LENTIVIRAL-BASED RNA INTERFERENCE OF GENES IN LEUKAEMIC CELLS

Supervisors: Dr. Hugh Brady, Dr. Jasper de Boer
Student: Wen-Hsin, Sandy Lee

September 2006
**ABSTRACT**

Childhood leukemia is a common pediatric cancer in the developed world and the biologically diverse subtypes of this disease are characterized by specific chromosomal translocations that alter the normal proliferative and survival signals of hematopoietic cells. Despite greatly improved cure rate of childhood leukemias over the past years, younger patients with acute leukemias involving E2A-HLF and MLL-ENL translocations still confer a poor prognosis that is associated with a very unfavorable outcome. The incidence of relapse after complete remission seems to crucially depend on a small population of leukemic stem cells that survive from the initial therapy and sustain the disease.

So far it has been unsuccessful to induce long-term gene silencing using siRNA technology in the primary hematopoietic cell lines and leukemic stem cells. Thus, this project aimed to optimise the current 2nd generation of miR30-based shRNA lentiviral vector to achieve this. The silencing cassettes were delivered to the cells of interest by lentivirus and long-term expression was seen. The result revealed that effective E2A-HLF and MLL-ENL gene silencing was achieved while LMO2 gene expression was not significantly knocked down by the predicted LMO2 shRNA constructs. The most efficient lentiviral vectors against specific genes will then be used to infect leukemic cells to test the effect on aspects of the leukemic phenotype.

Understanding of these fusion genes and identification of their downstream target genes in initiating and maintaining transformation events in the leukemic stem cells may aid the development of revolutionary therapeutics that specifically target leukemic stem cells. In conjunction with standard therapies, this approach could be more effective in treating MLL-ENL and E2A-HLF patients who tend to have an extremely unfavorable prognosis.
ACKNOWLEDGEMENTS

First, I would like to give my heartfelt thanks to Dr Hugh Brady, for all his help and encouragement, advice as well as for providing lab space, material and the idea of this project over these months. Special thanks to Jasper de Boer for his patience, support, guidance and experimental assistance. Many thanks also to the colleagues in the Molecular Haematology and Cancer Biology Unit for their helpful suggestions and being source of information and protocols. I appreciate Stephen J. Elledge, Didier Trono and Yasuhiro Ikeda for the kind gifts of lentiviral vector p201, psPAX2 & pMD2.G, and CSGW, respectively as well as Jenny Yeung and Tanzina Chowdhury for providing immortalised cell lines.
CONTENTS

ABSTRACT ..............................................................................................................................................................................1
ACKNOWLEDGEMENTS ..........................................................................................................................................................2
CONTENTS ................................................................................................................................................................................3
ABBREVIATIONS ......................................................................................................................................................................6

CHAPTER 1. INTRODUCTION ..................................................................................................................................................9

1.1. HAEMATOPOIESIS .............................................................................................................................................................10
1.2. CHROMOSOME TRANSLOCATIONS IN CHILDHOOD ACUTE LEUKAEMIAS .........................................................12
  1.2.1. E2A-HLF translocation ................................................................................................................................................14
  1.2.2. MLL-ENL translocation ...............................................................................................................................................17
1.3. FAILURE OF CURRENT TREATMENT ............................................................................................................................18
1.4. LEUKAEMIC STEM CELLS ..............................................................................................................................................19
1.5. PREVIOUSLY IDENTIFIED TARGETS OF E2A-HLF .......................................................................................................21
1.6. LENTIVIRAL-BASED RNA INTERFERENCE (RNAI) TECHNOLOGY ..............................................................................21
1.7. OVERVIEW OF PROJECT ..................................................................................................................................................26

CHAPTER 2. MATERIALS AND METHODS ..........................................................................................................................28

2.1. BUFFERS AND SOLUTIONS ..............................................................................................................................................29
2.2. CELL CULTURE .................................................................................................................................................................30
  2.2.1. 293 FT ........................................................................................................................................................................30
  2.2.2. Baf-3, 32D ..................................................................................................................................................................30
  2.2.3. C11, SEMK2 ............................................................................................................................................................30
  2.2.4. E2A-HLF, LMO2/BCL2 primary immortalized haematopoietic cells .................................................................31
  2.2.5. MLL-ENL primary immortalised haematopoietic cells .........................................................................................31
2.3. GENERAL MOLECULAR BIOLOGY TECHNIQUES ........................................................................................................31
  2.3.1. Restriction digests ....................................................................................................................................................31
  2.3.2. PCR-quick purification .............................................................................................................................................32
2.3.3. Alkaline phosphatase treatment..................................................................................32
2.3.4. Gel preparation and running....................................................................................32
2.3.5. UV exposure............................................................................................................32
2.3.6. Gel purification.........................................................................................................32
2.3.7. Generation of blunt ends by Large Klenow Fragment.............................................33
2.3.8. Transformation........................................................................................................33
2.3.9. Ligation....................................................................................................................33
2.3.10. Small-scale plasmid preparation (Miniprep)..........................................................33
2.3.11. Large-scale plasmid preparation (Maxiprep)..........................................................34
2.3.12. Polymerase chain reaction (PCR)..........................................................................34
2.3.13. DNA sequencing for shRNAs................................................................................36

2.4. TRANSDUCTION OF HAEMATOPOIETIC CELLS.........................................................37
2.4.1. Lentivirus generation and harvesting.......................................................................37
2.4.2. Lentivirus infection and titration.............................................................................38

2.5. FLUORESCENCE ACTIVATED CELL SORTING ANALYSIS OF TRANSDUCED CELLS........39
2.5.1. eGFP expression analysis.......................................................................................39
2.5.2. eGFP-positive transduced cell sorting....................................................................39

2.6. DETECTION OF PROTEIN EXPRESSION LEVEL.........................................................40
2.6.1. Protein assay............................................................................................................40
2.6.2 Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).................................................................40
2.6.3. Protein transfer to polyvinylidene fluoride membrane (PVDF) membrane...........41
2.6.4. Western blotting analysis.......................................................................................41

2.7. DETECTION OF mRNA EXPRESSION LEVEL..........................................................42
2.7.1 Isolation of total RNA.............................................................................................42
2.7.2. Detection of E2A-HLF and GAPDH transcripts by RT-qPCR...............................43
2.7.3. Calculation of LMO2 mRNA expression levels relative to the GAPDH............44

CHAPTER 3. RESULTS ....................................................................................................................45

3.1. Low-expression activity of CMV promoter in haematopoietic cells.................46

3.2. Replacement of CMV promoter with SFFV promoter........................................49

3.3. High-level transduction and long-term gene expression in haematopoietic cells using 2nd generation of lentiviral vector p201 incorporating SFFV promoter.................................................................56

3.4. Cloning of shRNAs into lentiviral vector SFFVp201.............................................62

3.5. Virus titration for achieving maximal infection efficiency in haematopoietic cells........................................................................................................................................................................................................64

3.6. Establishment of knockdown efficiency.................................................................70

3.6.1. MLL-ENL protein detection.................................................................................71

3.6.2. LMO2 protein and mRNA detection.................................................................72

3.6.3. E2A-HLF protein detection....................................................................................75

CHAPTER 4. DISCUSSION.............................................................................................................76

4.1. Efficient infection of quiescent cells by HIV-based lentiviral vector........................77

4.2. Low-level activity of CMV promoter in haematopoietic cell lines......................77

4.3. High-level activity of SFFV promoter in haematopoietic cell lines....................78

4.4. Virus titration...............................................................................................................79

4.5. Establishment of knockdown efficiency.................................................................80

4.6. Downstream targets of E2A-HLF............................................................................80

4.7. Future works...............................................................................................................81

CHAPTER 5. CONCLUSION...........................................................................................................83

REFERENCES.............................................................................................................................86

APPENDIX.................................................................................................................................98
ABBREVIATIONS

ALL    acute lymphoblastic leukaemia
AML    acute myeloid leukaemia
bHLH   basic helix-loop-helix
bp     base pairs
bZIP   basic leucine zipper
cDNA   complementary DNA
CMP    common myeloid progenitor
CMV    cytomegalovirus
CLP    common lymphoid progenitor
CMP    common myeloid progenitor
dd H₂O double distilled water
DEPC   diethyl pyrocarbonate
DNA    deoxyribonucleic acid
dNTP   dioxynucleotide triphosphate
dsRNA  double-stranded RNA
DTT    dithiothreitol
EDTA   ethylamine diamine tetra acetic acid
EGFP   enhanced green fluorescent protein
FACS   fluorescence activated cell sorting
FCS    foetal calf serum
Flt-3L fms-like tyrosine kinase-3 ligand
GAPDH  glyceraldehyde-3-phosphate dehydrogenase
GMP    granulocyte-macrophage progenitor
h      hour(s)
HLF    hepatic leukaemia factor
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>HOX</td>
<td>homeobox</td>
</tr>
<tr>
<td>HPC</td>
<td>haematopoietic progenitor cell</td>
</tr>
<tr>
<td>HSPC</td>
<td>haematopoietic/stem progenitor cell</td>
</tr>
<tr>
<td>HSC</td>
<td>haematopoietic stem cell</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>kb</td>
<td>kilobases</td>
</tr>
<tr>
<td>kDa</td>
<td>kilo Daltons</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>MEP</td>
<td>megakaryocyte-erythroid progenitor</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
</tr>
<tr>
<td>Min</td>
<td>minute(s)</td>
</tr>
<tr>
<td>miRNA</td>
<td>microRNA</td>
</tr>
<tr>
<td>MLL</td>
<td>mixed lineage leukaemia</td>
</tr>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>MPP</td>
<td>multipotent progenitor</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NK</td>
<td>natural killer</td>
</tr>
<tr>
<td>PAR</td>
<td>proline and acidic amino acid rich</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PKR</td>
<td>protein kinase RNA-activated</td>
</tr>
<tr>
<td>Pol II</td>
<td>polymerase II</td>
</tr>
<tr>
<td>Pol III</td>
<td>polymerase III</td>
</tr>
<tr>
<td>PPT</td>
<td>polypurine tracts</td>
</tr>
<tr>
<td>PRIME</td>
<td>potent RNAi using iR expression</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene fluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>reverse transcriptase-quantitative PCR</td>
</tr>
<tr>
<td>RCL</td>
<td>replication-competent lentivirus</td>
</tr>
<tr>
<td>RRE</td>
<td>rev-responsive element</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA induced silencing complex</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>SAP</td>
<td>shrimp alkaline phosphatase</td>
</tr>
<tr>
<td>rpm</td>
<td>revolutions per minute</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>sec</td>
<td>second(s)</td>
</tr>
<tr>
<td>SIN</td>
<td>self inactivating vectors</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin RNA</td>
</tr>
<tr>
<td>siRNA</td>
<td>short interfering RNA</td>
</tr>
<tr>
<td>SFFV</td>
<td>spleen focus forming virus</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-acetate EDTA</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris-buffered saline</td>
</tr>
<tr>
<td>TBS-T</td>
<td>Tris-buffered saline-Tween</td>
</tr>
<tr>
<td>TCR</td>
<td>T-cell receptor</td>
</tr>
<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TF</td>
<td>transcription factors</td>
</tr>
<tr>
<td>U</td>
<td>unit(s)</td>
</tr>
<tr>
<td>UV</td>
<td>ultraviolet</td>
</tr>
<tr>
<td>VSV-G</td>
<td>vesicular stomatitis virus glycoprotein</td>
</tr>
<tr>
<td>WPRE</td>
<td>woodchuck hepatitis virus posttranscriptional regulatory element</td>
</tr>
</tbody>
</table>
CHAPTER 1

INTRODUCTION
1.1. HAEMATOPOIESIS

Blood cell formation, termed haematopoiesis, is a exquisitely orchestrated and complicated process in which the generation of mature blood cells occurs from the differentiation and proliferation of a rare pluripotent haematopoietic stem cell (HSC) that resides in the bone marrow (Smith 2003). This allows the continuous replacement of the mature blood cells that have a short life span, sustaining the blood cell production. Haematopoiesis was found to occur in two waves at different organs. Primitive haematopoiesis occurs in the extraembryonic yolk sac followed by definitive haematopoiesis in the fetal liver region (Smith 2003). Primitive haematopoiesis is a transient phase of blood cell development that predominantly generates nucleated erythroid cells while definitive haematopoiesis is characterised by the production of cells of the myeloid and lymphoid lineages (Smith 2003). However, recent studies of yolk sac haematopoiesis have debated over several aspects of this paradigm (Smith 2003).

After birth, the bone marrow replaces the fetal liver as the dominant site of haematopoiesis and the number of HSCs is maintained in steady-state by continuous low-level turnover throughout adult life (Cheshier et al. 1999; Kondo et al. 2003). It is popularly viewed that during haematopoiesis, commitment to either the lymphoid or the myeloid lineage is the first step of lineage restriction from primitive HSCs. As illustrated in the Figure 1.1, the common myeloid progenitor (CMP) are the clonogenic precursors of the granulocyte-monocyte restricted progenitors (GMPs) and the megakaryocyte-erythrocyte restricted progenitors (MEPs) that give rise to granulocytes and monocytes or platelets and erythrocytes whereas common lymphoid progenitor (CLP) develops into T cells, B cells, and natural killer (NK) cells. However, bipotential progenitors have been identified in both murine foetal liver and murine bone marrow which generated B cells and macrophages (Cumano et al. 1992; Montecino-Rodriguez, Leathers, & Dorshkind 2001). More recently, characterisation of multipotent progenitor (MPP) subsets challenges the widely-known model of haematopoiesis and suggests that the divergence of the
lymphoid and myeloid lineages during early hematopoiesis occurs asymmetrically at distinct progenitor stages (Lai & Kondo 2006). Disturbances in normal hematopoiesis have been known to lead to blood disorders including leukemia, characterised by the clonal proliferation of immature hematopoietic blood cells.

![Diagram of hematopoietic stem cell lineages](image)

**Figure 1.1** All the cells of the hematopoietic system originate from HSCs. HSCs differentiate into committed multipotent progenitors, CMPs and CLPs. CMPs give rise to erythrocytes, megaryocytes, monocytes and granulocytes whereas CLPs develop into B and T cells, also NK cells. Cells mature from the CMP through two intermediates, the MEP and the GMP (Janeway & Travers 1997).
1.2. CHROMOSOME TRANSLocations IN CHILDHOOD ACUTE LEUKAEMias

Leukaemia is the most common paediatric cancer and the major morphological subtypes of leukemia, acute lymphoblastic leukaemia (ALL) and acute myeloblastic leukaemia (AML), are characterised by chromosomal translocations involving over 200 genes. Human acute leukaemias arise from blood cell progenitors that are developing in the lymphoid or myeloid pathway or from primitive stem cells with multilineage potential. The biological heterogeneity of childhood leukaemia is well documented (Biondi et al. 2000; Kersey 1997), with the most prevalent type being ALL which accounts for over 80% of all childhood cases in the population (Parkin et al. 1988). ALL is a stem cell disorder, characterised by the clonal proliferation and accumulation of lymphoblasts in the bone marrow that eventually spill into circulation, producing lymphocytosis (Randolph 2004). The precise pathogenetic events leading to the development of ALL are still unknown, but they are likely to affect genes that control lymphoid cell homoeostasis, resulting in dysregulated clonal expansion of immature progenitor cells. It is postulated that in most cases, childhood leukaemias may initially occur at prenatal stage (Bonnet & Dick 1997; Greaves & Wiemels 2003).

