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Lentiviral vectors as tools for gene manipulation

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Abstract

In this thesis the potential for lentiviral vectors as tools for gene manipulation was investigated. This thesis aims to answer the question of whether lentiviral vectors can be used to infect human stem cells with high efficiency without affecting their ability to differentiate and whether these vectors are able to deliver short hairpin RNA interference targeting a clinically relevant gene.

The introduction covers first the basics of the areas involved in this thesis, starting with stem cell biology and RNA interference before discussing vector biology and then Kaposi's sarcoma-associated herpesvirus.

Human stem cell biology is a novel and rapidly moving field. The applications of lentiviral vectors range from determining mechanisms of cell growth and differentiation to tissue engineering. The first application for these vectors investigated was therefore optimisation of the infection of human stem cells and determining that these cells retain their biological function after infection.

Lentiviral vectors to deliver short hairpin RNA were developed and a knockdown in human stem cells demonstrated. Kaposi sarcoma-associated herpesvirus (KSHV), a cause of virally driven malignancies in humans, was chosen as a target to demonstrate the therapeutic potential of these lentiviral vectors. Lentiviral shRNA vectors knocked down latent genes within KSHV *in vitro* and showed promising therapeutic potential.

The final section of this thesis documents the progression of this work to form an *in vivo* therapeutic against a KSHV-driven malignancy, primary effusion lymphoma (PEL) in a murine model.

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Dla Tygrysa, ktora zawsze błya tam kiedy potrzebowałem jej...

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Abbreviations used in this thesis

AAV	Adeno-associated virus
ALV	Avian leukaemia virus
APS	Ammonium persulphate
BCBL	Body cavity based lymphoma
bFGF	Basic fibroblastic growth factor
BL	Burkitt's lymphoma
CAEV	Caprine arthritis encephalitis virus
CDK	Cyclin dependent kinase
CL2	Containment level 2
CL3	Containment level 3
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagles medium
DMSO	Di methyl sulphoxide
DMVEC	Dermal microvascular endothelial cells
DNA	Deoxyribonucleic acid
DoH	Department of health
dsRNA	Double stranded RNA
EBNA	EBV nuclear antigen
EBV	Epstein-Barr virus
EC	Embryonic carcinoma
EDTA	Ethylene di-amino tetra acetic acid
EGF	Epidermal growth factor
EGM2-MV	Endothelial cell growth medium 2 (microvascular)
EIAV	Equine infectious anaemia virus
ERV	Endogenous retrovirus
ES	Embryonic stem cell
FACS	Fluorescence activated cell sorting
FCS	Fetal calf serum
FISH	Fluorescent in-situ hybridisation
FIV	Feline immunodeficiency virus

FLICE	Fas associated death domain-like interleukin-1β-converting enzyme
GM-CSF	Granulocyte monocyte colony stimulating factor
GTAC	Gene therapy advisory committee
HAART	Highly active antiretroviral treatment
HERV	Human endogenous retrovirus
hES	Human embryonic stem cell
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPV	Human papilloma virus
HSC	Haematopoietic stem cell
HSV	Herpes simplex virus
HTLV	Human T cell lymphotrophic virus
HUVEC	Human umbilical vein endothelial cells
HVS	Herpesvirus Saimirii
IGF	Insulin like growth factor
IKK	I KappaB kinase
IN	Integrase
IRF	Interferon responsive factor
KS	Kaposi's sarcoma
KSHV	Kaposi sarcoma-associated herpesvirus
LANA	Latency associated nuclear antigen
LCL	Lymphoblastoid cell line
LMP	Latent membrane protein
LNA	Locked nucleic acid
LTR	Long terminal repeat
MA	Matrix
MACS	Magnet assisted cell sorting
MAPC	Multipotent adult progenitor cell
MCD	Multicentric Castleman's disease
mES	Murine embryonic stem cell
MHV68	Murine herpesvirus 68

MLV	Murine leukaemia virus
MMTV	Mouse mammary tumour virus
MOI	Multiplicity of infection
MoMLV	Moloney MLV
MPMV	Mason-Pfizer monkey virus
mRNA	Messenger RNA
MSC	Mesenchymal stem cell
NF-κB	Nuclear factor κB
NLS	Nuclear localisation signal
NPC	Nasopharyngeal carcinoma
OTC	Ornithine transcarbamylase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PEL	Primary effusion lymphoma
PIC	Pre-integration complex
PKR	Protein kinase R
PTLD	Post transplant lymphoproliferative disorder
qRT-PCR	Quantitative RT-PCR
RAG	Recombination activating genes
RCL	Replication competent lentivirus
RCR	Replication competent retrovirus
RCV	Replication competent virus
RdRP	RNA dependent RNA polymerase
RIPA	Radio-Immunoprecipitation assay
RISC	RNA induced silencing complex
RNA	Ribonucleic acid
RNAi	RNA interference
RPMI 1640	Royal Park Memorial Institute medium 1640
RRE	Rev response element
RT	Reverse transcriptase
RT-PCR	Reverse transcriptase polymerase chain reaction

SARS-CoV	Severe acute respiratory syndrome- corona virus
SCID	Severe combined immunodeficiency
SDS	sodium dodecyl sulphate
shRNA	Short hairpin RNA
SIN	Self inactivating
siRNA	Short interfering RNA
SIV	Simian immunodeficiency virus
SV40	Simian virus 40
TEMED	N,N,N',N'-Tetramethylethylenediamine
UCH	University College Hospital
VEGF	Vascular endothelial growth factor
vFLIP	Viral FLICE inhibitory protein
vIL-6	Viral interleukin 6 analogue
vIRF	Viral interferon response factor
VSVg	Vesicular stomatitis virus G glycoprotein
VZV	Varicella zoster virus
WHO	World health organisation
WNT5	WNT oncogene family analogue 5

Chapter 1 – Introduction

This thesis aims to answer the question of whether lentiviral vectors can be used to infect human stem cells with high efficiency without affecting their ability to differentiate and whether these vectors are able to deliver short hairpin RNA interference targeting a clinically relevant gene.

The work presented here involves several fields including stem cell biology, RNA interference (RNAi) and Kaposi's sarcoma-associated herpesvirus (KSHV).

This introduction forms an overview of all of the fields investigated in this thesis, covers the current opinion in published literature and attempts to link these subjects together. The first section (1.1) is an introduction to stem cells, and explains first what constitutes a stem cell, what we can gain from investigation of stem cells and reviews the current vectors used to manipulate gene expression in these cells. This relates to Chapter 3.

The second section (1.2) covers RNA interference, a novel technique for knocking down gene expression in mammalian cells. The work presented in Chapter 4 involves the development of a lentiviral vector capable of knocking down gene expression using RNA interference.

Section 1.3 is an introduction to retroviral and lentiviral vector biology, including the life cycle, requirements and recent advances in the field. The potential for these vectors for *in vivo* therapeuics is also covered here.

The final section of the introduction (1.4) covers KSHV biology, it's associated morbidities and current opinion regarding the oncogenic abilities of the virus. Chapters 5 and 6 present the knockdown of KSHV latent genes both *in vitro* and *in vivo*, and this section of the introduction is relevant to these chapters.

1.1 Human Stem Cells

1.1.1 What is a stem cell?

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1.1.4 Manipulation of gene expression in stem cells

Human stem cell biology is a rapidly moving field. The ability of lentiviral vectors to infect human stem cells is showing importance in research involving these cells, and the results presented in Chapter 3 show that these vectors can express foreign transgenes without affecting the potential of these cells to differentiate. This section covers an introduction to human stem cells, their biological function and role in research and how we can manipulate these cells *in vitro*.

1.1 Human Stem Cells

1.1.1 What is a stem cell?

It is difficult to assign a universally understood meaning to the term 'stem cell'. In 1894, Bizzozero classified tissue in the human adult as belonging to one of three groups- renewing (such as the skin and epithelium), expanding (such as the liver) and static (such as the nervous system). These classifications are not completely comprehensive – we now recognise neural stem cells, and the fact that the adult nervous system is capable of renewing its cells when previously it was classified as static. This highlights the problems with trying to classify all 200 cell types within the human body.

There are three attributes which characterises a stem cell. A 'stem cell' is a cell found in either an embryo or an adult which is unspecialised, capable of long-term self renewal and also of differentiating into specialised daughter cells.

Stem cells are unspecialised - stem cells do not have any tissue-specific structures that allow them to perform specialised functions.

Stem cells are capable of dividing and renewing themselves for long periods. Unlike myocytes or neurons which do not normally replicate themselves or primary cells which have a limited life span, stem cells may proliferate indefinitely. If the daughter cells continue to be unspecialised, they are said to be capable of long-term self-renewal. The specific factors and conditions that allow stem cells to remain unspecialised *in vivo* are relatively unknown.

Stem cells can also give rise to specialized cells when grown under the correct conditions. Adult stem cells such as haematopoietic stem cells (HSC) and mesenchymal stem cells (MSC) are termed multipotent meaning that they can differentiate into cells from more than one lineage. When stimulated in culture, MSC can form adipocytes (fatty tissue), osteocytes (bone) and

chondrocytes (cartilage). Human embryonic stem cells (hESC, or hES) by comparison are termed pluripotent as they have the potential to differentiate into all tissue types, however cannot form a placental body and therefore cannot grow into a foetus. Totipotent stem cells are capable of forming a foetus when grown *in utero*. Although murine embryonic stem cells (mES) are able to form placental tissues *in vitro* they cannot perform this function *in vivo* and so are pluripotent. There are currently no embryonic cell lines which are totipotent.

1.1.1.1 The role of stem cells in growth and development

Knowledge of embryogenesis and the cellular functions of the human body have led to a view of the differentiating organism beginning with the ovum and progressing through fertilisation, zygote, morula, blastocyst and implantation. At each stage, cell division results in a larger number of more specialised cells. At the morula stage (12-16 cells), each cell is said to be totipotent. A totipotent stem cell is one which has the ability to form both any cell in an organism and the organism itself when implanted *in utero*. It is useful to classify cells at this stage into embryonic and extraembryonic. Embryonic cells go on to form the foetus and extraembryonic cells to form the placenta. This division helps when considering the concept of a pluripotent stem cell, which is embryonic, and cannot form extraembryonic tissues.

By the time a blastocyst is formed (16-64 cells), an outer layer which will become the placenta and an inner cell mass which will become the foetus is present. Cell lines established from the inner cell mass at this stage of embryogenesis are pluripotent, and include human embryonic stem cells. A pluripotent stem cell has the ability (theoretically) to give rise to every type of cell found in a living organism, however cannot form the organism itself if implanted *in utero*, because the cells cannot form the embryonic component of the placenta.

An important distinction to be made here is the difference between embryonic stem (ES) cells and embryonic carcinoma (EC) cells. EC cells hold all three of the properties assigned to stem cells, they are unspecialised, self-renew and they are pluripotent – yet they are not stem cells. They are usually derived from a teratocarcinoma, a malignant tumour of germ line cells. Although they hold properties associated with stem cells, EC lines behave very differently for unknown reasons. They differentiate in different directions in response to different cues (Cadrin et al., 1988; Jones-Villeneuve et al., 1983; McBurney et al., 1982). They are not stem cells because they are derived from an abnormal (malignant) tissue and often contain chromosomal abnormalities

such as translocations and deletions. This means they are not as useful to investigate stem cell behaviour.

As embryonic growth proceeds, cells within the blastocyst become gradually more specialised. It is unknown how many 'levels' of differentiation and specialisation which occur, how many intermediates, and where the definition 'stem cell' stops and 'differentiated cell' begins. There are several documented intermediates, including multipotent stem cells, progenitor cells, tissue stem cells and precursor cells (or satellite cells).

Multipotent stem cells have the ability to form more than one distinct type of daughter cell and include the mesenchymal and haematopoietic stem cells (MSC and HSC) (Pittenger et al., 1999; Marks and Kovach, 1966). These cells are all morphologically and immunohistochemically distinct. To make matters more complicated, it has been proposed that multipotent adult progenitor cells (MAPC) are pre-MSC/HSC derived from bone marrow (Jiang et al., 2002). They can form cells from any embryonic lineage and they have been classed as pluripotent, and are an intermediate between hES and the multipotent cells.

1.1.1.2 The role of stem cells in tissue regeneration

The presence of 'stem cells' in the growing embryo can be deduced without investigation - one cell becomes all of the cells of the human body, and some form of proliferation and differentiation must be required. However for cells which are constantly renewing, such as the epithelial lining of the gastrointestinal cavity, the presence of precursor cells is essential. These precursors are restricted to a single lineage and lack the ability to specialise further- they are therefore not stem cells. Human bone marrow contains a myriad of precursor and stem cells and there are even cells which give a hint *in vitro* at being pluripotent (Jiang et al., 2002). These cells are the precursors for many tissues - injected bone marrow cells have also been shown to integrate into tissues throughout the whole body (Liechty et al., 2000; MacKenzie and Flake, 2001).

Some stem cells are also known to migrate into the peripheral circulation in response to certain hormones. The use of granulocyte monocyte colony stimulating factor (GM-CSF) to mobilise HSC in order to harvest them for 'bone marrow transplant' is now preferred to traditional surgical methods. The presence of these cells in the periphery has led to theories about regeneration around wound sites involving stem cells. An animal study after bone injuries indicates that MSC indeed mobilise to the periphery and then form new osseous tissue (Tsuchida et al., 2003) and that MSCs improve healing in an animal model of diabetes (Sivan-Loukianova et al., 2003; Stepanovic et al., 2003).

Healing therefore is not only proliferation of cells surrounding an injury. Some mechanisms, such as hypoxic stimulation of angiogenesis to reperfuse the injured area (Knighton et al., 1981) are readily explained, however the complete regeneration of a deep wound requires a diverse range of cells growing in co-ordination. That stem cells may be passive in this – dividing only as required - is now called into question. A group in New York described signalling factors (IGF, insulin like growth factor and WNT5, *Wnt* oncogene

analogue 5) released by murine ES cells which rescue mouse embryos with cardiac defects (Fraidenraich et al., 2004). These cells manage this without integrating into the embryonic heart. Another report that introduction of MSCs into a damaged brain both promotes proliferation of existing cells (Lu et al., 2001) and induces expression of neuronal markers by MSCs (Mahmood et al., 2004) indicates that there is a co-stimulation between injured cells and stem cells to coordinate appropriate wound healing.

Stem cells would appear therefore not just to be a source of new differentiated cells during the healing process, but also to interact via chemical messengers with injured tissue to encourage appropriate and necessary cell proliferation. This property seems to be held by both embryonic and adult stem cells.

1.1.1.3 The link between stem cells, ageing and cancer

Human bodies lose certain functions as they age - elderly people are more prone to develop osteoporosis, even in nutritionally equivalent environments (Crockett, 1960), skin wounds take longer to heal as we age (Reed, 1998) and the functions of the immune system decline (Janssens and Krause, 2004).

Both the ageing process and development of cancer have been linked to stem cells (Bell and Van Zant, 2004). In the laboratory, HSC and MSC have limited lifespans – past around 15 passages, the cells will no longer divide or differentiate *in vitro* (Pittenger et al., 2000). There is also a significant difference in the ability of 'old' and 'young' stem cells cells to proliferate *in vivo* (Parsch et al., 2004), and there is evidence to suggest that people with osteoporosis or osteoarthritis (either young or old) have significantly decreased osteogenic function in their MSCs (Stenderup et al., 2001; Murphy et al., 2002).

Cancer may also be related to stem cells. Many cancer cells share some of the properties of stem cells. Although the theory behind stem cells initiating cancer is several years old (Reya et al., 2001), the idea of cancer as a stem cell disorder is gaining more popularity. Recent evidence has emerged linking haematopoietic (Huntly and Gilliland, 2005), brain (Singh et al., 2004) and breast (Al Hajj et al., 2003) cancers to tissue-specific stem cells. The knowledge that stem cells are involved in the initiation or the underlying pathology of a tumour may allow novel pathways for study and therapeutic intervention.

1.1.2 Control of stem cell differentiation

In humans, the exact mechanism by which stem cells can self-renew, and how they 'choose' to become differentiated are relatively unknown. We can assume much from studies of *Drosophila*, *Caenorhabditis elegans* and yeast growth.

Molecular cues from the external environment stimulate stem cell activity (Reddy et al., 1997) (Eaves et al., 1999). This *in vitro* stimulation causes cell growth at a rate comparable to *in vivo* results (Oostendorp et al., 2000). Cytokines have been shown to activate numerous cell signalling pathways which are linked to cell division including the important Jak/Stat pathway (Heim, 1999). Growth factors such as epidermal growth factor (EGF) and basic fibroblastic growth factor (bFGF) have also been linked to stem cell division and maintenance of stem cell identity (Moghal and Sternberg, 1999; Szebenyi and Fallon, 1999), and bFGF has been shown to increase proliferation and stimulate differentiation potential in MSCs (Tsutsumi et al., 2001; Sugi et al., 2003).

From a cell cycle point of view, it has been proposed that passage through the cell cycle determines the fate of the stem cell, and chromatin remodelling during S phase restores stem cell multipotency (McConnell and Kaznowski, 1991). Yeast studies indicate that chromatin remodelling factors are recruited during M/G_1 transition and the changes that occur in S phase become apparent during the subsequent G_1 phase (Cosma et al., 1999; Krebs et al., 1999). The genes that are involved in differentiation have yet to be fully characterised. Evidence exists that some genes are required for differentiation into a lineage (Yang et al., 2001), and other genes which play a vital role in the process can be used as markers for differentiation (Hong et al., 2004). An example is the POU family transcriptional regulator *oct3/4*, which shows expression restricted to germinal and embryonic tissue (Pesce et al., 1998) and is also essential for maintenance of pluripotency (Nichols et

al., 1998) and the undifferentiated state (Pesce and Scholer, 2001) in embryonic stem cells.

