactions between peg-ylated magnetic-fluid-loaded liposomes and macrophages. *Biomaterials,* 28(28), 4143-4153.

Mason, M., Lieberman, A. & Anderson, P. (2003) Corticospinal neurons up-regulate a range of growth-associated genes following intracortical, but not spinal, axotomy. *European Journal of Neuroscience,* 18(4), 789-802.

Mathonnet, G., Fabian, M. R., Svitkin, Y. V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W. C., Darzynkiewicz, E., Pillai, R. S., Filipowicz, W., Duchaine, T. F. & Sonenberg, N.

(2007) Microrna inhibition of translation initiation in vitro by targeting the cap-binding complex eif4f. *Science,* 317(5845), 1764-1767.

Matsukura, S., Jones, P. A. & Takai, D. (2003) Establishment of conditional vectors for hairpin sirna knockdowns. *Nucl. Acids Res.,* 31(15), e77-.

Mcbride, J. L., Boudreau, R. L., Harper, S. Q., Staber, P. D., Monteys, A. M., Martins, I. S., Gilmore, B. L., Burstein, H., Peluso, R. W., Polisky, B., Carter, B. J. & Davidson, B. L. (2008) Artificial mirnas mitigate shrna-mediated toxicity in the brain: Implications for the therapeutic development of rnai. *Proceedings of the National Academy of Sciences,* 105(15), 5868-5873.

Mccaffrey, A., Ohashi, K., Meuse, L., Shen, S., Lancaster, A., Lukavsky, P., Sarnow, P. & Kay, M. (2002) Determinants of hepatitis c translational initiation in vitro, in cultured cells and mice. *Mol Ther,* 5(6), 676-84.

Mccaffrey, A. P., Nakai, H., Pandey, K., Huang, Z., Salazar, F. H., Xu, H., Wieland, S. F., Marion, P. L. & Kay, M. A. (2003) Inhibition of hepatitis b virus in mice by rna interference. *Nat Biotech,* 21(6), 639-644.

Mccown, T., Xiao, X., Li, J., Breese, G. & Samulski, R. (1996) Differential and persistent expression patterns of cns gene transfer by an adeno-associated virus (aav) vector. *Brain Res.,* 713(1-2), 99-107.

Mclean, G., Rixon, F., Langeland, N., Haarr, L. & Marsden, H. (1990) Identification and characterization of the virion protein products of herpes simplex virus type 1 gene ul47. *J Gen Virol,* 71(12), 2953-2960.

Mcmanus, M. T., Petersen, C. P., Haines, B. B., Chen, J. & Sharp, P. A. (2002) Gene silencing using micro-rna designed hairpins. *RNA,* 8(6), 842-850.

Mcmenamin, M. M., Byrnes, A. P., Charlton, H. M., Coffin, R. S., Latchman, D. S. & Wood, M. J. A. (1998) A [gamma]34.5 mutant of herpes simplex 1 causes severe inflammation in the brain. *Neuroscience,* 83(4), 1225-1237.

Mcphail, L., Ward T. Plunet, Partha Das & Ramer, M. S. (2005) The astrocytic barrier to axonal regeneration at the dorsal root entry zone is induced by rhizotomy. *European Journal of Neuroscience,* 21(1), 267-270.

Mears, W. & Rice, S. (1998) The herpes simplex virus immediate-early protein icp27 shuttles between nucleus and cytoplasm. *Virology,* 242(1), 128-37.

Mehta, N. R., Lopez, P. H. H., Vyas, A. A. & Schnaar, R. L. (2007) Gangliosides and nogo receptors independently mediate myelin-associated glycoprotein inhibition of neurite outgrowth in different nerve cells. *J. Biol. Chem.,* 282(38), 27875-27886.

Meister, G., Landthaler, M., Patkaniowska, A., Dorsett, Y., Teng, G. & Tuschl, T. (2004) Human argonaute2 mediates rna cleavage targeted by mirnas and sirnas. *Molecular Cell,* 15(2), 185-197.

Meredith, M., Orr, A., Elliott, M. & Everett, R. (1995) Separation of sequence requirements for hsv-1 vmw110 multimerisation and interaction with a 135-kda cellular protein. *Virology,* 209(1), 174-87.

Mi, S., Lee, X., Shao, Z., Thill, G., Ji, B., Relton, J., Levesque, M., Allaire, N., Perrin, S., Sands, B., Crowell, T., Cate, R. L., Mccoy, J. M. & Pepinsky, R. B. (2004) Lingo-1 is a component of the nogo-66 receptor/p75 signaling complex. *Nat Neurosci,* 7(3), 221-228.

Michael, G. J. & Priestley, J. V. (1999) Differential expression of the mrna for the vanilloid receptor subtype 1 in cells of the adult rat dorsal root and nodose ganglia and its downregulation by axotomy. *J. Neurosci.,* 19(5), 1844-1854.

Michael, N. & Roizman, B. (1993) Repression of the herpes simplex virus 1 alpha 4 gene by its gene product occurs within the context of the viral genome and is associated with all three identified cognate sites. *Proc Natl Acad Sci,* 90(6), 2286-90.

Mikol, D., Alexakos, M., Bayley, C., Lemons, R., Le Beau, M. & Stefansson, K. (1990) Structure and chromosomal localization of the gene for the oligodendrocyte-myelin glycoprotein. *J. Cell Biol.,* 111(6), 2673-2679.

Miller, C. S., Danaher, R. J. & Jacob, R. J. (2006) Icp0 is not required for efficient stress-induced reactivation of herpes simplex virus type 1 from cultured quiescently infected neuronal cells. *J. Virol.,* 80(7), 3360-3368.

Miller, T., Brian K. Kaspar, Geert J. Kops, Koji Yamanaka, Lindsey J. Christian, Fred H. Gage & Cleveland., D. W. (2005) Virus-delivered small rna silencing sustains strength in amyotrophic lateral sclerosis. *Annals of Neurology,* 57(5), 773-776.

Milne, R. S. B., Nicola, A. V., Whitbeck, J. C., Eisenberg, R. J. & Cohen, G. H. (2005) Glycoprotein d receptor-dependent, low-ph-independent endocytic entry of herpes simplex virus type 1. *J. Virol.,* 79(11), 6655-6663.

Minakuchi, Y., Takeshita, F., Kosaka, N., Sasaki, H., Yamamoto, Y., Kouno, M., Honma, K., Nagahara, S., Hanai, K., Sano, A., Kato, T., Terada, M. & Ochiya, T. (2004) Atelocollagen-

mediated synthetic small interfering rna delivery for effective gene silencing in vitro and in vivo. *Nucl. Acids Res.,* 32(13), e109-.

Miyagishi, Hidetosh, Hiroyuki Miyoshi, Yutaka Kawakami & Taira, K. (2004) Optimization of an sirna-expression system with an improved hairpin and its significant suppressive effects in mammalian cells. *The Journal of Gene Medicine,* 6(7), 715-723.

Miyagishi, M., Hayashi, M. & Taira, K. (2003) Comparison of the suppressive effects of antisense oligonucleotides and sirnas directed against the same targets in mammalian cells. *Antisense and Nucleic Acid Drug Development,* 13(1), 1-7.

Montag, D., Giese, K., Bartsch, U., Martini, R., Lang, Y., Blüthmann, H., Karthigasan, J., Kirschner, D., Wintergerst, E. & Nave Ka., E. A. (1994) Mice deficient for the myelin-associated glycoprotein show subtle abnormalities in myelin. *Neuron,* 13(1), 229-46.

Montgomery, R., Warner, M., Lum, B. & Spear, P. (1996) Herpes simplex virus-1 entry into cells mediated by a novel member of the tnf/ngf receptor family. *Cell,* 87(3), 427-36.

Moriuchi, S., Krisky, D., Marconi, P., Tamura, M., Shimizu, K., Yoshimine, T., Cohen, J. & Glorioso, J. (2000) Hsv vector cytotoxicity is inversely correlated with effective tk/gcv suicide gene therapy of rat gliosarcoma. *Gene Ther,* 7(17), 1483-90.

Morrissey, D. V., Lockridge, J. A., Shaw, L., Blanchard, K., Jensen, K., Breen, W., Hartsough, K., Machemer, L., Radka, S., Jadhav, V., Vaish, N., Zinnen, S., Vargeese, C., Bowman, K., Shaffer, C. S., Jeffs, L. B., Judge, A., Maclachlan, I. & Polisky, B. (2005) Potent and persistent in vivo anti-hbv activity of chemically modified sirnas. *Nat Biotech,* 23(8), 1002-1007.

Moschos, S. A., Jones, S. W., Perry, M. M., Williams, A. E., Erjefalt, J. S., Turner, J. J., Barnes, P. J., Sproat, B. S., Gait, M. J. & Lindsay, M. A. (2007) Lung delivery studies using sirna conjugated to tat(48−60) and penetratin reveal peptide induced reduction in gene expression and induction of innate immunity. *Bioconjugate Chemistry,* 18(5), 1450-1459.

Mossman, K. L., Sherburne, R., Lavery, C., Duncan, J. & Smiley, J. R. (2000) Evidence that herpes simplex virus vp16 is required for viral egress downstream of the initial envelopment event. *J. Virol.,* 74(14), 6287-6299.

Mossman, K. L. & Smiley, J. R. (1999) Truncation of the c-terminal acidic transcriptional activation domain of herpes simplex virus vp16 renders expression of the immediate-early genes almost entirely dependent on icp0. *J. Virol.,* 73(12), 9726-9733.

Mourelatos, Z., Dostie, J. E., Paushkin, S., Sharma, A., Charroux, B., Abel, L., Rappsilber, J., Mann, M. & Dreyfuss, G. (2002) Mirnps: A novel class of ribonucleoproteins containing numerous micrornas. *Genes & Development,* 16(6), 720-728.

Mukhopadhyay, G., Doherty, P., Walsh, F., Crocker, P. & Filbin, M. (1994) A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron,* 13(3), 757-67.