Chromosome translocations or other genetic aberrations in the stem cells have been proposed to be responsible for early or initiating transforming events in leukaemogenesis (Bonnet & Dick 1997; Greaves & Wiemels 2003). The process of chromosomal translocation involves the detachment and reattachment of a piece of one chromosome to another chromosome, bring two unrelated genes together. The result, referred to as gene fusion, can wreak havoc on the cell: they can turn off genes that normally direct haematopoietic differentiation, or/and, activate genes that help cell proliferation, leading to uncontrolled growth of immature cells. In childhood acute leukaemias, a large number of genetic alterations have been identified, these provide important diagnostic as well as prognostic implications that can guide the selection of therapy (Greaves & Wiemels 2003). Chromosomal abnormalities are the microscopically
visible indicators of genetic changes, and up to 80% of children with ALL have abnormal karyotypes (Harrison 2000).

The most frequent targets of chromosomal translocations in the acute leukaemias are genes that encode regulatory transcription factors, playing critical role in the control of blood cell development (Shivdasani & Orkin 1996). Two distinct mechanisms by which chromosomal translocations aberrantly activate genes encoding transcription factors (TF) have been elucidated (Figure 1.2) (Look 1997). TF proto-oncogenes are activated by the juxtaposition of a cellular oncogene (e.g. *c-myc* or *BCL-2*) with the promoter of a lineage-specific gene such as an immunoglobulin (Ig) gene (in B-cell malignancies) or a T-cell receptor (TCR) gene (in T-cell malignancies) (Rabbitts 1994). More commonly, the modular structure of transcription factors--including discrete DNA-binding, dimerisation, and trans-effector domains--allows normally unrelated sequences from different chromosomes to be recombined into hybrid genes that encode fusion products with unique properties including constitutive kinase activation (e.g. BCR-ABL and TEL-JAK2), but frequently, alters transcriptional regulation (Look 1997). The chimeric transcription factors (e.g. E2A-HLF, PML-Rarα and TEL-AML1, MLL-ENL) resulting in the altered transcriptional regulation can lead to leukaemic transformation by disturbing stem cell development or lineage specification in haematopoiesis (Rabbitts 1994).
Figure 1.2. Two distinct mechanisms by which chromosomal translocations aberrantly activate genes encoding TF. (A) TF proto-oncogenes that are silent or expressed at low levels in the progenitor cells of a particular lineage may be activated when placed under the control of potent enhancer elements within the regulatory region (R) of a gene that is normally highly expressed. (B) More commonly, chromosomal breakpoints occur within introns, between the coding sequences of each of two transcription factor genes on different chromosomes, producing a fusion gene that encodes a chimeric transcription factor with altered function (Look 1997).

1.2.1. E2A-HLF fusion protein

The E2A-gene-encoded transcription factors, E12, E47 and E2-5 resulting from alternative splicing of exons, have been found to contain the basic helix-loop-helix (HLH) domain, which mediates binding to specific E-box elements, and is relevant in immunoglobulin and T-cell
receptor gene rearrangements during development (Murre et al. 1989). Homodimers of E2A proteins (Class I HLH proteins) are required for the proper E2A function in B cells, whereas tissue-specific Class II HLH proteins, such as MyoD (muscle), Tal1 and Ly11 (T cells), and NeuroD (neurons) heterodimerise with E2A to cooperatively mediate basic HLH functions (Seidel & Look 2001). Furthermore, activity of Class I HLH proteins is antagonised by heterodimerisation with Class V HLH proteins (Id proteins) as they lack a functional basic DNA binding region. E2A serves as a tumor suppressor *par excellence*, mediating essential functions in the regulation of differentiation, maturation, cell fate, and the cell cycle, so that modifications of its expression level tends to contribute in important ways to leukaemogenesis (Seidel & Look 2001). Moreover, several studies have indicated that E2A component appears to be sufficient to trigger oncogenic transformation if mis-expressed and dimerised due to its fusion partner (Bayly & LeBrun 2000; Inukai et al. 1998; Kamps, Wright, & Lu 1996; Monica et al. 1994). *E2A* gene has been reported to involve in transcriptional regulation of cysteine rich LIM-domain-only genes *LMO2*, encodes a nuclear protein that is necessary for embryonic erythropoiesis and for adult haematopoiesis (Grutz et al. 1998). These functions seem to be mediated through participation of LMO2 protein in the transcription factor complex which includes E2A, TAL1, GATA1 and LDB1 in erythroid cells (Osada et al. 1995; Wadman et al. 1997). Within this transcription complex, LMO2 mediates the protein-protein interactions by recruiting LDB1, whereas TAL1, GATA1, and E2A regulate the binding to specific DNA target sites (Grutz et al. 1998). Aberrant expression of LMO2 protein can block early stages of T cell differentiation, which in turn, contributes to the formation of the T cell lymphatic leukaemia. Of note, constitutive expression of LMO2 by itself is not enough for oncogenic transformation, and additional mutation, are required to induce malignant T-ALL clone (Rabbitts 1998).

Hepatic leukaemia factor (HLF) belongs to the PAR (proline and acidic amino acid-rich) subfamily of basic leucine zipper (bZIP) transcription factors which was first identified due to
nature of its fusion to E2A in ALL (Hunger et al. 1992; Inaba et al. 1992). HLF is normally expressed in human brain, lung, liver and kidney and was found to be closely related to the genes that regulate developmental stage-specific gene expression (Hunger, Ohyashiki, Toyama, & Cleary 1992; Inaba et al 1992). HLF is not initially believed to be expressed in haematopoietic and lymphoid cells, however a recent report showed that HLF was expressed in erythroid cells (Crable & Anderson 2003; Hunger et al. 1992; Inaba et al. 1992). Analysis of rat HLF expression revealed that the rat HLF gene encoded two major transcriptional activators HLF43 and HLF36, detected in the liver which had distinct tissue distribution and were expressed in different circadian fashion (Falvey, Fleury-Olela, & Schibler 1995). Furthermore, compelling evidence suggested that HLF regulates normal expression of liver-specific genes in the liver as HLF have different activation potentials for the promoters of the cholesterol 7α hydroxylase promoter, albumin promoter, Factor VIII and Factor IX promoters (Begbie et al. 1999; Falvey et al. 1995; Newcombe et al. 1998). HLF has also been shown to transactivate the LMO2 promoter in reporter assays in an erythroid cell line (Crable and Anderson, 2003).

E2A-HLF, the chimeric fusion protein resulting from the leukaemogenic translocation t(17;19)(q22;p13) gives rise to a distinct but fortunately rare form of high risk pro-B-cell ALL in adolescents, by acting as an anti-apoptotic factor, interfering with an early step in apoptotic signaling (Seidel & Look 2001; Yeung et al. 2006). The oncogenic ability of E2A-HLF has been observed in mice model which has been shown to require at least one of the two trans-activation domains of E2A, as well as the leucine zipper dimerisation domain of HLF (Yoshihara et al. 1995). The E2A-HLF fusion protein contains the N-terminal transactivation domains of E2A joined to the basic region and leucine zipper domain of HLF (Hunger et al 1992; Inaba et al. 1992). E2A-HLF fusion, via chromosomal translocation, might actively employ a survival signaling pathway to perform its oncogenic functions which could be the major leukaemic event in addition to the perturbation of normal E2A function (Seidel & Look 2001).
1.2.2. MLL-ENL fusion protein

The *MLL* (Mixed Lineage Leukaemia or Myeloid/Lymphoid Leukaemia) gene on chromosome 11q23 is frequently involved in chromosomal translocations associated with human acute leukaemias (Ziem-in-van der et al. 1991). These translocations lead to fusion genes generally resulting in novel chimeric proteins containing the amino terminus of MLL fused in-frame to one of about 40 distinct partner proteins, including AF6, AF9, AF10 and ENL (Mitterbauer-Hohendanner & Mannhalter 2004). Abnormalities involving the *MLL* gene are observed in infant leukaemias of myeloid and lymphoid lineage and in treatment-induced secondary leukaemias. In the majority of cases, chromosomal abnormalities involving the *MLL* gene are indicators of very poor prognosis, especially the ones associated with pro-B ALL found in infants (< 1 year). This gene has been shown to code for an unusually large protein (431 kDa), that is a homologue of *Drosophila trithorax* and is involved in homeotic gene regulation (Tkachuk, Kohler, & Cleary 1992). Studies in mice have revealed that disruption of the *MLL* gene abolished homeodomain (HOX) gene expression (Mitterbauer-Hohendanner & Mannhalter 2004). HOX TF genes are highly conserved in mammals, which are important for embryonic development as well as for the regulation of haematopoietic differentiation (Lawrence et al. 1996; Look 1997). A sizable amount of data suggested that MLL is not necessary for the initial regulation of HOX gene expression, but is required for the maintenance of gene expression necessary for embryonic development haematopoiesis (Hanson et al. 1999; Hess et al. 1997; Yagi et al. 1998; Yu et al. 1995; Yu et al. 1998).

In studies of a chimeric protein involving t(11;19) translocation, fusion of the amino-terminal AT-hook motifs of the *MLL* gene on chromosome 11 to a previously undescribed protein (62 kDa) chromosome 19 was identified, named ENL (Tkachuk et al. 1992). AT hooks was discovered to mediate specific binding to the minor groove of AT-rich DNA (Tkachuk et al. 1992). Through them, MLL might stabilise protein-DNA interactions by inducing conformational
changes, which then facilitate the binding of specific factors that regulate target gene transcription (Mitterbauer-Hohendanner & Mannhalter 2004). ENL was shown to function, in both myeloid and lymphoid cells, as a nuclear protein in which the minimal portion of ENL required for the activation of transcription was localised to the C-terminal 90 amino acids (Rubnitz et al. 1994).

A number of studies have demonstrated the potential transforming effects of MLL-ENL by different experimental models. Laveau et al. demonstrated a direct role for MLL-ENL, by retroviral transduction, in the immortalisation and leukaemic transformation of a myeloid progenitor and support a gain-of-function mechanism for MLL-ENL-mediated leukaemogenesis (Lavau et al. 1997). Cells transduced with MLL-ENL induced myeloid leukaemias in syngeneic and SCID mice. Moreover, wildtype ENL or a deletion mutant of MLL-ENL lacking the ENL component did not possess in vitro transforming capabilities. AT hook motifs and methyltransferase homology domain of MLL as well as the carboxyterminal 84 amino acids of ENL, which encode two predicted helical structures, are essential for the full transforming activity of MLL-ENL (Slany, Lavau, & Cleary 1998). It correlates the biological function of an MLL fusion partner with the transforming activity of the chimeric protein.

1.3 Failure of Current Treatment

Combination chemotherapy, involving vincristine, dexamethasone, asparaginase, daunoyubicvin, doxorubicin, methotrexate etc, is the most common regimen in treating the childhood leukaemias. Although optimisation of existing treatment modalities result in long-term survival in approximately 80% of the cases (Pui, Relling, & Downing 2004), the prognosis still remains poor in some subgroups (e.g. involving MLL-ENL and E2A-HLF chromosomal translocations) of patients, owing to the clinical problems like considerable toxicity, morbidity, emergence of secondary complications etc. Clearly, there is a need to identify novel
drug targets whose expression is dysregulated by these translocations, in hope to bring significant improvement in treatment options.

The treatment outcome is age-dependent and infants with ALL have not benefited from the greatly improved treatment regimens that have developed over recent decade. Although morphological complete remission is achieved in the vast majority (c. 95%) of these very young children (Frankel et al. 1997; Reaman et al. 1999), a favourable outcome usually is hampered by an exceedingly high relapse rate. Numerous data suggested that cellular drug resistance seems to significantly contribute to the dismal prognosis of childhood acute leukaemias involving E2A-HLF and MLL-ENL translocations (Dordelmann et al., 1999; Riehm et al., 1987). Although current anticancer therapies have produced dramatic response at the early stage of the treatment by directly against and reducing the bulk of the tumour mass (i.e. the fast dividing cells), a small numbers of slow-/non-dividing cells (known as leukaemia stem cells, LSCs) that are presumably even more resistant than the bulk of the leukaemic cell, as a result of selection, may contribute to the re-emerging leukaemia.

1.4 Leukaemic Stem Cells

LSCs seem to be surprisingly different from the normal blast and are presumed to arise from mutations in normal haematopoietic stem cells or progenitor cells. They are difficult to be eradicated as their quiescent property renders them remain unresponsive to the cell cycle-specific cytotoxic agents commonly used to treat leukemia and survive from the initial therapy, thus contributes to failure of the treatment. LSCs may undergo mutations and epigenetic changes, further leading to drug resistance and relapse. Reflecting this point, most patients who experience a relapse do not survive their disease (e.g. Reaman et al., 1999). Recent data suggest that mature leukemia cells may acquire LSC characteristics, thereby evading chemotherapeutic treatment and sustaining the disease. Thus, therapy directed at stem cell-specific targets, rather than
cancer-specific targets, may offer the best chance of eliminating the LSCs as a new goal of therapy in leukemia.

It is generally accepted that self-renewal is the hallmark property of stem cells in both normal and neoplastic tissues, thus recent research has been in attempt to identify the genes, transcription factors, and cell cycle regulators that modulate the self renewal and differentiation of HSCs by over-expression and knockout experiments. Genes (such as SCL, GATA-2, LMO-2, and AML-1) deregulated in leukaemia are those govern the transcriptional regulation of early hematopoiesis (Ravandi & Estrov 2006). For example, SCL is normally expressed in HSCs and immature progenitors and is down-regulated as differentiation proceeds (Ravandi & Estrov 2006). Aberrant expression of SCL, seen in children T-cell ALL, results from collaboration with other oncoproteins through chromosomal aberrations and initiates malignant transformation (Ravandi & Estrov 2006). Deregulated expression of Hox family members such as HoxA9 is commonly observed in AML (Huntly & Gilliland 2005). The importance of Wnt signaling pathway has been recently determined in the regulation of haematopoietic stem and progenitor cell (HSPCs) functions (Huntly & Gilliland 2005). Activation of Wnt signaling has been shown to expand the transplantable HSC pool in long-term cultures, also, to increases the expression HoxB4 and Notch-1 that are important in HSC self-renewal (Huntly & Gilliland 2005). The Notch/Jagged pathway is important in regulating the integration of extracellular regulatory signals controlling HSC fates (Huntly & Gilliland 2005). Of interest, the gene encoding the Notch receptor was originally identified as the gene rearranged by recurrent chromosomal translocations in some patients with T-cell ALL (Ravandi & Estrov 2006). Other transcription factors and cell cycle regulators associated with oncogenesis, such as Bmi-1 and Sonic hedgehog, may play roles in the regulation of proliferation of both HSCs and LSCs (Huntly & Gilliland 2005). Further studies of these transcription factors in HSCs and the mechanisms causing their deregulation are likely to provide us with better targets for development of disease-specific therapies.
1.5. **PREVIOUSLY IDENTIFIED TARGETS OF E2A-HLF**

The changes in the global expression of genes resulted from chromosomal aberrations and other genetic lesions have been determined by the use of microarray technology. Microarray technology, evolved from southern blotting, enables tens of thousands of genes to be analysed simultaneously in a single experiment to delineate differential expression of genes in the cells that have been subjected to a particular treatment or express a specific protein. The characteristic gene expression profiles for the different chromosomal abnormalities can provide information regarding the underlying biology of the leukaemia and identification of molecular targets for therapeutic intervention. Expression of E2A-HLF has been proposed to alter the transcriptional profile of pro-B cells that carry the t(17;19) translocation. To elucidate the role of E2A-HLF in leukaemic phenotype of ALLs, studies have been previously carried out by Jenny Yeung in Brady's lab (J. Yeung PhD thesis, UCL 2005 and Yeung et al. manuscript in preparation) to identify its downstream targets which may play a vital role in inhibiting apoptosis or other leukaemogenic processes by microarrays.