The restriction of stem cells to one lineage is thought to be determined by epigenetics or chromatin remodelling (Fisher, 2002). There are some indications that adult stem cells are capable of forming tissue not within their lineage. This may occur by the cells de-differentiating to a more pluripotent cell. An example of this is oligodendrocyte precursors which can revert to multilineage neural stem cells (Kondo and Raff, 2000). Another process is termed transdifferentiation, or the differentiation of a cell directly to a different lineage. Haematopoietic stem cells have been shown to regenerate hepatocyte populations, despite this being a mesodermal lineage (Jang et al., 2004), and mesenchymal stem cells can give rise to neurons and glia (Mezey et al., 2000).

In theory, all that would be required for a stem, or even a terminally differentiated cell, to become pluri- or even toti- potent would be a reversal of the chromatin remodelling which resulted in it being lineage restricted (Kondo and Raff, 2004). Stem cell research would benefit from the ability to create pluripotent stem cells from an adult sample as this would avoid many ethical problems currently associated with embryonic stem cells.

1.1.3 Research into the clinical applications of human stem cells

Because the scope of differentiation for stem cells is unlimited the potential uses for stem cells in clinical settings is diverse. Stem cells have the ability to accelerate healing (Lee et al., 2000) and generate tissue (Kassem, 2004). Since they are the precursors of cells throughout the body, stem cell manipulation may also prove to be the key to treatment of many hereditary conditions. Genetic alteration of stem cells has led to human therapy for X chromosome linked severe combined immunodeficiency (X-linked SCID) (Cavazzana-Calvo et al., 2000) and treatment in animal models for haemophilia (Karlsson et al., 2002), β thalassaemia (Puthenveetil et al., 2004) and Parkinsons disease (Levy et al., 2004), amongst others. Both multipotent and pluripotent stem cells have been considered for use in a clinical setting, although currently most research concentrates on the multipotent stem cells, because they are easier to obtain and culture and because of ethical implications. One of the barriers to the use of hES has been the difficulty of propagating and differentiating these cells *in vitro*. When cells from a blastocyst are isolated, they tend to differentiate spontaneously. If all the undifferentiated cells are not removed from a sample of ES cells prior to use *in vivo*, the sample is tumourigenic. In 1998, the University of Wisconsin (Thomson et al., 1998) became the first group to culture hES successfully in an undifferentiated state. Along with several other groups who were also investigating this, they used irradiated murine feeder cells to provide the growth factors essential to keep the cells undifferentiated. This has become a concern because of the possibility of transmission of infectious particles from animal feeder cells. Recently, feeder free growth of hES using conditioned medium (Xu et al., 2001) or supplemented medium (Amit et al., 2004) has been demonstrated. These cells lines still required the use of animal feeder cells for derivation, although hES have been derived which are completely free from contact with animal cells (Klimanskaya et al., 2005). Currently, we are also facing a lack of understanding of the processes which govern the differentiation of hES. We can stimulate MSC, HSC and MAPC to differentiate into specific lineages (Pittenger et al., 1999; Eaves et al., 1992; Sutherland et

al., 1989; Jiang et al., 2002), but controlling the division of hES into pure cell populations that could be used therapeutically still eludes us.

One of the barriers to the use of stem cells in a clinical setting is the possibility of an adverse immune response (Bradley et al., 2002). It is not known exactly how immunologically active transplanted stem cells will be. An example of this is MSC, which appear to express different human leukocyte antigen (HLA) molecules according to the culture environment (Gotherstrom et al., 2004). hES cells do not seem to express HLA molecules, although the reason or biological role for this is unknown. HLA molecules may also upregulate upon differentiation (Li et al., 2004). Certainly, if we are to use these cells to create tissues and therapies then these issues will need to be addressed.

1.1.4 Manipulation of gene expression of stem cells

Although using stem cells for research by manipulation of culture conditions holds much promise, this does not represent their full potential. Driving stem cells towards a pure population by regulating gene expression (Furumatsu et al., 2004; Tai et al., 2004), investigation of the mechanisms of differentiation and unlocking the secrets of why some stem cells are immortal require the ability to alter gene expression within stem cells.

Like other primary cells, stem cells are refractory to transfection by standard laboratory methods- although hES do seem to show some amenity to transfection with lipid based agents (Zwaka and Thomson, 2003). MSCs, HSCs and MAPCs are difficult to transfect with high efficiency, however introduction of transgenes using viral vectors has been demonstrated. Many viral vectors have been tested, including adenoviral (Cournoyer et al., 1991; Mitani et al., 1995), adeno-associated virus (AAV) (Lebkowski et al., 1988), herpesvirus (Dilloo et al., 1997), retroviral (Mitani et al., 1993), and lentiviral (Akkina et al., 1996) vectors.

Much work has been done with all these vectors, and some vectors hold more promise than others. The most efficient transfer to stem cells is with retroviral or lentiviral vectors, however these methods have safety and packaging difficulties which may make adenoviral or AAV vectors more suitable. For hES, only adenoviral (Smith-Arica et al., 2003) and lentiviral (Ma et al., 2003; Gropp et al., 2003) vectors have shown efficient transduction *in vitro*.

These vectors can be used in hES without a loss of ability to differentiate (Gropp et al., 2003). There is evidence that vector used at high multiplicity of infection (MOI) of above 20 to achieve high transduction efficiency reduces the ability of hES to differentiate (Clements et al., 2006). This may be a barrier to lentiviral vector use in these cell types.

1.2 RNA interference

1.2.1 Introduction

1.2.2 The mechanism of RNAi

1.2.3 Applications of RNAi

1.2.4 Synthesis and Delivery

1.2.5 Prospects for RNAi as a therapy in humans

The use of primary and stem cells as models for diseases and as therapeutics is currently limited by our ability to manipulate them. The recent discovery of RNA interference (RNAi) in mammalian cells gives us the ability to reduce the expression of endogenous genes. In Chapter 4, the use of lentiviral vectors to deliver functional RNAi is shown and then demonstrated in human primary and stem cells. Chapters 5 and 6 then show the potential of lentiviral shRNA as a therapeutic both *in vitro* and *in vivo*. This introduction covers the principles behind RNA interference and explains also why it is of great significance to scientists in a broad range of fields.

1.2.1 Introduction

The ability to disrupt gene expression using long strands of antisense RNA *in vivo* was first reported in 1985 (Rosenberg et al., 1985). Not long after, its application to human pathogenic viruses and cancer began: antisense RNA was used to study the biological role of the human papillomavirus transcripts in cervical cancer (Schwarz et al., 1985), to knockdown the expression of p53 (Shohat et al., 1987) and c-*myc* (Nepveu et al., 1987), and to inhibit the replication of simian virus 40 (SV40) (Jennings and Molloy, 1987). In 1988, the potential to treat human cancers with antisense oligonucleotides was described as an approach, which

"although interesting, is paved with considerable conceptual and technical difficulties"

(Paoletti, 1988).

These difficulties included an incomplete understanding of the complex cellular processes driving human oncogenesis, unstable and incomplete silencing of genes using antisense or DNA oligonucleotides and difficulties delivering these oligonucleotides *in vivo*.

'RNA interference' (RNAi), or post-transcriptional gene silencing, utilizes small 21-22 nucleotide fragments (Elbashir et al., 2001b) and has emerged as a promising new hope to silence genes *in vitro* and *in vivo*. Although dsRNA fragments were shown to inhibit gene expression in *C. elegans* with effects an order of magnitude more efficient than antisense RNA (Fire et al., 1998), it was not known whether mammalian cells would also undergo the phenomenon of RNAi. It is well documented that mammalian cells elicit an interferon reaction to exogenous long double stranded RNA (>30bp) molecules as part of the natural cellular antiviral response. Tuschl and colleagues showed that short dsRNA molecules escape the interferon response in mammalian cells (Elbashir et al., 2001a).

1.2.2 The mechanism of RNAi

The term 'RNAi' actually describes a cascade of cellular responses which results in the selective degradation of homologous target mRNAs (Figure 1.2.1). RNAi initiates when dsRNA is cleaved into 21- to 23-bp fragments called small interfering (si) RNAs by an RNase III-like cellular enzyme called Dicer. These resulting small dsRNA molecules (or small interfering RNA, siRNA) associate with cellular proteins forming a complex known as the RNA-induced silencing complex (RISC). RISC is known to include members of the Argonaute family, Qde2 and Rde1 (Fagard et al., 2000). This silencing complex is phosphorylated and the inactive strand is released. This now activated RISC-RNA complex hybridizes to target mRNAs containing a complementary sequence to the active strand of the siRNA. The target mRNAs and the RISC are cleaved and subsequently degraded.

It is thought that the dsRNA molecule is reconstituted (Sijen et al., 2001) or amplified, either by degradation of RISC, or the action of RNA-dependent RNA polymerases on the released strand. This may explain why dsRNA is more robust than antisense RNA at silencing gene expression. In Drosophila, 35 dsRNA molecules per cell can efficiently silence a gene whose mRNA copy number is over 1000 (Kennerdell and Carthew, 1998).

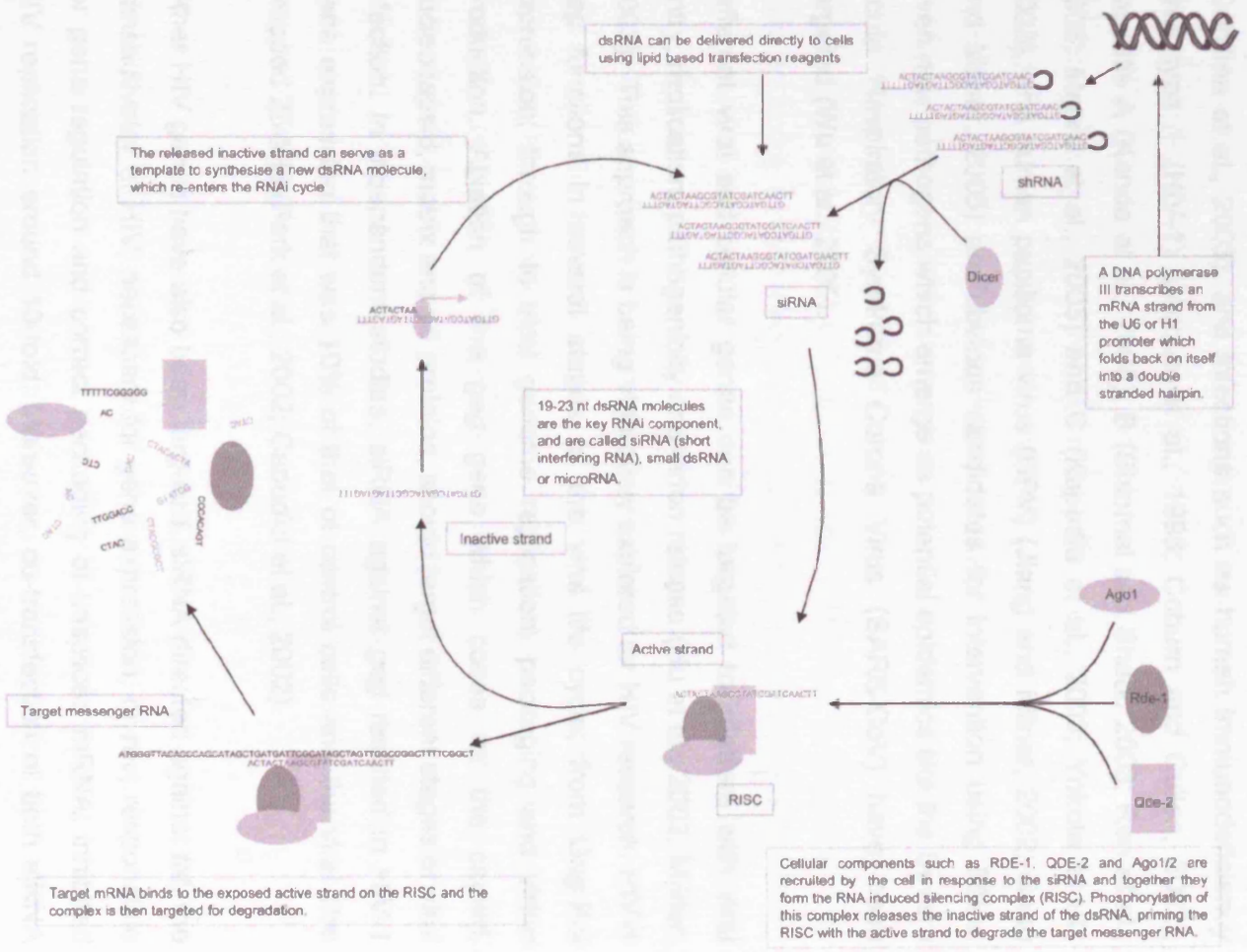
Although the proposed mechanism of RNAi means it should be specific to the mRNA sequence being targeted, several groups have called into question the specificity of RNAi and whether or not there are more widespread downstream effects. Adverse effects of siRNA molecules on non-targeted genes have been suspected through transitive RNAi or non-specific activity of dsRNA molecules.

Transitive RNAi is a process during which a cellular RNA dependent RNA polymerase (RdRP) produces non-specific off target dsRNA molecules (Sijen et al., 2001). This effect is theoretically unlikely to occur in human cells

because of the lack of RdRP, however there is a recent report of RdRP-like activity within human cells (Cheng et al., 2005).

Specificity of the effect on the targeted gene have been investigated more thoroughly, however the reports have fallen on both sides of the fence- some indicating specificity (Chi et al., 2003; Elbashir et al., 2001c; Du et al., 2005; Bilanges and Stokoe, 2005), others suggesting non-specific silencing at the RNA (Jackson et al., 2003) and protein levels (Scacheri et al., 2004). These conflicts have yet to be resolved, and there are reports emerging that stable RNAi can be used in stem cells without affecting their biological potential (Vallier et al., 2004). This implies that any off-target actions may be minimal or unimportant.

Figure 1.2.1 Proposed mechanism of RNA interference in mammalian cells



(Godfrey et al., 2003)

1.2.3 Applications of RNAi

Because this technology is sequence-specific, cancers that arise from gene point mutations (Brummelkamp et al., 2002b) and translocations such as chronic myeloid leukaemia (Wilda et al., 2002), Ewing's sarcoma (Dohjima et al., 2003), and infections such as human immunodeficiency virus type 1 (HIV-1) (Jacque et al., 1998; Coburn and Cullen, 2002), hepatitis A (Kanda et al., 2005), B (Shlomai and Shaul, 2003; Ren et al., 2005; Moore et al., 2005) and C (Kapadia et al., 2003; Yokota et al., 2003), and human papilloma virus (HPV) (Jiang and Milner, 2002; Jiang and Milner, 2005) are obvious candidates for intervention using RNAi. Even new pathogens which emerge as potential epidemics like the Severe Acute Respiratory Syndrome Corona Virus (SARS-CoV) have been targeted (Wu et al., 2005).

Different viral and cellular genes can be targeted to interfere with viral entry, replication, pathogenicity and virion release (Hu et al., 2002; Milner, 2003). This approach is being vigorously explored for HIV research. HIV-1 *gag* functions in several stages of the viral life cycle; from Gag-Pol expression, through to viral genome replication, packaging and virion production. Ablation of the *gag* gene which codes for the capsid, nucleocapsid, matrix and p6 proteins, should target different stages of viral infection. In independent studies, siRNA against *gag* resulted in HIV-1 gene expression that was 10% of that of control cells and the viral titre dropped 25-fold (Park et al., 2002; Capodici et al., 2002).

Other HIV genes have also been targeted. siRNA directed against *tat*, the transactivator of HIV, necessary for gene expression, or *rev*, responsible for gene regulation and correct packaging of unspliced mRNA, inhibited HIV replication around 10-fold. Moreover, co-transfection of both siRNA constructs into 293 cells resulted in a 10,000-fold reduction in viral titre in the supernatant (Lee et al., 2002). Suppression of the co-receptors CXCR4 and CCR5 in HIV susceptible cell lines also resulted in resistance to infection when delivered both by small molecules (Anderson and Akkina, 2005) and viral vectors (Lee et al., 2003). Inhibiting cellular factors

required for infection has also proved successful (Chiu et al., 2004; Ping et al., 2004). Reduction of the expression of CD4, one of the HIV-1 cellular receptors, resulted in a 4-fold decrease in the expression of that protein (Novina et al., 2002). Targeting different viral sequences may be synergistic and prevent the development of resistance ('escape mutants'), in a similar way to the use of a cocktail of anti-HIV therapies in HAART (highly active antiretroviral therapy).

dsRNA against hepatitis B RNA has shown a decrease in both hepatitis B surface and core antigen *in vivo* (McCaffrey et al., 2003). Similarly, targeting Hepatitis C virus inhibits viral replication *in vitro* (Yokota et al., 2003; Kapadia et al., 2003).

Viruses have not been the only focus of RNAi-driven technology. The ability to inhibit a component of a cellular pathway to investigate the effect of its loss and determine its function has also been explored (Nair et al., 2005; Rodda et al., 2005). Novel techniques involving screening libraries of dsRNAs targeting known genes allows us to characterise pathways in more depth by discovering new functional components (Berns et al., 2004), and to establish completely novel functions for known genes (Paddison et al., 2004). Techniques for producing such libraries is also now advancing (Cleary et al., 2004), as is the number of applications. New components of the pathways involved in endocytosis (Pelkmans et al., 2005) and regulators of apoptosis (Mackeigan et al., 2005) have been identified using RNAi screens.

RNAi has shown potential benefits in disease models of neurological conditions (Xia et al., 2004; Lu, 2004; Singer, 2004; Burton, 2005) and regulation of gene expression with RNAi is also proving valuable in cancers which are not virally driven (Hata et al., 2005; Wilda et al., 2002).