Murashov, A. K., Chintalgattu, V., Islamov, R. R., Lever, T. E., Pak, E. S., Sierpinski, P. L., Katwa, L. C. & Van Scott, M. R. (2007) Rnai pathway is functional in peripheral nerve axons. *FASEB J.,* 21(3), 656-670.

Muratovska, A. & Eccles, M. (2004) Conjugate for efficient delivery of short interfering rna (sirna) into mammalian cells. *FEBS Lett,* 558(1-3), 63-8.

Myers and Ferrell, J. (2005) Silencing gene expression with dicer-generated sirna pools. *Methods Mol Biol,* 309(93-196.

Myers, J. W. & Ferrell, J. E. (2005) Silencing gene expression with dicer-generated sirna pools. *Methods Mol Biol,* 309(93-196.

Naldini, L., Blömer, U., Gage, F., Trono, D. & Verma, I. (1996) Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector. *Proc Natl Acad Sci,* 93(21), 11382-8.

Neumann, S., Bradke, F., Tessier-Lavigne, M. & Basbaum, A. I. (2002) Regeneration of sensory axons within the injured spinal cord induced by intraganglionic camp elevation. 34(6), 885-893.

Neumann, S. & Woolf, C. J. (1999) Regeneration of dorsal column fibers into and beyond the lesion site following adult spinal cord injury. 23(1), 83-91.

Nicola, A. V., Hou, J., Major, E. O. & Straus, S. E. (2005) Herpes simplex virus type 1 enters human epidermal keratinocytes, but not neurons, via a ph-dependent endocytic pathway. *J. Virol.,* 79(12), 7609-7616.

Niederost, B., Oertle, T., Fritsche, J., Mckinney, R. A. & Bandtlow, C. E. (2002) Nogo-a and myelin-associated glycoprotein mediate neurite growth inhibition by antagonistic regulation of rhoa and rac1. *J. Neurosci.,* 22(23), 10368-10376.

Nishina, K., Unno, T., Uno, Y., Kubodera, T., Kanouchi, T., Mizusawa, H. & Yokota, T. (2008) Efficient in vivo delivery of sirna to the liver by conjugation of [alpha]-tocopherol. *Mol Ther,* 16(4), 734-740.

Noma, K.-I., Sugiyama, T., Cam, H., Verdel, A., Zofall, M., Jia, S., Moazed, D. & Grewal, S. I. S. (2004) Rits acts in cis to promote rna interference-mediated transcriptional and post-transcriptional silencing. *Nat Genet,* 36(11), 1174-1180.

Noonberg, S. B., Scott, G. K. & Benz, C. C. (1996) Evidence of post-transcriptional regulation of u6 small nuclear rna. *J. Biol. Chem.,* 271(18), 10477-10481.

Nottrott, S., Simard, M. J. & Richter, J. D. (2006) Human let-7a mirna blocks protein production on actively translating polyribosomes. *Nat Struct Mol Biol,* 13(12), 1108-1114.

O'hare, P., Goding, C. & Haigh, A. (1988) Direct combinatorial interaction between a herpes simplex virus regulatory protein and a cellular octamer-binding factor mediates specific induction of virus immediate-early gene expression. *EMBO J.,* 7(13), 4231-8.

O'hare, P. & Hayward, G. S. (1985a) Evidence for a direct role for both the 175,000- and 110,000-molecular-weight immediate-early proteins of herpes simplex virus in the transactivation of delayed-early promoters. *J. Virol.,* 53(3), 751-760.

O'hare, P. & Hayward, G. S. (1985b) Three trans-acting regulatory proteins of herpes simplex virus modulate immediate-early gene expression in a pathway involving positive and negative feedback regulation. *J. Virol.,* 56(3), 723-733.

Oertle, T., Van Der Haar, M. E., Bandtlow, C. E., Robeva, A., Burfeind, P., Buss, A., Huber, A. B., Simonen, M., Schnell, L., Brosamle, C., Kaupmann, K., Vallon, R. & Schwab, M. E. (2003) Nogo-a inhibits neurite outgrowth and cell spreading with three discrete regions. *J. Neurosci.,* 23(13), 5393-5406.

Oh, J. & Fraser, N. W. (2008) Temporal association of the herpes simplex virus genome with histone proteins during a lytic infection. *J. Virol.,* 82(7), 3530-3537.

Okada, S., Nakamura, M., Katoh, H., Miyao, T., Shimazaki, T., Ishii, K., Yamane, J., Yoshimura, A., Iwamoto, Y., Toyama, Y. & Okano, H. (2006) Conditional ablation of stat3 or socs3 discloses a dual role for reactive astrocytes after spinal cord injury. *Nat Med,* 12(7), 829-834.

Okamura, K., Chung, W.-J., Ruby, J. G., Guo, H., Bartel, D. P. & Lai, E. C. (2008) The drosophila hairpin rna pathway generates endogenous short interfering rnas. *Nature,* 453(7196), 803-806.

Okamura, K., Hagen, J. W., Duan, H., Tyler, D. M. & Lai, E. C. (2007) The mirtron pathway generates microrna-class regulatory rnas in drosophila. *Cell,* 130(1), 89-100.

Okamura, K., Ishizuka, A., Siomi, H. & Siomi, M. C. (2004) Distinct roles for argonaute proteins in small rna-directed rna cleavage pathways. *Genes & Development,* 18(14), 1655-1666.

Olsen, P. H. & Ambros, V. (1999) The lin-4 regulatory rna controls developmental timing in caenorhabditis elegans by blocking lin-14 protein synthesis after the initiation of translation. *Developmental Biology,* 216(2), 671-680.

Omidi, Y., Hollins, A. J., Benboubetra, M., Drayton, R., Benter, I. F. & Akhtar, S. (2003) Toxicogenomics of non-viral vectors for gene therapy: A microarray study of lipofectin- and oligofectamine-induced gene expression changes in human epithelial cells. *Journal of Drug Targeting,* 11(6), 311-323.

Ong, S. T., Li, F., Du, J., Tan, Y. W. & Wang, S. (2005) Hybrid cytomegalovirus enhancerâ€“h1 promoter-based plasmid and baculovirus vectors mediate effective rna interference. *Human Gene Therapy,* 16(12), 1404-1412.

Orr, H. T., Chung, M.-Y., Banfi, S., Kwiatkowski, T. J., Servadio, A., Beaudet, A. L., Mccall, A. E., Duvick, L. A., Ranum, L. P. W. & Zoghbi, H. Y. (1993) Expansion of an unstable trinucleotide cag repeat in spinocerebellar ataxia type 1. *Nat Genet,* 4(3), 221-226.

Oudega, M., Rosano, C., Sadi, D., Wood, P., Schwab, M. & Hagg, T. (2000) Neutralizing antibodies against neurite growth inhibitor ni-35/250 do not promote regeneration of sensory axons in the adult rat spinal cord. *Neuroscience,* 100(4), 873-83.

Paddison, P. J., Caudy, A. A. & Hannon, G. J. (2002) Stable suppression of gene expression by rnai in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America,* 99(3), 1443-1448.

Paddison, P. J., Silva, J. M., Conklin, D. S., Schlabach, M., Li, M., Aruleba, S., Balija, V., O'shaughnessy, A., Gnoj, L., Scobie, K., Chang, K., Westbrook, T., Cleary, M., Sachidanandam, R., Richard Mccombie, W., Elledge, S. J. & Hannon, G. J. (2004) A resource for large-scale rna-interference-based screens in mammals. *Nature,* 428(6981), 427-431.

Pak, J. & Fire, A. (2007) Distinct populations of primary and secondary effectors during rnai in c. Elegans. *Science,* 315(5809), 241-244.

Pal-Bhadra, M., Leibovitch, B. A., Gandhi, S. G., Rao, M., Bhadra, U., Birchler, J. A. & Elgin, S. C. R. (2004) Heterochromatic silencing and hp1 localization in drosophila are dependent on the rnai machinery. *Science,* 303(5658), 669-672.

Palella, T. D., Silverman, L. J., Schroll, C. T., Homa, F. L., Levine, M. & Kelley, W. N. (1988) Herpes simplex virus-mediated human hypoxanthine-guanine phosphoribosyltransferase gene transfer into neuronal cells. *Mol. Cell. Biol.,* 8(1), 457-460.

Palliser, D., Chowdhury, D., Wang, Q.-Y., Lee, S. J., Bronson, R. T., Knipe, D. M. & Lieberman, J. (2006) An sirna-based microbicide protects mice from lethal herpes simplex virus 2 infection. *Nature,* 439(7072), 89-94.

Palmer, J. A., Branston, R. H., Lilley, C. E., Robinson, M. J., Groutsi, F., Smith, J., Latchman, D. S. & Coffin, R. S. (2000) Development and optimization of herpes simplex virus vectors for multiple long-term gene delivery to the peripheral nervous system. *J. Virol.,* 74(12), 5604-5618.

Pan, B., Fromholt, S., Hess, E., Crawford, T., Griffin, J., Sheikh, K. & Schnaar, R. (2005) Myelin-associated glycoprotein and complementary axonal ligands, gangliosides, mediate axon stability in the cns and pns: Neuropathology and behavioral deficits in single- and double-null mice. *Exp Neurol,* 195(1), 208-17.

Papp, I., Mette, M. F., Aufsatz, W., Daxinger, L., Schauer, S. E., Ray, A., Van Der Winden, J., Matzke, M. & Matzke, A. J. M. (2003) Evidence for nuclear processing of plant micro rna and short interfering rna precursors. *Plant Physiol.,* 132(3), 1382-1390.

Park, J. B., Yiu, G., Kaneko, S., Wang, J., Chang, J. & He, Z. (2005) A tnf receptor family member, troy, is a coreceptor with nogo receptor in mediating the inhibitory activity of myelin inhibitors. 45(3), 345-351.

Park, W., Li, J., Song, R., Messing, J. & Chen, X. (2002) Carpel factory, a dicer homolog, and hen1, a novel protein, act in microrna metabolism in arabidopsis thaliana. *Current Biology,* 12(17), 1484-1495.