The chimaeric nature of the E2A-HLF transcription factor suggested that the genes that it regulates are likely to be different to those normally regulated by either E2A or HLF. Microarray studies showed significant changes (>1.3-fold) of 46 genes in pro-B cells expressing E2A-HLF. Of 46 genes, 26 genes were up-regulated by the expression of E2A-HLF, including BCL2 antiapoptotic protein and LMO2 transcriptional factor. The co-expression of both genes have been found to immortalise haematopoietic progenitor cells while either LMO2 or BCL2 expression alone did not confer cell survival.

1.6 **LENTIVIRAL-BASED RNA INTERFERENCE (RNAi) TECHNOLOGY**

While conventional methods (such as generation of knockout and transgenic animals) to decode the function of novel genes still remain laborious, time-consuming, and expensive, RNAi
is rapidly becoming the preferred system for targeted genetic manipulation by inducing posttranscriptional gene silencing in which gene function can be approached on a single-gene or genome-wide level (Hannon 2002; Silva et al. 2005). The RNAi in combined with the lentiviral vector-mediated gene delivery to a wide range of cell types offers great possibility of long-term down-regulation of specific target genes that are abnormally up-regulated in the diseased cells both in vitro and in vivo. Thus, the use of silencing lentivectors allows for a rapid and convenient way of assessing how the potential candidate genes contribute to the development of leukaemia in eukaryotic cells and to further determine if the genes are potential drug target candidates.

RNAi is an evolutionarily conserved mechanism that has been exploited in organisms ranging from plants to fungi to animals, mediates resistance to both endogenous parasitic and exogenous pathogenic nucleic acids, and regulates the expression of protein-coding genes (Hannon 2002). The RNAi machinery can be programmed by exogenous or endogenous sources of double-stranded RNA (dsRNA). RNAi with long dsRNA (500 bp or more) works well for Caenorhabditis elegans and Drosophila melanogaster, in most mammalian cells, however, this provokes a strong cytotoxic response, presumably because of the activation of antiviral responses, which results in the general inhibition of protein synthesis (Hunter et al. 1975). This aspecific interferon response was circumvented when it was discovered that short (<30 bp) synthetic dsRNAs, namely short interfering RNA (siRNA), can trigger silencing of gene expression with exquisite specificity, but small enough to evade significant detection by the interferon responses (Elbashir et al. 2001). Briefly, endogenous RNAi pathway is harnessed by introduction of synthetic dsRNA into the mammalian cell, which is then processed into siRNA with specific structure (short duplexes of about 19–21 nt in length, bearing 2 nucleotide (nt) 3’ overhangs) by cytoplasmic RNase III enzyme Dicer (Fire et al. 1998; Tomari & Zamore 2005). siRNA is subsequently taken up by RNA induced silencing complex (RISC). The antisense strand (complementary to the target gene) of siRNA then associates with the mRNA target for
destruction by Ago 2 nuclease, while the "sense" strand (the strand that has exactly the same sequence as a target gene) is released and degraded (Elbashir et al. 2001; Hamilton et al. 2002; Martinez et al. 2002).

An important development in RNAi technology was the finding that siRNAs could be genetically encoded in an organism by expressing a short hairpin RNA (shRNA), consisting of a sequence of 21-29 nt, a short loop region, and the reverse complement of the 21- to 29-nt region driven by a polymerase III (pol III) such as those that drive expression of the endogenous U6 small nuclear RNA or the H1 RNA (Brummelkamp et al. 2002; Lin et al. 2004; Paddison et al. 2002). When transcribed in vivo, this short transcript folds back on itself to form a hairpin structure, which is converted by endogenous nuclease into short RNAs that are recognised by the RISC and used to target mRNA for destruction. This breakthrough discovery provided the base for the design of 1st-geneartion shRNA plasmids for long-lasting gene silencing in the mammalian cells (Figure 1.3).

More recently, 1st-generation shRNA design has been optimised to achieve highly efficient target gene knockdown by creating an expression cassette that embeds the sequence of shRNA into the larger fold of a ubiquitous microRNA (miRNA), thus shRNA is processed by the endogenous-like miRNA to perform gene silencing (Figure 1.3). During the process, miRNAs are initially transcribed by polymerase II (pol II) as long pre-miRNA and then processed by the nuclear RNase III enzyme Drosha to give the ~60 nt pre-miRNA hairpin intermediate, which are exported to the cytoplasm (Cullen 2006). The subsequent processing steps of pre-miRNAs involving Dicer cleavage and RISC-mediated mRNA degradation are indistinguishable to that of shRNAs in human cells. Since the design of miRNA-based shRNA mimics endogenous trigger of RNAi pathway, 2nd generation siRNA vector containing pol II promoters for expression of shRNAs in the context of miRNA precursors can result in the production of a mature siRNA with continuous expression and subsequent degradation of the target mRNA, thus stable gene
knockdown can be established (Grishok et al. 2001; Hutvagner et al. 2001). 2nd generation siRNA design has been experimentally proven to outperform the 1st generation (Stegmeier et al. 2005).

Figure 1.3. Design of 1st and 2nd generation of lentiviral-based siRNA vectors. In the 1st generation, the expression cassettes have 19-29 nt stem loop shRNA within minimal flanking RNA sequence and are traditionally expressed from pol III promoters, such as U6 whereas hCD2 surface marker is expressed from another promoter. In contrast, 2nd generation design contains the naturally occurring primary miRNA precursors fold extensively beyond the core stem-loop shRNA structure, which is co-expressed with eGFP under the control of the high-copy Pol II promoter. Unlike 1st generation, promoter in 2nd generation can be changed with alternative promoter.

Over the past decade, considerable progress has been accomplished in the design of gene delivery vehicles based on HIV-1, the best characterised of the lentiviruses. The HIV-1 genome contains 9 open reading frames encoding at least 15 distinct proteins involved in the infectious cycle, including structural and regulatory proteins. In addition, there are a number of cis-acting elements required at various stages of the viral life cycle. These include the long
terminal repeats (LTRs), the TAT activation region (TAR), splice donor and acceptor sites, packaging and dimerisation signal (\(\Psi\)), Rev-responsive element (RRE) and the central and terminal polypurine tracts (PPT) (Salmon & Trono 2002). Lentiviral vectors derived from HIV-1 offer several advantages over other vectors, including stable integration into the host-cell genome, effective infection of a wide variety of dividing and nondividing cells, resulting in sustained expression of the transgene (Kafri et al. 1997; Miyoshi et al. 1997; Miyoshi et al. 1999; Naldini et al. 1996; Uchida et al. 1998). Thus, lentiviral-based RNAi experiment is becoming a useful to effectively transduce quiescent cells and induce long-term gene silencing (Figure 1.4).

**Figure 1.4. Lentiviral infection and expression in the mammalian RNAi experiment.** The HIV-1 like virus enters the cell cytoplasm by a process in which the viral envelope merges, via recognition of receptors/co-receptors displayed on the host cell surface, with a host cell
membrane while viral RNA are released into the cells and imported into the nucleus compartment where the viral RNA are converted to viral DNA by HIV enzyme reverse transcriptase. Viral transfer vector used to generate the HIV-1 like virus has been self-inactivated, thus, they are replication-defective. Upon stable integration of viral DNA into host cell genome, two modes of RNAi action i.e. siRNA and miRNA can occur, depend on whether 1st generation or 2nd generation shRNA vectors, respectively, is used. With 1st generation, shRNA duplex derived from the host genome is exported, by Exportin5, to the cytoplasm where the loop is cut by Dicer (blue). Duplex siRNAs are unwound by an ATP-dependent helicase (orange) and one strand (red) is incorporated into a large multiprotein nuclease complex, RISC, while the other strand (black) is removed. Incorporation of small RNA activates RISC (green). Guided by the antisense strand of siRNA, the Ago2 endonuclease (pink) in the activated RISC recognises and cleaves the corresponding mRNA (brown), with a resultant decrease in the steady-state levels of mRNA. With 2nd generation vector, the individual miRNA-containing hairpin precursor (pre-miRNA) is excised from the primary miRNA by Drosha in the nucleus that is then exported to the cytoplasm. The subsequent pathway of miRNA action mode (2nd generation vector) in the cytoplasm is indistinguishable to that of siRNA (1st generation vector).

1.7 Overview of Project

Altered gene expression resulted from chimeric fusion proteins, such as E2A-HLF and MLL-ENL, is associated with the subtypes of childhood acute leukaemias. Despite of the overall cure rate has significantly increased to nearly 80%, the subtypes of childhood acute leukaemias characterised by E2A-HLF and MLL-ENL translocations are still of poor prognosis. Thus much effort has gone towards identifying the novel drug candidate targets that either exhibit unwanted or inadequate activity in the diseased tissues, to improve the current treatment options. Brady, H.J.M. and his colleagues have previously identified, by Affimetrix experiment, a number of candidate target genes whose expression is up-regulated by certain translocations,
including LMO2 and BCL2 by E2A-HLF in pro-B cells. They also compared the effectiveness of the 1st and 2nd generation of lentiviral vectors (Figure 1.3) in establishing a knock down phenotype. In first generation vector, shRNA expression driven by pol III (U6) promoter is located upstream of the hCD2 surface marker that is expressed from a separate promoter, SV40. On the other hand, shRNA and expression marker, eGFP (enhanced green fluorescent protein) are transcribed from the same promoter, CMV (human cytomegalovirus immediate-early). In contrast to 1st generation vector, 2nd generation vector is a miRNA-based shRNA vector in which shRNA is embedded in a miRNA fold like endogenous miRNA-30, more efficient knockdown was observed. An added bonus of the 2nd generation vector is the ease of cloning that allows quick insertion of any shRNA into the miRNA fold, together with a promoter of choice (i.e. pol II CMV promoter can be changed with other promoters). They discovered that both 1st and 2nd generation vectors could not achieve long-term expression in primary haematopoietic cells, thus it was a must to change the promoter for more effective knockdown. Since pol III promoter in the 1st generation vector can not be changed, 2nd generation vector was preferred for the RNAi experiment in the primary haematopoietic cells.

The aim of this project was to establish long-term gene silencing in HSCs and primary haematopoietic cells with the use of re-engineered 2nd generation of lentiviral vector as well as to establish knock down efficiency in the primary immortalised haematopoietic cell lines. The strategy involved replacing the CMV with a more effective promoter and then cloned the miR30-based shRNAs that specifically target LMO2, HLF, MLL and ENL genes into the vector. Not all rationally designed shRNAs will knock down gene expression at the same level, and therefore, the knockdown efficiency of the shRNA vectors generated were evaluated and established by western blot and RT-qPCR to detect the expression levels of exogenous protein and mRNA, respectively.
CHAPTER 2

MATERIALS AND METHODS
Chemicals were purchased from VWR, Poole, Dorset, UK unless otherwise stated.
All tissue culture reagents were from Invitrogen, Paisley, UK unless otherwise stated.
Enzymes were purchased from Promega, Madison, Wisconsin, USA.
Oligonucleotides were purchased from Sigma Genosys, Haverhill, UK.
All recombinant cytokines were purchased from Peprotech EC, London, UK.

2.1. BUFFERS AND SOLUTIONS

Table 2.1. Buffers and solutions

<table>
<thead>
<tr>
<th>Buffer or solution</th>
<th>Final concentration of components</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luria-Bertani (LB)-agar</td>
<td>LB broth medium plus 15 g/l agar.</td>
</tr>
<tr>
<td>LB broth</td>
<td>1.0% tryptone, 0.5% yeast extract, 1.0% NaCl (pH 7.0).</td>
</tr>
<tr>
<td>FACS buffer</td>
<td>0.1% BSA and 0.1% azide suspended in PBS</td>
</tr>
<tr>
<td>RIPA lysis buffer</td>
<td>150 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl (pH 8.0), 5 mM EDTA. One Complete Mini protease inhibitor cocktail tablet (Roche, Lewes, UK) was dissolved per 10 ml lysis buffer.</td>
</tr>
<tr>
<td>2× Sample Buffer</td>
<td>100 mM Tris-HCl (pH 6.8), 4% SDS, 0.2% bromophenol blue, 20% glycerol (Sigma). Dithiothreitol (DTT) was added to protein samples to final concentration of 200 mM.</td>
</tr>
<tr>
<td>SDS-PAGE electrophoresis buffer</td>
<td>25 mM Tris-HCl (pH 7.5), 192 mM glycine, 0.1% SDS.</td>
</tr>
<tr>
<td>Protein transfer buffer</td>
<td>5 mM Tris-HCl (pH 8.3), 192 mM glycine, 20% methanol.</td>
</tr>
<tr>
<td>Tris-buffered saline (TBS)</td>
<td>20 mM Tris-HCl (pH 7.5), 150 mM NaCl.</td>
</tr>
<tr>
<td>Tris-buffered saline –</td>
<td></td>
</tr>
<tr>
<td>Tween 20 (TBS-T)</td>
<td>20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20 (sigma)</td>
</tr>
<tr>
<td>TAE buffer</td>
<td>40 mM Tris-acetate and 1 mM EDTA at pH 8.3.</td>
</tr>
</tbody>
</table>
2.2. **CELL CULTURE**

Cells were maintained at 37 °C with 5% CO₂ in a humidified atmosphere. Viable-cell counts were performed and determined by trypan blue dye exclusion with a Hemacytometer. Complete medium was sterile filtered using 0.22 μm filter (Millipore, Watford, UK) and stored at 4°C until use.

2.2.1. **293 FT**

293 FT cells, human embryonic kidney epithelial cells, are ecotropic lentivirus packaging cell line. They stably and constitutively express the SV40 large T antigen and were cultured in D-MEM, 10% foetal bovine serum (FBS), 0.1 mM MEM Non-Essential Amino Acids (NEAA), 2 mM L-glutamine, 1% Pen-Strep (penicillin, streptomycin) and 0.5 mg/ml Geneticin. Additional 1 mM MEM Sodium Pyruvate was added for virus production.