1.2.4 Synthesis and Delivery

The easiest and most common way to induce RNAi is to synthesise small dsRNA molecules *in vitro* and introduce them into cells in culture, using cationic lipid carriers. They can even be injected directly into mouse tail veins (Song et al., 2003) or into embryos (Calegari et al., 2002). Although the effects of siRNA can be amplified intracellularly, introducing RNA still remains a transient, self-limiting effect. DNA-based hairpin approaches offering stable expression have been engineered (Brummelkamp et al., 2002a; Sui et al., 2002). These DNA constructs are designed to transcribe an mRNA molecule containing internal complementary sequences, which anneal to each other to form a hairpin. Dicer is thought to cleave this hairpin into small dsRNA molecules (Bernstein et al., 2001), which are capable of RNAi (Figure 1.2.1). Using DNA to induce RNAi allows for the possibility of stable integration of DNA into the target cell's genome and thus permanent knock-down of the gene of interest. These DNA constructs benefit from the advances made in traditional gene based delivery systems, including the use of viral vectors to deliver the DNA-based silencing construct (Xia et al., 2002; Stewart et al., 2003; Grimm et al., 2005; Hosono et al., 2005; Hurtado et al., 2005; Nicholson et al., 2005). These systems even allow the delivery of stable RNAi to stem cells (Zaehres et al., 2005). The recent discovery that RNA polymerase II promoters and inducible polymerase III promoters can deliver short hairpins reliably enough to enable short hairpin production (Zhou et al., 2005; Matthess et al., 2005) allows both a much greater scope for targeting these vectors to a specific cell type and much greater plasticity for controlling levels of expression.

Delivery of RNA interference as dsRNA has also been extensively investigated with modifications to the chemical structure of the dsRNA molecule. These modifications mostly centre on improving both serum stability (Czauderna et al., 2003) and duration of action (Amarzguioui et al., 2003) of these molecules. A recent attempt to prepare dsRNA for *in vivo* use involved producing conformationally stable nucleotide analogues

in a dsRNA template. The chemical structure of the nucleotides is altered so that the 2'-oxygen and the 4'-carbon atoms are linked with a methylene chemical group. This gives them better thermal and chemical stability and increases the avidity with which they bind complementary RNA sequences (Vester and Wengel, 2004). These locked nucleic acids (LNA) have previously been used for antisense RNA (Wahlestedt et al., 2000), but were shown to improve duration and potency of action and minimal toxicity as dsRNA templates for RNAi (Elmen et al., 2005).

1.2.5 Prospects for RNAi as a therapy in humans

Exploiting RNAi as a therapy is plagued with similar problems to those previously demonstrated for antisense: the delivery of double stranded RNA to all infected cells *in vivo* is currently only a dream. Although massive doses of dsRNA have been injected into the tail veins of mice, and were successfully delivered to hepatocytes (McCaffrey et al., 2003), such systemic delivery in humans is not currently feasible. Double stranded RNA has a short half life and repeated delivery will not be practical, although one advantage of targeting foreign or mutated genes is that cytotoxicity *in vivo* should be limited. The development of DNA plasmids or viral vectors that lead to stable dsRNA delivery may achieve long-term silencing. Targeting dsRNA to a specific cell type may be possible by generating pseudoviruses with envelopes for specific cell types, or by expressing short hairpin RNA (shRNA) with tissue-specific promoters. dsRNA molecules which are resistant to degradation (Layzer et al., 2004; Elmen et al., 2005) and the development of new delivery systems such as use of peptide-based approaches (Kichler et al., 2003; Simeoni et al., 2003) and gel based application (Jiang et al., 2004) hold promise for translating RNAi from laboratory to the clinic.

1.3 Lentiviral vectors

1.3.1 Viral vectors as gene delivery tools

1.3.2 Retroviral and lentiviral vectors

1.3.2.1 The retroviral life cycle

1.3.2.2 The unique characteristics of lentiviruses

1.3.2.3 Development of retroviral and lentiviral vectors

1.3.2.4 Infection of primary and stem cells

1.3.2.5 Limitations of HIV-1 as a lentiviral vector

1.3.2.6 Vector induction of interferon responsive genes

1.3.3 Current gene therapy trials

Development of lentiviral vectors for efficient infection of stem cells and for delivery of shRNA is the basis of the work presented here. This section aims to cover the basic biology of retroviruses and lentiviruses and the application of lentiviral vectors as gene delivery vectors.

1.3.1 Viral Vectors as Gene Delivery Tools

Many of the advances that have been made in science have called for the ability to enhance, alter, inhibit or complement the expression of the genetic code within a system. As our models of human systems improve, and our abilities to culture new and exotic cells such as primary and human embryonic stem (hES) cells advance, traditional methods are no longer sufficient for the purpose. Most primary and stem cells are refractory to transfection, electroporation or even mechanical manipulation to enable gene delivery. Promoters which function strongly in cell lines are silenced in stem cells when they differentiate. These difficulties have driven the development of new methods of gene delivery.

One rapidly advancing field is the use of viral vectors. An extraordinary variety of viruses exist- almost all cell types in animal, human and even prokaryotic systems are the host or target for a virus. We are able to use the mechanisms by which they propagate their genetic material within their hosts to deliver novel genetic material.

Different viral vectors within the laboratory setting exploit the weaknesses and strengths of the viruses upon which they were based (see Table 1.3.1 for an overview). This Table is by no means comprehensive, with many vectors based on different viruses reported in the literature; however a detailed discussion of these vectors available for gene transfer is beyond the scope of this introduction. The remainder of this introduction centres on the development of retroviruses as viral vectors. These viral vectors are of special interest because of their ability to express transgenes reliably and stably.

Table 1.3.1 Comparison of some commonly used types of viral vectors for gene delivery

Virus	Titre (Approx.)	Envelope	Tropism	Capacity	Immune Response	Expression	Advantages	Disadvantages
Adeno-associated Virus	10^8	-	Broad	<5kb	Weak	Episomal (90%) and integrated	Non-immunogenic and non pathogenic, stably integrates at specific location	Relatively small packaging capacity will limit gene transfer
Adeno-virus	10^{13}	-	Broad	8 kb	Strong	Episomal	Very efficient delivery in most tissues, very high titre production possible	Induces strong T cell response.
Herpes Simplex Virus	10^{10}	+	Narrow, but vectors are strong for neurons	40kb	Strong	Episomal	Large packaging capacity. Strongly neurotropic.	Immune response. Transient expression in non neural cells.
Retrovirus	10^7	+	Dividing cells only	9kb	Weak	Integrated	Persistent gene expression in infected cells	Only transduces dividing cells and potentially oncogenic
Lentivirus	10^9	+	Broad	9kb	Weak	Integrated	Stable expression in almost all tissues	Potentially oncogenic. Safety concerns regarding recombination

1.3.2 Retroviral and lentiviral vectors

Retroviruses are unique in their ability to integrate their genetic code reliably into the host cell. AAV's will only typically integrate in 5-10% of infected cells (Huser et al., 2002). Retroviruses require the integration of their genetic material to complete their life cycle. Because of this, they are natural vectors which are ideal for the purpose of both *in vitro* and *in vivo* gene transfer. They have in fact been delivering genetic material to humans for millions of years in the form of human endogenous retroviruses (HERV, ERV, or retrotransposons). One recent report indicates that over 10% of our DNA may in fact be retroviral in origin (Bannert and Kurth, 2004).

There are many known retroviruses, although their hosts are mostly animals - the first human exogenous pathogenic retrovirus was not identified until 1981 (Poiesz et al., 1981). Classification of retroviruses can be according to genetic similarity (Table 1.3.2) (Hunter et al., 2000) or morphologically (Table 1.3.3).

The most commonly used vectors are based upon the gammaretrovirus murine leukaemia virus (MLV, a C type retrovirus) or the lentivirus human immunodefiency virus type 1 (HIV-1). Unless otherwise stated, when discussing retroviral and lentiviral vectors, I refer to MLV and HIV based vectors, respectively. The next section covers the life cycle and properties of retroviruses and lentiviruses and then discusses the production of safe gene transfer vectors from these viruses.

Table 1.3.2 Taxonomy of retroviruses

Family	Genus	Type Species	Hosts
Retroviridae	Alpharetrovirus	Avian leukosis virus (ALV)	Vertebrates
	Betaretrovirus	Mouse mammary tumour virus (MMTV)	Vertebrates
	Gammaretrovirus	Murine leukaemia virus (MLV)	Vertebrates
	Deltaretrovirus	Bovine leukaemia virus	Vertebrates
	Epsilonretrovirus	Walleye dermal sarcoma virus	Vertebrates
	Lentivirus	Human immunodeficiency virus 1 (HIV-1)	Vertebrates
	Spumavirus	Chimpanzee foamy virus	Vertebrates
Metaviridae	Metavirus	Saccharomyces cerevisiae Ty3 virus	Fungi
	Errantivirus	Drosophila melanogaster gypsy virus	Invertebrates
Pseudoviridae	Pseudovirus	Saccharomyces cerevisiae Ty1 virus	Invertebrates
	Hemivirus	Drosophila melanogaster copia virus	Invertebrates

Table 1.3.3 Morphological classification of retroviruses

A-type	Non-enveloped, immature particles only seen inside cells. Exist possibly as a result of endogenous retrovirus-like (ERV) genetic elements.
B-type	Enveloped virions with a condensed, acentric core and prominent envelope spikes, e.g. MMTV.
C-type	As B-type, but with a central core and barely visible spikes - e.g. most mammalian and avian retroviruses (MLV, ALV)
D-type	Usually slightly larger (to 120nm) and spikes less prominent, e.g. Mason-Pfizer monkey virus (MPMV)

(Adapted from Coffin et al. 1997)

1.3.2.1 The retroviral life cycle

A typical retroviral genome (Fig 1.3.1, top) contains three genes which code for the core proteins ('*gag*' from **g**roup specific **a**nti**g**en), the viral enzymes (*pol*, from **pol**ymerase) and the **env**elope (*env*). The *pol* gene is sometimes divided from the viral protease (*pro*) in diagrams. These genes are present in all retroviruses, and are grouped according to function. They do not necessarily show strong sequence homology between different retroviruses. Some retroviruses contain a gene which is known to cause cancer in its host (viral oncogene) – examples include the human T cell lymphotrophic viruses I and II (HTLV-I and II) (Fig 1.3.1, bottom). Other retroviruses, such as the spumaviruses encode additional genes which are not always essential for viral replication. Lentiviruses also contain regulatory genes (such as *tat* and *rev*) and accessory genes (in HIV there are four – *nef, vif, vpr,* and *vpu*).

In mature virions, the retroviral genome is always carried in the form of two plus strand RNA sequences (except in spumaviruses where reverse transcription occurs in the virion and a significant amount of DNA is present) and is surrounded by a lipid bilayer derived from the plasma membrane of the previous host cell. The virion also contains the products of both *gag* and *pol* genes, with *env* products embedded in and across the lipid bilayer.

The life cycle of a retrovirus is shown in figure 1.3.2. During infection, mature virions bind to a surface receptor on the cell membrane or through interaction with cell surface proteins. The viral core is released into the cell by fusion of the viral and host cell membranes. The viral core is transported to the cytoplasm by binding to cytoskeletal elements. During transport through the cytoplasm reverse transcription of the viral RNA into cDNA begins through the action of the viral enzyme reverse transcriptase (RT). The viral RNA, DNA and proteins are together termed the pre-integration complex (PIC) until it integrates into the host genome.

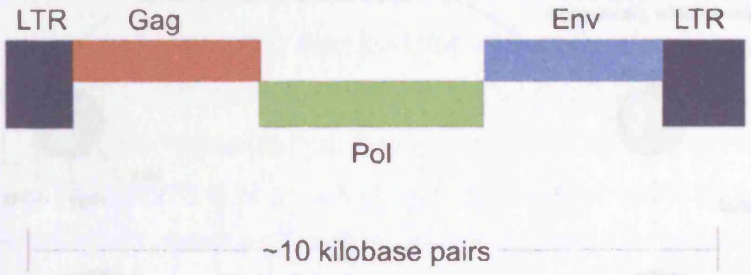
The retrovirus enters the nucleus during S phase transition when the nuclear membrane breaks down (Lewis and Emerman, 1994). Once inside the nucleus, viral integrase (IN) begins incorporating the new cDNA into the host genome. After integration into the host genome, the viral DNA becomes known as a provirus, and when the cell becomes transcriptionally active, viral genes are transcribed and translated by the host cell. Although integration has previously been believed to be random, it is now thought that different retroviruses integrate more frequently in different areas of the genome. An example is HIV-1, which appears to integrate more frequently in transcriptionally active areas of the genome (Reviewed in Mok et al. 2005).

Transcription of the viral genome is driven by the viral promoter in the 5' (upstream) long terminal repeat (LTR). The poly-A signals in the 3' LTR cause transcription to terminate (Guntaka, 1993). The entire genome is transcribed on a single mRNA transcript and retroviruses employ a variety of tactics such as splice variation and frameshift translation to allow this single transcript to code for multiple gene products.

In the cytoplasm, spliced mRNA is translated into viral gene products. *Gag* and *pol* are translated into a single protein molecule known as a Gag-Pol precursor. The viral proteins and genomic RNA undergo assembly at the cell membrane (in B and D type viruses this occurs in the cytoplasm), when they are packaged with the *env* gene products (SU and TM) to form immature virions. Retroviruses rarely cause cells to lyse, with budding being the primary method of virion release. This budding gives the virus its lipid bilayer envelope. After immature virions bud from the cell membrane they undergo maturation. This involves viral protease cleaving Gag-Pol protein products into the core proteins and enzymes involved in the life cycle. These virions are now said to be mature.

Fig 1.3.1 The retroviral genome

(a) Common retroviral elements



LTR – long terminal repeat

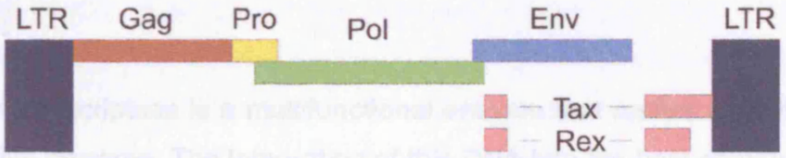
Gag – group specific antigens gene

Pro – codes for viral protease

Pol – codes for viral enzymes (integrase and polymerase)

Env – viral envelope gene

(b) The genome of HTLV-I

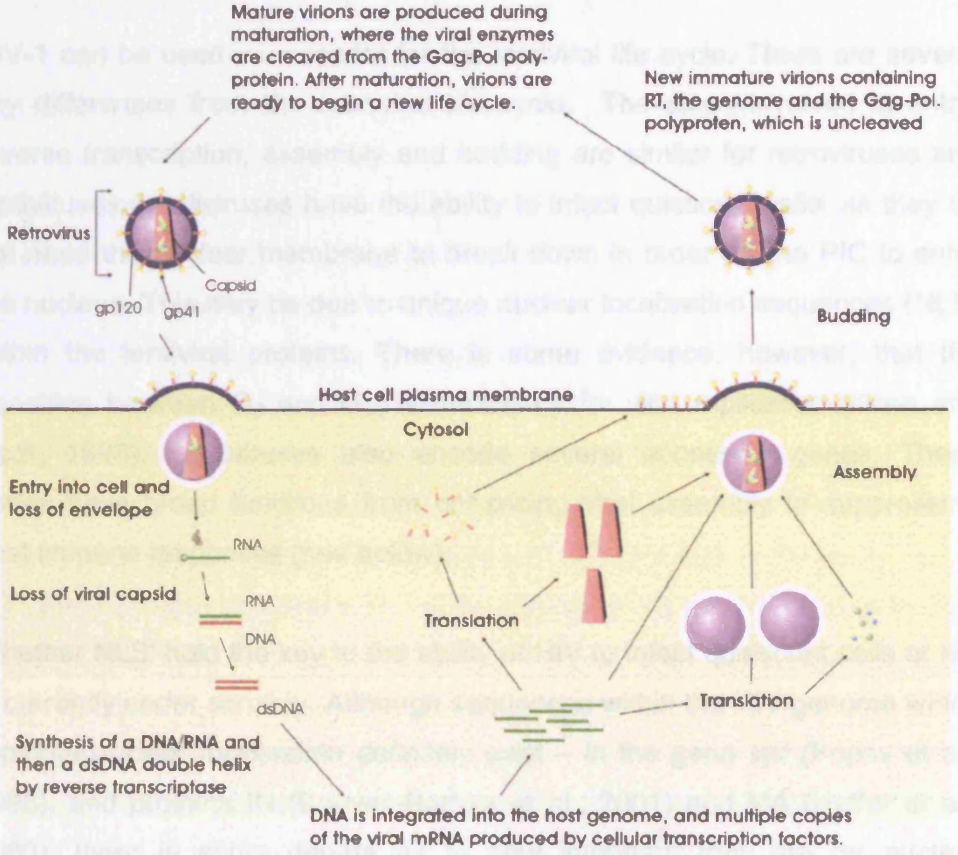


Tax – transactivator/ x-region (oncogene)

Rex – codes for RexRE binding protein

(Adapted from Coffin et al., 1997)

Fig 1.3.2 The retroviral life cycle



Legend:

Reverse transcriptase is a multifunctional enzyme that makes a dsDNA copy of the RNA genome. The integration of this DNA into the host chromosome is catalyzed by a viral protein called integrase. Retroviruses are examples of enveloped viruses, in which the protein shell is further enclosed by an outer lipid bilayer membrane. The envelope contains proteins that enable the virus to bind to cells, and that aid its entry into a cell. The virus is released from the cell by a process of budding from the plasma membrane, taking some of this membrane with it.

(Adapted from Alberts et al., 1994)

1.3.2.2 The unique characteristics of lentiviruses

HIV-1 can be used as a model for the lentiviral life cycle. There are several key differences from the retroviral life cycle. The steps involved in entry, reverse transcription, assembly and budding are similar for retroviruses and lentiviruses. Lentiviruses have the ability to infect quiescent cells, as they do not need the nuclear membrane to break down in order for the PIC to enter the nucleus. This may be due to unique nuclear localisation sequences (NLS) within the lentiviral proteins. There is some evidence, however, that the transition between G_0 and G_{1b} is necessary for viral replication (Korin and Zack, 1998). Lentiviruses also encode several accessory genes. These genes have broad functions from enhancing viral assembly to suppressing host immune responses (see below).

Whether NLS' hold the key to the ability of HIV to infect quiescent cells or not is currently under scrutiny. Although sequences within the HIV genome which promote nuclear localisation definitely exist – in the gene *vpr* (Popov et al., 1998), and proteins IN (Bouyac-Bertoia et al., 2001) and MA (Haffar et al., 2000), there is some debate as to how important they are for nuclear transport of the PIC. A recent report of the effect of adding a NLS to a retroviral vector showed that this did not enhance nuclear transport in nondividing cells (Caron and Caruso, 2005), and another that mutations of the NLS within MA did not diminish nuclear transport (Mannioui et al., 2005).