Parker, J., Roe, S. & Barford, D. (2004) Crystal structure of a piwi protein suggests mechanisms for sirna recognition and slicer activity. *EMBO J.,* 23(4727–4737.

Parry, C., Bell, S., Minson, T. & Browne, H. (2005) Herpes simplex virus type 1 glycoprotein h binds to {alpha}v{beta}3 integrins. *J Gen Virol,* 86(1), 7-10.

Paterna, J., Moccetti, T., Mura, A., Feldon, J. & Büeler, H. (2000) Influence of promoter and whv post-transcriptional regulatory element on aav-mediated transgene expression in the rat brain. *Gene Ther,* 7(15), 1304-11.

Paul, C. P., Good, P. D., Winer, I. & Engelke, D. R. (2002) Effective expression of small interfering rna in human cells. *Nat Biotech,* 20(5), 505-508.

Pebernard, S. & Iggo, R. (2004) Determinants of interferon-stimulated gene induction by rnai vectors. *Differentiation,* 72(103-11.

Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S. & Stoeckli, E. T. (2003) Screening for gene function in chicken embryo using rnai and electroporation. *Nat Biotech,* 21(1), 93-96.

Peng, T., Ponce-De-Leon, M., Jiang, H., Dubin, G., Lubinski, J. M., Eisenberg, R. J. & Cohen, G. H. (1998) The gh-gl complex of herpes simplex virus (hsv) stimulates neutralizing antibody and protects mice against hsv type 1 challenge. *J. Virol.,* 72(1), 65-72.

Perez, M. C. P., Hunt, S. P., Coffin, R. S. & Palmer, J. A. (2004) Comparative analysis of genomic hsv vectors for gene delivery to motor neurons following peripheral inoculation in vivo. *Gene Ther,* 11(13), 1023-1032.

Perng, G.-C., Jones, C., Ciacci-Zanella, J., Stone, M., Henderson, G., Yukht, A., Slanina, S. M., Hofman, F. M., Ghiasi, H., Nesburn, A. B. & Wechsler, S. L. (2000) Virus-induced neuronal apoptosis blocked by the herpes simplex virus latency-associated transcript. *Science,* 287(5457), 1500-1503.

Perng, G., Ghiasi, H., Slanina, S., Nesburn, A. & Wechsler, S. (1996) The spontaneous reactivation function of the herpes simplex virus type 1 lat gene resides completely within the first 1.5 kilobases of the 8.3- kilobase primary transcript. *J. Virol.,* 70(2), 976-984.

Perng, G. C., Dunkel, E. C., Geary, P. A., Slanina, S. M., Ghiasi, H., Kaiwar, R., Nesburn, A. B. & Wechsler, S. L. (1994) The latency-associated transcript gene of herpes simplex virus type 1 (hsv-1) is required for efficient in vivo spontaneous reactivation of hsv-1 from latency. *J. Virol.,* 68(12), 8045-8055.

Petersen, C. P., Bordeleau, M.-E., Pelletier, J. & Sharp, P. A. (2006) Short rnas repress translation after initiation in mammalian cells. *Molecular Cell,* 21(4), 533-542.

Pfeffer, S., Zavolan, M., Grasser, F. A., Chien, M., Russo, J. J., Ju, J., John, B., Enright, A. J., Marks, D., Sander, C. & Tuschl, T. (2004) Identification of virus-encoded microrнas. *Science,* 304(5671), 734-736.

Pillai, R., Artus, C. & Filipowicz, W. (2004) Tethering of human ago proteins to mrna mimics the mirna-mediated repression of protein synthesis. *RNA,* 10(10), 1518-1525.

Pillai, R. S., Bhattacharyya, S. N., Artus, C. G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E. & Filipowicz, W. (2005) Inhibition of translational initiation by let-7 microrna in human cells. *Science,* 309(5740), 1573-1576.

Pomonis, J. D., Harrison, J. E., Mark, L., Bristol, D. R., Valenzano, K. J. & Walker, K. (2003) N-(4-tertiarybutylphenyl)-4-(3-cholorphyridin-2-yl)tetrahydropyrazine -1(2h)-carbox-amide (bctc), a novel, orally effective vanilloid receptor 1 antagonist with analgesic properties: Ii.

In vivo characterization in rat models of inflammatory and neuropathic pain. *J Pharmacol Exp Ther,* 306(1), 387-393.

Post, L. & Roizman, B. (1981) A generalized technique for deletion of specific genes in large genomes: Alpha gene 22 of herpes simplex virus 1 is not essential for growth. *Cell,* 25(1), 227-32.

Poy, M. N., Eliasson, L., Krutzfeldt, J., Kuwajima, S., Ma, X., Macdonald, P. E., Pfeffer, S., Tuschl, T., Rajewsky, N., Rorsman, P. & Stoffel, M. (2004) A pancreatic islet-specific microrna regulates insulin secretion. *Nature,* 432(7014), 226-230.

Prakash, T. P., Allerson, C. R., Dande, P., Vickers, T. A., Sioufi, N., Jarres, R., Baker, B. F., Swayze, E. E., Griffey, R. H. & Bhat, B. (2005) Positional effect of chemical modifications on short interference rna activity in mammalian cells. *Journal of Medicinal Chemistry,* 48(13), 4247-4253.

Preston, C. M., Mabbs, R. & Nicholl, M. J. (1997) Construction and characterization of herpes simplex virus type 1 mutants with conditional defects in immediate early gene expression. *Virology,* 229(1), 228-239.

Qiu, J., Cai, D., Dai, H., Mcatee, M., Hoffman, P. N., Bregman, B. S. & Filbin, M. T. (2002) Spinal axon regeneration induced by elevation of cyclic amp. 34(6), 895-903.

Ralph, G. S., Radcliffe, P. A., Day, D. M., Carthy, J. M., Leroux, M. A., Lee, D. C. P., Wong, L.-F., Bilsland, L. G., Greensmith, L., Kingsman, S. M., Mitrophanous, K. A., Mazarakis, N. D. & Azzouz, M. (2005) Silencing mutant sod1 using rnai protects against neurodegeneration and extends survival in an als model. *Nat Med,* 11(43), 429-433.

Ramer, M., Bishop, T., Dockery, P., Mobarak, M., O'leary, D., Fraher, J., Priestley, J. & Mcmahon, S. (2002) Neurotrophin-3-mediated regeneration and recovery of proprioception following dorsal rhizotomy. *Mol Cell Neurosci,* 19(2), 239-49.

Ramer, M. S., Duraisingam, I., Priestley, J. V. & Mcmahon, S. B. (2001) Two-tiered inhibition of axon regeneration at the dorsal root entry zone. *J. Neurosci.,* 21(8), 2651-2660.

Ramer, M. S., Priestley, J. V. & Mcmahon, S. B. (2000) Functional regeneration of sensory axons into the adult spinal cord. *Nature,* 403(6767), 312-316.

Rand, T. A., Ginalski, K., Grishin, N. V. & Wang, X. (2004) Biochemical identification of argonaute 2 as the sole protein required for rna-induced silencing complex activity. *Proceedings of the National Academy of Sciences of the United States of America,* 101(40), 14385-14389.

Randall, G., Lagunoff, M. & Roizman, B. (1997) The product of orf o located within the domain of herpes simplex virus 1 genome transcribed during latent infection binds to and inhibits in vitro binding of infected cell protein 4 to its cognate dnaâ€‰site. *Proceedings of the National Academy of Sciences of the United States of America,* 94(19), 10379-10384.

Randall, G., Lagunoff, M. & Roizman, B. (2000) Herpes simplex virus 1 open reading frames o and p are not necessary for establishment of latent infection in mice. *J. Virol.,* 74(19), 9019-9027.

Randall, G. & Roizman, B. (1997) Transcription of the derepressed open reading frame p of herpes simplex virus 1 precludes the expression of the antisense gamma(1)34.5 gene and may account for the attenuation of the mutant virus. *J. Virol.,* 71(10), 7750-7757.

Raoul, C., Abbas-Terki, T., Bensadoun, J.-C., Guillot, S., Haase, G., Szulc, J., Henderson, C. E. & Aebischer, P. (2005) Lentiviral-mediated silencing of sod1 through rna interference retards disease onset and progression in a mouse model of als. *Nat Med,* 11(43), 423-428.

Reddy, R. (1988) Transcription of a u6 small nuclear rna gene in vitro. Transcription of a mouse u6 small nuclear rna gene in vitro by rna polymerase iii is dependent on transcription factor(s) different from transcription factors iiia, iiib, and iiic. *J. Biol. Chem.,* 263(31), 15980-15984.

Reier, P., Perlow, M. & Guth, L. (1983) Development of embryonic spinal cord transplants in the rat. *Brain Res.,* 312(2), 201-19.

Reinhart, B. J. & Bartel, D. P. (2002) Small rnas correspond to centromere heterochromatic repeats. *Science,* 297(5588), 1831-.

Reinhart, B. J., Weinstein, E. G., Rhoades, M. W., Bartel, B. & Bartel, D. P. (2002) Micrornas in plants. *Genes & Development,* 16(13), 1616-1626.

Reynolds, A., Anderson, E. M., Vermeulen, A., Fedorov, Y., Robinson, K., Leake, D., Karpilow, J., Marshall, W. S. & Khvorova, A. (2006) Induction of the interferon response by sirna is cell typeâ€“ and duplex lengthâ€“dependent. *RNA,* 12(6), 988-993.

Reynolds, A., Leake, D., Boese, Q., Scaringe, S., Marshall, W. S. & Khvorova, A. (2004) Rational sirna design for rna interference. *Nat Biotech,* 22(3), 326-330.

Rice, S., Long, M., Lam, V., Schaffer, P. & Spencer, C. (1995) Herpes simplex virus immediate-early protein icp22 is required for viral modification of host rna polymerase ii and establishment of the normal viral transcription program. *J. Virol.,* 69(9), 5550-5559.

Rice, S. A. & Lam, V. (1994) Amino acid substitution mutations in the herpes simplex virus icp27 protein define an essential gene regulation function. *J. Virol.,* 68(2), 823-833.