2.2.2. **Baf-3, 32D**

Baf-3 cells (early precursors of B lymphocytes from murine bone marrow) and 32D cells (murine myeloid cell line) are dependent on the cytokine interleukin-3 (IL-3) for proliferation. They were grown at concentrations of 1 x 10^5 – 5 x 10^5 cells/ml and maintained in RPMI 1640 containing 10% foetal calf serum (FCS) (GlobePharm, Surrey, UK), 10% murine IL-3 supplement, 0.5 mg/ml geneticin, 1% Pen-Strep, and 2 mM L-glutamine.

2.2.3. **C11, SEMK2**

32D-derived C11 cells (MLL-ENL) and human leukemia cell line SEMK2 (MLL-AF4) were cultured in RPMI 1640 medium containing 10% FCS, 1% Pen-Strep and 2 mM L-glutamine. Cultures were maintained by the addition of fresh medium or replacement of medium. To split confluent cells at regular time interval, cell density between 2 x 10^5 and 1 x 10^6/ml cells were used for seeding.
2.2.4. *E2A-HLF, LMO2/BCL2* primary immortalised haematopoietic cells

They are lymphoid lineage cell lines, cultured in IMDM medium containing 10% FCS, 2mM L-glutamine, 100 U/ml penicillin 100 µg/ml streptomycin, 50 µM 2-ME with 100 ng/ml stem cell factor (SCF), 200 ng/ml IL-7 and 10 ng/ml *fms*-like tyrosine kinase-3 ligand (Flt-3-L). To subculture confluent cells at regular time interval (approximately 3 to 4 days), cell density between 1 x 10⁵ and 1 x 10⁶/ml cells were seeded. Cells were cultured until confluent.

2.2.5. *MLL-ENL* primary immortalised haematopoietic cells

It is myeloid lineage cell line, cultured in IMDM medium containing 10% FCS, 2mM L-glutamine, 100 U/ml penicillin 100 µg/ml streptomycin, 50 µM 2-ME with 100 ng/ml stem cell factor (SCF), 10 ng/ml IL-3 and 10 ng/ml IL-6. To subculture confluent cells at regular time interval (approximately 3 to 4 days), cell density between 1 x 10⁵ and 1 x 10⁶/ml cells were seeded. Cells were cultured until confluent.

2.3. General molecular biology techniques

2.3.1 Restriction digests

1 or 5µg of plasmid DNA were digested in a total reaction volume of 20 µl, or 100 µl for diagnostic digests and opening a vector/excising an insert, respectively. 1X restriction enzyme, 10X restriction enzyme buffer (refer to *Promega* catalogue), and 10X BSA were added (if applicable) to the DNA. Sterile double distilled water (dd H₂O) was added to make up the total volume. The digest was incubated at the appropriate temperature (refer to *Promega* catalogue) for 1 hour (h) diagnostic digest or at least 2 h in preparation for a ligation. The efficiency of the digest reaction was checked by loading a tenth of the reaction (or the whole diagnostic digest) onto an agarose gel for electrophoresis.
2.3.2 **PCR-quick purification**

PCR products and vector DNA were purified by *QIAquick PCR Purification Kit (Qiagen)* and eluted in 30 μl dd H2O. Please refer to the manufacturer’s instructions for the detailed protocol.

2.3.3 **Alkaline phosphatase treatment**

2μl of shrimp alkaline phosphatase (SAP) was added to dephosphorylate the 5’ phosphorylated ends of the purified vector DNA (30μl) after the reaction mixture had been heat-inactivated. After incubation at 37 °C for 2 h, the SAP was heat-inactivated for 40 minutes (min) at 65 °C.

2.3.4 **Gel preparation and running**

Appropriate amount of agarose (*Invitorgen*) was heat-dissolved in 150mL 1X TAE buffer (Table 2.1). Ethidium bromide (final concentration 0.5 ug/ml, *Invitrogen*) was added when the gel was hand-warm, then gel poured. Migration was performed under a voltage of ~150V, 20 to 30 min for diagnostic gels and 60 to 90V, 1 to 1.5 h for purification gels. Ready-to-use 1 kb Plus DNA ladder (*Invitrogen*) was added with 10X BlueJuic Gel Loading Buffer (*Invitrogen*) at a concentration of 2X for electrophoresis of DNA standards was loaded on agarose gels to determine the length of the DNA fragments.

2.3.5 **UV exposure**

To visualise the DNA bands, the gels were exposed to UV light and photographed.

2.3.6 **Gel purification**

DNA fragments for ligation were extracted from the gel using the *QIAGEN MinElute Gel Extraction Kit*, eluting the DNA in 20μl of dd H2O. Please refer to the manufacturer’s
instructions for the detailed protocol.

2.3.7 Generation of blunt ends by Large Klenow Fragment

4 µg of DNA (in a volume of 20 µl) was digested with an appropriate restriction enzyme that generated a 5' - overhang, and were then purified by QIAquick PCR Purification Kit. The optimal reaction conditions for filling 5'-protruding ends were: 50 mM Tris-HCl (pH7.2), 10 mM MgSO4, 0.1 mM DTT, 40 µM of each dNTP, 20 µg/ml acetylated BSA and 1 unit of Klenow Fragment per microgram of DNA. The reaction was incubated at room temperature for 10 min, and stopped by heating for 75 °C for 10 min.

2.3.8 Ligation

The ligation reactions were set up with 100ng of dephosphorylated vector. Purified insert was added according to the vector/insert molar ratios of 1:3 and a control reaction for SAP activity with a ratio of 1:0. Ligation reactions were set up with the vector DNA, insert DNA and 2µl 10X ligation buffer, adjusted to 20 µl with dd H2O, 1 µl of T4 DNA ligase was added and left for 2-4 hours at the room temperature or at 16°C over night.

2.3.9 Transformation

2 µl of a ligation reaction was gently mixed with 50 µl competent E. coli (strain: DH5α, STBL3, Invitrogen) and incubated on ice for 5 to 30 min. Cells were heat shocked at 42 °C for 30 seconds (sec). 250 µl room temperature SOC medium was added to the cells and incubated at 37 °C for 1 to 2 h with vigorous shaking. 100 µl to 200 µl of bacterial culture was plated on to LB-agar plates (Table 2.1) containing ampicillin (50 µg/ml) and incubated at 37°C overnight.

2.3.10 Small-scale plasmid preparation (Miniprep)

5 ml of LB broth (Table 2.1) containing ampicillin (50 µg/ml) was inoculated with a
bacterial colony and cultured overnight at 37 °C with vigorous shaking. Plasmid DNA was extracted from 1.5 ml of bacterial culture using QIAprep Spin Miniprep kit (Qiagen, Crawley, UK) according to manufacturer’s instructions. DNA was eluted in 50 μl of sterile dd H2O and stored at -20 °C.

2.3.11 Large-scale plasmid preparation (Maxiprep)

3 ml of LB broth containing ampicillin (50 μg/ml) was inoculated with a bacterial colony or left-over culture from Miniprep and cultured for 6 h. 400 ml of LB broth containing ampicillin was inoculated with 3 ml of the starter culture and cultured overnight at 37 °C with vigorous shaking. Plasmid DNA was extracted from 2 ml of bacterial culture using a Plasmid Maxi kit (Qiagen) according to manufacturer’s instructions. DNA was eluted in 500 μl of dd H2O.

2.3.12. Polymerase chain reaction (PCR)

PCR was used to incorporate XhoI/EcoRI flanking restriction sites to the hairpin oligo prior to cloning into appropriate vectors. PCR was carried out in a 50 μl reaction volume containing 100 ng template oligo (Table 2.2, Sigma), 1× thermoPol Buffer (NEB), 0.2 mM deoxynucleotide triphosphate (dNTP) mix (Promega), 2 mM MgSO4, 0.5μM of pSMS2C forward and reverse primers (Table 2.3) and 1 U VENT polymerase (NEB). All components were added on ice. Cycling conditions were 94 °C for 5 min, 12 cycles of 94 °C for 30 sec, 54 °C for 30 sec and 75 °C for 32 sec followed by final extension at 75°C for 2 min. Following PCR amplification, PCR products were purified with PCR purification columns (QIAquick PCR Purification Kit) and eluted with 50 μl elution buffer.
<table>
<thead>
<tr>
<th>Oligo name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>miRNA_ENL.1</td>
<td>TGC TGT TGA CAG TGA GCG CGC GAG AAG CTC ACC TTC AAC ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT GTT GAA GGT GAG CTT CTC GCA TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_ENL.2</td>
<td>TGC TGT TGA CAG TGA GCG AGC AAA GAC TCC GAG AGC AAG ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT CTT GCT CTC GGA GTC TTT GCG TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_ENL.3</td>
<td>TGC TGT TGA CAG TGA GCG AGC CAC TTC AAT GTC ACC AAC ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT GTT GGT GAC ATT GAA GTG GCC TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_mLMO2_2</td>
<td>TGC TGT TGA CAG TGA GCG ACC CAC AGA TTG TTT CTA TAC ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT GTA TAG AAA CAA TCT GTG GGG TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_mLMO2_4</td>
<td>TGC TGT TGA CAG TGA GCG CTG GAC TAG AGTGT GCA ATT ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT AAT TGC ACA ACT CTA GTC CAT TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_HLF_1</td>
<td>TGC TGT TGA CAG TGA GCG CGC AAG AAC ATA CTT GCC AAG TTA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAA CTT GGC AAG TAT GTT CTT GCA TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_HLF_2</td>
<td>TGC TGT TGA CAG TGA GCG CGA ACA TAC TTG CCA AGT ATG ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT CAT ACT TGG CAA GTA TGT TCT TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_HLF_3</td>
<td>TGC TGT TGA CAG TGA GCG CGG GCA AGG CGC AGA A AG AAC ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT GTT CTT TCT GCG CCT TGC CCA TGC CTA CTG CCT CGG A</td>
</tr>
<tr>
<td>miRNA_HLF_4</td>
<td>TGC TGT TGA CAG TGA GCG AGC TGG GCA AAT GCA AGA ATA GTG AAG</td>
</tr>
<tr>
<td></td>
<td>CCA CAG ATG TAT TCT TGC ATT TGC CCA GCT CCT TGC CTA CTG CCT CGG A</td>
</tr>
</tbody>
</table>
Table 2.3 PCR primers

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSM2C Forward</td>
<td>5'-GATGGCTG-CTCGAG-AAGGTATAT-TGCTGGACAGTGAGCG-3'</td>
</tr>
<tr>
<td>pSM2C Reverse</td>
<td>5'-GTCTAGAG-GAATTC-CGAGGCAGTAGGCA-3'</td>
</tr>
<tr>
<td>EF1-α Forward</td>
<td>5'-GAATGCAATAATGGCTCCGTGCCCGTC-3'</td>
</tr>
<tr>
<td>EF1-α Reverse</td>
<td>5'-CTGCTAGCTACGACACCTGAATGGAAG-3'</td>
</tr>
</tbody>
</table>

Table 2.4 Sequencing Primer for shRNA

<table>
<thead>
<tr>
<th>Primer name</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>pSM2sequencing</td>
<td>5'-ACAGAATCGTTGCCTGCACA-3'</td>
</tr>
</tbody>
</table>

2.3.13. DNA sequencing for shRNAs

The samples for performing cycle sequencing were prepared by mixing 1.5 μl of DMSO, 1 μl of primer (3.2 pmol, Table 2.4), 6μl of Terminator Ready Reaction Mix (Applied Biosystems), and 6.5 μl of DNA (200-500ng) to give a final volume of 15 μl per reaction.

The conditions used to sequence DNA were 25 cycles of 94 °C for 30 sec, 50 °C for 15 sec and 60 °C for 4 min. Following this, the extension products were purified. Briefly, each extension reaction was mixed with 80 μl of 75% isopropanol in 1.5 ml microcentrifuge tube and
left at room temperature for 15 min to precipitate the extension products. They were then spun for 20 min at maximum speed and the supernatants were aspirated carefully. 250 µl of 70% ethanol was added to the DNA pellets and were then washed by spinning for 10 min at maximal speed. Supernatants were carefully aspirated, allowed the samples to dry. The sample pellets were suspended in 3 µl of sequencing loading buffer (containing deionised formamide and EDTA with blue dextran at 5:1 ratio), and heated at 100 °C for 2 min before carrying out sample electrophoresis. Please refer to the manufacturer’s instructions for the detailed protocol.

2.4. Transduction of Haematopoietic Cells

2.4.1. Lentivirus generation and harvesting

The evening before transfection, 293 FT cells were subcultivated and plated in a 10 cm tissue culture dish such that they could be transfected at 90% to 95% confluency (i.e. 4 x 10^6 cells in 10 ml of growth medium containing serum). On the morning of the day of transfection, culture medium was removed from the 293 FT cells and was replaced with 5ml of appropriate growth medium supplemented with serum, but no antibiotics was contained. In the evening, transient transfections in 293 FT cells were carried out using the thee-plasmid system with Lipofectamin 2000 (Invitrogen) transfection reagent. For each transfection sample, DNA-Lipofectamine 2000 complexes were prepared by gently mixing 5 µg of transfer vector (viral plasmid), 3.75µg of packaging plasmid (psPAX2; a gift from Trono Didier, Laboratory of Virology and Genetics, Switzerland) and 1.5µg of envelope plasmid (pMD2G; a gift from Trono Didier, Laboratory of Virology and Genetics, Switzerland) with 1.5 ml of Opti-MEM medium (Invitrogen). In a separate sterile tube, 30 µl of Lipofectamine 2000 was diluted in 1.5 ml of Opti-MEM medium without serum, and was gently mixed and incubated for 5 min at room temperature. Following this, diluted DNA with the diluted Lipofectamine 2000 were combined and gently mixed. DNA-Lipofectamin 2000 mix was then incubated at room temperature for 20
min at room temperature to allow the DNA Lipofectamine 2000 complexes to form. 3ml of DNA-Lipofectamine 2000 complexes were added drop-wise to each plate of 293 FT cells and incubated overnight at 37 °C/5% CO₂ incubator. the next day, medium containing the DNA-Lipofectamine 2000 complexes were removed and replaced with complete culture medium containing sodium pyruvate, providing energy for viral production. At 48 h post-transfection, virus-containing supernatants were harvested and cleared of cellular debris by low-speed centrifugation (1200 rpm for 5 min at 4°C). Viral particles were filtered through 0.45μm membrane and concentrated using Centricon® Plus-70 unit 10-fold by centrifugation at 1500 rpm at 15 °C for 30-45 min and resuspended in 2 ml of DMEM medium containing 10% FCS, 2 mM, L-glutamine and 100 U/ml penicillin 100 μg/ml streptomycin.