Rev (one of the accessory genes) also encodes a NLS, however the function of this is to allow entry of Rev/Importin-β into the nucleus prior to assembly. This complex binds to the factor Crm1, releasing importin-β. Rev binds the *rev*-response element (RRE) in unspliced HIV-1 mRNA and transports this into the cytoplasm (Askjaer et al., 1998). Rev therefore enhances the export of unspliced HIV-1 mRNA. After nuclear export, assembly budding and maturation happen exactly as for retroviral vectors.

Throughout the life cycle, the accessory genes *tat*, *nef*, *vif*, *vpu*, *vpr*, (and *vpx* for HIV-2) perform their roles in the life cycle. Only the Vif, Vpr and Nef proteins are present in the mature virions. The other accessory genes are expressed from the viral mRNA at other times in the life cycle.

Tat (**t**ranscriptional **a**ctivator) helps HIV overcome a limitation in its transcription. Normally, transcription driven by the LTR is relatively slow and a hairpin structure within the genome prevents efficient production of full length mRNA. Once a small amount of Tat is produced, this activates cellular factors and allows HIV to overcome this limitation (Kim and Sharp, 2001). It causes a positive feedback effect on its own protein levels. Tat may also have a more direct role in the pathogenicity of HIV. The protein is released by cells in culture, and is often found in the blood of HIV positive individuals. Tat is sufficient to cause apoptosis in T cells in culture without infection with HIV (Xiao et al., 2000).

Nef, or **ne**gative regulatory **f**actor, is a gene involved in T cell activation to ensure continued production of viral progeny and downmodulation of MHC class II, CD4 and CD28. It has also been recently documented that *Nef* may have a role in the transcription and expression of viral proteins (Reviewed in Joseph et al., 2005).

Vif, or **v**iral **i**nfectivity **f**actor, encodes for a small protein whose exact function is unclear. It is known to be important for viral replication and determines how the viral RNA and Gag bind together. It also inhibits various cellular proteins involved in RNA processing (Reviewed in Strebel, 2003).

VpR, or **v**iral **p**rotein **R**, plays an important role in regulating nuclear import of the HIV-1 pre-integration complex (Popov et al., 1998), and is required for virus replication in non-dividing cells. Vpr has an immunomodulatory effect, possibly due to its ability to induce cell cycle arrest in proliferating cells (Bukrinsky and Adzhubei, 1999).

Vpu, or **v**iral **p**rotein **u**nknown, is involved in enhancing viral budding from an infected cell (Klimkait et al., 1990). Vpu also causes downregulation of CD4 on infected T cells (Geleziunas et al., 1994).

For viral vectors, these accessory genes are mostly unnecessary, as they are involved in immune system modulation or enhanced LTR driven transcription and are not usually included. This adds to the safety of these vectors.

1.3.2.3 Development of retroviral and lentiviral vectors

The most basic retroviral vectors have the entire gag-pol-env component of the genome removed. The cDNA of interest is placed after the packaging signal (ψ) and its expression driven by the 5' LTR. These vectors require co-transfection with other plasmids to provide the viral proteins, and they usually have a high rate of recombination. Viral preparations usually contain small quantities of replication competent virus (RCV, or replication competent retrovirus, RCR). These vectors are usually produced with packaging cells to make production easier. These packaging lines often provide the retroviral gag-pol and sometimes env sequences, eliminating the need to transfect these to produce vectors.

More complex vectors have emerged from an understanding of the structure of the retroviral genomic RNA (Fig 1.3.4). It is now known which components of the genome are essential for retroviral vectors, and which can be removed to increase packaging size and vector safety.

Efficient integration requires the following components (Fig 1.3.5):

- A promoter and polyadenylation signal in the viral genome
- Viral packaging signal (ψ)
- The primer binding site (PBS) and poly purine tract (PPT) which initiate first and second strand DNA synthesis (reverse transcription) respectively
- The R region at both ends of the RNA
- Short partially inverted terminal repeats (ITR) in the U5 and U3 regions (which are required for integration)

Extensive deletions of the elements which are not required for efficient integration has led to a second and third generation of vectors. The most recent retroviral backbones have been termed self-inactivating (SIN). They were constructed by deleting transcriptional enhancers in the U3 region. As

the viral RNA is reverse transcribed, this deletion is copied into both 3' and 5' LTRs and therefore the provirus is transcriptionally inert (Fig 1.3.5). An internal promoter cloned into the provirus will still be able to express a transgene, and the retained presence of ψ, the PBS and PPT, the R regions and ITRs mean that reverse transcription and integration still occur (Dougherty and Temin, 1987; Hawley et al., 1987). This means that they are much less likely to recombine with elements of wild type viruses or transcriptionally activate an endogenous oncogene. The drawback to these vectors is that the viral titre is markedly reduced.

There are many vector systems currently available based on viruses from most animal families. On of the most common retroviral vectors is based upon the gammaretrovirus Moloney murine leukaemia virus (MoMLV). It is an ecotropic virus, infecting only murine cells. Both replication competent (Lobel et al., 1985) and defective vectors (Gruber et al., 1985) have been reported. This virus has also been pseudotyped with the envelope from the vesicular stomatitis virus G (VSVG). This envelope binds non-specifically to cell membranes, increasing the tropism of the vectors, and also has the advantage that it is stable enough for ultracentrifugation to concentrate vector stocks (Burns et al., 1993).

The increased difficulty of synthesising lentiviral vectors due to their more complex genome has been successful mostly because of their ability to infect nondividing cells and their efficiency when infecting slowly growing cells such as stem cells.

The first HIV-1 based lentiviral vectors were crude vessels with virtually intact genomes which could only infect T cells and were dangerous to produce and handle. They contained only a marker gene driven by an internal or heterologous viral promoter (Page et al., 1990; Buchschacher, Jr. and Panganiban, 1992). All HIV-1 genes were provided *in cis* (from the same construct), whereas the *env* gene was provided *in trans* (expressed from a different construct). Packaging cells for HIV-1 based lentiviral vectors are

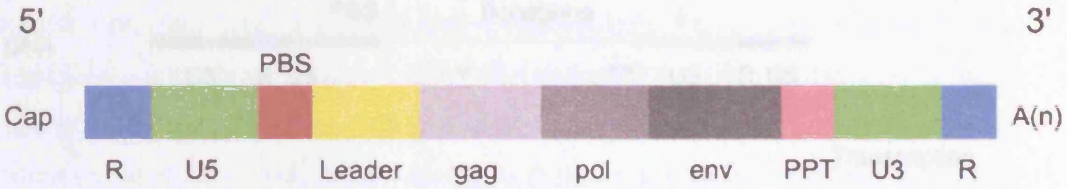
difficult to produce, as many HIV-1 proteins themselves are toxic. There has been some success in manufacturing producer cells expressing HIV-1 Gag-Pol on its own (Haselhorst et al., 1998) or in combination with the amphotrophic MLV envelope (Ikeda et al., 2003). The role of some accessory genes such as *tat* and *rev* in vector function complicates the requirements of lentiviral vectors.

Breakthroughs in our understanding of HIV biology have resulted in far more versatile vector systems. The cell range of these virions was increased by pseudotyping with the VSVG envelope (Yee et al., 1994) or the amphotrophic MLV envelope (Page et al., 1990). The first 'usable' lentiviral vector was based on HIV-1 (Naldini et al., 1996b). This vector was shown to transduce retinal cells, hepatocytes and neural tissue (Blomer et al., 1997; Naldini et al., 1996a; MacKenzie et al., 2002; Miyoshi et al., 1997).

The parts of the HIV-1 genome required for functional vector production have been characterised. The packaging signal is now better understood (McBride et al., 1997), meaning that *gag* is no longer required for vector function. The inclusion of other viral promoters eliminates the need for *tat*, which is required for transcription of the viral genomes driven by the HIV-1 LTRs. In some vectors, even *rev* and the RRE can be replaced with similar sequences from the Mason-Pfizer monkey virus although this sequence may not be as efficient at nuclear export of mRNA (Srinivasakumar et al., 1997). Hybrid vectors mean that the possibility of recombination with wild type HIV in humans can become vanishingly small. Most lentiviral vectors do not include the HIV-1 accessory genes as these are not required for the vector function.

The self-inactivating (SIN) lentiviral vectors utilise this technology from retroviral vectors. This can fortunately be performed in lentiviral vectors without a significant decrease in the final titre of the vectors, and the resulting vectors are considered safer (Chang et al., 1999; Logan et al., 2004).

Fig 1.3.4 The retroviral genomic RNA structure

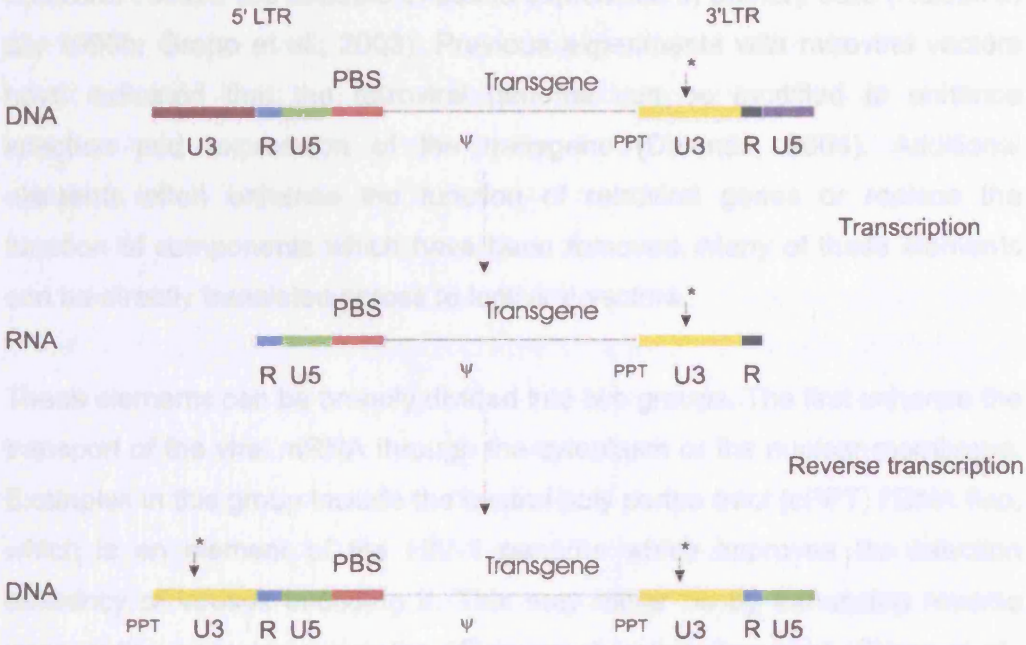


The retroviral RNA structure, from 5' Cap to 3' Poly-A tail.

R region	A short (18-250nt) sequence which forms a direct repeat at both ends of the genome. Responsible for transfer of DNA synthesis between first and second strand reverse transcription
U5	A unique non coding region which is the first part of the genome to be reverse transcribed
PBS	Primer binding site- 18nt complementary to the 3' end of the tRNA used by the virus to initiate transcription
Leader	An untranslated region (90-500nt) downstream of the transcription site present at the 5' end of viral mRNAs
PPT	Poly-purine tract. A short (~15-20) purine nucleotide sequence which initiates + strand synthesis during reverse transcription
U3	A unique noncoding region which forms part of the LTRs of the provirus, and contains the promoter elements which drive transcription of the provirus

(Adapted from Coffin et al., 1997)

Figure 1.3.5 Essential elements in the retroviral genome and SIN vectors



The elements shown (U3, R, U5, PBS, ψ) are essential components of plasmid DNA (top) for production of retroviral vectors. The U3 and U5 regions needs to contain an ITR (not shown). This diagram also demonstrates the principal of self-inactivation. Deletions in the U3 region (marked *) are copied to the RNA strand during transcription and then during reverse transcription are duplicated at both 5' and 3' LTR, resulting in a transcriptionally inactive provirus.

(Adapted from Coffin et al., 1997)

1.3.2.4 Infection of primary and stem cells

Lentiviral vectors are capable of stable expression in primary cells (Naldini et al., 1996b; Gropp et al., 2003). Previous experiments with retroviral vectors have indicated that the retroviral genome can be modified to enhance infection and expression of the transgene (Delenda, 2004). Additional elements often enhance the function of retroviral genes or replace the function of components which have been removed. Many of these elements can be directly translated across to lentiviral vectors.

These elements can be broadly divided into two groups. The first enhance the transport of the viral mRNA through the cytoplasm or the nuclear membrane. Examples in this group include the central poly purine tract (cPPT) / DNA flap, which is an element of the HIV-1 genome which improves the infection efficiency of viruses encoding it. This may either be by enhancing reverse transcription or by improving the efficiency of nuclear transport (Sirven et al., 2000; Fuentes et al., 1996). The second group enhances the expression of the transgene. This group includes post-transcriptional regulatory elements of hepatitis B virus (HBRE) or, more commonly, the woodchuck hepatitis virus (WPRE) (Zufferey et al., 1999). The precise mechanism of action of these elements is unknown, although is has been theorised that they lengthen the poly-A tail on the mRNA which allows the accumulation of mRNA in the cell (Huang and Liang, 1993). These elements can also function in the producer cells, increasing the titre of the virus produced. The constitutive transport element (CTE) (Mautino et al., 2000) is thought to enhance expression by replacing or enhancing the function of Rev and the RRE which can then be left out of vectors to increase safety (Gasmi et al., 1999).

Until a few years ago, very few promoters were widely used. In adherent cells, the cytomegalovirus immediate-early (CMV) promoter was universally used. Other promoters such as the CAG (a combination of the chicken β-actin and cytomegalovirus immediate early promoters), phosphoglycerokinase (PGK) and thymidine kinase (TK) were in use, but recent tailoring of gene

expression to target cell groups (De Palma et al., 2003; Follenzi et al., 2004; Nash et al., 2004) shows just how versatile these vectors can be. The first reports of manipulation of human embryonic stem (hES) and haematopoietic stem cells (HSC) with retroviral vectors show that elements such as the cPPT and WPRE are essential for efficient infection and expression in these cells, and that even then some degree of silencing is usually seen during differentiation (Klug et al., 2000; Cheng et al., 1998). Infection of primary and stem cells with retroviral vectors have also shown low efficiencies when compared to lentiviral vectors (Leurs et al., 2003; Serrano et al., 2003). Lentiviral vectors also do not experience silencing in stem cells (Gropp et al., 2003).

There has been continuous refinement of lentiviral vectors since their discovery. New features such as the cPPT have been better characterized (De Rijck et al., 2005), while novel methods of producing virus using adenoviral vectors (Kuate et al., 2004) or optimising titre by inhibiting protein kinase R in target cells (Pernod et al., 2004) are being developed. With safety an ever present consideration, viruses which are integrase deficient and persist episomally have emerged (Vargas, Jr. et al., 2004). There are also groups developing lentiviral vectors which are easier to use. One of the practical barriers with these vectors has been the size of the resulting plasmid (some >10kb). This has left few cloning options since restriction sites are limited and blunt end cloning has extremely low efficiency with plasmids this size. One vector of note (Fussenegger et al., 1998) has been created using two different internal ribosomal entry sites (IRES) to allow expression of three genes from a single promoter. The vector contains individual cloning sites for each gene locus. This type of vector is now also becoming commercially available.

1.3.2.5 Limitations of HIV-1 as a lentiviral vector

The advantages of lentiviruses over other viral vectors have increased their use *in vitro*. Vectors have now been derived from simian immunodeficiency virus (SIV) (Rizvi and Panganiban, 1992), equine infectious anaemia virus (EIAV) (Olsen, 1998) , feline immunodeficiency virus (FIV) (Poeschla et al., 1998b), caprine arthritis encephalitis virus (CAEV), (Olsen, 2001) HIV-2 (Poeschla et al., 1998a) or even hybrids of different lentiviruses (Strappe et al., 2005; Khan et al., 2005; Corbeau et al., 1998; Rizvi and Panganiban, 1993) or hybrids of a retrovirus and a lentivirus (Steidl et al., 2004) . One of the main concerns regarding these vectors remains safety. The first generation of lentiviral vectors contained all the viral accessory genes and intact LTRs. These vectors readily recombined, both during vector production and in the wild and there were unacceptably high amounts of replication competent lentivirus (RCL) in the vector preparations. Removal of the accessory genes and the development of SIN vectors has also increased their safety.

In the laboratory, second generation and self-inactivating vectors in the UK are handled in a containment level 2 (CL2) laboratory, however in the USA regulation is different and vectors derived there often contain intact LTRs. These vectors are considered containment level 3 (CL3) in the UK. In addition, all cell lines infected with these vectors should be handled in CL3 facilities.

Contamination of vector stocks with replication competent lentivirus (RCL) is not usually a problem since all modern vectors are produced using plasmid transfection systems. Problems exist *in vivo* surrounding concomitant infection. Although the components required for production of RCL are not present in the plasmids, evidence from studies suggests that coinfection with wild-type HIV-1 might supply the missing sequences (Vanin et al., 1994; Purcell et al., 1996; Bukovsky et al., 1999), and that surprisingly little homology (10 identical base pairs) is required for this to happen (Otto et al.,

1994). This may result in the production of replication competent viruses containing the transgene, and is therefore undesirable.

Insertional mutagenesis or oncogenesis from the action of reverse transcription on the host DNA raises questions for both gene therapy and for *in vitro* experiments. Viral vectors often integrate multiple times into target cells (Woods et al., 2003), and the site of integration which was thought to be random may show preference. MoMLV may show preferential integration *in vivo* near promoter regions. This has been highlighted by the recent demonstration of retroviral integration near the promoter for the oncogene LM02 *in vivo* (Hacein-Bey-Abina et al., 2003). LM02 has a role in haematopoiesis (McCormack et al., 2003), and is associated with T cell acute lymphoblastic leukaemias (T-ALL) (Hammond et al., 2005). Recently, evidence that *in utero* injection of lentiviral vectors raise the rate of tumours in mice has emerged (Themis et al., 2005). HIV-1 is currently believed to show a preference for transcriptionally active sequences (Mok and Lever, 2005).