Richardson, P. & Issa, V. (1984) Peripheral injury enhances central regeneration of primary sensory neurones. *Nature,* 309(791-3.

Richardson, P., Mcguinness, U. & Aguayo, A. (1980) Axons from cns neurons regenerate into pns grafts. *Nature,* 284(264-5.

Rivas, F. V., Tolia, N. H., Song, J.-J., Aragon, J. P., Liu, J., Hannon, G. J. & Joshua-Tor, L. (2005) Purified argonaute2 and an sirna form recombinant human risc. *Nat Struct Mol Biol,* 12(4), 340-349.

Rodriguez, A., Griffiths-Jones, S., Ashurst, J. L. & Bradley, A. (2004) Identification of mammalian microrna host genes and transcription units. *Genome Research,* 14(10a), 1902-1910.

Roop, C., Hutchinson, L. & Johnson, D. C. (1993) A mutant herpes simplex virus type 1 unable to express glycoprotein l cannot enter cells, and its particles lack glycoprotein h. *J. Virol.,* 67(4), 2285-2297.

Rubinson, D. A., Dillon, C. P., Kwiatkowski, A. V., Sievers, C., Yang, L., Kopinja, J., Rooney, D. L., Zhang, M., Ihrig, M. M., Mcmanus, M. T., Gertler, F. B., Scott, M. L. & Van Parijs, L. (2003) A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by rna interference. *Nat Genet,* 33(3), 401-406.

Ruby, J. G., Jan, C. H. & Bartel, D. P. (2007) Intronic microrna precursors that bypass drosha processing. *Nature,* 448(7149), 83-86.

Sabbioni, S., Callegari, E., Manservigi, M., Argnani, R., Corallini, A., Negrini, M. & Manservigi, R. (2006) Use of herpes simplex virus type 1-based amplicon vector for delivery of small interfering rna. *Gene Ther,* 14(5), 459-464.

Sacks, W. R. & Schaffer, P. A. (1987) Deletion mutants in the gene encoding the herpes simplex virus type 1 immediate-early protein icp0 exhibit impaired growth in cell culture. *J. Virol.,* 61(3), 829-839.

Sadowski, I., Ma, J., Triezenberg, S. & Ptashne, M. (1988) Gal4-vp16 is an unusually potent transcriptional activator. *Nature,* 335(6190), 563-4.

Saeki, Y., Fraefel, C., Ichikawa, T., Breakefield, X. O. & Chiocca, E. A. (2001) Improved helper virus-free packaging system for hsv amplicon vectors using an icp27-deleted, oversized hsv-1 DNA in a bacterial artificial chromosome. *Mol Ther,* 3(4), 591-601.

Saenz, D. T., Loewen, N., Peretz, M., Whitwam, T., Barraza, R., Howell, K. G., Holmes, J. M., Good, M. & Poeschla, E. M. (2004) Unintegrated lentivirus DNA persistence and accessibility

to expression in nondividing cells: Analysis with class i integrase mutants. *J. Virol.,* 78(6), 2906-2920.

Saiki, R., Scharf, S., Faloona, F., Mullis, K., Horn, G., Erlich, H. & Arnheim, N. (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science,* 230(4732), 1350-4.

Saito, K., Nishida, K. M., Mori, T., Kawamura, Y., Miyoshi, K., Nagami, T., Siomi, H. & Siomi, M. C. (2006) Specific association of piwi with rasirnas derived from retrotransposon and heterochromatic regions in the drosophila genome. *Genes & Development,* 20(16), 2214-2222.

Samaniego, L., Webb, A. & Deluca, N. (1995) Functional interactions between herpes simplex virus immediate-early proteins during infection: Gene expression as a consequence of icp27 and different domains of icp4. *J. Virol.,* 69(9), 5705-5715.

Samaniego, L., Wu, N. & Deluca, N. (1997) The herpes simplex virus immediate-early protein icp0 affects transcription from the viral genome and infected-cell survival in the absence of icp4 and icp27. *J. Virol.,* 71(6), 4614-4625.

Samaniego, L. A., Neiderhiser, L. & Deluca, N. A. (1998) Persistence and expression of the herpes simplex virus genome in the absence of immediate-early proteins. *J. Virol.,* 72(4), 3307-3320.

Sandri-Goldin, R. M. (1998) Icp27 mediates hsv rna export by shuttling through a leucine-rich nuclear export signal and binding viral intronless rnas through an rggâ€‰motif. *Genes & Development,* 12(6), 868-879.

Sandvig, A., Martin Berry, Lee B. Barrett, Arthur Butt & Ann Logan (2004) Myelin-, reactive glia-, and scar-derived cns axon growth inhibitors: Expression, receptor signaling, and correlation with axon regeneration. *Glia,* 46(3), 225-251.

Sapru, M., Yates, J., Hogan, S., Jiang, L., Halter, J. & Bohn, M. (2006) Silencing of human alpha-synuclein in vitro and in rat brain using lentiviral-mediated rnai. *Exp Neurol,* 198(2), 382-90.

Sasaki, T., Shiohama, A., Minoshima, S. & Shimizu, N. (2003) Identification of eight members of the argonaute family in the human genome[small star, filled]. *Genomics,* 82(3), 323-330.

Sato, Y., Murase, K., Kato, J., Kobune, M., Sato, T., Kawano, Y., Takimoto, R., Takada, K., Miyanishi, K., Matsunaga, T., Takayama, T. & Niitsu, Y. (2008) Resolution of liver cirrhosis using vitamin a-coupled liposomes to deliver sirna against a collagen-specific chaperone. *Nat Biotech,* 26(4), 431-442.

Sawtell, N. M. & Thompson, R. L. (1992) Rapid in vivo reactivation of herpes simplex virus in latently infected murine ganglionic neurons after transient hyperthermia. *J. Virol.,* 66(4), 2150-2156.

Saydam, O., Glauser, D. L., Heid, I., Turkeri, G., Hilbe, M., Jacobs, A. H., Ackermann, M. & Fraefel, C. (2005) Herpes simplex virus 1 amplicon vector-mediated sirna targeting epidermal growth factor receptor inhibits growth of human glioma cells in vivo. *Mol Ther,* 12(5), 803-812.

Saydam, O., Saydam, N., Glauser, D. L., Pruschy, M., Dinh-Van, V., Hilbe, M., Jacobs, A. H., Ackermann, M. & Fraefel, C. (2007) Hsv-1 amplicon-mediated post-transcriptional inhibition of rad51 sensitizes human glioma cells to ionizing radiation. *Gene Ther,* 14(15), 1143-1151.

Schachner, M., Udo Bartsch, (2000) Multiple functions of the myelin-associated glycoprotein mag (siglec-4a) in formation and maintenance of myelin. *Glia,* 29(2), 154-165.

Schecterson, L. C. & Bothwell, M. (1992) Novel roles for neurotrophins are suggested by bdnf and nt-3 mrna expression in developing neurons. *Neuron,* 9(3), 449-463.

Scherer, L. J., Frank, R. & Rossi, J. J. (2007) Optimization and characterization of trna-shrna expression constructs. *Nucl. Acids Res.,* 35(8), 2620-2628.

Schnell, L. & Schwab, M. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature,* 343(269 - 272.

Schramke, V. & Allshire, R. (2003) Hairpin rnas and retrotransposon ltrs effect rnai and chromatin-based gene silencing. *Science,* 301(5636), 1069-1074.

Schratt, G. M., Tuebing, F., Nigh, E. A., Kane, C. G., Sabatini, M. E., Kiebler, M. & Greenberg, M. E. (2006) A brain-specific microrna regulates dendritic spine development. *Nature,* 439(7074), 283-289.

Schreyer, D. & Skene, J. (1993) Injury-associated induction of gap-43 expression displays axon branch specificity in rat dorsal root ganglion neurons. *Journal of Neurobiology,* 24(7), 959-970.

Schwab, M. & Caroni, P. (1988) Oligodendrocytes and cns myelin are nonpermissive substrates for neurite growth and fibroblast spreading in vitro. *J. Neurosci.,* 8(7), 2381-2393.

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

Sciabica, K., Dai, Q. & Sandri-Goldin, R. (2003) Icp27 interacts with srpk1 to mediate hsv splicing inhibition by altering sr protein phosphorylation. *EMBO J.,* 22(7), 1608-19.

Sears, A. E., Halliburton, I. W., Meignier, B., Silver, S. & Roizman, B. (1985) Herpes simplex virus 1 mutant deleted in the alpha 22 gene: Growth and gene expression in permissive and restrictive cells and establishment of latency in mice. *J. Virol.,* 55(2), 338-346.

Sears, A. E., Hukkanen, V., Labow, M. A., Levine, A. J. & Roizman, B. (1991) Expression of the herpes simplex virus 1 alpha transinducing factor (vp16) does not induce reactivation of latent virus or prevent the establishment of latency in mice. *J. Virol.,* 65(6), 2929-2935.

Seggerson, K., Tang, L. & Moss, E. G. (2002) Two genetic circuits repress the caenorhabditis elegans heterochronic gene lin-28 after translation initiation. *Developmental Biology,* 243(2), 215-225.

Sekulovich, R. E., Leary, K. & Sandri-Goldin, R. M. (1988) The herpes simplex virus type 1 alpha protein icp27 can act as a trans-repressor or a trans-activator in combination with icp4 and icp0. *J. Virol.,* 62(12), 4510-4522.

Selkoe, D. J. & Schenk, D. (2003) Alzheimer's disease: Molecular understanding predicts amyloid-based therapeutics. *Annual Review of Pharmacology and Toxicology,* 43(1), 545-584.

Seltzer, Z., Dubner, R. & Shir, Y. (1990) A novel behavioral model of neuropathic pain disorders produced in rats by partial sciatic nerve injury. *Pain,* 43(2), 205-18.

Semizarov, D., Frost, L., Sarthy, A., Kroeger, P., Halbert, D. N. & Fesik, S. W. (2003) Specificity of short interfering rna determined through gene expression signatures. *Proceedings of the National Academy of Sciences of the United States of America,* 100(11), 6347-6352.

