The Therapeutic Potential of Lentivector-delivered RNAi

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Abstract

Many forms of leukaemia are caused by chromosomal translocations, which result in specific and characteristic genomic sequences. Where these sequences are unique to the leukaemic cells, they represent good candidates for targeting by sequence-specific techniques, such as RNA-Interference (RNAi). RNAi is a mechanism inherent in eukaryotic cells which silences target mRNAs based on homology to a dsRNA template. This template may be introduced artificially by a number of methods, and so this mechanism can be manipulated to regulate the expression of target genes. One of the most efficient methods of introducing RNAi templates is by expression of short-hairpin RNA (shRNA) cassettes from DNA plasmids or vectors.

Lentiviral vectors are based on viruses that integrate into the DNA of the host cell, and are a highly efficient class of vectors for transducing and providing stable transgene expression in a range of cell types. They are particularly effective at tansducing haematopoietic cells, which have proven difficult to transduce by other methods. By fine-tuning the methods of vector production and transduction, a range of human leukaemic cells lines were able to be transduced with unprecedented efficiency in the present study. Lentiviral transduction was combined with rapid puromycin selection to generate a pure population of transduced cells with minimal expansion of the cell population.

The strategy of expressing shRNAs from retroviral and lentiviral vectors combined with puromycin selection was used to target three well-characterised fusion genes; Bcr-Abl, PML/RARα and RUNX1/ETO, in three human leukaemic cell lines. In two of these, the shRNA was able to efficiently and effectively down-regulate the target mRNA, and inhibit the proliferation of the transduced leukaemic cells. In the third case, RUNX1/ETO, no effective shRNA design could be identified.

Finally, concerns over the safety of integration targeting by current gene therapy vectors motivated an investigation of the activity of a novel integrase enzyme from the Ty3 retrotransposon found in yeast. In yeast cells, the integration-mediating enzyme of this retrotransposon has very specific targeting characteristcs, which, if retained in human cells, would provide a very safe gene therapy vector. It was found that this enzyme is indeed active in human cells, and therefore has potential in the context of human gene therapy.

Declaration

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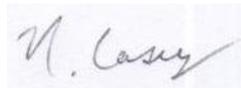
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Publications

Part of the work contained in this thesis has been published or submitted for publication as detailed below:

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Statement of Ethical Conduct

The research associated with this thesis abides by the international and Australian codes on human and animal experimentation, the guidelines of the Australian Government's Office of the Gene Technology Regulator and the rulings of the Safety, Ethics and Institutional Biosafety Committees of the University.

List of Abbreviations

6-FAM	6-CarboxyFluorescein-Aminohexyl Amidite
AAV	Adeno-Associated Virus
Abl	Abelson Leukemia
AE(1/2/3)	AML1-ETO shRNA design (1/2/3)
AIDS	Acquired Immuno Definicency Syndrome
ALL	Acute Lymphoblastic Leukaemia
AML	Acute myeloid Leukaemia
AML1	Acute myeloid Leukaemia-1
AP-1	Activator Protein 1
APL	Acute Promyelocytic Leukemia
ASLV	Avian Sarcoma Leukosis Virus
ASV	Avian Sarcoma Virus
ATCC	American Type Culture Collection
ATO	Arseninc Tetroxide
ATP	Adenosine Triphosphate
ATRA	All Trans-Retinoic Acid
B2M	Beta-2-microglobulin
Bcr	Breakpoint cluster region
BLAST	Basic Local Alignment Search Tool
bp	base pairs
CAG	Cytomegalovirus early enhancer element and chicken beta-actin promoter
CBFA2	Core-Binding Factor, runt domain, Alpha subunit 2
CBP	CREB (cAMP response element-binding)-Binding Protein
CD(nn)	Cluster Differentiation marker number (nn)
CML	Chronic Myelogenous Leukemia
CMV	Cytomegalovirus
CpG	C-phosphate-G
DAI	dsRNA-Activated Inhibitor
DMEM	Dulbecco's Modified Eagle Medium

DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
ECL	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
EF1α	Elongation Factor 1 α
EGFP	Enhanced Green Fluorescent Protein
EGTA	Ethylene Glycol Tetraacetic Acid
EIAV	Equine Infectious Anemia Virus
ERK	Extracellular-signal-Regulated Kinases
ETO	Eight Twenty-One
FAB	French-American-British
FACS	Fluorescence-Activated Cell Sorting
FCS	Foetal Calf Serum
FISH	Fluorescent In Situ Hybridization
FIV	Feline Immunodeficiency Virus
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
G-CSF	Granulocyte Colony-Stimulating Factor
gDNA	Genomic Deoxyribonucleic Acid
GFP	Green Fluorescent Protein
GRB2	Growth Factor Receptor-Bound protein 2
HAT	Histone Acetyl Transferase
HBS	Hepes Buffered Saline
HDAC	Histone Deacetylase
HEK	Human Embryonic Kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HERV	Human Endogenous Retrovirus
HI-FCS	Heat Inactivated Foetal Calf Serum
HIV	Human Immunodeficiency Virus
HSV	Herpes Simplex Virus
IFN	Interferon
IL2	Interleukin-2
IL2RG	Interleukin-2 Receptor subunit gamma

IN	Integrase
IRES	Internal Ribosomal Entry Site
ISG	Interferon-Stimulated Gene
JAK	Janus Kinase
kb	kilo-bases
kDa	kilo-Daltons
K-RAS	Kirsten Rat Sarcoma viral oncogene homolog,
LAM-PCR	Linear Amplification Mediate-Polymerase Chain Reaction
LMO2	LIM domain Only 2
LTR	Long Terminal Repeats
Lys	Lysine
MAPK	MAP (Mitogen-Activated Protein) Kinase
MEK	MAPK/ERK Kinase
MFI	Mean Fluorescence Intensity
miRNA	micro-Ribonucleic Acid
MLV	Murine Leukemia Virus
MoMLV	Moloney Murine Leukemia Virus
mRNA	messenger Ribonucleic Acid
MTG8	Myeloid Translocation Gene 8
N-CoR	Nuclear receptor Co-Repressor 1
nef	negative regulatory factor
NHR	Nuclear Hormone Receptor
NS	Nonsense shRNA design
OAS	Oligoadenylate Synthetase
ORF	Open Reading Frame
PAGE	Poly-Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PEBP2αB	Polyomavirus Enhancer Binding Protein 2 alpha B
(p)ERK	(phosphorylated) Extracellular signal-Regulated Kinase
PIWI	P-element Induced Wimpy testis
PKR	Protein Kinase R

PLZF	Promyelocytic Leukemia Zinc Finger
PML	Promyelocytic Leukemia
PNPP	p-Nitrophenyl Phosphate
POD	PML Oncogenic Domain
PolIII	Polymerase class III
PR(1/2)	PML/RARα shRNA design (1/2)
PUROr	Puromycin resistance gene
PVDF	Polyvinylidene Fluoride
qRT-PCR	Quantitative Reverse Transcript Polymerase Chain Reaction
RA	Retinoic Acid
RARα	Retinoic Acid Receptor alpha
RARE	Retinoic Acid Responsive Element
RAS	Rat Sarcoma
RING	Really Interesting New Gene
RISC	RNA-Induced Silencing Complex
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute
RUNX1	Runt-related transcription factor 1
SDS	Sodium Dodecyl Sulfate
shRNA	short-hairpin Ribonucleic Acid
siRNA	short-interfering Ribonucleic Acid
SMRT	Silencing Mediator for Retinoid and Thyroid-hormone receptors
SNFW	Sterile Nuclease-Free Water
SRC	Sarcoma
SSC	Saline Sodium Citrate
STAT(1/5)	Signal Transducers and Activators of Transcription
SUP2	Suppressor 2
SYBR	Synergy Brands
TBS	Tris Buffered Saline
TFIII	Transcription Factor III
TIF1α	Transcriptional Intermediary Factor 1 alpha
tRNA	transfer RNA

vif	viral infectivity factor
vpr	viral protein R
VSV-G	Vesicular Stomatitis Virus G
WPRE	Woodchuck hepatitis post-transcriptional regulatory element
X-SCID	X-linked Severe Combined Immunodeficiency

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1 Literature Review

1.1 Outline

Cancer is a class of diseases characterised by uncontrolled growth and spread of cells. Cancer therapies ideally inhibit the abnormal proliferation of cancerous cells, or selectively kill the cancerous cells, or both. Leukaemias are cancers of the white blood cells and are frequently caused by chromosomal translocations. They typically arise from stem cell populations resident in the bone marrow. These stem cells are also essential for proper immune function, and targeting this stem cell population without disrupting immune activity is very difficult. Many therapies for leukaemia utilise stem cell transplantation, using cells from either the patient or a closely matched donor. Such strategies allow ablation of stem cells in the marrow, followed by reconstitution of normal function with the transplanted cells.

The number of leukaemias that have been characterised, with sequencing of the molecular lesion, is continually increasing. In many cases, the ultimate cause of the disease is a mutation (translocation) that gives rise to a unique genetic sequence. Thus many leukaemias represent a class of disease caused by specific and characteristic genetic sequences. Molecular therapies are an emerging class of cancer therapy that acts at the genetic level, responding to and influencing the genetic milieu of diseased cells. RNA interference (RNAi), an enzymatic mechanism that is able to specifically degrade the mRNA of unique sequences, has in recent years proven to be both powerful and widely applicable (reviewed in Caplen 2003).

The nature of the cancer causing genomic lesions requires ongoing therapy, and at present, effective and efficient long-term delivery of RNAi represents a major challenge. RNAi may be effected by either short-interfering RNA's (siRNA) or by short-hairpin RNA's (shRNA) (Rao *et al.* 2009). siRNA molecules must be synthesised, and the systemic delivery of these molecules is both expensive and wasteful, as synthesis is expensive, and they have a short half-life *in vivo*. Better targeting of these molecules will enhance efficiency and efficacy.

shRNA molecules are very similar to siRNAs, but may be formed by expression of linear DNA within the target cell (Brummelkamp *et al.* 2002; Yu *et al.* 2002),

providing a cheaper and potentially more efficient alternative (reviewed in Rao *et al.* 2009). The shRNAs may be expressed from cassettes integrated into the genome of the host cell, enabling stable expression of the hairpin and offering long-term protection from the effects of disease-causing genetic sequences (Damm-Welk *et al.* 2003). These cassettes are also compatible with the method of stem cell transplantation commonly used as part of the treatment regimen for leukaemias. This method may be adapted to 'pre-treat' potentially oncogenic stem cells before transplantation, protecting them against the deleterious effects of subsequent expression of the fusion gene. Presently, lentiviral vectors offer the most effective and efficient option for integrating shRNA expression cassettes into the genome of haematopoietic stem cells, and development to enhance the safety and efficiency of these vectors is ongoing.

1.2 Leukaemia

The production of blood cells, or haematopoiesis, begins in embryogenesis and continues throughout life. This complicated process is tightly regulated and requires coordination between extracellular elements, such as cytokines and transcriptional regulators, as well as intracellular factors (reviewed by Alcalay *et al.* 2001). Dysregulation of the haematopoietic processes can lead to leukaemia. Frequently, further aberrations multiply and accumulate, ultimately resulting in blockage of terminal differentiation of the cells, and consequently the blood is overpopulated with immature, proliferative, non-functional blood cells. The broadest classifications of leukaemia are based of the rate of progression of the disease - acute versus chronic - and the origin of the cell population involved; lymphocytic or myelogenous (reviewed by Crans and Sakamoto 2001).

Chromosomal abnormalities such as translocations or inversions are frequently associated with particular types or subtypes of leukaemia, lymphoma or sarcoma. Cloning and characterisation of these breakpoints has greatly facilitated our understanding of the molecular biology of cancer, and has allowed more accurate tailoring of available treatments (Rowley 1998). It can also present novel and cancer-specific targets for the latest gene-silencing therapies (Damm-Welk *et al.* 2003).

Cell lines have been derived from a number of forms of leukaemia and these provide excellent models for *in vitro* studies. In some leukaemias, a large proportion of

cases are due to a common breakpoint location. Cell lines of such leukaemias represent ideal models for testing new molecular therapies. Three such cancers, CML, APL and AML, were examined in this thesis.

1.2.1 Chronic Myelogenous Leukaemia (CML)

Chronic myelogenous leukaemia (CML) is a chronic myeloproliferative disorder of haematopoietic stem cells (Shet *et al.* 2002) resulting in proliferation of myeloid and erythroid cells and platelets in the peripheral blood (Sawyers 1999). CML typically progresses from a benign chronic phase to a rapidly fatal blast crisis within three to five years (Sawyers 1999). CML is associated with an acquired genetic abnormality, the Philadelphia chromosome, which is present in 95 percent of patients. This mutation was first described as a shortened chromosome 22 in 1960 (Nowell and Hungerford 1960) and then as a t(9;22) translocation in 1973 (Rowley 1973). Another 5 percent of patients have various translocations involving additional chromosomes, but with the same end result; the fusion of the *Bcr* gene on chromosome 22 to the *Abl* gene on chromosome 9 (reviewed in Sawyers 1999). Depending on the site of the break point, the translocation results in fusion proteins varying in size from 185 kd to 230 kd. However, patients with chronic-phase CML typically express a 210-kd fusion protein (Sawyers 1999). This 210 kDa protein is necessary and sufficient for malignant transformation (McLaughlin *et al.* 1987; Daley *et al.* 1990; Elefanty *et al.* 1990; Heisterkamp *et al.* 1990; Gishizky and Witte 1992; Gishizky *et al.* 1993).

The *Bcr* or 'Breakpoint cluster region' gene encodes a ubiquitously expressed, 160 kDa protein (Laneuville 1995). Bcr contains a serine threonine kinase near the N-terminus (Figure 1.1), with only one substrate identified to date (Reuther *et al.* 1994), however, there may also be some autophosphorylation activity (Laneuville 1995). The coiled coil domain near the N-terminus allows dimer formation (McWhirter *et al.* 1993), and the *Dbl*- and pleckstrin-homology domains near the middle of Bcr stimulate guanidine exchange on Rho guanidine exchange factors (Denhardt 1996). The C-terminus has also guanidine triphosphatase activity (Diekmann *et al.* 1991). Bcr may be phosphorylated on any of several tyrosine residues (Wu *et al.* 1998), with phosphorylation of the tyrosine at 177 involved in Ras activation (Ma *et al.* 1997). Mice with only one copy of Bcr are phenotypically normal, indicating that loss of a single copy of this gene has minimal effect (Shet *et al.* 2002).

Figure 1.1 Domains of the Bcr, Abl, and Bcr-Abl proteins
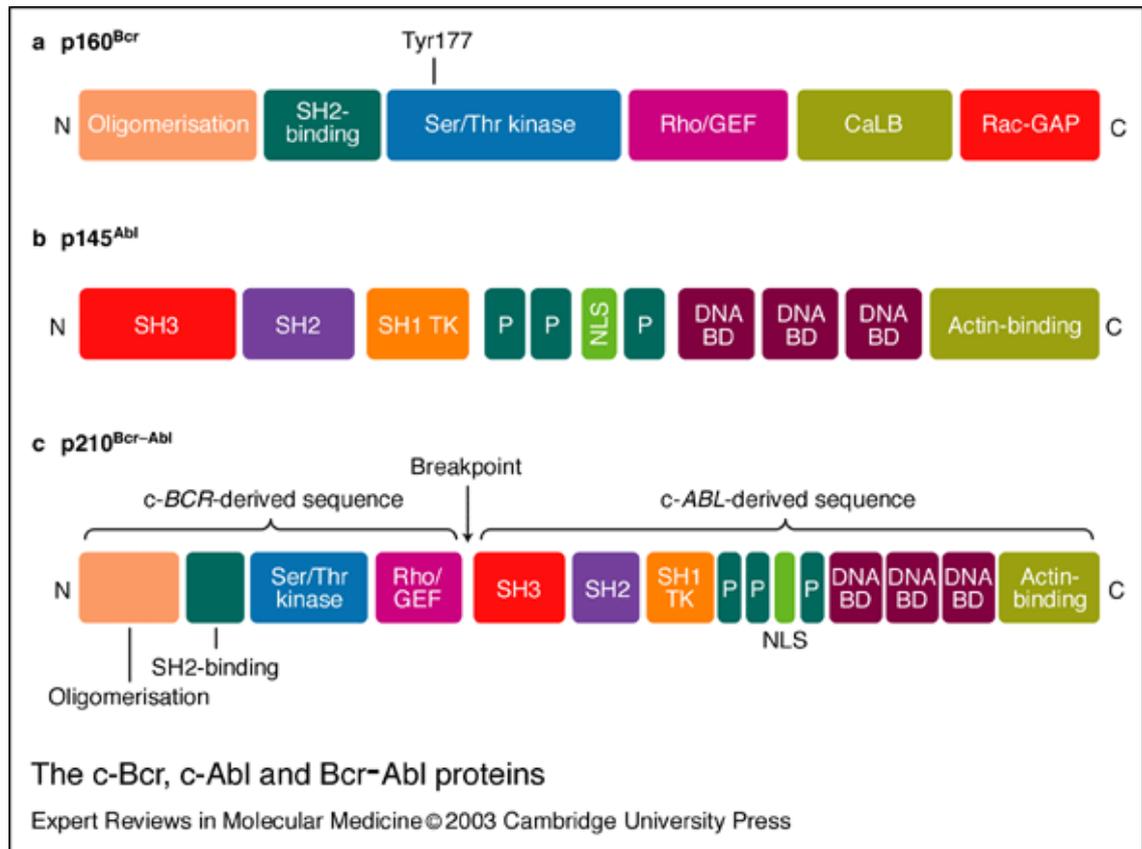
Figure 1.1 - The c-Bcr, c-Abl and Bcr–Abl proteins. Structural motifs have been highlighted in p160[Bcr], p145[Abl] and the fusion protein p210[Bcr–Abl], which is formed following breakpoint translocation. (a) c-Bcr comprises an oligomerisation domain, a domain thought to mediate binding to Src-homology 2 (SH2)-domain-containing proteins, a serine/threonine kinase domain, a region with homology to Rho guanine-nucleotide-exchange factor (Rho-GEF), a region thought to facilitate calcium-dependent lipid binding (CaLB) and a region showing homology to Rac GTPase activating protein (Rac-GAP). Bcr is phosphorylated on many tyrosine residues, notably Tyr177, which when phosphorylated mediates the binding of the adaptor molecule Grb2. (b) c-Abl comprises an SH3 and SH2 domain, an SH1 tyrosine kinase (TK) domain, several proline-rich domains (P), a nuclear localisation signal (NLS), several DNA-binding domains (DNA BD) and an actin-binding domain. (c) The Bcr–Abl fusion protein comprises the first four domains of Bcr and all the c-Abl domains except the N-terminal SH3 domain. From Smith *et al.* (2003).

The *Abl* or Abelson gene was first described in the Abl leukemia virus (Abelson and Rabstein 1970) and encodes a ubiquitously expressed 145 kDa tyrosine kinase (Laneuville 1995). The protein has two isoforms, resulting from alternative splicing (Laneuville 1995). Three SRC homology (SH) domains are located towards the N-terminus of the protein, of which SH1 bears the tyrosine kinase function, with SH2 and SH3 responsible for interactions with other proteins (Cohen *et al.* 1995). The roles of ABL are complex, but it is involved in cell signalling (Kipreos and Wang 1990; Sawyers *et al.* 1994), integrating signals from the internal and external cellular environments (Lewis and Schwartz 1998; Yuan *et al.* 1999), and thus influencing cell cycling and apoptosis (reviewed in Van Etten 1999)). Mice lacking one copy of *Abl* are normal (Shet *et al.* 2002), however, double knockouts die shortly after birth (Tybulewicz *et al.* 1991).

1.2.1.1 Bcr/Abl Breakpoint

Breakpoints within the *Abl* gene occur within a 300 kb region at the 5' end (Melo 1996). Thus the fusion may include both exons 1a and 1b, only the 1b exon, or neither 1a or 1b (Melo 1996). Nevertheless, splicing of the transcript results in mRNA with *Abl* exon 2 attached directly to the *Bcr* fragment (Melo 1996). Thus *Abl* exon 1, even if retained in the genomic fusion, never constitutes part of the chimeric mRNA (Melo 1996; Deininger *et al.* 2000b). By contrast, breakpoints in the *Bcr* gene almost always localise to one of three 'breakpoint cluster regions' (Melo 1996). In most cases of Chronic Myelogeous Leukaemia (CML), and one third of Philadelphia-chromosome-positive Acute Lymphoblastic Leukaemias (ALL), the break occurs in a 5.8 kb region of *Bcr*, known as the major breakpoint cluster region (M-*bcr*). This covers exons 12-16 of *Bcr* (originally named b1-b5). After splicing, fusion transcripts with either e13a2 or e14a2 (b2a2 or b3a2) are formed. Both transcripts give rise to the 210 kDa fusion protein (Melo 1996). In the remaining cases of ALL, and in some cases of CML, the breakpoint occurs upstream in a 54.4 kb region, between Bcr exons e2' and e2. This is the minor breakpoint cluster region (m-*bcr*) and gives rise to e1a2 mRNA, and the 190 kDa fusion protein (Melo 1996). A third breakpoint cluster region (μ-*bcr*) occurs downstream of exon 19, and is found in some cases of the rare Philadelphia-positive chronic neutrophilic leukaemia (Pane *et al.* 1996; Wilson *et al.* 1997). This results in a

230 kDa fusion protein (Melo 1996). In many patients, alternate fusion transcripts may be found in addition to the initial variant (van Rhee *et al.* 1996; Melo 1997; Leibundgut *et al.* 1999). Mutational analysis has identified numerous features of the chimeric protein that are essential for transformation, including the SH1, SH2, actin-binding domains of Abl, the coiled-coil motif, the tyrosine at 177, and the phosphoserine-threonine-rich regions between residues 192-242 and 298-413 of BCR (reviewed in Deininger *et al.* 2000b).

The Bcr/Abl fusion protein gains autophosphorylative activity due to dysregulation of the ABl tyrosine kinase activity resulting from loss of the inhibitory SH3 domain in the Abl region (Muller *et al.* 1991) and oligomerisation of the coiled coil domain in the Bcr region (McWhirter *et al.* 1993). Autophosphorylation of the Y-177 tyrosine residue (Pendergast *et al.* 1993) recruits GRB2 (Ma *et al.* 1997), activating the RAS pathway (Egan *et al.* 1993; Reuther *et al.* 1994; Tauchi *et al.* 1994; Sawyers *et al.* 1995). JAK and STAT1/STAT5 are also phosphorylated (Carlesso *et al.* 1996; Frank and Varticovski 1996; Ilaria and Van Etten 1996; Shuai *et al.* 1996), leading to independence from growth factors (Shet *et al.* 2002). Loss of the Abl nuclear-localisation signal leads to increased amounts of the fusion protein in the cytosol and increased binding of actin (McWhirter and Wang 1993; Van Etten *et al.* 1994; Salgia *et al.* 1997). Subsequent phosphorylation of cytoskeletal proteins disrupts adhesion receptor function, likely causing the premature entry of progenitor cells into the circulation (Gordon *et al.* 1987; Bhatia and Verfaillie 1998).

The Bcr/Abl protein also enhances the resistance of cells to apoptosis caused by DNA damage (Bedi *et al.* 1994; Bedi *et al.* 1995), and phosphorylation of Bad inactivates the pro-apoptotic of this protein (Wang *et al.* 1996a; Zha *et al.* 1996). The Bcr/Abl protein also activates the PI(3)K/Akt pathway (Skorski *et al.* 1995; Carpino *et al.* 1997; Franke *et al.* 1997; Jain *et al.* 1997), increases expression of BCL-2 (Sanchez-Garcia and Grutz 1995), and phosphorylates STAT5 (de Groot *et al.* 2000; Horita *et al.* 2000), all of which increase resistance to apoptosis. In addition to, the Bcr/Abl protein may trigger mitogenesis by numerous paths, such as the Ras/MAP-kinase, Jak-Stat, PI3 or the Myc pathways (reviewed in Deininger *et al.* 2000b). While the role of the fusion protein in differentiation is not fully elucidated, it is known to cause a block in blast phase CML (Wetzler *et al.* 1993a; Wetzler *et al.* 1993b; Gordon *et al.* 1999).

1.2.1.2 Neutralisation of the Bcr/Abl Fusion Gene

Specific targeting of the Bcr/Abl fusion gene and protein has been attempted via many routes. STI571 has proven successful and is widely used in the clinic, however, relapse or resistance occurs in a significant number of cases and a number of targeted molecular therapies are also under investigation.

STI571 (= CGP57418B = Gleevec = Imanitib) is a competitive inhibitor of the ATP-binding cleft of Abl and selectively inhibits the kinase activity of both Abl and Bcr/Abl (Buchdunger *et al.* 1995; Druker *et al.* 1996; le Coutre *et al.* 1999). This inhibition results in the apoptotic death of Philadelphia-positive cells (Deininger *et al.* 2000a) and the selective suppression of proliferation of CML primary cells and cell lines, both *in vitro* and *in vivo* (Druker *et al.* 1996; Deininger *et al.* 1997; le Coutre *et al.* 1999). The relationship between c-Abl, Bcr-Abl and differentiation is not completely understood as STI571 has been reported to induce apoptosis without differentiation (Deininger *et al.* 1997; Gambacorti-Passerini *et al.* 1997) while elsewhere it induced haemoglobin and the expression of the myeloid differentiation marker CD11b (Fang *et al.* 2000). Interestingly while STI571 has no effect on Bcr/Abl-negative cells, when these same cells are made to express Bcr/Abl, STI571 induces apoptosis and growth arrest (Dan *et al.* 1998; Fang *et al.* 2000; Blagosklonny *et al.* 2001; Nimmanapalli *et al.* 2002). It is suggested that this results from an accumulation of proapoptotic stimuli behind the Bcr/Abl block, which when removed, releases the apoptotic cascade, flooding the cell (Blagosklonny 2004).

Unfortunately clinical resistance to STI571 is common, particularly in the later stages of CML (Druker *et al.* 2001; Talpaz *et al.* 2002). In addition to over expression of Bcr/Abl and mutations such as the His396Pro Bcr/Abl kinase mutant (Corbin *et al.* 2003), resistance to STI571 may develop due to the acquisition of mutations in other genes and independence from the Bcr-Abl fusion gene (Nimmanapalli *et al.* 2002).

Short-interfering RNA's designed to target the Bcr/Abl fusion gene were found to reduce Bcr/Abl mRNA by up to 87% in Bcr/Abl-positive cells lines as well as primary cells from CML patients. c-Bcr and c-Abl mRNA levels in these cells were unaffected (Scherr *et al.* 2003b). Knockdown of fusion mRNA was demonstrated by quantitative PCR and down-regulation of the fusion protein was confirmed by Western blotting (Wilda *et al.* 2002; Scherr *et al.* 2003b; Wohlbold *et al.* 2003). This down-

regulation of Bcr/Abl protein levels relieved Bcr-Abl-dependent effects on cell cycle regulation and anti-apoptotic proteins, selectively inhibited cell growth (Wohlbold *et al.* 2003), and induced apoptosis (Wilda *et al.* 2002). RNAi took 24 hours longer than STI571 for full induction of apoptosis (Wilda *et al.* 2002), which is presumably due to the different modes of action of each, with STI571 acting directly on the protein, while RNAi prevents the synthesis of new fusion protein, which has a half-life of 48 hours (Dhut *et al.* 1990; Spiller *et al.* 1998). RNAi produces no additive effect with STI571 in K562 cells (Wilda *et al.* 2002), although this cell line is inherently resistant to STI571 (Wohlbold *et al.* 2003). In other cell lines, RNAi enhanced sensitivity to gamma-radiation and STI571, and restored STI571 sensitivity in cells expressing the STI571-resistant, His396Pro Bcr-Abl kinase mutant (Wohlbold *et al.* 2003). The Bcr-ABl fusion transcript has also been targeted with ribozymes, resulting in, variously, downregulation of the fusion mRNA, reduced expression of the fusion protein, suppressed clonogenicity of the cells, and increased apoptosis (Snyder *et al.* 1993; Snyder *et al.* 1997; Wright *et al.* 1998; Wu *et al.* 1998; Wu *et al.* 2003). However some of the most active ribozyme designs also cleaved non-target transcripts (Wright *et al.* 1993; Kearney *et al.* 1995)

A few studies have also investigated the potential of antisense oligonucleotides designed to target Bcr/Abl, however, these have given conflicting results with respect to specificity and functionality (Skorski *et al.* 1994; Smetsers *et al.* 1994) and have not been pursued further.

1.2.2 Acute Promyelocytic Leukaemia (APL)

Acute promyelocytic leukaemia (APL) is a common subtype of acute myeloid leukaemia (AML) (Bennett *et al.* 1976) that was first identified in 1957 (Hillestad 1957). APL is characterised by a distinct blockage of myeloid differentiation and the accumulation of immature promyelocytes in patient bone marrow and the peripheral blood (Zhou *et al.* 2005). The majority of APL patients possess a translocation involving the RARα gene on chromosome 17, with five fusion partners far identified to date (Warrell *et al.* 1993). All translocations in APL are reciprocal but only the X-RARα fusion products are implicated in the block to myeloid differentiation and the development of APL (Lin *et al.* 1999; Melnick and Licht 1999; Pandolfi 2001). The

RARα-X proteins appear to play some role in modulating the disease phenotype (Lin *et al.* 2001a).

The leukaemogenic effects of these fusion proteins are exerted through dominant negative inhibition of the transcription regulatory pathways (Lin *et al.* 2001a). This results in enhanced binding of corepressor units leading in turn to the repression of target genes and the generation of the promyelocytic differentiation block (Lin and Evans 2000; Minucci *et al.* 2000). In contrast to other types of AML, APL patients initially respond to pharmacological concentrations of retinoic acid (RA), which triggers differentiation of the leukaemic blasts into granulocytes (Huang *et al.* 1988; Warrell *et al.* 1991).

The PML gene is involved in 98 % of APL cases and is located on chromosome 15q22. Alternative splicing gives rise to approximately 20 different forms of the protein, with the longest predicted to be 70 kDa (Grignani *et al.* 1994). PML is expressed ubiquitously and contains a RING domain, B1 and B2 boxes, and an α-helical coiled coil domain (Mu *et al.* 1994; Borden *et al.* 1996). PML also appears to have tumour-suppressive activity, probably via involvement in the RA-pathway (Wang *et al.* 1998; Piazza *et al.* 2001). PML also acts as a transcription coactivator, and can potentiate the transactivating functions of AP-1 (Jun/Fos) (Vallian *et al.* 1998) by targeting transcriptional accessory factors such as CBP and TIF1α (Vallian *et al.* 1998; Doucas *et al.* 1999). PML also forms cellular coactivating complexes with TIF1α and CBP, and these complexes are recruited to RA-responsive elements (RARE's), indicating that PML is also a coactivator of RARs (Zhong *et al.* 1999).

RARα is a member of the nuclear hormone receptor superfamily (Evans 1988) and helps to mediate myeloid differentiation induced by retinoic acid signalling (de The *et al.* 1989; Gallagher *et al.* 1989; Largman *et al.* 1989). This role is auxiliary, however, as RAR-α and -γ double knockout mice are normal with respect to myeloid lineage distribution and early differentiation (Labrecque *et al.* 1998; Kastner *et al.* 2001). Nevertheless X-RARα fusion proteins are still able to block the RA-independent differentiation of primary myeloid progenitors, suggesting a general inhibitory effect on such differentiation. It is also possible that the reciprocal RARα-X fusion proteins cooperate in deregulating myeloid target genes (Tsai and Collins 1993; Du *et al.* 1999).

RARα normally forms a heterodimer with the retinoid X receptor (RXR), and in the absence of retinoic acid, this heterodimer recruits a repression complex containing nuclear receptor corepressors (SMRT or N-CoR), mSin3, and histone deacetylases (Chen and Evans 1995; Horlein *et al.* 1995; Heinzel *et al.* 1997). Retinoic acid induces conformational change in RARα that leads to dissociation of the HDAC complex and recruitment of coactivators, in turn leading to a permissive chromatin structure and transcriptional activation (Minucci *et al.* 2001). Retinoic acid also promotes the association of a coactivator complex containing nuclear receptor coactivator p160 family members, and histone acetylases CBP/p300 (Chen *et al.* 1997; Torchia *et al.* 1997). Core histone acetylation leads to chromatin relaxation, promoter clearance, and activation of gene transcription.

1.2.2.1 PML-RARα Breakpoint

The t(15;17) translocation contains multiple cluster regions (Figure 1.2), with variation in the contribution of the PML gene while the contribution of RARα is invariant (Pandolfi *et al.* 1992). Individual APL patients usually possess a unique set of fusion products that reflect alternative splices derived from a single breakpoint (Pandolfi *et al.* 1992). The breakpoint variants each show different sensitivity to ATRA (Gallagher *et al.* 1995) and incidence of relapse (Huang *et al.* 1993).

The fusion protein when it forms is more abundant than the wild-type RARα (Pandolfi *et al.* 1992; Jansen *et al.* 1995), and whereas the wild-type RARα forms a heterodimer with RXR, the mutant X-RARα's are able to form homodimers and/or oligodimers (Lin *et al.* 2001a). In PML-RARα, for example, this is due to the self-associating coiled coil domain of PML (Minucci *et al.* 2001). Consequently, whereas the normal RARα-RXR heterodimer binds only one unit of the corepressor complex, the PML-RARα homodimer or oligomers are able to bind multiple corepressor units (Lin and Evans 2000; Minucci *et al.* 2000). Mutant X-RARα proteins also retain the DNA- and ligand-binding domains of wild-type RARα, and have comparable affinity for RA (Dong *et al.* 1996; Benedetti *et al.* 1997; Minucci *et al.* 2001). The enhanced stoichiometry of the interaction between NCoR/SMRT and PML-RARα results in an increased local concentration of HDAC on the target DNA, and enhanced

Figure 1.2 Domains of the PML and RARα proteins
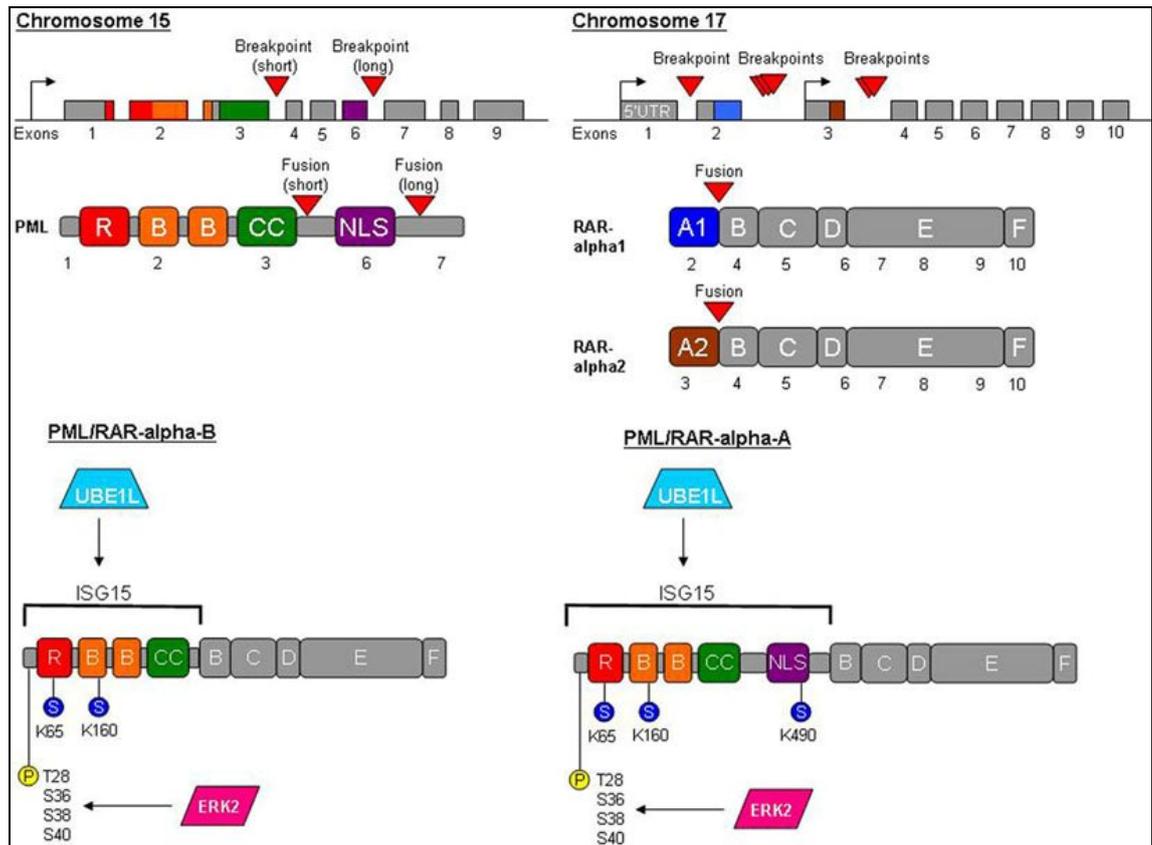
Figure 1.2 - Schematic of the PML and RAR-alpha genes, the two PML-RAR-alpha fusion protein isoforms and their modifications. Red triangles denote fusion points between the PML and RAR-alpha sequences. Based on the genomic breakpoint in PML gene, either a short (PM/RAR-alpha-B) or long (PML/RAR-alpha-A) isoform is generated. From Nichol *et al.* (2009).

transcriptional repression, at physiological concentrations of RA (Perez *et al.* 1993; Dong *et al.* 1996; Minucci *et al.* 2001).

Many proteins involved in transcriptional regulation interact directly with PML, or colocalise with PML in so-called 'PML oncogenic domains' (POD's), in which PML is normally found (Dyck *et al.* 1994; and reviewed in Li *et al.* 2000; Zhong *et al.* 2000). These nuclear bodies are also involved in the regulation of apoptosis and replicative senescence, through regulation of p53 function (Minucci *et al.* 2001). It is unclear by which mechanism, or mechanisms, the POD complexes are able to regulate transcription, nor is it clear how the displacement of PML from this complex by PML-RARα affects the genes targeted by this transcriptional regulation (Dyck *et al.* 1994; Weis *et al.* 1994; reviewed Lin *et al.* 2001a). PML-RARα also triggers the release of PML from STAT3, restoring the activity of the latter and promoting the growth and survival of myeloid cells (Kawasaki *et al.* 2003).

1.2.2.2 Neutralisation of the PML-RARα Fusion Gene

Retinoic acid is the natural ligand for the Retinoic Acid Receptor α, yet at physiological concentrations of RA the fusion proteins PML-RARα and PLZF-RARα interact strongly with SMRT or N-CoR, recruit HDACs, blocking the RA activated differentiation of myeloid leukemic cells (see above). At higher, 'pharmacological' doses of RA, corepressor dissociation from PML-RARα is induced, and thus mediated transcription and differentiation is activated. This does not occur with PLZF-RARα (Hong *et al.* 1997; Grignani *et al.* 1998; Guidez *et al.* 1998; Lin *et al.* 1998). Also, a subset of APL patients (eg. t(11;17)) will fail to respond to the pharmacological doses of RA, and many responsive patients eventually relapse and develop RA resistance (Warrell 1993). In some cases this acquisition may be due to a point mutation in the ligand-binding domain of the RARα portion, which decreases sensitivity to retinoic acid (Imaizumi *et al.* 1998). Accordingly, alternate therapies are under investigation.

Transcriptional repression mediated by Histone Deacetylases (HDAC's) is common with translocation-associated oncogenes, with the deregulated chromatin structure potentially leading to neoplasia (reviewed in Lin *et al.* 2001a). Consistent with this is the recruitment of the HDAC complex, which is crucial for the oncogenic activity of X-RARα. Specific HDAC inhibitors combined with RA can overcome the

repressor activity of X-RARα and trigger terminal differentiation, even in RA-resistant APL cells (Hong *et al.* 1997; Grignani *et al.* 1998; Guidez *et al.* 1998; Lin *et al.* 1998). HDAC-inhibitors in combination with ATRA have also proved effective in a relapsed case of t(15;17) (Warrell *et al.* 1998).

Arsenic trioxide (ATO) was used as an early form of treatment for CML, prior to the advent of radiation and cytotoxic chemotherapy, and interest in the therapeutic potential of ATO has been revived (see Zhou *et al.* 2007). ATO was shown to be effective in bringing about complete remission in the majority of newly-diagnosed and/or relapsed APL patients (Wang 2003), including some who had relapsed after ATRA (Shen *et al.* 1997). Furthermore, it was effective when delivered alone, or in combination with low-dose chemotherapeutics or ATRA (Shen *et al.* 1997). It also proved to be relatively safe (Shen *et al.* 1997).

The PML-RARα fusion has also been targeted by a number of molecular techniques. Hammerhead ribozymes targeted to the breakpoint found in NB4 cells were able to down-regulate the fusion mRNA, and the resulting knockdown of the fusion protein inhibited growth and induced apoptosis in this cell line. It was also found to increase the sensitivity of the cells to ATRA (Nason-Burchenal *et al.* 1998a). Knockdown of the PML-RARα protein did not overcome the maturation block found in these leukemic cells (Nason-Burchenal *et al.* 1998b). An antisense oligonucleotide targeting the PML-RARα fusion resulted in transient down-regulation of the fusion mRNA (Chen *et al.* 1999b), accompanied by a reduction in levels of the fusion protein, with partial differentiation of NB4 cells at day 5 and apoptosis at day 7 (Chen *et al.* 1999a). Finally, a short-hairpin RNA of 30 bp length down-regulated the fusion protein in HeLa cells transfected with PML-RARα (Oshima *et al.* 2003). Despite the length of the hairpin construct, there was no evidence of a non-specific interferon-mediated response (Oshima *et al.* 2003). Effects on cell proliferation were not investigated in this study.

1.2.3 Acute Myeloid Leukaemia (AML) – RUNX1/ETO

The RUNX1/ETO fusion gene is usually found in Acute Myeloid Leukaemia (AML) of the FAB M2 subtype, in the granulocytic lineage (Rowley 1984; Brunning and McKenna 1994). This fusion gene is often found in early adulthood, and is

associated with a good prognosis (Choi *et al.* 2006). Secondary mutations occurring in other genes are important in the development of AML (Elagib and Goldfarb 2007).

The *Runx1* gene (or AML1, CBFA2, PEBP2αB) is essential for the development of the haematopoietic system during embryogenesis (Okuda *et al.* 1996; Wang *et al.* 1996b), and is one of the most common targets of chromosomal translocation in human leukaemias (Look 1997; Speck and Gilliland 2002). Three Runx splice variants are possible, and all contain a 'Runt homology domain'. The two longer isoforms (numbers 1 and 2) also have a transactivation domain that is absent on the shorter isoform (Meyers *et al.* 1993; Bae *et al.* 1994; Takahashi *et al.* 1995). This short form may still have a regulatory role, as it binds more efficiently to regulatory regions of DNA, but does not initiate transcription following binding (Meyers *et al.* 1993; Bae *et al.* 1994; Licht 2001). Runx1 combines with CBFβ to form a heterodimer that co-operates with other transcription factors and is thus able to regulate a range of haematopoietic lineage-specific genes (Lutterbach and Hiebert 2000). The RUNX-1/CBF-β heterodimer may regulate gene expression by recruiting proteins with histone acetyl transferase (HAT) activity, thus acetylating nearby histone tails and in turn switching on gene expression (reviewed Licht 2001).

The human and murine Eight Twenty-One (ETO) gene (also known as 'Myeloid Translocation Gene 8' (MTG8)) belongs to a family of three myeloid transforming genes (Gamou *et al.* 1998; Kitabayashi *et al.* 1998) and was identified as the partner of *AML-1* in the t(8;21) fusions (Miyoshi *et al.* 1993; Erickson *et al.* 1996). Much information about the co-repressor function of ETO has been gleaned from studies of the repressive activity of AML1/ETO (Hiebert *et al.* 2001). The zinc-finger domains of ETO (NHR2 and NHR4) are required for AML1/ETO repression of transcription and suggest a co-repressor role for ETO (Lenny *et al.* 1995). Numerous co-repressor binding partners have been identified, including nuclear receptor co-repressor (N-CoR) (Wang *et al.* 1998), the silencing mediator of retinoic acid and thyroid hormone receptors (SMRT) (Chen and Evans 1995; Gelmetti *et al.* 1998), mSin3A and HDAC1 (Gelmetti *et al.* 1998; Wang *et al.* 1998; Lutterbach *et al.* 1998a), as well as the promyelocytic leukaemia zinc-finger (PLZF) and Growth factor independence-1 (Glf1) (Melnick *et al.* 2000). The NHR2 domain mmediates dimerisation, and multiple ETO domains are required for maximal repression (Lutterbach *et al.* 1998b)

MTG8 also plays an important role in development of the gastro-intestinal system as indicated by gene-targeting in mice in which inactivation of the MTG8 gene resulted in reduced viability of heterozygotes, evidently stemming from gross disruption of the gut architecture, including absence of the midgut in many cases (Calabi *et al.* 2001).

1.2.3.1 RUNX1-ETO Breakpoint

The RUNX1-ETO translocation, t(8;21)(q22;q22), is one of the most common in acute myeloid leukaemia, and comprises the N-terminal end of the RUNX-1 protein, and the c-terminus of the ETO protein (Miyoshi *et al.* 1991, and see Figure 1.3). Where Runx1 is involved in acute myeloid leukaemia translocations, the Runt homology domains are usually intact, but the transactivation domain is often replaced (Nucifora and Rowley 1995; Speck and Gilliland 2002).

It is thought that AML1-ETO fusion leads to the development of leukaemias because the RUNX1 transcriptional activator, after fusion with ETO, becomes a transcriptional repressor of the RUNX1 activation targets (reviewed in Elagib and Goldfarb 2007), that is, those required for haematopoiesis and differentiation (Sakakura *et al.* 1994; Gelmetti *et al.* 1998). RUNX1/ETO may also selectively bind co-repressor proteins (eg. N-CoR, SMRT) with deacetylating activities and then prevent the binding of proteins that stimulate acetyl transferase activity, resulting in the repression of target genes (Wang *et al.* 1999). This binding appears to be regulated by the ETO domain (Gelmetti *et al.* 1998). However it is becoming clear that rather than causing the indiscriminate inhibition of RUNX-1 targets, specific genes, such as G-CSF, are upregulated by RUNX1-ETO (Shimizu *et al.* 2000). Similarly microarray studies demonstrate that a range of genes can be upregulated or downregulated (Shimada *et al.* 2000).

1.2.3.2 Neutralisation of the RUNX1-ETO Fusion Gene

AML is conventionally treated with chemotherapies, such as a combination of an anthracycline (eg. daunorubicin or idarubicin) and cytarabine (Lowenberg *et al.* 1999). As permanent remission is rare after this treatment alone (Cassileth *et al.* 1988), it is usually followed with consolidating treatments. Depending on the patient's prognosis this may consist of further chemotherapy if the prognosis is good, or

Figure 1.3 Domains of the RUNX1/ETO protein
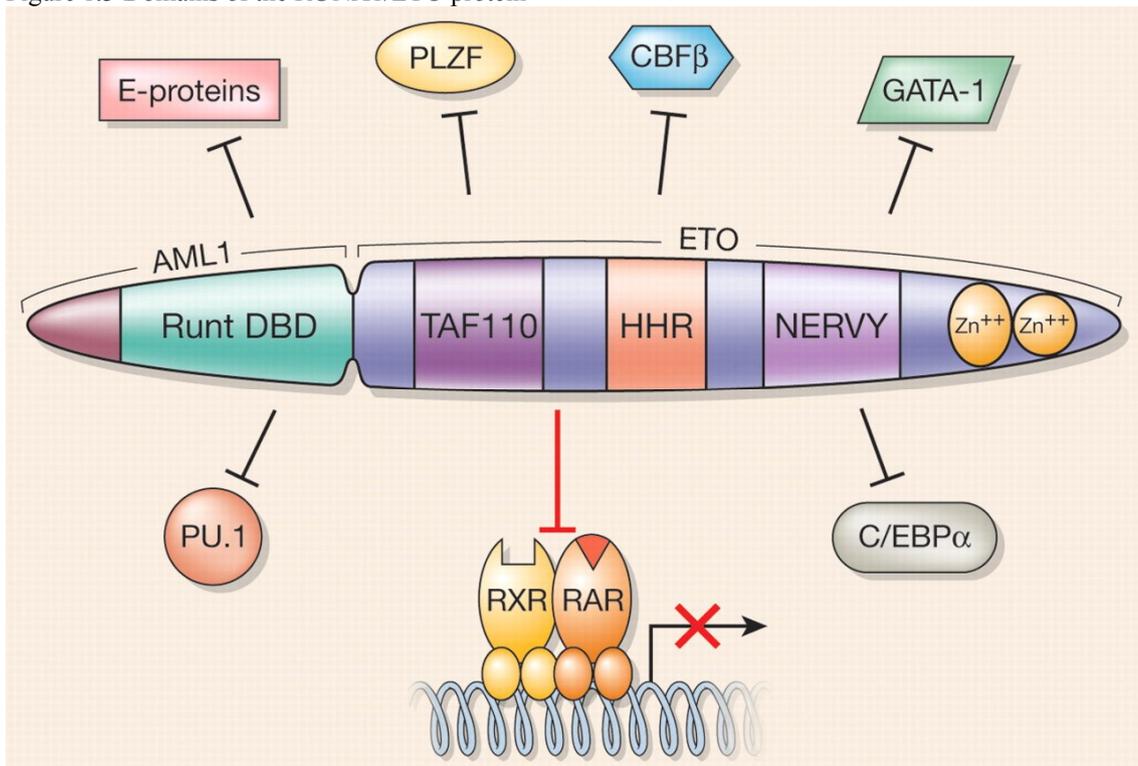
Figure 1.3 - AML1/ETO (RUNX1/ETO) promiscuously targets and blocks the activities of RXR/RARα and other hematopoietic transcription factors. Different functional domains of the AML1/ETO fusion protein are indicated as follows: the Runt DNA-binding domain (DBD) of the AML1 moiety; a region that shares homology with TAF110 and other related TAF proteins; a heptad repeat of hydrophobic amino acids (HHR); a region that shares homology with *Drosophila* nervy protein; and a C-terminal region containing 2 nonclassical zinc fingers. Positions of indicated transcription factors relative to AML1/ETO do not reflect regions of interaction with the fusion protein. From Petrie and Zelent (2007).

allogeneic or autologous stem cell transplantation for patients with a poor prognosis (Appelbaum *et al.* 2000).

Alternatively, as abnormal recruitment and altered activity of histone deacetylases (HDAC's) are a common theme in acute myeloid leukaemias (Minucci *et al.* 2001), the development of various HDAC inhibitors (HDACi) offers the potential for differentiation therapy in a range of acute myeloid leukaemias. However, while this is able to revert the malignant phenotype in some cases (Marks *et al.* 2000), inhibition of the enzymatic activity of the HDAC complex on its own is not sufficient to restore the ability of AML cells to differentiate (Minucci *et al.* 2001). Combination with retinoic acid is emerging as the superior option, even in non-APL cases of AML (ie. those with the RUNX1-ETO translocation) (Ferrara *et al.* 2001).

In spite of these developments, no specific and effective therapy has thus far been found for AML, yet molecular therapies have achieved some promising results. The RUNX1-ETO fusion mRNA has successfully been targeted *in vitro* with hammerhead ribozymes, resulting in protein knockdown (Szyrach *et al.* 2001), and inhibition of cell growth (Kozu *et al.* 2000). Similarly the use of siRNAs targeted against the RUNX1-ETO selectively downregulated the fusion mRNA, without effect on normal RUNX1 (Heidenreich *et al.* 2003). This resulted in increased sensitivity to differentiation agents and reduced clonogenicity of the cells (Heidenreich *et al.* 2003). Microarrays showed that these effects were accompanied by upregulation of antiproliferative genes and genes associated with differentiation, but down-regulation of genes associated with drug resistance and a poor prognosis (Dunne *et al.* 2006). Finally, the transient delivery of the siRNA molecules proved sufficient to delay tumour formation *in vivo* (Martinez Soria *et al.* 2009).

1.3 RNA Interference

RNA Interference (RNAi) is an enzymatic mechanism triggered by double-stranded RNA and found in eukaryotes (Fire *et al.* 1998) by which mRNA molecules are specifically degraded in the cytoplasm. Some form of RNA interference has been found in all eukaryotic phyla, from plants and fungi to mammals (reviewed in Dykxhoorn *et al.* 2003) and is thought to have developed as a form of intracellular defence against viruses (Gitlin *et al.* 2002).

An RNAse III enzyme, Dicer mediates cleavage of long, double-stranded RNA (dsRNA) (Bernstein *et al.* 2001; Billy *et al.* 2001; Ketting *et al.* 2001, and see Figure 1.4) in an ATP-dependent reaction (Zamore *et al.* 2000). The resulting short interfering RNA's (siRNAs) are 21-23-nt double-stranded RNA duplexes with 2-3-nt 3' overhangs, and 5'-phosphate and 3'-hydroxyl groups (Elbashir *et al.* 2001a). These siRNAs are incorporated into an RNA-induced silencing complex (RISC), where the duplex is unwound in an ATP-dependent reaction (Hammond *et al.* 2000) and the antisense strand guides the RISC to homologous target mRNA. The target mRNA is enzymatically cleaved at a single site in the centre of the duplex region, 10 nt from the 5' end of the siRNA (Elbashir *et al.* 2001a). This process takes place in the cytoplasm (Hutvagner and Zamore 2002; Zeng and Cullen 2002; Kawasaki and Taira 2003).

Long dsRNA may be cleaved into multiple siRNAs however in mammalian cells dsRNA longer than 30 nt induces a non-specific interferon response (Elbashir *et al.* 2001b). Interferon activates two enzymes; the protein kinase PKR, which acts indirectly to shut down all protein synthesis, and 2',5'-oligoadenylate synthetase (OAS), which indirectly activates RNAseL, a non-specific enzyme that targets mRNA (reviewed in Bass 2001). However the introduction of 21 nt siRNAs into mammalian cells results in sequence-specific silencing of the target gene, and does not trigger the non-specific interferon response (Elbashir *et al.* 2001b). This has allowed experimental manipulation of the RNAi pathway.