Another practical consideration for the use of retroviral and lentiviral vectors *in vivo* is the titre of the virus produced. Adenovirus and adeno-associated virus' can be produced in high quantities- both in volume and titre. Retroviral and lentiviral vectors cannot easily be produced in quantities high enough for *in vivo* use in humans. Many of the applications proposed for lentiviral vectors are therefore currently impractical in man. Some groups are attempting to address this with new production techniques (Ni et al., 2005; Sena-Esteves et al., 2004; Ikeda et al., 2003). Current ongoing clinical trials with retroviral vectors therefore involve *ex vivo* manipulation of cells.

The possibility of producing an AIDS-like syndrome by using lentiviral vectors has been discounted as the mechanisms of both these vectors and wild type HIV-1 are better understood. This has not stopped a drive away from HIV-1 based vectors towards animal viruses which are considered non-pathogenic in man. These systems may offer safety advantages, and in some cases have been shown to be more efficient than HIV-1 based vectors (Beutelspacher et

al., 2005), however they still suffer from many of the limitations of HIV-1-based vectors.

For both *in vitro* and *in vivo* experiments, the expression of multiple genes is often desired for optimum results. With plasmid transfection systems, coexpression with two plasmids is easily guaranteed, however infection with viral vectors does not follow this. One solution is to place two promoters in the same construct. The size of the lentiviral backbone can make this approach difficult. An option which links expression and is more convenient in terms of size is the use of an internal ribosomal entry site (IRES), although the expression levels from the IRES are not guaranteed.

IRES are mRNA hairpins which have a structure allowing ribosomes to attach to the mRNA strand and then translate the second gene (Borman et al., 1994). The first and second genes are translated from the same strand, the transcription of which is driven by the same promoter. Some older systems showed cell specific efficacy due to the requirement of co-factors for IRES function (Jang et al., 1990). However, newer IRES function across a broader cell range (Hellen and Wimmer, 1995).

An important limitation of all viral vectors is the undesirable non-specific effects on target cells. This is discussed in the next section.

1.3.2.6 Vector induction of interferon responsive genes

Interferon refers to a cytokine which is produced as part of the immune response to viral infection. There are five kinds of interferons, four type 1 proteins ($\alpha, \beta, \omega, \tau$) and one type 2 interferon (γ). Interferons act at a cell surface receptor and stimulate a host of intracellular pathways (Dubreuil et al., 1988). Importantly, a group of genes called the interferon response factors (IRF) are upregulated (Fujita et al., 1988). The upregulation of IRFs acts to push cells into an anti-viral state, and forms part of the natural anti-viral response. The anti-viral activity is mediated in part by upregulation of protein kinase R (PKR) and RNAse L (Samuel, 2001; Taniguchi et al., 1988). These enzymes degrade the viral RNA and inhibit infection. Other cellular pathways have also been linked to IRF upregulation, including inhibiting the activity of mitochondria (Shan et al., 1990).

Important for viral vectors, *de novo* production of interferon and activation of the interferon receptor both activate the IRFs (Fujita et al., 1989), and cause cells to enter an 'anti-viral state'.

The effect is rapid (within an hour), transient and short lived (~10 hours) (Lewis et al., 1989). The level of activation varies greatly between subtypes of virus, and many viruses have evolved mechanisms to inhibit production of the IRFs (e.g. *tat* for HIV-1) (Roy et al., 1990).

This anti-viral state is undesirable. The pathways activated cloud results, and cells which are in an anti-viral state are difficult to transfect and are refractory to further infection. It is not known if cells suffer long term effects from this, and whether this effect represents a barrier to the use of these vectors for gene therapy (Tan et al., 2005).

1.3.3 Current gene therapy trials

The number of gene therapy trials has been increasing since the 1980s, and until 2001 looked set to continue that trend (Figure 1.3.6). Many different vectors have been chosen (Table 1.3.4), each exploiting the strengths of the vectors for their particular application. There have been some notable successes and setbacks using viral vectors for gene therapy. There are currently 261 retroviral and 260 adenoviral trials underway. The two most widely publicised gene therapy trials were both successful in their goals but suffered unexpected setbacks. The first involved the treatment of ornithine transcarbamylase deficiency (OTC) with an adenoviral vector. Unfortunately, the result was the death of an 18 year old called Jesse Gelsinger, who suffered a fatal anaphylactic reaction after being given $4x10^{13}$ IU of adenovirus (Marshall, 1999; Raper et al., 2003).

The more recent of the two trials involved the treatment of children with X-linked SCID with a retroviral vector. It was carried out first by Alain Fischer of the Necker Institute for Sick Children in Paris and then at Great Ormond Street Hospital., London (Cavazzana-Calvo et al., 2000; Qasim et al., 2004). The results of this showed a significant improvement in the health of the treated children, however three years after the trial began two of the children in the French cohort developed leukaemia, and genetic analysis showed a preferential integration of the vector near the oncogene LMO2 (Hacein-Bey-Abina et al., 2003).

Gene therapy is currently controlled in the United Kingdom by the Department of Health. The DoH is advised on current advances in technology and the ethical and practical issues involved in proposals by the Gene Therapy Advisory Committee (GTAC). The committee is composed of scientists from institutions around the country. The original group (The Clothier Committee) recommended in 1992 that gene therapy be "limited to life threatening diseases and disorders". Since then, advice on the advances and ethics of gene therapy has kept the UK at the forefront of both technology and safety.

Lentiviral vectors have great potential for gene therapy. They can infect almost any cell in the human body, and offer stable expression. Lentiviral vectors are not used more extensively at the moment mostly due to fears over the safety of these vectors.

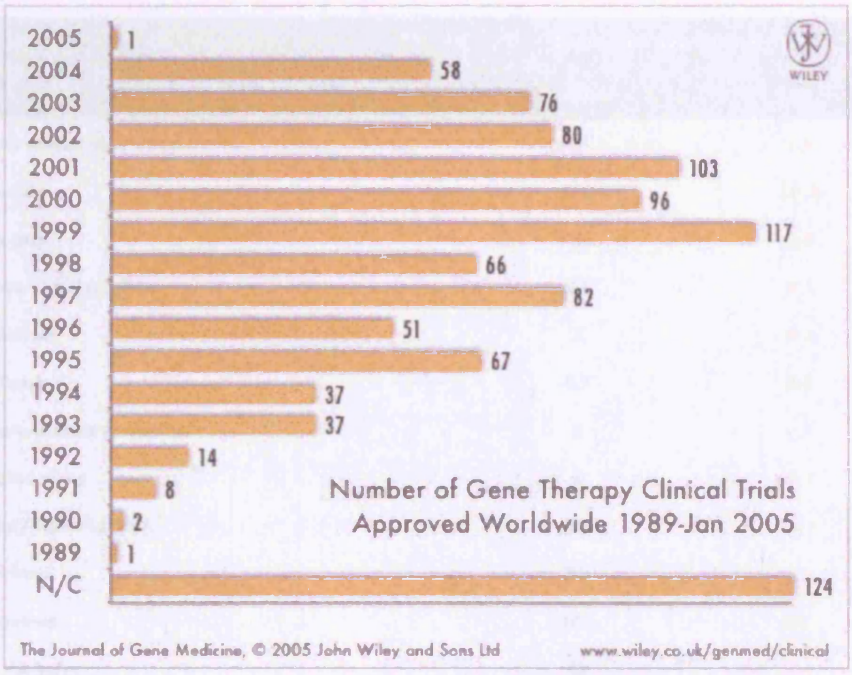
There are applications for these vectors in therapeutic models. Animal models for Friedreichs Ataxia (Fleming et al., 2005), Parkinsons disease (Lo et al., 2004), Wiskott-Aldrich syndrome (Charrier et al., 2005), small cell lung carcinoma (Sumimoto et al., 2005), β thalassaemia major (Puthenveetil et al., 2004), and haemophilia B (Waddington et al., 2004) are examples where lentiviral vectors have shown benefit. These diseases are all conditions which are debilitating, potentially life threatening and for which current traditional therapies are insufficient.

The use of lentiviral vectors is increasing and research to overcome the barriers to their use is inevitable. Unfortunately, as we search for evidence of their safety, we are uncovering potentially worrying complications of gene therapy with these viruses. One recent report indicates that *in utero* injection of an EIAV based vector increases the background rate of tumours in mice (Themis et al., 2005).

This evidence adds weight to the arguments that lentiviral vectors for *in vivo* therapy need to be used wisely and as more knowledge about these vectors becomes available then their potential will be realised.

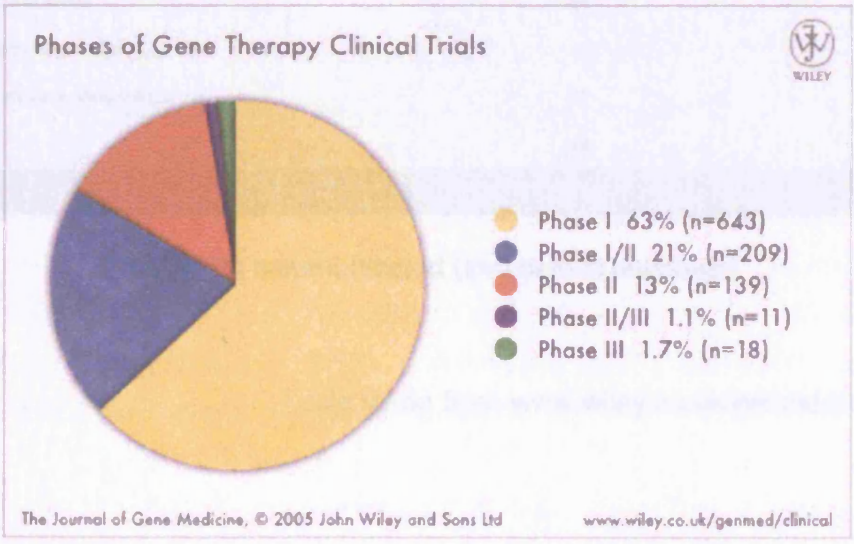
Figure 1.3.6 The progression of gene therapy in recent years

a)



N/C = not communicated (trial details unknown)

b)



from www.wiley.co.uk/genmed/clinical

Table 1.3.4 The choice of vector for registered gene therapy trials in 2004

Vector	Gene Therapy Clinical Trials	
	Number	%
Adeno-associated virus	27	2,6
Adenovirus	260	25,5
Gene gun	5	0,5
Herpes simplex virus	32	3,1
Lentivirus	3	0,3
Lipofection	87	8,5
Listeria monocytogenes	1	0,1
Measles virus	1	0,1
Naked/Plasmid DNA	162	15,9
Pox virus	56	5,5
Retrovirus	261	25,6
RNA transfer	12	1,2
Salmonella typhimurium	2	0,2
Semliki forest virus	1	0,1
Vaccinia virus	36	3,5
Adenovirus + Retrovirus	2	0,2
Pox virus + Vaccinia virus	17	1,7
N/C	45	4,4
Total	1020	

N/C = not communicated (trial details unknown)

Table taken from www.wiley.co.uk/genmed/clinical

1.4 Kaposi's Sarcoma-Associated Herpesvirus (KSHV)

1.4.1 Related gamma herpesviruses

1.4.1.1 Epstein-Barr virus

1.4.1.2 Herpesvirus saimiri

1.4.1.3 Murine herpesvirus 68

1.4.2 KSHV associated morbidity

1.4.3 KSHV gene expression profiles

1.4.4 Targets for therapeutic intervention

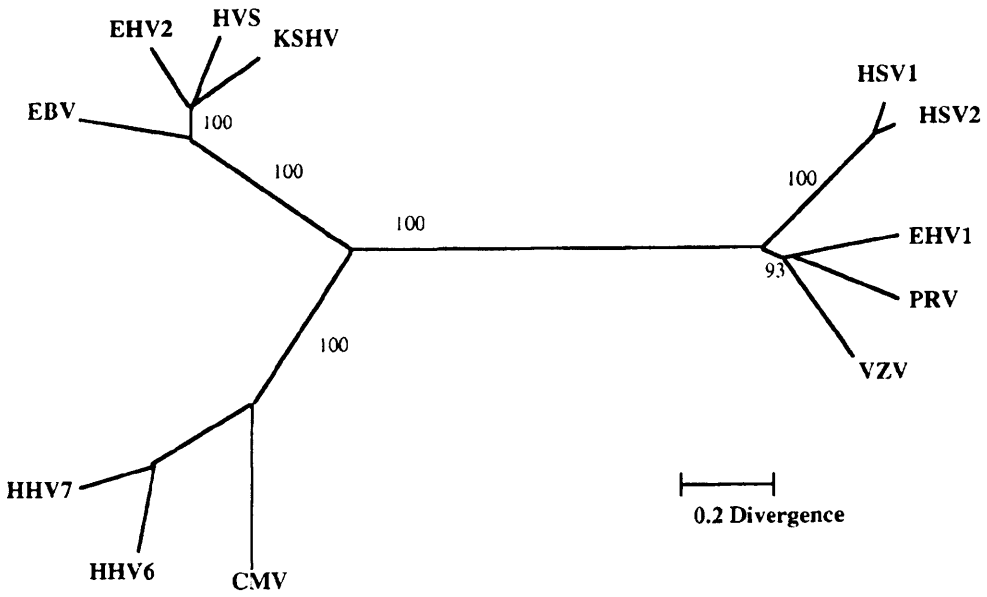
KSHV is a gammaherpesvirus which causes significant morbidity and mortality in immunocompromised individuals. Although it is not common in the United Kingdom, probably due to the widespread availability of effective antiretroviral drugs, it is problematic in Africa and Asia. KSHV was chosen as a therapeutic target for lentiviral delivered shRNA (Chapters 5 and 6) and this section covers the biology of KSHV and the reasons why disrupting the latent genes was expected to have therapeutic potential.

1.4.1 Related gamma herpesviruses

The human herpesviruses (HHVs) are a family of enveloped, double stranded DNA viruses. Their genomes vary greatly in size with cytomegalovirus (CMV) having the largest at about 230 kb. They are broadly classified into alpha-, beta- or gamma- herpesviruses originally based upon their target cell during latency and characteristic life cycle (Figure 1.4.1). The alpha herpesviruses include herpes simplex virus (HSV) -1 and 2 and varicella zoster virus (VZV). They have a short replicative life cycle (12-18 hours), a broad cell tropism, remain latent in neurons and induce cytopathology in monolayer cell cultures. The beta herpesviruses - cytomegalovirus (CMV), herpes lymphotrophic virus (HHV-6) and human herpesvirus 7 have a longer replicative cycle (several days) and a narrower cell tropism. They remain latent in lymphocytes (particularly T) and monocytes. The gammaherpesviruses are Epstein-Barr virus (EBV) and Kaposi's sarcoma-associated herpesvirus (KSHV, HHV-8). They have a very restricted host cell type. Their latent cell type has not been conclusively determined, although for EBV this is believed to be B lymphocytes (Ooka, 1985).

The herpesvirus' life cycle involves receptor-mediated binding, nucleocapsid transport to the nucleus, transcription, synthesis and assembly. Transcription is complex, with three types of protein being produced, categorised by their appearance within the life cycle as immediate-early, early or late proteins. (Alberts et al., 1994).

Figure 1.4.1: Phylogenetic tree of some related herpesviruses



(Divergence refers to the percent of base pairs which differ between genomes, numbers on graph indicate the number of times, as a percent, that this branch appeared in 100 replicates)

EHV2	Equine herpesvirus 2
HVS	Herpesvirus saimiri
HSV	Herpes simplex virus types 1 and 2
EHV1	Equine herpesvirus 1
VZV	Varicella zoster virus
HHV6	Human herpesvirus 6
HHV7	Human herpesvirus 7
CMV	Cytomegalovirus
PRV	Pseudo rabies virus

(Adapted from Moore et al., 1996)

1.4.1.1 Epstein-Barr virus

EBV is a gammaherpesvirus which infects B lymphocytes both *in vitro* and *in vivo*. *In vivo* infection with EBV has been associated with infectious mononucleosis, hairy leucoplakia and several lymphoproliferative neoplastic disorders, including Burkitts lymphoma and nasopharyngeal carcinoma (NPC) (Gunven et al., 1970), Hodgkin's lymphoma (Gledhill et al., 1991) and post-transplant lymphoproliferative disease (PTLD) (Nalesnik et al., 1988). *In vitro*, where a small proportion of infected cells establish latent infection, the virus induces cellular transformation and these cells contain multiple copies of the EBV genome (Adams and Lindahl, 1975). The DNA is circular (episomal), and is tethered to the host cell DNA via the protein Epstein Barr virus nuclear antigen 1 (EBNA-1) (Kaschka-Dierich et al., 1976), which is essential for the stability and proliferation of the episome (Yates et al., 1985). Latently infected cells *in vitro* are termed lymphoblastoid cell lines (LCLs). LCLs only express a handful of the 100 genes present in the EBV genome including EBNAs 1-6, the latent membrane proteins (LMP) 1-3 and two non-translated RNAs termed EBER1 and 2. This is in contrast to latent infection *in vivo*, where the only expressed gene is often EBNA-1. (Rowe et al., 1987). EBNA-1 is unique amongst the EBV genes in that it does not invoke an immune response when expressed on HLA. This is believed to be due to glycine-alanine repeats in it's molecular structure (Murray et al., 1988). EBV shares some characteristics with KSHV in terms of it's gene expression profile and cellular tropism, but although results from EBV studies hold some correlation with KSHV, EBV has limited use as a model for KSHV

1.4.1.2 Herpesvirus Saimiri (HVS)

HVS is a relatively close viral relative of KSHV on the phylogenetic tree (Figure 1.4.1). Its natural reservoir is squirrel monkeys (Melendez et al., 1968). It was isolated following observation of squirrel monkey cells in culture, and was just one of several novel herpesviruses isolated from primate tissues during this time. HVS remains latent in lymphocytes (like other gammaherpesviruses) and in permissive hosts it produces a life long infection. It has never been linked to any neoplastic disorder in squirrel monkeys, however it has been documented to cause both diverse lymphoproliferative disorders in other new world monkeys (Fleckenstein and Desrosiers, 1982).