Sen, G. L. & Blau, H. M. (2005) Argonaute 2/risc resides in sites of mammalian mrna decay known as cytoplasmic bodies. *Nat Cell Biol,* 7(6), 633-636.

Shao, Z., Browning, J. L., Lee, X., Scott, M. L., Shulga-Morskaya, S., Allaire, N., Thill, G., Levesque, M., Sah, D., Mccoy, J. M., Murray, B., Jung, V., Pepinsky, R. B. & Mi, S. (2005) Taj/troy, an orphan tnf receptor family member, binds nogo-66 receptor 1 and regulates axonal regeneration. 45(3), 353-359.

Shen, B. & Goodman, H. M. (2004) Uridine addition after microrna-directed cleavage. *Science,* 306(5698), 997-.

Shieh, M., Wudunn, D., Montgomery, R., Esko, J. & Spear, P. (1992) Cell surface receptors for herpes simplex virus are heparan sulfate proteoglycans. *J. Cell Biol.,* 116(5), 1273-1281.

Shimeld, C., Whiteland, J., Williams, N., Easty, D. & Hill, T. (1997) Cytokine production in the nervous system of mice during acute and latent infection with herpes simplex virus type 1. *J Gen Virol,* 78(12), 3317-3325.

Shukla, D., Liu, J., Blaiklock, P., Shworak, N., Bai, X., Esko, J., Cohen, G., Eisenberg, R., Rosenberg, R. & Spear, P. (1999) A novel role for 3-o-sulfated heparan sulfate in herpes simplex virus 1 entry. *Cell,* 99(1), 13-22.

Sijen, T., Steiner, F. A., Thijssen, K. L. & Plasterk, R. H. A. (2007) Secondary sirnas result from unprimed rna synthesis and form a distinct class. *Science,* 315(5809), 244-247.

Silva, J. M., Li, M. Z., Chang, K., Ge, W., Golding, M. C., Rickles, R. J., Siolas, D., Hu, G., Paddison, P. J., Schlabach, M. R., Sheth, N., Bradshaw, J., Burchard, J., Kulkarni, A., Cavet, G., Sachidanandam, R., Mccombie, W. R., Cleary, M. A., Elledge, S. J. & Hannon, G. J. (2005) Second-generation shrna libraries covering the mouse and human genomes. *Nat Genet,* 37(11), 1281-1288.

Simonen, M., Pedersen, V., Weinmann, O., Schnell, L., Buss, A., Ledermann, B., Christ, F., Sansig, G., Van Der Putten, H. & Schwab, M. E. (2003) Systemic deletion of the myelin-associated outgrowth inhibitor nogo-a improves regenerative and plastic responses after spinal cord injury. 38(2), 201-211.

Singer, O., Marr, R. A., Rockenstein, E., Crews, L., Coufal, N. G., Gage, F. H., Verma, I. M. & Masliah, E. (2005) Targeting bace1 with sirnas ameliorates alzheimer disease neuropathology in a transgenic model. *Nat Neurosci,* 8(10), 1343-1349.

Sinha, S., Anderson, J. P., Barbour, R., Basi, G. S., Caccavello, R., Davis, D., Doan, M., Dovey, H. F., Frigon, N., Hong, J., Jacobson-Croak, K., Jewett, N., Keim, P., Knops, J., Lieberburg, I., Power, M., Tan, H., Tatsuno, G., Tung, J., Schenk, D., Seubert, P., Suomensaari, S. M., Wang, S., Walker, D., Zhao, J., Mcconlogue, L. & John, V. (1999) Purification and cloning of amyloid precursor protein [beta]-secretase from human brain. *Nature,* 402(6761), 537-540.

Sledz, C. A. & Williams, B. R. G. (2004) Rna interference and double-stranded-rna-activated pathways. *Biochem. Soc. Trans.,* 32(Pt 6), 952-956.

Smiley, J. & Duncan, J. (1997) Truncation of the c-terminal acidic transcriptional activation domain of herpes simplex virus vp16 produces a phenotype similar to that of the in1814 linker insertion mutation. *J. Virol.,* 71(8), 6191-6193.

Smiley, J. R., Johnson, D. C., Pizer, L. I. & Everett, R. D. (1992) The icp4 binding sites in the herpes simplex virus type 1 glycoprotein d (gd) promoter are not essential for efficient gd transcription during virus infection. *J. Virol.,* 66(2), 623-631.

Smith, C. A., Bates, P., Rivera-Gonzalez, R., Gu, B. & Deluca, N. A. (1993) Icp4, the major transcriptional regulatory protein of herpes simplex virus type 1, forms a tripartite complex with tata-binding protein and tfiib. *J. Virol.,* 67(8), 4676-4687.

Sodeik, B., Ebersold, M. W. & Helenius, A. (1997) Microtubule-mediated transport of incoming herpes simplex virus 1 capsids to the nucleus. *J. Cell Biol.,* 136(5), 1007-1021.

Soliman, T., Sandri-Goldin, R. & Silverstein, S. (1997) Shuttling of the herpes simplex virus type 1 regulatory protein icp27 between the nucleus and cytoplasm mediates the expression of late proteins. *J. Virol.,* 71(12), 9188-9197.

Song, E., Lee, S.-K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P. & Lieberman, J. (2003) Rna interference targeting fas protects mice from fulminant hepatitis. *Nat Med,* 9(3), 347-351.

Song, E., Zhu, P., Lee, S.-K., Chowdhury, D., Kussman, S., Dykxhoorn, D. M., Feng, Y., Palliser, D., Weiner, D. B., Shankar, P., Marasco, W. A. & Lieberman, J. (2005) Antibody mediated in vivo delivery of small interfering rnas via cell-surface receptors. *Nat Biotech,* 23(6), 709-717.

Song, J.-J., Smith, S. K., Hannon, G. J. & Joshua-Tor, L. (2004a) Crystal structure of argonaute and its implications for risc slicer activity. *Science,* 305(5689), 1434-1437.

Song, X.-Y., Zhong, J.-H., Wang, X. & Zhou, X.-F. (2004b) Suppression of p75ntr does not promote regeneration of injured spinal cord in mice. *J. Neurosci.,* 24(2), 542-546.

Sørensen, D. R., Leirdal, M. & Sioud, M. (2003) Gene silencing by systemic delivery of synthetic sirnas in adult mice. *Journal of Molecular Biology,* 327(4), 761-766.

Soutschek, J., Akinc, A., Bramlage, B., Charisse, K., Constien, R., Donoghue, M., Elbashir, S., Geick, A., Hadwiger, P., Harborth, J., John, M., Kesavan, V., Lavine, G., Pandey, R. K., Racie, T., Rajeev, K. G., Rohl, I., Toudjarska, I., Wang, G., Wuschko, S., Bumcrot, D., Koteliansky, V., Limmer, S., Manoharan, M. & Vornlocher, H.-P. (2004) Therapeutic silencing of an endogenous gene by systemic administration of modified sirnas. *Nature,* 432(7014), 173-178.

Spaete, R. R. & Frenkel, N. (1982) The herpes simplex virus amplicon: A new eucaryotic defective-virus cloning-amplifying vector. 30(1), 295-304.

Spillantini, M. G., Schmidt, M. L., Lee, V. M. Y., Trojanowski, J. Q., Jakes, R. & Goedert, M. (1997) [alpha]-synuclein in lewy bodies. *Nature,* 388(6645), 839-840.

Stark, A., Brennecke, J., Bushati, N., Russell, R. B. & Cohen, S. M. (2005) Animal micrornas confer robustness to gene expression and have a significant impact on 3'utr evolution. *Cell,* 123(6), 1133-1146.

Stark, G. R., Kerr, I. M., Williams, B. R. G., Silverman, R. H. & Schreiber, R. D. (1998) How cells respond to interferons. *Annual Review of Biochemistry,* 67(1), 227-264.

Steiner, I., Spivack, J. G., Deshmane, S. L., Ace, C. I., Preston, C. M. & Fraser, N. W. (1990) A herpes simplex virus type 1 mutant containing a nontransinducing vmw65 protein establishes latent infection in vivo in the absence of viral replication and reactivates efficiently from explanted trigeminal ganglia. *J. Virol.,* 64(4), 1630-1638.

Steinmetz, M. P., Horn, K. P., Tom, V. J., Miller, J. H., Busch, S. A., Nair, D., Silver, D. J. & Silver, J. (2005) Chronic enhancement of the intrinsic growth capacity of sensory neurons combined with the degradation of inhibitory proteoglycans allows functional regeneration of sensory axons through the dorsal root entry zone in the mammalian spinal cord. *J. Neurosci.,* 25(35), 8066-8076.

Stevens, J. G., Wagner, E. K., Devi-Rao, G. B., Cook, M. L. & Feldman, L. T. (1987) Rna complementary to a herpesvirus $\alpha $ gene mrna is prominent in latently infected neurons. *Science,* 235(4792), 1056-1059.

Steward, O., Sharp, K., Yee, K. M. & Hofstadter, M. (2008) A re-assessment of the effects of a nogo-66 receptor antagonist on regenerative growth of axons and locomotor recovery after spinal cord injury in mice. *Experimental Neurology,* 209(2), 446-468.

Stow, N. D. & Stow, E. C. (1986) Isolation and characterization of a herpes simplex virus type 1 mutant containing a deletion within the gene encoding the immediate early polypeptide vmw110. *J Gen Virol,* 67(12), 2571-2585.

Stow, N. D. & Wilkie, N. M. (1976) An improved technique for obtaining enhanced infectivity with herpes simplex virus type 1 DNA. *J Gen Virol,* 33(3), 447-458.

Su, L. & Knipe, D. (1989) Herpes simplex virus alpha protein icp27 can inhibit or augment viral gene transactivation. *Virology,* 170(2), 496-504.

Suh, M.-R., Lee, Y., Kim, J. Y., Kim, S.-K., Moon, S.-H., Lee, J. Y., Cha, K.-Y., Chung, H. M., Yoon, H. S., Moon, S. Y., Kim, V. N. & Kim, K.-S. (2004) Human embryonic stem cells express a unique set of micrornas. *Developmental Biology,* 270(2), 488-498.