In designing siRNA molecules for triggering the RNAi pathway, there is general consensus that a GC content of approximately 50%, or at least within the range of 30-70%, is optimal (Dykxhoorn *et al.* 2003). Secondary structure of the target mRNA does not appear to affect silencing (Harborth *et al.* 2001; Yoshinari *et al.* 2004). Analysis of a panel of active siRNAs has resulted in eight criteria which, when combined into an algorithm, have vastly improved the probability of selecting an effective siRNA (Reynolds *et al.* 2004). These criteria included; a G/C content of 30-52%, the presence of an 'A' or 'U' in positions 15-19, low internal repeat stability, an 'A' at position 19 and position 3, and a 'U' in position 10, the absence of 'G' or 'C' at position 19 of the sense strand, and a 'G' at position 13 of the sense strand (Reynolds *et al.* 2004). Another analysis found that the following features - A/U at the 5' end of the antisense strand, G/C at the 5' end of the sense strand, at least five A/U residues in the 5' terminal

Figure 1.4 The RNA Interference Pathway.
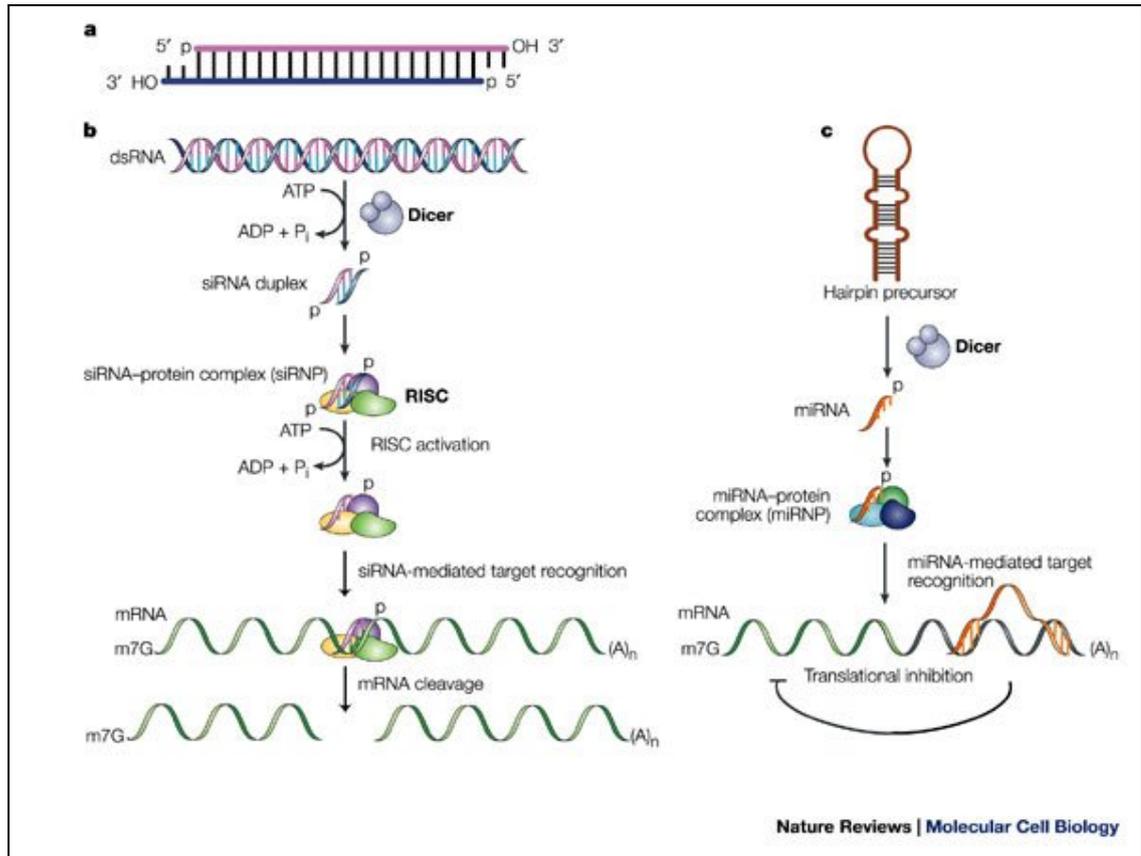
Figure 1.4 – The RNA Interference Pathway; (a) Short interfering (si)RNAs. Molecular hallmarks of an siRNA include 5' phosphorylated ends, a 19-nucleotide (nt) duplexed region and 2-nt unpaired and unphosphorylated 3' ends that are characteristic of RNase III cleavage products. (b) The siRNA pathway. Long double-stranded (ds)RNA is cleaved by the RNase III family member, Dicer, into siRNAs in an ATP-dependent reaction. These siRNAs are then incorporated into the RNA-inducing silencing complex (RISC). Although the uptake of siRNAs by RISC is independent of ATP, the unwinding of the siRNA duplex requires ATP. Once unwound, the single-stranded antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA. (c) | The micro (mi)RNA pathway. Although originally identified on the basis of its ability to process long dsRNA, Dicer can also cleave the ⁓70-nt hairpin miRNA precursor to produce ⁓22-nt miRNA. Unlike siRNAs, the miRNAs are single stranded and are incorporated into a miRNA–protein complex (miRNP). Caenorhabditis elegans let-7 and lin-4 miRNAs pair with partial sequence complementarity to target mRNA leading to translational repression. In addition to Dicer, the two pathways require other PAZ/PIWI domain proteins (PPD), including eukaryotic translation initiation factor 2C 2 (eIF2C2). From Dykxhoorn *et al.* (2003).

one-third of the antisense strand, and the absence of any GC stretch of more than 9 nt in length - were associated with effective RNAi in mammalian cells and chick embryos (Ui-Tei *et al.* 2004)   While these and similar analyses have formed the basis of algorithms for siRNA design developed by various groups (eg. Tuschl et al. Elbashir *et al.* 2002; Paddison and Hannon 2002) and commercial entities (eg. Ambion, Dharmacon, GenScript), the selection and/or design of siRNAs remains something of an empirical process, and it is recommended to test at least three siRNAs for any given target sequence (Dykxhoorn *et al.* 2003).

It was found that RNA interference may be triggered by molecules expressed as inverted repeats that spontaneously fold into a hairpin structure (Yu *et al.* 2002). These short-hairpin RNA's (shRNAs) are processed by the Dicer enzyme (Paddison *et al.* 2002b) and enter the RNAi pathway (Paddison *et al.* 2002a). While it has generally been assumed that features desirable for siRNA design also apply to shRNA design, it is becoming increasingly apparent that many sequences that are active as siRNAs are inactive as shRNAs (Kolykhalov *et al.* 2005), and that functional shRNAs and siRNAs exhibit similar but not identical sequence preferences (Li et al. 2007). Algorithms developed specifically for shRNA design have only recently been developed but are proving quite effective (Li et al. 2007).

1.3.1 RNAi and Antisense RNA

The gene silencing abilities of antisense RNA's have been under investigation for some time, and there is evidently some overlap of pathways used by RNA-interference and antisense RNA (Martinez *et al.* 2002; Holen *et al.* 2003). However in direct comparison the potency of RNA-interference was found to be approximately an order greater than that of antisense RNA (Aoki *et al.* 2003; Cioca *et al.* 2003). Furthermore, siRNAs were quantitatively more efficient *in vitro*, with a longer-lasting effect than oligonucleotides targeted against the same sequence (Bertrand *et al.* 2002). Furthermore, following 'Cytofectin' transduction of both molecule types in mice, siRNA was found to be effective, but no activity was observed with the oligonucleotides (Bertrand *et al.* 2002).

1.3.2 Non-Specific Activities of siRNAs

While many of the non-specific effects that can occur with long dsRNA's are not observed with siRNAs and shRNAs, potential affects on off target genes still remain. The three major categories on non-specific effects recognised to date are described below.

1.3.2.1 miRNA-like Activity

Micro-RNAs (miRNAs) are endogenous molecules that are structurally identical to siRNAs, being small (~22 nt) non-coding, regulatory RNA's (Lagos-Quintana *et al.* 2001; Hannon 2002; Pasquinelli and Ruvkun 2002) with widespread roles in development and disease (see Marquez and McCaffrey 2008). miRNA's may suppress their target mRNA's by a number of mechanisms. Where perfect complementarity exists with the target mRNA, the miRNA's act as siRNAs and trigger mRNA cleavage via the RNAi pathway, although this is not thought to be common in mammals (Marquez and McCaffrey 2008). However where the miRNA guide strand has only partial complementarity to the 3' untranslated region of the target mRNA, suppression generally results from translational repression (see Marquez and McCaffrey 2008), but may also result from methylation of the target gene (Kawasaki and Taira 2004; Morris *et al.* 2004). This repression is cumulative, with more miRNA molecules bound to the target gene producing greater transcriptional repression of that gene (Doench *et al.* 2003). Thus siRNAs designed to target one gene may have partial complementarity with non-target genes and trigger suppression of those genes (Doench *et al.* 2003).

1.3.2.2 Interferon Response

The interferon response is a non-specific anti-viral response that occurs in mammals, and can be triggered by dsRNA longer than 30 nt (Elbashir *et al.* 2001b). However there have also been many reports of interferon-stimulated gene (ISG) induction by siRNAs and shRNAs (reviewed in Schlee *et al.* 2006), with some ISG's responding to dsRNA a short as 15 bp (Sarkar *et al.* 1999). In a study of shRNAs, shRNAs of 21-25 nt's consistently induced a non-specific response (*OAS1*), whereas shRNAs of 19nt did not (Fish and Kruithof 2004). Aside from length, immunorecognition of RNA depends on certain molecular features such as double- versus single-strand configuration, sequence motifs (eg. 'AA' at the transcription start site), and nucleoside modifications such as triphosphate residues (Pebernard and Iggo

2004; Schlee *et al.* 2006). This immunorecognition is also dose-dependent (Sledz *et al.* 2003; Persengiev *et al.* 2004). Similarly it is thought that shRNAs excess to the processing capacity of the RNAi machinery contribute to the triggering of IFN expression (Bridge *et al.* 2003). Strategies that appear to ameliorate the interferon response include the use of an miRNA-like backbone (Bauer *et al.* 2009), and reducing the length of the dsRNA stem to 14 bp (Pebernard and Iggo 2004).

1.3.2.3 Overload Toxicity

One final non-specific effect of RNAi emerged after systemic delivery and sustained high-level expression of a range of shRNAs in mice (Grimm *et al.* 2006). Liver damage and death were associated with down-regulation of endogenous, liver-derived miRNA's. Evidence suggested overloading of part of the miRNA/shRNA processing mechanism, namely exportin-5, therefore it is expected that the siRNA form of the molecule will not trigger this effect (Grimm *et al.* 2006). Furthermore, more accurate targeting of shRNA/siRNA delivery would reduce the dose required and should ameliorate many of the potential adverse effects (eg. Ghatak *et al.* 2008)

1.4 Expression of shRNAs

Short-hairpin RNA's (shRNAs) may be expressed as inverted repeats from linear DNA cassettes using a Pol-III promoter (Brummelkamp *et al.* 2002b). When transcribed, these inverted repeats spontaneously form a hairpin RNA molecule, which is then processed by Dicer as per the processing of long-dsRNA or miRNA's (Siolas *et al.* 2005).

The choice of Pol-III promoter affects the potency of RNA interference (Boden *et al.* 2003). Two most commonly used Pol-III promoters are U6 and H1. The level of hairpin expression from U6 promoters in human lymphocytes expression was higher than from H1 promoters, though this expression was less stable in the long term, possibly due to cytotoxicity of the U6 promoter itself (An *et al.* 2006). The lower level of shRNA expression from the H1 promoter would also reduce the risk of cytotoxic effects (Grimm *et al.* 2006). Furthermore, expression from the U6 promoters also resulted in higher induction of ISG's in some conformations (Pebernard and Iggo 2004). These factors suggest that the H1 promoter is preferable for use in human cells.

1.5 RNA Interference Targeting Oncogenes

Many of the human cancer genes identified to date involve chromosomal translocations (Futreal *et al.* 2004). Where the chromosomal translocation results in a chimeric mRNA that contains a tumour-specific sequence at the fusion site, there is potential to target the tumour-specific sequence without affecting normal genes (Damm-Welk *et al.* 2003). This is also true of point mutations (Brummelkamp *et al.* 2002a) and there are many studies utilising various methods to deliver short-interfering RNA (siRNA) to cancerous cells, and a number of these have proven effective (for example, see summary in Devi 2006). Unfortunately, the emergence of point mutations within the RNAi target region may abolish the sequence-specific silencing. Alternatively, mutations in the RNAi machinery would engender resistance (Damm-Welk *et al.* 2003). Furthermore, many chromosomal translocations bear alternatively spliced transcripts, and any single hairpin would only be effective against a subset of any given oncogene (Damm-Welk *et al.* 2003). There are also certain hurdles to be overcome in delivery of RNAi, whether it be via siRNA or shRNA molecules (siRNA or shRNA) (Heidenreich 2004).

1.5.1 Delivery of siRNA

With respect to the delivery of synthetic siRNAs, while the development of vesicle-mediated forms of delivery continues, high concentrations are required for systemic delivery and the rate of turnover *in vivo* is high, making this option very expensive. Furthermore, where the oncogene is harboured in quiescent stem cells, these siRNAs must be applied repeatedly and patients must be monitored closely for signs of relapse.

1.5.2 Vector Delivery of shRNA

The expression of shRNAs from Pol-III promoters means they are compatible with delivery by viral vectors. For example, when a cassette comprising the Pol-III promoter, the inverted repeat, and the appropriate termination signal, is expressed from a stably-integrated, self-inactivating retroviral vector (Brummelkamp *et al.* 2002b), oncogenes can be specifically and stably inactivated in human cancer, even though the oncogenic allele differs from wild-type by only one nucleotide, as in the case of *K-RAS*

(Brummelkamp *et al.* 2002a). Similarly lentivector delivery of shRNA molecules targeting fusion oncogenes has been successfully demonstrated against various forms of fusion oncogenes, such as the Bcr-Abl fusion gene (Li *et al.* 2003b; Scherr *et al.* 2005), and the ALK fusion gene (Piva *et al.* 2006), leading to cell-cycle arrest and apoptosis, *in vitro* and *in vivo* (see also summary in Devi 2006). The stable transduction mediated by integrating vectors largely eliminates the need for repeated administration of treatments. Another advantage of shRNA is that the expression cassettes may be inserted into the lentiviral U3 LTR, resulting in duplication of shRNA transcription unit during reverse transcription (Scherr *et al.* 2003a). Finally, the production of retroviral and lentiviral vectors is much cheaper than the production of synthetic siRNAs.

There are, however, some potential disadvantages with vector delivery of shRNA molecules. While transgene expression may be stable in the short- to medium-term, transgenes expressed from stably-integrated vectors are subject to the effects of transgene silencing in the longer term (eg. Mok *et al.* 2007), although this would be less of a problem in quiescent cells. Finally, where integrating vectors are used for delivery of shRNA, the widespread, systemic use of these vectors there carries the risk of insertional mutagenesis (O. Heidenreich pers. comm. Scherr and Eder 2004; Nienhuis *et al.* 2006). This point is explored further below (Section 1.6.7).

More recently, miRNA-based systems for delivery of RNAi have become more common, and are commercially available. The expression of miRNAs from PolII promoters (Boden *et al.* 2004) allow tissue-specific and inducible expression (reviewed in /Marquez and McCaffrey 2008). However, at the time that this study was undertaken, these systems were not readily available. Furthermore, the objectives of the present study were oriented more towards the discovery of effective shRNA designs, and so modulated expression of shRNAs was not necessary.

1.5.3 Stable Transduction of shRNA as a Therapy for Leukaemia

In leukaemias in which the haematopoietic stem cells carry a chromosomal translocation, stable transduction of quiescent cells with a hairpin targeted to the breakpoint sequence might protect against future activation of the fusion gene, allowing vector transduction combined with transplantation of the haematopoietic stem cells (Pavletic *et al.* 2005) as an alternative form of therapy avoiding many of the problems

associated with current stem cell transplantation therapies (Khouri *et al.* 1994; Pavletic *et al.* 1998; Dreger and Montserrat 2002; Kufe *et al.* 2006; Bruno *et al.* 2007). Viral vectors are the most efficient form of delivery for stably integrated shRNA, however, selection of vector type depends on a number of factors.

1.6 Gene Therapy

1.6.1 Vector Delivery of therapeutic transgenes

The main objective of gene therapy is the expression of therapeutic genes - or 'transgenes' - in target cells, however, this may be achieved in many ways. For some applications, transient expression of a transgene is sufficient, in which case a number of non-viral (eg. liposomes, 'envelope-interacting protein' vesicles, plasmids), and/or non-integrating viral (eg. Adenovirus, Epstein-Barr virus) delivery systems might be utilised. Where efficient and stable transgene expression is required the options are more limited. Vectors designed to bring about stable expression of transgenes often depend upon insertion of a transgene cassette into the host genome. Such vectors may be based on viral vectors or liposomes, but in each case, integration of the transgene cassette will depend upon some form of integrating enzyme, or 'integrase'. To date a number of virus types have undergone extensive development as viral vectors. The major classes of vectors capable of stable transgene expression are summarised below.

1.6.1.1 Adeno-associated viruses

Adeno-associated Viruses (AAV's) are small, single-stranded DNA viruses that can integrate into the genome of infected cells (Coffin *et al.* 1997). The packaging capacity of AAV's is limited, and large-scale production is inefficient (Lundstrom 2003). Pre-existing immunity to AAV vectors is common and can impair transduction rates however use of alternative serotypes can avoid this (Lundstrom 2003). AAV's depend on the presence of a helper virus for replication, usually adenovirus or herpesvirus. In the absence of these, adeno-associated virus establishes latency by integrating into the host genome (reviewed in Lai *et al.* 2002) specifically to human chromosome 19 q13.3-qter (Kotin *et al.* 1990; Samulski *et al.* 1991; Kotin *et al.* 1992). However this site-specificity is mediated by adeno-associated virus Rep protein, which is absent from the recombinant adeno-associated virus vector (Russell *et al.* 1994).

Recombinant adeno-associated virus may persist in non-dividing cells as an episome, or less frequently as an integrated concatemer (Nakai *et al.* 2001). Adeno-associated virus, by preferentially integrating into genes rather than non-coding regions (Nakai *et al.* 2003), presents a risk of genetic disruption and oncogenic transformation (Check 2003), with recent mapping of AAV insertions identifying a number of insertions with oncogenic potential (Nakai *et al.* 2005; Donsante *et al.* 2007).

1.6.1.2 Herpes Simplex Virus

Vectors based on Herpes Simplex Virus-1, the α-Herpesvirus vectors, have a natural tropism for cells of the nervous system, and are able to transduce dividing and non-dividing cells. (Latchman 2002). While expression levels are modest (Lundstrom 2003), they have a large transgene capacity and are the subject of much interest in delivery of gene therapy to neural tissues (Coffin *et al.* 1997), however, the inability of these vectors to integrate into the host cell genome limits their potential in dividing cell populations.

γ-Herpesvirus vectors such as Epstein-Barr virus are attractive candidates for stable gene therapy as they are able to persist as non-integrated latent episomes in dividing cell populations by active episomal maintenance (Yates *et al.* 1985; Yates and Guan 1991). Unfortunately the Epstein-Barr virus persists asymptomatically in >90% of the population, raising the potential for recombination between Esptein-Barr based vectors and wild-type Epstein-Barr viruses. There is therefore much interest in an alternate γ-HSV, the Herpesvirus saimiri, though considerable development of this virus vector is still required (Griffiths *et al.* 2006).

1.6.1.3 Retroviruses

Retroviruses are RNA viruses that replicate through an integrated DNA intermediate (Coffin 1996). The lipid envelope of the infectious 'virion' contains two RNA copies of the viral genome (see Figure 1.5). After binding of the viral envelope to receptors on the target cell surface, fusion of the virion and cell membranes allows entry of the virion into the target cell cytoplasm (step 1). Within the cell, viral RNA is reverse transcribed into DNA (step 2), then transported and integrated into the host genome by integrase respectively (step 3). These viral enzymes enter the cell as part of the virion particle. The integrated 'provirus' is transcribed back to RNA by the cellular

Figure 1.5 Retroviral Life Cycle
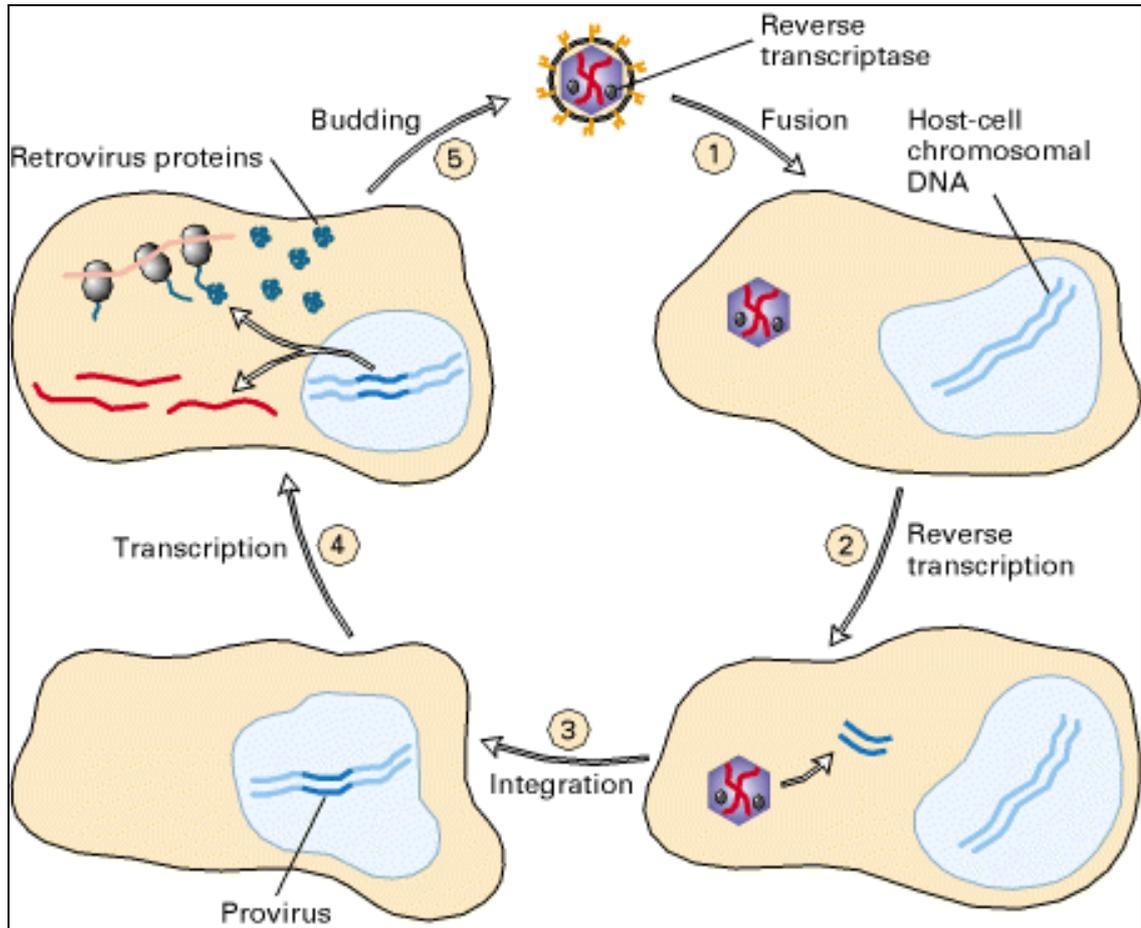
Figure 1.5 - Retroviruses have two identical copies of a plus single-stranded RNA genome and an outer envelope containing protruding viralglycoproteins. After envelope glycoproteins on a virion interact with a specific host-cell membrane protein or group of proteins, the retroviral envelope fuses directly with the plasma membrane without first undergoing endocytosis (step 1). Following fusion, the nucleocapsid enters the cytoplasm of the cell; then deoxynucleoside triphosphates from the cytosol enter thenucleocapsid, where viral reverse transcriptase and other proteins copy the ssRNA genome of the virus into a dsDNA copy (step 2). The viral DNA copy is transported into the nucleus (only one host-cell chromosome is depicted) and integrated into one of many possible sites in the host-cell chromosomal DNA (step 3). The integrated viral DNA, referred to as a provirus, is transcribed by the host-cell RNA polymerase, generating mRNAs (light red) and genomic RNA molecules (dark red). The host-cell machinery translates the viral mRNAs into glycoproteins and nucleocapsid proteins (step 4). The latter assemble with genomic RNA to form progeny nucleocapsids, which interact with the membrane-bound viral glycoproteins, as illustrated inFigure 6-17. Eventually the host-cell membrane buds out and progeny virions are pinched off (step 5). See Figures 9-20 and9-21 for details of the reverse transcription process and the transcription and processing of viral RNA. From Darnell *et al.* (1990).

machinery, driven by signals in the U3 region of the long terminal repeats (LTR's) of the proviral genome (step 4). Some copies of the transcribed genome are spliced and processed to express the viral genes necessary for formation of new virions. The relevant viral proteins and the full-length RNA genome assemble to form new viruses (step 5) (summarised by Coffin 1996). Spumaviruses differ from other retroviruses in that reverse transcription occurs before the virus leaves the host cell, such that the infectious virus particle contains a DNA genome (Yu *et al.* 1999).

Their efficient integration into the genomic DNA of host cells makes retroviruses attractive as gene therapy vectors. As they are replicated with the host DNA they are transmitted to all daughter cells (Coffin *et al.* 1997). Furthermore, they are able to bring about long term transgene expression in a variety of human cell types (Austin *et al.* 2000).

1.6.1.4 Lentiviral Vectors and Transduction of Non-mitotic Cells

While lentiviruses are a subgroup of retroviruses and share the general features of the group, their ability to transduce non-mitotic cells has important implications for gene therapy. Whereas nuclear transport of the preintegration complex of other retroviruses is dependent upon the breakdown of the nuclear envelope that occurs at mitosis (Weinberg *et al.* 1991; Roe *et al.* 1993; Lewis and Emerman 1994), the karyophilic properties of the lentiviral preintegration complex enable active transport through the nucleopore, and thus they are able to mediate efficient delivery, integration, and long-term transgene expression of non-mitotic cells (Naldini *et al.* 1996a; Naldini *et al.* 1996b; Blomer *et al.* 1997). This allows transduction of terminally differentiated and non-dividing quiescent cells (Lewis *et al.* 1992; Bukrinsky *et al.* 1993; Srinivasakumar 2001; Scherr and Eder 2002), ultimately resulting in higher rates of transduction (Chang *et al.* 1999; Park and Choi 2004).

1.6.2 Enhancing the Safety of Retroviral and Lentiviral Vectors

While retroviruses, including lentiviruses, hold great potential as gene therapy vectors, the wild type viruses are often highly pathogenic and have undergone and continue to undergo extensive modifications in order to make them safer.

1.6.2.1 Self-Inactivating Retrovectors

To minimise the risk of replication and inadvertent spread of vectors to non-target tissues, vectors were designed to self-inactivate after gene delivery. In these vectors, after integration certain *cis*-acting elements necessary to efficiently complete another round of replication were deleted (Hu and Pathak 2000). U3-negative vectors were the first such self-inactivating vectors to be developed, and are still the most commonly used form of self-inactivating vector (Hu and Pathak 2000). In such vectors, the U3 region of the 3' LTR is deleted. Because this forms the template for the U3 region of the 5' LTR during reverse transcription, the resulting integrated provirus lacks the strong U3 promoter (Yu *et al.* 1986; Dougherty and Temin 1987; Zufferey *et al.* 1998). Elimination of this promoter also reduces the risk of aberrant gene expression and genotoxicity, with cell-culture assays demonstrating a significantly reduced transforming capacity from self-inactivating vectors when compared with corresponding LTR-intact vectors (Modlich *et al.* 2006).

1.6.2.2 Fragmentation of the Retrovirus Genome and Use of Packaging Cells

The risk of vector replication has been further reduced by deletion of non-essential and pathogenic genes fragmentation of the genome into separate elements (Ni *et al.* 2005). Packaging cells lines that stably express the retroviral accessory protein have been developed that facilitate the safe production of replication-deficient virus carrying only the genes of interest (Mann *et al.* 1983; Watanabe and Temin 1983). The development of packaging cells able to stably express lentiviral accessory proteins has proved challenging and transient co-transfection of helper cells with multiple plasmids is more common (Ni *et al.* 2005). By these methods, high-titre, replication-defective retrovirus or lentivirus vectors may be safely produced (Landau and Littman 1992; Pear *et al.* 1993; Finer *et al.* 1994; Soneoka *et al.* 1995).

The fragmentation and development of the lentiviral genome has continued beyond the original designs, and progressed through various generations of packaging systems. The 'first generation' lentiviral vectors were initially developed as a three-plasmid (transgene, packaging and envelope plasmids) expression system (Naldini *et al.* 1996b), as per the retroviral strategy. The HIV-1 core proteins, enzymes and accessory

genes are expressed in transiently co-transfected helper cells. Various deletions or substitutions were made in order to minimise pathogenicity and the likelihood of genetic recombination (Naldini *et al.* 1996b).

To address biosafety issues, the *vif*, *vpr*, and *nef* accessory genes were also deleted, to create the 'second generation' lentiviral vectors (Zufferey *et al.* 1997). These deletions were found to reduce transduction efficiency in some cell types, for example primary macrophages (Dull *et al.* 1998). This impairment can be overcome by replacing certain elements, for example by pseudotyping with the G surface protein from the Vesicular Stomatitis Virus (VSV-G) (Scherr and Eder 2002).

'Third generation' vectors also have a tat deletion, as this gene is unnecessary when replaced by alternate promoters from other viruses (Dull *et al.* 1998). Some variants of the third generation vectors express the *rev* gene from a separate, fourth plasmid (Dull *et al.* 1998).

The deletion of the accessory proteins, as in the second and third generation vectors, minimises the risk of producing replication-competent lentiviruses (Scherr and Eder 2002). Deletion of these various regions also reduces the risk of interaction with host genes near the integration site (Scherr and Eder 2002). The development of vectors from lentiviruses, such as feline immunodeficiency virus (FIV) (Poeschla *et al.* 1998b), and HIV-2 (Poeschla *et al.* 1998a) that are less pathogenic to humans, should also reduce the potential for adverse side-effects (Scherr and Eder 2002).

1.6.3 Markers for Elimination of Non-transduced Cells

Marker genes are widely used in gene therapy as they allow the identification of cells that have been successfully transduced with the vector, and can allow selection for or against the transduced cells by various methods. Two common forms of marker gene are fluorescent proteins, or growth selection markers such as puromycin resistance.

The Green Fluorescent Protein was originally isolated from the jellyfish *Aequorea victoria* and fluoresces green when exposed to blue light (Tsien 1998). It is a widely used and well-understood marker gene, and is frequently used as a reliable marker of cellular transduction, and may also be used semi-quantitatively. There has been some suggestion of cellular toxicity of the GFP protein (Hanazono *et al.* 1997; Liu *et al.* 1999), however, this may be due to specific sequences in the multiple cloning site of the vectors involved (Endemann *et al.* 2003).

Puromycin is an aminonucleoside antibiotic isolated from *Streptomyces alboniger* that inhibits translation in prokaryotes and eukaryotes (reviewed in Vázquez 1979). Expression of puromycin N-acetyl-transferase (PAC), originally derived from *S. alboniger*, confers resistance to puromycin upon mammalian cells (Vara *et al.* 1986). Puromycin is one of the fastest growth selection markers available, eliminating non-transduced cells in a matter of days (Kallifatidis *et al.* 2008). For some applications, puromycin selection is superior to FACS sorting (Kallifatidis *et al.* 2008), however, the ultimate use of growth selection markers in a therapeutic context has a limited future, with the European Union ruling that antibiotic resistance genes cannot be used therapeutically (Products 2001).

1.6.4 A Comparison of Retrovectors and Lentivectors

While both vector types have undergone extensive development to enhance safety, a direct comparison of both vector types in a tumour prone mouse model found that retroviral vector transduction triggered a dose-dependent acceleration of tumour onset, subsequent to vector LTR activation. By contrast, transduction with the lentiviral vector did not accelerate tumourigenesis in transplant recipients, even at high integration loads. In addition to the advanced vector design of the lentiviral vector, analysis suggested that the integration profile of the lentiviral vector also contributed to this enhanced safety (Montini *et al.* 2006). In addition, lentiviral vectors appear to be less prone to suppression of transgene expression than retroviral vectors (Svoboda *et al.* 2000; Tiscornia *et al.* 2003; Anderson and Akkina 2005). In one study, transgene expression from a lentiviral vector persisted for over 6 months in the central nervous system (Blomer *et al.* 1997). Finally, lentiviral vectors do not induce interferon-stimulated genes (Bridge *et al.* 2003; Fish and Kruithof 2004; Pebernard and Iggo 2004), and the lentiviral Tat element exerts an inhibitory effect on the interferon-stimulated dsRNA-activated inhibitor (DAI) protein kinase (Gunnery *et al.* 1990; Roy *et al.* 1990).

1.6.5 Transduction of Haematopoietic Stem Cells

The ability of different vector types to transduce certain cell types also varies markedly. Lentiviral vectors were able to efficiently transduce CD34+ cells whereas vectors based on the Murine Leukaemia Virus (MLV) were relatively inefficient

(Salmon *et al.* 2000). Furthermore, efficient transduction with the HIV vectors was achieved at a relatively low multiplicity of infection (Salmon *et al.* 2000).

The promoter used to express the transgene must be chosen with consideration of the cell type targeted. Commonly used promoters, such as CMV and CAG were inefficient and vulnerable to suppression in embryonic stem cells (Xia *et al.* 2007). CMV promoters also had low transcriptional activity in most lympho-haematopoietic cell lines (Case *et al.* 1999; Miyoshi *et al.* 1999; An *et al.* 2000). In contrast, the EF-1α promoter is highly efficient in CD34+ cells and several differentiated haematopoietic cell lines the, and there was no evidence of transgene suppression (Salmon *et al.* 2000).

1.6.6 Retroviral Integrases and Targeting of Integration

The integration of gene therapy vectors is crucial to the long term expression of therapeutic transgenes from these vectors, and this integration is mediated by specialised and characteristic enzymes known as integrases (Coffin *et al.* 1997). Retroviral integrase (IN) contains three physically distinct domains; an N-terminal domain, including three α-helices and a zinc-binding motif; a central domain containing the conserved central triad D,D(35)E; and the C-terminal domain, which contributes to oligomerisation and has non-specific DNA-binding activity (reviewed in Sandmeyer 2003; Zhu *et al.* 2003). The retroviral preintegration complex consists of integrase bound to the ends of the full-length DNA. This complex mediates the integration of the DNA into the host genome (Sandmeyer 2003). Integrase is also responsible for nuclear import of viral cDNA in HIV (Ikeda *et al.* 2004).

Traditionally, retroviral integration was considered to be targeted primarily towards active genes, with access dependent upon the open chromatin associated with transcription (Coffin *et al.* 1997). Bending of the target DNA and hairpin structures also favour integration (Sandmeyer 2003) while a high level of transcriptional activity disfavours integration at that location, probably due to stearic hindrance with the polymerase machinery (Weidhaas *et al.* 2000; Mitchell *et al.* 2004; Maxfield *et al.* 2005). Beyond these trends, however, integration biases exist with respect to whole chromosomes that cannot be entirely accounted for by gene density or activity, suggesting the influence of other factors (Mitchell *et al.* 2004). Such results are more compatible with a 'tethering' model, in which the pre-integrative complex (PIC) associates with specific elements of the target genome (Bushman 2003). Tethering

would allow more specific association with promoters of factors found in transcription units, for example (Bushman 2003).

1.6.6.1 Targeting Preferences of Currently-Used Vectors

Integration of retroviral integrases is clearly not targeted to specific sequences, and it is evident that different selection pressures apply to different integrating retroviruses, resulting in characteristic integration profiles for each virus group (Bushman 2003). Unfortunately, the integration strategies that might be useful for a pathogenic virus, which routinely cause the death of the host cell in the course of replication and transmission, are often not desirable in most gene therapy application. For example, HIV infection which quickly leads to pathogenic cell death, and in this context transcription and virion production would be maximised by integration into transcriptionally active regions (Bushman 2003; Jordan *et al.* 2003). As such, the integration profiles of vectors currently used for gene therapy are coming under closer scrutiny.

The wild-type adeno-associated virus (AAV) integrates preferentially in a specific region of human chromosome 19 (Russell *et al.* 1994). AAV gene therapy vectors in human cells exhibited a significant integration preference for CpG islands, and the first 1 kb of genes. However overall, there was only a slight preference for transcribed sequences (Miller *et al.* 2005)

Over one third (39%) of Murine Leukemia Virus (MLV) integrations in the human genome were within transcription units (Wu *et al.* 2003). Within these transcription units, there was a preference for promoter regions over downstream regions. Up to 17% of all integration sites were within 1kb either side of the transcriptional start point, dropping off to background levels within 5kb on either side (Wu *et al.* 2003). MLV integration was also positively associated with CpG islands (Wu *et al.* 2003; Mitchell *et al.* 2004).

The distribution of foamy virus integration sites in the human genome was similarly non-random, but they did not preferentially integrate within genes. There was however a modest preference for integration start sites, and a significant preference for CpG islands (Trobridge *et al.* 2006).

Human Immunodeficiency Virus (HIV*)* vector integrations appear to show a strong bias, with up to 69% of integration sites occurring within transcription units, though there was no bias within these units (Schroder *et al.* 2002; Wu *et al.* 2003). Areas of very high-level transcription were actually disfavoured, as were CpG islands, which usually correspond with transcription factor binding sites (Mitchell *et al.* 2004). More broadly, integration site selection correlated with transcriptional activity. Similarly, genes activated by HIV infection were subject to higher rates of integration (Schroder *et al.* 2002). Despite the preference of HIV for insertion into transcriptionally active sites, direct oncogenic transformation of human cells by HIV is rare (Beatty *et al.* 2002), with only one probable case recorded (Herndier *et al.* 1992). However, while a causative association was not demonstrated, a number of non-Hodgkins lymphomas from late-stage AIDS patients were found to have HIV inserted in a uniform location, within the *fur* gene. This insertion evidently upregulated the c-*fes/fps* protooncogene (Shiramizu *et al.* 1994). Furthermore, it is probable that the lytic nature of wild-type HIV may obscure any oncogenic tendencies (Mok and Lever 2005).

1.6.6.2 Integration Targeting Characteristics of Other Retroviral Groups

A series of other lentiviral and retroviral vectors are under investigation for gene therapy, and the integration profiles of these have also come under scrutiny. The integration profile of Feline immunodeficiency virus (FIV) favoured actively-transcribed genes, with integrations occurring across the entire length of the transcriptional units. As such, FIV integration preferences were more similar to those of other primate lentiviruses, and distinct from those of Moloney murine leukemia virus, Foamy Virus, and ASLV (see below) (Kang *et al.* 2006). It is worth noting that FIV was associated with an instance of B-cell lymphoma, with vector insertion occurring in a gene in a region of tumour-specific deletions (Beatty *et al.* 2002).

Vectors based on the equine infectious anaemia virus (EIAV) preferentially integrated within active genes, but showed no preference within the transcription unit (Hacker *et al.* 2006).

Avian Sarcoma-Leukosis Virus (ASLV) showed no preference for transcription units or active genes in human cells, and integration was only slightly correlated with

CpG islands (Mitchell *et al.* 2004). These characteristics may result from ALSV having strict reactive preferences with chicken cells (Mitchell *et al.* 2004).

Avian Sarcoma Virus (ASV) also shows only a slight preference for genes, and transcriptional activity does not appear to influence site selection (Narezkina *et al.* 2004).

1.6.7 Safety Concerns and Insertional Mutagenesis

Retroviral vectors are widely used, and a number of clinical trials have been conducted. One such trial involved a replication-defective Moloney murine leukemia retrovirus vector designed to express the IL2 receptor γ chain (IL2RG) successfully corrected X-linked severe combined immunodeficiency (Hacein-Bey-Abina *et al.* 2002). Unfortunately a number of X-linked severe immunodeficiency patients receiving viral therapy developed leukaemia (Bonetta 2002; Hacein-Bey-Abina *et al.* 2003a; Marshall 2003). Two of these cases were examined in detail and the *IL2RG*-containing vector was found to have integrated in proximity to *LMO2*, a known T-cell oncogene (Boehm *et al.* 1991; Royer-Pokora *et al.* 1991) resulting in aberrant transcription and expression of *LMO2* in monoclonal T-cells (Hacein-Bey-Abina *et al.* 2003a; Hacein-Bey-Abina *et al.* 2003b). The oncogenic interaction of these genes has previously been recorded in a database of retrovirally induced hematopoietic tumours in mice (Davé *et al.* 2004). It was likely that many patients received a high number of cells each with insertions in similar locations, suggesting that this event was likely accompanied by other acquired somatic mutations, possibly from other vector insertions (Hacein-Bey-Abina *et al.* 2003b; Davé *et al.* 2004; Howe *et al.* 2008). Furthermore, there are a number of factors unique to X-SCID that may facilitate a high rate of transformation (Shou *et al.* 2006).

Detailed studies with another retrovirus vector, derived from the Harvey murine sarcoma retrovirus found clonal amplification caused by insertional mutagenesis following vector integration (Modlich *et al.* 2005). The leukemic clones had an over-representation of insertional mutations in proto-oncogenes and/or other signalling genes (Modlich *et al.* 2005). Some cases also exhibited additional chromosomal translocations, indicating secondary genetic instability (Modlich *et al.* 2005).

In light of these findings of retrovector genotoxicity, attention has focussed on strategies to minimise the risks of insertional mutagenesis. These include the generation

of more efficient vectors to minimise the number of copies required per cell (Bonetta 2002; Scherr *et al.* 2005), and improved vector design, in order to minimise the vectors' ability to transactivate neighbouring genes (Baum *et al.* 2003; Kohn *et al.* 2003; Sadelain 2004). Another avenue for enhancing the safety of gene therapies is the development of site-specific recombinases, or the identification and utilisation of novel integrase enzymes from non-retroviral integrating elements that might have safer integration profiles (Glover *et al.* 2005).

1.6.8 Selection Pressures on Other Integrating Elements

Different selection pressures apply to different integrating retroviruses and this is true of other integrating elements as well however many groups of integrating elements have lost the ability to infect new hosts, and in such cases, the selection pressures are dramatically different (Bushman 2003). For example; yeast LTR-retrotransposons replicate within a single, gene-dense cell, and must integrate without suicidal disruption of genes necessary for host cell survival. Similarly, most HERVs present in the human genome are found outside transcription units, and where they do occur within genes, they are usually in a reverse orientation, such that they do not interfere with expression of that gene (Smit 1999). These characteristics suggest selection after integration (Bushman 2003).

Alternate integration systems should allow modulation of integration target sites and offer the potential for safer gene therapy options (Schroder *et al.* 2002; Wu *et al.* 2003; Mitchell *et al.* 2004).

1.6.9 Retrotransposons

Retrotransposons represent one of the major groups of 'retroelements', units that are able to amplify to new locations within the eukaryotic genome via an RNA intermediate. The 'LTR retrotransposons', aside from possessing long terminal repeats, share other structural characteristics with retroviruses, and encode proteins necessary for retrotransposition (eg. integrase and reverse transcriptase). However, they lack the genetic components required for forming a functional viral capsule (reviewed Deininger and Batzer 2002; Bowen *et al.* 2003). They are therefore dependent upon vertical transmission from their host cell to their vertically derived progeny (Craigie 1992).

Retrotransposons occur in many groups, including humans (reviewed in Miki 1998) however perhaps some of the most interesting, and certainly among the best studied, are the retrotransposons found in yeast cells. Compared with the genomes of many eukaryotes, the genomes of yeast, such as that of *Saccharomyces cerevisiae*, are densely packed with open reading frames (ORFs). These genomes appear to be intolerant of excessive junk DNA, containing relatively few transposon copies (Boeke and Devine 1998). From this it is concluded that there exists strong selection pressure on retrotransposon integration to minimise adverse effects upon their host. accordingly, the integration targeting of yeast retrotransposons is highly specific to 'safe' regions of the host genome, where they are least likely to cause damage (Boeke and Devine 1998).

A number of retrotransposons found in *Saccharomyces cerevisiae* have been examined in considerable detail. The Ty5 retrotransposon preferentially inserts into the heterochromatic regions of the yeast genome, at the telomeres, and at silent mating loci (Brady *et al.* 2008). This is also the case for most eukaryotic retrotransposons (Zou *et al.* 1995). Targeting is mediated by an interaction between Ty5 integrase and the heterochromatin protein silent information regulator 4 (Sir4) (Xie *et al.* 2001; Brady *et al.* 2008). The Ty1 retrotransposon integrates within a window located 100-700bp upstream of genes transcribed by RNA polymerase III (Devine and Boeke 1996). The integration targeting of Ty3 also occurs upstream of PolIII genes, however, the targeting of Ty3 integration is specific to the 1-4 nucleotides immediately upstream of the transcription initiation sites (Chalker and Sandmeyer 1992). This targeting results from tethering of the Ty3 integration machinery to the Pol III transcription apparatus, specifically via protein-protein interaction with the transcription factors TFIII-B and -C (Kirchner *et al.* 1995; Connolly and Sandmeyer 1997).

As locations for integration, the areas upstream of Pol III genes are thought to be excellent locations for retrotransposon integration, as they are gene-poor, and most Pol III genes, including the tRNA genes that are the targets of Ty3 integrase, possess only internal promoters. Thus insertions in such locations are expected to have a minimal effect on gene expression levels (Chalker and Sandmeyer 1992; Devine and Boeke 1996; Zhu *et al.* 2003). It should be noted, however, that there is some evidence that levels of SUP2 pre-tRNA were moderately increased when SUP2 was associated with the Ty3 LTR, in either orientation (Kinsey and Sandmeyer 1991).

1.6.10 Ty3 Integrase and Human Genes

The Ty3 integrase has numerous features that are desirable in human gene therapy, although the activity of Ty3 integrase in human cells has not yet been examined directly. Nevertheless, two early studies have examined aspects of the interaction between this enzyme and human genes, and provide a benchmark for further study.

In the first of these experiments, a plasmid containing the human tRNA-Lys gene was introduced into yeast cells. The Ty3 integrase was found to preferentially interact with the transcription start site of these genes, although this interaction was presumably mediated by the recognition of the human gene by the yeast transcription factor subunits, TFIIIB and TFIIIC (Dildine and Sandmeyer 1997).

The second study was conducted with a chimeric integrase in which the C-terminal domain of MoMLV integrase was replaced with the C-terminal domain of Ty3 integrase. This chimeric integrase was found to be active in human cells, however, sequence analysis of integration sites indicated that it did not target human tRNA genes (Dildine *et al.* 1998). It should be noted however that in a range of viral systems, while the N-terminus had no real influence both the C-terminus and the central domain determine the activity and substrate specificity of the enzyme (Berger *et al.* 2001).

Another observation relevant to the potential of Ty3 integrase in human cells is the high degree of homology between the *S. cerevisiae* and human TFIIIB subunits (Huang and Maraia 2001). The homology extends as far as the three initiation-related subunits of TFIIIC that are associated with the proximal promoter element, but there is markedly less homology in the more distal TFIIIC subunits (Huang and Maraia 2001).

1.7 Aims

The hypotheses and aims of this project are as follows:

Hypothesis 1: That an optimised vector design and arrangement of shRNA cassettes within the lentiviral vectors can enhance delivery and expression of shRNA molecules

Aim 1: To optimise the structure of lentiviral vectors for the most efficient and effective knockdown of target genes.

Hypothesis 2: That down-regulation of the Bcr/Abl fusion gene and protein will trigger cell differentiation in surviving cells

Aim 2: To analyse the effects of knockdown of the Bcr/Abl fusion gene by retroviral delivery of shRNAs on cell differentiation.

Hypothesis 3: That appropriately-designed shRNAs will down-regulate PML-RARα mRNA, with subsequent effects on biology of the cancer cells

Aim 3: To test lentivector-delivered shRNAs designed to downregulate the PML-RARα fusion gene, and assess their effects on the biology of the cells.

Hypothesis 4: That appropriately-designed shRNAs will down-regulate RUNX1-ETO mRNA, with subsequent effects on biology of the cancer cells

Aim 4: To test lentivector-delivered shRNAs designed to downregulate the RUNX1-ETO fusion gene, and assess their effects on the biology of the cells.

Hypothesis 5: That the integrase enzyme of the Ty3 yeast retrotransposon will facilitate integration in human cells

Aim 5: To investigate the rate and nature of transgene integration mediated by the Ty3 yeast retrotransposon integrase in human cells.

2 General Materials and Methods

2.1 Cell Culture

The 293T cells were cultured in 10% DMEM (see list of buffers and recipes, Section 2.13). To passage or replate the 293T cells, they were trypsinised by washing with sterile PBS, followed by the addition of approximately 5 ml Trypsin/EDTA per T75 flask. Culture vessels were gently rocked until cells detached.

K562 and NB4 cell lines were cultured in 10% RPMI. Kasumi cells were cultured in 20% RPMI, which was made up in small batches such that the L-glutamine never exceeded two weeks of age. Doubling time of the K562 and NB4 cell lines was approximately two days, whereas the Kasumi's doubled in approximately three days. Cells were maintained at densities in the range of 5-10 x 10^5 cells/ml, except Kasumi cells were split at approximately 12 x 10^5 cells/ml. For maintenance of the culture, cells were split by diluting the culture volume with fresh medium. Cells were cultured at 37 $^{\circ}$C and 5% CO_2 in a humidified incubator.

Cell density was measured with a haematocytometer. In the case of cell viability counts, cells were first mixed 1:1 with Trypan blue, with the number of Trypan positive and negative cells counted.

All cell lines were spun at 1000 g, except for Kasumi cells, which were spun at 300 g. Cells were frozen at 10^6 cells per ml of 10% DMSO (Sigma) and 90% FCS, and stored at -80 $^{\circ}$C or in liquid nitrogen.

2.2 Cloning

Custom vectors were generated by modifying existing plasmid and vector backbones, as indicated. The principal methods used in cloning were Polymerase Chain Reaction (PCR) amplification, restriction digestion and ligation.

To generate genes flanked by useful restriction sites, primers were designed using one of three programs; MacVector software, or the Primer 3 (http://frodo.wi.mit.edu/primer3/) or Primer BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHome) online tools. Useful restriction sites were added to the 5' ends of these primers as

appropriate. PCRs were performed using iTaq DNA Polymerase (BioRad) or Taq DNA Polymerase (Invitrogen), as per the manufacturers instructions.

Restriction maps of existing vectors were generated from available vector maps entered into the NEBcutter online tool (http://tools.neb.com/NEBcutter2/). Restriction digestions were performed with restriction enzymes (New England Biolabs), as per the supplier's instructions. Dephosphorylation of digested fragments, when required, was performed using Alkaline or Antarctic Phosphatases (New England Biolabs), as per the manufacturers instructions.

The products of PCRs and restriction digests were separated by electrophoresis on 1% agarose gels in TAE buffer. DNA was visualised with 0.85 ng of Ethidium Bromide (Sigma) per 50 ml gel, or with 1.7 µl SYBR-safe stock (Invitrogen) per 50 ml gel. Gels were electrophoresed at 70-120 V, then examined using a UV light-box. Gels images were recorded using the Gel Doc system (Bio-Rad).

Selected bands were excised and purified using a Wizard SV Gel and PCR Clean-up kit (Promega), as per manufacturer's instructions.

Ligation of DNA fragments was performed using T4 DNA Ligase (New England Biolabs), based on the manufacturers instructions. Ligation incubation times and volumes were sometimes varied to improve reaction efficiency.

2.3 Culture and Transformation of Bacteria

2.3.1 Transformation, Culture and Screening of Bacteria

Competent *E. coli* were transformed with custom plasmids by mixing the plasmid with 100 µl of recently thawed competent *E. coli* in an Eppendorf tube. Tubes were placed on ice for 40 minutes, 'heat shocked' at 42 ^{o}C for 90 seconds, then returned to ice for 10 minutes. *E. coli* were then plated onto agar plates containing the appropriate antibiotic, and grown overnight at 37 ^{o}C. Top 10 *E. coli* (Invitrogen) were used for general cloning and ER2925 *E. coli* (New England Biolabs) were used for Dam-negative culture.

Where possible, candidate colonies were screened by PCR. This was done by introducing a small sample of the colony into a PCR reaction that included appropriate primers. The PCR reaction was then visualised by gel electrophoresis.

Candidate colonies were subsequently transferred via clean pipette tip to Luria Broth containing ampicillin or kanamycins, as appropriate, at 0.05 mg/ml. For routine cloning, 5 ml of broth was incubated at 37 ^{o}C overnight with shaking. Cultures were then prepared with either a Wizard Plus SV Miniprep DNA Purification System kit (Promega), or a PureLink Quick Plasmid Miniprep kit (Invitrogen), as per the manufacturer's instructions. For larger cultures, bacteria were as above, but in 200 ml of Luria Broth. These cultures were prepared with a PureYield Plasmid Midiprep System kit (Promega), as per manufacturer's instructions.

2.3.2 Glycerol Stocks

Select cultures of *E. coli* were frozen for later use in glycerol by adding 850µl of overnight culture to 150 µl of glycerol. This was mixed thoroughly and stored at − 80 ^{o}C.

2.4 Hairpin Design, Annealing and Nonsense Control

All shRNA designs were generated from one of two online algorithm tools, provided by Ambion (http://www.ambion.com/techlib/misc/siRNA_finder.html) and GenScript (https://www.genscript.com/ssl-bin/app/rnai). Output sequences were 20 and 21 nucleotides respectively, however both tools presented the output in an inverted repeat format suitable for expression of shRNAs from pSilencer vectors. The 3.1-H1 neo pSilencer vector (Ambion) provided a H1 PolIII promoter for shRNA expression, and facilitated subsequent cloning of the expression cassette into other backbones (see below). While it is usual to test at least five different hairpin designs, the need to include the breakpoint restricted the target sequence to 40 nucleotides (20 nucleotides either side of the breakpoint) which in turn greatly reduced the number of candidate designs that could be tested.

The sense and antisense strands provided by the online tools were ordered as custom oligos from Geneworks, and the 2 µl of each of the complementary single-stranded oligonucleotides (at 30 µM) were mixed with 5 µl of '10x annealing buffer' in a 50 µl reaction (made up with dH$_2$O). This mixture was heated to 100 ^{o}C in a heating block and allowed to cool slowly. Alternatively, a thermocycler was used, in which case the mixture was cooled from 100 ^{o}C at a rate of 1 ^{o}C per minute. The resulting

dsDNA fragments were purified and ligated into a BamHI- and HindIII-digested pSilencer backbone.

A variety of controls are recommended for experiments involving shRNAs ("Whither RNAi?", Nature Cell Biology (2003), v.5 (6) pp. 489-490), and selection of appropriate controls is discussed in the relevant sections. However the 'Nonsense' control was used as the default control throughout these experiments, and consisted of a scrambled shRNA design which was provided with the pSilencer vector (Ambion), which corresponded to no known sequence.

2.5 Vector Construction

The pHIV-7-GFP vector and the plasmids described below were used throughout many sections of this thesis. Additional vectors used in this study are described where appropriate.

The pHIV-7-GFP lentivirus vector was donated by Professor J. J. Rossi (City of Hope, Duarte). This vector has been used and validated previously, and resulted in >98 % transgene expression in K562 cells (Li *et al.* 2003b). Of the unique restriction sites available in this vector, the BamHI site has been used and validated for expression of shRNA cassettes (Li *et al.* 2003b). Immediately adjacent to this site there was also an XmaI restriction site. As the BamHI site was also present in the pSilencer shRNA expression cassette, the XmaI site was used for insertion of the shRNA cassette. The shRNA cassettes from the pSilencer vectors (see above) were amplified by PCR. The primers used for this amplification were based on the pSilencer sequencing primers recommended by Ambion, with XmaI linker sequences added to the 5' end of each to allow cloning into the pHIV-7-GFP backbone in either orientation. The final arrangement is shown in Figure 2.1.