2.4.2. Lentivirus infection and titrating

To establish knockdown efficiency, immortalised MLL-ENL and primary immortalised LMO2/BCL2 and E2A-HLF haematopoietic progenitor cells were cultured in a 6-well plate in medium containing recombinant murine cytokines for 24 h prior to infection. For lymphoid cell assays, cells were cultured in DMEM medium containing 10% FCS, 2mM, L-glutamine, 100 U/ml penicillin 100 μg/ml streptomycin, 50 μM 2-ME with 100 ng/ml stem cell factor (SCF), 200 ng/ml IL-7 and 10 ng/ml fms-like tyrosine kinase-3 ligand (Flt-3-L). For myeloid assays, cells were cultured in DMEM medium containing 10% FCS, 2 mM L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin with 100 ng/ml SCF, 10 ng/ml IL-3 and 10 ng/ml IL-6.

Infections were performed in 96-well plates (1 × 10⁴ cells/100 μl/well) with concentrated lentivirus resuspended in DMEM medium containing 5 μg/ml polybrene (Sigma), 20% FCS, L-glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-ME and appropriate cytokines. Presence of polybrene during virus adsorption enhanced transduction by acting as a
small neutraliser that binds to the negatively charged cell surfaces and thus allows the viral glycoproteins to bind more efficiently to their receptors by reducing the repulsion between sialic acid-containing molecules. HPCs were spininfected by centrifugation at 1800 rpm at 25 °C for 45 minutes. Fresh medium containing appropriate cytokines was added to infected cells 24 h after infections.

To achieve maximal infection efficiency, MOI (Multiplicity Of Infection = ratio of infectious virus particles to cells) was approximated by titrating different viral supernatants on BAF3 by 5 fold serial dilutions while SEM2K, 32D, C11 and LMO2/BCL2 cells were infected, by serial dilutions, with the same virus. MOI was estimated from mathematical calculation and line graph.

2.5. FLUORESCENCE ACTIVATED CELL SORTING (FACS) ANALYSIS OF TRANSduced CELLS

2.5.1. eGFP expression analysis

Transduced cells were removed from the flat-bottom 96 well plate to the conical-bottom 96 well plate and were then washed with PBS by centrifugation for 5 min at 1200 rpm. Following the removal of PBS, the transduced cells were resuspended in FACS buffer (Table 2.1) in the FACS tubes. FACS was performed using Beckman-Coulter Epics ALTRA flow cytometer and expression of eGFP was analysed using accompanying software, which revealed percentage of GFP positive cells, mean fluorescence intensity (MFI) of the GFP positive cell population and cell viability. Appropriate uninfected controls were used for all FACS analyses.

2.5.2. eGFP-positive transduced cell sorting

The transduced cells were collected in the FACS tubes, and suspended in the FACS buffer before carrying out cell sorting analyses using DakoCytomation CyAn ADP flow cytometer. The infected cells were separated from the uninfected cells and were collected according to the
expression of GFP. The eGFP-positive cells (1x10^6 cells) were plated and grown in the appropriate medium.

2.6. Detection of Protein expression level

2.6.1. Protein assay

BSA (Promega) was used to prepare serial 1:2 dilution of protein standard starting from 10 μg/μl to 0.156μg/μl. 2 μl of protein sample or protein standard was added to 500 μl of 1 X BioRad protein assay reagent in each 1.5 ml tubes. 150 μl of mixture was transferred into a well of a 96-well microtitre plate. Absorbance was measured at 595 nm and proteins were quantified using BioRad GS-800 densitometer and software.

2.6.2. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

Cells were centrifuged at 1200 rpm for 5 min at 4 °C to remove the medium, then washed twice in cold PBS and lysed in RIPA lysis buffer (Table 2.1) containing protease inhibitors (Roche). Cells were transferred into a 1.5 ml tube and left on ice for 30 min before another centrifugation at 13.2 k rpm for 10 min at 4°C to remove the cellular debris. Supernatant were transferred into a fresh tube and stored at -20 °C for short term storage or at -80°C for long term storage.

Protein concentration of cell lysates was determined from the protein standard curve by protein assay. To prepare sample for SDS-PAGE, appropriate amount of lysis buffer was added to 10 μg protein from the cell lysate to give a final volume of 10 μl, mixed with an equal volume of 2X sample buffer (Table 2.1). Samples were boiled for 5 min at 100°C before loading on to a SDS-PAGE gel.

Cell lysates containing 10μg protein was resolved by electrophoresis in 10% SDS-PAGE gels. SDS-PAGE was carried out using Mini Protean III apparatus (BioRad) according to
manufacturer's instructions. 10% resolving gels contained 10% acrylamide (National Diagnostics, Atlanta, Georgia, USA), 375 mM Tris-HCl (pH 8.8), 0.1% SDS, 0.1% ammonium persulphate (Sigma) and 0.4% N, N, N', N'-tetramethylethylenediamine (TEMED) (Sigma). Stacking gels contained 5% acrylamide, 125 mM Tris-HCl (pH 6.8), 0.1% SDS, 0.1% ammonium persulphate and 0.1% TEMED. Gels were electrophoresed in SDS-PAGE electrophoresis buffer (Table 2.1) at 80 V.

2.6.3. Protein transfer to polyvinylidene fluoride membrane (PVDF) membrane

Proteins in SDS-PAGE gels were transferred on to PVDF membrane (Millipore) in protein transfer buffer (Table 2.1) using a BioRad Mini Trans-Blot Electrophoretic Transfer system according to the manufacturer's instructions. Gels were transferred for 1 h 100 V.

2.6.4. Western blotting analysis

All antibodies were diluted in 5% milk powder in TBS-T (Table 2.1). Working dilutions of antibodies used for Western blotting analysis are given in Table 2.3. Membranes were incubated for 1 h at room temperature with gentle agitation in 5% milk powder in TBS-T. Blocking solution was discarded. Membranes were subsequently incubated with primary antibody (Table 2.5) for 1 h at room temperature with gentle agitation. Membranes were washed in TBS-T for three 10 min washes with agitation at room temperature. Membranes were subsequently incubated for 1 h with the relevant secondary antibody (Table 2.5) with agitation at room temperature. Membranes were washed as previously described followed by a final 5 min wash with TBS buffer (Table 2.1). Membrane was washed once for 5 min at room temperature with 1 X TBS. Enhanced Chemiluminescence (ECL™) reagent (Amersham Biosciences, Little Chalfont, UK) was prepared by mixing an 1 ml of solution A to an equal volume of solution B. Excess 1 X TBS was drained off from membrane and the protein-side was placed down on to ECL™ reagent,
left for 1 min. Excess ECL™ reagent was drained off from membrane, which was then wrapped in fresh cling film and placed in a film cassette. Proteins were visualised by ECL™ reagent when exposed to film and developed in the dark room.

Table 2.5 Antibodies Used for Western Blot Analysis

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Manufacture</th>
<th>Isotype</th>
<th>Working solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse anti-β Actin</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:1000</td>
</tr>
<tr>
<td>Mouse anti-Myc 9B11</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:1000</td>
</tr>
<tr>
<td>Rabbit anti-Hsp90</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:2000</td>
</tr>
<tr>
<td>Donkey anti-mouse-HRP conjugate</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:2000</td>
</tr>
<tr>
<td>Donkey anti-rabbit-HRP conjugate</td>
<td>Santa Cruz Biotechnology</td>
<td>IgG</td>
<td>1:2000</td>
</tr>
</tbody>
</table>

2.7. DETECTION OF mRNA EXPRESSION LEVEL

2.7.1 Isolation of total RNA

Total RNA was extracted from 5 x 10^5 cells using RNeasy® Mini Kit (Qiagen) following the recommended manufacturer’s procedure. Briefly, pelleted cells were lysed in 600 μl buffer RLT and the lysate was transferred into a QIAshredder spin column placed in a 2ml collection tube and centrifuged for 2 min at full speed. 1 volume of 70% ethanol was added to the homogenised lysate, it was then spun through the RNeasy spin column for 15s at 10,000 rpm. After flow through was discarded, 700 μl Buffer RW1 was added to the column to wash the membrane by centrifugation for 1 min at 10,000 rpm. RNA was eluted in 30–50 μl RNase-free water by centrifugation for 1 min at 10,000 rpm and stored at -80 °C until use.
2.7.2 Detection of E2A-HLF and GAPDH transcripts by reverse transcriptase-quantitative PCR (RT-qPCR)

RT reaction was set up by mixing 10 μg RNA, 2 μl DNase1 enzyme, 2 μl DNase buffer (x10) and DEPC water was added to make up a total volume of 40 μl. The mixture was incubated at room temperature for 15 min. To stop the reaction, the mixture was added with 2 μl of 25 mM EDTA and incubated at 65°C for 5 min then placed on ice. To perform cDNA synthesis, 8 μl first strand buffer (x5, Invitrogen), 4 μl DTT (Invitrogen), 2 μl random primers (0.32 μg/μl, Invitrogen), 2 μl dNTP (10 mM for each dNTP, Invitrogen), 2 μl RNase Out (Invitrogen), 2 μl MMLV-RT (Invitrogen) were added to the RT reaction mixture and first incubated at 37°C for 2 h then at 70°C for 15 min. Control tubes contained all of the reagents for cDNA synthesis except RT.

To conduct q-PCR, 5 ng of total cDNA was used in 20 μl per PCR reaction (in MicroAmp™ Fast Optical 96-Well Reaction Plate, Applied Biosystem) containing 10 μl of 1x TaqMan(R) Fast Universal PCR Master Mix (Applied Biosystem) and 1μl of the primer either amplifying LMO2 or GAPDH (Applied Biosystem), using 7900HT Fast Real-Time PCR machine. A negative control contained no template DNA was included to make sure there was no RNA or DNA contamination to the qPCR reagents. GAPDH was used as a reference gene. Cycling conditions were 95 °C for 20 sec followed by 40 cycles of 95 °C for 1 sec and 60 °C for 20 sec.
2.7.3 Calculation of LMO2 mRNA expression levels relative to the GAPDH housekeeping gene

The calculation was based on the assumption that the level of expression of the reference molecule (GAPDH gene) must not vary under the different experimental conditions being studied. This level was used as a reference value for quantification. qPCR Ct value is the point where the growth curve crosses the threshold, reflecting the amounts of cDNA produced by the reverse transcriptases, with total RNA from the cells.

Relative quantification (ratio between the amount of LMO2 and GAPDH molecules) was determined by the following equations:

1) \[ \Delta Ct = C_{target} - C_{GAPDH} \]
2) \[ \Delta Ct_1 - \Delta Ct_{control} = \Delta \Delta Ct \]
3) \[ 2^{\Delta \Delta Ct} = \text{relative expression level} \]
3.1 Low-Expression Activity of CMV Promoter in Haematopoietic Cells

The construction of basic vector p201 (a gift from Stephen J. Elledge, Appendix 1) of pPRIME (potent RNAi using iR expression) series has been previously described (Stegmeier, Hu, Rickles, Hannon, & Elledge 2005). Lentiviral vector p201 is of “minimal lentiviral genomes” containing RRE, cPPT and woodchuck hepatitis virus posttranscriptional regulatory (WPRE) elements, in the other words, dispensable lentiviral virulence/accessory genes have been eliminated. During transcription of HIV, the RRE sequence is bound by the viral Rev, an essential HIV-1 regulatory protein, tethering the viral RNA to the CRM1-mediated nuclear export pathway. In the absence of Rev or RRE the genomic RNA of HIV is either spliced or degraded and does not efficiently reach the cytoplasm, a prerequisite for packaging (Cullen 1998). WPRE and cPPT elements are downstream from the transgene in an effort to increase nuclear export and to provide high gene expression levels in haematopoietic cells (Demaison et al. 2002). U3 region of the 3’ LTR is deleted to improve the safety of the vector system. The vector also contains eGFP marker gene driven by an internal promoter CMV. To optimise gene knockdown effectiveness, primary microRNA transcripts, which are endogenous triggers of RNAi, has been included in the vector. miR30 was chosen because processing of the larger pre-miRNA transcript (70 nt) to its functional miRNA has been well characterised in vitro and in vivo (Cullen 2004).

To investigate the CMV promoter activity of p201 vector, 293FT cells were transfected with this viral vector together with the relevant packaging and envelop plasmids to produce the virus carrying CMV promoter. psPAX2 packaging construct (kindly provided by Didier Trono, Appendix 2) expresses gag, pro and pol, which encode the structural proteins as well as the enzymes (reverse transcriptase, RNase H, integrase and protease), tat, rev and vpu responsible for increasing virus production are also expressed (Logan, Lutzko, & Kohn 2002; Tang, Kuhen, & Wong-Staal 1999). pMD2G envelop construct (kindly provided by Didier Trono, Appendix 3)
expresses vesicular stomatitis virus glycoprotein (VSV-G) that confers the viral particle with the stability to transduce a broad range of cell types, including primary cells and stem cells (Logan, Lutzko, & Kohn 2002; Tiscomia et al. 2003). Primary immortalised haematopoietic cell lines, primary HSCs in myeloid and lymphoid conditions as well as immortalised haematopoietic cell line (BAF3) were then infected and analysed for eGFP expression by fluorescent microscopy 3 days (Figure 3.1) and by FACS 3 days, 7 days after infection (Figure 3.11, 3.12). Uninfected cells were used as a negative control, so that the strength of CMV promoter in the infected cells could be quantitatively determined by FACS analysis by measuring the level of eGFP reporter gene expression (MFI). When the CMV promoter activity was examined under the inverted fluorescence microscopy, eGFP expression, of different intensity, was detectable in many infected cell whereas no fluorescent cells were observed in the negative control cells, proving that this vector was functional in the haematopoietic cells.

However, fluorescence microscopy lacked of providing the precise information on the promoter activity and transduction efficiency. Thus, FACS analysis was carried out. Using an eGFP marker transgene under the transcriptional control of a CMV, the level of transduction as determined by the gating has achieved 85.5% on average in the input cells at 3 days post-infection, confirming that high efficiency of transduction of the haematopoietic cells, even the hard-to-transfect primary cells and HSCs, was feasible using 2nd generation of HIV-1-based lentiviral vector. As expected, well-established haematopoietic cell line (BAF3) was most infectable which also showed relatively high gene expression in comparison to the primary haematopoietic cell lines. Nevertheless, the gene expression levels in all the transduced cell lines, including BAF3 cells were considerably low. Furthermore, MFI on an average of 22 in the input cells has further reduced by 36% at 7 days post-infection, suggesting that the gene expression driving by CMV promoter was not only undesirable but also rather unstable in the haematopoietic cell lines. Altogether, the activity of internal CMV promoter has proven to be
short-termed and weak in haematopoietic cell lines in vitro, although this is one of the most popular promoters that has been used to over-express foreign genes in many different cell lines, revealing a cell specificity of the CMV promoter activity. Thus, there was a need to replace CMV promoter with an alternative promoter that could drive strong and long-term expression in haematopoietic cell and thereby to allow a more effective gene knock-down in the haematopoietic cells.