Sui, G., Soohoo, C., Affar, E. B., Gay, F. D. R., Shi, Y., Forrester, W. C. & Shi, Y. (2002) A DNA vector-based rnai technology to suppress gene expression in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America,* 99(8), 5515-5520.

Sullivan, C. S., Grundhoff, A. T., Tevethia, S., Pipas, J. M. & Ganem, D. (2005) Sv40-encoded micrornas regulate viral gene expression and reduce susceptibility to cytotoxic t cells. *Nature,* 435(7042), 682-686.

Sun, D., Melegari, M., Sridhar, S., Rogler, C. & Zhu, L. (2006) Multi-mirna hairpin method that improves gene knockdown efficiency and provides linked multi-gene knockdown. *Biotechniques,* 41(1), 59-63.

Szallasi, A., Cortright, D. N., Blum, C. A. & Eid, S. R. (2007) The vanilloid receptor trpv1: 10 years from channel cloning to antagonist proof-of-concept. *Nat Rev Drug Discov,* 6(5), 357-372.

Tabara, H., Yigit, E., Siomi, H. & Mello, C. C. (2002) The dsrna binding protein rde-4 interacts with rde-1, dcr-1, and a dexh-box helicase to direct rnai in c. Elegans. *Cell,* 109(7), 861-871.

Takeshita, F., Minakuchi, Y., Nagahara, S., Honma, K., Sasaki, H., Hirai, K., Teratani, T., Namatame, N., Yamamoto, Y., Hanai, K., Kato, T., Sano, A. & Ochiya, T. (2005) Efficient delivery of small interfering rna to bone-metastatic tumors by using atelocollagen in vivo. *Proceedings of the National Academy of Sciences of the United States of America,* 102(34), 12177-12182.

Tal-Singer, R., Peng, C., Ponce De Leon, M., Abrams, W., Banfield, B., Tufaro, F., Cohen, G. & Eisenberg, R. (1995) Interaction of herpes simplex virus glycoprotein gc with mammalian cell surface molecules. *J. Virol.,* 69(7), 4471-4483.

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. (2008) Pseudogene-derived small interfering rnas regulate gene expression in mouse oocytes. *Nature,* 453(7194), 534-538.

Tam, W. & Dahlberg, J. (2006) Mir-155/bic as an oncogenic microrna. *Genes, Chromosomes and Cancer,* 45(2), 211-212.

Tan, P. H., Yang, L. C., Shih, H. C., Lan, K. C. & Cheng, J. T. (2004a) Gene knockdown with intrathecal sirna of nmda receptor nr2b subunit reduces formalin-induced nociception in the rat. *Gene Ther,* 12(1), 59-66.

Tan, W., Zhu, K., Segal, D. J., Barbas, C. F., Iii & Chow, S. A. (2004b) Fusion proteins consisting of human immunodeficiency virus type 1 integrase and the designed polydactyl zinc finger protein e2c direct integration of viral DNA into specific sites. *J. Virol.,* 78(3), 1301-1313.

Tanabe, K., Bonilla, I., Winkles, J. A. & Strittmatter, S. M. (2003) Fibroblast growth factor-inducible-14 is induced in axotomized neurons and promotes neurite outgrowth. *J. Neurosci.,* 23(29), 9675-9686.

Tang, G., Reinhart, B. J., Bartel, D. P. & Zamore, P. D. (2003) A biochemical framework for rna silencing in plants. *Genes & Development,* 17(1), 49-63.

Taylor, L., Jones, L., Tuszynski, M. H. & Blesch, A. (2006) Neurotrophin-3 gradients established by lentiviral gene delivery promote short-distance axonal bridging beyond cellular grafts in the injured spinal cord. *J. Neurosci.,* 26(38), 9713-9721.

Tazi, J., Forne, T., Jeanteur, P., Cathala, G. & Brunel, C. (1993) Mammalian u6 small nuclear rna undergoes 3' end modifications within the spliceosome. *Mol. Cell. Biol.,* 13(3), 1641-1650.

Tetzlaff, W., Alexander, S., Miller, F. & Bisby, M. (1991) Response of facial and rubrospinal neurons to axotomy: Changes in mrna expression for cytoskeletal proteins and gap-43. *J. Neurosci.,* 11(8), 2528-2544.

Thakker, D. R., Natt, F., Husken, D., Van Der Putten, H., Maier, R., Hoyer, D. & Cryan, J. F. (2005) Sirna-mediated knockdown of the serotonin transporter in the adult mouse brain. *Mol Psychiatry,* 10(8), 782-789.

Thermann, R. & Hentze, M. W. (2007) Drosophila mir2 induces pseudo-polysomes and inhibits translation initiation. *Nature,* 447(7146), 875-878.

Thomas, C. E., Schiedner, G., Kochanek, S., Castro, M. G. & Lã¶Wenstein, P. R. (2000) Peripheral infection with adenovirus causes unexpected long-term brain inflammation in animals injected intracranially with first-generation, but not with high-capacity, adenovirus vectors: Toward realistic long-term neurological gene therapy for chronic diseases. *Proceedings of the National Academy of Sciences of the United States of America,* 97(13), 7482-7487.

Thomas, S. K., Gough, G., Latchman, D. S., R. S. & Coffin (1999a) Herpes simplex virus latency-associated transcript encodes a protein which greatly enhances virus growth, can compensate for deficiencies in immediate-early gene expression, and is likely to function during reactivation from virus latency. *J. Virol.,* 73(8), 6618-6625.

Thomas, S. K., Lilley, C. E., Latchman, D. S. & Coffin, R. S. (1999b) Equine herpesvirus 1 gene 12 can substitute for vmw65 in the growth of herpes simplex virus (hsv) type 1, allowing the generation of optimized cell lines for the propagation of hsv vectors with multiple immediate-early gene defects. *J. Virol.,* 73(9), 7399-7409.

Thomas, S. K., Lilley, C. E., Latchman, D. S. & Coffin, R. S. (2002) A protein encoded by the herpes simplex virus (hsv) type 1 2-kilobase latency-associated transcript is phosphorylated, localized to the nucleus, and overcomes the repression of expression from exogenous promoters when inserted into the quiescent hsv genome. *J. Virol.,* 76(8), 4056-4067.

Thompson, R. & Sawtell, N. (1997) The herpes simplex virus type 1 latency-associated transcript gene regulates the establishment of latency. *J. Virol.,* 71(7), 5432-5440.

Thompson, R. L. & Sawtell, N. M. (2006) Evidence that the herpes simplex virus type 1 icp0 protein does not initiate reactivation from latency in vivo. *J. Virol.,* 80(22), 10919-10930.

Tiscornia, G., Singer, O., Ikawa, M. & Verma, I. M. (2003) A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering rna. *Proceedings of the National Academy of Sciences of the United States of America,* 100(4), 1844-1848.

Tiscornia, G., Tergaonkar, V., Galimi, F. & Verma, I. M. (2004) Cre recombinase-inducible rna interference mediated by lentiviral vectors. *Proceedings of the National Academy of Sciences of the United States of America,* 101(19), 7347-7351.

Tolia, N. H. & Joshua-Tor, L. (2007) Slicer and the argonautes. *Nat Chem Biol,* 3(1), 36-43.

Tom, V. J., Steinmetz, M. P., Miller, J. H., Doller, C. M. & Silver, J. (2004) Studies on the development and behavior of the dystrophic growth cone, the hallmark of regeneration failure, in an in vitro model of the glial scar and after spinal cord injury. *J. Neurosci.,* 24(29), 6531-6539.

Tomari, Y., Du, T. & Zamore, P. D. (2007) Sorting of drosophila small silencing rnas. *Cell,* 130(2), 299-308.

Tominaga, M., Caterina, M. J., Malmberg, A. B., Rosen, T. A., Gilbert, H., Skinner, K., Raumann, B. E., Basbaum, A. I. & Julius, D. (1998) The cloned capsaicin receptor integrates multiple pain-producing stimuli. 21(3), 531-543.

Tonges, L., Lingor, P., Egle, R., Dietz, G. P. H., Fahr, A. & Bã¤Hr, M. (2006) Stearylated octaarginine and artificial virus-like particles for transfection of sirna into primary rat neurons. *RNA,* 12(7), 1431-1438.

Towbin, H., Staehelin, T. & Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proceedings of the National Academy of Sciences of the United States of America,* 76(9), 4350-4354.

Trousdale, M. D., Steiner, I., Spivack, J. G., Deshmane, S. L., Brown, S. M., Maclean, A. R., Subak-Sharpe, J. H. & Fraser, N. W. (1991) In vivo and in vitro reactivation impairment of a herpes simplex virus type 1 latency-associated transcript variant in a rabbit eye model. *J. Virol.,* 65(12), 6989-6993.

Turner, G. C. & Varshavsky, A. (2000) Detecting and measuring cotranslational protein degradation in vivo. *Science,* 289(5487), 2117-2120.

Unwalla, H. J., Li, M.-J., Kim, J. D., Li, H. T., Ehsani, A., Alluin, J. & Rossi, J. J. (2004) Negative feedback inhibition of hiv-1 by tat-inducible expression of sirna. *Nat Biotech,* 22(12), 1573-1578.

Uprichard, S. & Knipe, D. (1996) Herpes simplex icp27 mutant viruses exhibit reduced expression of specific DNA replication genes. *J. Virol.,* 70(3), 1969-1980.

Urban-Klein, B., Werth, S., Abuharbeid, S., Czubayko, F. & Aigner, A. (2004) Rnai-mediated gene-targeting through systemic application of polyethylenimine (pei)-complexed sirna in vivo. *Gene Ther,* 12(5), 461-466.

Vagin, V. V., Sigova, A., Li, C., Seitz, H., Gvozdev, V. & Zamore, P. D. (2006) A distinct small rna pathway silences selfish genetic elements in the germline. *Science,* 313(5785), 320-324.