Although the pHIV-7-GFP vector was a 'third-generation' lentiviral vector, and was supplied with three accessory plasmids (pCHGP-2, pCMV-rev and pCMV-G, Li *et al.* 2003b), it was used here in conjunction with the 'second-generation' accessory plasmids supplied by the Tronolab (Lausanne, Switzerland). These accessory plasmids were the pMD2.G and psPAX2 plasmids.

Diagnostic restriction digestions were used for initial screening vector constructs. Finally, Beckman sequencing was carried out by Macrogen (Seoul, Korea).

Figure 2.1 pHIV-7-GFP-NSf
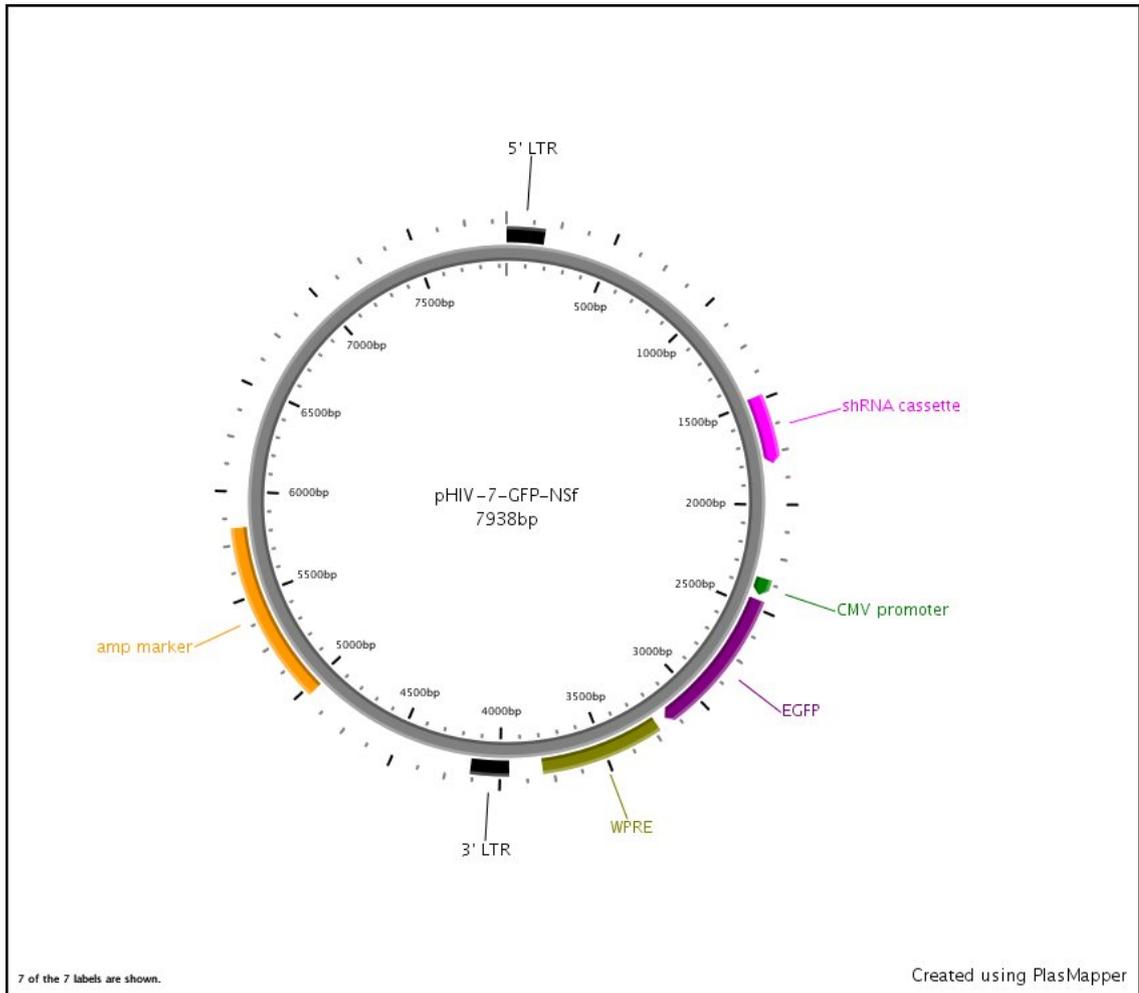
Figure 2.1 – The pHIV-7-GFP-shf vector construct, with the (NS) shRNA cassette inserted upstream of the GFP marker gene, and oriented in the same direction.

2.6 Production of Infectious Vector Particles

293T cells were seeded at 5×10^5 cells per well, in a 6-well plate, with 2 ml complete DMEM two days prior to transfection.

For each well, 4 µg of vector, 3 µg of psPAX2 plasmid, and 1 µg of pMDG2 plasmid in 50 µl of sterile dH$_2$O was mixed with 50 µl of sterile 2 M CaCl$_2$. This mixture was added dropwise to 100 µl sterile 2x HBS (pH 7.06), with bubbling, then incubated at room temperature for 20 minutes. During the incubation, the culture medium was removed from the packaging cells and replaced with 1 ml of serum-free DMEM. After incubation, the mixture was added dropwise to the cells (200 µl/well) with gentle swirling. Cells were returned to the incubator, then after 1 hour, 1 ml of DMEM with 20% FCS was added to each well.

After a further 5-7 hours (at 6-8 hours), the media was replaced with 2 ml per well of complete DMEM plus 20 µl of 0.6 M butyric acid (sterile, in PBS). This media was harvested and frozen at 24 hours, and replaced with same. Media was finally harvested and frozen at 36-48 hours, after which the cells were discarded.

The frozen supernatant was thawed and filtered through 0.4 µm filter cartridges. 1 ml aliquots of the filtrate were ultracentrifuged in autoclaved Eppendorf tubes for 5 hours at 21,640 g and 4 ^{o}C. Tubes were then opened and the supernatant gently decanted to avoid disturbing the loose pellet, and excess liquid removed by touching the rim to a clean tissue. The pellet was then resuspended in an appropriate volume (0.5 ml for NB4 cells, 0.25 ml for Kasumi cells) of the appropriate complete medium, and frozen until required.

2.7 Transduction of target cells with purified vector

The following protocol was found to give reliably high titres in the refractory leukaemic cell lines used in these experiments (see Chapter 3).

Vector aliquots were thawed and reconstituted with the addition of an equal volume of fresh medium, restoring the original vector concentration. 2 ml total of reconstituted vector medium was added to 5×10^5 cells per well in a 6-well plate, with 'Polybrene' (hexadimethrine bromide, Sigma) added at 250 ng/ml. This was 'spinoculated' at 1000 g (300 g for Kasumi cells) for 100 minutes at room temperature.

Plates were then returned to the incubator. This transduction procedure was repeated after 6 hours.

Rates of transduction were assessed by measurement of expression of the GFP marker at 48 hours post-transduction.

For attached 293T cells, the percentage of fluorescent cells was estimated by visual examination of cells using a Leica DM ARB inverted fluorescent microscope with a blue filter. This was sufficient to reliably distinguish fluorescent from non-fluorescent cells.

For more detailed analysis, flow cytometry analysis of cellular fluorescence was performed with a Coulter EPIGS ELITE ESP fluorescence activated cell sorter (FACS) machine. Where appropriate, cells were fixed in 'FACS Fix' and stored in the dark at 4 $^{\circ}$C prior to FACS analysis. The proportion of transduced cells was measured as the percentage of cells greater than a threshold fluorescent level. The mean or median fluorescence intensity was measured on an arbitrary scale, with all appropriate control samples included in each run.

2.8 Quantitative Reverse Transcription Polymerase Chain Reactions (qRT-PCRs)

2.8.1 RNA Isolation

RNA was isolated using Tri Reagent (Sigma) from 2-5x10^6 cells per sample. Cells were pelleted then lysed by repeated pipetting with 1 ml Tri-reagent (per 5-10 x 10^6 cells). After a 5 minute incubation at room temperature, 0.1 ml of 1-Bromo-3-chloro-propane was added to each homogenate, which were then shaken vigorously for 30 seconds, then incubated for 15 minutes at room temperature. Samples were then centrifuged at 12,000 g and 4 $^{\circ}$C for 15 minutes, and the upper, aqueous phase was carefully transferred to a fresh tube, avoiding the intermediate phase. 0.5 ml isopropanol was added to this, tubes were mixed by inversion, and RNA was allowed to precipitate at room temperature for 10 minutes. Samples were centrifuged at 12,000 g for 10 minutes at 4 $^{\circ}$C, then the supernatant removed. The pellet was washed by vortexing with 1 ml 75% ethanol, then centrifuged at 12,000g for 5 minutes at 4 $^{\circ}$C. the ethanol was removed, and the pellet briefly air-dried. The RNA was then dissolved in

sterile, nuclease-free water by pipetting followed by incubation for 10-15 minutes at 60 $^{\circ}$C.

Prior to qRT-PCR, 1000 ng of RNA was made up to 8 μl with SNFW, 1 μl of 10x DNase Reaction Buffer (Sigma) was added to each sample, then samples were each treated with 1 μl of Amplification Grade DNase I (1 unit/μl, Sigma). Samples were mixed without vortexing, then incubated for 15 minutes at room temperature. Subsequently 1 μl of Stop Solution (Sigma) was added and the mixture heated at 70 $^{\circ}$C for 10 minutes. After chilling on ice, samples were ready for downstream applications.

2.8.2 qRT-PCR Chemistry

qRT-PCRs were performed using the one-step iScript SYBR Green RT-PCR Kit (Bio-Rad) or the Plexor One-Step qRT-PCR System (Promega), according to the manufacturers instructions. The Plexor system required the use of labelled primers (Biosearch Technologies).

qRT-PCR programmes were based on the recommendations of the manufacturers of the various kits used, and were modified with respect to annealing temperatures, acquisition temperatures, and cycle number, as appropriate.

2.8.3 'House-keeping' Genes

Multiple house-keeping genes were used as standards in this study.

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), has long been used as a house-keeping standard for qRT-PCR, however due to the presence of pseudogene copies and variability under certain conditions, alternative housekeeping genes have been sought (Gabert *et al.* 2003). Beta-2-microglobulin (B2M) is considered a more reliable house-keeping gene (Schmittgen and Zakrajsek 2000), and so was used in preference throughout this study.

The Beta-2-microglobulin house-keeping gene (Beillard *et al.* 2003) used with the SYBR Green chemistry were as follows:

B2MFP

    5'-GAGTATGCCTGCCGTGTG-3'

B2MRP

    5'-AATCCAAATGCGGCATCT-3'

    Product size = 110 bp

The primers used for 2'5'-oligoadenylate synthetase (OAS) (Bridge *et al.* 2003) were as follows:

OASFP

    5'-AGGTGGTAAAGGGTGGCTCC-3'

OASRP

    5'-ACAACCAGGTCAGCGTCAGAT-3'

    Product size = 70 bp

Due to the unique chemistry of the Plexor system, specific Beta-2-microglobulin primers were designed using the Plexor Primer Design System (Promega website). These primers were as follows:

B2MFP

    5'-TGCTTACATGTCTCGATCCCACTTAAC-3', labelled with a 5' CAL Fluor Red 610 fluorophore

B2MRP

    5'-CTGGTCTTTCTATCTCTTGTACTACACTGAAT-3'

    Product size = 127 bp.

2.8.4 Standard Curves

qRT-PCRs were performed using a RotorGene 3000 (Qiagen).

To generate standard curves, PCR products obtained with the relevant primers were gel purified, extracted, quantified, and then used at 10-fold serial dilutions, ranging from 10^7 to 10^1 copies per 10 µl reaction. Reaction conditions for the standard curves were identical to conditions used for the samples, including the presence of the other primer pairs used in the qRT-PCR run. After generation of the standard curves, one standard was included in each run as an internal control, allowing normalisation to the standard curve during analysis. In this way, a 'relative copy number' value per tube was obtained for each gene. Values for the gene of interest (ie. the fusion gene) were then normalised against the house-keeping gene to obtain a relative value of fusion gene mRNA levels for that sample.

A standard threshold value of 0.05 was used for all qRT-PCR runs.

2.8.5 Analysis of Rotorgene Results

For each sample, relative copy number estimates of the house-keeping gene and the gene of interest were obtained. Where the SYBR Green system was used, these readings were obtained from separate tubes, however the Plexor system reduced variability by allowing both readings to be taken from the same tube. The copy number of the gene of interest was normalised against the copy number of the house-keeping gene providing a 'relative copy number' for each sample.

Due to variation between RotorGene runs, relative copy numbers for NS controls and the experimental shRNA designs for each run were treated as paired values, and analysed using paired t-tests from a minimum of four runs (n=4), and p-values less than 0.05 were considered significant.

2.9 Western Blotting

2.9.1 Isolation of Nuclear and Cytosol Extracts

All buffers and reagents used for the isolation of nuclear and cytosol extracts were chilled on ice before commencing the protocol. The following volumes are appropriate for up to 1.25×10^7 cells.

Cells were spun down at 500 g for 5 minutes at 4°C, then the supernatant removed by aspiration. The side of the tube was gently tapped to loosen the cell pellet, which was then resuspended in 1 ml ice-cold PBS. A further 9 ml PBS was added, then the were spun down again at 500 g for 5 minutes at 4 ^{o}C. The supernatant was removed and the cell pellet placed on ice. Again, the pellet was loosened, then resuspended in 1 ml of Buffer A containing Igepal, and transferred to an Eppendorf tube and incubated on ice for 5 minutes. The tubes were then spun at 1500 g for 5 minutes at 4 ^{o}C. The supernatant was removed and transferred to a clean Eppendorf tube. These cytoplasmic extracts were stored at −80 ^{o}C until required.

The pellet was resuspended in 1 ml Buffer A (no Igepal), then spun for 5 minutes at 1500 g. During this spin, 20 µl of Protease Inhibitor Cocktail (Sigma) and 1 µl of dithiorithrol (Bio-Rad) were added to 1 ml of Buffer C. After spinning, the supernatant was removed and the pellet resuspended in 25 µl of Buffer C. This was incubated on ice with shaking for 15 minutes at 4 ^{o}C, then spun at 13,000 g for 5 minutes at 4 ^{o}C. The supernatant was removed to a clean tube, and these nuclear extracts were stored at − 80 ^{o}C until required.

Protein content of these samples was assayed in duplicate using Bradford reagent (Bio-Rad) at 490 μm.

2.9.2 Polyacrylamide Gel Electrophoresis (PAGE)

Proteins were separated by polyacrylamide gel electrophoresis (PAGE). For proteins in the size range of 10-100 kDa, 12 % gels were used. 12% separating gels were cast into plastic cassettes (Invitrogen) and overlayed with 1 ml saturated butanol solution (butyl-alcohol plus dH_2O) until set (approximately 20 minutes). After setting, the butanol solution was removed, the cassette was washed with dH_2O, and the stacking gel poured over the separating gel, the comb was inserted, and all bubbles removed.

50 μg of cytosolic samples, and 30 μg of nuclear samples were run per lane. Prior to loading, samples were mixed with 3x Laemmli sample loading buffer and boiled for 5 minutes.

The cassette was placed into a Novex XCell II mini cell (Invitrogen), the cell filled with running buffer, and the comb removed. Samples and Protein-Pure ladder (Bio-Rad) were loaded, then run at 30 mA until sufficient separation of the ladder dye bands was observed.

2.9.3 Electro-Blotting

Samples were electroblotted onto nitrocelullose membranes (Amersham or Bio-Rad). the membrane and filter papers were trimmed to the size of the gel, and pre-wet in a shallow dish of electro-blot buffer. Gels were carefully removed from the cassette and layered with a blotting pad on the bottom (negative side), and on top of this, in order, a filter paper, the gel, the nitrocellulose membrane, another filter paper, and finally, another blotting pad. All bubbles were carefully removed from the sandwich before assembling blot module in the dH_2O-rinsed Novex XCell II mini cell (Invitrogen). The cell was filled with electroblot buffer, completely submerging the sandwich, and then the blot was run overnight at 30 V and 4 ^{o}C.

2.9.4 Antibody Staining

After electroblotting, membranes were removed from electroblot buffer and blocked with 5% low-fat skim-milk powder in TBS-Tween for 30 minutes at room temperature with rocking. Primary antibody was applied at a suitable dilution (eg.

1:1000) in 5% milk powder in TBS-Tween, and incubated at room temperature for 1 hour with rocking. Membranes were washed four times in TBS-Tween for 5 minutes each at room temperature with rocking. Secondary antibody was applied at 1:5000 in 5% milk powder in TBS-Tween at room temperature for 1 hour with rocking. Membranes were again washed four times in TBS-Tween. The membrane was kept in TBS-Tween to prevent drying out.

2.9.5 Enhanced Chemi-Luminescent Detection

Washed membranes were incubated in a 1:1 mixture of ECL Buffer and ECL Reagent (Sigma) for one minute. Membranes were exposed to X-ray films (Amersham) in a light proof container, in darkroom conditions. Timing of exposure was adjusted as appropriate.

Films were developed in Kodak Polymax RT Developer and Replenisher (Sigma), washed ten times briefly in running tap water, and then fixed for 10 minutes in Kodak Polymax RT Fixer and Replenisher (Sigma). Films were then washed for a minimum of 10 minutes in running tap water before air-drying.

2.9.6 Semi-Quantitative Analysis of Western Blots

Films of the Western Blots were scanned into the Quantity One program (Bio-Rad) and the peak intensity of the bands representing the fusion protein and the β-actin loading control were measured. To account for minor differences in the amount of protein loaded, the staining intensity of the fusion protein was expressed as a proportion of the staining intensity of the loading control. This analysis is considered to be semi-quantitative.

2.9.7 Cell Proliferation and Viability Assays

Cell proliferation assays were performed with a colorimetric 'Cell Titer 96' cell proliferation assay (Promega). 10 µl of 'Cell Titer 96' reagent was added to 200 µl of treated cells in a flat-bottom 96-well plate. The plate was returned to the incubator for 2 hours, and then absorbance was read at 490 nm using a Spectra max M2 plate reader, and SoftMax Pro 4.2 software (Molecular Devices, Surrey Hills, Australia). Raw

absorbance was converted to total cell number using a set of standard curves for each cell type.

2.9.8 Statistical Analyses

For many of the preliminary studies, where the sample size was less than three, statistical analysis was not performed. For the qRT-PCR and cell proliferation assays, there was considerable variation between replicate data sets, and analysis consisted of paired, one-tailed t-tests.

2.10 List of Buffers, Media

10% DMEM:

- Dulbecco's Modified Eagles Medium (DMEM) (Gibco)
- 10% heat-inactivated foetal calf serum (HI-FCS),
- 200 mM L-glutamine and
- 0.04 mg/ml Gentamicin (Pfizer)

10% RPMI:

- RPMI 1640 Medium (Gibco)
- 10% heat-inactivated foetal calf serum (HI-FCS),
- 200 mM L-glutamine
- 0.04 mg/ml Gentamicin (Pfizer)

20% RPMI:

- RPMI 1640 Medium (Gibco)
- 20% heat-inactivated foetal calf serum (HI-FCS),
- 200 mM L-glutamine
- 0.04 mg/ml Gentamicin (Pfizer)
- Medium made up fresh in small batches so that L-glutamine never exceeds two weeks of age

Phosphate Buffered Saline (PBS):

- 2.7 mM KCl,
- 4.3 mM dibasic Sodium Phosphate (Na_2HPO_4),
- 1.8 mM monobasic Potassium Phosphate (KH_2PO_4), and
- 137 mM NaCl
- Adjust pH to 7.2

Trypsin/EDTA:

- 0.125% Trypsin,
- 0.05 % EDTA,
- Made up in PBS and filter sterilised

Standard PCR Program:

The following PCR program reaction was used throughout this study, except where variations are indicated.

1. 94 $^{\circ}$C   2 minutes
2. 94 $^{\circ}$C   15 seconds
3. 60 $^{\circ}$C   15 seconds
4. 72 $^{\circ}$C   30 seconds
5. Go to Step 2, 30 times
6. 72 $^{\circ}$C 2 minutes

TAE Buffer:

- 4.84 g Tris base,
- 1.142 ml glacial acetic acid
- 2 ml 0.5 M Na2 EDTA (pH 8.0)
- Made up to 1 l with dH$_2$O

Luria Broth (LB):

- 1 g of tryptone (Oxoid Australia, Pty. Ltd.),
- 0.5 g of yeast extract (Oxoid),
- 0.5 g NaCl,
- This was made up to 100 ml with dH$_2$O.
- The pH was balanced by adding 20 µl of 10 M NaOH. The vessel was then autoclaved.

Agar plates:

Agar plates were made by adding 1.5 g of agar (Oxoid) to 100 ml of Luria broth. This mixture was autoclaved, then allowed to cool to between 40 and 50 $^{\circ}$C before addition of ampicillin or kanamycin at 0.05 mg/ml. This was poured into 90 mm plates in a laminar flow hood and allowed to cool. Plates were then stored at 4 $^{\circ}$C.

Competent *E. coli*:

- Grow E. coli bacteria in 6 ml Luria broth overnight with shaking at 37 °C
- Add this culture to 300 ml of Luria Broth a culture a further two hours to an optical density of 0.45 to 0.55 (at 550 nm).

- Decant this cell suspension into six 50 ml tubes and cool on ice for 15 minutes with occasional swirling
- Centrifuge tubes at 2,500 rpm for 15 min in a table-top centrifuge.
- Completely remove the supernatant and resuspend each pellet in 15 ml of cold 0.1 M $MgCl_2$ with gentle vortexing.
- Combine resuspended cells into two tubes and centrifuge again at 2,500 rpm for 15 minutes.
- Completely remove supernatant and resuspend pellets in 20 ml each of cold 0.1 M $CaCl_2$ and incubate on ice for 20 minutes
- Centrifuge again at 2,500 rpm for 10 minutes.
- Remove supernatant, resuspend each pellet in 6 ml of 0.1 M $CaCl_2$/15 % Glycerol, and combine into one tube.
- Transfer 100 μl aliquots of these competent cells to Eppendorf tubes and store at −80 °C.

10x Annealing Buffer:

- 1 ml of 1 M Tris.HCl (pH 7.5),
- 2 ml of 5 M NaCl,
- 200 μl of 0.5 M EDTA
- 6. 8ml dH_2O

Tris/EDTA (TE) Buffer:

- 1 ml of 1 M Tris-HCl, (pH 7.4),
- 0.2 ml of 0.5 M EDTA (pH 7.8)
  pH to 7.4 and make up to 100 ml with dH_2O.

2x Hepes Buffered Saline (HBS):

- 280 mM NaCl
- 50 mM HEPES
- 1.5 mM NaPO4
- pH 7.06

FACS Fix:

- 2 g glucose
- 0.67 ml 15% NaN_3
- 8 ml 40% (w/v) formaldehyde
- Made up to 100 ml with PBS

Buffers for Preparation of Nuclear Extracts

Buffer A:

- 100 μl 1 M Tris (pH 7.4)

- 100 µl 1 M NaCl
- 30 µl 1 M MgCl$_2$
- 2 µl 0.5 M EDTA (pH 8.0)
- 0.5 ml 10% Igepal (in MilliQ water)
- 9.298 ml MilliQ water

Buffer A (no Igepal):

- 50 µl 1 M Tris (pH 7.4)
- 50 µl 1 M NaCl
- 15 µl 1 M MgCl$_2$
- 1 µl 0.5 M EDTA (pH 8.0)
- 4.884 ml MilliQ water

Buffer C:

- 400 µl 1 M NaCl
- 7.5 µl 1 M MgCl
- 0.4 µl 0.5 M EDTA
- 591.1 µl MilliQ water
- Immediately before use, add 1 µl 1 M dithiorethrol

Polyacrylamide Gel Electrophoresis

5x Separating buffer:

- 112.5 g Tris
- 2.5 g SDS
- Make up to 500 ml with dH$_2$O
- Adjust pH to 8.8

10x Stacking Buffer:

- 75.5 g Tris
- 5 g SDS
- Make up to 500 ml with dH$_2$O
- Adjust pH to 6.8

12% separating gel:

- 4 ml of 30% acrylamide (Bio-Rad)
- 3 ml of '5 x separating buffer'
- 8 ml dH$_2$O
- 50 µl of 10% Ammonium Persulphate (in dH$_2$O), added immediately before pouring
- 20 µl Temed (Bio-Rad) added immediately before pouring

Stacking gel:

- 1 ml of 30% acrylamide (Bio-Rad)
- 1 ml of '10 x stacking buffer'
- 8 ml dH$_2$O
- 30 μl of 10% Ammonium Persulphate (in dH$_2$O), added immediately before pouring
- 12 μl Temed (Bio-Rad) added immediately before pouring

Laemmli Sample Loading Buffer (3x):

- 2.4 ml of 1 M Tris-Cl, pH 6.8
- 3 ml of 20% SDS
- 3 ml of glycerol
- 1.6 ml of Beta-mercaptoethanol
- 6 mg Bromophenol blue

Running Buffer:

- 3.028 g Tris
- 14.414 g glycine
- 1 g SDS
- Made up to 1 l with dH$_2$O

Electroblot Buffer:

- 4 l dH$_2$O
- 1 l methanol
- 15.1 g Tris
- 72 g glycine

10x Tris-Buffered Saline (TBS):

- 12.11 g Tris-base
- 87.66 g NaCl
- Made up to 1 l with ddH$_2$O
- Adjust pH to 7.6

TBS-Tween:

- 0.5 ml Tween 20 (BDH)
- 500 ml Tris-buffered Saline

2.11 List of Reagents and Suppliers

Product/Brand	Supplier	Address

Ambion	Applied Biosystems	Austin, TX., USA
Amersham	GE Healthcare Bio-Sciences	Rydalmere, NSW Australia
BDH Laboratory Supplies	Biolab Australia	Scoresby Vic., Australia
Corbett Research	Qiagen	Doncaster Vic., Australia
Geneworks	Geneworks Pty. Ltd.	Hindmarsh, SA, Australia
Gibco	Invitrogen	Mulgrave Vic., Australia
New England Biolabs	Genesearch	Arundel Qld Australia
Sigma	Sigma-Aldrich	Castle Hill NSW, Australia
	Bio-Rad	Gladesville NSW, Australia
	Biosearch Technologies	Novata, CA., USA
	Crown Scientific	Brisbane, Qld., Australia
	Molecular Devices	Surrey Hills, Vic., Australia
	Promega	Alexandria NSW, Australia
	Quantum Scientific	Murarrie, Qld, Australia

3   Vector Adaptation

3.1 Introduction

Integrating vectors of the retroviral class provide efficient and stable expression of transgenes in a range of cell types. The efficiency of vector transduction, and by extension, the safety of these vectors (Baum *et al.* 2003; Woods *et al.* 2003) is influenced by many factors including vector type, but is also a consequence of vector production and transduction methods.

The production of retroviral vectors is a multistep process, and many of the methods involved require specific equipment, notably the high-speed centrifuges required for vector concentration. There are, however, a number of alternative methods that may be employed that provide some flexibility with respect to equipment, but also allow customisation and optimisation for maximal rates of vector production and transduction.

The characteristics of the chosen vector used for transduction also influence the efficiency and reliability of transduction and subsequent gene therapy. Retroviruses have formed the basis for many of the earliest gene therapies, but more recently lentiviral vectors have been shown to provide highly efficient transduction of a range of cell types, and improved rates of transduction in a number of recalcitrant cell types, for example primary cells, haematopoietic stem cells and numerous leukaemic cell lines (Bai *et al.* 2003; Zhang *et al.* 2004).

The lower titre of retroviral vectors can be enhanced *in vitro* by a number of methods, such as multiple application of vector to the target cells, and low-speed 'spinoculation' of cells after application of the vector. The effect of these methods on titre of lentiviral vectors on recalcitrant leukaemic cells lines will be examined in this chapter.

In the therapeutic model examined in this thesis, autologous transplantation of therapeutically transduced haematopoietic stem cells, transfusion of a pure population of transduced cells is one of the major objectives. Consequently, post-transduction options for purification of the transduced cell population were also examined in this chapter.

The purification methods examined here were both dependent on marker genes expressed by the lentiviral vectors themselves. Specifically, cells transduced with fluorescent marker genes can be positively sorted with by flow cytometry. The vectors tested here expressed an enhanced green fluorescent protein (Persons *et al.* 1998; Li *et al.* 2003b).

Antibiotic resistance genes can also allow elimination of non-transduced cells by growth selection. A range of antibiotic resistance markers are available, however, the puromycin resistance marker gene (PUROr) was tested here as it can effectively eliminate non-transduced cells within approximately 48 hours (Kallifatidis *et al.* 2008).

Expression of shRNAs from retroviral or lentiviral vectors is usually accomplished by use of Pol-III class promoters (see Section 1.4.4), however, as in the vectors tested here, a number of other genes are expressed from the vectors. Such genes are typically expressed from stronger, Pol-II class promoters. Due to the limited capacity of integrating vectors consideration must be given to the possibility of promoter interference, an effect whereby expression of either or both of the shRNA and the Pol-II class genes might be impaired. Reversing the orientation of a gene relative to other genes can reduce the effects of promoter interference, however this strategy must be tested empirically, as it is not always successful (Curtin *et al.* 2008). The effect of orientation of the shRNA cassettes, relative to neighbouring transgenes, was also examined in this chapter. Another option that can reduce promoter interference is the bicistronic expression of two genes from a single promoter (Wong *et al.* 2002).

The aims of this study, therefore, were to optimise vector production and vector titre in refractory cell types. This chapter also aimed to identify the optimal arrangements for efficient expression of transgenes, and to construct vectors allowing purification of transduced cells.

3.2 Materials and Methods

3.2.1 Transfection of Packaging Cells

Unless indicated otherwise, the GFP-expressing, pHIV-7-GFP vector backbone, without hairpin cassettes, were used throughout these experiments.

3.2.1.1 293T Packaging Cells

The HEK-293T cell line (hereafter 293T cells) was used as the packaging cell line for these experiments. The standard calcium phosphate precipitation protocol (Section 1.11, and Sambrook and Russell 2000) was used to transfect these cells, with variations as indicated. All the vectors used in this chapter carried the GFP marker gene, and the rates of transfection of these cells were measured by microscopy using blue fluorescent light on an inverted florescent microscope. This method allowed recording of the percentage of fluorescent cell *in situ*, permitting ongoing culture of the attached packaging cells.

3.2.1.2 Culture Vessels

Standard transfection protocols recommend transfections be carried out in 90 mm petri dishes. To examine whether efficiency may be improved by use of alternate culture vessels, 293T cells were seeded in 90 mm petri dishes, 6-well plates, and T75 culture flasks at equivalent densities, based on surface area of the vessel. This density was in turn based on the cell number needed to achieve at least 50% coverage by 48 hours, as indicated in the standard method.

At 48 hours, the culture medium was removed and replaced with serum free medium. The DNA/calcium phosphate mixtures were also added, as per the standard method, and in proportion to the surface area of the vessels. Serum was replenished after one hour, and transfection rates were assessed by fluorescent microscopy at 24 and 48 hours.

3.2.1.3 Comparison of Three-plasmid and Four-plasmid Packaging Systems

Transfection rates using the calcium phosphate precipitation method are usually high, however a transfection rate of 100% of cells is rarely achieved. As the genomes of retroviral and lentiviral vectors are always fragmented into separate plasmids to improve safety (Ni *et al.* 2005), any shortfalls in transduction rates are amplified by the number of plasmids involved.

Many lentiviral vectors, such as those used here, are compatible with both second and third generation packaging systems, and both were available for use in this study. In order to generate infectious vector particles, the packaging cells need to be transfected with all necessary plasmid components; three in the case of the second generation vector, and four in the case of the third generation vector. To calculate the proportion of cells transfected with all plasmids necessary for vector production, it was assumed that the transfection rate for each component was the same, and then the transfection rate of the vector, measured as the proportion of packaging cells expressing the GFP marker, was cubed or quadraticised as appropriate (for the three- and four-plasmid systems respectively).

3.2.1.4 Concentration of Vector

Two different types of centrifuge tubes were compared for their ability to concentrate the vector particles. 'Oak Ridge' tubes, of 36 ml capacity and a rounded bottom, were used with a swing bucket rotor, while 1.5 ml 'Eppendorf' tubes were centrifuged in a fixed-angle rotor. Oak Ridge tubes were filled with filtered supernatant, and topped up with sterile PBS where necessary to eliminate bubbles. Filtered supernatant was added to autoclaved Eppendorf tubes in 1 ml aliquots. Both sets of samples were then spun at 21,640 g for five hours at 4 $^{\circ}$C. After centrifugation the supernatant was gently decanted, with great care taken not to dislodge any pellet that had formed. Pellets were then frozen in the fluid remaining after decanting.

The titre of vector prepared in each tube was tested on 293T cells in 6-well plates. Tubes were thawed, and the pellets were resuspended in the remaining volume and volumes added to the 293T cells, diluted into 1 ml complete DMEM, with 5 μl

Polybrene stock per well. Transfection rates were assessed by fluorescent microscopy 48 hours after transduction.

3.2.1.5 Storage and Reconstitution of Vector Pellet

The effect of storage volume on vector titre was examined by pipetting off the supernatant after ultracentrifugation to leave 10 or 100 μl of DMEM. Samples were frozen in these volumes for a minimum of 24 hours, then aliquots were thawed and added to 2.5×10^5 K562 or NB4 cells in one well of a 24-well plate, with culture volume made up to 1000 μl with complete RPMI, and 5 μl of Polybrene stock (50 μg/ml) added. Preliminary observations were made at 48 hours, then cells were prepared for examination by flow cytometry at 72 hours to determine the percentage of fluorescent cells and mean fluorescent intensity (MFI).

The effect of storage volume on vector titre was examined further by resuspending pellets in 500 μl complete RPMI before freezing. After freezing for at least 24 hours, two of these 500 μl aliquots were thawed and applied to 5×10^5 NB4 cells in one well of a 6-well plate, with 1 ml fresh complete RPMI and 10 μl Polybrene stock. The percentage of fluorescent cells and mean intensity of fluorescence were examined by flow cytometry at 48 hours post-transduction.

3.2.2 Methods for Transduction of Refractory Cell Types

Although not normally required for lentiviral transduction, 'spinoculation' and repeated application of vector are routinely used to improve retroviral vector titres. The effects of these methods on lentiviral vector titre in refractory cell types were examined separately.

The effect of spinoculation on lentiviral titre was examined by applying 500 μl aliquots of vector to NB4 cells, with 1 ml fresh RPMI and 10 μl Polybrene as described above. The 6-well plates were then centrifuged at 1000 g for 100 minutes at room temperature. The percentage of fluorescent cells and mean intensity of fluorescence were examined by flow cytometry at 48 hours post-transduction.

To examine the effect of repeated applications of vector, this treatment and spinoculation was repeated two or three times with different samples, at six hour intervals. The percentage of fluorescent cells and mean intensity of fluorescence were examined by flow cytometry at 48 hours post-transduction.

3.2.3 Transduction of Kasumi Cells

As the Kasumi cells were more sensitive to physical stresses, and should not be centrifuged at speeds greater than 300 g, a separate study was conducted in order to determine if spinoculation at 300 g also improved vector titre in these cells. As Kasumi cells have a lower rate of proliferation than other cells examined in this thesis, the effect of repeating treatment and spinoculation at a longer interval was also examined. Both these factors were examined simultaneously in a four-way study.

In order to maintain Kasumi cells at a suitable density throughout this trial, the purified vector aliquots were resuspended in 250 μl of complete RPMI (with 20% FCS), rather than 50 μl. These aliquots were added to

2.5×10^5 Kasumi cells in one well of a 24-well plate, with 250 μl fresh RPMI (with 20% FCS) and 10 μl Polybrene. The first group was spinoculated at 300 g, with the transduction repeated at 6 hours. The second group was also spinoculated at 300 g, but transduction was repeated at 12 hours. The third group was not spinoculated, with application of vector repeated at 6 hours.   The fourth and final group was not spinoculated, with application of the vector repeated at 12 hours. The percentage of fluorescent cells and mean fluorescence intensity were examined by flow cytometry at 72 hours post-transduction.

3.2.4 Orientation of shRNA cassette

The influence of orientation of the shRNA cassette on the expression of the shRNA was tested indirectly using the PR2 shRNA. Preliminary testing demonstrated consistent activity of this shRNA design in down-regulating levels of the PML-RARα mRNA target that could be reliably measured by qRT-PCR. Accordingly, qRT-PCR was used to measure expression of the PR2 shRNA cassette in the forward and reverse orientation relative to the vector backbone. The shRNA cassettes were cloned into the pHIV-7-GFP vector as per Section 2.8, and NB4 cells were transduced with these vectors, or control vectors bearing the NS shRNA in forward (see Figure 2.1) or reverse orientations (Figure 3.1) for comparison. RNA was collected from transduced cells at 48 and 72 hours and PML-RARα mRNA levels assessed by qRT-PCR (see Section 2.13)

Figure 3.1 pHIV-7-GFP-NSr
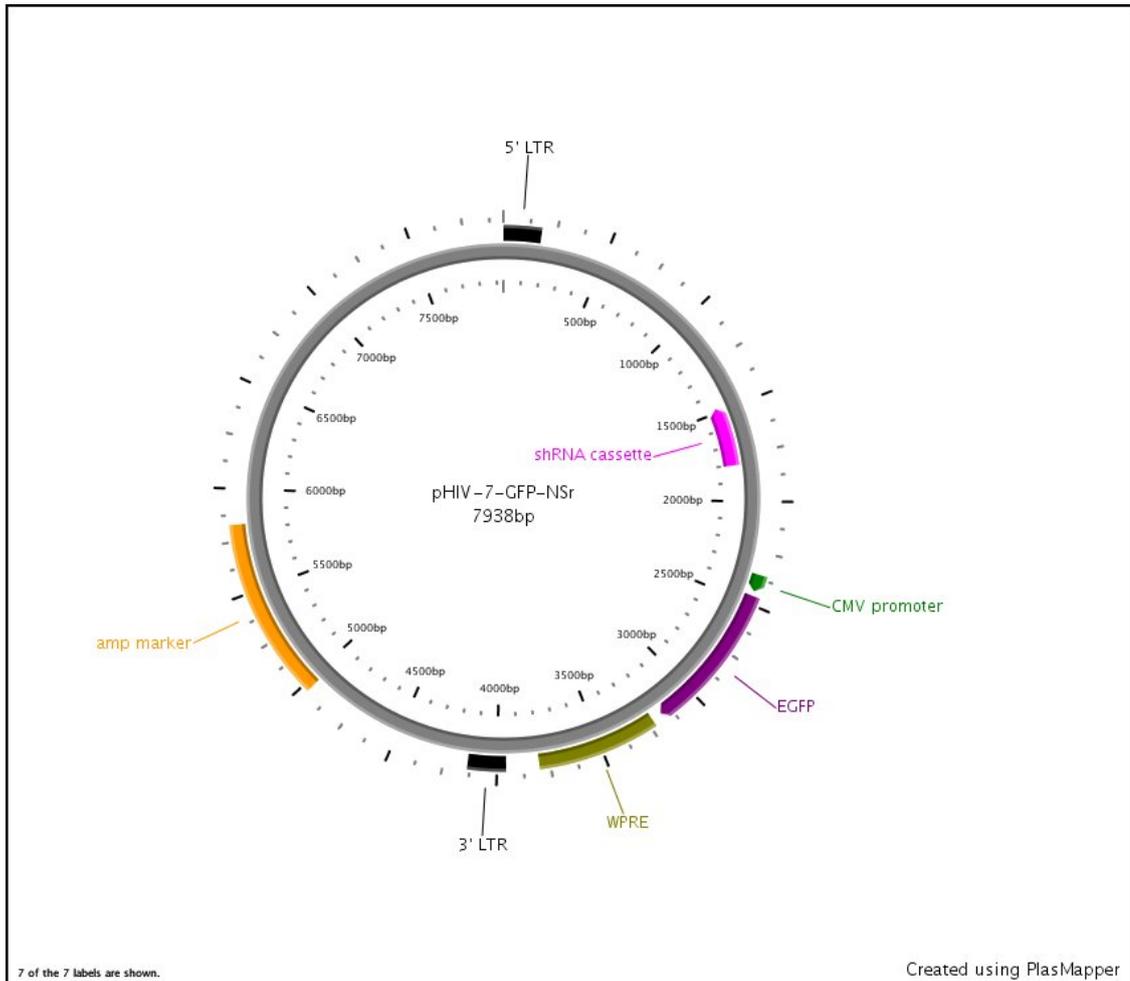
Figure 3.1 –The pHIV-7-GFP-shr vector construct, with the (NS) shRNA cassette inserted upstream of the GFP marker gene, but oriented in the reverse direction.

3.2.5 Vector Backbones Incorporating Antibiotic Resistance

The puromycin resistance gene (PUROr) can allow positive growth selection of transduced cells, and was obtained from the pBABE-puro vector (AddGene). The objective was to express the shRNAs, as well as the GFP and PUROr markers from a single vector, allowing puromycin selection of transduced cells, as well as monitoring of transgene expression by flow cytometry. Multiple strategies were used to achieve this, namely insertion of the PUROr marker into the pHIV-7-GFP used thus far, and the construction of a novel vector (based on pLV-tTRKRAB-Red) expressing GFP and PUROr bicistronically, with shRNA expressed separately from the vector backbone. The steps involved in each strategy are outlined below.

3.2.5.1 PUROr into pHIV-7-GFP

The pHIV-7-GFP vector has been shown to reliably express shRNA cassettes from positions upstream of the GFP marker (this study, see also Li *et al.* 2003b). The AgeI and AfeI restriction sites immediately upstream of the GFP marker promoter were also considered sufficiently distal from the location of the shRNA cassettes (see Section 2.8) to avoid promoter interference. Due to the proximity of this location to the CMV promoter driving GFP expression, the PUROr gene, along with SV40 promoter (from pBABE-puro) was inserted in the reverse orientation. The forward and reverse primers designed to amplify the SV40 promoter and PUROr gene from the pBABE-puro vector were AgeI-linked and AfeI-linked respectively. The primer sequences were as follows:

AgeSV40FP

> 5'-CGACGCGTACCGGTCCCTGTGGAATGTGTGTCAGTTAGGGTG-3'

AfeRP

> 5'-CGAGCGCTGCATGGGGTCGTGCGCTCCTTTCGG-3'

The resulting amplicon was double digested with AgeI and AfeI, and ligated into a similarly digested pHIV-7-GFP backbone to form the 'pHIV-PUROr-GFP' vector.

3.2.5.2 Bicistronic Vectors

A bicistronic vector, pLV-tTRKRAB-Red, was obtained from the laboratory of Professor Didier Trono (http://tronolab.epfl.ch/page58115.html). This vector originally contained an EF-1α promoter, a KRAB gene, an internal ribosomal entry site (IRES), a

gene for the dsRed fluorescent protein, and a WRPE insulator element. The PUROr and GFP marker genes were substituted for the KRAB and dsRed genes, respectively, by the steps described below.

The IRES fragment was first sub-cloned into a pGEM-T-easy vector (Promega) to facilitate subsequent cloning steps. The primers used for this sub-cloning step included a number of additional restriction sites, and were also designed to reintroduce the more efficient wild-type IRES start codon (Martin *et al.* 2006). The sequences of these primers, designed by Dr Stephen Frankenberg (University of Melbourne), were as follows:

IRES-FP (42 nt containing, in order, MluI, BamHI, XmaI, and SpeI restriction sites)

5′-ACGCGTGGATCCCGGGACTAGTTCCGCCCCCCCCCCCCTAAC-3′ IRES-RP (38 nt containing, in order, XbaI, EcoRI, and BglII restriction sites. The complement of the wild-type IRES start codon is underlined)

5′-TCTAGAATTCAGATCTCC<u>CAT</u>ATTATCATCGTGTTTTTC-3′

The purified amplicon was ligated directly into the pGEM-T-easy vector by Dr Stephen Frankenberg, creating the 'pGTE-IRES' vector.

The GFP fragment from the pEGFP-N2 vector (Clontech) was excised with BamHI and XbaI restriction enzymes and ligated into a BglII- and XbaI-cut pGTE-IRES vector, creating the 'I-G-pGTE' intermediate vector. This cloning step was also performed by Dr Stephen Frankenberg. The IRES-GFP fragment from this I-G-pGTE vector was then excised by double-digestion with MluI and XbaI. The pLV-tTRKRAB-Red was double-digested with MluI and SpeI, creating compatible overhangs, and the IRES-GFP fragment was ligated into this backbone, creating the 'I-G-pLV' intermediate.

The PUROr gene was copied from the pBABE-puro vector by PCR amplification using BamHI- and SpeI-linked forward and reverse primers respectively. The sequence of these primers was as follows:

PUROFP2

5′-GGGATCCGCATGACCGAGTACAAGCCCACGG-3′

PURORP2

5′-GACCGGTACCGGCGCGCCTCTAGAGCATGGGGTCGTGCGCTCCTTTCGG-3′

The resulting 686 bp amplicon was double-digested with BamHI and XbaI, and ligated into an I-G-pLV vector backbone digested with BamHI and SpeI to create the 'pPIG-LV' vector. This vector construct became the basis for further studies. shRNA cassettes were inserted in two different locations to identify the optimal arrangement for expression of the shRNA, GFP and PUROr transgenes. to reflect the different locations of the shRNA cassettes in the following constructs, the relative position of the cassettes was represented by 'sh' in the name of the vector. The construction of each vector is described below.

3.2.5.2.1 psh-PIG-LV

The pPIG-LV vector retains two unique restriction sites, SalI and ClaI, upstream of the EF-1α promoter. These sites were used to insert shRNA cassettes in the reverse orientation relative to the EF-1α promoter, as per the strategy used with the pHIV-7-GFP vector. The primers used to amplify the shRNA cassettes comprised the pSilencer forward and reverse sequencing primers linked with SalI and ClaI restriction sites respectively. The primer sequences were as follows:

SalFP

    5'-ACGCGTCGACGTTTTCCCAGTCACGACGTTG-3'

ClaRP

    5'-CCATCGATGAGTTAGCTCACTCATTAGGC-3'

The amplicons and the vector backbone were each double-digested with SalI and ClaI, and ligated to form the 'psh-PIG-LV' vector.

3.2.5.2.2 pPIG-2sh-LV

The original pLV-tTRKRAB-Red vector possessed a LoxP reaction site to facilitate Cre-Lox recombinase-mediated recombination of shRNA cassettes into the 3' LTR region of the vector. The location of the shRNA cassette in the 3' LTR results in two copies of the cassette after integration of the vector into the host genome. This LoxP location, retained in the pPIG-LV vector, formed the basis of two strategies to insert shRNA cassettes into this location. The first strategy used the Cre-Lox recombinase (LoxP reactions), whereas in the second strategy, unique restriction sites were introduced to the same location, generating the 'pPIG-MUT-LV' intermediate vector. The details of each strategy are outlined below.

3.2.5.2.2.1 LoxP Reactions

To generate shRNA cassettes with LoxP overhangs, the pSilencer sequencing primers were designed to amplify the shRNA cassette from the existing pSilencer vectors with LoxP linkers at each end. The sequence of each primer was as follows:

LoxFP

```
5'-
ATAACTTCGTATAGCATACATTATACGAAGTTATGTTTTCCCAGTCACGACGTTG-3'
```

LoxRP

```
5'-
ATAACTTCGTATAATGTATGCTATACGAAGTTATGAGTTAGCTCACTCATTAGGC-3'
```

The first shRNA cassette used in the LoxP reaction was the PR1 cassette, as it contained a unique BglII restriction site that would facilitate identification of recombinants by diagnostic digestion. Cre-Lox recombinase reactions with Lox-linked PR1 cassettes were performed as per the manufacturer's recommendations (NEB), however no successful recombinants were able to be identified by this method.

To allow growth selection of recombinants, the Kanamycin gene from the pEGFP-N1 vector (Clontech) was cloned, also with LoxP linkers. These linker primers also included BamHI and XmaI restrictions sites designed to facilitate subsequent cloning steps independent of the LoxP recombinase. The design of these primers was as follows:

LOXKFP

```
5'-
ATAACTTCGTATAGCATACATTATACGAAGTTATCGGGATCCTCCTGAGGCGGAAAGAA
CCAGC-3'
```

LOXKRP

```
5'-
ATAACTTCGTATAATGTATGCTATACGAAGTTATCCCCCGGGACAAACGACCCAACACC
GTGCG-3'
```

No successful recombinants were able to be identified following this method.

3.2.5.2.2.2 pPIG-MUT-LV

As an alternative to utilising the LoxP sites, a novel XmaI unique restriction site was introduced into the 3' LTR, in approximately the same location as the LoxP site.

To introduce these restriction sites, the regions of the 3' LTR upstream and downstream of the target site were amplified separately by PCR reactions using custom primers with appropriate linkers.

The upstream region of the LTR included an XbaI restriction site, and the forward primer was designed to include this site to allow cloning of the mutant LTR back into the vector backbone. The reverse primer for the upstream region of the LTR introduced the new XmaI restriction site. The primers were as follows:

GTELTRFP

    5'-TCCCAGTCACGACGTTGTAAAACG-3'

MUTRP

    5'-CCCCCGGGATACATTATACGAAGTTATGCTGCTTTTT-3'

As an intetrmediate step, this upstream amplicon was digested with XbaI and XmaI and ligated into an XmaI- and XbaI-cut pGem-T Easy vector backbone ('pGTE-UP').

The downstream region of the LTR included an NdeI site, and the reverse primer was designed to include this site to allow cloning of the mutant LTR back into the vector backbone. The forward primer for the downstream region of the LTR introduced the new XmaI restriction site. The primers were as follows:

MUTFP

    5'-CCCCCGGGAATTCATCTTGTCTTCGTTGGGAGTGAATTAG-3'

GTELTRRP

    5'-GCTATGACCATGATTACGCCAAG-3'

The resulting amplicon was digested with XmaI and NdeI. These same enzymes were used to digest the pGTE-UP intermediate vector, and the amplicon and backbone were then ligated, creating the pGTE-MUT vector. This vector now contained the lentiviral LTR, with the original XbaI and NdeI sites at either end, and a novel XmaI site near the original LoxP site. This 'mutant' LTR was excised with XbaI and NdeI restriction enzymes, and ligated back into an XbaI- and NdeI-cut pPIG-LV backbone, creating the 'pPIG-MUT-LV' vector. The new XmaI restriction site in the 3' LTR of this vector was available for insertion of XmaI-linked shRNA cassettes. As integrated forms of this vector would possess two copies of the shRNA cassette, after insertion of the shRNA cassette the pPUT-MUT-LV, the resulting vector was referred to as pPIG-

2sh-LV (Figure 3.2), where 'sh' represents the shRNA cassette. Except where indicated, the pPIG-2sh-LV vector was used throughout this thesis.

3.2.6 Puromycin Kill Curves

To determine the appropriate dose of puromycin to use for growth selection, a series of kill curves was performed for both NB4 and Kasumi cells. Cells at a density of $5x10^5$ cells/ml in complete RPMI, were treated with puromycin at a range of concentrations spanning the effective dose range indicated by the supplier (0, 0.75, 1, 1.5, 2, 3 and 6 μg/ml) and returned to culture. At 48 hours, the number of viable NB4 cells was counted by Trypan blue exclusion. Trypan blue counts of Kasumi cells were performed at 48 and 72 hours.

3.2.7 Activity of PUROr in the Alternate Lentiviral Backbones

To test the activity of the PUROr gene in the pHIV-PUROr-GFP construct, cells were transduced with the vector and then treated with 1.5 μg/ml at 48 hours post-transduction to allow recovery of cells from the transduction protocol, and to allow time for expression of the transgenes. Trypan blue counts for viable cells were carried out at 96 and 120 hours post-transduction.

The activity of the PUROr gene in the psh-PIG-LV and pPIG-2sh-LV vector constructs was examined by transducing NB4 cells with the NS form of each vector (ie. pNS-PIG-LV or pPIG-2NS-LV), then cells were treated with 2.5, 5 or 12.5 μg/ml puromycin to ensure rapid growth selection, again at 48 hours post-transduction. Non-transduced cells were cultured in each plate, with and without puromycin selection, as controls. Transduction and subsequent cell culture took place in 96-well plates, but cells were not spinoculated. To allow assessment of the long term effects of puromycin selection, CellTiter cell proliferation assays were performed at 24 hour intervals, commencing at 48 hours after treatment with puromycin, with the last assay at 120 hours. Accordingly, four wells of a 96-well plate were seeded with 2.5×10^4 cells per well for each of the 48, 72 and 96 hour time points, and four wells were seeded with 1.25×10^4 cells per well, with volumes adjusted accordingly, for the 120 hour time point. Puromycin treatment was repeated at 96 hours for the 120 hour sample.

Figure 3.2 pPIG-2sh-LV Vector
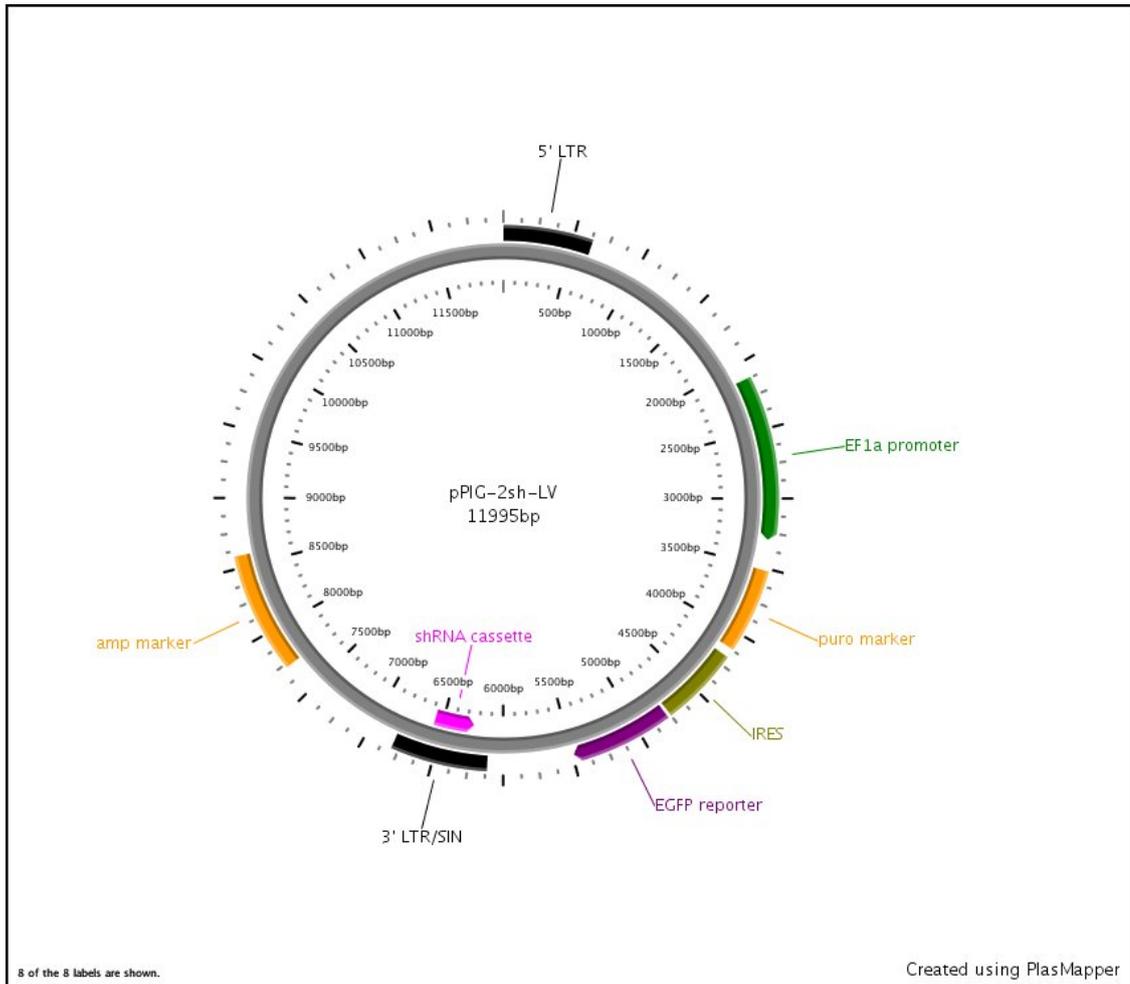
Figure 3.2 – The pPIG-2sh-LV vector construct, with the EGFP marker following the puromycin resistance marker in the bicistronic cassette, and with the shRNA cassette inserted in the reverse direction within the 3' LTR.