**Figure 3.1 Analysis of eGFP expression of p201CMV in haematopoietic cell lines by inverted fluorescence microscopy**

<table>
<thead>
<tr>
<th>Uninfected haematopoietic cells, 3 days after infection</th>
<th>Infected haematopoietic cells, 7 days after infection</th>
</tr>
</thead>
</table>

eGFP Images

eGFP images show the detection of eGFP expression in the cells transduced with p201CMV lentiviral vector whereas no fluorescent cells are visible in the uninfected control cells.
3.2 Replacement of CMV Promoter with SFFV Promoter

As suggested from the previous experiment, lentiviral vectors p201 using an internal CMV promoter to transcribe the gene was not yet optimised for efficient expression in haematopoietic cells. It was then aimed to replace CMV promoter with an alternative one to achieve long-term expression in (primary) haematopoietic cell lines. It has been shown that significantly higher levels of GFP expression (3- to 5-fold) were achieved with EF1α promoter than that of CMV promoter in the human haematopoietic stem/progenitor cell (HSPC) line KG1a (Ramezani, Hawley, & Hawley 2000), unfortunately, cloning of this promoter was unsuccessful. Efficient transduction by spleen focus forming virus (SFFV) promoter in haematopoietic repopulating cells has been reported (Demailson, Parsley, Brouns, Scherr, Battmer, Kinnon, Grez, & Thrasher 2002).

Cloning strategy for the replacement of CMV promoter by SFFV involved blunting. Due to the limitation of enzyme recognition sites available in the vector, the more effective sticky-ended ligation followed by double digestion was thus not employed. A unique EcoRI site at position 7749 in the donor vector (CSGW) and XbaI site at position 2576 in the recipient vector (p201) were blunted. Thus, the EcoRI site in the basic p201 vector remained unique and available for the subsequent cloning of shRNA. Different cloning strategies have been employed, and the resultant construct was obtained by the strategy described as follows (Figure 3.4).

5μg of vector p201 was restriction digested in a total volume of 100 μl for 2 hours with 5% of XbaI enzymes (Figure 3.2, Figure 3.4 stage A). 5'end overhangs produced from XbaI enzyme cleavage were then incubated with Large Klenow Fragment to generate blunt ends by filling in recessed 3'ends of DNA fragments. The linearised vector with blunt ends was further digested with NotI enzyme, resulted in two DNA fragments, 1.35 kb and 7.15 kb (Figure 3.3, Figure 3.4 stage B), that both consisted of one sticky and one blunt end. The vector DNA upon the removal of CMV promoter and eGFP transgene was recovered after gel purification of
the 7.15 kb band, using the *Qiagen MinElute Gel Extraction kit* and stored at -20°C.

Figure 3.2- Digest of p201 with XbaI.
Lane 1: sample
Lane 2: ladder marker

Figure 3.3- Digest of p201 with NotI.
Lane 1: ladder marker
Lane 2 & 3: sample 1, 2
Figure 3.4 - Cloning Strategy for Changing of the Promoter (not drawn in scale)

Stage A)

**CSGW Vector**

- EcoRI
- Digest with EcoRI
- 5' overhangs fill-on

**p201 Vector**

- XbaI
- Digest with XbaI
- 5' overhangs fill-on

<table>
<thead>
<tr>
<th>Vector</th>
<th>LTR</th>
<th>SFFV</th>
<th>eGFP</th>
<th>LTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSGW</td>
<td>LTR</td>
<td>SFFV</td>
<td>eGFP</td>
<td>LTR</td>
</tr>
<tr>
<td>p201</td>
<td>LTR</td>
<td>CMV</td>
<td>eGFP</td>
<td>miR30</td>
</tr>
</tbody>
</table>

Stage B)

- Blunt
- Digest with NotI
- Gel Purification

<table>
<thead>
<tr>
<th>Vector</th>
<th>LTR</th>
<th>SFFV</th>
<th>eGFP</th>
<th>LTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSGW</td>
<td>LTR</td>
<td>SFFV</td>
<td>eGFP</td>
<td>LTR</td>
</tr>
<tr>
<td>p201</td>
<td>LTR</td>
<td>CMV</td>
<td>eGFP</td>
<td>miR30</td>
</tr>
</tbody>
</table>

Stage C)

- Blunt
- NotI
- Digest with NotI
- Gel Purification

<table>
<thead>
<tr>
<th>Vector</th>
<th>LTR</th>
<th>SFFV</th>
<th>eGFP</th>
<th>LTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSGW</td>
<td>SFFV</td>
<td>eGFP</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p201</td>
<td>LTR</td>
<td></td>
<td>miR30</td>
<td>LTR</td>
</tr>
</tbody>
</table>

Stage D)

**Ligation**

**p201SFFV Vector**

<table>
<thead>
<tr>
<th>Vector</th>
<th>LTR</th>
<th>SFFV</th>
<th>eGFP</th>
<th>miR30</th>
<th>LTR</th>
</tr>
</thead>
<tbody>
<tr>
<td>CSGW</td>
<td>LTR</td>
<td>SFFV</td>
<td>eGFP</td>
<td>miR30</td>
<td>LTR</td>
</tr>
<tr>
<td>p201</td>
<td>LTR</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
The SFFV element to be inserted into the linearised p201 construct was recovered from another vector, CSGW, (see Appendix 4). Vector CSGW is a self-inactivating HIV-1 packageable vector, encodes eGFP expressed from a SFFV 5'LTR promoter. A unique endonuclease-EcoRI site within the SFFV promoter sequence was eliminated by cutting (Figure 3.5, Figure 3.4 stage A), filling in with Large Klenow Fragment. The linearised vector was then cleaved by NotI enzyme (Figure 3.4 stage B), which gave rise to two bands appearing on the agarose gel, one at 1.3 kb and one at 8.4 kb (Figure 3.6). The SFFV element fused with eGFP open-reading frame was recovered after gel purification of the 1.3 kb band, using the Qiagen MinElute Gel Extraction kit.

The recovered insert and vector p201 were then ligated (Figure 3.4 stage C) for 2 hours at room temperature according to the following vector/insert molar ratios: 1:3, 1:0. The ligation products were then transformed into competent E.coli cells. The transformation cultures were spread on to the gel plate containing ampicillin and were incubated overnight at 37°C. Colonies were picked the next morning and grown overnight at 37°C in 5ml of LB medium containing
ampicillin. The DNA was extracted from the cells when the bacteria were at the exponential growth phase. A series of diagnostic digests was performed in order to identify which clones contained the desired vector. Each of the extracted DNA samples were first restriction digested with two combinations of restriction enzymes, EcoRI/XbaI and NotI/BamHI, which was able to tell us whether the blunting reaction by Large Klenow Fragment worked and whether the ligation was successful. Since there should be no XbaI site present in the recombinant vector after blunting and only one EcoRI site in the vector backbone, clone 2, 5, 10, 13 and 14 that showed single band seemed to be promising (Figure 3.7). Nevertheless, appearance of single band on the gel after EcoRI/XbaI digestion could also indicate an empty vector, thus NotI/BamHI digestion was carried out as empty vector did not have BamHI cutting site while recombinant vector did. Two bands, one at 7758 bp and the other at 742 bp, were expected from NotI/BamHI digestion as NotI cut once at position 3825 and BamHI is the unique site at position 3084 within the insert part (Figure 3.8). By combining the results from EcoRI/XbaI and NotI/BamHI digests, clone 14 appeared to be the only clone that contained the right vector. Clone 14 was then further subjected to several diagnostic digests with BamHI/EcoRI, BamHI/XbaI, NheI/EcoRI and NsiI/EcoRI to make sure this was the desired vector for later cloning. As expected, two bands showed on the gel from the BamHI/EcoRI (Figure 3.9), NheI/EcoRI and NsiI/EcoRI digests (Figure 3.10) while only one digested band showed from the BamHI/XbaI digest (Figure 3.9). DNA sequencing analysis has verified sequence of this vector. The new vector generated was named p201SFFV (Figure 3.4 stage D).
Figure 3.7- Digest of miniprep DNA with EcoRI and XbaI to eliminate the clones that definitely have undestroyed EcoRI and XbaI sites.
Lane 1-3, 5-10, 12-17: sample 1 to 15
Lane 11, 19: ladder marker

Figure 3.8- Digest of miniprep DNA with BamHI and NotI to eliminate the clones that definitely have no inserts.
Lane 1-3, 6-17: sample 1-15
Lane 4, 5, 18: ladder marker
Figure 3.9- Digest of DNA from clone 14 with BamHI/EcoRI (lane 1) and BamHI/XbaI (lane 3). Lane 1, 3: sample 1, 2, Lane 2: ladder marker.

Figure 3.10- Digest of DNA from clone 14 with NheI/EcoRI (lane 1) and NsiI/EcoRI (lane 3). Lane 1, 3: sample 1, 2, Lane 5: ladder marker.
3.3 High-Level Transduction and Long-Term Gene Expression in Haematopoietic Cells Using 2nd Generation of Lentiviral Vector p201 Incorporating SFFV Promoter

Following the successful cloning of SFFV promoter into p201 vector in replacement of CMV promoter, the effect of the SFFV promoter on transgene expression in haematopoietic cells in vitro was then evaluated using FACS analysis. The virus bearing p201SFFV expression cassette was used to transduce primary immortalised cell lines (E2A-HLF, MLL-ENL, and LMO2/BCL2), primary haematopoietic cell lines (myeloid and lymphoid conditions) and established haematopoietic cell line (BAF3). Uninfected cells were used as the negative controls. The efficiency of transcription of the eGFP reporter gene from p201SFFV lentiviral vector after stable incorporation into the target cell genome was measured 3 days and 7 days after infection, as illustrated in Figure 3.11. The levels of eGFP reporter gene expression from the transduced cells were directly examined for the efficiency of transcription after stable integration into the host genome. It is shown that the levels of eGFP reporter gene expression of all the haematopoietic cells transduced with p201SFFV at 3 days post-infection were exceptionally high compared to that of p201CMV-transduced cells and the transduction efficiency was almost 100% as determined by the gating. Percentage of eGFP-positive cells stayed constant at 7 days post-infection, MFI values have significantly increased in all the transduced cells (Figure 3.13). Highest MFI was observed in BAF3 cell line, which was of 2-4 folds more than that of primary haematopoietic cells. The result (Figure 3.11, 3.12, 3.13) demonstrated that the newly derived vector p201SFFV not only enhanced gene transfer efficiency, as measured by the percentage of eGFP-positive cells, but also showed greatly improved stability of gene expression, as measured by the MFI of eGFP, over the parent vector p201CMV in the infected cells. The result definitely suggested that SFFV promoter can induce
long-term gene silencing in a variety of haematopoietic cells, including primary and non-dividing HSCs in vitro.

Figure 3.11. Comparison of transduction efficiency and gene expression between p201 CMV and p201 SFFV by FACS analysis

Infection of Haematopoietic Cells (BAF3)
Infection of E2A-HLF Primary Immortalised Cells

p201CMV

- 3 Days
- 7 Days

Relative count

Infection of LMO2/BCL2 Primary Immortalised Cells

p201SFFV

- 3 Days
- 7 Days

Relative count
Infection of MLL-ENL Primary Immortalised Cells

**p201CMV**

- 3 Days: 86% infected, MFI=24
- 7 Days: 20% infected, MFI=12

**p201SFFV**

- 3 Days: 99% infected, MFI=912
- 7 Days: 252% infected, MFI=3438

Infection of Primary HSCs in Lymphoid Condition

**p201CMV**

- 3 Days: 85% infected, MFI=24
- 7 Days: 1% infected, MFI=8

**p201SFFV**

- 3 Days: 99% infected, MFI=171
- 7 Days: 100% infected, MFI=1480
Infection of Primary HSCs in Myeloid Condition

Fig 3.11 Analysis of eGFP expression of p201CMV and p201SFFV in haematopoietic cell lines. p201CMV shows low-level and short term expression whereas p201SFFV shows high-level and long term expression in primary HSCs, primary immortalised haematopoietic cell lines and established haematopoietic cell lines. Transduction efficiency is enhanced in the cells transduced with p201SFFV lentiviral vector in comparison to that with p201CMV vector.
CMV promoter drives low-level gene expression in the transduced cell lines. Gene expression decreased 7 days after infection.

SFFV promoter drives high-level gene expression in the transduced cell lines, in BAF cell line in particular. Gene expression significantly increased 7 days after infection.
3.4. CLONING OF shRNAs INTO LENTIVIRAL VECTOR SFFVp201

Once long-term expression of lentiviral vector SFFVp201, derived from parent p201, has been confirmed by FACS analysis, construction of shRNA lentiviral vectors was completed by subcloning of the candidate shRNA oligo into p201SFFV vector. A selection of the shRNA target sequences (Table 2.2) were designed, by The BLOCK-iTTM RNAi Designer from Invitrogen website and RNAi Central shRNA Designer from Hannon’s lab (http://katahdin.cshl.org:9331/siRNA/RNAi.cgi?type=shRNA), against the candidate leukaemic fusion genes, include human MLL-ENL and human E2A-HLF as well as LMO2 mouse gene that was found to be abnormally up-regulated by transcriptional fusion protein E2A-HLF in leukaemic cells. Generally, at least 3 different oligos need to be generated and tested for every target gene as a success rate of one in three was seen in most experimental settings. A number of design criteria have been recommended to construct effective shRNA expression vectors. The target sequence should be 21-23 bases long while longer molecules can trigger a PKR (protein kinase activated by dsRNA) response. The G/C content should be 40-55%, also stretches of four or more nucleotide repeats and targeting introns should be avoided. Furthermore, to prevent the off-target effects, database search was performed to filter out the sequences that share a certain degree of homology with other related or unrelated genes, avoiding silencing of these loci.

To clone the hairpin insert into vector SFFVp201, they were first amplified from the oligos by PCR with low cycle numbers to minimise the chance of having mutation in the resultant products. pSM2C primers (Table 2.3) were used to perform amplification of all the shRNAs. The forward primer for amplifying the siRNA sequences incorporated XhoI restriction site also overlapped with the beginning of miR30 sequence while the EcoRI restriction site and last few bases of miR30 sequence were contained in the reverse primer. To accomplish the final step of construction of miR30-based shRNA lentiviral vectors, PCR products (110 bp) were first
digested with EcoRI and XhoI enzymes for 4 hours at 37°C, and then directly cloned into well-digested vector p201SFFV (Figure 3.14) flanked by the corresponding recognition sites. Presence of hairpin insert in the p201SFFV was checked on the 2% agarose gel by diagnostic digesting with XhoI and EcoRI enzymes. The result from diagnostic digest (Figure 3.15) indicated that all the clones contained the right vectors as a small band at 110 bp and one vector DNA band at 8.5 kb were revealed in each sample. The shRNA sequences have been confirmed by sequencing analysis. The newly generated shRNA plasmids (Appendix 5) were named p201SFFV-miR30-MLL, p201SFFV-miR30-ENL1, p201SFFV-miR30-ENL2, p201SFFV-miR30-HLF1, p201SFFV-miR30-HLF2, p201SFFV-miR30-HLF3, p201SFFV-miR30-HLF4, p201SFFV-miR30-LMO2.2, and p201SFFV-miR30-LMO2.4. Amplification of siRNA sequences human ENL3 and mouse LMO2.1, mouse LMO2.3 were never successful as point mutations always occurred.
3.5. **Virus Titration for Achieving Maximal Infection Efficiency in Haematopoietic Cells**

For the purpose of estimating functional virus titre in order to achieve high-level transduction in the haematopoietic cell lines as well as to get good reproducibility over the course of multiple viral preparations and experiments, it was then to define the MOI for different cell lines since MOI is cell-type dependent. MOI is the ratio of infectious virus particles to the number of cells being infected. The general theory behind MOI is to introduce one infectious virus particle to every host cell that is present in the culture. However, multiple copies of the viral integration may happen, which leaves a percentage of cells uninfected. This occurrence can be minimised by using a higher MOI to ensure that every cell is infected, such that the transduction can be optimised.