HVS is also capable of infecting human cells, and has been shown to form lytic infection in human fibroblasts and latent infection in human T cells. Both human CD4+ and CD8+ T cells can be infected, and in both a latent infection is formed (Johnson et al., 1980). The virus has a 155 kbp dsDNA genome which bears a strong similarity in structure to that of KSHV. Because of the evolutionary and genomic similarity, HVS is a better model for KSHV than EBV.

1.4.1.3 Murine Herpesvirus 68 (MHV68)

MHV68 is a rodent virus which was isolated from voles (Blaskovic et al., 1980). In a laboratory setting, the virus infects B lymphocytes and in experimental animals establishes latent infection in the spleen and causes acute interstitial pneumonia (Sunil-Chandra et al., 1992). Infection is also associated with lymphoproliferative disorders (Sunil-Chandra et al., 1994).

Genomic analysis indicates that MHV68 is closer to KSHV than EBV (Virgin et al., 1997), and there is evidence that, like KSHV, it does not infect exclusively B cells (Weck et al., 1996). The virus can also infect blood vessels containing smooth muscle (Weck et al., 1997). KSHV is the only other gammaherpesvirus which is known to infect vasculature. MHV68 infection is considered to be a direct animal model of KSHV and is, therefore, often used to draw direct comparisons with KSHV.

1.4.2 KSHV associated morbidity

1.4.2.1 Kaposi's Sarcoma

Moriz Kaposi defined an illness in 1872 occurring predominantly in elderly Mediterranean or East European men involving tumours affecting mainly the skin of the lower limbs. Although more aggressive forms presented, including lesions within pharynx, small intestine, stomach, liver and colon; the skin lesions were by far the most common symptom. He called this disease Kaposi's Sarcoma (KS).

KS-like lesions have been associated with several clinical groups, and four subgroups of KS are recognised:

- Classic KS affects predominantly elderly Mediterranean or East European men. Classic KS occurs more often in men than women with distinct geographical distributions (Franceschi and Geddes, 1995)

- Endemic KS is a more aggressive form found in sub Saharan Africa (D'Oliveira and Torres, 1972). It often affects lymph nodes and occurs in children and adults. Again, there is a distinct geographical distribution for endemic KS.

- Iatrogenic (or post-transplant) KS occurs in patients who have been immunosuppressed, especially post-transplant (Harwood et al., 1979).

- AIDS-related KS has been recognised since patients with HIV were noticed to develop KS-like lesions, and also have an increased risk of primary effusion lymphoma (PEL) and multicentric Castleman's disease (MCD) (Whitby et al., 1995; Ascoli et al., 2001). KSHV associated KS, PEL or MCD is considered AIDS defining in HIV positive individuals by the World Health Organisation (WHO).

(http://www.who.int/hiv/strategic/en /cdc_1993_hivaids_def.pdf).

The aetiological agent responsible for KS was discovered by representational difference analysis (RDA) in 1994 and was officially classified as human herpesvirus 8 (HHV-8) or Kaposi's Sarcoma-associated herpesvirus (KSHV) (Chang et al., 1994). Rapid research into detection, characterisation and epidemiology of KSHV linked it with at least two further conditions MCD (Soulier et al., 1995) and PEL (Nador et al., 1996).

1.4.2.2 Multicentric Castleman's Disease

Castleman's disease was characterised in 1956 as the development of solid growths within the lymphatic tissue (Castleman et al., 1956). These growths are benign and contrast strongly with multicentric Castleman's disease (MCD). MCD is a malignant lymphoproliferative disease with a poor prognosis. In HIV positive individuals, almost all cases of MCD are associated with KSHV infection. MCD does occur in HIV negative individuals where around 50% are associated with KSHV infection. In KSHV positive individuals, the severity of the disease correlates closely with KSHV viral load (Oksenhendler et al., 2000).

Castleman's disease has two subtypes- the hyaline vascular and the plasma cell variants. The hyaline vascular sub-type accounts for 90% of cases of Castleman's disease and patients are generally asymptomatic. Histologically, there is extensive capillary proliferation within the affected lymph nodes, and a lymphocyte predominant infiltrate surrounding small germinal centres.

The plasma cell variant typically has more generalized (thoracic, mesenteric, and retroperitoneal) lymph node involvement and the disorder tends to be disseminated. The capillary proliferation seen in the hyaline vascular sub-type is absent on examination of enlarged nodes. Dysregulation of interleukin-6 is implicated in the pathogenesis of plasma cell Castleman's disease, and anti-IL6 antibody therapy has been suggested (Beck et al., 1994). MCD is a severe form of plasma cell variant Castlemans disease. KSHV is present in plasmablastic MCD (both hyaline vascular and plasma ell subtypes) and this form of the disease is referred to as plasmablastic or KSHV associated MCD.

Treatment of MCD combines steroids, chemotherapeutic agents or even rituximab (anti CD-20 antibody) (Corbellino et al., 2001). Prognosis is poor with a median survival of 26 months from diagnosis. Patients with multicentric disease and an associated neuropathy have an extremely poor prognosis, despite treatment with steroids and chemotherapy. Disruption of KSHV within

the enlarged nodes of MCD may provide a therapeutic option for those who develop MCD, even with HAART and an adequate CD4 count and resistance to current treatment options.

1.4.2.3 Primary Effusion Lymphoma

PEL, a form of body cavity-based B-cell lymphoma (BCBL), is a rare non-Hodgkin's-like malignant lymphoproliferation found primarily in body cavities. PEL occurs primarily in the severely immunocompromised and is associated strongly with HIV infection. Although there is not usually any solid mass focus, there have been several documented cases of PEL with an extra-cavity tumour (Chadburn et al., 2004; Mate et al., 2004). Effusions can be found in pleural, peritoneal, pericardial or even the subarachnoid space (Ely et al., 1999). Development of PEL has been linked to infection with KSHV (Nador et al., 1996).

Cells found with the effusions are large immunoblast-like cells which do not express common B lymphocyte specific markers. This distinguishes these cells form those of MCD, which usually express CD20. These cells are thought to be derived from germinal or post-germinal B cells because of their Ig gene rearrangement. PEL cells contain 100-150 copies of the KSHV genome, which is significant because in KS lesions the copy number of KSHV is much smaller, less than 10 copies per cell (Ansari et al., 1996; Cesarman et al., 1995). The KSHV gene expression profile within PEL is distinct from other KSHV-associated tumours (Parravicini et al., 2000).

These cells are also frequently associated with EBV infection, however coinfection is not essential for development of PEL, and the role of EBV is not known (Fassone et al., 2000). There are not usually any rearrangements or alterations in c-myc, bcl-2, ras or p53 in PEL cells.

The prognosis of PEL is also very poor- with a median survival of less than 6 months. There are currently no effective protocols for the treatment of PEL, although a variety of antiviral and chemotherapeutic regimes have been tested. Patients who are not on HAART who develop PEL may benefit from anti-retroviral treatment (Hocqueloux et al., 2001). Alpha interferon (Monini et al., 1999) and rituximab (Perez and Rudoy, 2001) have also been tried as

therapeutics. Novel approaches that inhibit latent gene expression (Curreli et al., 2005; Godfrey et al., 2004) may offer new therapeutics.

Several cell lines for PEL exist, although many are coinfected with EBV (see Table 1.4.1), compared to very few KS cell lines and no MCD lines. Unfortunately, all currently available cell lines derived from KS lesions do not contain detectable copies of the genome (Flamand et al., 1996). There are also mouse models of PEL (Staudt et al., 2004; Boshoff et al., 1998), which means that PEL is more easily studied both *in vitro* and *in vivo*.

Table 1.4.1 The KSHV and EBV status of commonly used PEL cell lines

Cell line	Origin	Host HIV-1 serostatus	HHV8 DNA	EBV DNA
BC-1	AIDS-PEL	Positive	Positive	Positive
BC-2	AIDS-PEL	Positive	Positive	Positive
BC-3	PEL	Negative	Positive	Negative
BCBL-1	AIDS-PEL	Positive	Positive	Negative
JSC-1	AIDS-PEL	Positive	Positive	Positive
BCP-1	AIDS-PEL	Positive	Positive	Negative
CRO/AP3	AIDS-PEL	Positive	Positive	Negative
CRO/AP5	AIDS-PEL	Positive	Positive	Positive
CRO/AP6	AIDS-PEL	Positive	Positive	Negative
HBL-6	AIDS-BL	Positive	Positive	Positive

Table 1.4.1: Table showing the origin of commonly used primary effusion lymphoma (PEL) cell lines. These cells are believed to have a B cell lineage, and are derived from PEL samples in HIV positive (AIDS-PEL) or negative (PEL) individuals, or from a Burkitt's Lymphoma biopsy shown to be HHV-8 positive (HBL-6, marked BL). The EBV status is significant as some viral genes between EBV and HHV-8 have conserved function, and so is shown in the final column.

1.4.3 KSHV gene expression profiles

The KSHV genome is about 140 kbp long and contains around 85 open reading frames (Fig 1.4.2). Around 15 of these are currently thought to be unique to KSHV (designed *K1-K15*), and many of the others are pirated cellular genes, or cellular homologues like *vIL-6* (viral interleukin 6 homologue), *vIRF* (viral interferon responsive factor) and *vFLIP* (viral FLICE inhibitory protein) (Moore et al., 1996; Russo et al., 1996). The remainder are common to gammaherpesviruses, and often represent critical components of the life cycle or are involved in establishing latent infection (such as *LANA*, the latency associated nuclear antigen, which is functionally related to *EBNA-1*). The viral genes are divided in terms of their expression. They can be divided into latent and lytic, depending on which phase of the life cycle they are expressed in, or immediate early, early, late-early, and late depending on when they are expressed after infection (Jenner et al., 2001). The latent genes are suspected to be involved in tumourigenesis.

The lytic phase is characterised by the release of new viral progeny and the latent phase by limited gene expression. KSHV encodes a number of cellular homologues, and it is thought that these genes determine the pathogenicity of KSHV (Neipel et al., 1997).

Initial infection involves lytic replication, followed by establishment of latency in some cells. During latency, only a handful of genes are expressed, and only three are detectable in all latent infections (Sarid et al., 1999). Reactivation from latency into lytic replication can be stimulated with sodium butyrate or 12-o-tetradecoylphorbol 133-acetate (TPA) (Yu et al., 1999) or induction of the lytic cycle promoter ORF-50 (Rta) (Lukac et al., 1998).

The association of the latent life cycle with oncogenesis stems from the transcription pattern in KSHV-related tumours and the fact that acute infection does not induce tumours. The interactions and cellular functions of the latent genes are also evidence for their role in oncogenesis. Although no single

KSHV gene is sufficient for oncogenesis - like HTLV-1 *tax*, the biological roles of the latent genes together have these abilities (Table 1.4.2).

LANA has many roles in the pathogenesis of KSHV and has been functionally linked to *EBNA-1* in EBV. It binds to p53, downregulating its activity and preventing it from driving cells into apoptosis (Friborg, Jr. et al., 1999). LANA binds to and inactivates pRb (Radkov et al., 2000). LANA also associates with a whole range of other cellular factors involved in cell cycle dysregulation (Komatsu et al., 2001). After its interaction with p53 and pRb, perhaps it's most important role is its ability to tether the viral episome to cellular DNA, allowing latent infection (Ballestas et al., 1999). It also seems to allow the latent life cycle by inhibiting lytic replication, and exists antagonistically with Rta (the major promoter in the lytic life cycle) (Lan et al., 2004).

The viral cyclin (vcyclin) is a cellular cyclin D homologue. Its sequence shows around 50% sequence similarity with cyclin D2 (Li et al., 1997) and it can bind to and activate Cdk4 and Cdk6 and direct their kinase activity towards Rb (Chang et al., 1996). There is evidence that vcyclin binds preferentially with Cdk6 (Godden-Kent et al., 1997). The vcyclin is also much less susceptible to inactivation by cellular factors which normally govern the cell cycle, including pRb and p21 (Direkze and Laman, 2004; Verschuren et al., 2004). These factors give the viral cyclin a key role in oncogenesis.

vIL-6, vBcl-2 and vFLIP all act on apoptosis and cellular transcription factors such as I KappaB kinase (IKK) leading to NF-κB activation (Field et al., 2003; Weiss et al., 1998). These abilities together allow KSHV to cause malignant cell proliferation.

Recent evidence that disruption of the latent genes leads to senescence, apoptosis or interference in cell pathway interaction in latently infected PEL cells (Guasparri et al., 2004; An et al., 2004) offer the potential to treat KSHV-driven malignancies. Methods to inhibit gene expression which are feasible in a clinical setting are now needed.

Table 1.4.2 Latent genes and their main biological roles

Gene name	Gene location	Gene classification	Life cycle	Major function
LANA-1	*ORF 73*	1	Latent	Binds p53 and pRB
vcyclin	*ORF 72*	1	Latent	Cell cycle progression
vFLIP	*ORF 71*	1	Latent	Inhibition of *Fas*-induced apoptosis. Activation of NFκB
Kaposin A	*K-12/T0.7*	2	Latent	Unknown
vIRF1	*K 9 2*	2	Latent in KS	Inhibits interferon-induced gene expression, MYC activation interacts with p53
LANA-2/ vIRF3	*ORF 10.5*	3	Latent in PEL, MCD	Interacts with p53

LANA, latency associated nuclear antigen; *v-FLIP*, viral Fas-associated death domain-like interleukin 1 β converting enzyme; vIRF, viral interferon regulatory factor; *vGPCR*, viral G-protein-coupled receptor; *vIL-6*, viral interleukin 6; *vMIP*, viral macrophage inflammatory protein; ORF, open reading frame; K, genes unique to KSHV/HHV-8; KS, Kaposi's sarcoma; PEL, primary effusion lymphoma; MCD, multicentric Castleman's disease. Gene classification as determined by Sarid et al, 1998. Data for table taken from Cathomas, 2003.

Figure 1.4.2 Structure of the KSHV Episome. (Sharp and Boshoff, 2000)

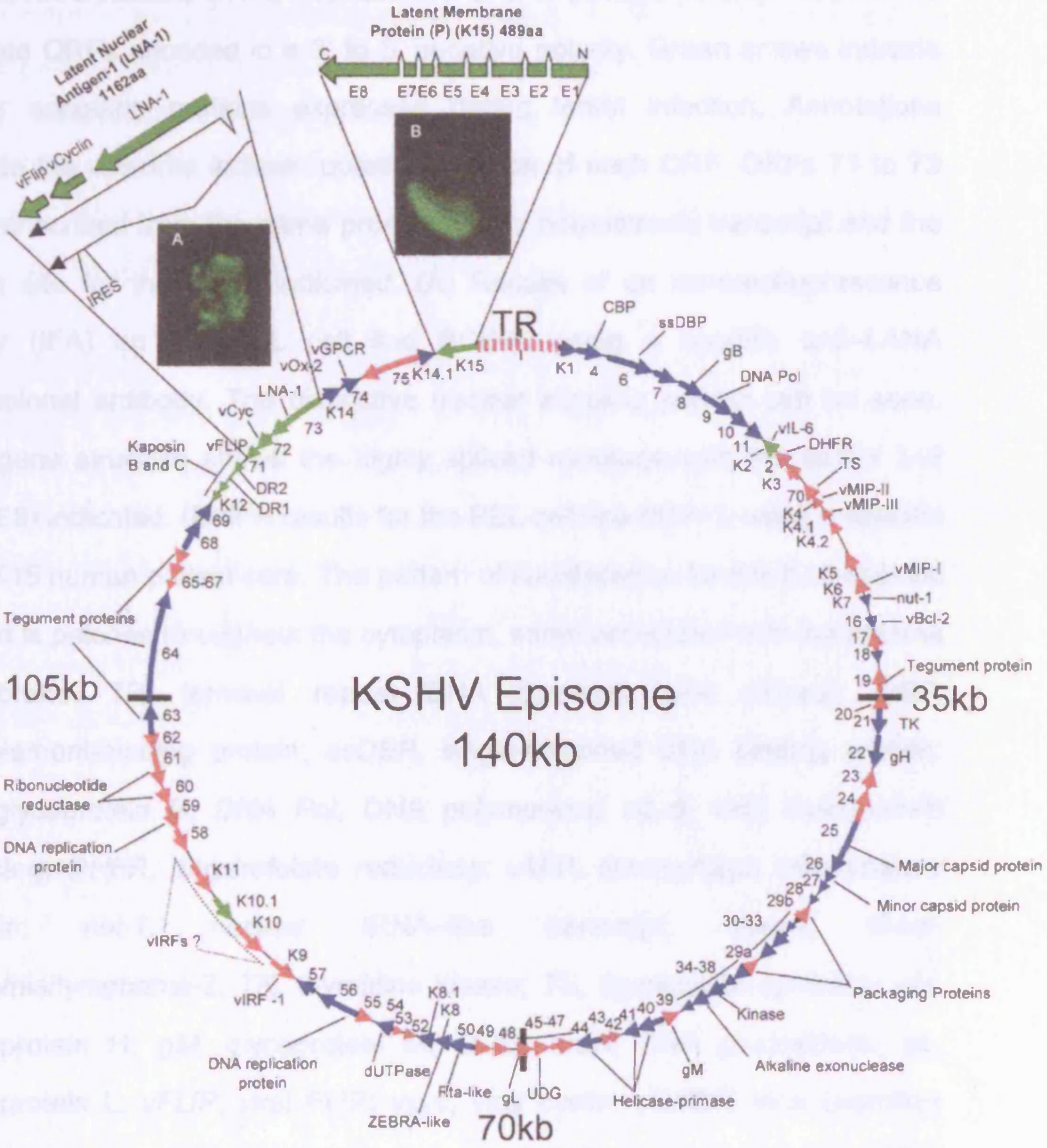


Figure 1.4.2 Numbers outside of episome represent nucleotide base pairs in kilobases (kb). Numbers within the episome indicate KSHV-encoded ORFs. Novel ORFs not present in other herpesviruses were designated *K1* to *K15*. Blue arrows indicate ORFs encoded in a 5' to 3' positive polarity. Red arrows indicate ORFs encoded in a 3' to 5' negative polarity. Green arrows indicate ORFs encoding proteins expressed during latent infection. Annotations outside the episome indicate putative function of each ORF. ORFs 71 to 73 are transcribed from the same promoter on a polycistronic transcript and the splice site for the intron indicated. (A) Results of an immunofluorescence assay (IFA) on the PEL cell line BCP-1, using a specific anti–LANA monoclonal antibody. The distinctive nuclear stippling pattern can be seen. K15 gene structure shows the highly spliced message with the exons 1–8 (E1–E8) indicated. (B) IFA results for the PEL cell line BCP-1, using a specific anti-K15 human patient sera. The pattern of fluorescence for this K15-specific serum is patches throughout the cytoplasm, some associated with the plasma membrane. TR, terminal repeat DNA domains (pink stripes); *CBP*, complement-binding protein; *ssDBP*, single-stranded DNA binding protein; *gB*, glycoprotein B; *DNA Pol*, DNA polymerase; *vIL-6*, viral interleukin-6 homolog; *DHFR*, dihydrofolate reductase; *vMIP*, macrophage inflammatory protein; *nut-1*, nuclear tRNA–like transcript; *vBcl-2*, B-cell leukemia/lymphoma-2; *TK,* thymidine kinase; *TS*, thymidylate synthase; *gH*, glycoprotein H; *gM,* glycoprotein M; *UDG*, uracil DNA glucosidase; *gL*, glycoprotein L; *vFLIP*, viral FLIP; *vcyc*, viral cyclin; *vGPCR*, viral G-protein coupled receptor; (P), predominant form of K15; aa, amino acids; IRES, internal ribosomal entry site.