Valyi-Nagy, T., Deshmane, S. L., Spivack, J. G., Steiner, I., Ace, C. I., Preston, C. M. & Fraser, N. W. (1991) Investigation of herpes simplex virus type 1 (hsv-1) gene expression and DNA synthesis during the establishment of latent infection by an hsv-1 mutant, in1814, that does not replicate in mouse trigeminal ganglia. *J Gen Virol,* 72(3), 641-649.

Van De Wetering, M., Oving, I., Muncan, V., Pon Fong, M., Brantjes, H., Van Leenen, D., Holstege, F., Brummelkamp, T., Agami, R. & Clevers, H. (2003) Specific inhibition of gene expression using a stably integrated, inducible small-interfering-rna vector *EMBO reports,* 4(6), 609-615.

Van Den Haute, C., Eggermont, K., Nuttin, B., Debyser, Z. & Baekelandt, V. (2003) Lentiviral vector-mediated delivery of short hairpin rna results in persistent knockdown of gene expression in mouse brain. *Human Gene Therapy,* 14(18), 1799-1807.

Vasudevan, S. & Steitz, J. A. (2007) Au-rich-element-mediated upregulation of translation by fxr1 and argonaute 2. *Cell,* 128(6), 1105-1118.

Vasudevan, S. & Steitz, J. A. (2008) Cell-cycle control of microrna-mediated translation regulation. *Cell Cycle,* 7(11), 1545-9.

Vasudevan, S., Tong, Y. & Steitz, J. A. (2007) Switching from repression to activation: Microrna can up-regulate translation. *Science,* 318(5858), 1931-1934.

Vazquez, F., Vaucheret, H., Rajagopalan, R., Lepers, C., Gasciolli, V., Mallory, A. C., Hilbert, J.-L., Bartel, D. P. & Crété, P. (2004) Endogenous trans-acting sirnas regulate the accumulation of arabidopsis mrnas. *Molecular Cell,* 16(1), 69-79.

Venkatesh, K., Chivatakarn, O., Lee, H., Joshi, P. S., Kantor, D. B., Newman, B. A., Mage, R., Rader, C. & Giger, R. J. (2005) The nogo-66 receptor homolog ngr2 is a sialic acid-

dependent receptor selective for myelin-associated glycoprotein. *J. Neurosci.,* 25(4), 808-822.

Ventura, A., Meissner, A., Dillon, C. P., Mcmanus, M., Sharp, P. A., Van Parijs, L., Jaenisch, R. & Jacks, T. (2004) Cre-lox-regulated conditional rna interference from transgenes. *Proceedings of the National Academy of Sciences of the United States of America,* 101(28), 10380-10385.

Verdel, A., Jia, S., Gerber, S., Sugiyama, T., Gygi, S., Grewal, S. I. S. & Moazed, D. (2004) Rnai-mediated targeting of heterochromatin by the rits complex. *Science,* 303(5658), 672-676.

Verma, U. N., Surabhi, R. M., Schmaltieg, A., Becerra, C. & Gaynor, R. B. (2003) Small interfering rnas directed against {beta}-catenin inhibit the in vitro and in vivo growth of colon cancer cells. *Clin Cancer Res,* 9(4), 1291-1300.

Volpe, T. A., Kidner, C., Hall, I. M., Teng, G., Grewal, S. I. S. & Martienssen, R. A. (2002) Regulation of heterochromatic silencing and histone h3 lysine-9 methylation by rnai. *Science,* 297(5588), 1833-1837.

Vyas, A. A., Patel, H. V., Fromholt, S. E., Heffer-Lauc, M., Vyas, K. A., Dang, J., Schachner, M. & Schnaar, R. L. (2002) Gangliosides are functional nerve cell ligands for myelin-associated glycoprotein (mag), an inhibitor of nerve regeneration. *Proceedings of the National Academy of Sciences of the United States of America,* 99(12), 8412-8417.

Wagner, E., Rice, M. & Sutherland, B. (1975) Photoreactivation of herpes simplex virus in human fibroblasts. *Nature,* 254(5501), 627-8.

Wagstaff, M., Lilley, C., Smith, J., Robinson, M., Coffin, R. & Latchman, D. (1998) Gene transfer using a disabled herpes virus vector containing the emcv ires allows multiple gene expression in vitro and in vivo. *Gene Ther,* 5(11), 1566-70.

Wakiyama, M., Takimoto, K., Ohara, O. & Yokoyama, S. (2007) Let-7 microrna-mediated mrna deadenylation and translational repression in a mammalian cell-free system. *Genes & Development,* 21(15), 1857-1862.

Walker, J. R., Mcgeagh, K. G., Sundaresan, P., Jorgensen, T. J., Rabkin, S. D. & Martuza, R. L. (1999) Local and systemic therapy of human prostate adenocarcinoma with the conditionally replicating herpes simplex virus vector g207. *Human Gene Therapy,* 10(13), 2237-2243.

Wang, K. C., Kim, J. A., Sivasankaran, R., Segal, R. & He, Z. (2002) P75 interacts with the nogo receptor as a co-receptor for nogo, mag and omgp. *Nature,* 420(6911), 74-78.

Wang, Q.-Y., Zhou, C., Johnson, K. E., Colgrove, R. C., Coen, D. M. & Knipe, D. M. (2005) Herpesviral latency-associated transcript gene promotes assembly of heterochromatin on viral lytic-gene promoters in latent infection. *Proceedings of the National Academy of Sciences of the United States of America,* 102(44), 16055-16059.

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. (2008) Endogenous sirnas from naturally formed dsrnas regulate transcripts in mouse oocytes. *Nature,* 453(7194), 539-543.

Wechsler, S. L., Nesburn, A. B., Watson, R., Slanina, S. M. & Ghiasi, H. (1988) Fine mapping of the latency-related gene of herpes simplex virus type 1: Alternative splicing produces distinct latency-related rnas containing open reading frames. *J. Virol.,* 62(11), 4051-4058.

Weinheimer, S. P., Boyd, B. A., Durham, S. K., Resnick, J. L. & O'boyle, D. R., 2nd (1992) Deletion of the vp16 open reading frame of herpes simplex virus type 1. *J. Virol.,* 66(1), 258-269.

Wightman, B., Ha, I. & Ruvkun, G. (1993) Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in c. Elegans. *Cell,* 75(5), 855-862.

Wilusz, C. J., Wormington, M. & Peltz, S. W. (2001) The cap-to-tail guide to mrna turnover. *Nat Rev Mol Cell Biol,* 2(4), 237-246.

Winton, M. J., Dubreuil, C. I., Lasko, D., Leclerc, N. & Mckerracher, L. (2002) Characterization of new cell permeable c3-like proteins that inactivate rho and stimulate neurite outgrowth on inhibitory substrates. *J. Biol. Chem.,* 277(36), 32820-32829.

Wiznerowicz, M. & Trono, D. (2003) Conditional suppression of cellular genes: Lentivirus vector-mediated drug-inducible rna interference. *J. Virol.,* 77(16), 8957-8951.

Wolfrum, C., Shi, S., Jayaprakash, K. N., Jayaraman, M., Wang, G., Pandey, R. K., Rajeev, K. G., Nakayama, T., Charrise, K., Ndungo, E. M., Zimmermann, T., Koteliansky, V., Manoharan, M. & Stoffel, M. (2007) Mechanisms and optimization of in vivo delivery of lipophilic sirnas. *Nat Biotech,* 25(10), 1149-1157.

Wong, E. V., David, S., Jacob, M. H. & Jay, D. G. (2003) Inactivation of myelin-associated glycoprotein enhances optic nerve regeneration. *J. Neurosci.,* 23(8), 3112-3117.

Wong, S. T., Henley, J. R., Kanning, K. C., Huang, K.-H., Bothwell, M. & Poo, M.-M. (2002) A p75ntr and nogo receptor complex mediates repulsive signaling by myelin-associated glycoprotein. *Nat Neurosci,* 5(12), 1302-1308.

Wright, D. & Snider, W. (1995) Neurotrophin receptor mrna expression defines distinct populations of neurons in rat dorsal root ganglia. *J Comp Neurol,* 351(3), 329-38.

Wu, K. Y., Hengst, U., Cox, L. J., Macosko, E. Z., Jeromin, A., Urquhart, E. R. & Jaffrey, S. R. (2005) Local translation of rhoa regulates growth cone collapse. *Nature,* 436(7053), 1020-1024.

Wu, L., Fan, J. & Belasco, J. G. (2006) Micrornas direct rapid deadenylation of mrna. *Proceedings of the National Academy of Sciences of the United States of America,* 103(11), 4034-4039.

Wu, N., Watkins, S., Schaffer, P. & Deluca, N. (1996) Prolonged gene expression and cell survival after infection by a herpes simplex virus mutant defective in the immediate-early genes encoding icp4, icp27, and icp22. *J. Virol.,* 70(9), 6358-6369.

Wudunn, D. & Spear, P. G. (1989) Initial interaction of herpes simplex virus with cells is binding to heparan sulfate. *J. Virol.,* 63(1), 52-58.

Xia, H., Mao, Q., Eliason, S. L., Harper, S. Q., Martins, I. H., Orr, H. T., Paulson, H. L., Yang, L., Kotin, R. M. & Davidson, B. L. (2004) Rnai suppresses polyglutamine-induced neurodegeneration in a model of spinocerebellar ataxia. *Nat Med,* 10(8), 816-820.

Xia, H., Mao, Q., Paulson, H. L. & Davidson, B. L. (2002) Sirna-mediated gene silencing in vitro and in vivo. *Nat Biotech,* 20(10), 1006-1010.

Xia, X. G., Zhou, H., Ding, H., Affar, E. B., Shi, Y. & Xu, Z. (2003) An enhanced u6 promoter for synthesis of short hairpin rna. *Nucl. Acids Res.,* 31(17), e100-.

Xiao, X., Li, J. & Samulski, R. J. (1998) Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus. *J. Virol.,* 72(3), 2224-2232.