Titreing lentiviral vectors were performed by assessment of expression of eGFP transgene following 48 h post-transduction of E2A-HLF and LMO2/BCL2 primary immortalised haematopoietic cell lines as well as BAF3, 32D, C11 (MLL-ENL) and SEMK2 haematopoietic cell lines with the a unrelated viral vector, which allowed us to estimate MOI for different cell types. Meanwhile, BAF3 cells were also transduced with a variety of viral vectors, including the 9 shRNA constructs (obtained from chapter 3.4.), which allowed us to make sure that different viruses infect the same cell line with more or less the same efficiency. To obtain MOI, it was first to work out the actual amount of the virus particles that have infected 1 ml of 1x10^4 cells per 96-well. Based on the assumption that each cell was infected by one virus, 1x10^4 cells that presented 100% transduction was multiplied by the percentage of eGPF-positive cells obtained from the FACS analysis. The derived answer was then divided by the amount of virus supernatant (µl) (5-folds serial dilution) used to infect the cells and then multiplied by 1000 to estimate the amount of the viral particles per ml. The virus titre of 1 MOI value for most of the
viruses that transduced BAF3 cells were approximately $3 \times 10^7$ cells per ml, as shown in Table 3.1 and Figure 3.16 (the representative sample, BAF3 infected with MLL shRNA construct) whereas LMO2 construct-containing viruses showed an 1 MOI of 10-folds less (Table 3.2 and Figure 3.17), suggesting that LMO2 lentiviral construct is relatively inefficient to infect the cells.
% Infected cells | viral supernatant (ul) | viral particles/ml
--- | --- | ---
85.20% | 50 | $1.70 \times 10^5$
87.50% | 10 | $8.75 \times 10^5$
72.70% | 2 | $3.64 \times 10^6$
56.70% | 0.4 | $1.42 \times 10^7$
25.10% | 0.08 | $3.14 \times 10^7$
9.00% | 0.016 | $5.63 \times 10^7$
2.40% | 0.0032 | $7.50 \times 10^7$

Table 3.1 Infection of BAF3 cells with p201SFFV-miR30-MLL. 5-folds serial dilution of the viral supernatant is correlated with the decreased level of transduction efficiency. Virus titres (viral particles per ml) were calculated.

Figure 3.16. Plot of % infected cells against MOI shows S-shaped trend line. Since MOI=1 was estimated to lie within the range (green line) where the gradient is constant, the midpoint (red circle) along the green straight line was taken as the point for MOI=1 ($3.14 \times 10^7$ virus particles/ml).
Table 3.2 Infection of BAF3 cells with p201SFFV-miR30-LMO2.4. 5-folds serial dilution of the viral supernatant is correlated with the decreased level of transduction efficiency. Virus titres (viral particles per ml) were calculated.

<table>
<thead>
<tr>
<th>% Infected cells</th>
<th>viral supernatant</th>
<th>viral particles/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>69.00%</td>
<td>50</td>
<td>$1.38 \times 10^5$</td>
</tr>
<tr>
<td>69.70%</td>
<td>10</td>
<td>$6.97 \times 10^5$</td>
</tr>
<tr>
<td>37.50%</td>
<td>2</td>
<td>$1.88 \times 10^6$</td>
</tr>
<tr>
<td>19%</td>
<td>0.4</td>
<td>$4.75 \times 10^6$</td>
</tr>
<tr>
<td>9.00%</td>
<td>0.08</td>
<td>$1.13 \times 10^7$</td>
</tr>
<tr>
<td>2.50%</td>
<td>0.016</td>
<td>$1.56 \times 10^7$</td>
</tr>
<tr>
<td>0.90%</td>
<td>0.0032</td>
<td>$2.81 \times 10^7$</td>
</tr>
</tbody>
</table>

Figure 3.17. Plot of % infected cells against MOI shows S-shaped trend line. Since MOI=1 was estimated to lie within the range (green line) where the gradient is constant, the midpoint (red circle) along the green straight line was taken as the point for MOI=1 (4.75 $x 10^6$ virus particles/ml).
To compare the infectability of different cell lines, percentage of eGFP-positive cells were plotted against amount of virus supernatant (in Log scale) used to transduce the cells, as illustrated in Table 3.3 and Figure 3.18. The p201SFFV-miR30-MLL vector (representative sample) produced significant levels of transduction at an MOI of 10 in most of the cell lines except for LMO2/BCL2 primary immortalised cell lines. SEMK2 and C11 cell lines consistently produced higher transduction levels (approximately 98%) compared to BAF3 (85.2%). Apparently, SEMK2 cell line is the most infectable as high level of transduction can be reached with a low MOI of 0.5 (meaning that you inoculate 5 virus particle for every 10 cells) whereas LMO2/BCL2 primary immortalised cell line is the least as higher MOI (> 100) is required to achieve effective transduction. Interestingly, loss of infection efficiency was observed at an MOI greater than 10 in BAF3 cell line, suggesting that there is certain range of MOI for optimal transduction in this cell line as BAF3 cell line might be sensitive to the purity of the virus supernatant. The result thus demonstrates that infectability, in terms of MOI, is in a cell-type dependent manner.
Table 3.3. Decreased percentage of infected cells is correlated with the 5-fold serial dilution of the virus supernatant. Virus titres (amount of virus particles per ml) were calculated.

<table>
<thead>
<tr>
<th>Amount of virus (ul)</th>
<th>Amount of virus particles (ml)</th>
<th>BAF3</th>
<th>32D</th>
<th>C11</th>
<th>SEMK2</th>
<th>LMO2/BCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>$1.57 \times 10^6$</td>
<td>85.20%</td>
<td>97.20%</td>
<td>93.50%</td>
<td>98.70%</td>
<td>61.00%</td>
</tr>
<tr>
<td>10</td>
<td>$3.14 \times 10^5$</td>
<td>87.50%</td>
<td>94.70%</td>
<td>78.20%</td>
<td>99.10%</td>
<td>51.10%</td>
</tr>
<tr>
<td>2</td>
<td>$6.28 \times 10^4$</td>
<td>72.70%</td>
<td>83.40%</td>
<td>54.90%</td>
<td>98.60%</td>
<td>21.20%</td>
</tr>
<tr>
<td>0.4</td>
<td>$1.26 \times 10^4$</td>
<td>56.70%</td>
<td>72%</td>
<td>38.20%</td>
<td>96.60%</td>
<td>12.30%</td>
</tr>
<tr>
<td>0.08</td>
<td>$2.51 \times 10^3$</td>
<td>25.10%</td>
<td>49.50%</td>
<td>19.30%</td>
<td>72.40%</td>
<td>4.80%</td>
</tr>
<tr>
<td>0.016</td>
<td>$5.02 \times 10^2$</td>
<td>9.00%</td>
<td>28.60%</td>
<td>5.90%</td>
<td>30.50%</td>
<td>4.60%</td>
</tr>
<tr>
<td>0.0032</td>
<td>$1.00 \times 10^2$</td>
<td>2.40%</td>
<td>25.30%</td>
<td>2.90%</td>
<td>16.70%</td>
<td>1.50%</td>
</tr>
</tbody>
</table>

Figure 3.18. Plot of % infected cells against MOI and amount of virus shows the infectability is cell-type dependent. SEMK2 cell line is the most infectable while LMO2/BCL2 cell line is the least. Most cell lines, except for LMO2/BCL2, show significant level of transduction at MOI=10 to 100 that is $10^5$ to $10^6$ of the virus particles. Higher level of transduction in LMO2/BCL2 cell line is expected to be achieved at an MOI $> 100$ ($>10^6$ of the virus particles)
Since the assumption of infecting $1 \times 10^4$ cells in each 96-well by $1 \times 10^4$ viral particles with 100% transduction can not be true in practical, higher titre i.e. more than $1 \times 10^4$ viral particles must be used to transduce the cells for effective infection. The estimation of virus titre ($3.14 \times 10^7$ virus particles per ml) for MOI=1 (Table 3.1, Figure 3.16) allowed us to figure out the amount of virus particles that is required to yield, for example, an MOI of 10 in BAF3 cell line for high-level transduction. As illustrated in table 3.3, calculation of amount of virus particles for the most concentrated virus supernatant (50μl) was derived from $3.14 \times 10^4$ virus particles per μl times 50μl that gave $1.57 \times 10^6$ virus particles in total. Due to 5-fold dilution, the number of $1.57 \times 10^6$ virus particles was thus divided by 5 each time, and obtained the amount of virus particles of $1 \times 10^2$ for the least concentrated virus supernatant. Table 3.3 and Figure 3.18 show an estimation of the amount of virus particles to achieve high-level transduction in different cell lines. For instance, to achieve almost 100% transduction in SEMK2 cell line, $1 \times 10^4$ to $1 \times 10^5$ virus particles should be used. In contrast, much higher virus titre (more than $1 \times 10^7$ virus particles) must be used to achieve high-level transduction in LMO2/BCL2 cell line. Therefore, titreing gives the viral concentration of the supernatant, which allows one to know how many infectious particles are added in a given experiment that is important to define functional titre for efficient transduction as well as to provide reproducible data.

3.6. ESTABLISHMENT OF KNOCKDOWN EFFICIENCY

Having designed shRNAs that were predicted to load RISC efficiently with siRNAs specific for those sequences and constructed a set of miR30-based shRNA lentiviral vectors targeted to gene of interest, it was then important to determine whether effective knock-down of the target mRNA and its encoded gene product could be obtained. Following the transduction of the cells with lentiviral expression vector encoding a shRNA specific for the gene of interest, eGFP-positive cells were sorted and isolated for having high purity of the infected cells. 1- to 2-
week’s duration was given to allow the infected cells to proliferate so that substantial reduction in the level of expression of stable proteins encoded by the targeted mRNA can be observed.

### 3.6.1 MLL-ENL protein detection

It has been difficult to obtain a good knock-down in transduced cells by western blotting. All the proteins of interest to be detected by western blotting were myc-tagged. As illustrated in Figure 3.19, introduction of the p201SFFV-miR30-MLL and p201SFFV-miR30-ENL1 into MLL-ENL immortalised haematopoietic cell line (C11) resulted in a marked and specific reduction in the level of exogenous MLL-ENL fusion protein compared to the control cells that were transduced with an irrelevant p201SFF-miR30-HLF. In theory, unrelated virus does not exert specific effect on the target, thus serve as a negative control. In contrast, no reduction was seen in the cells transduced with 201SFFV-miR30-ENL2. Authenticity of knockdown efficiency was confirmed by detection of unchanged expression levels of HSP90 loading control protein among the samples.

**Figure 3.19.** MLL-ENL immortalised haematopoietic cell line (C11) was transduced with the indicated lentiviruses (MOI = 10, cells transduced with nonspecific p201SFFV-miR30-HLF virus serve as control). GFP-expressing cells were sorted 7 days after infection. Whole-cell extract were analysed by western blotting for the indicated proteins.
3.6.2 LMO2 protein and mRNA detection

Detection of LMO2 protein in LMO2/BCL2 immortalised primary haematopoietic cell line has been difficult. Having repeated this experiment several times, it has been assured that compatibility, affinity and activity of the primary and secondary antibody, transfer of the protein from the gel to the membrane as well as the amount of the SDS in the gel and buffer were not the possible causes. Loss of either BCL2 or LMO2 expression in the immortalised cells would have caused cell death and therefore the possible explanations for the absence of the band indicating LMO2 protein expression are the instability and low expression of LMO2 protein in the cells. Finally, detection of LMO2 protein was achieved by boiling the whole-protein extract in the RIPA buffer, gently mixed and span them immediately prior to loading. Figure 3.20 shows detection of both BCL2 (25 kDa) and LMO2 (18 kDa). Clearly, the result showed no significant knock down in the cells transduced with p201SFFV-miR30-LMO2.2 and p201SFFV-miR30-LMO2.4 compared to the control uninfected cells and cells transduced with irrelevant virus.

Figure 3.20. LMO2/BCL2 primary immortalised haematopoietic cell line was transduced with the indicated lentiviruses (MOI = 100, uninfected cells and cells transduced with nonspecific virus serve as controls). It shows relatively weak detection of LMO2 protein expression in comparison to that of BCL2. Markedly, LMO2 is expressed at exceedingly low level compared to BCL2 in the immortalised cells.
RT-qPCR experiments were performed on RNA samples extracted from LMO2/BCL2 primary immortalised haematopoietic cells transduced with p201SFFV-miR30-LMO2.2, p201SFFV-miR30-LMO2.4 and the uninfected cells to assess the amount of knockdown of LMO2 on an mRNA level. Expression levels of LMO2 transcripts were quantified relative to the expression level of the endogenous housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). In addition to the transduction with specific LMO2 shRNA constructs, the cells were also transduced with the irrelevant viral vector, p201SFFV-miR30-HLF. **Figure 3.21** shows an up-regulation of relative mRNA expression level in all the transduced cells compared to the uninfected cells. To establish substantial knockdown at mRNA level, cells treated in the same condition were preferred for the comparison, thus, cells transduced with the irrelevant viral vector, rather than the uninfected cells, were used as the control for the analysis of the knockdown efficiency. Result from RT-qPCR indicated that RNAi did not succeed to induce significant degradation (<20% knockdown) of LMO2 mRNA in the cells transduced with p201SFFV-miR30-LMO2.2 and p201SFFV-miR30-LMO2.4, which is concordant with the result obtained from western blotting analysis (**Figure 3.20**).
Analysis of LMO2 Knockdown by Quantitation of LMO2 RNA Expression Level

Figure 3.21 RT-qPCR Analysis for the LMO2 mRNA Degradation. LMO2 mRNA up-regulation was observed in the cells transduced with both specific and irrelevant shRNA lentiviral vectors in comparison to the uninfected cells. Having p201SFFV-miR30-HLF transduced cells as a control (i.e. relative mRNA expression level = 1), mRNA expression levels in the cells transduced with p201SFFV-miR30-LMO2.2 and p201SFFV-miR30-LMO2.4 are at 0.84 and 0.93, respectively, in relation to the control (relative expression level = 1).
3.6.3. E2A-HLF protein detection

Different viruses that bearing different designs of HLF shRNAs for targeting E2A-HFL transcript were used to transduce the E2A-HLF primary immortalised cell line. To confirm the knockdown efficiency of p201SFFV-miR30-HLF1, p201SFFV-miR30-HLF2, p201SFFV-miR30-HLF3, and p201SFFV-miR30-HLF4, western blotting analysis was performed. Uninfected cells served as a negative control. Among the four different viruses, p201SFFV-miR30-HLF1 and p201SFFV-miR30-HLF4 seemed to down-regulate E2A-HLF gene effectively, in comparison to the negative control (Figure 3.22). E2A-HLF knockdown seems to be true as the expression levels of loading control (β-Actin) are the same.