1.4.4 Targets for therapeutic intervention

There are currently no good therapies against KSHV. Although the antivirals ganciclovir, cidofovir and foscarnet effectively inhibit the lytic life cycle (Kedes and Ganem, 1997), the transforming properties of KSHV involve latent gene expression and these antiviral drugs do not have any effect. Treatment for KS often involves surgical removal, radiotherapy or chemotherapy. Topical treatment with 10% docosanol cream is effective in some KS lesions, and treatment of underlying immunosuppression either with HAART or regulation of immunosuppressant drugs is important.

The latent genes *LANA*, *vFLIP* and *vcyclin* are obvious targets when considering the oncogenic potential of KSHV (Staudt and Dittmer, 2003). They are the only three genes expressed in all latently infected cells. Disrupting LANA should theoretically cause expulsion of the virus. Inhibition of LANA may be an effective therapeutic, however LANA has other roles, and reducing *LANA* expression may reactivate the lytic life cycle. In KS, where the copy number of the virus is small this may be desirable, however in PEL and MCD this could cause major complications.

Chapter 2:

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2.1 Standard Methods

2.1.1 Plasmid preparation

Plasmid preparation was performed broadly according to the Qiagen guidelines for use of miniprep, maxiprep or endotoxin free maxiprep. Endotoxin free maxipreps were performed to produce viral packaging plasmids which require high transfection efficiency.

Minipreps were performed for routine screening for inserts or to test plasmids for production of virus. Colonies grown on an $LB^{antibiotic}$ plate (usually ampicillin or kanamycin) were taken and inoculated into 5 mls of LB^{amp} or LB^{kan} for overnight culture. The number of colonies chosen for individual minicultures varied according to the ligation efficiency. Between 4 and 8 colonies per reaction was usually sufficient.

The minicultures were grown overnight (16 hours) at 37^0c shaken at 225 rpm. 1.5 mls of this culture was taken and placed into a 1.5 ml eppendorf, where it was spun at 6,000 rpm in a desktop centrifuge for one minute. The LB was removed, and the pellet resuspended, lysed and neutralized by alkaline lysis method in accordance with the guidelines in the kit. The cloudy liquid was then centrifuged at 13,000 rpm in a desktop centrifuge. This was decanted into the miniprep columns, spun at 13,000 rpm for one minute. The wash steps (endonuclease removal, PB; column wash, PE; column clean) were performed according to the kit guidelines.

Resuspension was in 50 μl of water and plasmids were then kept on ice during use or at -20^0c for long term storage. DNA concentration and purity was assessed using a UV spectrophotometer measuring the A_{260} and A_{260}/A_{280} ratio.

Maxicultures for maxiprep were prepared by inoculating 500mls (for high copy plasmids) or 2000 mls (for low copy plasmids) of LB^{amp} or LB^{kan} with 1 ml of

miniculture and growing overnight at 37^{0}c shaken at 225 rpm. This broth was centrifuged at 4,000 rpm for 10 minutes using a JA10.5 rotor and 2x250 ml or 6x350 ml centrifuge bottles. These mixtures were resuspended, lysed and neutralized according to the Qiagen kit guidelines. After centrifugation for 20,000g for 30 minutes using a JA25.5 rotor, maxiprep/ endotoxin free maxiprep columns were equilibrated (15mls buffer QBT), and the clear supernatant added to the column. Washes were performed according to the Qiagen kit guidelines.

Resuspension was in 0.5 ml water, and plasmid concentration and purity was assessed using a UV spectrophotometer measuring the A_{260} and A_{260}/A_{280} ratio.

Where necessary, phenol/chloroform extraction precipitation was used to increase the purity of plasmid DNA. A volume equal to that of the sample of phenol/ chloroform/ iso-amyl alcohol in a ratio of 25:24:1 followed by vigorous mixing for 30 seconds and then phase separation by centrifugation at 13,000g for 5 minutes. The aqueous phase was extracted. This procedure was repeated using chloroform/ iso-amyl alcohol at a ratio of 24:1. Ethanol precipitation, where necessary, was performed by adding 3.5 volumes of 100% ethanol to the DNA mixture and 0.1 volumes of sodium acetate. This resulting mixture was incubated at -20^{o}c for 1 hour and the precipitated DNA pelleted using a desktop centrifuge at 13,000g for 15 minutes. The supernatant was carefully discarded and the pellet resuspended in sterile nuclease free water or a suitable buffer.

2.1.2 Restriction digestion

Restriction digests were used to clone fragments of DNA, determine correct insertion, orientation of inserts and confirm plasmid integrity.

Restriction digestion was performed at 37^{0}c unless otherwise indicated in the Promega notes (25^{0}c for Sma I and 65^{0}c for BstB I) in a total volume of 20 μl.

Incubation was normally for 1 hour, but overnight incubations were used for some polymerase chain reaction (PCR) products containing short overhangs (as advised by the New England Biolabs digestion handbook). The buffer used was determined by consulting the Promega enzyme buffer compatibility chart, when performing multiple digests the most appropriate buffer was chosen. 0.5 μl (generally 5-6 units of enzyme) was incubated with 1-2 μg plasmid DNA for each reaction, and a non-digested plasmid and positive control plasmid containing the target restriction site were always used. After restriction, 4 μl of 6x DNA loading buffer (see below) was added and the mixture stored at 4^0c or -20^0c until agarose gel analysis/ extraction.

2.1.3 Agarose gel analysis

1% weight/ volume (w/v) agarose in TAE (tris-acetate 0.4M, ethylene di-amino tetraacetic acid (EDTA) 0.01M) gels were standardly used for analysis throughout this thesis. Smaller fragments (such as PCR products <100bp) used higher w/v gels, usually 2% or 3.5%. Agarose gels were prepared by heating the agarose (AMRESCO) / 0.5x TAE mixture until the agarose fully dissolved. Between 50 and 100mls of the solution was poured into a gel cast, an appropriate comb added and left to cool for 5-10 minutes. Before the gel became solid, 2-4 μl of 10 mg/ml ethidium bromide (Sigma) was added and mixed gently into the liquid. Gels were placed in a tank containing 0.5x TAE. Samples were loaded with a Promega 1kb ladder in lane 1 and electrophoresed at 5-10 V/cm for 30 minutes-2 hours depending on the size of fragments and resolution required. If fragments were to be used for DNA ligation, gel extraction was performed using a Qiagen gel extraction kit in accordance with the manufacturers guidelines. Up to 100 mg of gel was placed in an eppendorf and 300 μl buffer QG added. Mixture was incubated at 65°C for 10 minutes and purified using supplied column, centrifugation at 13,000g and washing using buffer QG and PE. Elution was in 50 μl (or 30 μl if quantity of DNA was low or high concentration required) sterile water.

2.1.4 Ligation

Ligation reactions were performed using New England Biolabs T4 DNA ligase and NEB T4 DNA ligase buffer in a total volume of 10 μl. 100-400ng of backbone DNA was mixed in a 1:3 vector:insert ratio with 20 units (1 μl) of enzyme per ligation reaction. The mixture was incubated for 30 minutes at room temperature (sticky ends), 1 hour at room temperature (blunt ends) or overnight at 4^0c. 1 μl of reaction mixture was used for transformation.

2.1.5 Competent cell preparation

Electrically competent cells were prepared by inoculating a 50 μl aliquot of appropriate cells (HB101 or DH5α) into 250 mls of LB under sterile conditions. After 6-8 hours at 37^0c shaken at 225 rpm, when the cells reached an A_{600} of 0.5-0.8 using a UV spectrophotometer, the bacteria are in logarithmic growth phase and are ready for harvest.

The bacteria, 375 mls of distilled autoclaved water and 5.5 mls of 10% glycerol were placed at 4^0c. After 1hr, the medium was centrifuged at 4,000g at 4^0c for 15 minutes using a JA10.5 rotor to pellet the bacteria. The pellet was resuspended gently in 250mls of cold distilled water, and then centrifuged at 4,000g at 4^0c for 15 minutes. The pellet was resuspended in 125 mls distilled, autoclaved water. This mixture was centrifuged at 4,000g at 4^0c for 15 minutes and resuspended in 5 mls glycerol. The mixture was transferred to a 15 ml falcon. This mixture was centrifuged at 4,000g at 4^0c for 10 minutes and resuspended in 500 μl cold glycerol. 50 μl aliquots were snap frozen in a dry ice-ethanol bath and kept at -80^0c for up to 6 months. Chemically competent cells were purchased as Top 10's from Invitrogen BV, Groningen.

2.1.6 Bacterial transformation

Chemical transformation (heat shock) was performed by placing the competent cells (individual aliquots) on ice for 30 minutes, and then adding 1 μl of the ligation mixture or 100 ng of intact plasmid. This mixture was heated to 42^0c in a water bath for 35 seconds, and then 1 ml of LB (no antibiotic) added and incubated at 37^0c for 1 hour. 100 μl was then plated onto an LB plate containing suitable antibiotic (usually ampicillin or kanamycin).

Electrical transformation was performed when increased efficiency was required, such as for blunt end or low yield ligations. Aliquots of electrically competent bacteria were placed at 4^0c for 15 minutes prior to electroporation. After 15 minutes, the cells (50 μl) were placed in a chilled 1 mm cuvette. 1 μl of the ligation reaction was added to the cells and mixed by gentle agitation. Electroporation was performed in a BioRad 100 electroporator, using 200 ohms, 2.5 μF and 1.25 kV. Time constants for successful electroporation were between 4 and 5 ms.

2.1.7 Blunting sticky DNA fragments

Two methods were used to blunt sticky DNA fragments when compatible sticky ends could not be found in the vector and insert. Fill-in reactions, to preserve the restriction sites were performed using T4 DNA polymerase Large (Klenow) fragment, and exonuclease reactions to destroy the restriction site and generate flush DNA ends were performed using Mung Bean Nuclease. In both cases, DNA was extracted (see section 2.1.1) and eluted in 30 μl water.

For Klenow treatment, the DNA was mixed with 5 μl 10x DNA polymerase buffer (Promega), a final concentration of 1 mM dNTPs, 1 unit/ μg DNA of Klenow (Promega) and water to a total volume of 50 μl. This was incubated at 37^0c for 30 minutes and then 65^0c for 30 minutes.

Mung Bean nuclease treatment was performed in a total volume of 100 µl, using 10 µl of 10x Mung Bean buffer and 50-70 units of Mung Bean nuclease. Water was used to make up the final volume. This was incubated at 37^0c for 2 minutes, or room temperature for 15 minutes.

Enzyme was removed by agarose gel analysis and extraction or Qiagen PCR clean-up kit (used according to the manufacturers guidelines). After blunting, alkaline phosphatase treatment is required to prevent backbone self-ligation. 5 units of Shrimp Alkaline phosphatase (Promega) was added to the DNA and 5 µl of SAP 10x buffer. Final volume was made up to 50 µl with water. This mixture was incubated at 37 ^{0}c for 15 minutes and enzyme removed by phenol/ chloroform extraction.

2.1.8 PCR diagnosis/ cloning

Polymerase chain reaction (PCR) was carried out either to amplify fragments for cloning or to detect presence of DNA inserts. PCR was performed on a Primus 96-plus thermocycler (MWG Biotech). Reaction conditions were optimized based upon the Tm of the primers, the GC content and length of the amplified fragment. Typically, reaction volume was 50 µl. 5 µl of complete PCR buffer (Roche), 1 µl Taq DNA polymerase (Roche) or Expand high fidelity polymerase for cloning (Roche), 1 µl 10 mM dNTP's and 100 ng of each primer were added to the target DNA and the volume made up by UV Stratalinker treated water. 10 ng (plasmid) – 1 µg (whole cellular) DNA was used depending on the quality of the sample. Typical amplification conditions were 35-40 cycles of 95^0c for 1 minute, 55^0c for 1 min, 72^0c for 2 minutes, followed by a further 1 minute at 72^0c. Controls included a no polymerase reaction, no template reaction and if appropriate, internal controls for the β-actin gene. If no product was seen, the annealing step (55^oc) temperature was lowered, if multiple nonspecific products were seen, this temperature was increased. If the product was large (>1kb), the length of the extension step

(72^0c) was increased to 4 or 6 minutes. After cycling, the product was mixed with 4x DNA loading buffer and placed on agarose gel for analysis.

2.1.9 Site directed mutagenesis

Site directed mutagenesis was performed using a Stratagene QuickChange Site Directed Mutagenesis kit in accordance with the manufacturers guidelines.

PCR primers overlapping the region containing the new mutation were designed as indicated by the manufacturer. 1, 10 and 100 ng of original plasmid were incubated in parallel with 1 unit of high fidelity Taq polymerase, the primers and 1 μl of 10 mM dNTPs. 40 rounds of PCR amplification were used with an annealing temperature of 55^0c and an extension time of 2-5 minutes (20 seconds per kilobase). After PCR, the original plasmid was enzyme digested and resulting mixture transformed into electrocompetent bacteria. 10-1000 colonies were seen after overnight incubation at 37^0c.

2.1.10 Nucleic acid extraction

Nucleic acid extraction was required to analyse DNA (or RNA) from cells in tissue culture or for samples from mice in *in vivo* experiments.

DNA extraction was carried out using a Qiagen Blood and Cell Culture Mini kit in accordance with the manufacturer's guidelines. Tissue samples were suspended in tissue lysis buffer: 50 mM KCl, 10 mM Tris pH 8.3, 2.5 mM $MgCl_2$, 0.01% gelatin, 1% tween and 200 μg/ml proteinase K (Sigma). Samples were incubated at 55^oc for 24 hours and then at 94^oc for 10 minutes to inactivate the proteinase K. Phenol chloroform precipitation was performed.

Cells from tissue culture or ascitic samples were centrifuged at 1200g for 5 minutes and resuspended in DNA lysis buffer: 100 mM NaCl, 10 mM Tris-

HCL pH 8.0 and 10 mM EDTA. Solution was mixed thoroughly and incubated at room temperature for 10 minutes. DNAse free RNAse A was added to a final concentration of 100μg/ml. This mixture was incubated at room temperature for 2 hours. Proteinase K was added and the mixture incubated for 14-18 hours at 56°C. Phenol chloroform extraction was then performed (see section 2.1.1).

RNA for RT-PCR (reverse transcriptase polymerase chain reaction) and qRT-PCR (quantitative RT-PCR) was extracted from tissue samples and cells from tissue culture. Whenever RNA extraction was performed, all work surfaces, gloves and containers were treated with RNAseZAP (Ambion) to remove RNAse from the environment. RNA purification was performed using the RNEasy kit (Qiagen) in accordance with the manufacturers guidelines. Briefly, up to 5 x 10^6 cells were used for each extraction. Cells were centrifuged at 3000 rpm in a desktop centrifuge and then resuspended in 350 μl of buffer RLT and vortexed until no clumps were visible. This lysate was placed in a QiaShredder column to homogenize and centrifuged at 13000 rpm for 2minutes. 350μl of 70% ethanol was added to the flowthrough and this mixture passed over an RNEasy column. The column was washed with both buffer RW1 and buffer RPE and the RNA eluted in 30 μl of sterile RNAse free water.

2.1.11 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR)

RT-PCR was performed on RNA extracted from cells as a two stage process consisting of cDNA synthesis (RT step) and PCR. RNA was diluted to a final concentration of 0.1 mg/ml in sterile, nuclease free water. 10 μl of this solution was mixed with 1 μl RNAse inhibitor (Promega) by gentle tapping. A positive control (RNA known to contain gene of interest) and a negative control (nuclease free water) were added at this step. An internal control (primers to β-actin) was used if results were poor. 2 tubes for each sample, each containing 2 μl of this solution was mixed with 0.5mM dNTP and 10mM

DTT and diluted to a final volume of 19μl with sterile nuclease free water were prepared. 1μl of Superscript RT II (Invitrogen) diluted to 40 U/μl was added (final concentration 2 U/μl) to one tube and 1 μl of nuclease free water to the other for each sample (no RT control for each sample). These mixtures were incubated on a Primus 96-plus thermocycler (MWG Biotech) at 42°C for 5 minutes, 50°C for 50 minutes and then 70°C for 15 minutes. Mixtures were kept on ice if for immediate use and at -20°C if for use later.