3.3 Results

3.3.1 Transfection of Packaging Cells

3.3.1.1 Effect of Culture Vessels on Transfection Rates

Transfection rates of the vector in 293T cells were compared in 90 mm petri dishes, 6-well plates and T75 culture flasks. The percentage of fluorescent cells was examined by fluorescent microscopy 48 hours after calcium phosphate transfection and indicated that transfection rates in 90 mm Petri dishes and 6-well plates were comparable, however transfection rates in T75 flasks was much lower (Table 3.1). Packaging cells were subsequently cultured in 6-well plates.

Table 3.1 Packaging Cell Transfection Rates in Different Culture Vessels

Vessel Type	90 mm Petri Dish	6-well Plate	T75 Flask
% Fluorescent Cells	>90%	>90%	10-15%

Table 3.1 – 293T packaging cells were grown in different culture vessels and transfected with lentiviral vectors expressing GFP by calcium phosphate precipitation. The percentage of fluorescent cells was examined at 48 hours post-transfection by fluorescent microscopy.

3.3.1.2 Second vs Third Generation Packaging Systems

To calculate the percentage of packaging cells transfected with all components necessary for each vector system, the accessory plasmids were assumed to be transfected at the same rate as the GFP vector component. The transfection rate for this vector, 90%, was then cubed for the three-plasmid, second generation system, and quadraticised for the four-plasmid, third generation system. This calculation suggests that with a 90% transfection rate, the percentage of packaging cells transfected with all elements of the second generation system was 72.9% (ie. 90% x 90% x 90%). The same value for the third generation system was 65.61% (ie. 90% x 90% x 90% x 90%). Therefore the rate of production of complete vector particles with the second generation system was higher than the rate of production of complete vector particles with the third generation system. The second generation vector system was subsequently used.

3.3.2 Tubes for Ultracentrifugation

Packaging cell supernatant was ultracentrifuged in Oak Ridge tubes or Eppendorf tubes, to compare their ability to concentrate the vector particles. After ultracentrifugation and decanting of the packaging cell supernatant, no pellet was evident in the Oak Ridge, however pellets were visible in the Eppendorf tubes. 10 µl of remaining concentrated supernatant was applied to 293T cells in 6-well plates and these cells were examined by fluorescent microscopy 48 hours later. The percentage of fluorescent 293T cells transduced with vector ultracentrifuged in the Oak Ridge tubes was 15%. The proportion of cells transduced with vector ultracentrifuged in the Eppendorf tubes was 65%. Therefore, despite the grater initial volume of vector supernatant added to the Oak Ridge tubes, the rate of transduction from the resulting preparation was much lower than for the Eppendorf tubes. Eppendorf tubes were subsequently used to concentrate vector supernatant.

Table 3.2 Vector Titre After Ultracentrifugation in Different Tubes

Tube Type	Oak Ridge	Eppendorf
% Fluorescent Cells	15%	65%

Table 3.2 – Vector supernatant was ultracentrifuged in Oak Ridge or Eppendorf tubes. The concentrated supernatant from each was used to transduce 293T cells, which were examined by fluorescent microscopy 48 hours later.

3.3.3 Transduction of Target Cells

3.3.3.1 Resuspension and Storage of Vector Supernatant

The effect of storage volume was examined using ultracentrifuged samples that were resuspended in 10 µl or 100 µl DMEM after removal of supernatant. These samples were applied to K562 and NB4 cells, and the culture volumes made up with fresh complete RPMI 1000 µl with 250ng/ml of Polybrene. Fluorescent microscopic observations at 48 hours showed that greater than 90% of K562 cells were expressing GFP in both treatment groups, although the intensity of fluorescence was higher in the cells receiving the vector stored in 100 µl. In NB4 cells at 48 hours, both the percentage of fluorescent cells, and the fluorescent intensity in those cells were low, regardless of the vector storage conditions. At 72 hours, flow cytometric analysis showed that both

vector storage conditions resulted in a high titre and high fluorescence intensity in the K562 cells (Table 3.3). Flow cytometric analysis of NB2 cells at 72 hours demonstrated that fluorescence intensity in both groups was similarly low (Table 3.3), however the percentage of cells expressing the transgene was considerably higher when transduced with vector stored in 100 μl than with vector stored in 10 μl. It was clear that storage in larger volumes of media improved vector titre.

Table 3.3 The Effect of Storage Volume on Vector Titre

Cell Type	K562		NB4	
Storage Volume	10 μl	100 μl	10 μl	100 μl
Fluorescent Cells	96%	97%	63%	80%
MFI	111	136	3.61	4.55

Table 3.3 – Fluorescence of K562 and NB4 cells 72 hours after transduction with vector stored in 10 or 100 μl of DMEM.

These results were pursued further, by resuspending the vector in 500 μl fresh complete RPMI before freezing. NB4 cells were transduced with these 500 μl aliquots, made up to 1000 μl with fresh RPMI, and Polybrene added. For compatibility with other assays, fluorescence was assayed by flow cytometry at the earlier 48 hour time point. Nevertheless, the percentage of fluorescent cells at this time point (see below, Table 3.4, Column 1) was greater than at 72 hours after transduction with vector stored in 10 or 100 μl volumes (Table 3.3). The intensity of fluorescence was similar. From these results it was clear that resuspension and storage of the ultracentrifuged vector supernatant in 500 μl of RPMI prior to freezing resulted in an increase in the proportion of transduced cells.

3.3.3.2 Spinoculation and Repeated Transductions

To test the effect of spinoculation after application for the vector, NB4 cells were subsequently centrifuged at 2200 rpm for 100 minutes, with fluorescence assessed at 48 hours. The increase in the percentage of fluorescent cells after spinoculation was only minor however it resulted in a doubling of the mean fluorescence intensity (Table 3.4).

The effect of repeated transductions, including spinoculation, was also tested. Vector application and spinoculation was repeated after six hours for the '2x' treatment and then a third time after another six hours for the '3x' treatment (Table 3.4). The second transduction increased the percentage of transduced cells to over 90%, but also improved levels of transgene expression almost three fold (Table 3.4). A third application of vector showed no real improvement over these levels, but resulted in a minor decrease in mean fluorescence intensity.

Both spinoculation and repeated transduction improved effective vector titre (percent fluorescent cells and fluorescent intensity) and were subsequently adopted.

Table 3.4 The Effect of Spinoculation and Repeated Transduction

48 hours post-transduction	Not Centrifuged, 1x	Centrifuged, 1x	Centrifuged, 2x	Centrifuged, 3x
%	84.8	86.5	92.3	92.4
MFI	2.52	5.7	14.2	12.8

Table 3.4 – NB4 cells were transduced with vector stored in 500 μl RPMI. The effect of spinoculation and repeated transduction were examined by flow cytometry at 48 hours.

3.3.3.3 Transduction of Kasumi Cells

Kasumi cells proliferate more slowly than NB4 cells, and are more sensitive to physical stresses and cell density. To test the value of low speed spinoculation after vector application, Kasumi cells were spinoculated at 300 g and titre was compared with cells that were not spinoculated. The value of a greater interval before the second application of vector was also tested by comparing titre in cells transduced at 6 hours interval with cells transduced after a 12 hours interval.

In the sensitive Kasumi cells, cells were compared with and without centrifugation (at 300 g) after vector application. The effect of a greater period of recovery before the second application was also tested. Table 3.3 clearly demonstrates that higher rates of transduction and higher levels of transgene expression were achieved with low speed centrifugation after vector application. It is also clear that a longer interval between applications had a detrimental effect on vector titre. Therefore

the conditions used for the NB4 cells, with a lower centrifugation speed, were also appropriate for the Kasumi cells also with a reduced spinoculation speed.

Table 3.5 The Effects of Low Speed Spinoculation and Interval between Vector Applications

	Spinoculated		Not Spinoculated	
Interval	6 hours	12 hours	6 hours	12 hours
% Fluorescent Cells	91%	84%	75%	70%
MFI	3.19	2.73	1.93	1.69

Table 3.5 – Kasumi cells were treated with vector then spinoculated at 300 *g* or not spinoculated. In the same trial, the effect of a 12 hour interval prior to the second vector application was also examined.

3.3.4 Orientation of shRNA cassette

The effect of orientation of the shRNA cassette on shRNA activity was tested by transducing NB4 cells with vectors bearing shRNA cassettes in the forward or reverse orientations, relative to the strong CMV promoter (compare Figures 2.1 and 3.1). The activity of the PR2 shRNA in each orientation as measured by qRT-PCR was compared with NS control shRNAs in the same orientation. It was clear that the activity of the PR2 shRNA in the reverse orientation was higher both at 48 and 72 hours post transduction (Figure 3.3). This orientation was subsequently used in all experiments.

3.3.5 Puromycin Kill curves

Non-transduced NB4 and Kasumi cells were treated with a series of puromycin concentrations and returned to culture. Trypan counts of viable NB4 cells were conducted at 48 hours, and counts of viable Kasumi cells were condicted at 48 and 72 hours. It is clear from the results (Table 3.6) that a dose of 1 μg/ml was sufficient for complete elimination of NB4 cells after 48 hours of treatment. None of the tested doses was sufficient to eliminate all Kasumi cells by 48 hours, however by 72 hours the 1 μg/ml dose had eliminated all viable cells. Therefore the 1 μg/ml dose was considered sufficient for further use in these experiments.

Figure 3.3 Activity of shRNA Cassettes in the Forward and Reverse Orientations
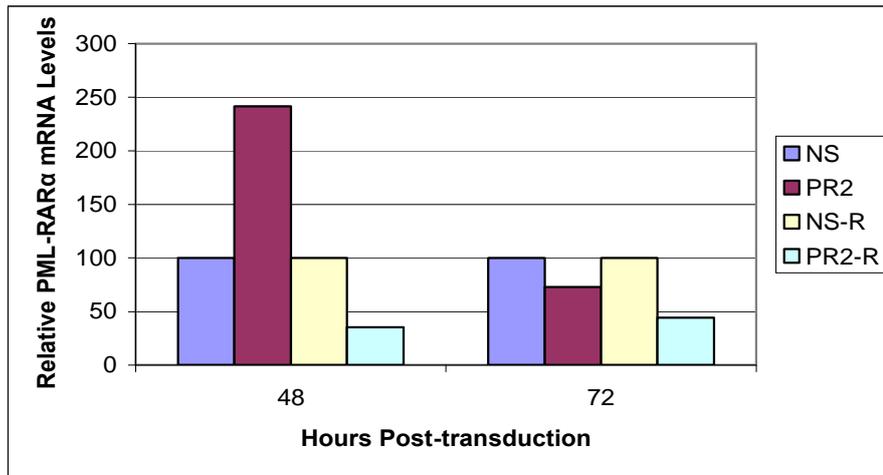
Figure 3.3 – The activity of the PR2 shRNA in the forward and reverse orientations relative to neighbouring transgenes was analysed by qRT-PCR and compared with the NS control shRNA in the same orientation. PML-RARα mRNA levels were normalised to the β2m house-keeping gene. This normalised value is presented relative to the NS control, and this graph presents the average of two separate experiments.

Table 3.6 Viable Cell Counts Following Puromycin Treatment

Puromycin (μg/ml)	48 hours		72 hours
	NB4 cells (x10^4/ml)	Kasumi cells (x10^4/ml)	Kasumi cells (x10^4/ml)
0	41	72	101
0.75	1	6	<1
1	0	3	0
1.5	0	1	0

Table 3.6 – NB4 and Kasumi cells were treated with puromycin over a range of doses based on the manufacturer's recommendations. Viable NB4 cells were counted at 48 hours, and the more slowly proliferating Kasumi cells were counted at 48 and 72 hours.

3.3.6 Activity of PUROr Marker Gene in pHIV-PUROr-GFP

NB4 cells were transduced with the pHIV-PUROr-GFP vector and treated with 1 μg/ml puromycin at 48 hours post-transduction. After 48 hours of puromycin treatment (i.e. 96 hours post-transduction) a count of viable cells revealed that none remained. Thus the activity of the PUROr marker gene in this construct was insufficient to allow growth selection using puromycin.

3.3.7 PUROr Activity in the Bicistronic Constructs

Alternative lentiviral vectors bearing a puromycin resistance (PUROr) marker gene, a GFP marker gene and the NS control shRNA cassette, were tested in NB4 cells. The two constructs, pNS-PIG-LV and pPIG-2NS-LV ('pNS' and '2NS' respectively), differed with respect to the position of the shRNA cassette. Transduced cells were treated with high doses of puromycin (2.5, 5, and 12.5 μg/ml) to provide strong and rapid selection of transduced cells. Two sets of non-transduced cells were included as controls. The 'P' controls were treated with puromycin at the same concentrations as the transduced cells, while the 'K' controls received no puromycin. Puromycin was added at 48 hours, and again at 96 hours for the 120 hour sample. CellTiter cell proliferation assays were performed at 96 and 120 hours post-transduction.

It was clear from the results (Figure 3.4) that the puromycin treatment reduced the number of viable cells, at all concentrations, relative to the untreated cells. It was also clear that the pNS-PIG-LV ('pNS') vector had no protective effect on cell survival

Figure 3.4 The Effect of Puromycin on the Number of Viable Cells Transduced with
Biscistronic Vectors
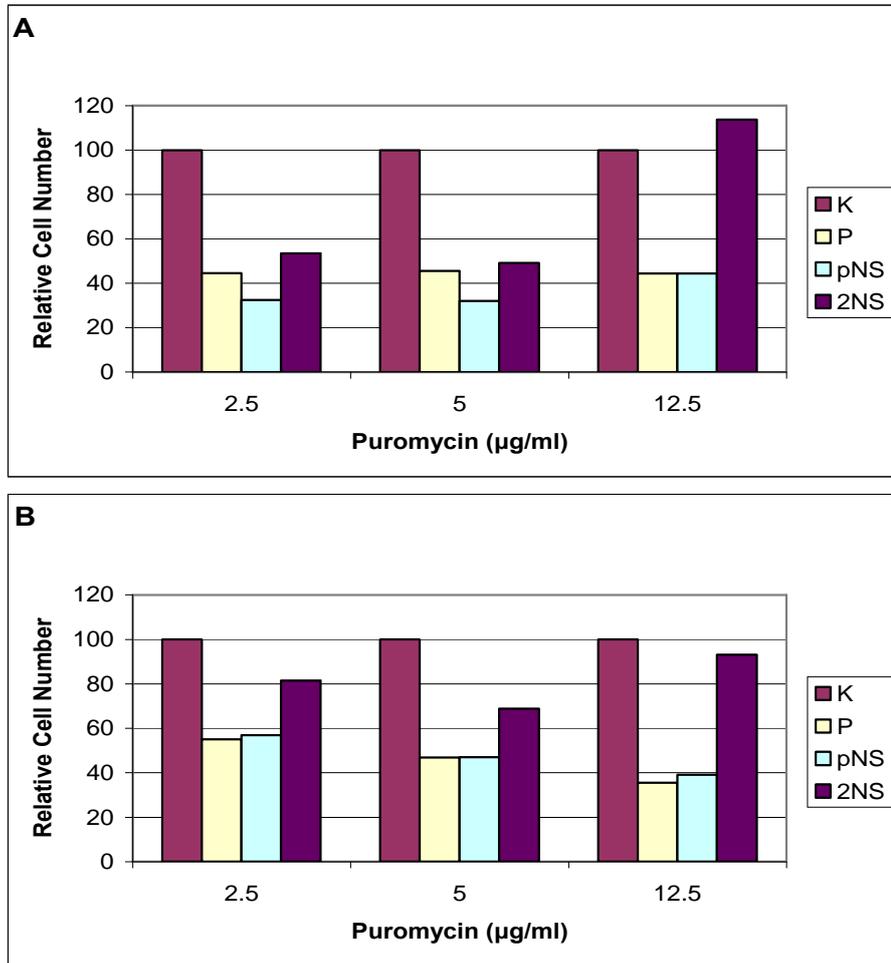
Figure 3.4 – NB4 cells were transduced with pNS-PIG-LV ('pNS') and pPIG-2NS-LV ('2NS') vectors
and treated with puromycin at 48 hours. Two sets on non-transduced cells were included as controls, the
first group ('K') was not treated with puromycin, while the second set of controls ('P') received the same
doses of puromycin as the transduced cells. CellTiter assays were conducted at 96 (A) and 120 hours (B)
post-transduction. These graphs represent the averages of four replicates from a single experiment.

against puromycin treatment. By contrast, the pPIG-2NS-LV vector ('2NS') did protect the NB4 cells against puromycin. This effect appeared to be stronger at higher doses of puromycin, and at 12 µg/ml, the number of viable cells bearing this vector approached or even exceeded the number of viable untreated cells ('2NS' vs 'P').

A number of general trends were apparent from the results of the proliferation assays (Figure 3.4). It was also clear that the relative effects of each vector on cell proliferation were comparable between the three different doses of puromycin. That is, the relative proliferation of each group receiving a given vector were similar compared to groups receiving the same vector bearing the other shRNA, regardless of puromycin concentration received. There were also trends associated with each of the vectors tested, which are outlined below.

3.3.7.1 Dose and Effect of Puromycin

A comparison of the 'K' and 'P' samples confirmed that the puromycin doses were effective at restricting growth of the NB4 cells at each time points (after 48 hours). However, in contrast to the Trypan blue counts above (Table 3.4), it is clear that there remains some cellular activity, and thus some viable cells, at each time point.

In connection with this point, it is interesting that a number of the transduced samples actually contained a lower number of viable cells than the non-transduced control treated with puromycin ('P').

3.3.7.2 Activity of PUROr in Alternate Vector Constructs

At all puromycin concentrations and at all time points the cells transduced with both forms of the psh-PIG-LV vector (ie. pNS-PIG-LV or pPR2-PIG-LV) showed levels of cellular activity approximating that of the 'P' controls. It is therefore clear that the PUROr marker gene in this arrangement/construct did not offer protection against puromycin treatment.

The levels of cellular activity in samples transduced with the two pPIG-2sh-LV vectors were higher than the 'P' samples at almost all points (except after 72 hours of 5 µg/ml), and the difference was most pronounced in the samples treated with 12.5 µg/ml puromycin. This demonstrates that the PUROr gene in this construct is protecting the cells from the effects of puromycin.

The pHIV-2sh-LV vector construct therefore provides good protection against puromycin, and allows positive selection of transduced cells.

3.4 Discussion

Efficient and stable gene therapy depends on high titre integrating vectors, such as retroviral or lentiviral vectors. The final titre of such is strongly affected by the methods of preparation, including production and storage, of the infectious vector particles. This chapter has identified a number of methods to improve final titre of lentiviral vectors.

Vector titre also influences the safety of gene therapy, as minimising the number of vectors required to deliver a therapeutic effect reduces the risk of adverse integration events (Baum *et al.* 2003; Woods *et al.* 2003). Similarly, the number of vectors required for a therapeutic effect can be minimised by optimising the efficiency of expression of the therapeutic transgene from the gene therapy vector. Factors influencing the efficiency of transgene expression, and ways to maximise this expression, were also examined in this chapter.

Finally, the therapeutic strategy explored in this thesis is dependent upon obtaining a pure population of cells transduced with the gene therapy vector. Vector marker genes may be used to select transduced cells in a population, and the potential of one such marker gene was also explored in this chapter.

Inefficient transduction of packaging cells can lead to low titre vectors and an exponential increase in the number of 'empty' vector coats that can compete against complete vector particles for binding sites on the surface of the target cells. This competition impairs the titre of the vector. Two aspects affecting the efficiency of production of complete vectors particles were examined in this chapter.

In third generation vector packaging systems, the viral elements essential for transduction of the target cell and genomic integration and transgene expression are divided amongst four separate plasmids, reducing the chances of pathogenic recombination of the viral elements and improving vector biosafety. The greater number of plasmids, however, has an adverse effect of the production of complete vector particles. The transfection rate of each component plasmid in packaging cells is generally below 100%, therefore each additional plasmid required to produce of complete vector particles reduces the number of cells receiving all plasmids, increasing the proportion of incomplete vector particles. Therefore, in applications where the

biosafety of vector production is not of primary importance, the analysis from this chapter demonstrates that higher vector titres would be achieved with a second generation vector packaging system.

Petri dishes are used for the culture of packaging cells used in standard vector production protocols, however they have a limited surface area when compared with other culture vessels. Examination of other culture vessels found that packaging cell transfection rates were comparable between 90 mm petri dishes and 6-well plates, however transfection was greatly impaired in T-75 culture flasks. This difference was likely explained by the superior mixing possible in the circular dishes, and therefore more efficient dispersal of the calcium phosphate and DNA mixture over the culture surface, highlighting the importance of this aspect in efficient transfection. While transfection rates in 6-well plates and petri dishes were similar, however, the 6-well plates had a greater culture surface area, were easier to handle, and could be stacked with greater stability. Therefore the 6-well plates provide a better solution for the culture of packaging cells for vector production.

The supernatant collected from the packaging cells is usually concentrated by ultracentrifugation however alternate protocols offering greater flexibility in the methods of purification of harvested vector supernatant have been established and published (Geraerts *et al.* 2005), and have provided a useful starting point for further explorations.

A comparison of vessels used for ultracentrifugation found that choice of vessel had a dramatic effect on vector titre. Oak Ridge centrifuge tubes are generally recommended for their large capacity and tolerance of the forces involved in high-speed centrifugation, however distinct pellets of concentrated vector did not form in these tubes. By contrast, distinct pellets readily formed in Eppendorf tubes, despite the much lower capacity. These results correlated with vector titre, as the samples prepared in the Eppendorf tubes had a much higher titre than those prepared in the Oak Ridge tubes. Furthermore the narrow, conical bottom of the Eppendorf tubes also facilitated decanting of the supernatant without disturbing the pellet whereas removal of supernatant from the Oak Ridge tubes by either decanting or pipetting had no effect on final titre, indicating that the hemispherical shape of the tubes, and not accidental disturbance of any nascent pellet, was the main factor in the low titres observed from

vectors prepared in these tubes. Finally, the smaller Eppendorf tubes allow storage of vector in aliquots, minimising exposure of stored vector to excessive thaw cycles. Consequently, Eppendorf tubes, despite their smaller capacity, have many advantages of over Oak Ridge tubes with respect to vector preparation, and provide a useful alternative where vector titre needs to be enhanced.

Following production and ultracentrifugation of vector particles, the conditions of storage have a marked effect on the final titre (Zhang *et al.* 2004). Accordingly a study examining the effect of storage volume identified a novel protocol resulting in a large improvement in vector titre. Standard protocols indicate that the pellet collected by ultracentrifugation can be stored without resuspension in only a few microlitres of medium. This method was found to be sufficient to achieve high titres in certain commonly used cell lines, namely 293T and K562 cells. By contrast, vector produced by this method had much lower titres in NB4 and Kasumi cells, whereas results from this chapter demonstrated that, following ultracentrifugation and decanting of the supernatant, immediate resuspension of the pellet in 250 or 500 μl of cell culture medium greatly improved vector titre in these refractory cell types. The reasons for this effect were unclear, nevertheless this method results in an improved vector titre and therefore provides a further option for enhancing vector titre in cell types that are difficult to transduce.

Retroviral vectors have been in use for some time however the titre of these vectors is generally much lower than for lentiviral vectors. Consequently additional methods have been used with retroviral vectors that are generally considered unnecessary with lentiviral vector. Without these methods, for example, lentiviral vectors can reliably transduce more than 90% of K562 cells (Bai *et al.* 2003). By contrast, transduction rates of NB4 cells under the same conditions are typically between 1 and 25% (Bai *et al.* 2003). Kasumi cells are also difficult to transduce by standard methods (P. Oakford pers. comm.). Consequently the impact of the additional methods used for retroviral vectors on lentiviral titre in these refractory cell types was examined in this chapter. The results form this chapter clearly demonstrated that both 'spinoculation' and the repeated application of vector greatly improved vector titre in both NB4 and Kasumi cell types, with transduction rates exceeding 90% in each. These

methods, therefore, may be used to improve vector titre in other refractory cell types, such as other leukaemic cell lines, haematopoietic stem cells, or primary cell types.

Efficient expression of therapeutic transgenes from gene therapy vectors results in efficient gene therapy from minimal vector copy numbers. The vectors developed in this study were designed to deliver therapeutic shRNA cassettes. The pHIV-7-GFP vector used throughout this study originally contained an shRNA cassette upstream from the CMV promoter used to drive the GFP marker gene. This location allowed strong expression of the shRNAs (Li *et al.* 2003b) and was used for insertion of the novel shRNA cassettes used in the present study. In positioning the shRNA cassettes, however, attention was given to the possibility of promoter interference from the CMV promoter. It has been shown that positioning genes in a reverse orientation relative to neighbouring genes can ameliorate the effects of promoter interference (Curtin *et al.* 2008), and the insertion of novel shRNA cassettes into the vector backbone was taken as an opportunity to identify to optimal orientation for expression of shRNAs relative to the CMV. While there was evidence of some expression of shRNAs from cassettes in both orientations, efficacy of the shRNAs from cassettes of the reverse orientation was greater. This was consistent with the effects of promoter interference, and the reverse orientation was subsequently used for all shRNAs.

For many gene therapy applications, such as the autologous transplantation strategy examined in this thesis, it is necessary to obtain a pure population of transduced cells. It is unlikely that this will be achieved by improvements in transduction rates alone however there are a number of methods are available for purifying populations of transduced cells. One of these is the use of growth selection markers, such as the puromycin resistance marker examined in this chapter. The vectors used in this study already carried the GFP marker, necessitating inclusion of the PUROr marker in such a way as to avoid promoter interference. Two alternative locations were tested, each designed to avoid promoter interference by a different method.

When the PUROr marker was expressed from an SV40 promoter and located upstream of the GFP marker driven by a CMV promoter in the existing pHIV-7-GFP vector, the puromycin resistance marker gene was inactive, as transduced cells showed no resistance to puromycin and did not appear to be active as the cells did not survive low doses of puromycin. By contrast, the GFP marker gene in this vector remained

active. This suggested that promoter interference was suppressing expression of the PUROr marker.

One strategy to avoid promoter interference is the bicistronic expression of two PolII genes from a single promoter, with the genes separated by an internal ribosomal entry sites (IRES) (Curtin *et al.* 2008). This approach was tested in this chapter, with the GFP and PUROr markers expressed in series from an EF-1α promoter. This arrangement allowed strong expression of both genes, as demonstrated by rates of cell growth from puromycin-treated cells that approximated normal rates of growth from untreated cells.

In addition to the two marker genes, however, the vectors were intended to express an shRNA cassette, and the positioning of this cassette had a strong influence on expression of the bicistronic marker genes. In one vector, psh-PIG-LV, the shRNA cassette was located upstream of the EF-1α promoter. The PUROr marker in this construct was also inactive, as transduced cells had no survival advantage over non-transduced cells under puromycin selection. This indicated a promoter interference effect, which was interesting as it involved suppression of a PolII promoter by a PolIII promoter. However, in the pPIG-2sn-LV, where the shRNA cassette was located distally from the bicistronic arrangement, the vectors did confer puromycin resistance. This design was therefore superior with respect to expression of the marker genes, and had the added benefit of expressing two copies of the shRNA cassette from the integrated vector.

While antibiotic selection remains a viable option for purifying transduced cell populations, it should be used in conjunction with optimised transduction rates, in order to minimise proliferative stress. Furthermore a great deal of fine tuning is required to avoid promoter interference and other disruptive effects on transgene expression.

Although not available at the time of the present study, the flexibility offered by Pol II-, miRNA-based vectors may provide a range of possibilities for addressing the challenges encountered here, in the development of gene therapy vectors (Marquez and McCaffrey 2008)

3.4.1 Summary

The methodological modifications identified in this chapter resulted in improvements at all stages of vector production and transduction, with improvements in

vector titre and transgene expression. Finally, this chapter confirmed the suitability of growth selection markers in purifying populations of transduced cells for gene therapy applications.

4 Down-regulation of Bcr-Abl by RNAi leads to erythroid differentiation

4.1 Introduction

During normal haematopoietic differentiation pluripotent stem cells commit to various lineages and differentiate into distinct blood cell types (Shet *et al.* 2002). The MEK/ERK signalling pathway has been implicated in differentiation of numerous lineages of haematopoietic cells (Melemed *et al.* 1997; Sevinsky *et al.* 2004), and evidence suggests that *in vitro* differentiation of several erythroid cell lines is accompanied by the down-regulation of ERK1/2 mitogen activated protein kinase activities (Kang *et al.* 1999; Kolonics *et al.* 2001; Woessmann and Mivechi 2001; Kawano *et al.* 2004; Kucukkaya *et al.* 2006), however, the changes in ERK1/2 activity in erythroid differentiation depend on the inducing agent (Woessmann *et al.* 2004). More generally, erythroid differentiation is marked by a decrease in PU.1 levels (Back *et al.* 2004) and the increased expression of β-globin (Stamatoyannopoulos and Nienhuis 1994; Bulger and Groudine 1999; Crusselle-Davis *et al.* 2006).

The Bcr/Abl chromosomal translocation occurs in many cases of Chronic Myelogenous Leukaemia (CML) (Heisterkamp *et al.* 1985) and selective inhibition of this fusion gene results in apoptosis, but also causes erythroid differentiation (Fang *et al.* 2000; Jacquel *et al.* 2003). While the constitutive tyrosine kinase activity of the Bcr/Abl fusion protein leads to abnormal signalling of growth and survival signals along multiple signalling pathways, including ERK mitogen-activated protein kinase (MAPK) cascade (Zou and Calame 1999), the mechanisms of these responses have yet to be clearly defined (Shet *et al.* 2002).

The K562 cell line is used as an *in vitro* model of chronic myelogenous leukaemia, and was originally isolated from an adult patient with CML (Lozzio and

Lozzio 1975). These are undifferentiated blast cells expressing the phenotypic markers of an erythroid lineage (Koeffler and Golde 1980). The b3a2 Bcr/Abl breakpoint is commonly amplified in K562 cells, with amplification ranging from four-fold (Grosveld *et al.* 1986) to 22-24 fold, apparently increasing with the age of the cell line (Wu *et al.* 1995). Targeting of the fusion mRNA with RNAi has been shown to induce specific and significant down-regulation of the Bcr-Abl fusion gene and its fusion protein (Wilda *et al.* 2002; Li *et al.* 2003b; Scherr *et al.* 2003b; Wohlbold *et al.* 2003; Scherr *et al.* 2005). Consequently, the main aims of this chapter were to examine the effects of knocking out the upstream Bcr-Abl kinase by retroviral delivery of anti-Bcl-Abl shRNA on the dynamics of the ERK1/2 pathway, and to study the downstream effects on erythroid differentiation by assessing changes to a number of differentiation associated markers.

4.2 Methods

4.2.1 Retrovirus-vector construction

Retroviral vectors were designed and constructed as described in the General Materials and Methods, with the following variations. The anti-Bcr-Abl shRNA sequence ('BA1') used in this experiment was derived from a sequence validated and published previously (Li *et al.* 2003b). The nonsense shRNA design ('NS', see General Materials and Methods, Section 2.6.3.2) was used as a control. The shRNA sequence was cloned into the pSilencer vector, as outlined in the General Materials and Methods, but with AgeI linkers added to the primers.

The retro-vector backbone was pLPCX (Clontech, discontinued), a 'RetroPack'-compatible vector derived from the Moloney murine leukemia and sarcoma retroviruses (MoMuLV and MoMuSV). The unique AgeI restriction site of pLPCX, located between the extended viral packaging signal and the puromycin resistance gene, was considered the optimal location for the shRNA cassette, to minimise the risk of interference from other promoters in the vector. Enhanced Green Fluorescent Protein was amplified from the pEGFP-N1 plasmid (BD Biosciences), and cloned into the multiple cloning site (MCS) of the pLPCX vector using the XhoI and NotI restriction sites in the donor plasmid and recipient vector. The final arrangement of the resulting pLPCX-sh-GFP is shown in Figure 4.1.

4.2.2 Virus Production

Stable transduction of K562 cells was achieved by using a double packaging cell system Phoenix / PG13 (Ujhelly *et al.* 2003). The Phoenix-eco packaging cell line (Orbigen) was transfected by calcium phosphate coprecipitation. Supernatant from these cells was collected 48 hours after transfection, filtered, then used to transduce the PG13 packaging cell line (ATCC CRL 10686). At 48, 72 and 96 hours, PG13 supernatant was harvested and fresh culture medium applied to the cells. The harvested supernatant was frozen at -80°C. Supernatant was thawed, filtered through 0.4 μm filter cartridges, and centrifuged at 4 °C at 180,000 g for 1 hour. Centrifuged filtrate was resuspended in 1 ml fresh medium per 50 ml of original harvest, and stored at –80 °C until use.

Figure 4.1 pLPCX-sh-GFP Vector
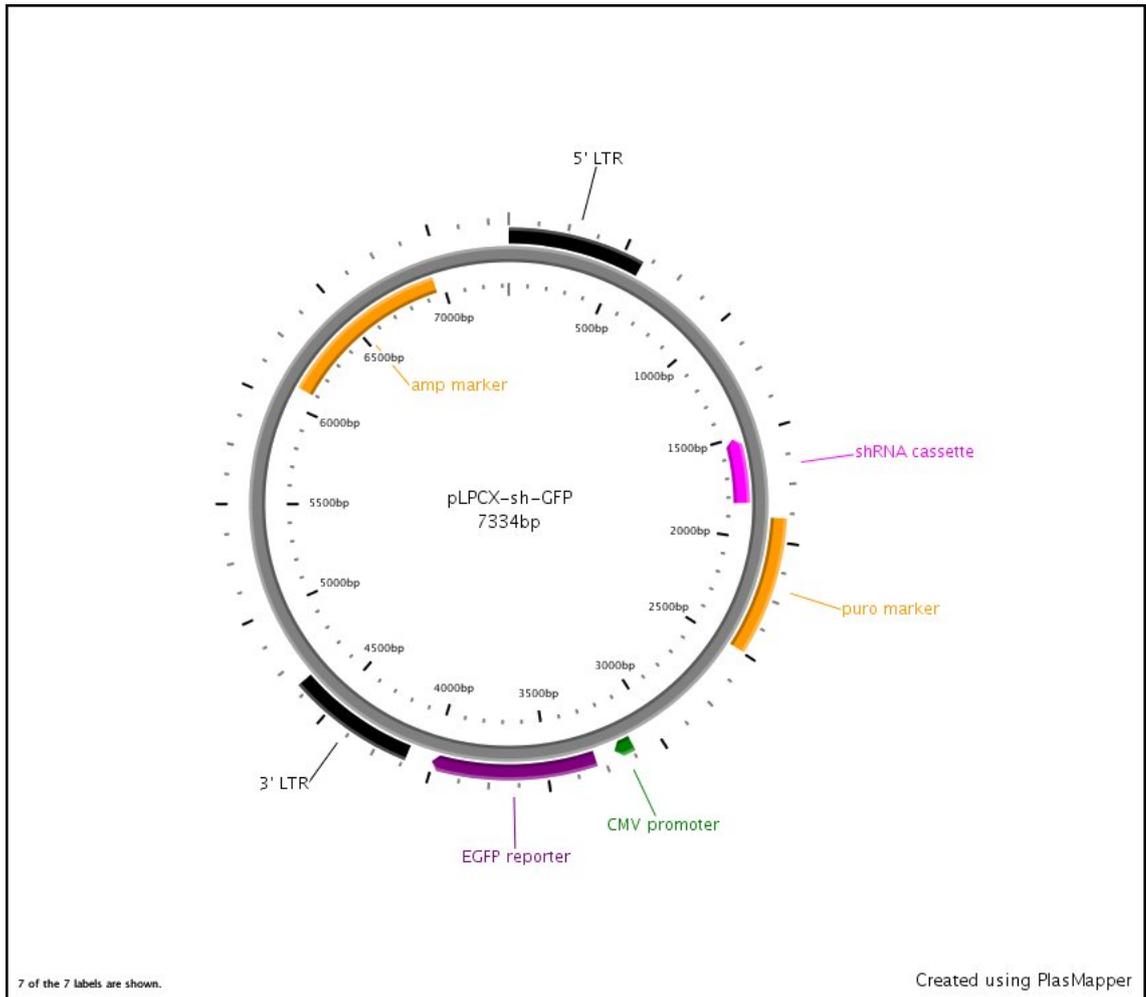
Figure 4.1 – The pLPCX-sh-GFP has a EGFP marker, with a separate CMV promoter, inserted downstream of the puromycin resistance gene, and the shRNA cassette inserted upstream of the puromycin resistance gene, and the reverse orientation.

K562 cells were transduced as per the General Materials and Methods (Section 2.11), with the following variations. Due to the high speed ultracentrifugation,only 50 µl of concentrate was necessary per 1 ml total medium for 10^5 cells in each well of a 24-well plate. Cells were transduced three times, and one hour after the final transduction, the virus supernatant was removed and replaced with fresh medium for ongoing culture. Expression of the GFP marker gene was assessed by flow cytometric analysis 48 hours after final transduction

4.2.3 Preparation of cell extracts

Cells were collected by centrifugation at 1200 g and lysed for 10 minutes on ice with gentle shaking in buffer containing; 250 mM NaCl, 50 mM HEPES pH 7.4, 1 mM EDTA, 1 mM EGTA, 1.5 mM $MgCl_2$, 0.1% Nonidet P-40, 40 mM β-glycero-phosphate, 1 mM Na^3VO_4, 1 mM phenyl-methyl-sulphonyl-fluoride, 10 mM benzamidine, 20 mM NaF, 1mM PNPP, 10 mM Na-pyrophosphate, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 10 µg/ml antipain. After centrifugation for 12 minutes at 13,000 g at –4 ^{o}C, clear supernatants were mixed with 1/3 volume of Laemmli gel loading buffer.

4.2.4 Western Blotting

Western blotting was carried out as per the General Materials and Methods (Section 2.13), with the following variations. 50 µg of lysed protein was separated by SDS-PAGE and transferred to PVDF membrane using the Mini-Protean III system (Bio-Rad). Anti-cAbl (Santa Cruz, #sc-887), anti-ERK1 (Santa Cruz, #sc-93) and anti-PU.1 (Santa Cruz, #sc-352) polyclonal antibodies, and anti-phosphorylated-ERK1/2 (Santa Cruz, #sc-101760) and anti-phosphorylated-Tyrosine (Upstate #16-184) monoclonal IgGs were used. Western blotting was performed by A. Brozik.

4.2.5 Preparation of total RNA and quantitative measurement of β-globin mRNA levels by the LightCycler System

Total RNA was prepared as per the General Materials and Methods (Section 2.12.1). Total cDNA was prepared by reverse transcription with random hexamers using 1 µg RNA. 1 µl of total cellular cDNA was amplified using the LightCycler FastStart DNA Master SYBR Green I kit (Roche). β-globin mRNA copy number was calculated relative to β-2-microglobulin copy number. β-globin primers were designed to detect

the elements of the β-globin gene cluster, (β, δ, ε, γ1, γ2) but not the elements of the α-globin cluster. The sequences of these primers were as follows:

β-globinFP

     5'-TACCCTTGGACCCAGAGGTTCTTT-3'

β-globinRP

     5'-CCCAGGAGCCTGAAGTTCTC-3'

4.2.6 Puromycin Selection

A range of concentrations of puromycin was tested to find the optimal level of growth selection. Preliminary results indicated that a final concentration of 1.5 μg/ml was most the suitable, consequently this dose was used to select PG13 cells before collection of the virus supernatants. K562 cells were also selected with 1.5 μg/ml puromycin from day two post-transduction, and this dose was repeated every 48 hours.

4.2.7 Flow-cytometric Analysis

The rate of vector transduction was interpreted from the number of cells expressing the GFP marker, and the level of transgene expression in each cell was estimated from intensity of fluorescence. Both were measured by flow cytometry (General Materials and Methods, Section 2.11.1). To assess the rate of cell death, cells were washed twice in ice cold PBS, then pelleted again and resuspended in Propidium Iodide (40 μg propidium iodide per ml of PBS) and incubated at 37 ^{o}C for 30 minutes. The proportion of cells positive for propidium iodide was measured by flow cytometry.

4.2.8 Fluorescent *In Situ* Hybridisation

Cells were prepared for Fluorescent *In Situ* Hybridisation (FISH) analysis as per standard protocols (Pardue and Gall 1969). Cells were fixed in a 3:1 mixture of ethanol-acetic acid, then transferred to a drop of 45% acetic acid on a slide coated with 0.1% gelatine and 0.01% chrome alum. Slides were fixed in 95% ethanol, air dried, then dipped in 0.5% agar at 60°C. Slides were air dried in a vertical position until the agar had gelled, then placed in 0.07 M NaOH for 5 minutes at room temperature. Slides were next washed in two changes of 70% ethanol and two changes of 95% ethanol before air drying. The slide-mounted samples were hybridised with labelled DNA at

66 ^{o}C for 12-15 hours. Slides were then washed five times with SSC (0.15 M NaCl, 0.015 M Na citrate, pH 7.0) at 60°C, with a final wash in SSC at room temperature. The slides were then rinsed in 70 and 95% ethanol and air dried.

4.2.9 Statistical Analyses

Many of the results presented below are semi quantitative and from preliminary trials with only one or two replicates. Quantitative assays with at least three replicates were analysed by paired, one-tailed t-tests.

4.3 Results

The efficacy of the anti-Bcr-Abl shRNA design has been demonstrated numerous times (Wilda *et al.* 2002; Li *et al.* 2003b; Scherr *et al.* 2003b; Wohlbold *et al.* 2003), but the efficacy of the shRNA expression cassette and the retroviral construct used here was confirmed in in observations of down-regulation of the Bcr/Abl fusion protein (see below).   Therefore the main aim of this chapter was to evaluate the downstream effects of this shRNA in transduced cells.   Changes in cell number, viability, apoptosis, erythroid differentiation as well as alterations in cellular signalling were followed in transduced cells from day 3 up to day 21.

4.3.1 Fluorescent In Situ Hybridisation

FISH analysis of the K562 cells revealed multiple copies of the Bcr-Abl fusion gene.   A total of 10 copies were evident in most cells (Figure 4.2), indicating amplification and increased copy number of the chromosomal translocation in these cells.

4.3.2 Transduction efficiency of the retroviral vector

K562 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs, as well as GFP and puromycin resistance markers.   Transduction rates were assessed by flow cytometry utilising the GFP marker, with fluorescence recorded at 4, 5 and 7 days post-transduction.

The percentage of fluorescent cells transduced with the NS-expressing vector was stable at approximately 80% (80 to 84%), with median fluorescence peaking at approximately 1000 units on day 5 post-transduction (Figures 4.3A, B).   Following transduction with the BA1 vector, the percentage of fluorescent cells fluctuated between 65 and 71% with median fluorescence intensity reaching 410 units by day 5 (Figures 4.3A, B).   The percentage of propidium iodide positive cells in each population did not exceed 1.5% (Figure 4.3C).   These results clearly show that the NS shRNA design allows both a greater percentage of fluorescent cells, and a higher intensity of fluorescence from those cells.

Figure 4.2 FISH Probing for Bcr/Abl Chromosomal Translocations
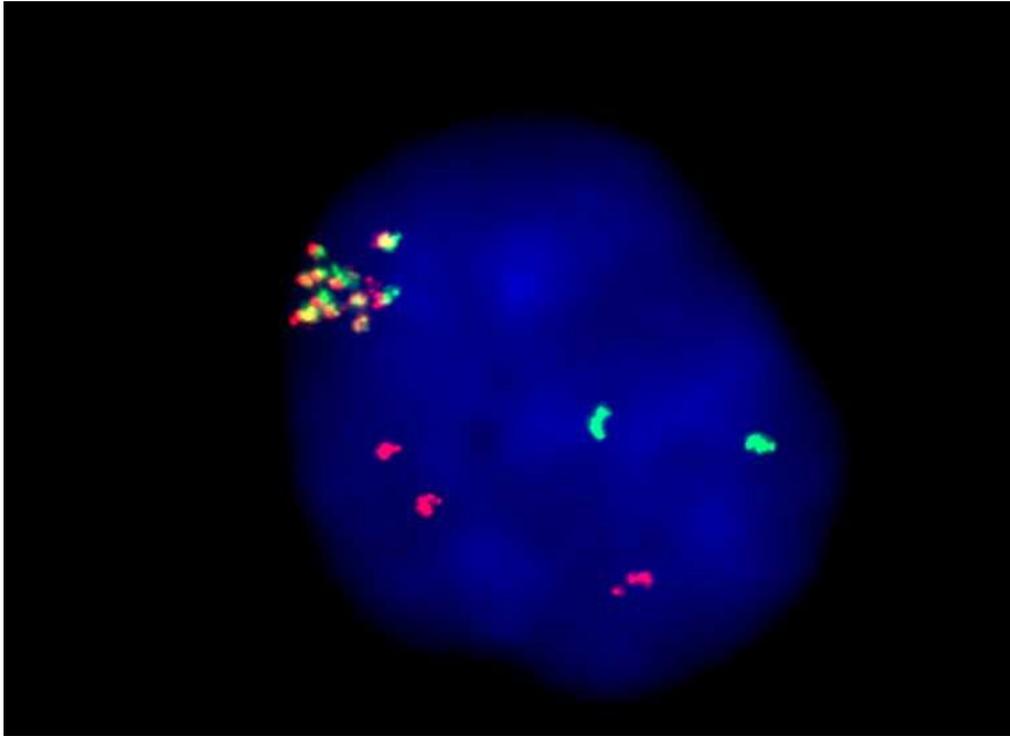
Figure 4.2 – K562 cells were prepared and probed by Fluorescent In Situ Hybridisation for Bcr (green) and Abl (red) genes. This fluorescent micrograph shows one K562 cell, with multiple copies of the Bcr/Abl translocation (yellow).

Figure 4.3 Flow Cytometry of Transduced Cells
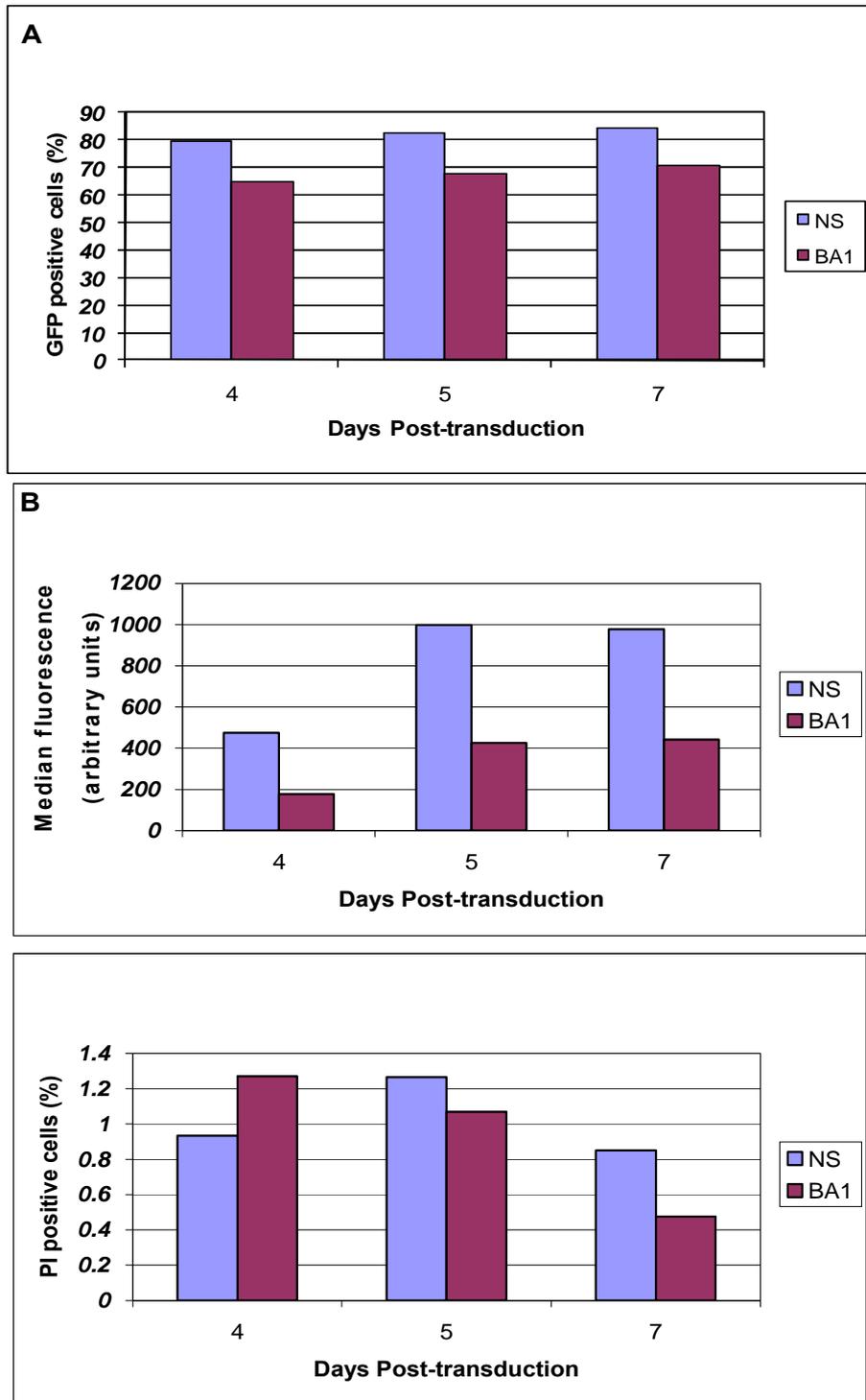
Figure 4.3 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs then analysed by flow cytometry on days 4, 5 and 7 post-transduction. (A) The percentage of GFP positive cells indicates the proportion of cells successfully transduced with the vectors. (B) The median fluorescence intensity reflects the level of transgene expression in transduced cells. (C) Live staining with propidium iodide identified apoptotic cells. These graphs represent the results of a single flow cytometric assay.

4.3.3 Puromycin Treatment of Transduced Cells

In a separate trial, transduced cells were treated with 1.5 μg/ml puromycin, commencing at day 2 post-transduction. Puromycin treatment resulted in a decrease in the percentage of fluorescent cells in the NS shRNA treated population, dropping from 70% at day 4 to 42% by day 7 (Figure 4.4A). This decrease was accompanied by an increase in median fluorescence intensity, peaking at over 1400 units at day 7 (Figure 4.4B), and an increase in propidium iodide positive cells, with a maximum of 25% at day 5 (Figure 4.4C). Puromycin treatment of cells transduced with the BA1 shRNA resulted in an increase in the percentage of fluorescent cells, increasing from 70 at day 4 to 91% at day 7 (Figure 4.4A). Median fluorescence intensity also increased over this period, from 194 to 449 units (Figure 4.4B), while the percentage of propidium iodide positive cells did not exceed 3% (Figure 4.4C). From these results it was clear that puromycin treatment had a marked effect on the transduced cells. Puromycin treatment reduced the percentage of fluorescent cells in the NS population, while there was also a correlation between the increased fluorescence intensity and the increased rate of cell death. The affect of puromycin selection on the BA1 transduced cells was less dramatic, resulting only in an increase in the percentage of fluorescent cells, without major impact on fluorescence intensity or cell death.

4.3.4 MEK-ERK Signalling Pathway

Following transduction with retroviral vectors expressing NS or BA1 shRNA, K562 cells were growth-selected with puromycin commencing on day 2 post-transduction. Cells were collected at various intervals and samples prepared for RT-PCR and Western blotting to assess the effects of the BA1 shRNA on the differentiation of transduced cells.

To confirm the effect of the BA1 shRNA on levels of the fusion gene, Western blots were probed with an antibody to the Abl component of the fusion protein (Figure 4.5). The relative amount of the fusion protein was calculated from Western-blots using ERK1 as a reference protein and optical density values were normalized to the non-transduced control. The amount of Bcr-Abl protein was significantly lower in cells transduced by the BA1 shRNA vector than those transduced by the nonsense shRNA or in the non-transduced cells, at days 3 and 4 after transduction (Figure 4.6). This

Figure 4.4 Flow Cytometry of Transduced Cells Following Puromycin Selection
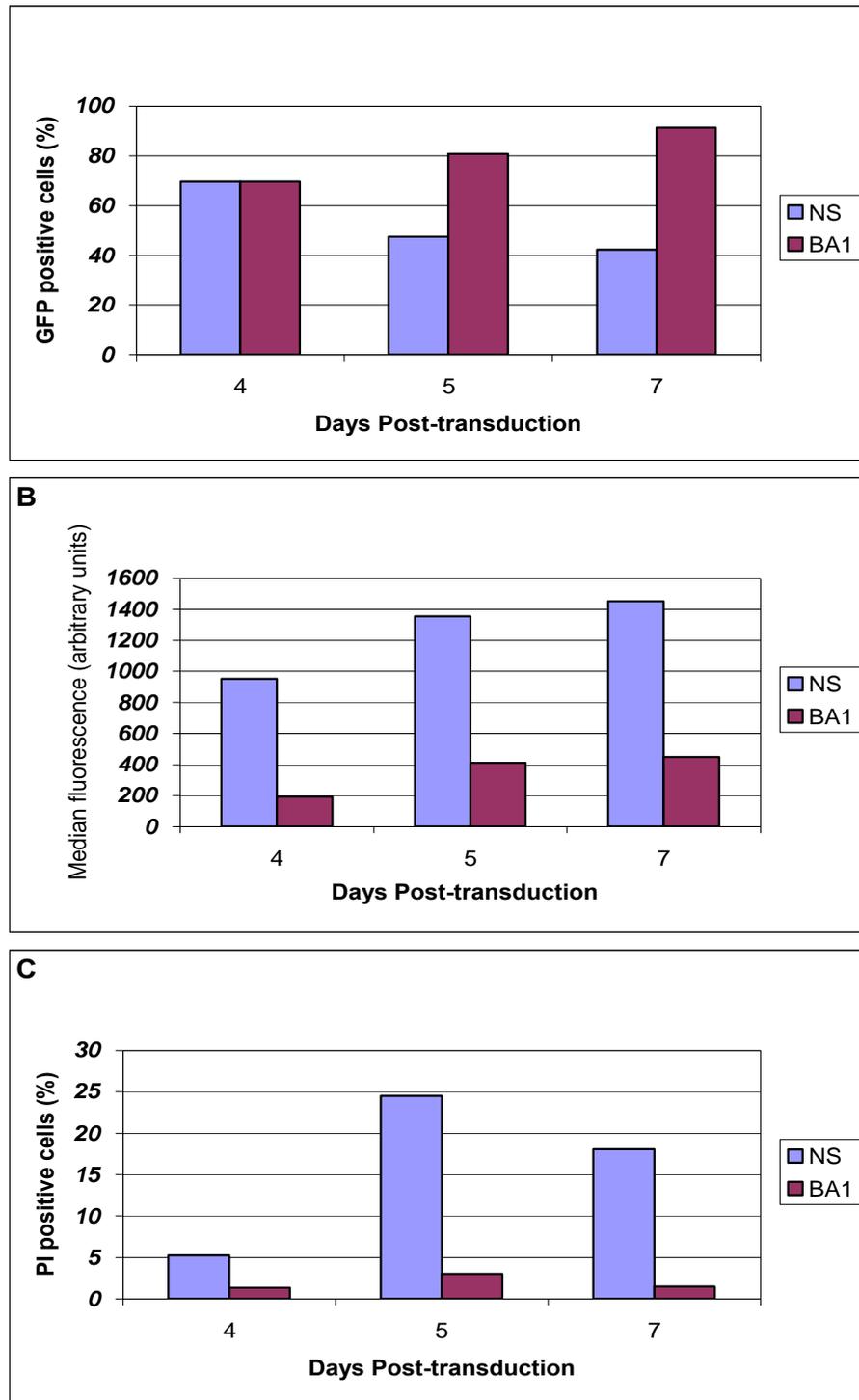
Figure 4.4 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs, selected with puromycin from day 2, then analysed by flow cytometry at days 4, 5 and 7 post-transduction. (A) The percentage of GFP positive cells indicates the proportion of cells successfully transduced with the vectors. (B) The median fluorescence intensity reflects the level of transgene expression in transduced cells. (C) Live staining with propidium iodide identified apoptotic cells. These graphs represent the results of a single flow cytometric assay.