![Western Blot Image]

**Figure 3.22** E2A-HLF primary immortalised haematopoietic cell line was transduced with the indicated lentiviruses (MOI = 10, uninfected cells serve as control). It shows efficient knockdown of E2A-HLF gene by p201SFFV-miR30-HLF1 and p201SFFV-miR30-HLF4.
CHAPTER 4

DISCUSSION
4.1. Efficient Infection of Quiescent Cells by HIV-Based Lentiviral Vector

Since the discovery that an RNAi pathway was conserved in mammals, the exploitation of this silencing response as a reverse genetic tool has evolved in concert with better understanding of its biochemical mechanism. In mammalian RNAi experiments, the long-term expression in the transduced cells is cell-type and promoter dependent. Lentiviral vectors based on HIV-1 achieve entry through the nucleopores by utilising active transport mechanisms that delivering intron-containing sequences through the binding of the viral Rev protein to a cis-acting RRE whereas commonly used vectors based on murine oncoretroviruses rely on the passive mechanism of nuclear envelope breakdown during the cell cycle cell division prior to integration in host cell genome. Hence, in this study, HIV-1-based lentiviral vectors were preferred to oncoretroviral for infection especially because they stably integrate in nondidving quiescent cells such as HSCs, avoiding the use of prolonged cytokine to trigger cell cycling of HSCs that may induce differentiation together with proliferation, thereby leading to the loss of fundamental properties of HSCs during the transduction process.

4.2. Low-Level Activity of CMV Promoter in Haematopoietic Cell Lines

This study revealed that while efficient gene transfer to HSCs was achieved, the in vitro levels of transgene expression from internal CMV immediate early region enhancer– promoter were low, indicating 2nd generation of lentiviral vector was not yet optimised for long-term expression of gene of interest in mouse HSCs. Our finding is supported by a recent study showed that whereas the CMV promoter gave high levels of GFP gene expression in two nonhaematopoietic cell lines (293T and HT1080 cells), MFI has decreased by 10-12 fold in haematopoietic cell line (KG1a cells). Similar findings have also been reported that lentiviral vectors using a CMV immediate early region enhancer–promoter to transcribe the gene of interest does not perform well in human HSCs (An et al. 2000;Ramezani, Hawley, & Hawley 2000;Scharffmann, Axelrod, & Verma 1991). Such problem might be owing to the CMV
promoter is often subject to extinction of expression and silencing \textit{in vivo}, resulted from the natural defense strategies of the eukaryotic cells, such as presence of large stretches of compact heterochromatin serving as transcriptional barriers against integrated retroviruses in the cells and methylation of viral transcriptional element (An, Wersto, Agricola, Metzger, Lu, Amado, Chen, & Donahue 2000; Scharffmann, Axelrod, & Verma 1991). Thus, despite of the utility of HIV-1 based lentiviral vectors for gene transfer into human HSPC subset have been established, nonetheless, the lentiviral vectors using internal CMV promoter presently in general use are not optimised for gene expression in HSPCs.

4.3. \textbf{HIGH-LEVEL ACTIVITY OF SFFV PROMOTER IN HAEMATOPOIETIC CELL LINES}

High level activity of the SFFV enhancer has been shown to sustain throughout all stages and lineages of haematopoietic development, particularly in the context of oncoretroviral vectors (Baum et al. 1997). To augment transgene expression in HSCs, and to facilitate the analysis of transduced cells, the SIN HIV-1-based vector p201 containing WPRE, cPPT, RRE and eGFP genes under the transcriptional regulation of the internal CMV promoter was modified by replacing CMV promoter with the U5 part of the SFFV strain P long terminal repeat sequence. The result showed a significant increase of eGFP expression driven by SFFV in transduced haematopoietic cells, including primary immortalised cells and primary HSCs, with optimal transduction efficiency. Furthermore, effective activity of SFFV in the transduced cells has been observed 7 days post-transduction, interestingly, MFI (mean fluorescence index) has increased from 3 days post-infection to 7 days. The data has confirmed that the choice of the promoter manifests significant influence on the long-term expression of genes introduced in primary haematopoietic cells by new generation lentiviral vectors. Establishment of high-level and stable long-term expression of the lentiviral vector containing SFFV promoter in HSCs is in consistent with a recent study. They found that the incorporation of the U3-LTR of SFFV used as an internal promoter, together with WPRE, cPPT and termination sequences in the vector
backbone, provided between a 20- and 73-fold increase in transgene expression in engrafted cell lineages compared to a basic vector containing the CMV promoter (Demaison, Parsley, Brouns, Scherr, Battmer, Kinnon, Grez, & Thrasher 2002).

### 4.4. Virus Titration

Virus titration was performed in order to determine the optimal concentration of the lentiviral particles used for achieving high-level transduction in the (primary) haematopoietic cells. In principle, poor infection can be improved by increasing virus titreing. HIV-1-based lentiviral vector containing an internal SFFV promoter has exhibited high-level transduction in haematopoietic cells, unfortunately, the infection rate of all the cells that have transduced at estimated MOI has declined from 100% to as low as 20% after the candidate shRNAs were cloned into the viral vector. It was first thought that inefficient transduction might due to bad DNA quality, unhealthy cells for transduction, stale cytokines for cells growth, or inappropriate timeframe for the transduction process. However, favourable percentage of eGFP-positive cells was not yielded from the technical improvement. Furthermore, the limitations of virus titreing are two-fold: it assumes that the level of expression of all integrated vectors is above the detection threshold of the assay and it may not distinguish cells with multiple copies of vector. Of note, it is important to consider transduction rate. A respectable 30% transfection efficiency could give no more than a 30% drop in target mRNA or protein expression levels even if the shRNA was 100% effective. The transduction efficiency problem was overcome by cell sorting that yielded high purity of eGFP-positive cells so that interpretable mRNA and protein knockdown data can be obtained. Furthermore, higher titreing is likely to induce apoptosis in some cell lines. The toxicity possibly arises from the VSV-G envelope protein, from the high-level transgene expression, from copurified contaminants in the vector preparation, or from a combination of these factors (Demaison, Parsley, Brouns, Scherr, Battmer, Kinnon, Grez, & Thrasher 2002).
4.5. **Establishment of Knockdown Efficiency**

Once the silencing capability of several candidates of shRNA for every target gene has been evaluated by western blotting or/and RT-qPCR at the level of exogenous protein or/and mRNA, respectively. Western blotting analysis showed that p201SFFV-miR30-MLL and p201SFFV-miR30-ENL1, but not p201SFFV-miR30-ENL2, effectively knocked down MLL-ENL expression; p201SFFV-miR30- HLF1 and p201SFFV-miR30-HLF4, but not p201SFFV-miR30- HLF2 and p201SFFV-miR30-HLF3, significantly down-regulated E2A-HLF expression. It suggested that even if shRNA is optimally designed by the computer algorithms and efficiently incorporated into RISC, there is still no guarantee that it will be effective. Current evidence suggest that fully functional shRNA can be entirely ineffective due to the fact that RISC complexes are not able to access mRNA target sites that are sequestered by another macromolecular interaction or by even relatively weak RNA secondary structure (Dallas & Vlassov 2006). Instability and low expression of LMO2 proteins in the BCL2/LMO2 immortalised cell line were demonstrated by western blotting analysis, which made the LMO2 protein detection difficult. Both RT-qPCR and western blotting analysis showed ineffective LMO2 knockdown by p201SFFV-miR30-LMO2.2 and p201SFFV-miR30-LMO2.4, thus, new LMO2 shRNA oligos need to be designed.

4.6. **Downstream Targets of E2A-HLF**

Several downstream targets of E2A-HLF have been previously identified by other groups using cDNA representational difference analysis and include *Annexin VIII, SRPUL, Slug, Groucho-related genes and Runx1/AML-1* and *Annexin II* (Dang et al. 2001; Inukai et al. 1999; Kurosawa et al. 1999; Matsunaga et al. 2004). *Annexin VIII, SPRUL* and *Slug* were identified as E2A-HLF targets in the E2A-HLF-expressing UOC-B1 leukaemic cell line induced to express a dominant negative form of E2A-HLF that lacks transactivational activity. Of the previously identified targets of E2A-HLF, only over-expression of Slug, a mammalian
homologue of CES-1, confers some resistance to IL-3 withdrawal-induced apoptosis in Baf-3 cells, suggesting its antiapoptotic activities in pro-B cells (Kurosawa, Goi, Inukai, Inaba, Chang, Shinjyo, Rakestraw, Naeve, & Look 1999). However it is not expressed in all leukaemia cell lines that express E2A-HLF (Inukai et al. 1999). Further study using microarray experiment by Jenny Yeung assessed expression of these E2A-HLF target genes. However, Slug was not expressed or over-expressed by the inducible E2A-HLF clones, also Annexin II and Runx1, were expressed, but were not changed by the expression of E2AHLF, suggesting these genes are not essential for the leukaemogenic activities of E2A-HLF. Thus, it remains to unravel whether LMO2 contributes to the development of leukaemia by further studies.

4.7. Future Works

The most efficient lentiviral vectors against specific genes will be used to infect leukaemic cells and the effect on aspects of the leukaemic phenotypes such as the cell cycle, apoptosis and immortalisation will be tested, which in turn, to determine the importance of E2A-HLF and its target gene, LMO2 as well as MLL-ENL during leukaemogenesis, also the functional role played by MLL-ENL during normal haematopoiesis. Phenotypic changes upon introduction of shRNAs into the target leukaemic cells could be assessed by using antibodies to identify cell surface markers (e.g. B220, cKit, CD19, Mac-1 and Gr-1), by colony-forming assay to trace cell fate or by Annexin V assay to detect apoptosis at a very early stage. Inducible and conditional RNAi systems can be used to study gene function in vivo in animal model. GFP-miR30-shRNA vector can be used in Tet-on system to study biological consequences of inactivation of leukaemogenic gene in vivo. Primary immortalised HPCs transduced with Tet-on lentivector carrying specific silencing cassette can be transplanted into mice, which in turn, can initiate the development of leukemia. In order to know if the knockdown of abnormally expressed genes renders the leukaemic cells to undergo cellular differentiation or apoptosis, doxycycline can be introduced into the mice by oral administration or direct injection. Presence of doxycycline
relieves transcriptional repression, thus, allowing transcription of shRNA from promoter to induce silencing effect. The identification and characterisation of dysregulated genes in rare LSCs using lentiviral-based RNAi together with a series of approaches will benefit the design of the antileukaemic treatments to specially target these LSCs, and to effectively cure and prevent childhood ALL relapse.
CHAPTER 5

CONCLUSION
Childhood leukaemias have traditionally been treated on the basis of phenotypic characteristics, such as morphology, cell-surface markers and cytogenetic aberrations by reducing the bulk population. Nevertheless, lack of durable response occurs in most cases, suggesting that the existing treatment used may not effectively target the LSC population within a leukemia. Indeed, the failure of the current therapeutic regimens is likely related to the resistance and persistence of LSCs.

The microarray technology has enabled comparison of expression profiles in malignant cells and their normal cellular counterparts, thus allowed for identification of new diagnostic or therapeutic targets. A set of genes have been found to be dysregulated by E2A-HLF and MLL-ENL fusion in pro-B cells. To obtain better understanding of the biological and molecular features of the cells that initiate and sustain ALL, a number of experiments, including RNAi-mediated gene knockdown, are required to fully elucidate the role of E2A-HLF, MLL-ENL and their target genes in leukaemogenesis and inhibition of apoptosis, which is an essential step in the development of novel agents effective against the childhood ALL.

Since inhibition of the abnormally up-regulated target gene, LMO2, of E2A-HLF by chemotherapeutic agents may improve treatment and clinical outcome, lentivirus-based RNAi was used to perform gene silencing in the immortalised HPCs. E2A-HLF and MLL-ENL gene knockdown were also performed to determine their importance in HPCs. In this study, HIV-based lentiviral vector for high-level transduction of the primary HSPCs have been optimised by replacing internal CMV promoter with the U5-LTR of SFFV. The miR30-based shRNAs could very efficiently be expressed from the promoter such that single-copy proviral integrants provide high-level knockdown. The shRNA oligos against human MLL, ENL, HLF and mouse LMO2 were subcloned into this p201SFFV miR30-based lentiviral vector. Furthermore, GFP fusion downstream from the SFFV promoter not only was used to sort for
reporter-positive cells but also to normalise and monitor transduction levels to accurately assess knockdown efficiencies. pSFFV-miR30-MLL and pSFFV-miR30-ENL1 showed efficient and sequence-specific gene silencing by endogenously expressed shRNAs in mammalian haematopoietic cells while pSFFV-miR30-ENL2 failed to reduce MLL-ENL gene expression. pSFFV-miR30-HLF1 and pSFFV-miR30-HLF4 but not pSFFV-miR30-HLF2 and pSFFV-miR30-HLF3 have knocked down E2A-HLF gene expression. Unfortunately, high-level knockdown was not observed in the LMO2/BCL2 primary immortalised cells transduced with pSFFV-miR30-LMO2.2 or pSFFV-miR30-LMO2.4. It has been generally accepted that knock-down of a target gene by > 80% will only be achieved with about one-third of the shRNAs tested despite of a series of design criteria and technologies that should allow effective shRNA expression vectors specific for mRNA have been followed. It remains to be seen if novel principles and insight will lead to future shRNA design criteria that give efficient target gene knock-down with every shRNA analysed. Gene delivery by infecting haematopoietic cells with a lentiviral vector bearing a miR30-based shRNA sequence expressed from SFFV promoter seems to be a very useful tool to perform RNAi experiment in the mammalian haematopoietic cells.

Over the past decades, the mainstay of leukemia therapy has been to induce complete remission and to consolidate this with further courses of chemotherapy. Validation of the downstream targets of E2A-HLF and MLL-ENL and elucidation of the pathogenic pathways in childhood leukaemias may hold great promise for further improving cure rates by developing drugs against specific target molecules required for the initiation and progression of leukaemia.
REFERENCES


Hunger, S. P., Ohyashiki, K., Toyama, K., & Cleary, M. L. 1992, "Hlf, a novel hepatic bZIP protein, shows altered DNA-binding properties following fusion to E2A in t(17;19) acute lymphoblastic leukemia", *Genes Dev.*, vol. 6, no. 9, pp. 1608-1620.


APPENDIX 2: VIRAL PACKAGING VECTOR

psPAK2

10703 bp
APPENDIX 3: VIRAL ENVELOP VECTOR
XbaI fragment is about 1100bp and 8600bp.
NheI fragment is about 2200bp and 7500bp.
Appendix 5: P201SFFV Vector

SFFVp201

8402 bp