Xie, Z., Allen, E., Wilken, A. & Carrington, J. C. (2005) Dicer-like 4 functions in trans-acting small interfering rna biogenesis and vegetative phase change in arabidopsis thaliana. *Proceedings of the National Academy of Sciences of the United States of America,* 102(36), 12984-12989.

Xie, Z., Johansen, L., Gustafson, A., Kasschau, K., Lellis, A., Zilberman, D., Jacobsen, S. & Carrington, J. (2004a) Genetic and functional diversification of small rna pathways in plants. *PLoS Biol,* 2(5), E104.

Xie, Z., Johansen, L., Gustafson, A., Kasschau, K., Lellis, A., Zilberman, D., Jacobsen, S. & Carrington, J. (2004b) Genetic and functional diversification of small rna pathways in plants. *PLoS Biol,* 2(5), E104.

Yamashita, T., Higuchi, H. & Tohyama, M. (2002) The p75 receptor transduces the signal from myelin-associated glycoprotein to rho. *J. Cell Biol.,* 157(4), 565-570.

Yang, Y., Nunes, F., Berencsi, K., Furth, E., Gönczöl, E. & Wilson, J. (1994a) Cellular immunity to viral antigens limits e1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci,* 91(10), 4407-11.

Yang, Y., Nunes, F. A., Berencsi, K., Gonczol, E., Engelhardt, J. F. & Wilson, J. M. (1994b) Inactivation of e2a in recombinant adenoviruses improves the prospect for gene therapy in cystic fibrosis. *Nat Genet,* 7(3), 362-369.

Yang, Z., Ebright, Y. W., Yu, B. & Chen, X. (2006) Hen1 recognizes 21-24 nt small rna duplexes and deposits a methyl group onto the 2' oh of the 3' terminal nucleotide. *Nucl. Acids Res.,* 34(2), 667-675.

Ye, G.-J., Vaughan, K. T., Vallee, R. B. & Roizman, B. (2000) The herpes simplex virus 1 ul34 protein interacts with a cytoplasmic dynein intermediate chain and targets nuclear membrane. *J. Virol.,* 74(3), 1355-1363.

Yekta, S., Shih, I.-H. & Bartel, D. P. (2004) Microrna-directed cleavage of hoxb8 mrna. *Science,* 304(5670), 594-596.

Yi, R., Doehle, B. P., Qin, Y., Macara, I. G. & Cullen, B. R. (2005) Overexpression of exportin 5 enhances rna interference mediated by short hairpin rnas and microrna. *RNA,* 11(2), 220-226.

Yi, R., Qin, Y., Macara, I. G. & Cullen, B. R. (2003) Exportin-5 mediates the nuclear export of pre-microrna and short hairpin rnas. *Genes & Development,* 17(24), 3011-3016.

Yigit, E., Batista, P. J., Bei, Y., Pang, K. M., Chen, C.-C. G., Tolia, N. H., Joshua-Tor, L., Mitani, S., Simard, M. J. & Mello, C. C. (2006) Analysis of the c. Elegans argonaute family reveals that distinct argonautes act sequentially during rnai. *Cell,* 127(4), 747-757.

Yin, H. & Lin, H. (2007) An epigenetic activation role of piwi and a piwi-associated pirna in drosophila melanogaster. *Nature,* 450(7167), 304-308.

Yiu, G. & He, Z. (2006) Glial inhibition of cns axon regeneration. *Nat Rev Neurosci,* 7(8), 617-627.

Yokota, T., Miyagishi, M., Hino, T., Matsumura, R., Andrea, T., Urushitani, M., Rao, R., Takahashi, R., Bredesen, D., Taira, K. & Mizusawa, H. (2004) Sirna-based inhibition specific for mutant sod1 with single nucleotide alternation in familial als, compared with ribozyme and DNA enzyme. *Biochem. Biophys. Res. Commun.,* 314(1), 283-291.

York, I., Roop, C., Andrews, D., Riddell, S., Graham, F. & Johnson, D. (1994) A cytosolic herpes simplex virus protein inhibits antigen presentation to cd8+ t lymphocytes. *Cell,* 77(4), 525-35.

Yu, J.-Y., Deruiter, S. L. & Turner, D. L. (2002) Rna interference by expression of short-interfering rnas and hairpin rnas in mammalian cells. *Proceedings of the National Academy of Sciences of the United States of America,* 99(9), 6047-6052.

Zeng, Y. & Cullen, B. R. (2003) Sequence requirements for micro rna processing and function in human cells. *RNA,* 9(1), 112-123.

Zeng, Y., Wagner, E. J. & Cullen, B. R. (2002) Both natural and designed micro rnas can inhibit the expression of cognate mrnas when expressed in human cells. 9(6), 1327-1333.

Zhang, H., Kolb, F., Brondani, V., Billy, E. & Filipowicz, W. (2002) Human dicer preferentially cleaves dsrnas at their termini without a requirement for atp. *EMBO J.,* 21(21), 5875–5885.

Zhang, H., Kolb, F. A., Jaskiewicz, L., Westhof, E. & Filipowicz, W. (2004a) Single processing center models for human dicer and bacterial rnase iii. *Cell,* 118(1), 57-68.

Zhang, N., Inan, S., Cowan, A., Sun, R., Wang, J. M., Rogers, T. J., Caterina, M. & Oppenheim, J. J. (2005) A proinflammatory chemokine, ccl3, sensitizes the heat- and capsaicin-gated ion channel trpv1. *Proceedings of the National Academy of Sciences of the United States of America,* 102(12), 4536-4541.

Zhang, Y., Paul A. Dijkhuizen, Patrick N. Anderson, A. Robert Lieberman & Verhaagen, J. (1998) Nt-3 delivered by an adenoviral vector induces injured dorsal root axons to regenerate into the spinal cord of adult rats. *Journal of Neuroscience Research,* 54(4), 554-562.

Zhang, Y., Tohyama, K., Winterbottom, J. K., Haque, N. S. K., Schachner, M., Lieberman, A. R. & Anderson, P. N. (2001) Correlation between putative inhibitory molecules at the dorsal root entry zone and failure of dorsal root axonal regeneration. *Molecular and Cellular Neuroscience,* 17(3), 444-459.

Zhang, Y., Zhang, X., Wu, D., Verhaagen, J., Richardson, P. M., Yeh, J. & Bo, X. (2007) Lentiviral-mediated expression of polysialic acid in spinal cord and conditioning lesion promote regeneration of sensory axons into spinal cord. *Mol Ther,* 15(10), 1796-1804.

Zhang, Y., Zhang, Y.-F., Bryant, J., Charles, A., Boado, R. J. & Pardridge, W. M. (2004b) Intravenous rna interference gene therapy targeting the human epidermal growth factor receptor prolongs survival in intracranial brain cancer. *Clin Cancer Res,* 10(11), 3667-3677.

Zheng, B., Atwal, J., Ho, C., Case, L., He, X.-L., Garcia, K. C., Steward, O. & Tessier-Lavigne, M. (2005) Genetic deletion of the nogo receptor does not reduce neurite inhibition in vitro or promote corticospinal tract regeneration in vivo. *Proceedings of the National Academy of Sciences of the United States of America,* 102(4), 1205-1210.

Zheng, B., Ho, C., Li, S., Keirstead, H., Steward, O. & Tessier-Lavigne, M. (2003) Lack of enhanced spinal regeneration in nogo-deficient mice. 38(2), 213-224.

Zheng, J.-Q., Kelly, T. K., Chang, B., Ryazantsev, S., Rajasekaran, A. K., Martin, K. C. & Twiss, J. L. (2001) A functional role for intra-axonal protein synthesis during axonal regeneration from adult sensory neurons. *J. Neurosci.,* 21(23), 9291-9303.

Zhou, H., Xia, X. G. & Xu, Z. (2005) An rna polymerase ii construct synthesizes short-hairpin rna with a quantitative indicator and mediates highly efficient rnai. *Nucl. Acids Res.,* 33(6), e62-.

Zhu, Z., Cai, W. & Schaffer, P. A. (1994) Cooperativity among herpes simplex virus type 1 immediate-early regulatory proteins: Icp4 and icp27 affect the intracellular localization of icp0. *J. Virol.,* 68(5), 3027-3040.

Zhu, Z., Deluca, N. & Schaffer, P. (1996) Overexpression of the herpes simplex virus type 1 immediate-early regulatory protein, icp27, is responsible for the aberrant localization of icp0 and mutant forms of icp4 in icp4 mutant virus-infected cells. *J. Virol.,* 70(8), 5346-5356.

Zimmermann, T. S., Lee, A. C. H., Akinc, A., Bramlage, B., Bumcrot, D., Fedoruk, M. N., Harborth, J., Heyes, J. A., Jeffs, L. B., John, M., Judge, A. D., Lam, K., Mcclintock, K., Nechev, L. V., Palmer, L. R., Racie, T., Rã¶Hl, I., Seiffert, S., Shanmugam, S., Sood, V., Soutschek, J. R., Toudjarska, I., Wheat, A. J., Yaworski, E., Zedalis, W., Koteliansky, V., Manoharan, M., Vornlocher, H.-P. & Maclachlan, I. (2006) Rnai-mediated gene silencing in non-human primates. *Nature,* 441(7089), 111-114.

Zufferey, R., Donello, J. E., Trono, D. & Hope, T. J. (1999) Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.,* 73(4), 2886-2892.

Zwaagstra, J. C., Ghiasi, H., Nesburn, A. B. & Wechsler, S. L. (1991) Identification of a major regulatory sequence in the latency associated transcript (lat) promoter of herpes simplex virus type 1 (hsv-1). *Virology,* 182(1), 287-297.

Zwaagstra, J. C., Ghiasi, H., Slanina, S. M., Nesburn, A. B., Wheatley, S. C., Lillycrop, K., Wood, J., Latchman, D. S., Patel, K. & Wechsler, S. L. (1990) Activity of herpes simplex virus type 1 latency-associated transcript (lat) promoter in neuron-derived cells: Evidence for neuron specificity and for a large lat transcript. *J. Virol.,* 64(10), 5019-5028.