Figure 4.5 Western Blots
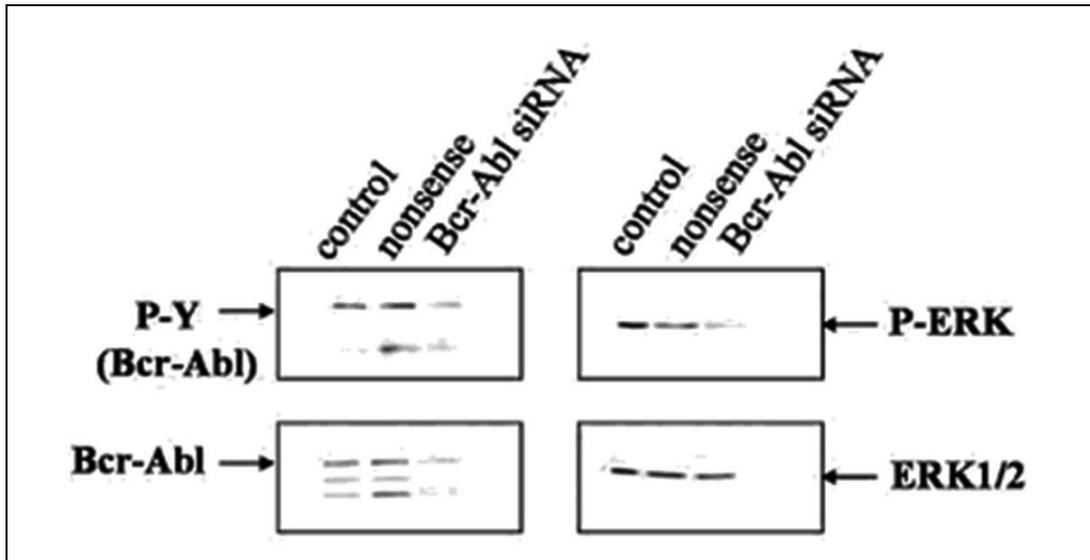
Figure 4.5 - K562 cells were transduced with retroviral vectors expressing NS ('nonsense') or BA1 shRNAs ('Bcr/Abl siRNA'), then prepared for Western blotting. Western blots were stained and developed with antibodies against Bcr-Abl, phosphorylated tyrosine ('P-Y'), phosphorylated ERK ('P-ERK'), and ERK1/2 proteins. The gel shown here was representative of three independent transductions. (Figure reproduced with permission from Brozik et al. (2006). Western blotting was performed by A. Brozik).

Figure 4.6 Normalised Levels of Bcr/Abl Protein
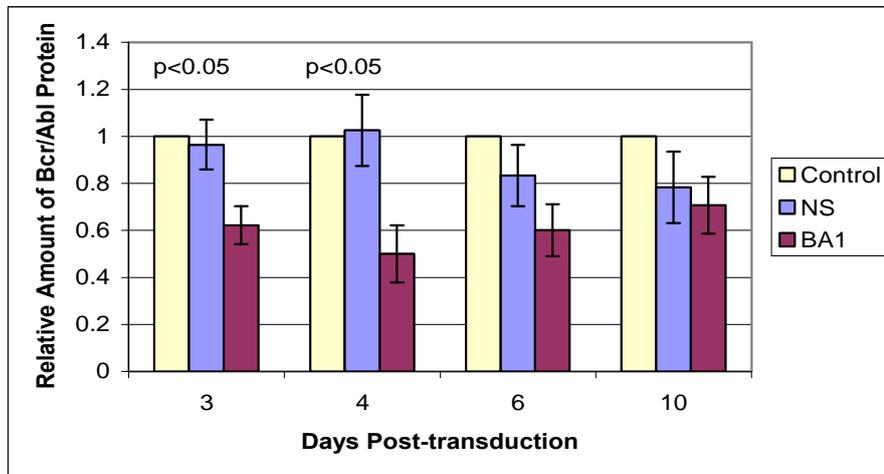
Figure 4.6 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs then sampled for Western blotting on days 3, 4, 6 and 10 post-transduction. Optical densities from the Bcr/Abl band of blots stained with the anti-c-Abl antibody were scanned and quantified with ERK1/2 as a loading control, and were normalized to protein levels from non-transduced cells. This graph presents the mean ± standard deviation from three independent transductions. NS and BA1 samples were compared with a paired t-test and were found to be significantly different ($p < 0.05$) at days 3 and 4.

reduction in levels of Bcr-Abl protein was transient, with some recovery of protein levels after 6-10 days.

The Western blots were stripped and reprobed with anti-phospho-ERK1/2 (pERK) antibody to assess the activity of the MEK/ERK pathway downstream of Bcr-Abl signalling, and optical densities were calculated (Figure 4.7). Levels of pERK in BA1 treated cells decreased to day 4, then showed recovery to day 10. These results correlate with the changes in Bcr-Abl protein levels, indicating that expression of the anti-*Bcr-Abl* shRNA also results in a significant decrease of ERK1/2 activities.

4.3.5 Differentiation Markers

Following transduction with NS or BA1 vectors, K562 cells were analysed for markers of erythroid differentiation, β-globin mRNA and PU.1 protein. β-globin mRNA levels in cells transduced with the BA1 vector increased markedly by day 6 compared with levels in cells transduced with the NS control vector (Figure 4.8). Semi-quantitative analysis of the PU.1 protein demonstrates a considerable and stable reduction in protein levels by day 12, lasting at least until day 17 (Figure 4.9). Both of these changes demonstrate that reduced Bcr/Abl function resulting from the BA1 shRNA leads to erythroid differentiation in K562 cells.

Figure 4.7 Normalised Levels of Phosphorylated ERK Protein
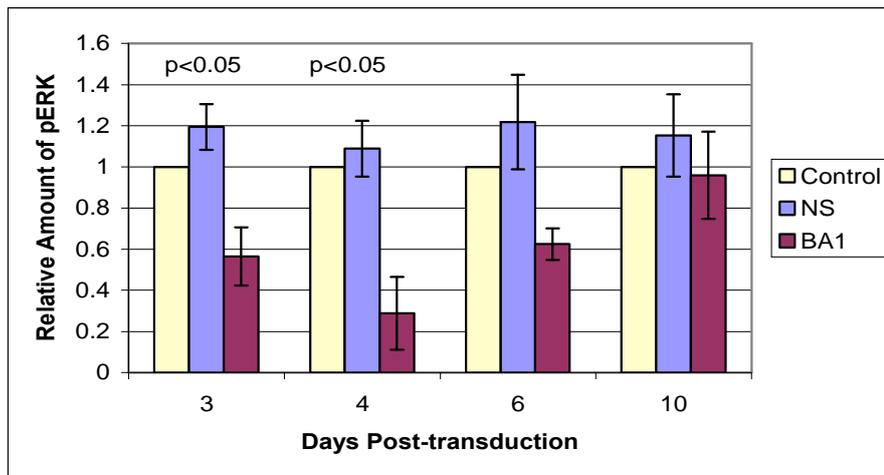
Figure 4.7 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs then sampled for Western blotting on days 3, 4, 6 and 10 post-transduction. Optical densities from blots stained with anti-pERK antibody were scanned and quantified with ERK1/2 as a loading control, and were normalized to protein levels from non-transduced cells. This graph presents the mean ± standard deviation from three independent transductions. NS and BA1 samples were compared with a paired t-test and were found to be significantly different ($p < 0.05$) at days 3 and 4.

Figure 4.8 Levels of β-Globin mRNA at Day 6 Post-Transduction
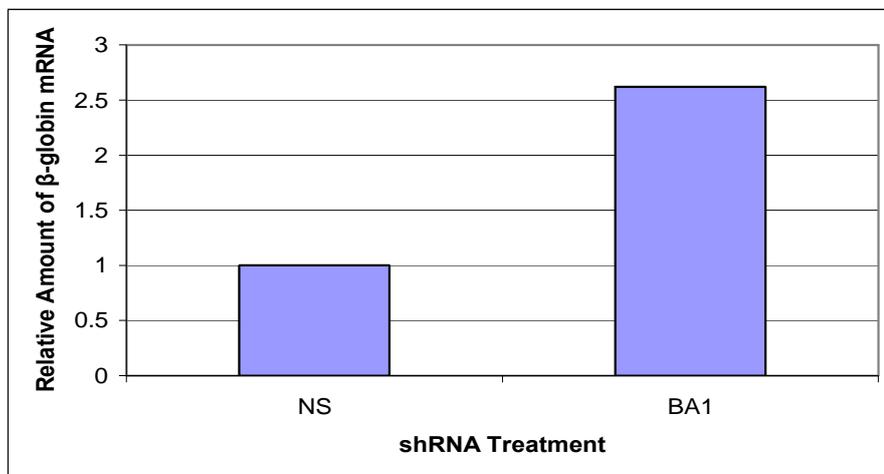
Figure 4.8 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs. mRNA was collected at day 6 post-transduction and quantified by qRT-PCR. β-globin mRNA levels were normalised to the β-2-microglobulin house-keeping gene, and results from BA1 transduced cells were normalised to results from NS control cells. This graph represents the average of two separate experiments.

Figure 4.9 Normalised Levels of PU.1 Protein
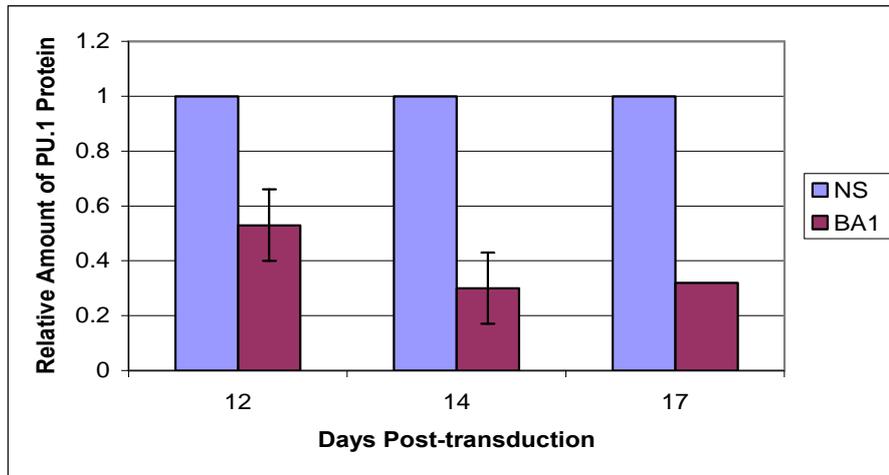
Figure 4.9 – K652 cells were transduced with retroviral vectors expressing NS or BA1 shRNAs then sampled for Western blotting on days 12, 14 and 17 post-transduction. Optical densities from blots stained with anti-PU.1 antibody were scanned and quantified with ERK1/2 as a loading control. Protein levels from BA1 transduced cells were normalised to protein levels from NS transduced cells. This graph represents the mean ± standard deviation from three independent transductions (only two transductions for day 17).

4.4 Discussion

The shRNA ('BA1') designed to target the Bcr/Abl fusion mRNA has previously been shown to reliably and effectively downregulate levels of this fusion transcript and the corresponding protein, leading to apoptosis and the inhibition of proliferation in the K562 cell line (Wilda *et al.* 2002; Li *et al.* 2003b; Scherr *et al.* 2003b; Wohlbold *et al.* 2003; Scherr *et al.* 2005). This effect, however, is dose-dependent, as low vector and shRNA copy numbers can allow cells to escape the effect of the shRNA, and continue to proliferate (Scherr *et al.* 2005), presenting a challenge to the use of this molecule in the context of cancer therapy. In addition to enhancing cell survival and proliferation, however, the Bcr/Abl fusion protein must also block differentiation of these cells (Fang *et al.* 2000). Therefore, as part of a broader study of erythroid differentiation in K562 cells (Brozik *et al.* 2006), the proliferating subpopulation of transduced cells was examined for evidence of erythroid differentiation. In order to minimise vector and shRNA copy number per cell, a lower-titre, retroviral vector was used to deliver the shRNA cassette in this study.

Initial screening of the K562 cells used in this study for genomic amplification of the Bcr/Abl fusion gene revealed a 10-fold amplification , which was moderate for this cell line (Wu *et al.* 1995). Nevertheless this amplification would be sufficient to confer enhanced resistance of these cells to the effects of the BA1 shRNA, compared with primary CML cells such as might be found in CML patients (Mahon *et al.* 2000). These cells therefore represented a good model of relatively low-levels of BA1 shRNA expression. The results from this chapter confirmed that transduction of K562 cells with retroviral vectors expressing the BA1 shRNA resulted in a decrease in Bcr/Abl protein levels, as has been reported previously (Li *et al.* 2003b; Scherr *et al.* 2005), however, a population of cells that continued to proliferate was also (Brozik *et al.* 2006). This population of cells continued to proliferate despite reduced levels of the Bcr/Abl fusion protein, and so were consistent with such populations identified previously, which were also reported to exhibit aberrant proliferation kinetics, and enhanced sensitivity to the Bcr/Abl-kinase inhibitor STI571 (Scherr *et al.* 2005).

The mechanisms by which the Bcr/Abl protein blocks the differentiation pathway have yet to be resolved, but it appears that the MEK/ERK signalling pathway

is involved (Melemed *et al.* 1997; Sevinsky *et al.* 2004), as reduced ERK1/2 activity has been associated with differentiation induced by certain agents, such as haemin, cisplatin, butyrate and ara-C (Woessmann *et al.* 2004). Key steps in this pathway were examined in the proliferating population of BA1-transduced cells.

ERK1/2 is one of many proteins phosphorylated and thus activated by the constitutive phosphorylation activity of the Bcr/Abl fusion protein. The results from this chapter demonstrate that the down-regulation of the Bcr/Abl fusion protein resulted in a decrease in the active, phosphorylated form of ERK (pERK). This decrease in pERK closely reflected the changes in Bcr/Abl protein levels, with the pattern of initial down-regulation followed by recovery of Bcr/Abl protein levels echoed after a short delay by a similar pattern of changes in pERK levels. This confirms the relationship between the Bcr/Abl and ERK proteins (Mizuchi *et al.* 2005), and demonstrates that such changes can also result from specific targeting of the fusion mRNA by shRNA.

One important finding from the present study, however, was the observation that despite the transience of the down-regulation of Bcr/Abl protein and pERK levels, these changes were nevertheless sufficient to irreversibly commit these cells to the erythroid differentiation pathway. This was demonstrated by changes to levels of twp differentiation markers. Increased expression mRNA levels of the β-globin precursor at day six indicated that the cells were preparing for expression of β-globin protein, despite the restored levels of Bcr/ABl and pERK protein seen at this time point. Similarly, levels of the PU.1 protein, a transcription factor associated with non-erythroid lineages, were dramatically reduced at every time point examined between 12 and 17 days post transduction.

Low levels of transgene expression are a constant challenge to gene therapies, and is perhaps more significant for cancer therapies, such as RNA interefence. However the results from this chapter have good implications for shRNA-based, anti-Bcr-Abl therapy, as it suggests that while some treated cells may initially escape apoptosis due to low vector and transgene copy number, they will nevertheless commit to differentiation and exit the proliferative phase of the cell cycle. Therefore, aside from the immediate effects on apoptosis and proliferation, the BA1 shRNA also has activity as a 'differentiation therapy', whereby agents are used to push cells past the

proliferative phase of the cell cycle towards more differentiated cell types, rather than simply triggering apoptosis of cancer cells.

The retroviral vectors used in this study carried two marker genes. The first of these, the puromycin resistance (PUROr) marker, was used simply to purify the transduced population of cells by growth selection. The second marker, enhanced green fluorescent protein (GFP), was included as an indicator of vector copy number per cell, and transgene activity. The interaction between these markers was more complex than expected, and must be considered in order to place the shRNA results in context.

In the absence of puromycin selection, the lower percentage and intensity of fluorescence in the cells transduced with the BA1 vector compared with the control. This result was consistent with the survival of cells bearing only a low copy number of the BA1 vector. A similar result has been reported previously with this shRNA design (Scherr *et al.* 2005). Furthermore, when puromycin selection was applied to these cells, there was no increase in fluorescence, as might be expected with selection of cells bearing a higher vector copy number. Instead, fluorescence intensity was similar to BA1 transduced cells without puromycin selection. From these results it can be concluded that cells with a vector copy number above a certain threshold were eliminated by the activity of the BA1 shRNA, and that only cells below this threshold were able to survive and proliferate.

By contrast, when puromycin selection was applied to the cells bearing the control vector, the outcome was more complex. Puromycin selection resulted in a decrease in the overall percentage of cells that expressed the GFP marker, but the fluorescence in those cells that did express GFP increased markedly. It is important to note that in the retrovector construct used here, the PUROr and GFP marker genes were driven by separate promoters. Consequently the dichotomy of cells within the puromycin treated NS control cells, with certain cells ceasing expression of the GFP marker, yet remaining resistant to puromycin selection, was most likely the result of promoter interference (Curtin *et al.* 2008), or perhaps the emergence of a GFP negative mutant.

Another change that accompanied puromycin selection in this population of cells that was a large increase in the number of apoptotic cells, peaking after three days of puromycin selection. This indicates that GFP expression may also have interfered with

expression of the puromycin resistance marker. Any such promoter interference in cells bearing the BA1 vector, which was identical to the NS apart from the shRNA cassette, was likely obscured by the effects of the BA1 shRNA itself. Given the temporal correlation between increased apoptosis and increased GFP expression, it is worth considering a direct effect of the GFP expression upon cell survival. Expression of GFP from this construct was highly efficient, so it is possible that the physiological burden of high level GFP expression simply overwhelmed the cells, and they were unable to survive. Alternately, it has been suggested that at least some transgenic forms of GFP may be directly toxic to cells (Liu *et al.* 1999). The details of such toxicity have yet to be elucidated, and it has been suggested that the particular constructs used to express GFP encode for toxic peptides (Endemann *et al.* 2003). Regardless, these results highlight the need for great care in designing gene therapy vectors for any application.

4.4.1 Conclusion

In summary, this chapter has demonstrated that in cases where the copy number 'dose' of anti-Bcr/Abl shRNA is below the threshold required to prevent proliferation and trigger apoptosis, it was nevertheless sufficient to bring about a transient lowering of Bcr-Abl protein levels. Although these levels of Bcr/Abl protein, and those of the downstream target, phosphorylated ERK recovered, and cells continued to proliferate, this transient change was sufficient to irreversibly commit cells to the erythroid differentiation pathway. These results also demonstrate that of the multiple signal transduction pathways influenced by Bcr/Abl down-regulation, the effect upon ERK1/2 activities might be a key determinant in overcoming the differentiation blockade that is characteristic of myeloproliferative disorders. Finally, these results also confirm the flexibility of delivery of shRNAs designed to target fusion oncogenes.

5   Down-regulation of PML-RARα by RNAi

5.1 Introduction

Acute promyelocytic leukaemias (APL's) are a subtype of acute myeloid leukaemias (Bennett *et al.* 1976) that usually result from chromosomal translocations involving the retinoic acid receptor α (RARα) and a range of fusion partners (Warrell *et al.* 1993), but 98 % of cases involve the PML gene (Grignani *et al.* 1994). The leukaemogenic effects of these translocations result from a dominant negative inhibition of transcription regulation (Lin *et al.* 2001a). In contrast to other forms of AML, APL patients initially respond to pharmacological concentrations of retinoic acid (RA) (Huang *et al.* 1988; Warrell *et al.* 1991).

In vitro studies of APL have been facilitated by the isolation of the NB4 cell line from the marrow of an APL patient in relapse (Lanotte *et al.* 1991). These cells express one of the larger isoforms of the PML-RARα fusion protein at 120 kDA (Jansen *et al.* 1995), with the breakpoint occurring after exon 6 of PML and before exon 2 of RARα (de The *et al.* 1990; Melnick and Licht 1999). The nucleotide sequence of the breakpoint region identified in NB4 cells has also been sequenced (de The *et al.* 1991).

Current treatment for APL primarily consists of retinoic acid therapy. The chimeric forms of the RARα receptor are insensitive to normal physiological concentrations of retinoic acid, however higher pharmacological doses are usually effective (Warrell 1993). Histone deacetylases provide another option for treatment (reviewed in Lin *et al.* 2001a), and can be effective in cases insensitive to retinoic acid (Warrell *et al.* 1998). Arsenic trioxide is another effective therapy and is currently undergoing a minor revival (Shen *et al.* 1997). While these therapies represent a range of options for the treatment of APL, there exists, as with all therapies, the threat of relapse (Warrell 1993)

A range of molecular strategies has been tested against the PML-RARα fusion gene. Hammerhead ribozymes were able to down-regulate PML-RARα mRNA, inhibiting growth and inducing apoptosis, but did not overcome the maturation block (Nason-Burchenal *et al.* 1998b). Antisense oligonucleotides targeted to the PML-RARα fusion sequence lead to a reduction in levels of the fusion mRNA, inducing partial

differentiation, and apoptosis (Chen *et al.* 1999b). Finally, a 30 nt short hairpin RNA (shRNA) has been shown to reduce levels of the fusion protein (Oshima *et al.* 2003). Effects on cell proliferation were not examined in this study.

Delivery of therapeutic molecules is a challenge in cancer therapy, but lentiviral transduction is an efficient method for systemic delivery of therapeutic transgenes and is suitable for therapeutic strategies involving autologous transplantation. While such vectors are also able to deliver hammerhead ribozymes, they are not suitable for delivery of antisense oligonucleotides and siRNA. By contrast, shRNAs are efficiently expressed from lentiviral vectors and consequently the primary aim of this chapter was to test shRNA designs delivered via lentiviral vectors and targeted to the PML-RARα fusion gene and to assess their efficacy in down-regulating levels of the PML-RARα fusion gene mRNA. Concomitant with this, the downstream biological effects on cell cycling and proliferation of the target cells was also monitored.

5.2 Methods

5.2.1 Transduction of NB4 cells with lentiviral vectors

The NB4 cells were transduced according to the general protocol outlined in Section 3.11. Briefly, two 0.5 ml aliquots of filtered and resuspended vector were applied to $5\text{x}10^5$ cells in one well of a 6-well plate. 1 ml of fresh complete RPMI and 10 µl of Polybrene stock (0.6 µg/ml) were also added and the cells centrifuged at 1000 g for 100 minutes at room temperature.

5.2.2 Hairpin Design

The shRNA designs were compared using the pHIV-7-GFPshr lentiviral vector backbone (Section 3.2.4).

5.2.2.1 PR1 and PR2 Hairpin Designs

The chromosomal fusion found in NB4 cells has been previously sequenced and lodged on the NCBI database with the accession number S50916 (de The *et al.* 1991). The target region for hairpin design spanned 20 nt on each side of the breakpoint, corresponding to nucleotides 20-60 of S50916. This sequence was as follows, with a forward slash indicating the breakpoint;

```
CAGTGGCGCCGGGGAGGCAG/CCATTGAGACCCAGAGCAGC
```

This sequence was submitted to both the Ambion and GenScript algorithms, however, neither identified a suitable shRNA design. It is worth noting that these algorithms are updated as new data is gathered, and that a previous version of the Ambion algorithm did identify a candidate shRNA for the PML-RARα breakpoint, although this design was not tested (Damm-Welk *et al.* 2003). Examination of the sequence showed that this sequence was optimised for minimal G/C content, and was used here as 'PR1'. The target sequence for this design, with the breakpoint indicated by a forward slash, was as follows:

```
5'-GGCAG/CCATTGAGACCCAGA-3'
```

A second sequence designed to target the PML-RARα breakpoint was tested as a 30 nt hairpin sequence (Oshima *et al.* 2003). This sequence was shortened to 21 nt, keeping the breakpoint in the middle of the target sequence, and adapted to an shRNA

format. The target sequence, designated 'PR2', was as follows, with the breakpoint indicated by a forward slash;

5'-CGGGGAGGCAG/CCATTGAGAC-3'

Both hairpin cassettes were cloned into the lentiviral vectors in the reverse orientation (see Section 3.3.4) and these vectors were transduced into NB4 cells, with vectors bearing the nonsense shRNAs acting as controls. The pHIV-7-GFPshr lentiviral backbone was used for testing shRNA designs.

5.2.2.2 Control shRNAs

A range of controls is available for testing hairpin activity although most were not appropriate here. Aside from the nonsense hairpin design, the remaining relevant control was the use of a modified hairpin bearing a 2- or 4-nt inversion at the central 'active site'. After preliminary experiments identified the PR2 hairpin as the more effective design, this design was used as the basis for a control hairpin with an inverted centre.

As per the recommendations of the Nature editorial panel ("Whither RNAi?", Nature Cell Biology (2003), v.5 (6) pp. 489-490), the control shRNAs were based on an inversion of the central nucleotides of the PR2 target sequence. Blasting of candidate control sequences bearing 2 and 4 nucleotide inversion indicated homology with non-target genes. For a 2 nt inversion, there was a 16 nt homology with a range of non-target genes. Furthermore this design would mimic a micro-RNA designed to target the same PML-RARα sequence. By contrast, the 4 nt inversion shared only 14 nt homology, and with fewer non-target genes. Therefore a 4 nt inversion hairpin was constructed and designated 'PRK'. This sequence, with the central 4 nt inversion underlined, was as follows:

5'-CGGGGAGGC<u>CCGA</u>ATTGAGAC-3'

5.2.3 qRT-PCR Assays

Total RNA was collected as per the General Materials and Methods. For the PML-RARα chapter, both SYBR Green and Plexor chemistries were used for the qRT-PCR's. SYBR Green was used for the preliminary comparison of PR1 and PR2 hairpin designs, as well as the OAS comparisons. All other results were based on the Plexor system.

β-2-microglobulin was used as the house-keeping gene throughout this chapter and the SYBR Green and Plexor primers for β-2-microglobulin, and the SYBR Green primers for OAS, were as per the General Materials and Methods.

5.2.3.1 SYBR Green Primers

The SYBR Green primers for the PML-RARα fusion gene were as follows:

PRAFP

    5'-GCCCAACAGCAACCACGT-3'

PRARP

    5'-GGAGGGCTGGGCACTATCTC-3'

Product size = 90 bp

5.2.3.2 Plexor Primers

The Plexor primers for the PML-RARα fusion gene were as follows:

PRAFP (5' labelled with the 6-FAM fluorophore)

    5'-CTGCTGCTCTGGGTCTCAATG-3'

PRARP

    5'-CCAGGAGCCCCGTCATAGGAA-3'

Product size = 97 bp

5.2.4 Puromycin Treatment

Cells were transduced with the pPIG-2sh-LV vectors (see Section 3.2.5.2.2.2) and growth-selected for 48 hours with 12.5 μg/ml puromycin.

5.2.5 Retinoic Acid treatment

All Trans Retinoic Acid (Sigma, cat. #R2625) was dissolved in DMSO at a stock concentration of 10^{-4} mol/ml, and stored at $-80\,^{\circ}$C. Before use, this was diluted to 10^{-6} mol/ml with complete RPMI, and added at the appropriate concentrations.

5.2.6 Cell Cycle Analysis

Samples of $5\text{-}10\text{x}10^{5}$ treated cells were washed in PBS then fixed in 70% ethanol at $4\,^{\circ}$C for at least 60 minutes, or overnight at $4\,^{\circ}$C. Cells were again centrifuged, and resuspended in 300 μl Hypotonic Propidium Iodide (40 μg propidium

iodide, 1 µg RNase per ml PBS) for 2 hours at RT, or 4 °C overnight. Analysis was performed with a Coulter EPICS ELITE ESP flow cytometer.

5.2.7 Differentiation Assays

Samples of 5×10^5 cells were pelleted at 1300 g for 5 minutes at room temperature then vortexed and resuspended in PBS. Cells were incubated in the dark for 15 minutes with 10 µl of either CD11b or CD14 antibody, conjugated to phycoerythrin (BD Biosciences, North Ryde NSW, Australia). Cells were washed with 100 µl PBS, pelleted, vortexed, then washed with another 100 µl of PBS. They were then pelleted, vortexed, and resuspended in 100 µl of FACS Fix (see General Materials and Methods). Cells were stored at 4 °C in the dark until analysed with a Coulter EPICS ELITE ESP flow cytometer.

5.3 Results

5.3.1 Selection of anti-PML-RARα and control shRNAs

The pHIV-7-GFP lentiviral backbone was used for this section.

5.3.1.1 PR1 vs PR2

To compare the PR1 and PR2 shRNA designs, NB4 cells were transduced with lentiviral vectors expressing each design. Control cells were transduced with vectors expressing the NS shRNA. For this preliminary trial, mRNA samples were collected 12 hours after the first transduction, and at 12 hour intervals thereafter up to 72 hours. The copy number of the PML-RARα fusion mRNA was assessed by qRT-PCR and normalised to β-2-microglobulin copy number. It was clear from this trial that the PR1 was not effective in down-regulating PML-RARα mRNA (Figure 5.1). Instead, cells transduced with the PR1 shRNA design had higher levels of PML-RARα mRNA than the NS controls. By contrast, expression of the PR2 shRNA resulted in a reduction in PML-RARα mRNA relative copy number relative to the NS controls, particularly over the first 36 to 48 hours. Although this preliminary data was derived from a single trial, the consistency of the effect was considered a sufficient demonstration of the efficacy of the PR2 shRNA design to pursue further testing of this design. The PR1 shRNA was not examined further.

5.3.1.2 Testing of 'inverted centre' control hairpin

Aside from the scrambled NS control shRNA, another control, based on the active hairpin but with an inversion of the central nucleotides is recommended where appropriate ("Whither RNAi?", Nature Cell Biology (2003), v.5 (6) pp. 489-490). Accordingly a control based on the PR2 shRNA, with an inversion of the 4 central nucleotides, was tested. NB4 cells were transduced with vectors expressing this 'PRK' shRNA, and compared with cells transduced with the NS and PR2 expressing vectors. Sampling of the PML-RARα mRNA at 72 hours post-transduction demonstrated that the effect of the PRK shRNA on PML-RARα mRNA levels was similar to that of the PR2 shRNA, in that both designs lead to a reduction in mRNA levels (reduced to 8% and 14% for the PR2 and PRK shRNA respectively), relative to the NS shRNA (Figure

Figure 5.1 NS vs PR1 vs PR2 shRNAs
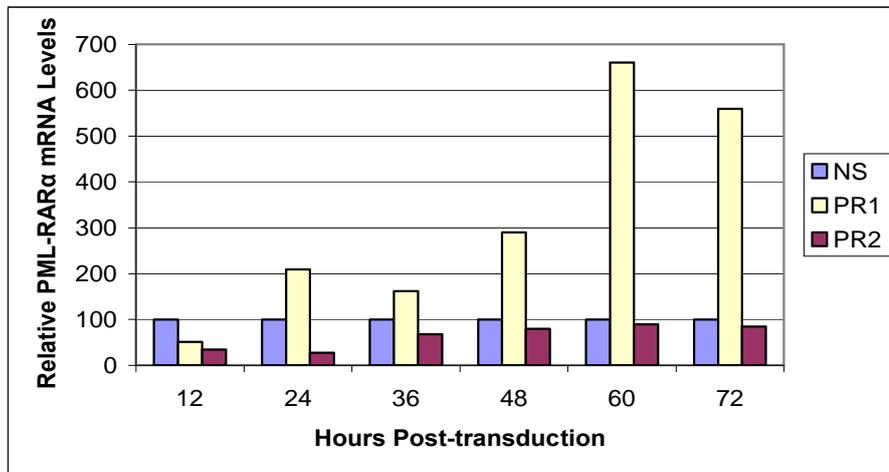
Figure 5.1 - NB4 cells were transduced with vectors expressing the NS, PR1 or PR2 shRNAs. mRNA was collected at 12 hour intervals and quantified against β-2-microglobulin mRNA. This normalised value is presented relative to the NS controls at each time point. This graph represents a single experiment.

Figure 5.2 Relative activity of NS, PR2 and the novel PRK control
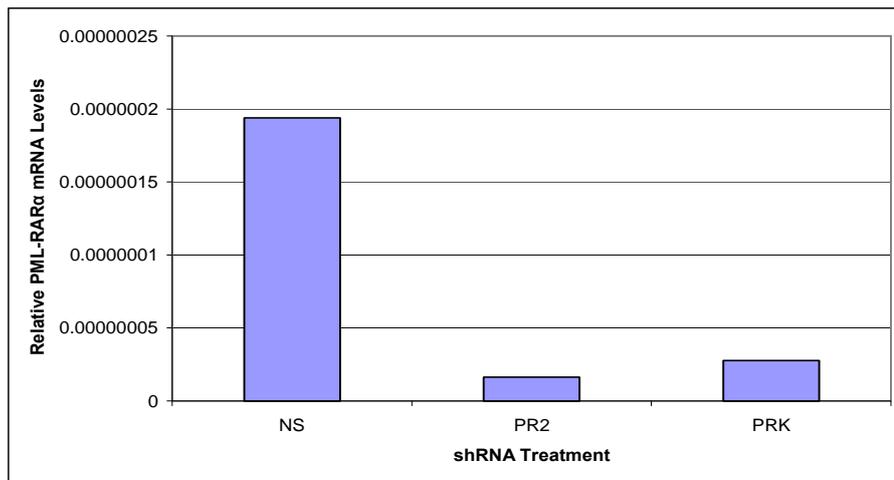
Figure 5.2 - NB4 cells were transduced with vectors expressing the NS, PR2 or PRK shRNAs. mRNA was collected at 72 hours and quantified against β-2-microglobulin mRNA. This normalised value is presented relative to the NS control. This graph represents a single experiment.

5.2). Due to this activity, the PRK shRNA was not considered an appropriate control and was subsequently discarded.

5.3.1.3 Testing for non-specific effects

After establishing the PR2 shRNA as the most active design, and NS shRNA as the most appropriate control, samples were re-analysed to look for stimulation of a non-specific interferon response. 2',5'-oligoadenylate synthetase (OAS), a gene upregulated as part of the interferon response, was used as a marker for such stimulation. Levels of OAS mRNA were quantified from samples collected 48 hours after transduction, and normalised against β-2-microglobulin mRNA.   There was no evidence of OAS stimulation by the PR2 shRNA design, when compared with the NS control (Figure 5.3).

5.3.2 Activity of PR2 Hairpin in Down-regulating the PML-RARα Fusion mRNA and Protein

5.3.2.1 qRT-PCR results

Preliminary trials with the lentiviral vector in NB4 cells indicated that expression of the transgene at 24 hours post-transduction was observed in approximately 65% of cells. Maximal expression and activity of the shRNA was therefore expected between 48 and 72 hours post-transduction. Therefore, in order to confirm the effects of the PR2 shRNA, RNA was collected from five replicates of transduced cells at these two time points, and PML-RARα mRNA copy number quantified against the β-2-microglobulin house keeping gene.   While there were differences in scale between replicates, there was a consistent trend of PML-RARα mRNA down-regulation in all samples at both time points (Figure 5.4). When these results were expressed relative to the NS control, average down-regulation was 30% at 48 hours, and 40% at 72 hours (Figure 5.5). The difference at 72 hours was significant (paired t-test; $p=0.052$ at 48 hours, and $p=0.025$ at 72 hours).

After the preliminary trials outlined above, NB4 cells were transduced with vectors expressing the NS control hairpin or the PR2 hairpin. qRT-PCR results (Figures 5.4 and 5.5) confirmed that the PR2 hairpin had a down-regulatory effect on PML-RARα mRNA levels at both 48 and 72 hours. The results of five replicates were

Figure 5.3 OAS levels at 48 hours after transduction
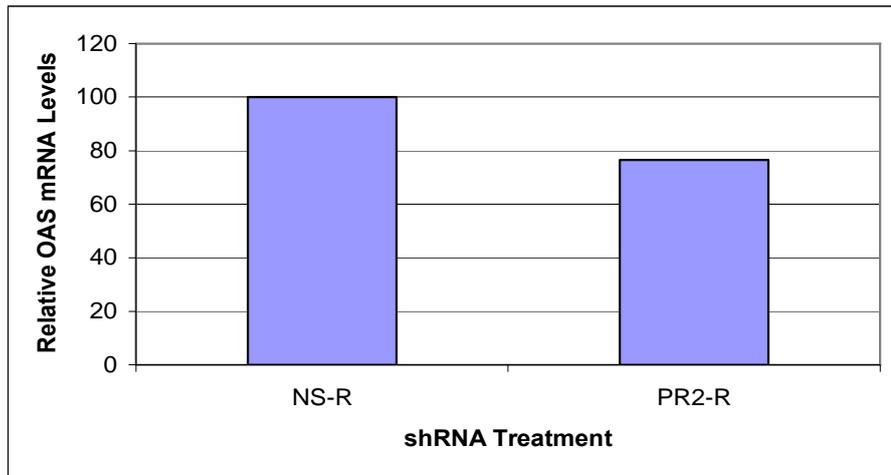
Figure 5.3 - NB4 cells were transduced with vectors expressing the NS, PR2 or PRK shRNAs. mRNA was collected at 48 hours and quantified against β-2-microglobulin mRNA. This normalised value is presented relative to the NS control. This graph presents the mean from two separate experiments.

Figure 5.4 PML-RARα mRNA levels following treatment with shRNAs
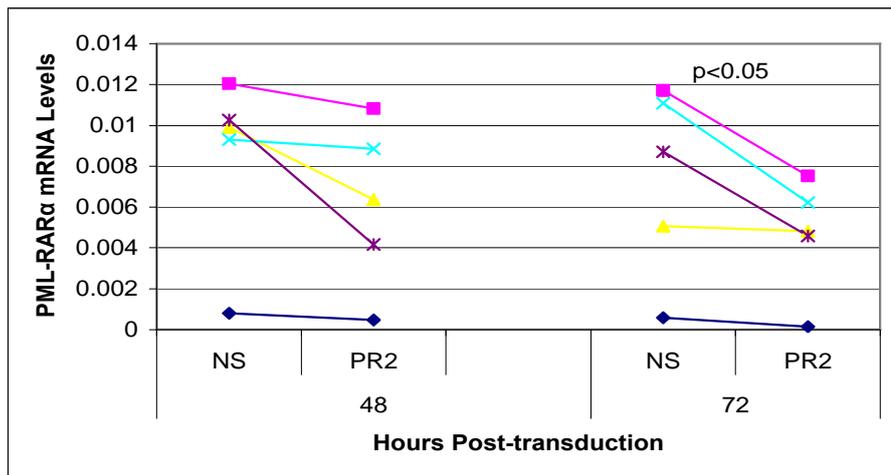
Figure 5.4 – NB4 cells were transduced with vectors expressing the NS or PR2 shRNAs. mRNA was collected at 48 and 72 hours and quantified against β-2-microglobulin mRNA. PML-RARα levels were significantly lower in the PR2 transduced cells at 72 hours. This graph presents the plots from five independent transductions.

Figure 5.5 Average down-regulation of PML-RARα mRNA relative to controls
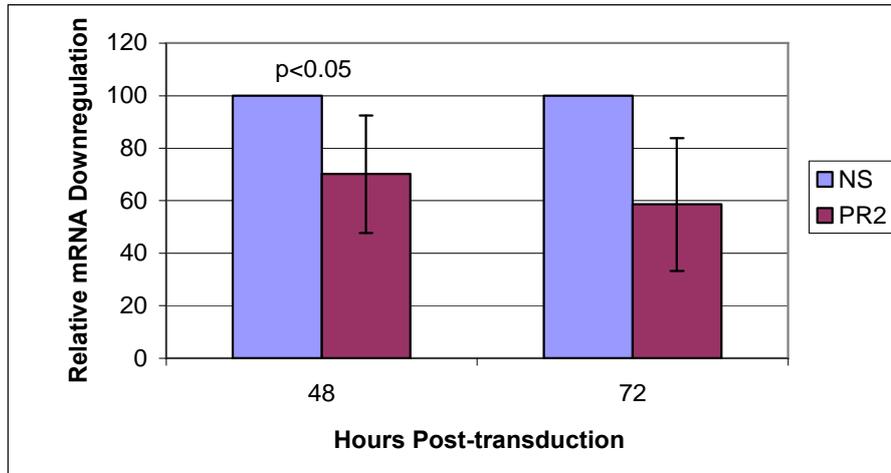
Figure 5.5 – NB4 cells were transduced with vectors expressing the NS or PR2 shRNAs. mRNA was collected at 48 and 72 hours and quantified against β-2-microglobulin mRNA. PML-RARα levels were significantly lower in the PR2 transduced cells at 72 hours. This graph presents the mean ± standard deviation from five independent transductions. NS and PR2 samples were compared with a paired t-test.

averaged (Figure 5.4) and at 48 hours post-transduction the fusion gene mRNA was reduced to approximately 75% by the PR2 hairpin, while at 72 hours the PML-RARα mRNA was reduced to approximately 60%. These results were analysed by paired t-tests, and despite the high standard deviations, the decrease in PML-RARα mRNA at 72 hours was significant (p=0.052 at 48 hours, and p=0.025 at 72 hours. n=5. See Appendix 5.3.2). Examination of the individual plots clearly demonstrates a consistent downward trend in PML-RARα mRNA levels in all samples transduced with the PR2 expressing vector (Figure 5.5). In summary these results clearly demonstrated that the PR2 hairpin was able to consistently down-regulate the level of PML-RARα mRNA in transduced NB4 cells.

5.3.2.2 Western Blots

Expression of the PR2 shRNA resulted in consistent down-regulation of PML-RARα mRNA levels. The half-life of the PML-RARα fusion protein is less that 24 hours (Grignani *et al.* 1999), therefore, after NB4 cells were transduced with the NS and PR2 expressing vectors as above, nuclear proteins were collected at 48 and 72 hours for Western blotting. Samples were probed with anti-RARα antibody, which recognised both the normal 55 kDa RARα protein, and the 210 kDa PML-RARα fusion protein found in NB4 cells (Figure 5.6A). The same blot was probed separately with anti-β-actin antibody as a loading control (Figure 5.6B). These blots were scanned and analysed semi-quantitatively using ChemiDoc software, and the results normalised to the β-actin loading control, and expressed relative to the NS control (Figure 5.7). PML-RARα fusion protein levels were similar between treatments at 48 hours (PR2 sample was 94% of control), however, the fusion protein was down-regulated at 72 hours post-transduction (69% of control). These results indicate that the PML-RARα mRNA down-regulation caused by expression of the PR2 shRNA also resulted in a decrease in levels of the PML-RARα fusion protein, however, this decrease was most prominent up to 48 hours later.

5.3.2.3 Cell Proliferation and Apoptosis

The PML-RARα fusion protein is involved in promoting proliferation and inhibiting apoptosis. To assess any effect of the PR2 shRNA in transduced cells, proliferation was assessed by a 'CellTitre' dye conversion assay. In the first trial,

Figure 5.6A Western blot of RARα and PML-RARα proteins
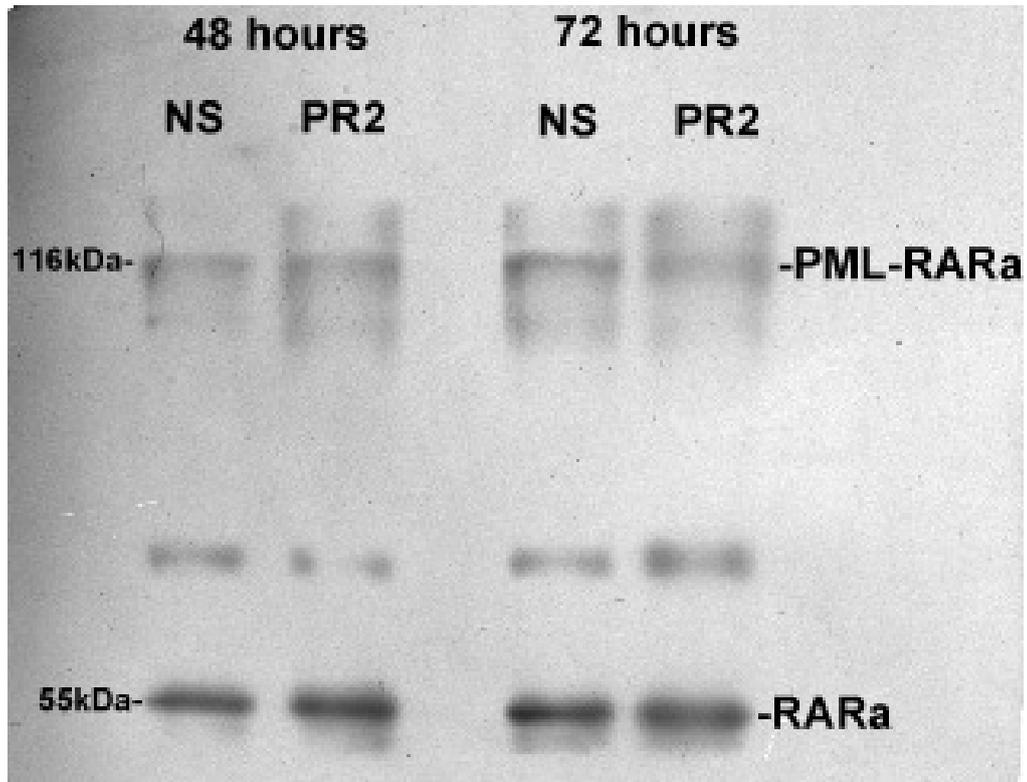
Figure 5.6B β-actin loading control

Figure 5.6 – Western blots of NS and PR2 transduced samples, collected at 48 and 72 hours post-transduction and stained with RARα antibody, which detected both the normal RARα protein, and the PML-RARα fusion protein (Figure 5.6A). The same blot was stained with the β-actin antibody as a loading control (Figure 5.6B). Additional bands evident on this figure are due to non-specific labelling. These figures are representative of three independent transductions and blots.

Figure 5.7 Relative Band Intensity
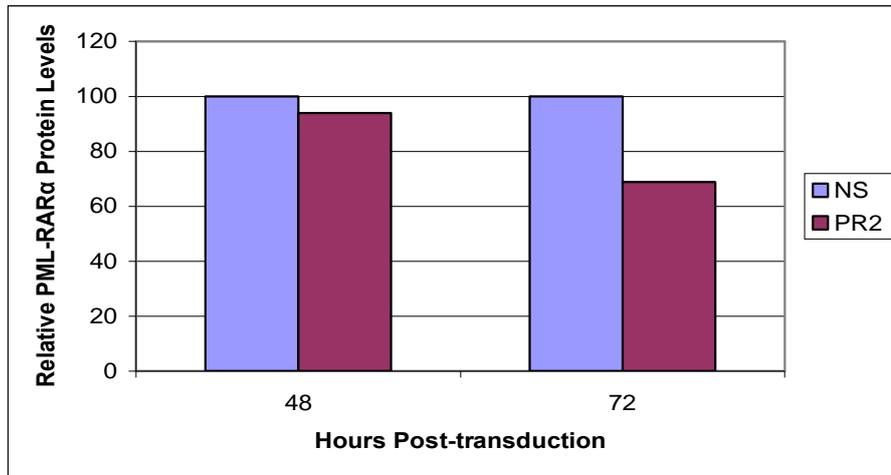
Figure 5.7 – The Western blots stained with the anti-RARα and anti-β-actin antibodies were scanned and quantified. The intensity of the PML-RARα bands was normalised against the β-actin loading control, and expressed here relative to the NS controls. This graph is based on the blots in Figure 5.6. This graph represents the two independent transductions.

proliferation assays were carried out before transduction, and at 24 hours intervals thereafter. Two additional assays were subsequently carried out at the time of maximal shRNA expression (48 and 72 hours). The results from these assays are combined in Figure 5.8, from which it was clear both that the transduction protocol had a pronounced adverse effect on cell survival, and recovery of the population was impaired in cells transduced with the PR2 vector, compared with cells transduced with the control. Furthermore, the difference in cell number was significant at 48 hours but not at 72 hours ($p=0.003$ and $p=0.201$ at 48 and 72 hours respectively). These results also indicate that the transduction protocol results in the death of many cells. There was also recovery of both cell populations, as cell number increased following both treatments. These results were examined in more detail in subsequent experiments (see Section 5.3.3). To complement the cell proliferation assays Trypan blue counts to assess viability were performed following transduction with NS or PR2 vectors. The percentage of Trypan blue positive cells did not reflect the adverse effects of the transduction protocol, suggesting that cell death occurred via a different pathway. The counts of Trypan blue positive cells (Figure 5.9) however, demonstrated a spike in cell death amongst PR2 transduced cells at 24 hours after transduction. It was clear from these results that the PR2 shRNA, in down-regulating both the PML-RARα mRNA and protein, also affected proliferation of the NB4 cells. This inhibition of proliferation was due at least in part to apoptosis.

5.3.2.4 Cell Cycling

The PML-RARα fusion gene disrupts the retinoic acid differentiation signal and effectively prevents the differentiation of the cells. This blockage is reflected in a greater proportion of cells in the M, S and G2 phases. Removal of this block should result in a greater percentage of cells remaining in the G_o/G_1 phase. To assess the effect of the PR2 shRNA on cell cycling, NB4 cells were transduced, and then stained with propidium iodide for cell cycle analysis by flow cytometry. Cells were sampled at 24, 48, 72 and 96 hours post-transduction. Transduction with the PR2 vectors resulted in only minor differences compared with the NS controls, and there was no consistent effect (Figure 5.10). These results provide no indication that the PR2 shRNA had any effect on cell cycling.

Figure 5.8 Proliferation of transduced cells
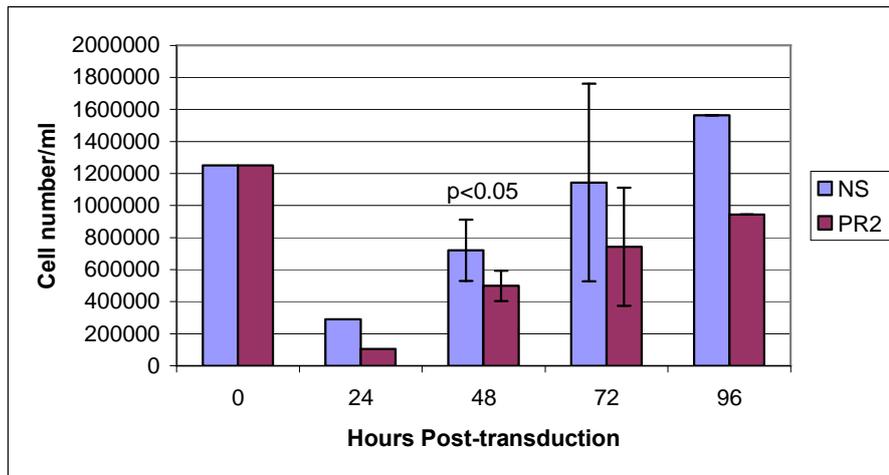
Figure 5.8 – NB4 cells were transduced with NS or PR2 vectors, and cell proliferation assays performed at 24 hour intervals. This graph presents the mean ± standard deviation from three independent transductions for the 48 and 72 hour standards. NS and PR2 samples were compared with a paired t-test and were found to be significantly different ($p < 0.05$) at 48 hours post transduction. The values at 0, 24 and 96 hours represent the average of four samples from a single experiment.

Figure 5.9 Cell death following transduction
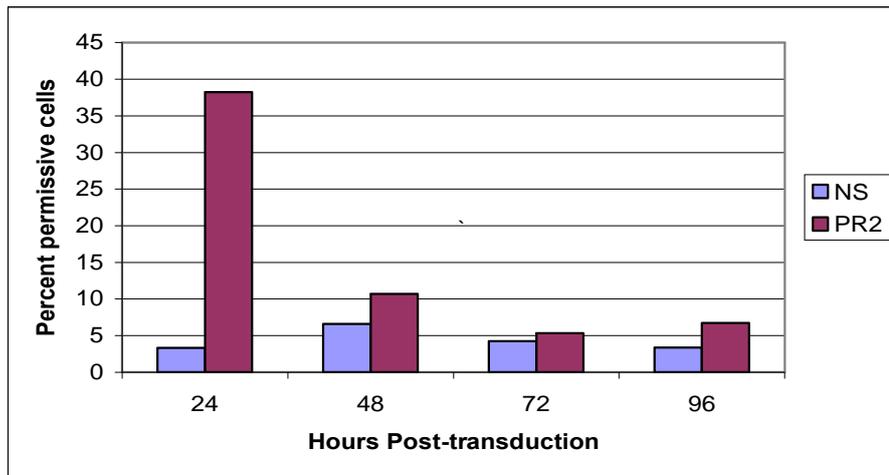
Figure 5.9 – NB4 cells were transduced with NS or PR2 vectors, and viability assessed with Trypan blue. The number of Trypan blue positive (dead) cells is expressed as a percentage of the total cell count at each time point. This graph represents the average of four samples from a single experiment.

Figure 5.10 Cell Cycling
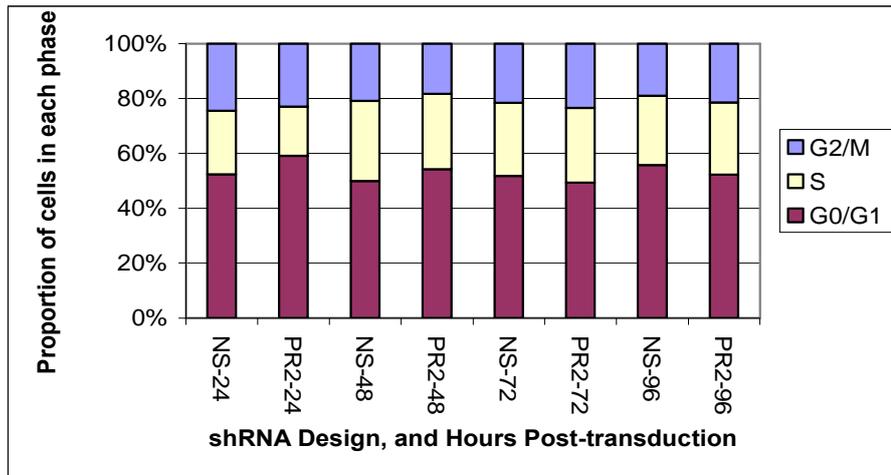
Figure 5.10 – NB4 cells were transduced with NS orPR2 vectors and then stained with propidium iodide to assess cell cycling. The number of cells in each phase of the cell cycle is presented as a proportion of the total number of analysed cells. This graph presents the average from two replicates.

5.3.3 Proliferation of Purified PR2 Transduced Cells

5.3.3.1 Activity of shRNA in the pPIG-2sh-LV vector

From the preceding results it was apparent that despite higher rates of cell death, especially at earlier time points, and despite lower total cell counts, there remained a certain level of proliferation in the PR2 transduced cells. As transduction rates were below 100%, it was thought that this proliferation might be the result of expansion of a sub-population of non-transduced cells. In order to test this explanation, non-transduced cells were eliminated by one of two methods. The NS and PR2 expression vectors used above also expressed a GFP marker that allowed positive selection of transduced cells by fluorescent activated cell sorting. The samples sorted by this method are hereafter referred to as 'sNS' and 'sPR2'. The second method was based on growth selection. Lentiviral vectors expressing a puromycin resistance marker and two copies of the shRNA cassettes were developed ('2sh' vectors, see Chapter 3). Growth selection with 12.5 μg/ml successfully eliminated non-transduced cells. Samples selected by this method are referred to hereafter as '2NS' and '2PR2', reflecting the different form of the vector.

Cells were transduced with each of these vectors, selected by the appropriate method, and grown as a purified population for at least another 48 hours. To confirm the activity of the shRNAs in these vectors under these conditions, RNA was collected and quantified, with PML-RARα copy number normalised against the β-2-microglobulin house-keeping gene. Although each method resulted in between 30 and 40% down-regulation of PML-RARα mRNA relative to the NS controls (Figure 5.11), only the difference in the growth selected population was significant (paired t-tests, p=0.052 and p=0.030 for the sorted and growth selected populations respectively).

Cell proliferation assays were also performed at 48 hours after sorting or growth selection. Non-transduced, unselected NB4 cells were included to indicate maximum growth under these conditions. These results show that transduced cells suffer some growth retardation relative to the non-transduced control, however, despite selection and purification of the respective transduced cell populations, there was no apparent effect from the PR2 shRNAs relative to the NS controls (Figure 5.12).

Figure 5.11 Levels of PML-RARα mRNA in purified cells
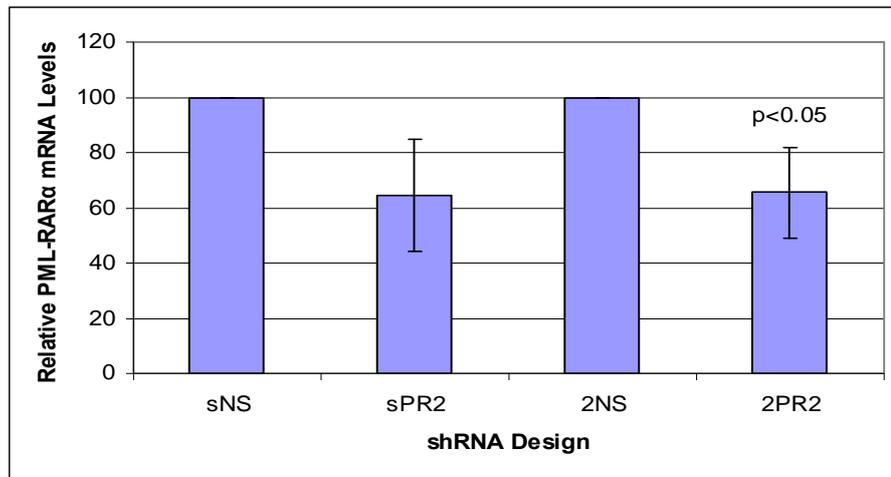
Figure 5.11 – NB4 cells were transduced with NS or PR2 forms of two vector types, then purified as appropriate. After a further 48 hours culture, mRNA was collected and PML-RARα copy number was normalised to the β-2-microglobulin house-keeping gene. PML-RARα mRNA levels are expressed relative to the appropriate NS control. This graph presents the mean ± standard deviation from four independent transductions, each the average of four samples. NS and PR2 samples with each vector type were compared with a paired t-test, and the difference between the 2NS and the 2PR2 samples was significant ($p < 0.05$).

Figure 5.12 Relative proliferation of purified cells
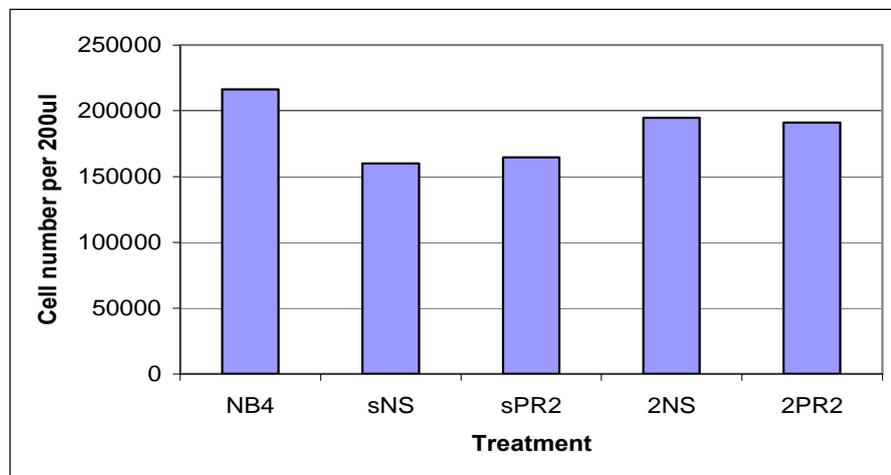
Figure 5.12 – NB4 cells were transduced with NS or PR2 forms of two vector types, then purified by sorting (sNS and sPR2), or with 12.5 μg/ml puromycin (2NS and 2PR2). Cell proliferation assays were conducted at 48 hours after selection, with non-transduced, unselected cells included as a control (NB4). This graph represents the average of four samples from a single experiment.

From these results it was apparent that some cells were surviving and proliferating despite expression of the PR2 shRNA, and despite the down-regulation of the PML-RARα mRNA.

5.3.3.2 Sensitisation to Stress of 'Spinoculation'

The inhibitory effect of the PR2 shRNA on proliferation that was observed immediately after transduction was not observed in the purified transduced cell populations. To determine if the initial inhibition of proliferation by the PR2 shRNA was the result of sensitisation to the stresses of the 'spinoculation' procedure, NB4 cells were transduced as before but without the spinoculation step, and proliferation was examined up to 72 hours. The results clearly demonstrate that when cells were transduced without the spinoculation step, the effect of the PR2 shRNA on cell proliferation remained (Figure 5.13). This effect was most pronounced at 24 hours post-transduction, with possible proliferative recovery apparent before 48 hours post-transduction. Therefore, the PR2 shRNA was affecting NB4 proliferation directly.

5.3.3.3 Effect of Addition of Fresh Medium

Given the timing of the period in which the PR2 shRNA affected cell proliferation, and the fact that the culture medium in the proliferation trials was usually replenished at 72 or 96 hours, the effect of replenishing the culture media was also examined. Cells were transduced with the NS and PR2 vectors without the spinoculation step, as above, however, at 48 hours an equal volume (2 ml) of fresh complete RPMI was added to one set of transduced cells (NS and PR2 samples), diluting them by half. Proliferation of these samples at 72 hours was compared with cells cultured in the original volume. The results of this trial clearly demonstrated that the addition of fresh culture medium at 48 hours after transduction eliminated the inhibitory effect of the PR2 shRNA on cell proliferation at later time points (Figure 5.14). These results suggested that the effects of the shRNA on proliferation remained in effect, but that some cells escaped these effects, and by proliferating were able to obscure the effects of the shRNA on cell proliferation.

Figure 5.13 Proliferation of cells without spinoculation
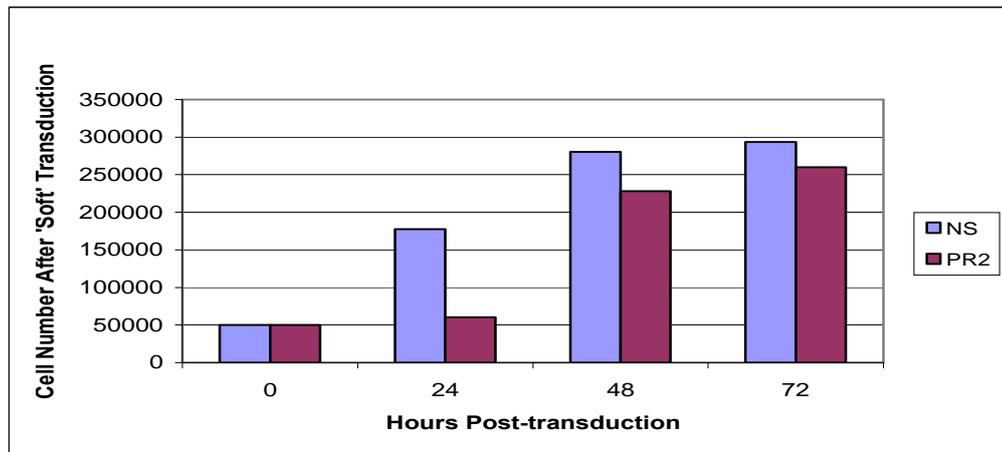
Figure 5.13 – Cells were transduced with NS or PR2 vectors without 'spinoculation', and cell proliferation assayed at 24 hour intervals. Proliferation of PR2 transduced cells is presented relative to the NS controls. This graph represents the average of four samples from a single experiment.

Figure 5.14 Effect of replenishing culture media
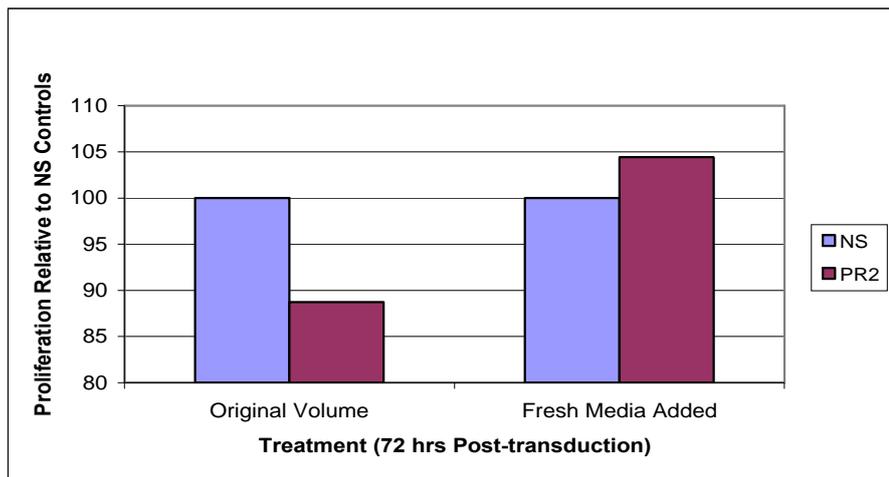
Figure 5.14 - Cells were transduced with NS or PR2 vectors without 'spinoculation', then cultured. An equal volume of fresh culture medium was added to one sample ("fresh") at 48 hours, and cell proliferation was assayed at 72 hours. This graph represents the average of four samples from a single experiment.

5.3.3.4 Threshold effect

From the results thus far, it was apparent that a subpopulation cells transduced with the PR2 vectors retained the ability to proliferate. Vector copy number is known to influence the efficiency of RNA interference triggered by shRNAs (Scherr *et al.* 2005). To determine the relative vector copy number in the proliferating PR2 transduced cells, levels of expression of the GFP marker were compared with the NS controls in each cell population were examined. Purified populations of sNS and sPR2 cells, and 2NS and 2PR2 cells (see above), were cultured for 48 hours, and the mean fluorescence intensity of each population was measured by flow cytometry (Figure 5.15 and Table 5.1). Regardless of the vector backbone and the method of purification, cells transduced with the PR2 shRNA had lower levels of GFP expression than the NS controls. These results suggest that the cells surviving after PR2 transduction were those cells in which expression of the shRNA was lower. By contrast, transduced cells were able to tolerate higher levels of NS shRNA expression. These findings confirm the suggestion that cells transduced with higher copy numbers of the PR2 vector were experiencing greater inhibition of proliferation, whereas cells with lower vector copy numbers were able to proliferate at a rate similar to the controls.

Table 5.1 Fluorescence intensity of purified transduced cells

Mean Fluorescent Intensity	FACS-sorted (pHIV-sh-GFP vectors)	Puromycin selected (12.5 µg/ml Puro., pPIG-2sh-LV vectors)
NS	28.4	46.7
PR2	16.1	23.8

Table 5.1 - NB4 cells were transduced with the indicated vectors and were purified as appropriate at 48 hours. After a further 48 hours culture, mean fluorescence intensity was measured by flow cytometry. Units are arbitrary fluorescence units.

5.3.3.5 Summary

While transduction with PR2 shRNAs inhibited proliferation or triggered apoptosis in some cells, other transduced cells were able to continue proliferating. The difference between these subpopulations was evidently related to the level of PR2 shRNA expression.

Figure 5.15 Fluorescence of purified transduced cells
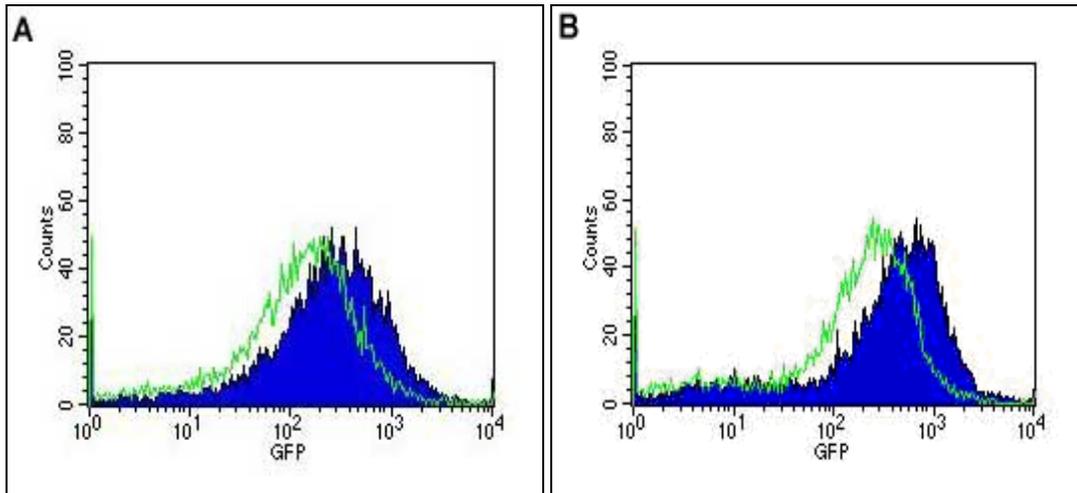
Figure 5.15 - NB4 cells were transduced with the pHIV-sh-GFP (A) or pPIG-2sh-LV (B) vectors, and purified as appropriate (by cell sorting or 12.5 ng/ml puromycin, respectively). After a further 48 hours culture, intensity of green fluorescence was measured by flow cytometry. In each experiment, levels of GFP expression were lower in cells transduced with the PR2 shRNAs (green overlay) compared with the NS controls (blue histogram).

5.3.4 Sensitivity to Retinoic Acid

Despite the ongoing proliferation of PR2 transduced cells, the shRNA still down-regulated levels of the PML-RARα mRNA and protein. Besides the effects on proliferation and apoptosis, the PML-RARα fusion gene is known to mediate the differentiation signalling of retinoic acid. The fusion gene desensitises cells to retinoic acid, necessitating a higher, 'pharmacological' dose for the same effect (see Introduction). To examine the effect of the PR2 shRNA on sensitivity of the NB4 cells to retinoic acid, purified cells were treated with retinoic acid. A consequence of retinoic acid induced differentiation is a decrease in cell proliferation, and therefore preliminary trials examined the combined effects of the PR2 shRNA and retinoic acid on NB4 cell proliferation.

In order to determine the effective dose of ATRA on the cells used in this study, non-transduced NB4 cells were treated at a range of concentrations for 72 hours, and cell proliferation was measured. At lower doses, ATRA stimulated cell proliferation, however, cell proliferation was inhibited at 50 nmol/l (Figure 5.16). This was taken as the minimum dose required to trigger differentiation under these conditions.

Accordingly, purified populations of sNS, sPR2, 2NS and 2PR2 transduced cells were treated with 50 nmol/l ATRA, and cell proliferation was measured after 72 hours of culture. The results show that cells transduced with the NS shRNAs proliferated at a rate comparable with the non-transduced controls (Figure 5.17). By contrast, proliferation of cells transduced with the PR2 shRNAs was strongly inhibited by the ATRA treatment, regardless of the vector backbone and the method of purification. This inhibition was significant with both vector backbones (paired t-tests, p=0.045 and p=0.010 for the sPR2 and 2PR2 vectors, respectively). When proliferation over time was examined (Figure 5.18), the number of both the NS controls and the non-transduced cells increased at least four fold over the course of the trial. By contrast, the population of cells transduced with the PR2 vectors approximately doubled after 72 hours of ATRA treatment. These results clearly indicate that the PR2 shRNAs sensitised the cells to the effects of ATRA.

To determine whether the inhibitory affect on proliferation was a result of cell differentiation in response to the retinoic acid treatment, purified cells were treated with retinoic acid for 72 hours, labelled with myeloid differentiation markers, CD11b and

Figure 5.16 Effect of ATRA on NB4 cell proliferation
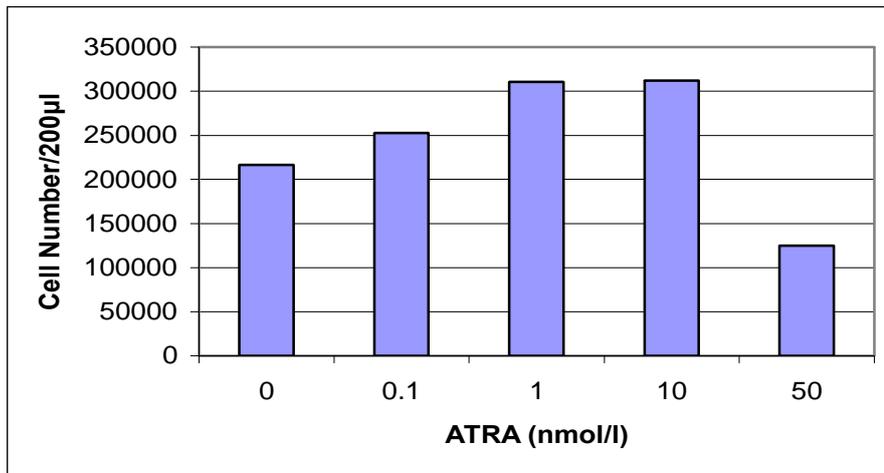
Figure 5.16 – NB4 cells were treated with increasing doses of ATRA and cell proliferation was measured by CellTitre assay at 72 hours. This graph represents total cell number from a single trial.

Figure 5.17 Sensitivity of PR2 transduced tells to ATRA treatment
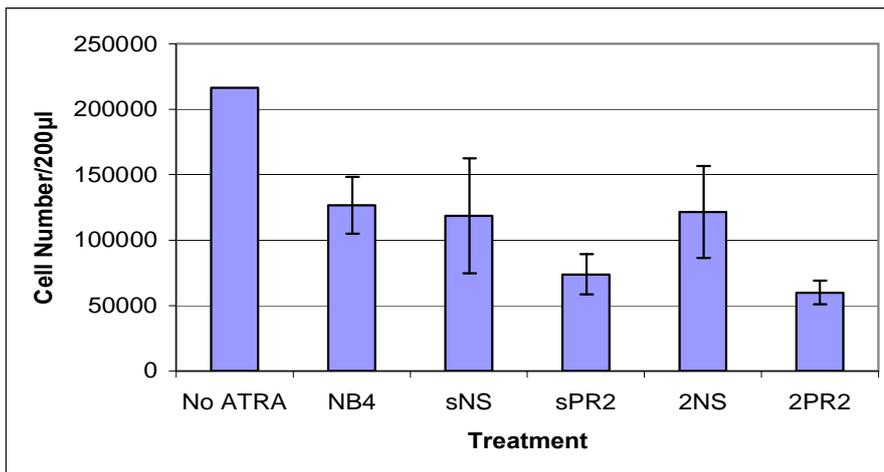
Figure 5.17 – NB4 were transduced as indicated ('NB4' is a non-transduced control) and purified as appropriate, then cultured for 48 hours. Purified cells were treated with 5×10^{-8} mol/l ATRA and cell proliferation measured by CellTitre assay 72 hours later. The differences between sPR2 and sNS, and between 2NS and 2PR2, were significant ($p < 0.05$). This graph represents total cell number, and presents the averages of four replicates. The "No ATRA" sample was a single experiment, conducted with the first of the four replicates, and is included as a baseline for NB4 cell proliferation in the absence of ATRA.

Figure 5.18 Proliferation of purified transduced NB4 cells after 72 hours ATRA treatment
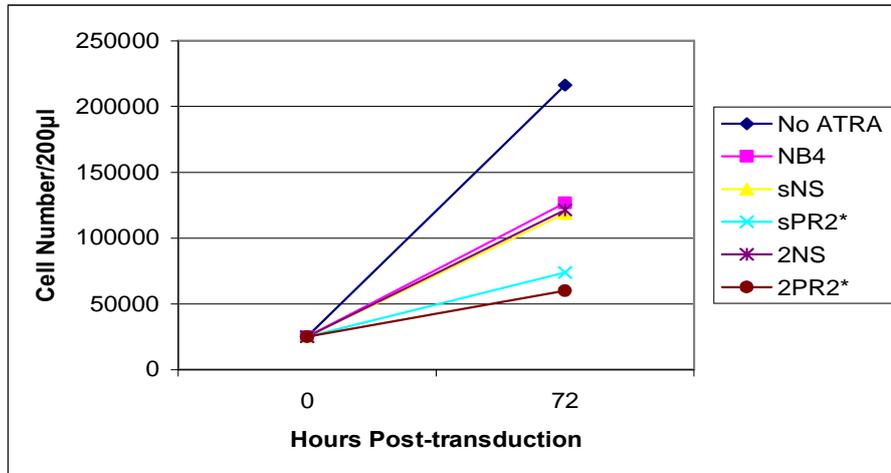
Figure 5.18 – NB4 were transduced as indicated ('NB4' is a non-transduced control) and purified as appropriate, then cultured for 48 hours. Purified cells were treated with $5x10^{-8}$ mol/l ATRA and cell proliferation measured by CellTitre assay 72 hours later. The differences between sPR2 and sNS, and between 2NS and 2PR2, were significant ($p < 0.05$). This graph presents average proliferation relative to the starting cell number ('0 hours') from four replicates. The "No ATRA" sample was a single experiment, conducted with the first of the four replicates, and is included as a baseline for NB4 cell proliferation in the absence of ATRA.

Figure 5.19 Labelling of sorted transduced NB4 cells for myeloid differentiation markers after 72 hours ATRA treatment
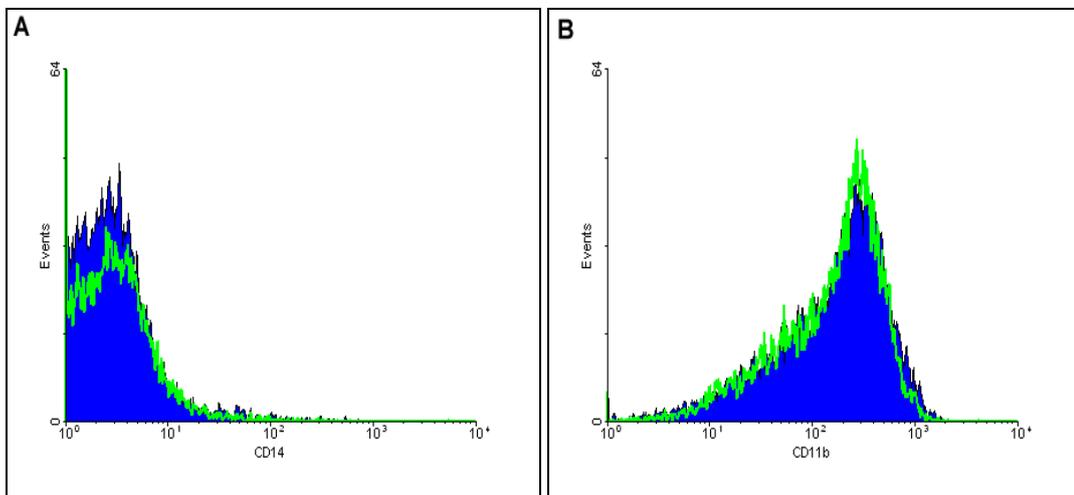
Figure 5.19 - NB4 cells were transduced with sNS (blue histogram) or sPR2 (green overlay) vectors, purified by cell sorting, then cultured for 48 hours. Cells were then treated with $5x10^{-8}$ mol/l ATRA, and cultured for another 72 hours. Finally cells were labelled with myeloid differentiation markers CD14 (A) or CD11b (B), then analysed by flow cytometry.

CD14, and then analysed by flow cytometry. This analysis showed that the retinoic acid treatment had very little effect on myeloid differentiation (Figures 5.19 and 5.20).

5.3.5 Conclusions

In conclusion, the lentiviral delivery of shRNA targeted against the PML-RARα fusion mRNA proved effective in down-regulating the copy number of the mRNA and levels of the fusion protein. The effects of this down-regulation were dose dependent, with higher doses resulting in apoptosis and/or inhibition of cell proliferation, while lower doses allowed continued proliferation of transduced cells, whilst sensitising them to the effects of ATRA.

Figure 5.20 Labelling of transduced, puromycin selected NB4 cells for myeloid differentiation markers after 72 hours ATRA treatment
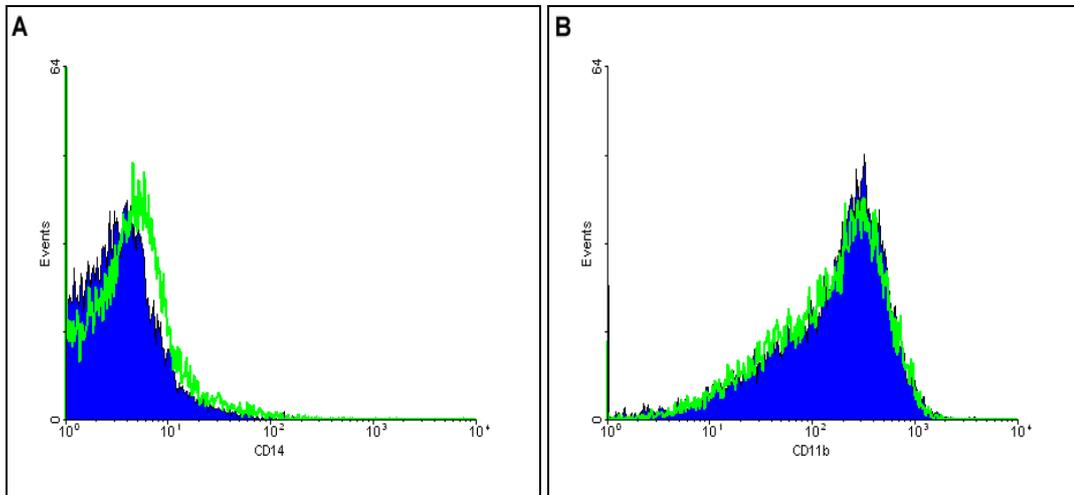
Figure 5.20 - NB4 cells were transduced with 2NS (blue histogram) or 2PR2 (green overlay) vectors, selected with 12.5 ng/ml puromycin, then cultured for 48 hours. Cells were then treated with 5×10^{-8} mol/l ATRA, and cultured for another 72 hours. Finally cells were labelled with myeloid differentiation markers CD14 (A) or CD11b (B), then analysed by flow cytometry.

5.4 Discussion

The PML-RARα fusion gene occurring in the NB4 cell line interferes with retinoic acid signalling (Huang *et al.* 1988; Warrell *et al.* 1991) and is a causative agent in many acute promyelocytic leukaemias (Jansen *et al.* 1995). As a consequence of the desensitisation of cells to retinoic acid caused by the PML-RARα fusion protein, current therapies commonly involve use of higher, pharmacological doses of retinoic acid (Warrell 1993).

Molecular strategies, including hammerhead ribozymes, antisense oligonucleotides and shRNAs, have been used to target and down-regulate PML-RARα mRNA (Nason-Burchenal *et al.* 1998b; Chen *et al.* 1999b; Oshima *et al.* 2003). The antisense oligonucleotides lead to apoptosis, growth inhibition and differentiation (Chen *et al.* 1999b) while the ribozymes affected apoptosis and proliferation only (Nason-Burchenal *et al.* 1998b). These aspects were not examined in the study involving shRNA (Oshima *et al.* 2003). In these previous studies, each molecular strategy was delivered transiently. However, shRNA molecules may also be delivered via integrating vectors, allowing stable transduction and presenting the possibility of stable therapy. Accordingly, the effects of the shRNA on apoptosis and proliferation, and the sensitivity of transduced cells to retinoic acid, require exploration. Furthermore, the effects and consequences of stable delivery of these shRNAs also need examination.

To address these questions, two shRNAs were designed to trigger RNA interference against the PML-RARα mRNA following delivery of the shRNAs via lentiviral vectors. The first shRNA design, 'PR1', was derived *de novo* from an siRNA design algorithm. The second design, 'PR2', was derived from a 30 bp shRNA used by Oshima *et al.* (2003) but reduced to 21 bp in accordance with optimal shRNA design criteria (Elbashir *et al.* 2001b). Results from this chapter demonstrated that the PR1 design did not down-regulate the target PML-RARα mRNA. Instead, cells treated with this shRNA had higher levels of PML-RARα mRNA than cells receiving the NS control shRNA. This apparent increase was unexpected, but might be explained by a mild immunostimulatory effect of the NS shRNA design. This possibility is discussed further below. By contrast, levels of PML-RARα mRNA were consistently and

significantly lower in cells treated with the PR2 design compared with cells treated with the NS shRNA.

Results from Western blots confirmed that the PR2-induced down-regulation of PML-RARα mRNA was accompanied by a reduction in levels of the PML-RARα protein. Protein down-regulation was evident 24 hours after mRNA down-regulation, which was consistent with the half-life of the PML-RARα protein (Grignani *et al.* 1999). Having demonstrated down-regulation of the PML-RARα protein, the downstream biological consequences were also examined.

The changes induced by the PR2 shRNA resulted in an increased number of dead cells at the early time points. This was consistent with increased apoptosis following targeting of the PML-RARα with hammerhead ribozymes and antisense oligonucleotides (Nason-Burchenal *et al.* 1998b; Chen *et al.* 1999b). Furthermore, analysis of cell cycling and differentiation indicated that the shRNA affected cell survival directly, without effecting these parameters. As such, the shRNA results described in this chapter closely mirror those described with hammerhead ribozymes (Nason-Burchenal *et al.* 1998b). By contrast, antisense oligonucleotides reportedly triggered apoptosis in combination with growth inhibition, although details were unavailable (Chen *et al.* 1999b).

Prolonged culture of the cells and supplementation with fresh culture medium resulted in some recovery of the PR2 transduced population. The timing of the wave of apoptosis suggested that it may have resulted from sensitisation of the cells by PR2 shRNA expression to the stress associated with the spinoculation transduction method. Experiments addressing this question demonstrated that the wave of apoptosis was also evident where cells were transduced without spinoculation

The recovering cell population also included non-transduced cells. Proliferation of transduced cells was therefore confirmed by eliminating non-transduced cells, by one of two methods. Positive selection by fluorescence activated cell sorting and puromycin selection were both effective in producing a purified population of transduced cells, and both purified populations continued to proliferate.

Finally, suppression of shRNA expression in the proliferating PR2-transduced cells was refuted by the persistent downregulation of PML-RARα mRNA levels, as measured by qRT-PCR, indicating that the PR2 shRNAs remained active. There was,

however, evidence for selection of cells bearing lower PR2 shRNA cassette copy numbers. The activity and efficacy of shRNAs expressed from lentiviral vectors is dependent upon vector copy number and thus the number of shRNAs copies present in the cell (Scherr *et al.* 2005). Vector copy number in transduced cells was interpreted by flow cytometric analysis of GFP expression, which revealed that the fluorescent intensity of the proliferating PR2 transduced cells was lower than for the comparable NS transduced controls, and this was true of both vector types. These results indicate that proliferating PR2 transduced cells have a lower vector copy number and consequently a lower copy number of the PR2 shRNA cassette. As the vectors were otherwise identical, and initial rates of transduction must be assumed to be the same, it must therefore be concluded that cells with a higher copy number of the PR2 vector were eliminated from the population. These results point to a threshold of PR2 shRNA expression; below which, cells are able to proliferate at a rate similar to the controls, while shRNA expression above the threshold triggers apoptosis.

Aside from its anti-apoptotic role the PML-RARα fusion protein also interferes with retinoic acid signalling due to the formation of RARα homodimers or oligodimers (Lin *et al.* 2001a) that enhance transcriptional repression at physiological concentrations of RA (Perez *et al.* 1993; Dong *et al.* 1996; Minucci *et al.* 2001).

Down-regulation of the PML-RARα fusion protein using hammerhead ribozymes has been shown to increase the sensitivity of the cells to ATRA (Nason-Burchenal *et al.* 1998a) and consequently the effects of the PR2 shRNA on retinoic acid signalling was also investigated.

If it could be shown that the PR2 shRNA, aside from triggering proliferation, was able to sensitise surviving cells to retinoic acid signalling, the shRNA would also have potential in differentiation therapy. Therefore, the effect of the PR2 shRNA on ATRA sensitivity of transduced cells was also examined.

Retinoic acid has been shown to affect a number of pathways in myeloid cells, resulting in differentiation of such cells combined with a decrease in cell proliferation (Otsuki *et al.* 2003; Jasek *et al.* 2008). When PML-RARα mRNA was targeted with hammerhead ribozymes and then the cells treated with ATRA, cell proliferation was inhibited, but this was the result of apoptosis, without any effect on the differentiation of the myeloid cells (Nason-Burchenal *et al.* 1998a). In the present study, both the

proliferation and differentiation of transduced cells treated with ATRA were examined. Consequently in this chapter, the effects of ATRA were initially examined with cell proliferation assays. These assays demonstrated that PR2-transduced cells, while able to survive and proliferate despite reduced levels of the PML-RARα mRNA, were more sensitive to the inhibitory effects of ATRA on cell proliferation, although differentiation was not affected. Therefore, these results again agree with studies targeting the PML-RARα mRNA with hammerhead ribozymes.

With regard to the therapeutic potential of the shRNA strategy, the results from this chapter have identified an shRNA design that is effective against the PML-RARα fusion mRNA, highlighting the therapeutic potential of these molecules. The beneficial effects of these molecules on activity of the fusion gene was dose dependent, and specific to the target gene only. In triggering apoptosis of the cells, or sensitising them to the pro-apoptotic effects of retinoic acid, the anti-PML-RARα shRNA was able to break the proliferative cycle of these leukaemic cells.

As regards the therapeutic delivery of these molecules, the results from this chapter indicated that the inclusion of selectable marker genes in lentiviral vectors provided an efficient and stringent means for positive selection of transduced cells, resulting in a pure population of transduced cells suitable for transplantation. The activity of the PR2 shRNA, combined with efficient selection of transduced cells, therefore, offers the ability to restore normal functioning of the haematopoietic stem cells, while eliminating the risk of cancerous growth, and thus, in combination with bone marrow ablation and autologous transplantation, provides an efficient method for replacing and reconstituting the myeloid cell population in PML patients.

Two technical points also arose from the results of this chapter. The results in this chapter hold implications for methods of the design of effective shRNAs. The PR2 shRNA was based on an empirical design, with the centre of the shRNA aligning with the centre of the target sequence. By contrast, the ineffective PR1 shRNA was designed according to the criteria of an siRNA design algorithm. Therefore, in this case, reliance on contemporary siRNA design algorithms alone would not have identified any effective shRNAs. These results also support the need for more advanced design algorithms that are appropriate for shRNAs. This is discussed further in the General Discussion (Chapter 8).

Another interesting result to emerge from this chapter relates to the selection and use of suitable controls in studies examining RNAi. Of the numerous forms of control recommended for studies examining RNAi ("Whither RNAi?", Nature Cell Biology (2003), v.5 (6) pp. 489-490), many were not applicable or not safe in the present study. The remaining strategies involved the use of a scrambled or 'nonsense' shRNA design, and the use of a control shRNA based on the active PR2 but with an inversion of the central nucleotides. This latter control was tested, however it proved to be inappropriate. Therefore the scrambled, 'nonsense' control was used throughout. A suitable 'nonsense' control will distinguish the specific effects of the experimental shRNA from non-specific effects, such as stimulation of non-specific innate immunity. One widely used control, designed to target GFP, has been criticised due to its unusually low immunostimulatory properties (Robbins *et al.* 2008). It was considered that use of this control would lead to non-specific effects of the experimental shRNA being attributed instead to sequence specific activity. In contrast to this, the nonsense control shRNA used in the present study may be superior to many alternate designs, as this nonsense design appeared to trigger some non-specific responses, such as upregulation of the OAS mRNA, part of the interferon pathway. This control shRNA may also have partially suppressed the super abundant expression of the PML-RARα mRNA, providing an explanation for the comparatively high levels of PML-RARα mRNA seen in PR1 transduced cells. If the nonsense shRNA was stimulating a low level non-specific response, it might consequently be considered as providing a more stringent test for the active shRNAs. That is, against the background of this non-specific response, any down-regulatory activity unique to the non-immunostimulatory PR2 shRNA must be considered to be specific. This interpretation was supported by the down stream effects of the PR2 shRNA, which were consistent with specific down-regulation of the PML-RARα fusion gene. Therefore, in cases where alternate controls are inappropriate or not applicable, it seems that use of this nonsense hairpin, combined with regular screening for expression of interferon stimulated genes, provides valid analysis of shRNA activity.

5.4.1 Summary

The PML-RARα translocation is a well defined genetic lesion responsible for many cases of APL. The results from this chapter demonstrate a number of points that form a path towards a potential therapy for this disease. Firstly, the experiments confirmed that the shRNA targeted to the breakpoint down-regulated expression of the target mRNA and inhibited proliferation of the cells in a dose-dependent manner. In cells where the level of shRNA expression was lower, cells were still able to proliferate, however sensitivity to retinoic acid signalling was restored, demonstrating multi-faceted effects of shRNA expression. The results also demonstrated that, using appropriate protocols, haematopoietic stem cells could be efficiently transduced with lentiviral gene therapy vectors. When such vectors are combined with puromycin growth selection, pure populations of transduced cells may rapidly be generated, minimising the need for excessive expansion of a small population of founder cells. Such purified populations of transduced cells would also be suitable for therapeutic transplantation and retransfusion.

6 Down-regulation of RUNX1-ETO by RNAi

6.1 Introduction

Acute myeloid leukaemias (AMLs) result from a range of chromosomal abnormalities including the RUNX1-ETO translocation. This fusion gene occurs in 10 to 15 % of AML cases, making it one of the most common translocations found in this disease (Rowley 1984). The fusion occurs between the RUNX1 (AML1) gene on chromosome 21, and the ETO (MTG, CBF2T1) gene on chromosome 8 (Miyoshi *et al.* 1993). This t(8;21)(q22;q22) fusion acts by changing the transcriptional activator, RUNX1, into a transcriptional repressor (reviewed in Elagib and Goldfarb 2007).

In vitro studies of AML have been facilitated by the establishment of a cell line from the peripheral blood of a young AML patient (Asou *et al.* 1991). This 'Kasumi' cell line carries the t(8;21)(q22;q22) fusion and has become an *in vitro* model for AML. The molecular fusion in this cell line has been sequenced and mapped to the same AML1 intron that is involved in all AML fusions (Miyoshi *et al.* 1993; Shiramizu *et al.* 1994).

Numerous attempts to interrupt the oncogenic capacity of Kasumi cells have been based on methods to directly reduce, or eliminate, the fusion gene mRNA. Hammerhead ribozymes and antisense oligonucleotides resulted in knockdown of the fusion protein and inhibition of cell growth (Kozu *et al.* 2000). siRNAs have also been used to target the RUNX1-ETO fusion gene, which selectively down-regulated the fusion mRNA, resulting in an increased sensitivity to differentiation agents, changes in cell shape and reduced clonogenicity of the cells (Heidenreich *et al.* 2003). The transient delivery of siRNA molecules was also sufficient to delay tumour formation *in vivo* (Martinez Soria *et al.* 2009) while repeated electroporation of the siRNAs eventually resulted in lower proliferation, which became apparent between days 4 and 7 of treatment (Martinez *et al.* 2004).

While the siRNAs are an effective means of down-regulating the RUNX1-ETO mRNA, delivery of these molecules with current methods is inefficient. Consequently siRNAs are not yet strong candidates for efficient cancer therapy. Lentiviral vector delivery of shRNAs has much greater transduction efficiency and one aim of this

chapter was to investigate the potential of shRNA molecules, delivered by lentiviral vectors, in down-regulating RUNX1-ETO mRNA. Furthermore, as Kasumi cells have to date proven difficult to transduce by conventional methods, and there are few, if any, reports of attempted retroviral or lentiviral transduction in this cell line, a concomitant aim for this chapter was to determine if modified lentiviral protocols can achieve efficient transduction of Kasumi cells.

6.2 Methods

6.2.1 Kasumi Cell Culture and Transduction

The Kasumi cells were cultured as per the General Materials and Methods (Section 2.1.2), with the following modifications to accommodate the characteristics of this cell line. The complete culture medium used for Kasumi culture contained 20 % foetal calf serum, and L-glutamine was added fresh before use. Medium was made up fresh every two weeks to ensure that the L-glutamine remained fresh. The doubling time of Kasumi cells is approximately three days, and cell cultures were diluted to half when they reached $1\text{-}1.2\text{x}10^{6}$ cells/ml.

Transduction of Kasumi cells was performed as per the modified method developed and outlined in Chapter 3, with the following modifications. As Kasumi cells are susceptible to high centrifugation speeds (P. Oakford, pers. comm.), these cells were never centrifuged at speeds above 300 g. In addition, total culture volume during transductions was carefully manipulated such that the density of the cells was always within the range of $5\text{-}10\text{x}10^{5}$ cells/ml. To achieve this the 1 ml aliquots of harvested vector supernatant (see Section 3.3.3.1) were resuspended in 0.25 ml complete medium. This volume was applied to $5\text{x}10^{5}$ Kasumi cells in one well of a 24-well plate. Finally, because of the slow doubling time of Kasumi cells, samples for both the qRT-PCR and the cellular proliferation assays were collected at 72 and 96 hours.

6.2.2 Hairpin Design

The nucleotide sequence of the RUNX1-ETO chromosomal breakpoint found in Kasumi cells has been sequenced previously (Miyoshi *et al.* 1993) and is available online (accession number D13979). In order to include the unique fusion sequence in the hairpin design algorithms, the region of interest included the 20 nucleotides on each side of the chromosomal breakpoint (see Section 2.6.2). This corresponded to nucleotide's 20-60 of D13979 (ie. `GGGCCCCGA GAACCTCGAA/ATCGTACTGA GAAGCACTCC`). This sequence was entered into both the Ambion and GenScript algorithms (also see Section 2.6.2). The output sequences are shown 5' to 3', and the location of the chromosomal breakpoint is indicated with a forward slash.

The output target sequences using the Ambion algorithm were:

Target sequence 1

    5'-AACCTCGAA/ATCGTACTGAG-3'

Target sequence 2

    5'-AA/ATCGTACTGAGAAGCACT-3'

The target sequences output from the GenScript algorithm were:

Target sequence 1 ("AE1")

    5'-CCTCGAA/ATCGTACTGAGAAG-3'

Target sequence 2 ("AE2")

    5'-CGAGAACCTCGAA/ATCGTACT-3'

Target sequence 3 ("AE3")

    5'-ACCTCGAA/ATCGTACTGAGAA-3'

The Ambion algorithm produced two sequences from the region of interest. Of these, the second was excluded because the breakpoint was too near the end of the target sequence. The first Ambion sequence was also very similar to GenScript sequences 1 and 3, beginning 2 nucleotides and 1 nucleotide respectively, upstream. The GenScript target sequence 1 also corresponded exactly with the sense sequence of the siRNA published previously (Damm-Welk *et al.* 2003), and later validated by Heidenreich and colleagues (Heidenreich *et al.* 2003). Furthermore, the second Ambion design possessed strong homology with the normal ETO gene, as the breakpoint distant from the active site of the shRNA. Therefore the three GenScript designs were selected for testing as they provided the widest range and included the design homologous to the validated design. Initial testing was performed with the AE1 hairpin because of the strong agreement with the validated sequence of Heidenreich *et al.* (2003). The other two GenScript sequences were subsequently also assembled and cloned into the lentiviral vectors. The shRNA designs were compared using the pHIV-7-GFPshr lentiviral vector backbone (Section 3.2.4).

6.2.3 Observations of Cell Shape and Appearance

In order to detect changes that may indicate entry into the differentiation pathway, Kasumi cells were observed at 24 hour intervals, starting on the day of transduction. Cells were observed at high magnification with an inverted light microscope, and cell shape was recorded as circular, irregular, or cylindrical.

6.2.4 qRT-PCR

Quantitative real-time PCR's were performed as per the General Materials and Methods, except for the following. Initial analyses indicated that there was considerable variation between replicate samples normalised to the β-2-microglobulin house-keeping gene (see below). Consequently, GAPDH was also used as a house-keeping gene. Assays were performed in parallel, with four replicates available for the β-2-microglobulin house-keeping gene, but only three were available for the GAPDH assay (only two for the AE3 hairpin). The Plexor system was used exclusively in this study, in order to minimise additional variation caused by pipetting error. The Plexor primers for the β-2-microglobulin were as per Section 2.12.6, but the GAPDH primers were obtained from Promega and the sequence of these primers was not made available, although the length of the product was 64 bp. The forward primer was labelled with 5' CAL Fluor Red 610 fluorophore label.

The primers for the RUNX1-ETO fusion gene were as follows (5'-3'). The forward primer was labelled with a 5' 6-FAM fluorophore.

RUNX/ETO-ForP

    5'-GGAGGTGGCATTGTTGGAGGA-3'

RUNX/ETO-RevP

    5'-CCTACCACAGAGCCATCAAAATCAC-3'

6.2.5 Cell Proliferation Assays

MTS cell proliferation assays for the Kasumi cells were performed as per the General Materials and Methods, except that assays were undertaken at additional time points, at 24 hour intervals from 72 hours to 144 hours. Cell density at the start of culture was modified such that cell density remained within the optimal range for Kasumi cells.

6.3 Results

6.3.1 Delayed Transgene Expression

Preliminary observations with an inverted fluorescent microscope indicated that expression of the GFP marker transgene was minimal at 48 hours post-transduction, but maximal at 72 hours post-transduction.

6.3.2 qRT-PCR

The figures in this section (Figures 6.1 to 6.6) show RUNX1-ETO fusion gene mRNA copy number relative to GAPDH or β-2-microglobulin, in Kasumi cells transduced with vectors expressing a range of short hairpin RNA's. The cells were transduced with vectors expressing a nonsense (NS) control hairpin sequence, or one of three shRNAs (AE1, AE2 or AE3) designed to reduce the copy number of RUNX1-ETO mRNA. To examine the effect of the various shRNAs on RUNX1-ETO mRNA copy number RNA samples were collected at 72 and 96 hours. The RUNX1-ETO mRNA copy numbers were measured by Plexor qRT-PCR and normalised against both the β-2-microglobulin (β2m) or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) house-keeping genes. Relative copy number of RUNX1-ETO mRNA from multiple replicates was compared between cells treated with the NS control and cells treated with each of the three anti-RUNX1-ETO hairpin designs.

6.3.2.1 AE1 Hairpin

The average relative copy number of RUNX1-ETO mRNA from four replicates of cells treated with the AE1 hairpin, and normalised to the β-2-microglobulin house-keeping gene, is shown in Figure 6.1A. At 72 hours after transduction, RUNX1-ETO mRNA levels in AE1-treated cells were more than 40 times greater (4345%) than levels found in NS-treated cells. By 96 hours after transduction, relative RUNX1-ETO copy number in the AE1 treated cells was approximately 1/8th (17%) of the level found in NS treated cells. It should be noted that relative RUNX1-ETO mRNA copy number in the NS treated controls was much higher at 96 hours than at 72 hours. Furthermore standard deviation between replicates was high, and there was no significant difference

Figure 6.1 RUNX1-ETO mRNA Levels from AE1 treated Kasumi cells
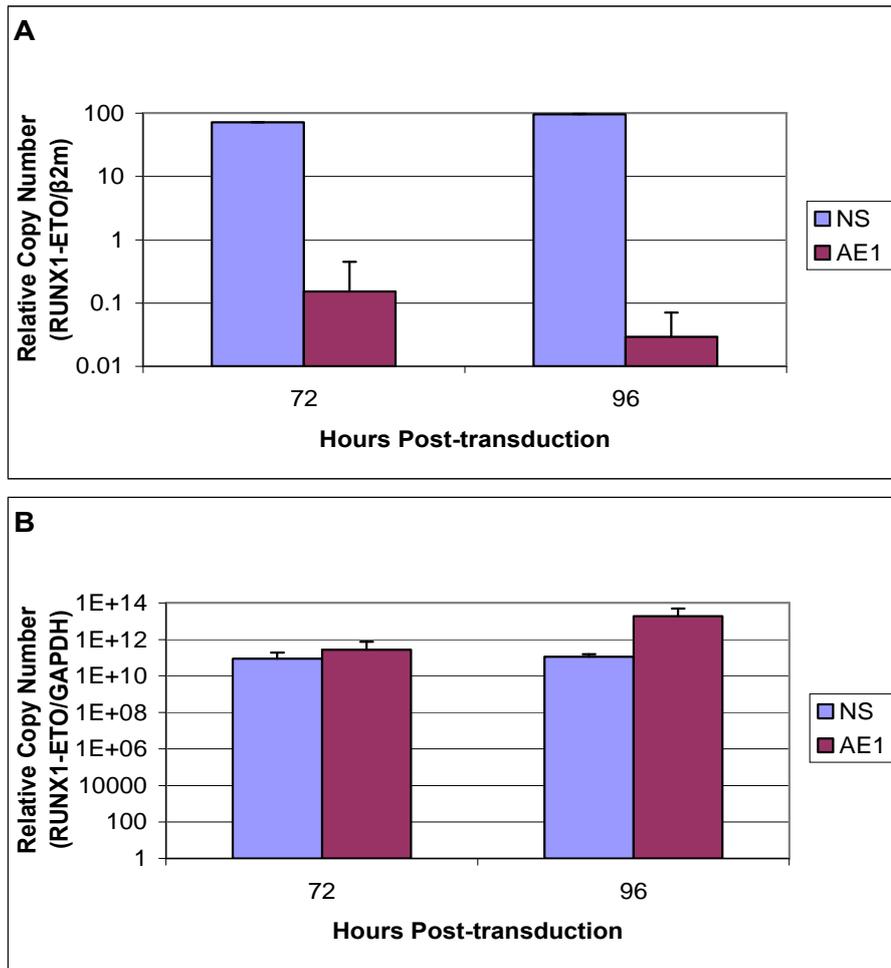
Figure 6.1 – Relative levels of RUNX1-ETO mRNA normalised to (A) the B2M and (B) the GAPDH house-keeping genes, comparing cells treated with AE1 or NS shRNAs, at 72 and 96 hours post-transduction. This graph presents, on a log scale, the mean ± standard deviation from four independent transductions for the β-2-microglobulin house-keeping genes, and three independent transductions for the GAPDH house-keeping gene. NS and AE1 samples were compared with a paired t-test.

between RUNX1-ETO mRNA levels in AE1 treated cells and controls (p = 0.190 and p = 0.163 at 72 and 96 hours respectively).

The qRT-PCR analysis of cells treated with the NS and AE1 vectors performed using GAPDH as the house-keeping gene is shown in Figure 6.1B. This assay was performed on the same RNA samples, although sufficient RNA was available from only three replicates. At 72 hours, RUNX1-ETO mRNA was three-fold higher (317%) in the AE1 treated cells than in the controls. At 96 hours, RUNX1-ETO mRNA was more than a thousand times higher (16523%) than the controls. Standard deviation between replicates was high, and no significant differences were present (p = 0.234 and p = 0.195 at 72 and 96 hours respectively).

Normalisation to the β-2-microglobulin house-keeping gene revealed a trend of down-regulation at the later time point, however there was high variation between samples and the results were not significant. Analysis of the remaining samples normalised to the GAPDH house-keeping gene contradicted this trend, suggesting a tendency to upregulation, however, there was high variation in these results also. These results did not provide any evidence for down-regulation of the RUNX1-ETO fusion mRNA by the AE1 hairpin.

6.3.2.2 AE2 Hairpin

When normalised to the β2m house-keeping gene, transduction with the AE2 hairpin resulted in a decrease in RUNX1-ETO relative copy number compared to NS transduced cells (Figure 6.2A). At 72 hours post-transduction, average relative copy number of the fusion mRNA in AE2 treated cells was approximately two orders of magnitude less (1%) than in NS transduced cells. At 96 hours, there was an overall increase in RUNX1-ETO relative copy number in both the control and AE2 treated samples compared with the 72 hour samples. Nevertheless, at 96 hours the RUNX1-ETO mRNA relative copy number in AE2 treated cells was little over half (52%) of the relative copy number in NS treated cells. There was a high standard deviation between replicates, especially in the 96 hours samples (Figure 6.2A). The differences between treatments were not significant at either 72 or 96 hours (p = 0.197 and p = 0.293 respectively).

Figure 6.2 RUNX1-ETO mRNA Levels from AE2 treated Kasumi cells
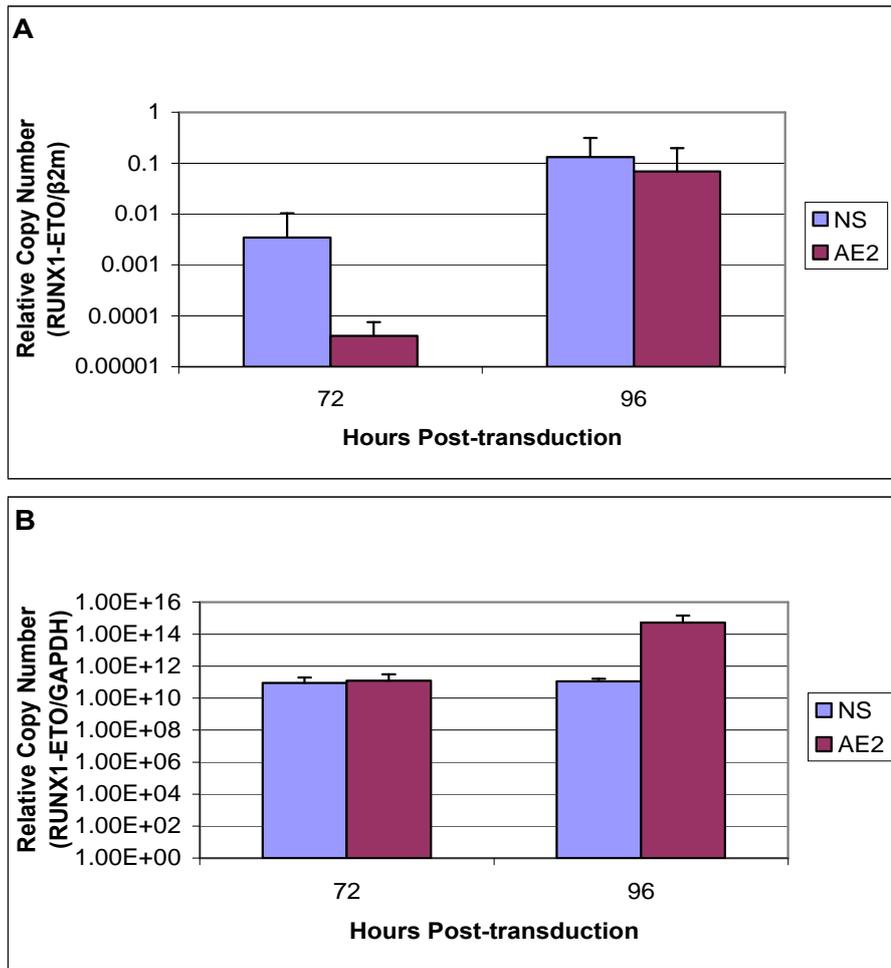
Figure 6.2 – Relative levels of RUNX1-ETO mRNA normalised to (A) the B2M and (B) the GAPDH house-keeping genes, comparing cells treated with NS or AE2 shRNAs, at 72 and 96 hours post-transduction. This graph presents, on a log scale, the mean ± standard deviation from four independent transductions for the β-2-microglobulin house-keeping genes, and three independent transductions for the GAPDH house-keeping gene. NS and AE2 samples were compared with a paired t-test.

The RNA collected from three replicates of NS vs AE2 treated cells was available for reanalysis using GAPDH as the house-keeping gene (Figure 6.2B). Use of this house-keeping gene produced results contrasting with those obtained using β2m as the house-keeping gene, despite use of the same RNA. Normalisation to the GAPDH house-keeping gene indicated a greater relative copy number of RUNX1-ETO mRNA in AE2 treated cells than in the NS treated controls at both 72 and 96 hours post-transduction. At 72 hours, RUNX1-ETO mRNA relative copy number in AE2 treated cells was greater than the relative copy number in controls by almost a half (140%). At 96 hours, the increase in RUNX1-ETO mRNA relative copy number was greater by many orders of magnitude (461862%). Despite this, standard deviation was high and the differences were not significant (p = 0.403 and p = 0.206 respectively).

The results obtained using the β2m house-keeping gene indicated down-regulation of the RUNX1-ETO target mRNA by the AE2 hairpin compared to the NS controls, however there was high variation between samples and this trend was not significant. Normalisation of the remaining samples to the GAPDH house-keeping gene contradicted this trend, suggesting a tendency of upregulation, however there was high variation in these results also. Therefore these results did not provide any evidence for down-regulation of the RUNX1-ETO fusion mRNA by the AE2 hairpin.

6.3.2.3 AE3 Hairpin

The AE3 hairpin appeared to increase the relative copy number of the RUNX1-ETO mRNA, with normalisation of samples against the β2m house-keeping gene (Figure 6.3A) indicating that the relative copy number of the RUNX1-ETO fusion gene at 72 hours was fifty times higher (5221%) in the AE3 treated cells than in the controls. At 96 hours, the relative copy number of the fusion gene was again higher in AE3 treated cells, although the difference was less (243%). Standard deviation was high, especially in the AE3 treated, 72 hour sample (Figure 6.3A), and there were no significant differences between hairpin treatments at 72 or 96 hours (p = 0.094 and p = 0.220 respectively).

Only two RNA samples from AE3 treated cells were available for reanalysis with the GAPDH house-keeping gene. Normalisation to this gene also indicated an increase in RUNX1-ETO mRNA following treatment with the AE3 hairpin, compared

Figure 6.3 RUNX1-ETO mRNA Levels from AE3 treated Kasumi cells

A

Relative Copy Number (RUNX1-ETO/β2m)

1

0.1

0.01

0.001

0.0001

NS
AE3

72   96

Hours Post-transduction

B

Relative Copy Number (RUNX1-ETO/GAPDH)

1.00E-07

1.00E-08

NS
AE3

72   96

Hours Post-transduction
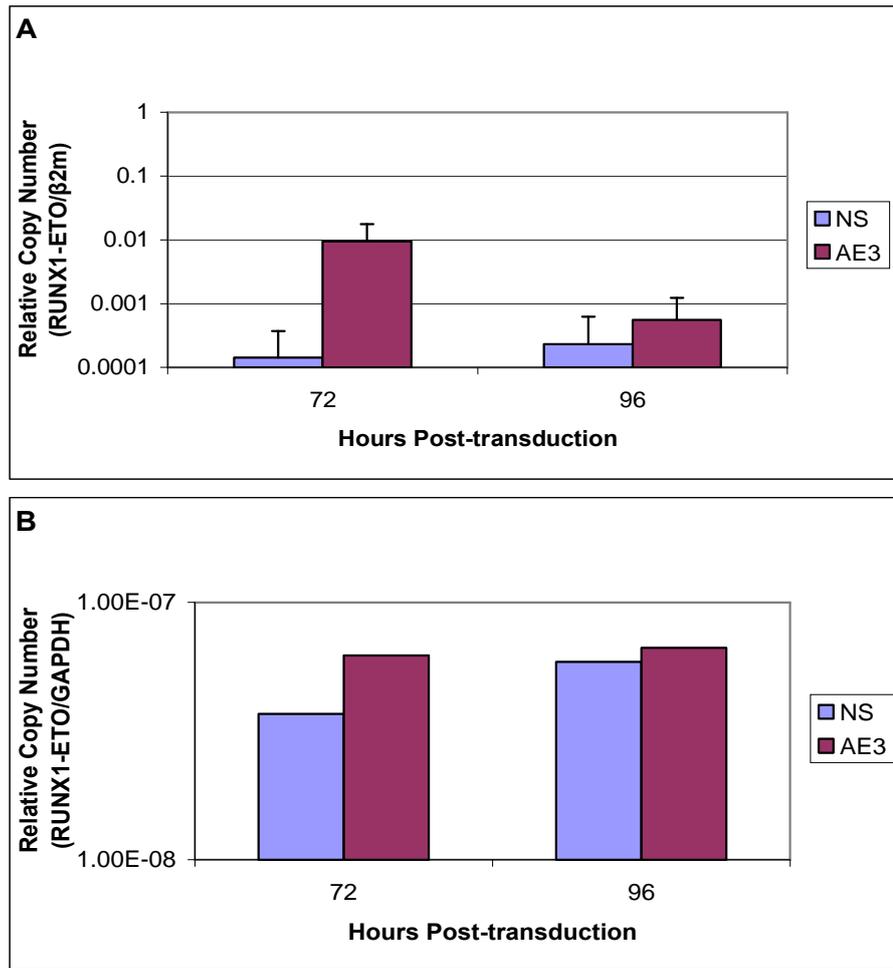
Figure 6.3 – Relative levels of RUNX1-ETO mRNA normalised to (A) the B2M and (B) the GAPDH house-keeping genes, comparing cells treated with NS or AE2 shRNAs, at 72 and 96 hours post-transduction. This graph presents, on a log scale, the mean ± standard deviation from four independent transductions for the β-2-microglobulin house-keeping genes, and two independent transductions for the GAPDH house-keeping gene. NS and AE3 samples normalised to the β-2-microglobulin house-keeping gene were compared with a paired t-test.

to the NS control. Relative copy number of the fusion gene at 72 hours post-transduction was higher by 2/3rds (168%) in the AE3 treated cells than in the controls (Figure 6.3B). At 96 hours, fusion gene levels in the AE3 treated cells were a little over 1/10th higher (113%) than in the controls. Due to the low sample size, statistical analyses were not performed.

Both analyses of RNA from AE3 treated cells indicated an upregulation of RUNX1-ETO fusion gene relative copy number, although this upregulation was not significant.

6.3.2.4 Summary of qRT-PCR Results

The qRT-PCR assays did not provide any evidence of a knockdown effect of the shRNAs designed to target the RUNX1-ETO fusion gene mRNA. The relative copy number of the fusion gene in each sample differed depending upon which house-keeping gene was used in the analysis. Furthermore, there was high variation in relative copy number between replicates of any given treatment.

6.3.3 Cell Proliferation assays

Because elimination of the RUNX1-ETO fusion gene and protein has been shown to have an inhibitory effect on Kasumi cell proliferation, cell proliferation assays were performed following transduction with the lentiviral vectors expressing the NS, AE1, AE2 or AE3 shRNAs. A range of time points was examined, beginning at 72 hours post-transduction, with the latest time point examined being 144 hours post-transduction.

Two assays at 72 hours (Figure 6.4) indicated that there was a minor increase in cell proliferation following treatment with the AE2 and AE3 shRNAs compared with the NS treated controls. Treatment with the AE1 hairpin had no apparent effect at this time point. Due to the low sample size, statistical analysis was not performed.

Four replicates were obtained for the 96 hour time point, as this period was considered most likely to demonstrate any inhibitory effect, given the proliferation rate of the Kasumi cells. These assays again indicated enhanced proliferation in the AE2 and AE3 treated cells, with no real effect of the AE1 hairpin on Kasumi proliferation (Figure 6.5). Statistical analysis indicates that the increase in proliferation of the AE3 treated cells was significant ($p = 0.009$).

Figure 6.4 Relative cell growth of Kasumi cells treated with AE1, AE2 or AE3 shRNAs
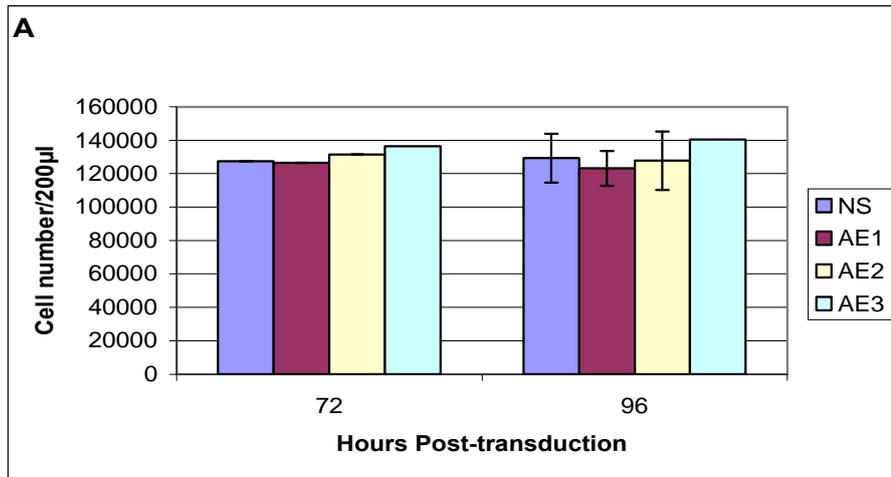
Figure 6.4 – Average cell number at 72 and 96 hours from two separate experiments following transduction with vectors expressing the NS, AE1, AE2 or AE3 shRNAs (n=2 except NS, AE1 and AE2 samples at 96 hours, n=4).

Figure 6.5 Relative cell growth of Kasumi cells treated with AE1 or AE2 shRNAs
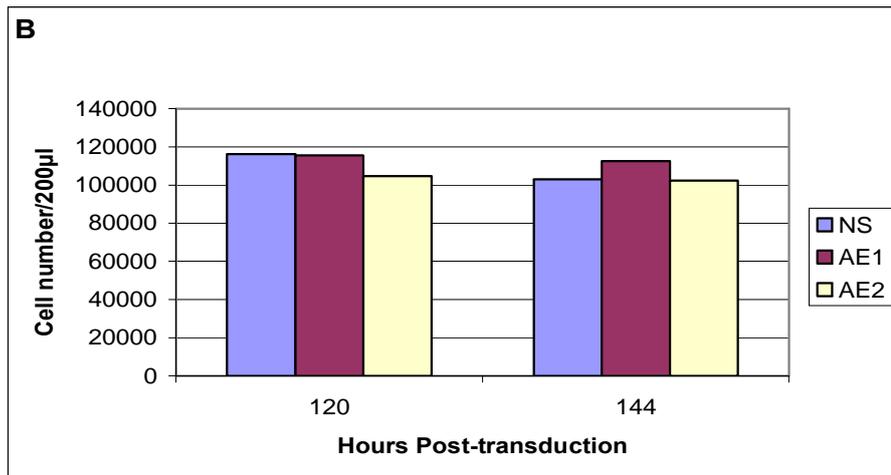
Figure 6.5 – Average cell number at 120 and 144 hours following transduction with vectors expressing the NS, AE1 or AE2 shRNAs. This graph represents a single replicate.

While 96 hours was considered optimal to observe any effects on cell proliferation, a single trial with the AE1 and AE2 shRNAs assessing cell proliferation to 196 hours was performed to examine any delayed effects from these shRNAs. While the number of cells transduced with the AE2 hairpin at 120 hours was lower than for the NS transduced controls, cell number in the control sample at 144 hours was very similar to the AE2 sample, indicating that the difference observed at 120 hours was not biologically significant.

For the cell proliferation assays, variation between replicates was low and the trends for each hairpin were consistent between time points, suggesting the assays themselves are reliable. From these assays it was concluded that there was no inhibitory effect on cell growth by any of the shRNAs designed to target the RUNX1-ETO fusion mRNA.

6.3.4 Appearance of Cells

As irregular cell shape can indicate entry of Kasumi cells into the differentiation pathway, regular observations of cellular appearance were made by light microscopy. From the first observation on the day of treatment up until the last time points examined (ie. 144 hours), all cells treated with the shRNAs remained regular in shape, indicating that none of the shRNAs were inducing differentiation.

6.4 Discussion

The RUNX1-ETO fusion gene found in Kasumi cells is responsible for many cases of acute myeloid leukaemia. Hammerhead ribozymes (Kozu *et al.* 2000; Szyrach *et al.* 2001) and siRNAs (Heidenreich *et al.* 2003) have been used to down-regulate the fusion mRNA, resulting in down-regulation of the protein, and impaired clonogenicity of Kasumi cells, although effects on cell proliferation were somewhat delayed (Martinez *et al.* 2004). Effective delivery of these molecules, however, has so far been inefficient, and one aim of this chapter was to enhance the delivery efficiency of effective gene therapy using lentiviral vectors. Transduction of Kasumi cells has in the past proved a challenge to delivery of therapeutics *in vitro*.

As has been outlined in Chapter 3, the modified lentiviral vectors and transduction techniques developed and used in this thesis reliably achieved high rates of transduction in Kasumi cells. To date there have been few if any reports of retroviral transduction of Kasumi cells, and this is the first report of successful transduction of these cells with lentiviral vectors. This strategy, combined with optional positive selection of transduced cells (see Chapter 3), provides a basis for effective and stable gene therapy of Kasumi cells and similar cell types. These results open the way for further investigations using compatible methodologies, such as hammerhead ribozymes and RNAi. The implications of these results extend beyond this, however, as many primary or patient-derived cell types have proven difficult to transduce with existing vector types, including lentiviral vectors. The methods and vectors developed here may therefore have application across a wide range of gene therapy protocols. This is discussed further in the General Discussion (Chapter 8).

Adapting RNAi molecules to delivery by lentiviral vectors required the identification of an shRNA molecule that was effective against the RUNX1-ETO fusion of Kasumi cells. A series of three different shRNAs molecules was designed using siRNA design algorithms, then cloned into lentiviral vectors for delivery and expression. Of the three shRNAs tested here, one bore homology with the effective siRNA design of Heidenreich *et al.* (2003), while the other two were novel designs. To test these designs, the shRNAs were delivered by lentiviral vectors, and the effects on levels of the RUNX1-ETO mRNA assessed by qRT-PCR.

The β-2-microglobulin house-keeping gene was used for normalisation as it was found to be one of the most stable in a comparison of commonly used house-keeping genes (Schmittgen and Zakrajsek 2000). When RUNX1-ETO mRNA levels were normalised to β-2-microglobulin, however, there was high variability between replicates. Consequently, samples were reanalysed and normalised against another commonly used house-keeping gene, GAPDH. This gene was selected as it was used previously to study RNAi against RUNX1-ETO (Heidenreich *et al.* 2003), however results normalised to GAPDH were also highly variable. Therefore the qRT-PCR results provided no evidence of a down-regulatory effect by any of the three shRNAs on RUNX1-ETO mRNA levels.

Despite widespread use as a house-keeping gene, GAPDH has been reported to be susceptible to perturbation by a range of factors (Schmittgen and Zakrajsek 2000), and qRT-PCR studies of RUNX1-ETO levels in Kasumi cells have previously proved unreliable when normalised against GAPDH (Krauter *et al.* 1999). While there have been no such reports in relation to β-2-microglobulin, problems with quantification of mRNA in the Kasumi cell line are frequently encountered (A. Holloway, pers. comm.). As such, future studies with this cell line must start with the identification of reliable housekeeping genes. One candidate housekeeping gene that has proven reliable after stringent testing is c-Abl (Beillard *et al.* 2003), and it may be worth examining the reliability of this housekeeping gene in Kasumi cells.

As the qRT-PCR results were unreliable, cells were examined for likely downstream effects of down-regulation of the RUNX-/ETO fusion mRNA, specifically induction of cell differentiation and inhibition of cell proliferation. Specific targeting and down-regulation of the RUNX1-ETO mRNA by catalytic ribozymes and antisense RNA results in rapid inhibition of proliferation, with changes in viable cell number evident within 48 hours (Kozu *et al.* 2000). Considering the expected delay in shRNA expression following vector delivery, changes in viable cell number might therefore be expected as soon as 72 or 96 hours. Despite examination of cell numbers from 72 hours to 144 hours, however, there was no evidence of any inhibitory effect of the shRNAs on Kasumi cell proliferation. Similarly there was no evidence of differentiation in the transduced cell populations. It should be noted, however, that when RUNX1-ETO mRNA was targeted by siRNAs, the inhibitory effect was delayed by comparison with

the ribozyme and antisense methods. Whereas the latter methods both inhibit cell proliferation within 48 hours, a single treatment with siRNA did not affect cell proliferation within 5 days, despite effective and sustained down-regulation of RUNX1-ETO mRNA and protein over this period (Heidenreich *et al.* 2003; Martinez *et al.* 2004). Instead, repeated treatments with siRNA were necessary to bring about changes to cell number, which was not evident until approximately day 5 (Martinez *et al.* 2004). Results from this thesis have demonstrated that despite effective transduction and knockdown of the fusion mRNA and proteins, a subpopulation of cells, that has received a lower effective dose of shRNA, may survive and lead to recovery of the transduced population (see Chapters 4, 5). This recovery was typically observed within a matter of days. Considering the delayed effect of the siRNAs on cell proliferation, it was therefore unlikely that any inhibitory effect of the shRNAs delivered by the lentiviral vectors would be observed before possible recovery of the transduced cell population.

6.4.1 Conclusion

From the results presented in this chapter it must be concluded that the three shRNAs designed to target the RUNX1-ETO mRNA were ineffective in down-regulating this target. This was despite the close homology of one of these designs with an effective siRNA design. Furthermore, it should be noted that an shRNA design based on this active siRNA has elsewhere been reported to successfully down-regulate the RUNX1-ETO mRNA target (Fazi *et al.* 2007). The design of this active shRNA has not been published, however, and a comparison with the shRNA designs used here has not been possible. Nevertheless the results from this chapter highlight the differences between optimal siRNA and shRNA designs, and emphasise the need for design algorithms more suitable for shRNA design.

7 Activity of Ty3 Integrase in Human Cells

7.1 Introduction

Stable gene therapy relies on long term expression of transgenes, which is best achieved by integration of the transgenes into the genome of the target cell. Use of viral vectors can mediate efficient and reliable integration of transgenes, however, such vectors present a potential risk due to insertional mutagenesis. Insertional mutagenesis is the disruption of normal genes by mutation or dysregulation by vector transgenes and their promoters. Integrase, the enzyme that mediates integration of the vector into the host genome, strongly influences the risk of insertional mutagenesis. Integrase enzymes from different vector types have characteristic integration profiles, but integration of many viruses is often targeted to areas of high transcriptional activity. Such targeting is due to selection pressures favouring higher expression of viral genes. Unfortunately, as speed of replication and transmission are priorities for many virus types, survival of the host cell rarely enters the equation for viral success. These priorities conflict with those of stable gene therapy. By contrast, retrotransposons are a group of integrating elements that share many characteristics with retroviruses, but are incapable of lateral transfer, and consequently their fate is closely tied to their host cells. Part of ensuring the long term survival of host cell is maintaining the genomic integrity of that host. Amongst retrotransposons, those found in yeast must navigate a gene-rich host genome without causing disruption, generating further selective pressure on targeting retrotransposon integration. Studies of a suite of yeast retrotransposons and their integrases have revealed a range of strategies to minimise the risk of genomic disruption (reviewed in Bushman 2003).

One of these yeast retrotransposons, Ty3, has evolved strict targeting characteristics. This retrotransposon is found in *Saccharomyces cerevisiae*, where integrated copies are always found between 1 and 4 nucleotides upstream of PolIII class tRNA genes (Chalker and Sandmeyer 1992). Such integration is mediated by direct interaction between the retrotransposon integrase and the yeast transcription factor subunits, TFIIIB and TFIIIC (Kirchner *et al.* 1995; Connolly and Sandmeyer 1997). Integration at this site is unlikely to cause disruption as tRNA genes possesses internal

promoters (Sharp *et al.* 1981; L'Etoile *et al.* 1994). Such integration targeting would be highly desirable in integrating vectors for human gene therapy. The Ty3 integrase has been shown to recognise the human tRNA-Lys gene present in a plasmid introduced into yeast cells, and bound upstream of this gene as it did for yeast genes (Dildine and Sandmeyer 1997), however, in this context the integrase would still be interacting with yeast TFIIIB and TFIIIC. Therefore it remains to be determined whether the Ty3 integrase possesses any integrative activity in human cells. There does exist a high degree of similarity between the TFIIIB and TFIIIC subunits of *S. cerevisiae* and humans, especially upstream near the site of Ty3 insertion (Huang and Maraia 2001).

In order to assess the integrative activity of the Ty3 integrase in human cells, experiments were carefully designed in consideration of the characteristics and varied roles of integrase enzymes. For example, the integration mechanism is dependent on interaction between the integrase and a reverse transcript of the Ty3 genome. This interaction has not been closely studies in retrotransposons, however, in retroviruses the region of the reverse transcript to which the retroviral integrase binds extends at least 21 base pairs from the end of the transcript, although sequences beyond the terminal 15 base pairs appear to play little role in substrate specificity (reviewed in Coffin *et al.* 1997). Furthermore, CA/GT dinucleotide pairs are consistently found at the ends of integrated transposable elements of many types, including Ty3 (Coffin *et al.* 1997; Sandmeyer *et al.* 2002), suggesting an important role for such pairs in the integration reaction (reviewed in Coffin *et al.* 1997). The integrase enzymes of HIV and MoMLV also carry out 'end processing', the cleavage of the terminal 2 nucleotides from each 3' end of the reverse transcripts, prior to insertion (Craigie *et al.* 1990; Engelman *et al.* 1991) and the same process evidently takes place in Ty3 (Kirchner and Sandmeyer 1996). Finally, Ty3 integrase also initiates reverse transcription (Nymark-McMahon *et al.* 2002), catalyses disintegration (Chow *et al.* 1992), and mediates nuclear localisation of the Ty3 genome, across the intact nuclear membrane (Lin *et al.* 2001b). These features have been incorporated into the experimental design. Similarly, the Ty3 integrase, normally expressed as part of the Pol polyprotein (Coffin *et al.* 1997), may instead be linked to a *Met* transcription start site immediately upstream of the coding region (Thyagarajan *et al.* 2001).

The main aim of this chapter, therefore, was to assess the integrative activity of Ty3 integrase in human cells. This aim relied on successfully expressing the integrase in these cells and providing this enzyme with a compatible substrate.

7.1.1 Methods

7.1.1.1 Expression Vectors

The pLV-tTRKRAB-Red lentiviral vector was selected as the basis for delivery and expression of the Ty3 integrase gene as it contained an EF-1α promoter, an internal ribosomal entry site, and a WPRE insulator element. To allow positive selection of cells expressing the Ty3 integrase (IN), this gene was linked to a puromycin resistance gene (PUROr) via the IRES. Accordingly the PUROr marker was inserted downstream of the IRES, from which position expression was expected to be lower than for the upstream gene. The PUROr gene was inserted first, and this form of the vector without the IN was used as the negative control. The details of the construction of these vectors are outlined below.

The original pLV-tTRKRAB-Red vector contained the tTRKRAB and dsRed genes upstream and downstream of the IRES, respectively. Of these, only the IRES was retained, being sub-cloned into a pGEM-T-easy vector (Promega) to facilitate subsequent cloning steps (see also Section 3.2.5.2). The primers used for this sub-cloning step included additional restriction sites, and also reintroduced the more efficient wild-type IRES start codon (Martin *et al.* 2006). The sequences of these primers, designed by Dr Stephen Frankenberg (University of Melbourne), were as follows:

IRES-FP (42 nt containing, in order, MluI, BamHI, XmaI, and SpeI restriction sites)

> 5'-ACGCGTGGATCCCGGGACTAGTTCCGCCCCCCCCCCCCTAAC-3' IRES-RP (38 nt containing, in order, XbaI, EcoRI, and BglII restriction sites. The complement of the wild-type IRES start codon is underlined)

> 5'-TCTAGAATTCAGATCTCCATATTATCATCGTGTTTTTC-3'

The purified amplicon was ligated directly into the pGEM-T-easy vector by Dr Stephen Frankenberg, creating the 'pGTE-IRES' vector.

The puromycin-resistance gene (PUROr) was amplified from the pBABE-PURO vector (supplied by AddGene) using primers with multiple restriction sites, including BamHI and XmaI on the forward and reverse primers respectively, which were used here. The sequences of the primers were as follows:

PUROFP2

    5'-GGGATCCGCATGACCGAGTACAAGCCCACGG-3'

PURORP2

    5'-GACCGGTACCGGCGCGCCTCTAGAGCATGGGGTCGTGCGCTCCTTTCGG-
3'

The resulting amplicon was digested with BamHI and XbaI restriction enzymes and ligated into a pGTE-IRES vector cut by BglII and XbaI restriction enzymes. The overhangs generated by BamHI digestion of the amplicon and BglII digestion of the vector were compatible, and when these were ligated, the BglII site in the vector was obliterated, leaving the BglII site in the amplicon as a unique restriction site for this enzyme. ligation of the compatible BamHI and BglII overhangs obliterated the BglII site of the vector, while retaining the XbaI site. From this 'pGTE-IRES-PURO' intermediate construct, the IRES-PURO fragment was excised with an MluI-XbaI double-digest. This fragment was then ligated into an MluI-SpeI-cut, pLV-tTR KRAB-Red backbone, to give the pLV-IRES-PURO vector. This was used as the negative control vector.

The pEGTy3-1 plasmid was provided by Professor Suzanne Sandmeyer (University of California, Irvine), and contained the complete genome of the Ty3 retrotransposon (accession number M23367). The integrase gene, as part of the Pol polyprotein, spanned nucleotides 3450-5060 of this sequence. The forward primer used for amplification of this sequence was also designed to introduce a Kozak consensus sequence and a Met/start codon immediately before the first integrase codon.

The forward primer was linked with an AscI restriction site, while the reverse primer was linked with an XmaI restriction site. The sequence of each primer was as follows:

KOZAKFP (Kozak consensus sequence in bold, start codon underlined)

    5'-CGGGCGC**GCCACC**<u>**ATG**</u>ACTATAACCCCCGAAACATCCCGACCT-3'

3INRP

    5'-TCCCCCCGGGAGGTTGTTTCATATGTGTTTTATGAAC-3'

The resulting amplicon was serially digested with AscI and XmaI and ligated into an MluI-XmaI-cut pLV-IRES-PURO vector backbone. The resulting expression vector was designated 'pLV-3IN-IRES-PURO' (Figure 7.1).

Figure 7.1 pLV-3IN-IRES-PURO
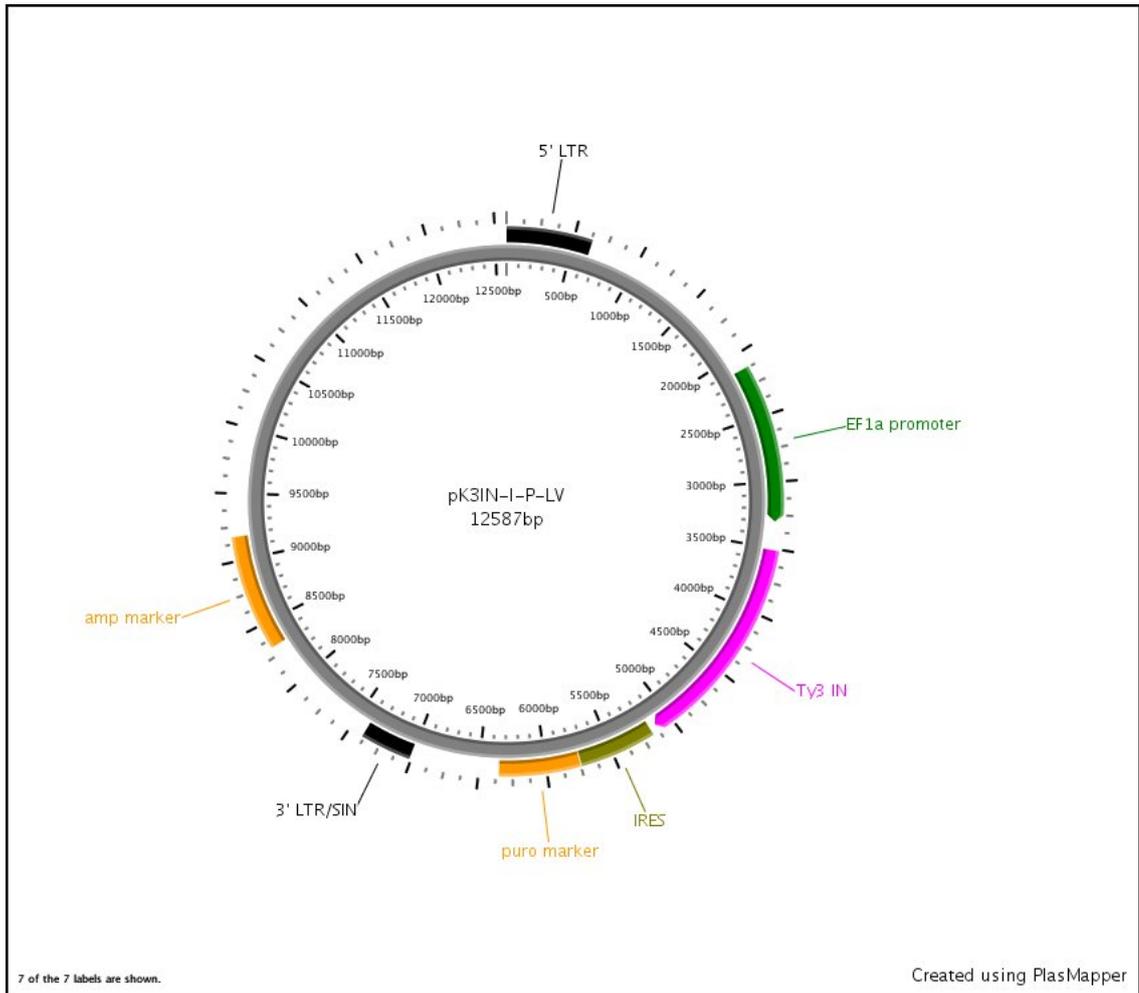
Figure 7.1 - Vector plasmid for expression of Ty3 integrase gene (Ty3 IN)

Figure 7.2 Overview of the Marker Integrant
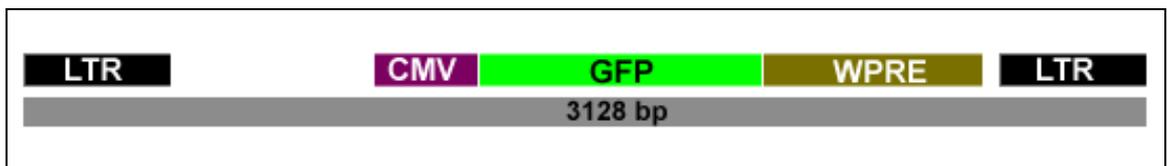
Figure 7.2 - The marker 'integrant' construct, with a CMV promoter (CMV), enhanced green fluorescent protein (GFP), and WPRE insulator (WPRE), flanked by wild-type long terminal repeats from the Ty3 retrotransposon (LTR).

7.1.2 Marker 'Integrant'

To substitute for the wild-type Ty3 genome, a marker 'integrant' was constructed, which consisted simply of a marker gene flanked by Ty3 LTRs. The GFP marker gene of the pHIV-7-GFP lentiviral vector (Li *et al.* 2003a) gave robust expression of the fluorescent protein and this vector was selected as the basis for the integrant. In this vector GFP expression was driven by a CMV promoter and followed by a WPRE insulator. These genes spanned nucleotides 2024 to 3653 of the pHIV-7-GFP vector and were flanked by a number of unique restriction sites that were used for insertion of Ty3 LTR sequences (see below). The restriction sites used for the cloning steps were BamHI and XmaI (cutting at positions 1467 and 1473 respectively), and KpnI and DraIII (cutting at positions 3860 and 4275 respectively).

The long terminal repeats of the wild-type Ty3 retrotransposon were also cloned from the pEGTy3-1 plasmid. The primers used to amplify the LTRs were linked with restriction sites compatible with those of the pHIV-7-GFP vector indicated above. That is, the forward primers were linked with BamHI and KpnI for the 5' and 3' LTRs respectively, while the reverse primers were linked with XmaI and DraIII, respectively. These primers allowed insertion of the LTRs in the forward orientation. The complete sequences for these primers were as follows:

LTRLINKBAMFP

    5'-CGGGATCCCGAAACACAAGACAACCCTGA-3'

LTRLINKXMARP

    5'-CCCCCGGGGAGCATCCAAAATGGAACTTT-3'

LTRLINKKPNFP

    5'-GGGGTACCCGAAACACAAGACAACCCTGA-3'

LTRLINKDRARP

    5'-CCCACTACGTGGAGCATCCAAAATGGAACTTT-3'

The resulting vector was designated 'pHIV-LTR'. This formed the template for amplification of the linear integrant by PCR. As the reverse transcript of the Ty3 genome is blunt-ended prior to end processing by the integrase enzyme, the integrant was amplified by blunt-ended PCR using the Phusion proofreading polymerase (Genesearch), as per the manufacturers' instructions. The annealing temperature was

60 °C. The primers used for this amplification were designed to anneal at each terminus of the Ty3 LTR sequence. The sequences of these primers were as follows:

LTRENDSFP

    5'-GATGTTGTATCTCAAAATGAGATATG-3'

LTRENDSRP

    5'-TCTGTTGTATTACGGGCTCGAGTAATA-3'

The full-length linear integrant amplicon was 3128 nt (Figure 7.2). As the primers bound at the termini of the LTRs, and the LTRs were complete at both ends of the template, a shorter, 344 nt product was also generated. This shorter product was dominant in the PCR reaction, however sufficient quantities of the 3128 nt product were generated for gel separation and extraction by standard methods. Preliminary testing by transient transfection of 293T cells demonstrated that low concentrations were sufficient for robust expression of the GFP marker gene.

7.1.3 Puromycin Selection

In order to determine the appropriate dose of puromycin from the transfected 293T cells, a series of dose-response experiments was performed. 293T cells were seeded at 5×10^5 cells per well of a 6-well plate. After 48 hours, cells were transfected with 1 μg per well of either the pLV-3IN-IRES-PURO vector or the negative control vector (pLV-IRES-PURO), as per standard Calcium Phosphate precipitation methods (see Chapter 2.10).

At 48 hours post transfection, cells were treated with puromycin at doses ranging from 0.5 to 50 μg/ml. The number of adherent 293T cells was assessed after 48 hours of puromycin treatment. The appropriate concentration of puromycin for each vector was taken as the dose at which approximately 10% of cells remained attached. For cells transduced with the pLV-3IN-IRES-PURO vector, the appropriate dose was 2.5 μg/ml puromycin, whereas for cells transduced with the negative control vector, the appropriate dose was 50 μg/ml.

7.1.4 Co-transfection

Preliminary testing indicated that transfection of 293T cells with 350 ng of the integrant marker per well (for a 6-well plate) was sufficient for widespread expression of the GFP marker. Accordingly, cells were seeded at 5×10^5 cells per well of a 6-well

plate, and after 48 hours, cells were co-transfected by calcium phosphate precipitation with 350 ng of the marker integrant and 1 μg of either the pLV-3IN-IRES-PURO vector or the negative control vector. At 48 hours post transfection, cells were treated with puromycin at the doses indicated above, with this dose repeated at 120 hours post transfection. At day 7, cells were trypsinised and counted. Cells were diluted to a density of 2 cells per 200 μl with fresh DMEM, and dispensed into 96-well plates at 200 μl per well.

7.1.5 Monitoring for Marker Gene Expression

At day 8, the 96-well plates were initially screened for adherent, fluorescent cells by inspection with an inverted fluorescent microscope. Fluorescent cells were identified, and their shape and location within the well were recorded. These fluorescent cells were checked every second day for two weeks with non-proliferating cells excluded from further observations. Similarly, where fluorescence was lost from proliferating colonies within this period, such colonies were also excluded. As colonies grew, they were passaged to 24-well plates then to 6-well plates. Where two or more proliferating colonies were present in the same original well, each colony was transferred separately such that after the first passage each well contained a single colony.

All colonies remaining in the study at week two were observed weekly up to 7 weeks post transfection, with the proportion and distribution of fluorescent cells within the colony recorded at each time point. Detailed records were kept and colonies fell into three distinct categories. In the first category, all cells within the colony retained fluorescence, and these colonies were recorded as 'fluorescent'. In the second category, colonies included fluorescent cells and cells from which fluorescence had been lost. These colonies were designated 'mosaic'. In the third category, fluorescence was lost from all cells in the colony, including the once-fluorescent progenitor cells. These colonies were recorded as 'fluorescence lost'.

Cells toward the centre of each colony would have limited opportunity for cell division, reducing the dilution or expulsion of non-integrated DNA. Therefore, colonies in which only a few cells toward the centre retained fluorescence were categorised as 'non-fluorescent' but with a note recording the presence of these few fluorescent cells. In all of these colonies, however, fluorescence from these few cells was lost after

passaging and replating. At the end of the 7 week culture period, gDNA was collected from each colony using a Wizard Genomic DNA Purification Kit (Promega).

7.1.6 Screening gDNA for integration of expression vectors

To ensure that results were not skewed by passive integration of the expression vectors and continued expression of the Ty3 integrase, gDNA from each colony was probed for the presence of the puromycin resistance gene using the PUROFP2 and PURORP2 cloning primers (see Section 7.3.1). PCR reactions were visualised on Agarose gels.

7.1.7 Identification of Integration targeting sites

Linear Amplification Mediated Polymerase Chain Reaction (LAM-PCR) was used to sequence the integration sites of the marker integrant in the gDNA of the transfected cells. The method used was that of Schmidt *et al.* (2007), except that high-resolution gels were not used. Furthermore, sequencing of Ty3 integration sites required the design of custom primers, and selection of an appropriate restriction enzyme. These steps are outlined below. The linker cassette and corresponding reverse primers described in the original protocol (Schmidt *et al.* 2007) were compatible with the 4-cutter restriction enzyme selected here.

7.1.7.1 Restriction Enzyme

As per the criteria set out in Schmidt *et al.* (2007), the BfaI 4-cutter restriction enzyme was selected. Within the Ty3 LTR, the BfaI restriction site occurred only at position 121 of the LTR sequence. From the 5' LTR, the next BfaI restriction site within the integrant sequence was at nucleotide 2390. Therefore, using primers designed to bind downstream of the BfaI restriction site, linear PCR from the 5' LTR, followed by BfaI digestion, should result in a fragment of no less than 2269 base pairs.

By contrast, the BfaI restriction enzyme cuts relatively frequently in the human genome. In human chromosome 21, for example, average 'fragment length' (chromosome length divided by the number of BfaI restriction sites) after BfaI digestion would be 398.7 nt (see www.bioinformatics.org/pipermail/bbb/2006-September/003548.html). Consequently, products amplified from the 3' LTR would be shorter on average, and should dominate the subsequent nested PCRs. The longer

products from the 5' LTR might also be easily identified and eliminated by gel separation.

7.1.7.2 Forward Primer Design

MacVector software was used to design nested forward primers to bind 3' of nucleotide 121 of the Ty3 LTR sequence. These primer sequences were 'Blasted' against the human genome to assess the probability of non-specific binding. The sequence of the primers is given below, along with the position of each primer in the 344 nt Ty3 LTR sequence and the degree of homology with the human genome.

LTI (biotinylated)

```
5'-GCTTCCACCACTTAGTATGATTC-3'
```

Positions 180-202 of Ty3 LTR. Primer length 23 nt, 17/17 nt homology with human genome.

LTII

```
5'-GTTTTGACAACTGGTTACTTCC-3'
```

Positions 258-279 of Ty3 LTR. Primer length 22 nt, 17/17 nt homology with human genome.

LTIII

```
5'- AACTGGTTACTTCCCTAAGACTG -3'
```

Positions 266-288 of Ty3 LTR. Primer length 23 nt, 17/17 nt homology with human genome.

The reverse linker cassette and reverse primers were used in the nested PCRs, as per the original protocol (Schmidt *et al.* 2007), and the products separated by electrophoresis on a 1% agarose gel.

7.1.7.3 Sequencing

Bands longer than 79 bp (the length from the LTIII primer to the end of the LTR) were excised and extracted using a Wizard SV Gel and PCR Clean-up kit (Promega). Extracted bands were cloned into pGEM-T-easy cloning vectors (Promega), which were then used to transform 'Top 10' *E. coli* (Invitrogen). The bacteria were grown overnight on Ampicillin agar plates that had been coated with X-gal 30 minutes before plating to enable blue/white screening. Blue colonies were probed with the LTIII and LCII primers in a 10 µl PCR reaction to confirm the presence of the insert, and

candidate colonies cultured overnight in Luria broth with Ampicillin. Cloning vector cultures were miniprepped (Promega) and sent to Macrogen for sequencing using both the T7 and SP6 universal primers.

Returned sequence data was filtered to exclude sequence from the cloning vectors and the primers. Remaining candidate sequences were then 'Blasted' against the human genome.

7.1.8 Screening of gDNA for Integrated Copies of the Marker Gene

To determine the genotype of colonies in each category, gDNA was also directly probed by PCR for integrated copies of the marker gene. Various combinations of existing primers (see above) that were specific to the marker sequence were tested, however, most combinations did not reliably discern positive from negative gDNA controls. Finally the LTRENDSFP primer was used in conjunction with a novel primer, 'PROBERP', designed to bind within the GFP sequence. These primers spanned a region extending from the 5' LTR to the GFP sequence, with an expected product size of 1271 bp. The sequence of the novel primer was as follows:

PROBERP

```
5'-AAGTCGTGCTGCTTCATGTG-3'
```

7.2 Results

7.2.1 Long-term Expression of Marker Transgene in Human Cell Colonies

In order to assess the influence of the Ty3 integrase on the stability of transgene expression, colonies of 293T cells were transfected with a marker integrant and either a vector expressing the integrase or a control vector. Cells were selected with puromycin and proliferating fluorescent colonies were observed every second day up to day 14. By this time the initial and widespread transient transgene expression had almost completely disappeared, and therefore only colonies with fluorescence at day 14, whether mosaic or completely fluorescent, were observed further. This left a total of 32 control colonies and 33 integrase-treated colonies.

At day 14 (week 2), at least one third of the control colonies had lost fluorescence from some of the cells (Figure 7.3A). By contrast, only one of the 33 integrase treated colonies had lost fluorescence from any of the cells. This trend continued to week 4, with most control colonies at this time point losing fluorescence from some (mosaic) or all cells (Figure 7.3B). One of the integrase treated colonies had lost all fluorescence, and some were categorised as mosaic, however two thirds of all colonies retained fluorescence in all cells.

At the end of the experiment (week 7), only one of 32 control colonies retained fluorescence in all cells, while half had lost all fluorescence, with the remaining 15 colonies categorised as mosaic (Figure 7.3C). For the integrase treated cells, the number of colonies in which all cells were fluorescent had decreased only marginally, from 22 at week 4 to 20 at week 7. The number of mosaic and non-fluorescent colonies had each increased by one.

These results clearly demonstrate that more integrase treated cells retained fluorescence than the controls, and in colonies where fluorescence was lost, it occurred later than in the controls. Thus the presence of integrase had a clear positive effect on the long term stability of transgene expression.

Figure 7.3 Relative numbers of fluorescent and mosaic colonies following treatment with Ty3 integrase
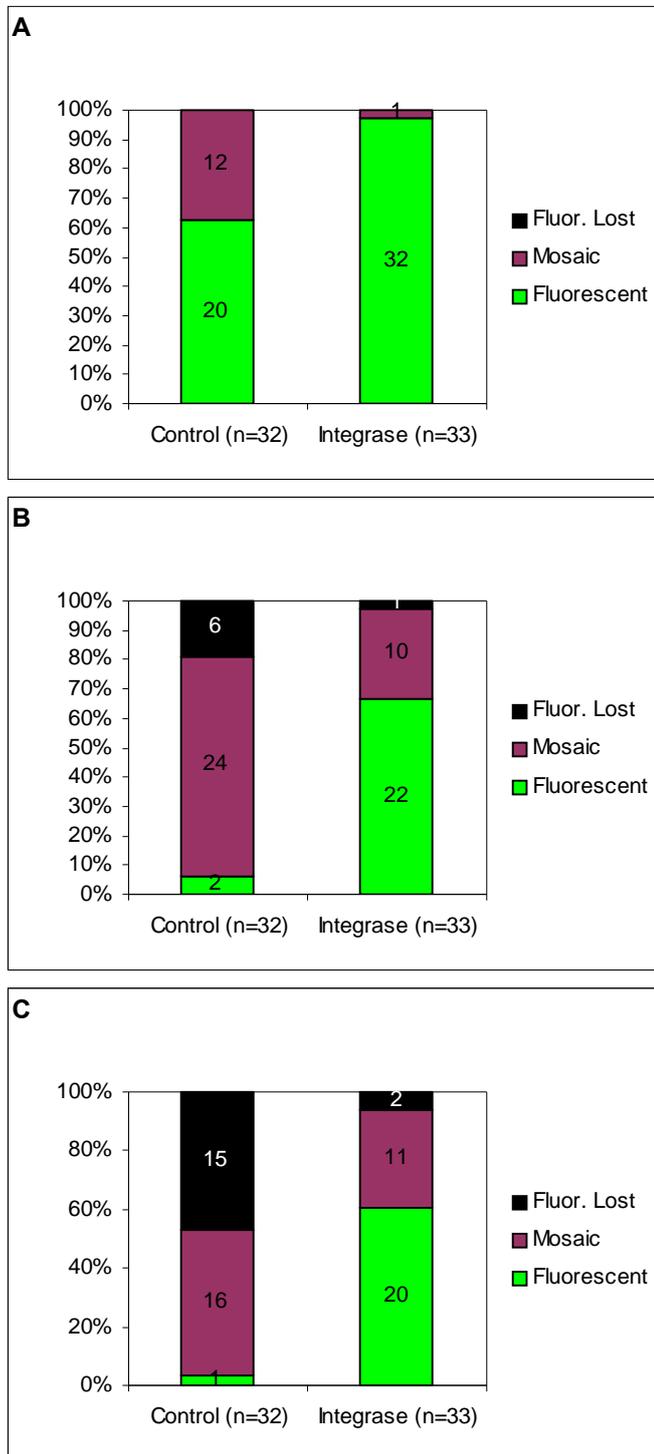
Figure 7.3 – 293T cells were co-transfected with an integrant marker, and the integrase vector or control. Graphs represent the proportion of colonies that were completely fluorescent, a mosaic of fluorescent and non-fluorescent cells, or completely non-fluorescent, at 2, 4 and 7 weeks (A, B and C respectively) after transfection. The results represent the total number of colonies from two separate experiments.

7.2.2 Screening for PUROr Marker Gene of the Expression Vectors

Genomic DNA samples representative of each group were screened by PCR for the presence of the puromycin resistance gene (PUROr), indicating the persistence of the expression vectors integrated into the genome. While a range of bands was present in all cells, there were no bands corresponding to the PUROr gene itself (Figure 7.4). From this it was concluded that the expression vectors had not integrated, and that the expression of the Ty3 integrase was only transient.

7.2.3 Gel-Separation of LAM-PCR Products

LAM-PCR reactions were performed on all available gDNA samples, then visualised by gel separation. Multiple bands were evident from most reactions, regardless of the treatment or the colony category (Figures 7.5 and 7.6). Most bands were up to 400 bp in length, but the maximum was approximately 800 bp. There were, however, some colonies from which no bands were produced (see Figures 7.5A and 7.6B), but again this bore no relation to the treatment or category of the colony. Similarly the number of bands produced bore no relation to the treatment or category of the colony. From these results it was clear that the number and size of PCR products from the LAM-PCR reactions did not accurately reflect the genomic status of the marker integrant in these colonies.

7.2.4 LAM-PCR Sequencing Results

The brightest and cleanest bands from the LAM-PCR products were extracted from the separation gels then cloned into cloning vectors and sequenced. Of the 29 sequences returned, few were of acceptable quality. Many sequences consisted only of the forward and reverse primers (6 sequences), or consisted of the primers plus sequence from the cloning vector (8 sequences). These sequences were ignored. Of the 15 remaining sequences that contained the primers and the marker LTR, 12 consisted of sequence from within the marker integrant. That is, the linear PCR step had extended from the 5' LTR rather than the 3' LTR.

Only three sequences contained human genomic DNA. Two of these were from the same control treated mosaic colony, while the third was from an integrase treated fluorescent colony. These sequences were 'Blasted' and the strongest homology for each sequence was as follows. Sequence A from the control treated mosaic colony

Figure 7.4 Probing for integrated expression vectors
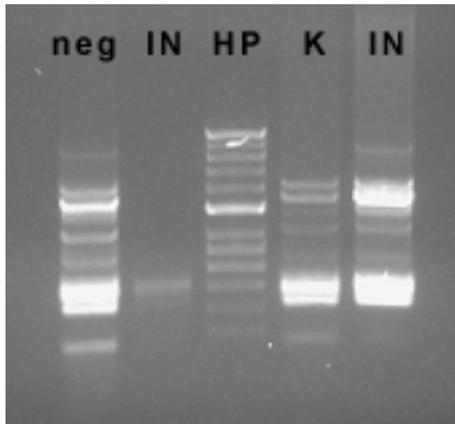
Figure 7.4 – Genomic DNA of cells treated with integrase ('IN') or controls ('K'), as well as gDNA of untreated controls ('neg'), were probed for integrated copies of the PUROr marker gene (expected size 686 bp) of the vectors. The PCR products were separated on a 1% agarose gel. The ladder ('HP') is a HyperLadder II (Appendix 7.X), with bright bands at 300 and 1000 bp (100 bp intervals, no band at 900 bp).

Figure 7.5 LAM-PCR products from integrase treated colonies
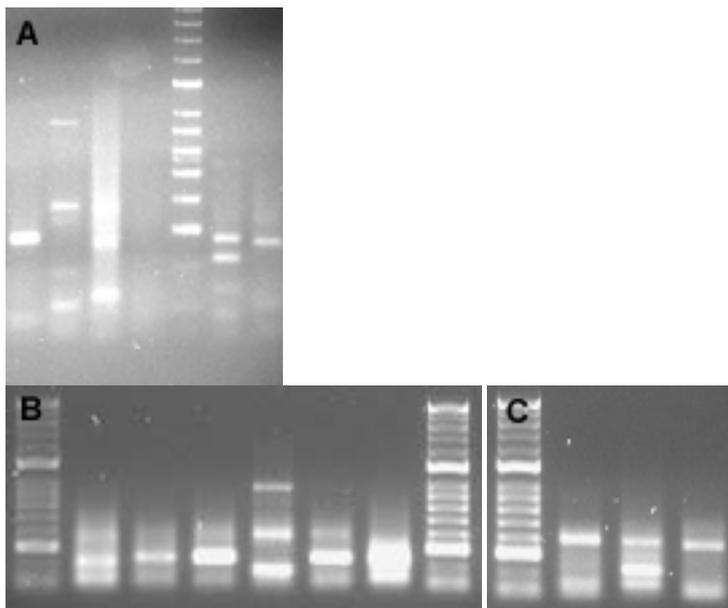
Figure 7.5 – LAM-PCR reactions were performed on gDNA from all colonies. The products of these reactions were visualised by gel separation. These images are of representative samples of integrase treated colonies that were all fluorescent (A), mosaic (B), or from which fluorescence had been lost entirely (C). The ladders are HyperLadder II ladders (Appendix 7.X), with brighter bands at 300 and 1000 bp (100 bp intervals, no band at 900 bp).

Figure 7.6 LAM-PCR Products from of control treated, fluorescent colonies
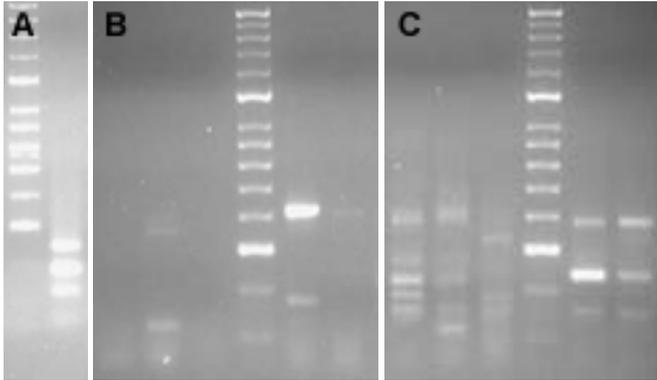
Figure 7.6 – LAM-PCR reactions were performed on gDNA from all colonies. The products of these reactions were visualised by gel separation. These images are of representative samples of control treated colonies that were all fluorescent (A), mosaic (B), or from which fluorescence had been lost entirely (C). The ladders are HyperLadder II ladders (Appendix 7.X), with brighter bands at 300 and 1000 bp (100 bp intervals, no band at 900 bp).

mapped to an intergenic region on chromosome 2 (genomic contig reference assembly). Sequence B from the control treated mosaic colony mapped to a solute carrier family 9 (sodium/hydrogen exchanger) gene, also on chromosome 2 (genomic contig reference assembly). The sequence from the integrase treated fluorescent colony mapped to an intergenic region on chromosome 20 (genomic contig, reference assembly). From these few results it was not possible to describe any general integration targeting characteristics of the Ty3 integrase in human cells.

Sequence A

TAACTGGTTACTTCCCTAAGACTGTTTATATTAGGATTGTCAAGACACTCCGGT
ATTACTCGAGCCCGTAATACAATTCAGATCTGGTCTAACCAGAGAGACCCAGTACAGGC
AAAAAGCAGATCTTGTCTTCTTTGGGAGTGAATTAGCCCTTCCAGTCCCCCCTTTTCTT
TTAAAAAGTGGCTAAGATCTACAGCTGCCTTGTAAGTCATTGATCTTAAAGGTACGGCT
ACCTAACTGCTGTGCCACTA

Name; Homo sapiens chromosome 2 genomic contig, reference assembly (flanking files only), (77-99)
Intergenic
Accession number; ref|NT_005403.17|
Homology; 66287470 – 66287448
(Blue highlighted text corresponds to vector sequence, while red highlighted text corresponds to the adaptor sequence).

Sequence B

TAACTGGTTACTTCCCTAAGACTGTTTATATTAGGATTGTCAAGACACTCCGGT
ATTACTCGAGCCCGTATTGTATCTCAAAATGAGATATGTCAGTATGACAATACGTCACC
CTAACTGCTGTGCCACTA

Name; solute carrier family 9 (sodium/hydrogen exchanger),chromosome 2 Genomic Contig reference assembly (8-29 (of unknown sequence))
Acession number; ref|NT_022171.15|
Homology; 7921799-7921779
(Blue highlighted text corresponds to vector sequence, while red highlighted text corresponds to the adaptor sequence).

Sequence from Integrase-treated colony

TAACTGGTTA CTTCCCTAAG ACTGTTTATA TTAGGATTGT CAAGACGAAC
AGGTTACTAA GAGCCTATGC TTCAACCAAT ATTCAGAATT GGCATGTATA
TTTACCACAA ATTGAATTTG TTTACAATTC TACACCTACT ACCTAACTGC
TGTGCCACTA

Name; Homo sapiens chromosome 20 genomic contig, reference assembly

138

Accession number; ref|NT_011387.8|
Homology; 22046670 – 22046648
(Blue highlighted text corresponds to vector sequence, while red highlighted text corresponds to the adaptor sequence).

As many of the LAM-PCR products were not genuine integrants, nor contained gDNA, these results indicated that the forward primers used for the LAM-PCR were binding non-specifically to areas of the human genome rather than to the LTR of the marker integrant. They were also disposed to primer-dimer formation. Therefore these LAM-PCR reactions need to be repeated with new, carefully designed primers.

7.2.5 Screening gDNA for GFP Transgene

Aside from generating unreliable sequence data, the LAM-PCR reactions also failed to provide any information regarding the integration status of the integrant marker in each colony. Therefore, to determine whether the colonies in each category contained integrated copies of the marker gene, the gDNA of colonies representative of all categories were screened by PCR for the marker integrant. Primer probes were designed to detect the LTR-GFP region of the marker integrant and the PCR products were visualised by gel separation. In integrase treated, fluorescent colonies, the marker was evident in most samples, however some colonies were negative for the integrant (Figure 7.7A). In integrase treated colonies with lower levels of fluorescence, the majority of colonies were negative for the integrant (Figure 7.7B). Similarly, integrase treated colonies that were mosaic or non fluorescent were also negative for the integrant (Figure 7.7C). By contrast, control colonies that were fluorescent or mosaic were positive for the integrant, while the non fluorescent colonies were negative (Figure 7.7D).

These results demonstrate that the fluorescent phenotype of each colony did not necessarily reflect the genotype of that colony. More specifically they suggest that in numerous colonies, the observed fluorescence was not derived from integrated copies of the marker gene.

Figure 7.7 Probing gDNA for Integrated Marker Sequence
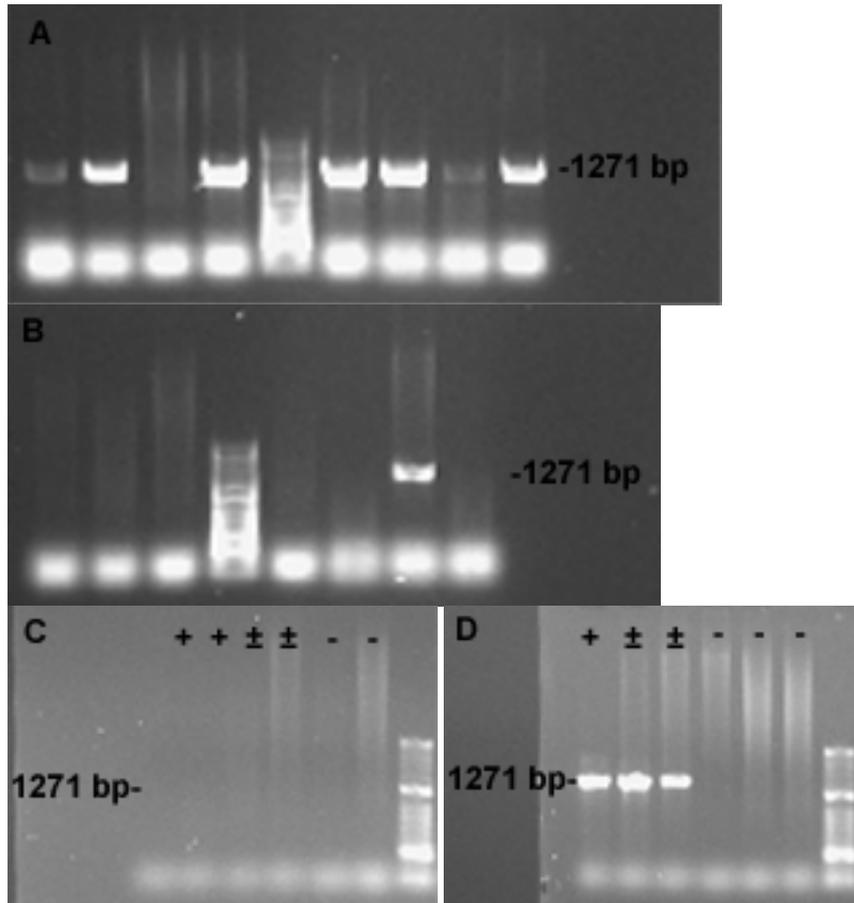
Figure 7.7 – Genomic DNA from each colony category was probed for integrated marker (expected product size 1271 bp). A number of integrase treated, fluorescent colonies were probed (A), as well as a number of integrase treated colonies with low fluorescence (B). Integrase treated colonies that were mosaic or that had lost fluorescence were also probed and compared with fluorescent colonies (C). A range of control colonies was also probed, including samples from fluorescent and mosaic colonies, and colonies that had lost fluorescence (D). The ladder ('HL') is a HyperLadder II (Appendix 7.X), with bright bands at 300 and 100 bp (100 bp intervals, no band at 900 bp). (Legend; '+' fluorescent, '±' mosaic, '-' fluorescence lost).

7.3 Discussion

The integrase from the Ty3 retrotransposon has very specific targeting characteristics in the genome of *Saccharomyces cerevisiae*, the normal host for this retrotransposon. Ty3 retrotransposons integrate four nucleotides upstream of the transcription initiation site of tRNA genes within the *S. cerevisiae* genome (Chalker and Sandmeyer 1992). Targeting characteristics such as these would be advantageous in human gene therapy, however, while the Ty3 integrase recognises human genes transfected into *S. cerevisiae* cells (Dildine and Sandmeyer 1997), there has been no investigation of the activity of this integrase in human cells. The purpose of this chapter was to examine this activity by co-transfecting the Ty3 integrase gene and a marker gene flanked by Ty3 LTRs into human 293T cells.

Previous studies of novel integrases in human cells have used growth selection markers to identify cells expressing transgenes (eg. Thyagarajan *et al.* 2001) however growth selection can promote passive integration of the marker genes, distorting assays of integrase activity. Furthermore, ongoing expression of the transgenes is difficult to monitor by growth selection, as cells in which transgene expression is diminished are excluded from the assay. Expression of the GFP transgene presents a burden on cellular metabolism and the GFP protein may be toxic under certain conditions (Liu *et al.* 1999). Consequently the GFP marker may be considered to have a neutral or negative effect on cell survival. In addition, the use of a GFP marker in adherent cell lines allows linear monitoring of individual cells through the course of the experiment. This experimental design allowed the identification of cells in which fluorescence was lost, sometimes after many weeks of observation, contributing greater detail regarding the long term effects of Ty3 integrase activity in human cells.

The total number of colonies in the control treatment was much higher than in the integrase treated sample. This was attributed to greater expression of the puromycin resistance marker from the vector lacking the Ty3 integrase gene, as genes upstream of internal ribosome entry sites have been shown to impair expression of genes downstream of the IRES (Mizuguchi *et al.* 2000). Nevertheless the percentage of GFP-positive colonies was similar in both the control and integrase treated groups. The bulk of the fluorescence in the control cells was lost within the first week or two, evidently

due to the loss of unintegrated GFP marker genes from the proliferating cells. Furthermore, many of the control colonies soon became heterogenous, with some cells losing fluorescence while neighbouring cells retained expression of the GFP marker. This was attributed to differential rates of ejection, dilution or digestion of the unintegrated GFP marker transgene. In the samples treated with the Ty3 integrase, however, more colonies, and more cells within the colonies, remained fluorescent.

Both the high overall percentage of fluorescent cells in the integrase treated samples, and the duration of GFP expression in these cells, demonstrate that the Ty3 integrase enzyme expressed in these cells was able to mediate stable expression of the co-transfected GFP marker gene. This was an important result as it not only demonstrated that the integrase was expressed in a functional conformation in human cells and that it recognised, bound and processed the co-transfected linear marker DNA, but it also recognised, bound and cleaved human genomic DNA. These are all essential steps in adapting the enzyme for use in human gene therapy.

Given these activities, it is of considerable interest to determine the integration targeting specificities of this enzyme in the human genome, and so LAM-PCR sequencing was undertaken with gDNA samples collected from each colony. The aims of the LAM-PCRs were to confirm the integration of the marker transgene, and to locate these integrations within the human genome. Although refined LAM-PCR protocols are available (Schmidt *et al.* 2007), the methodology remains complicated, and obtaining useful results with the published primers can be time consuming (J. Hyman, pers. comm.). Novel nested primers specific to the Ty3 LTRs were tested prior to undertaking the complete LAM-PCR reaction, however it was impossible to exactly replicate the conditions of the LAM-PCR, and the suitability of the primer design could not be determined until sequences were finally obtained. Unfortunately the primers were discovered to be unsuitable for the LAM-PCRs, however there was insufficient time to repeat the LAM-PCR reactions. Consequently the locations of the integrations could not be determined. Nevertheless, in light of the results confirming activity of the Ty3 integrase in human cells, and given the potential improvements to the safety of stable gene therapies of an integrase enzyme with characteristics such as those of the Ty3 integrase, sequencing of the integrations mediated by this enzyme would yield very interesting information, and should be pursued in future studies.

Another consequence of the unsuitable primers was that integration of the marker gene in the human gDNA could not be confirmed by LAM-PCR however, an alternate was available to answer this question. The gDNA was collected using a kit designed to remove non-genomic DNA from the sample. The gDNA collected in this way was screened by PCR using a pair of primers specific to the transgene marker itself. The characteristic size of the product expected from these primers meant that the presence of the transgene marker in the gDNA could be determined with some confidence.

It was found that in the majority of the samples, the fluorescence status of the colonies ('phenotype') did correlate with the results of the PCR (genotype), however this was not always the case. Of the colonies from which fluorescence had been lost that were probed were negative for copies of the marker gene in the gDNA. In most cases fluorescence was lost within two weeks of transfection and so this loss was evidently due to dilution or digestion of non-integrated copies of the marker. In some cells, however, fluorescence was not lost until after week 4. Unintegrated intracellular DNA usually only persists by forming circular episomes which, in proliferating cell populations, generally only survives a matter of days (see Cara and Klotman 2006) and often less than 48 hours, before degrading or being diluted out by cell proliferation (Sharkey *et al.* 2000; Butler *et al.* 2001). As such, the timing of the late loss of fluorescence, taking place between weeks 4 and 7, warrants consideration. Firstly, this result may have been due to 'disintegration' of previously integrated copies of the marker gene from the gDNA. In this case, such disintegration would most likely have been spontaneous, as the integrase, which can also mediate disintegration (Chow *et al.* 1992; Moore *et al.* 1995), was no longer present in the cells. However, given the conditions of these experiments, a simpler explanation for this delay might be that, despite repeated passaging of the cells, those cells towards the centre of the proliferating colonies would have been crowded, and thus their opportunities for proliferation limited, reducing the rate at which unintegrated DNA was lost via dilution.

The finding that all mosaic colonies tested positive for marker DNA was consistent with the presence of the marker gene in at least some cells. The presence of the marker DNA in daughter cells from which fluorescence had been lost could not be determined, as the screening performed in this thesis was not quantitative. However,

while it was not possible here, the fate of the marker DNA in the non-fluorescent cells, whether suppressed or lost, might easily be answered in future studies by subculturing non-fluorescent cells derived from fluorescent progenitors.

Most of the fluorescent colonies also tested positive for marker DNA, as expected, however a number of colonies with the fluorescent phenotype tested negative for marker DNA. This result could not be explained by the usual mechanisms outlined above, and so alternative explanations have been considered. Of the alternative explanations, the most likely are discussed below.

First, it was possible that that the screening for the marker DNA was negative simply because the gDNA samples had degraded after collection. This explanation seems unlikely, however, as there was no evidence for such degradation in the other samples, which were stored under the same conditions.

Secondly, it was possible that the marker DNA persisted as unintegrated episomes that were excluded by the gDNA purification protocol. While this explanation may seem unlikely, it was interesting to note that phenotypically positive but genotypically negative colonies only occurred in the integrase treated samples. This raised the intriguing possibility that the integrase itself was contributing to the persistence of unintegrated DNA. In viruses where episomal viral DNA persists in dividing cells as a normal part of the viral life cycle, for example in the herpes virus group, this persistence results from tethering of the viral genome to chromosomes of the host genome, which is mediated by DNA binding proteins produced by the virus (Griffiths *et al.* 2006). As such, it is interesting to note that one of the main functions of the Ty3 integrase is to bind to the Ty3 genome, then interact with the genome of the host (Lin *et al.* 2001b). There is, however, no direct precedent for an integrase enzyme performing such a role, and furthermore this explanation is dependent upon the long term stability of the Ty3 integrase enzyme in proliferating human cells.

The final, and perhaps simplest explanation for the negative result from gDNA probing of the marker gene in fluorescent colonies, was that cells in these colonies had a low copy number of the marker gene. Although this is unlikely, given the number of PCR cycles, it was consistent with the variations in fluorescence intensity often observed between some colonies as well as the variations in intensity of the bands in probes of some of the genotypically positive colonies.

7.3.1 Conclusions

The results from this chapter raise many questions for future investigation, particularly with respect to the interaction between the Ty3 integrase enzyme and the human genome. Nevertheless, in relation to the primary objective of this chapter, regarding the potential of the Ty3 integrase in human gene therapy, the results from this chapter clearly demonstrate that Ty3 integrase reliably facilitates the long term expression of transgenes in human cells via high rates of stable transgene integration. Given the potential of Ty3 integrase in mediating safer genomic integration, it is thus certainly worth the further effort to sequence the locations of the integrations mediated by this enzyme.

General Discussion

In the field of gene therapy, the therapeutic potential of RNA interference has emerged from an increasing number of studies. Whether mediated by siRNA or shRNA molecules, RNAi has proven effective against a wide range of targets, including numerous oncogenes and cancer associated genes (Devi 2006; Heidenreich 2009). RNAi can also be used to specifically target oncogenic mutants, resulting in apoptosis and/or inhibition of proliferation in mutant cells, leaving normal cells unaffected (see, for example, Brummelkamp *et al.* 2002a; Wilda *et al.* 2002; Wohlbold *et al.* 2003). While a range of options is available for the delivery of RNAi, with many of these options specific to either the siRNA or shRNA form of molecule, siRNAs have to date been the favoured form of molecule for experimental studies. Many options exist for synthesis and delivery of siRNAs (reviewed in Amarzguioui *et al.* 2005) and they have been validated against a wide range of oncogenes (reviewed in Heidenreich 2009). Nevertheless the main drawback for siRNAs is their transience (Holen *et al.* 2002), requiring repeated doses for a sustained effect. This is not only expensive, but also makes them incompatible with strategies requiring stable gene-correction, for example in transplanted stem cells. A more efficient and more effective option for such strategies is the delivery of RNAi by stably integrating viral gene therapy vectors. Numerous options exist for delivery of RNAi from such vectors, for example the separate expression of sense and antisense strands, or expression of chimeric miRNA's. Of the options available, the expression of shRNAs from inverted repeats is the best understood, and is arguably the most efficient (review Amarzguioui *et al.* 2005).

In light of these considerations, one of the major aims of this thesis was to develop and enhance methods for retroviral (including lentiviral) delivery of shRNAs. In pursuing these aims, chromosomal translocations that give rise to leukaemias are an ideal target for a number of reasons. Firstly, the sequences of chromosomal translocations are often unique to the leukaemic cells and are sufficient to cause pathology on their own. Secondly, the availability of leukaemic cells lines with well-characterised translocations, and existing therapeutic options such as *ex vivo* transduction followed by autologous transplantation, provide a meaningful and convenient model for *in vitro* studies. Finally, a number of such oncogenes have already been successfully targeted using conventional therapies, as well as siRNAs, and

thus provide a benchmark for development of shRNA alternatives. Furthermore, many haematopoietic cell types are difficult to transduce by existing methods, and so these cells also provide a challenging test for efficient transduction.

As regards the candidate integrating vectors to be used for delivery of these shRNAs, lentiviral vectors are one of the most efficient integrating vectors currently available, yet titres in many cell types remain low. A large part of this thesis examined many alternate methods with the aim of improving the final effective titre of lentiviral vectors in cell types that have been difficult to transduce. While many of the alternate methods explored here were ultimately not appropriate, this exploration nevertheless enabled the development of the protocols outlined in Chapters 2 and 3, which offer great improvements in effective vector titre over existing methods for the cell lines examined. These improvements have the potential to increase the efficiency and the safety of gene therapy in a wide range of human cells. Similarly, many of these methods remain applicable to more recent generations of shRNA vectors as they continue to develop.

The integrating vectors themselves, however, are not without their own risks, as the process of integration into the genome of the target cell presents the possibility of insertional mutagenesis, mutation or disruption of genes adjacent to the site of integration. As such, another aim of this thesis was to expand the potential of RNAi gene therapy in transplanted stem cells by enhancing the safety (including efficiency) of integrating vectors by testing the activity in human cells of Ty3 integrase, a novel integrase that possesses a safe integration profile in its normal host.

The specific chromosomal translocations studied in this thesis represent a cross-section of stages in the development of therapeutic shRNAs. The Bcr-Abl translocation has already been effectively targeted with shRNAs, and provides a platform for further examination and comparison. The RUNX1-ETO translocation represents an oncogene which may be treated with broad-spectrum conventional therapies, and has also been effectively targeted using siRNAs. No effective shRNA design is currently available. Finally, the PML-RARα translocation gives rise to a form of leukaemia that can often be effectively treated by conventional therapies, but is prone to relapse. Also, while there has been some success with molecular methods such as hammerhead catalytic ribozymes, no appropriate siRNA or shRNA designs are available.

The Bcr-Abl chromosomal translocation gives rise to a constitutively active tyrosine kinase which activates multiple signalling pathways and leads to the unregulated proliferation of cells (Zou and Calame 1999). RNAi directed against certain forms of the Bcr/Abl fusion oncogene has proven to be relatively successful *in vitro*, with validation of both siRNA and shRNA molecules (see Section 4.1 for summary). The shRNA molecule used in those trials was directly homologous to the siRNA form, and both have been shown to down-regulate mRNA levels and to inhibit proliferation of CML cells *in vitro* in a dose dependent fashion (Wilda *et al.* 2002; Li *et al.* 2003b; Wohlbold *et al.* 2003; Scherr *et al.* 2005). However, in addition to causing apoptosis, selective inhibition of the Bcr-Abl fusion protein by Gleevec also leads to differentiation of surviving cells towards the erythroid lineage (Fang *et al.* 2000; Jacquel *et al.* 2003; Kuzelova *et al.* 2005). In light of this, the effects of anti-Bcr/Abl shRNA on cell differentiation were examined in Chapter 4. The results from this chapter confirmed that down-regulation of Bcr/Abl by targeted shRNA caused differentiation of K562 cells in addition to apoptosis. These results further highlighted the potential of shRNA-mediated gene therapy and indicated that the specific activity of the shRNAs acts against the Bcr/Abl fusion gene in a manner similar to the action of Gleevec. That is, both treatments specifically inhibited tyrosine kinase activity, resulting in a cascade of downstream effects that ultimately lead to the inhibition of proliferation, as well as triggering differentiation via a pathway that involves the transient down-regulation of ERK (Kawano *et al.* 2004). The results from Chapter 4 confirmed the activity of the anti-Bcr-Abl shRNA, and demonstrated the effects of low dose treatment with this molecule, which had not been examined previously.

The PML-RARα fusion gene, by disrupting the normal function of the retinoic acid receptor-α (RARα), decreases the sensitivity of cells bearing this mutation to retinoic acid, in turn leading to uncontrolled proliferation of these cells (Warrell 1993). Many patients bearing this oncogene initially respond to higher, pharmacological doses of retinoic acid, however relapse, characterised by insensitivity to even these high doses, is common (Warrell 1993). Down-regulation of PML-RARα mRNA by catalytic ribozymes has been shown to lead to apoptosis of some cells, as well as enhanced sensitivity to RA in surviving cells (Nason-Burchenal *et al.* 1998a; Nason-Burchenal *et al.* 1998b). Targeting the PML-RARα fusion gene with a long form of hairpin RNA,

delivered by plasmid transfection, also lead to down-regulation of fusion mRNA and protein levels, however, downstream effects were not examined (Oshima *et al.* 2003). Results from this thesis (Chapter 5) indicated that delivery of shRNA by lentiviral vectors also down-regulated PML-RARα mRNA and protein levels, which led to one of two consequences, depending on dose. Those cells with a higher vector copy number were evidently eliminated from the population. By contrast, those cells bearing a lower vector copy number survived, and retained a level of proliferative potential. The reduced levels of the fusion gene in these surviving cells, however, combined with positive growth selection of transduced cells, resulted in a pure population of cells that resembled normal myeloid cells in their sensitivity to retinoic acid signalling. This resensitisation would be beneficial in a therapeutic context, particularly as the development of resistance to pharmacological doses of retinoic acid is a major problem for existing PML therapies, and so the strategy outlined here may provide additional treatment options. Finally, there have been numerous reports of cells escaping the apoptotic effects of various shRNAs after receiving only a low dose, for example with shRNAs directed against the Bcr-Abl fusion (Scherr *et al.* 2005, and see chapter 4). This response to lower doses of retinoic acid would allow regulation of those transduced cells that escaped the cell death triggered by higher doses of shRNA. This thesis represents the first report of transduced malignant cells returning to a phenotype that responds to lower doses of cellular signalling.

With regard to the design of the anti-PML-RARα shRNA used in chapter 5, the effective shRNA was related to a longer and, therefore, less suitable hairpin design used by Oshima *et al*. (2003). There was, however, another hairpin design that was tested in this study. This second design was based upon an siRNA design published by Damm-Welk *et al*. (2003) which was derived from an siRNA design algorithm. This design was tested here for the first time, in the form of an shRNA molecule, however this shRNA design was found to be ineffective in down-regulating PML-RARα mRNA levels. The implications of these findings for shRNA design are discussed further below. The effective anti-PML-RARα shRNA design detailed in Chapter 5 was a novel design and was shown to be effective in down-regulating levels of the target mRNA, as well as inhibiting proliferation and/or restoring sensitivity to retinoic acid, in a dose-dependent manner. From these results it was concluded that retransfusion of the

marrow stem cell pool with transduced cells bearing this shRNA may provide additional therapeutic options for the treatment of myeloid leukaemias, whether alone or in combination with existing therapies.

The RUNX1-ETO chromosomal translocation is one of the most common causes of acute myeloid leukaemias (Rowley 1984). While the oncogenic mechanism has yet to be fully detailed, the key action of the fusion protein appears to be the conversion of the transcriptional activator, RUNX1, into a transcriptional repressor (reviewed in Elagib and Goldfarb 2007). Transfection of the Kasumi cell line with siRNAs targeted against the RUNX1-ETO fusion gene carried by these cells has been shown to result in down-regulation of the fusion mRNA and protein with a resultant impaired cell proliferation and enhanced sensitivity to differentiation agents (Heidenreich *et al.* 2003; Martinez *et al.* 2004). In Chapter 6 a series of shRNA designs was generated using siRNA design algorithms, and delivered to Kasumi cells via lentiviral transduction. In total, three shRNA designs were tested, including one with strong homology to the siRNA design validated previously (Heidenreich *et al.* 2003). Unfortunately, standard assays of RUNX1-ETO mRNA levels and for the expected downstream biological effects of RUNX1-ETO down-regulation showed no beneficial effect from any of the three shRNAs tested. Thus the siRNA design algorithms again failed to identify an effective shRNA design.

The RUNX1-ETO mRNA has been successfully targeted by stably expressed shRNAs (Fazi *et al.* 2007) was derived from the active empirical siRNA design of Heidenreich *et al.* (2003). Unfortunately the sequence of the shRNA design was not published and so could not be compared to the designs used in this thesis. Such a comparison would be of great value, as differences of only a single nucleotide, not only in the sequence of the shRNA, but in the length of the strands and the size and identity of the overhangs, are crucial to the activity of RNAi molecules (Elbashir *et al.* 2001c; Elbashir *et al.* 2002).

These results further question the value of using current generation siRNA design algorithms for designing active shRNAs (Kolykhalov *et al.* 2005; Li *et al.* 2007). It is hoped that the next generation of design algorithms might be more effective in this role (see below). The results from chapters 5 and 6, involving the anti-PML-RARα and anti-RUNX1-ETO shRNAs, indicate that siRNA designs are often ineffective when

adapted directly to shRNA form, as none of the shRNA designs derived directly from siRNA algorithms, whether generated previously (Damm-Welk *et al.* 2003) or derived *de novo* in the present study, had any effect in the shRNA format. By contrast, the shRNA designs that were effective were not derived from any algorithm, but instead were originally designed by simply placing the chromosomal breakpoint at the centre of the corresponding shRNA (see Oshima *et al.* 2003; Scherr *et al.* 2005). It is clear, therefore, that the first generation siRNA design algorithms are not appropriate for the design of shRNA molecules. Second generation siRNA algorithms, 'trained' by results from large-scale screening of siRNAs and generated by artificial neural networks, are already proving more accurate than the first generation algorithms, which were based on thermodynamic features alone (reviewed Tilesi *et al.* 2009). Given the flexibility of delivery of the shRNA molecules, new algorithms should explicitly distinguish between optimal siRNA and shRNA designs, and generate designs specific for each form of molecule. Alternatively, separate algorithms for siRNA and shRNA design will be required. In any case, the results here highlight the value of empirical testing of all candidate designs as a first experimental step (Reynolds *et al.* 2004).

One of the great attractions of RNAi gene therapy against cancer is the potential to distinguish between normal genes and oncogenic mutants. Development of RNAi molecules against oncogenic mutants, however, must overcome a number of hurdles. Aside from the problems with designing shRNA molecules outlined above, the mutant sequence may simply not be unique. That is, while the sequence may be altered from the wild-type form of the gene, the mutant sequence may be identical to another wild-type gene, making the RNAi design unacceptable. The similarity between RNAi mechanisms and the structure and action of miRNA molecules compounds this problem, as si or shRNA molecules specific to the mutant sequence may still act as miRNA molecules by suppressing, rather than silencing, the normal sequence. The length of the siRNA or shRNAs also means that the necessity of including unique sequence of the oncogene within the 40-nt candidate target area for the siRNA or shRNA greatly reduces the probability of finding an effective design.

Potentially the first candidate application for therapies based on lentiviral vectors expressing shRNAs, however, would be an adaptation of the existing autologous stem cell transplantation protocol as the lentiviral vectors are able to transduce quiescent

cells, 'pretreating' any precancerous cells that can evade conventional therapies. Lentiviral vectors, by inclusion of include marker transgenes may allow selection of transduced cells prior to retransfusion, eliminating untreated cells from the transplanted population. Two such marker transgenes were were examined in this thesis. The use of vectors expressing a fluorescent marker gene allows purification of the population by fluorescence-activated cell sorting, resulting in a pure population of cells stably expressing the marker gene as well as the therapeutic shRNA. Elimination of non-transduced cells by puromycin growth selection also resulted in a pure population of transduced cells expressing the shRNA, however, growth selection would require stimulation of quiescent cells. Aside from complicating the protocol, stimulation of quiescent cells is preferably avoided, as there is a cumulative risk of mutation associated with unnecessary cell division, an effect known as 'proliferative stress'.

Successful retransfusion requires a minimum number of stem cells, however, expansion from a small population of transduced cells results in higher proliferative stress. This stress can be minimised with higher initial rates of transduction. In addition, more efficient vectors, requiring lower multiplicities of infection for the same rate of transduction, also minimise the risk of insertional mutagenesis. Lentiviral vectors typically have high rates of transduction in many commonly used cell lines (Li *et al.* 2003b), however, many leukaemic cell lines, and most primary cells, have proven difficult to transduce using standard lentiviral protocols (Bai *et al.* 2003). Results from Chapter 3 identified a number of techniques normally used in retroviral protocols that usefully increased lentiviral transduction efficiency in a number of recalcitrant cell lines. This improved efficiency minimised the number of cells that need to be transduced to obtain the number of cells necessary for reliable transplantation. This also reduced the need for *in vitro* amplification of the transduced population, thereby minimising the risks of proliferative stress, and so provides methodological improvements that may enhance the safety of gene therapy using integrating vectors.

Another aspect of integrating vector safety, the possibility of insertional mutagenesis, is strongly affected by the locations of the vector integrations. Modulating the targeting of integration might be expected to reduce this risk. There has consequently been much interest in novel integrase enzymes, which it is hoped might modify vector integration profiles in ways that enhance the safety of vector integration

(eg. Hackett *et al.* 2005). One novel integrase, from the Ty3 retrotransposon, was examined in Chapter 7. The Ty3 retrotransposon has an attractive integration profile in the yeast cells that are its natural host. The activity and integration profile mediated by this integrase has not yet been characterised in humans, but if this enzyme retains its native characteristics with respect to the human genome, it would greatly reduce the risk of insertional mutagenesis in human cells. The results from Chapter 7 indicate that Ty3 integrase from this retrotransposon was active in human cells, and facilitated stable integration and expression of a marker transgene. Although no integrations were mapped to PolIII tRNA promoters, probably as a result of technical issues, this integrase activity demonstrates an essential level of interaction between the integrase and the human genome. While the wild-type of this enzyme may ultimately prove inefficient by comparison with existing lentiviral integrase enzymes, the essential activity demonstrated here provides an advanced template for future studies, including possible manipulation of the integrase genetic sequence to optimise integration and targeting in human cells, and thereby lead to safer gene therapy in human cells.

These results suggest that the Ty3 integrase has potential in human gene therapy, and is worthy of further investigation. Specifically, the utilisation of next generation sequencing of genomic integrations should provide information about the integration profile of the native Ty3 integrase in human cells, thus providing a guidepost for future efforts to transfer the desirable characteristics of this integrase into a vector for human gene therapy.

8.1 Future Investigations

The results presented in this thesis represent advancements in many areas of gene therapy and RNA interference, and suggest a number of directions for future research.

The results relating to the anti-PML-RARα shRNA design point to the great potential of this design as an alternate or additional therapy for promyelocytic leukaemia, and one that is worthy of further investigation. Future research should test this design under a range of conditions, and also examine the signalling pathways involved more closely, especially in relation to retinoic acid signalling pathways. As a

candidate for human gene therapy, the next steps for this shRNA and the delivery mechanism would be testing on a larger scale, and experiments with *in vivo* models.

The studies relating to RUNX1-ETO mRNA levels were hampered by unreliable housekeeping genes for the qRT-PCR assay. Future work in this area must begin with identification of an appropriate standard for such assays involving the Kasumi cells. Also, the availability of improved shRNA design tools would invite repetition of these experiments with improved shRNA designs.

The experiments with the Ty3 integrase enzyme showed considerable promise in regard to the potential of this enzyme in human cells. The existing gDNA samples, containing marker gene integrations that were mediated by this enzyme, should be sequenced in order to locate these integrations within the genome. Larger scale sequencing, possibly utilising next generation technologies, would also be valuable. Further studies might focus on manipulating the sequence of the enzyme to optimise efficiency and targeting in the human genome.

8.2 Conclusions

In conclusion, the strategy of cancer gene therapy by lentiviral vector delivery of shRNA molecules has been demonstrated to be sound. The efficacy of the anti-Bcr-Abl shRNA was confirmed, and the mechanism of differentiation was shown to be via the ERK signalling pathway. This thesis has also identified a novel shRNA design that was effective against the PML-RARα fusion gene. This shRNA might potentially be used in therapy for promyelocytic leukaemia, whether delivered by lentiviral vectors or by other methods. No shRNA design was found to be effective against RUNX1-ETO. Any form of RNAi is dependent upon the design and selection of effective siRNA or shRNA designs, and that was the problem here. As such, these results merely highlighted the great need for better shRNA design tools, as distinct from siRNA algorithms.

The strategies explored here will ultimately be contingent upon improvements in vector safety that are currently under development, and were also explored in this thesis. The targeting of integrating vectors has a major impact on their overall safety and the results presented here suggest that the Ty3 integrase might form the basis for a modified integrating vector that can greatly enhance the safety of vector integration.

While the applications of these strategies in cancer gene therapy are not yet ready for the clinic, they remain as promising options in need of further exploration, especially where alternatives do not exist.

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Appendices

Table A-5.1 Data used in Figures 5.4 and 5.5.

	48 hours			72 hours	
Date	NS	PR2		NS	PR2
2/12/2007	0.000791	0.00048		0.000572	0.000141
22/2/2007	0.012045	0.010823		0.011711	0.007522
3/03/2007	0.009903	0.006368		0.005065	0.004812
8/3/2007 #1	0.009318	0.008862		0.011085	0.006219
8/3/2007 #2	0.010261	0.00417		0.008704	0.004589
Mean	0.008464	0.006141		0.007428	0.004657
StanDev	0.004408	0.004041		0.004635	0.002787
t-test (one-tailed, paired)	p=	0.051673		p=	0.025185

Table A-5.4 – The copy number values in this table were used for Figures 5.4 and 5,5. The numbers in this table are derived from the relative copy number for the PR2 fusion gene, which was normalised over the relative copy number of the $\beta 2M$ housekeeping gene.

Table A-5.2 Data used in Figures 5.17 and 5.18

Sample ID	0 ATRA	NB4	sNS	sPR2	2NS	2PR2
3-7A	216518.8	124611.9	97026.05	52170.6	76778.56	49029.42
10-7A		150903.1	170790.7	86225.94	155156.4	71269.14
10-7B		132482.7	136036	83119.63	111349.7	59602.11
10-7C		98636.52	70838.8	73830.65	142566.9	60108.88
Average	216518.8	126658.6	118672.9	73836.7	121462.9	60002.39
S.D.		21688.25	43872.67	15374.07	35021.9	9083.267
t-test				0.044746		0.009854

Table A-5.17 – The cell numbers in this table were used for Figures 5.17 and 5,18. The data point for each experiment is the average of four samples.

Table A-5.3 Data used in Figure 5.10

Sample	% G0/G1	% S	% G2/M
NS 48 hrs	47.9	34.1	16.4
PR2 48 hrs	55.6	27.9	13.2
NS 72 hrs	47.9	28.5	18.9

PR2 72 hrs	45.5	30.7	19.3
NS 96 hrs	56.3	27.1	13.8
PR2 96 hrs	51.3	26.4	15.5

Table A-5.3 – This table shows the percentages derived from FACS analysis of transduced cells (as indicated) stained with propidium iodide and gated according to DNA staining intensity into the G0/G1, S, or G2/M phases of the cell cycle.

Table A-6.1 Raw Data used in figures 6.1A, 6.2A and 6.3A

NS 72	A	B	C	D			
B2M	20,360.57	938,264.30	93,094.27	12,120,335.01			
AE	0.01	12,972.92	2.29	397.18	Average	S.D.	
AE/B2M	4.91145E-07	0.0138265 09	2.45987E-05	3.27697E-05	0.0034711	0.0069036	
NS 96							
B2M	396,815.23	258,663.70	134,705.73	3,290,660.33			
AE	151,970.68	38,071.83	5.2	212.94	Average	S.D.	
AE/B2M	0.3829759 26	0.1471865 98	3.86027E-05	6.47104E-05	0.1325665	0.1807752	
AE1 72							
B2M	222,188.81	646,913.92	85,577.93	3,965,149.88			
AE	2,681.56	382,411.12	1.59	44.82	Average	S.D.	t-test (vs. NS)
AE/B2M	0.0120688 35	0.5911313 83	1.85796E-05	1.13035E-05	0.1508075	0.2936042	0.1898328
AE1 96							
B2M	226,914.63	175,447.69	1.31	2,761,473.78			
AE	2,483.08	13,481.45	0.47	1.45	Average	S.D.	t-test (vs. NS)
AE/B2M	0.0109427 94	0.0768402 82	0.3587786 26	5.25082E-07	0.1116406	0.1682179	0.4489728
AE2 72							
B2M	96,869.71	49,860.96	104,152.87	11,738,582.69			
AE	8.72	1.8	3.09	63.64	Average	S.D.	t-test (vs. NS)
AE/B2M	9.00178E-05	3.61004E-05	2.96679E-05	5.42144E-06	4.03E-05	3.568E-05	0.1968528
AE2 96							
B2M	332,980.76	521,689.72	86,382.17	1,591,611.54			
AE	5,155.46	135,413.97	3.05	0.36	Average	S.D.	t-test (vs. NS)
AE/B2M	0.0154827 56	0.2595680 24	3.53082E-05	2.26186E-07	0.0687716	0.1274064	0.2925847

NS 72	A	B	C1	C2	D1	D2			
B2M	93,094.27	12,120,335.01	12,401.81	15,235.50	8,897.57	9,150.79			
AE	2.29	397.18	8.75	4.06	0.13	0.31	Average	S.D.	
AE/B2M	2.45987E-05	3.27697E-05		0.0004860 13		2.424E-05	0.0001419	0.0002294 38	
NS 96									
B2M	134,705.73	3,290,660.33	17,448.62	14,071.06	7,362.22	32,884.08			
AE	5.2	212.94	15	11.03	0.01	0.07	Average	S.D.	
AE/B2M	3.86027E-05	6.47104E-05		0.0008217 73		1.743E-06	0.0002317	0.0003942 24	
AE3 72									
B2M	Sample lost	10,128,837.05	95,296.92	57,103.77	26,027.16	18,029.32			
AE	Sample lost	397.18	268.16	1,633.92	221.26	300.94	Average	S.D.	t-test (vs. NS)
AE/B2M		3.92128E-05		0.0157135 57		0.0125964	0.0094497	0.0082974 38	0.0935073
AE3 96									
B2M	225,328.29	1,408,180.90	9,065.59	17,367.20	94,409.68	116,252.94			
AE	1.3	0.13	8.75	11.03	175.67	110.74	Average	S.D.	t-test (vs. NS)
AE/B2M	5.76936E-06	9.23177E-08		0.0008001 47		0.0014066	0.0005532	0.0006819 01	0.2195585

Table A-6.1 – This table presents the raw copy number values generated by the RotorGene software from a single set of standard curves. The copy number for both the β-2-microglobulin housekeeping gene (B2M) and the RUNX1-ETO gene (AE) are presented for each replicate (A-D). In an attempt to improve reliability, the final two replicates for the AE3 experiment were done in duplicate (C1, C2 and D1, D2)

and pooled. Each sample was normalised to the housekeeping gene (AE/B2M), and the average and standard deviation (S.D.) calculated. Finally, each AE hairpin treatment sample was compared with the NS control using a t-test.

Table A-6.2 Raw Data used in figures 6.1B, 6.2B and 6.3B

NS 72	A	B	C			
GAPDH	1,103,165.61	###########	###########			
AE	0.01	484,876.16	96,523.70	Average	S.D.	
AE/GAPDH	9.06482E-09	5.06633E-12	1.51979E-11	3.02836E-09	5.23E-09	
NS 96						
GAPDH	###########	###########	###########			
AE	247,484.95	1,740,150.07	725,905.44	Average	S.D.	
AE/GAPDH	1.53662E-11	6.39665E-12	8.61441E-12	1.01257E-11	4.67E-12	
AE1 72						
GAPDH	###########	###########	###########			
AE	1,952.35	21,981.30	56,359.65	Average	S.D.	t-test (vs. NS)
AE/GAPDH	2.10993E-10	1.22296E-12	7.2969E-11	9.50618E-11	1.07E-10	0.213089
AE1 96						
GAPDH	###########	###########	###########			
AE	154,557.96	110,420.66	144,505.10	Average	S.D.	t-test (vs. NS)
AE/GAPDH	3.39209E-13	1.89594E-14	1.36144E-11	4.65752E-12	7.76E-12	0.222623
AE2 72						
GAPDH	###########	###########	###########			
AE	34.52	144,505.10	1,329,700.67	Average	S.D.	t-test (vs. NS)
AE/GAPDH	8.53036E-11	3.87836E-11	3.00276E-12	4.23633E-11	4.13E-11	0.212019
AE2 96						
GAPDH	###########	###########	###########			
AE	34.52	1,861,207.98	888,187.52	Average	S.D.	t-test (vs. NS)
AE/GAPDH	6.52353E-16	3.8694E-14	1.93518E-10	6.4519E-11	1.12E-10	0.246264

NS 72	A1	A2	B1	B2			
GAPDH	###########	###########	53,826,321.80	102,357,928.78			
AE	2.19	3.22	0.19	0.14	Average	S.D.	
AE/GAPDH		7.13593E-08		2.44881E-09	3.69E-08	4.87E-08	
NS 96							
GAPDH	###########	###########	###########	26,260,508.18			
AE	27.74	29.96	0.06	0.81	Average	S.D.	
AE/GAPDH		1.02201E-07		1.54502E-08	5.88E-08	6.13E-08	
AE3 72							
GAPDH	###########	###########	###########	###########			
AE	119.60	7.51	129.15	393.89	Average	S.D.	t-test (vs. NS)
AE/GAPDH		9.49293E-08		2.93579E-08	6.21E-08	4.64E-08	0.02102442
AE3 96							
GAPDH	###########	###########	###########	###########			
AE	1.14	9.45	81.42	15.58	Average	S.D.	t-test (vs. NS)
AE/GAPDH		1.32765E-07		5.01466E-10	6.66E-08	9.35E-08	0.39479553

Table A-6.2 – This table presents the raw copy number values generated by the RotorGene software from a single set of standard curves. The copy number for both the GAPDH housekeeping gene (B2M) and the RUNX1-ETO gene (AE) are presented for each replicate (A-C). In an attempt to improve reliability, the two replicates for the AE3 experiment were done in duplicate (A1, A2 and B1, B2) and pooled. Each sample was normalised to the housekeeping gene (AE/B2M), and the average and standard deviation

(S.D.) calculated. Finally, each AE hairpin treatment sample was compared with the NS control using a t-test.

Figure A-5.1 FACS plot and cell-cycle gating of cells stained with propidium iodide 48 hours after transduction with NS vectors
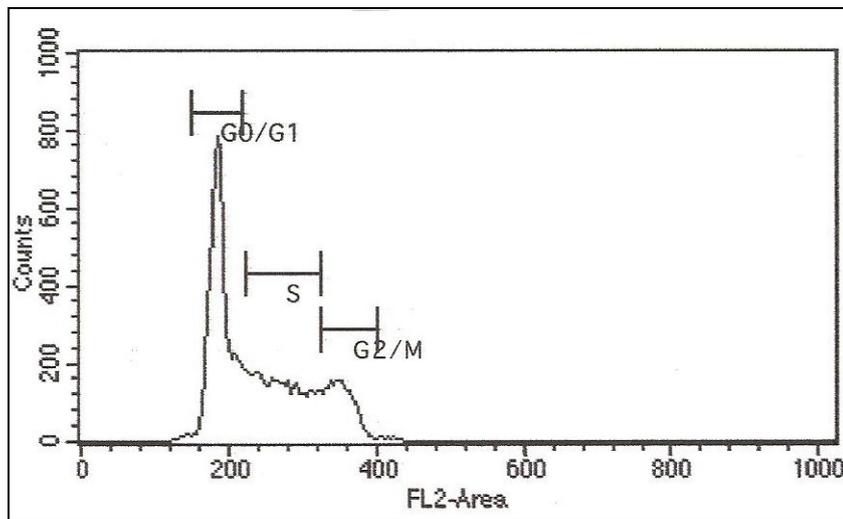

Figure A-5.1 – This FACS plot is derived from cells stained with propidium iodide 48 hours after transduction with NS vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-5.2 FACS plot and cell-cycle gating of cells stained with propidium iodide 48 hours after transduction with PR2 vectors
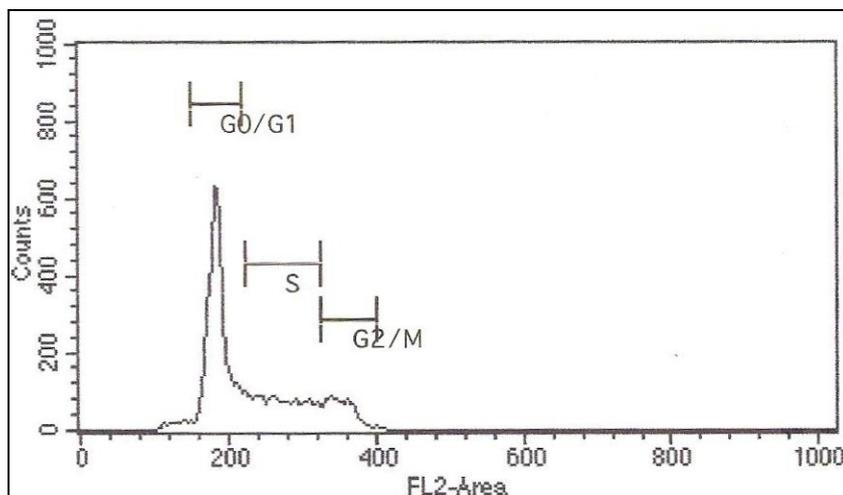

Figure A-5.2 – This FACS plot is derived from cells stained with propidium iodide 48 hours after transduction with PR2 vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-5.3 FACS plot and cell-cycle gating of cells stained with propidium iodide 72 hours after transduction with NS vectors
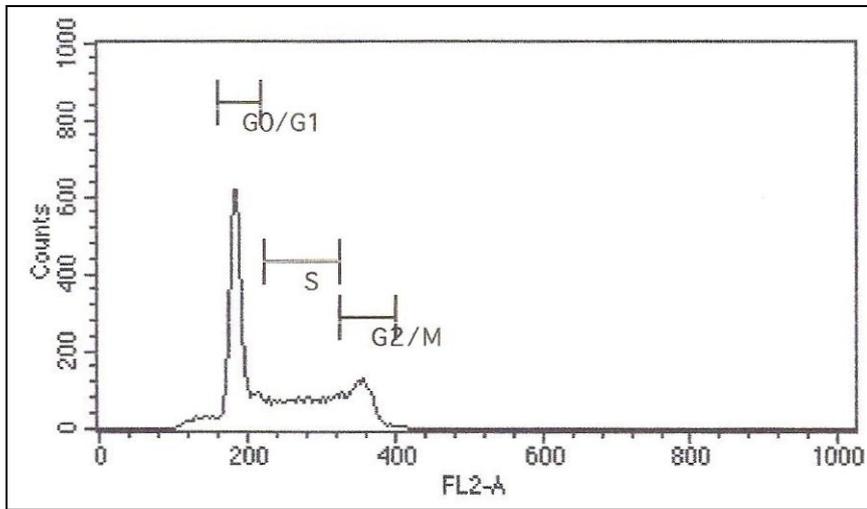
Figure A-5.3 – This FACS plot is derived from cells stained with propidium iodide 72 hours after transduction with NS vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-5.4 FACS plot and cell-cycle gating of cells stained with propidium iodide 72 hours after transduction with PR2 vectors
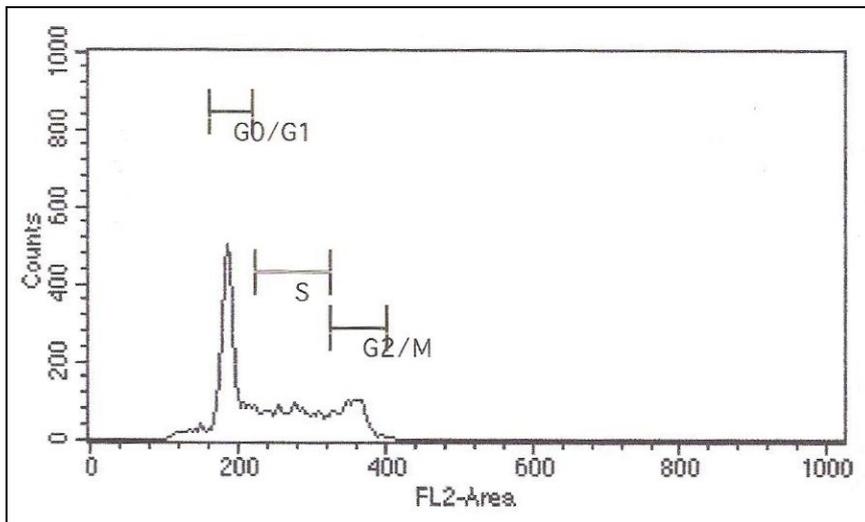

Figure A-5.4 – This FACS plot is derived from cells stained with propidium iodide 72 hours after transduction with PR2 vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-5.5 FACS plot and cell-cycle gating of cells stained with propidium iodide 96 hours after transduction with NS vectors
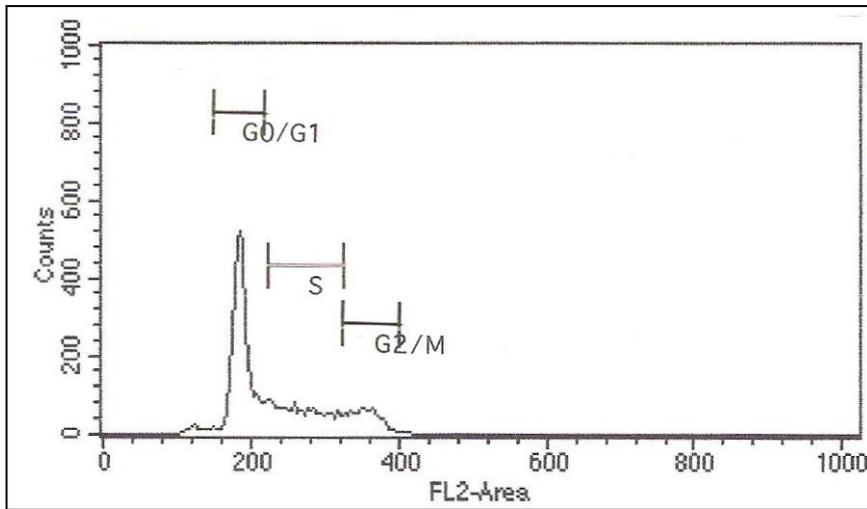
Figure A-5.5 – This FACS plot is derived from cells stained with propidium iodide 96 hours after transduction with NS vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-5.6 FACS plot and cell-cycle gating of cells stained with propidium iodide 96 hours after transduction with PR2 vectors
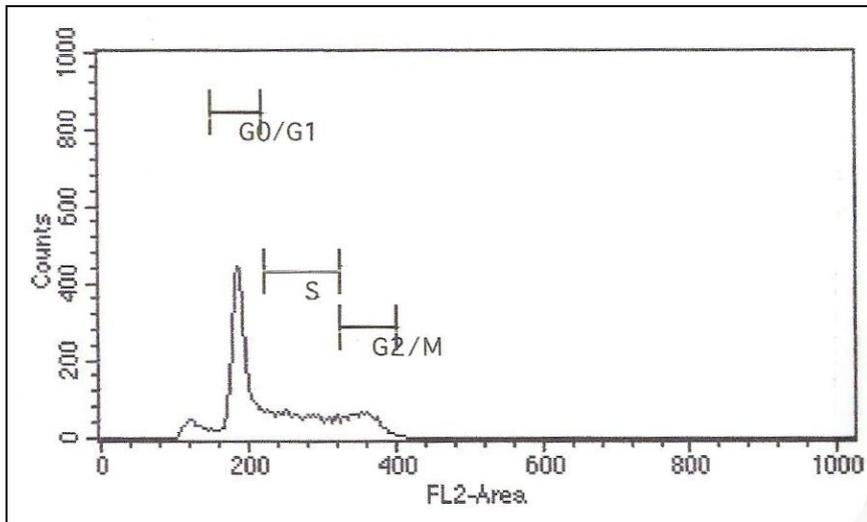

Figure A-5.6 – This FACS plot is derived from cells stained with propidium iodide 96 hours after transduction with PR2 vectors, and gated into the G0/G1, S, or G2/M phases of the cell cycle.

Figure A-7.1 HyperLadder II marker
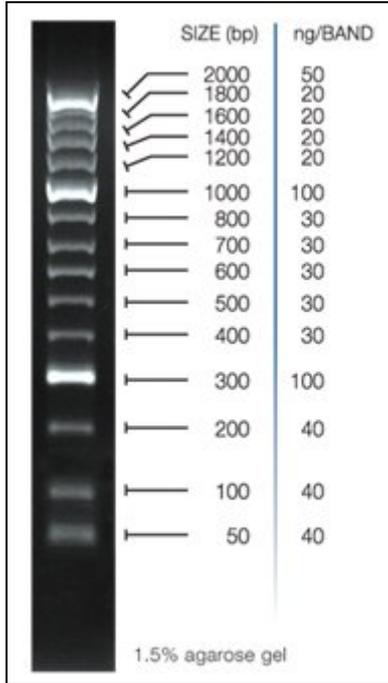
Figure A-7.1 – HyperLadder II DNA size marker (Bioline) used with agarose gels of LAM-PCR products in Figures 7.3 to 7.6 in Chapter 7.