PCR was performed using primers for the gene of interest and any further appropriate controls (see section 2.1.8)

2.1.12 TaqMan quantitative PCR / quantitative RT-PCR

Quantitative PCR and RT-PCR was performed using an ABI TaqMan Prism 7000 machine. Primers used for detection of lentiviral packaging cDNA and latent gene mRNA and DNA are listed in section 2.6.4. All reactions were performed using SYBR green at the concentrations indicated unless a probe is specified (for LANA). These conditions were shown through optimisation according to the manufacturers guidelines to be sensitive, specific and with no primer-dimer formation. Standard TaqMan cycling conditions were used throughout. Negative controls (no template and water only) and positive controls (known positive samples) were included in every reaction plate.

2.1.13 Western blotting (SDS-PAGE)

Protein was prepared by lysing 10^4 to 10^6 cells in Radio immunoprecipitation assay (RIPA) buffer (20 mM Tris pH 7.5, 50 mM NaCl, 1% SDS) and samples were quantified and equalized using a BCA assay. A BCA kit was purchased from Pierce and used to determine protein concentration. Standard protein concentrations of 1, 2.5, 5, 10 and 20 mg/ml protein were prepared using BSA (Promega) diluting the protein in RIPA buffer. 1 μl (representing total of 1, 2.5, 5, 10 or 20 μg protein) was used for each standard. Mixtures A and B were mixed at a 1:50 ratio and 1 ml of this mixture incubated with 1 μl of

standard or 5 μl of protein sample and incubated at 37^0c for 30 minutes. The A_{540} of the resulting mixture was used to create a standard line and thus determine the absolute protein concentration in the samples. Equalised samples were then mixed with 4X SDS loading buffer (see below), boiled for 2 minutes and centrifuged at 13,000 rpm in a desktop centrifuge for 1 minute.

Gels were prepared as 6% (LANA), 12% (eGFP) or 15% (vcyclin, vFLIP, β-actin) w/v polyacrylamide solution with 0.1% w/v SDS (sodium dodecyl sulphate) and 0.1% w/v APS (ammonium persulphate) buffered in pH 8.8 Tris HCl. Water was used to dilute to final volume and NNN'N'-Tetramethylethylenediamine (TEMED) was added at 0.05% final volume. Gels were poured with a 5% w/v acrylamide gel in Tris-HCl pH 6.8 buffer stacking layer for protein loading. 10 μl of Rainbow colour protein molecular weight ladder (BioRad) was loaded in lane 1 and 10 μg sample in lanes 2 onwards. Gels were electrophoresed at 100 volts until the marker/dye layer reached the bottom of the gel.

Protein was transferred to a HyBond-P (Amersham) membrane using semi-dry transfer for 30 minutes (small proteins) or 90 minutes (LANA) per membrane in standard transfer buffer (see below). After transfer, membrane was blocked with milk (Marvel) resuspended in PBS containing 0.05% v/v Tween 20 (PBS-Tween) for 1-2 hours. Membrane was incubated with primary antibody overnight at 4^0c at concentrations recommended by the manufacturer. Membranes were washed 4x for 15 minutes with PBS-Tween and then incubated for 30-60 minutes with secondary antibody used in accordance with the manufacturers guidelines. After incubation, a further 4x 15 minutes washes were performed and mixture incubated with ECL+ (Amersham) according to the manufacturers guidelines. 10 second, 1 minute and if necessary 10 minute exposures were taken and developed.

Where appropriate to ensure equal loading, the membrane was reprobed with β-actin antibody overnight and washed and developed as above.

2.2 Tissue Culture

2.2.1 Adherent cell line culture

Adherent cell lines were cultured in Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum (FCS). Antibiotics were not routinely used for tissue culture unless cryptic contamination was suspected. Eradication of suspected contamination was only carried out if the cell line was irreplaceable, and involved a two week culture period in penicillin and streptomycin or ciprofloxacin. Cells were ensured mycoplasma negative by culture testing every 3 months carried out by Mycoplasma Experience. When cells became > 70% confluent, or every three days (whichever was the sooner) cultures were passaged ('split'). Medium from plates was carefully poured into suitable disinfectant. Each 10 cm plate was washed 2x with 10 mls of pre-warmed PBS (Phosphate Buffered Saline) and then incubated for 2-5 minutes at 37°c with 1 ml of Trypsin-EDTA diluted in 4 mls of pre-warmed PBS. After this time, cultures were vigorously agitated. Cells would normally separate from the plate easily after this time. If cells did not separate from the culture dish, a longer incubation or using undiluted Trypsin-EDTA was sometimes necessary. The supernatant containing the cells was then centrifuged at 1200 rpm for 5 minutes in a 15 ml falcon. The resulting pellet was resuspended in 10 mls pre-warmed DMEM + 10% FCS. The cell density of this mixture was established using a haematocytometer and cells reseeded into new plates at an appropriate density. Trypan blue was used to determine live cell number once a week. This involved mixing an equal volume of trypan blue stain and cells. Cells were then counted using a haematocytometer, but strongly stained cells were considered to be dead or dying.

Frozen cell stocks were produced every month. 4 plates of cells were trypsinised as above, collected, centrifuged at 1200g for 5 minutes and the pellet resuspended in 1ml 90% FCS and 10% di-methyl sulphoxide (DMSO) and placed in cryotubes. These tubes were placed at -80°C overnight in

polystyrene containers to slow the freezing process. The morning after, cells were moved to storage in liquid nitrogen.

When counting cells for seeding experiments, a haematocytometer was used. Cells were diluted with an equal volume of trypan blue. Cells which were healthy excluded the dye from their membranes. Unhealthy or dead cells appeared blue under microscopy and were not counted. If large number of dead cells were seen in a culture, it was discarded.

Tissue culture microscopy was performed using a Nikon Eclipse E600 inverted microscope with Nikon fluorescent lamp. The aperture was 10, 20, 60 or 80x with total final magnification 100 – 800x. Unless otherwise stated in figures, magnification was 100x. Images were taken using a Zeiss Axiocam Camera and software.

2.2.2 Suspension cell line culture

All PEL cell lines used (BC-3, JSC-1, HBL-6, BCP-1) and the KSHV negative B cell line Ramos were suspension cells and were cultured as follows. This method also applies to the T cell lines H9 SF2 and H9 MN9. Cells were cultured in Royal Park Memorial Institute medium (RPMI 1640) supplemented with 10% (most lines) or 20% (BCP-1) FCS. See Table 1.4.1 for details of PEL cell lines used. Antibiotics were not routinely used for culture and cells were ensured mycoplasma negative by testing every 3 months by Mycoplasma Experience. Cells were split every three days by dilution into fresh medium to a cell density of $1x10^6$ cells/ml with 10mls of media in a T-25 flask and 25 mls of medium in a T-75 flask. BCP-1 cells were seeded at $2x10^6$ cells/ml. 30 mls of cells were centrifuged at 1200g for 5 minutes and the pellet resuspended in 1 ml 90% FCS and 10% DMSO and placed in cryotubes. These tubes were placed at -80°C overnight in polystyrene containers to slow the freezing process. The morning after, cells were moved to storage in liquid nitrogen.

2.2.3 Puromycin/ neomycin selection

For cells to be grown in puromycin or neomycin selection, fresh bottles of appropriate medium were supplemented with appropriate growth factors and serum and puromycin (Clontech) added to a final concentration of 3 μg/ml for selection and 2 μg/ml for continuing culture made from a stock solution of 2 mg/ml which was stored at -20°C. Neomycin (as G418 from Gibco) was used at a final concentration of 1 mg/ml for selection and 500 μg/ml for maintenance of resistant clones.

2.2.4 Ficoll gradient

Ficoll solution (Amersham) was used to separate live from dead cells in suspension cell culture. This is possible because the density of the Ficoll solution is carefully designed to allow dense dead cells to pellet at the bottom of the tube and less dense live cells to remain as a layer between the medium and the Ficoll solution. A Ficoll gradient separation was performed every two weeks prior to seeding suspension cells in culture, and for PEL cells was performed as cells were seeded the day before any experiment. Ficoll gradient separation was not routinely used on PEL cells for *in vivo* experiments as this would involve these cells remaining outside of a tissue culture situation for extended periods of time.

A Ficoll gradient was established by placing 15mls Ficoll solution (Amersham) into a 50ml tissue culture tube. 35mls of medium containing cells was added slowly to prevent mixing of the two liquids. A clear interface was visible between the two liquids. This container was carefully centrifuged at 1200g for 15 minutes with the centrifuge brake disengaged. After centrifugation, the white layer of cells between the medium (above) and the Ficoll solution (below) was carefully removed and resuspended in fresh medium for cell counting. The used Ficoll, dead cells and old medium were discarded.

2.2.5 Primary endothelial cell culture

Human dermal microvascular endothelial cells (DMVEC) were purchased in dry ice (Clonetics) at passage 2. If cells were not to be used immediately, they were placed in liquid nitrogen for storage. Endothelial cell growth medium 2 (microvascular) (EGM2-MV, Clonetics) was prepared in accordance with the manufacturers guidelines. This medium contained hEGF (human epidermal growth factor, final concentration 5 ng/ml), hydrocortisone (final concentration 200 ng/ml), gentamicin (final concentration 50 μg/ml), amphotericin B (final concentration 50 ng/ml), serum (FCS) 10 ml (2%), VEGF (vascular endothelial growth factor, final concentration 0.5 ng/ml), rhbFGF (recombinant human basic fibroblastic growth factor, final concentration 10 ng/ml), IGF-1 (insulin like growth factor, final concentration 20 ng/ml) and ascorbic acid (final concentration 1 μg/ml) as supplied by the manufacturer. For thawing cells, cells were warmed slowly in a water bath until almost completely thawed. Cells were added to 10mls of prewarmed DMEM + 10% FCS. Cells were centrifuged at 1200g for 5 minutes and supernatant discarded. Cells were then placed in 10 mls of prewarmed EGM2-MV (Clonetics) and into a 10 cm^2 dish or T-75 flask. The passage number was noted on the tissue culture vessel. After 48 hours, the medium was changed for fresh EGM2-MV to remove any dead cells and cell debris. Cells were examined using phase microscopy to determine if they required passaging. Cells which were > 75% confluent required passage. Cells were tested for the presence of mycoplasma every 3 months by Mycoplasma Experience.

Cells were passaged every 3 days. For passaging, medium was removed and cells washed with pre warmed (37°c) PBS. 5 mls pre warmed trypsin-EDTA was placed on the cells and incubated for 5 minutes at 37°c. Vigorous agitation was often necessary to remove cells completely from the plate, especially as cells became later passage. The trypsin-cell mixture was centrifuged at 1200g for 5 minutes and the trypsin then discarded. The pellet was resuspended in 1ml of fresh pre warmed EGM2-MV. Cell number was

estimated using trypan blue and a haematocytometer as for other cells, and the cells plated at a final density of 2-5 x 10^3 cells/ cm^2 ($3x10^5$ cells in a T-75 flask or 10 cm plate).

2.2.6 MSC/ HSC isolation

MSC and HSC were isolated from fresh bone marrow (bone marrow obtained from the molecular haematology unit, University College Hospital (UCH) in accordance with local ethical guidelines). This bone marrow was purified immediately after extraction and was not stored or frozen before purification. Purification was performed using the Magnet-assisted cell sorting (MACS) system (Miltenyi Biotech) in accordance with the manufacturers guidelines. The bone marrow was diluted into 5 mls of PBS (if the volume was less than 5 mls) and incubated with a MACS hapten anti-CD34 beads 30 minutes at 4°c. All steps were carried out with strict aseptic technique in tissue culture facilities. This solution was centrifuged at 1200g for 5 minutes and then pellet resuspended in magnetic anti-hapten beads for 15 minutes at 4°c. The MACS column was placed in the MACS magnetic separator and column equilibrated with 500 µl of wash buffer. Labeled cell solution was placed in the MACS column and the flow through collected. The column was rinsed three times with 500 µl wash buffer and the flow through collected in the same container. This flowthrough was collected and stored and was assumed to be the CD34 –ve fraction. The MACS column was removed from the magnetic separator, rinsed three times with 500 µl wash buffer and flow through collected. This flow through was treated for 10 minutes at 4°c with 20 µl of MACS multisort release buffer. A new MACS column was placed in the MACS magnetic separator and the cell/release buffer mixture passed through the column. The flow through was collected. The column was rinsed three times with 500 µl wash buffer and the flow through collected in the same container. This fraction was the CD34 +ve bone marrow fraction. The CD34 +ve and CD34 -ve fractions were centrifuged at 1200g for 15 minutes and the pellet resuspended in 90% FCS/ 10% DMSO for freezing in liquid nitrogen or plated

in appropriate medium. CD34 expression was not checked by fluorescence activated cell sorting (FACS) at this stage.

2.2.7 Mesenchymal stem cell culture

Frozen CD34-ve cells were thawed as described for endothelial cells (see section 2.2.5) or fresh cells used. All cells collected from a bone marrow sample were used at once. The cells were placed on a 10 cm dish in 10 mls of MesenCult (StemCell Technologies) and incubated at 37°c and 5% CO_2. After 48 hours cells were assessed by light microscopy. Some cells have adhered to the plate by this time, and there are numerous non-attached cells in the medium. The plate was washed gently with PBS and then 10mls MesenCult added. After 14 days, MSC should be clearly visible on the plate. After a further 14 days, the cells were checked for cell surface profile and if confirmed as being consistent with MSC were marked passage 2 and used as MSC for further experiments. If at any point the cells ceased to retain expression of SH2 or SH4 (see section 3.1) or became morphologically distinct from the expected picture of MSC the culture was assumed to have begun differentiation and was discarded.

Passaging and seeding density was as for endothelial cells, except MesenCult was used instead of EGM2-MV. MSC were passaged twice per week and the medium changed for fresh every day. Cells were tested for the presence of mycoplasma every 3 months by Mycoplasma Experience.

2.2.8 Differentiation of MSC and HSC

Differentiation of MSC was accomplished by seeding cells at high density (5 x 10^4 cells/cm^2) and replacing MesenCult with medium supplemented to stimulate differentiation into a particular lineage. The medium for adipogenic differentiation contained: 50 µg/ml ascorbic acid, 1 mM dexamethasone (Sigma), and 50 µg/ml indomethacin (Sigma). The medium for osteogenic differentiation contained: 200 µg/ml ascorbic acid, 0.1 mM dexamethasone

(Sigma), and 10 mM ß-glycerophosphate (Sigma). Cells were cultured in this medium for 28 days, changing the medium every second day. Differentiation usually became morphologically obvious by day 7-10. Oil Red O and alkaline phosphatase staining were performed as previously described (Pittenger et al., 1999).

HSC cannot be cultured *in vitro* without differentiation. Differentiation of CD34+ve cells into endothelial lineage was carried out by growing cells in EGM2-MV medium (see section 2.2.5 for information on EGM2-MV supplements) for 28 days. Were assessed for the presence of surface markers at 0, 14 and 28 days.

2.2.9 Cell doubling time measurement

Cell doubling time was measured by seeding $1x10^5$ cells (1×10^4 primary or stem cells) into 1 ml of appropriate medium in a 24 well plate and culturing for 24 hours. After this time, the number of cells in the well was estimated using a haemocytometer. The cell doubling time in days was calculated according to the formula

Doubling time (days)= $\log_{10}2 / \log_{10}(a)-\log_{10}(b)$
where a=number of cells after 24 hours and b= $1x10^5$

This process was performed in triplicate and repeated to allow the cell doubling time to be calculated over the course of the experiment.

2.2.10 Antibody staining for cell surface markers

Cells for antibody staining were trypsinised (if necessary) and washed twice in PBS + 1% FCS after pelleting by centrifugation at 1200g for 5 minutes. Infected cells or cell from medium potentially containing virus were fixed in 4% paraformaldehyde for 15 minutes before staining. Primary antibody was diluted to the working concentration in PBS + 1% FCS and the pellet

resuspended and incubated for 1 hour at 37°c. Cells were centrifuged at 1200g for 5 minutes and washed twice with PBS + 1% FCS. Secondary antibody was diluted to the working concentration in PBS + 1% FCS and incubated for 30 minutes at 37°c. If the secondary antibody was conjugated to a fluorescent tag, the tube was wrapped in foil to keep the mixture in the dark. Secondary antibody was removed by washing twice with PBS + 1% FCS and appropriate analysis (eg FACS) then performed.

2.2.11 Cell cycle FACS (PI staining)

Cells were trypsinised (if appropriate) and washed twice in PBS + 1% FCS. Approximately 1 x 10^6 cells were resuspended in PBS + 1% FCS and were fixed in 4% paraformaldehyde for 1 hour at 4°c. Cells were washed twice in PBS and resuspended in 1 ml of PI staining solution (3.8 mM sodium citrate, 50 μg/ml PI (Sigma)) and 50 μl of RNAse A 10 μg/ml solution (Promega). Cells were incubated in this solution for 3 hours at 4°c, washed twice in PBS and stored at 4°c until analysis by FACS. FACS analysis showed the position in the cell cycle according to FL2-H fluorescence. See figure 5.5d for details of cell gating to determine stage of cell cycle.

2.2.12 BrdU / MTT assay for cell proliferation

MTT assay (Roche) was performed in accordance with the manufacturers guidelines. Cells were seeded into a 96 well multiplate format. When adhered, cells were incubated for 4 hours with MTT (3-(4,5-dimethylthiazol-2-yl)-diphenyl-tetrazolium bromide). Cells were then incubated with formazen dye for 1 hour to solubilise and the colour density at A_{570} measured on a plate colorimeter.

BrdU Cell Proliferation ELISA (Roche) was performed according to the manufacturer's guidelines. Cells were cultured in a 96 well format, and when adhered were labelled with BrdU (bromodeoxyuridine) for 16 hours. FixDenat solution was added for 30 minutes, and then anti-BrdU peroxidase added.

After 1 hour, TMB (peroxidase substrate) was added and the colour density at A_{370}-A_{492} measured on a plate colorimeter.

2.2.13 Annexin V staining for apoptosis

Percentage of cells in apoptosis was determined by annexin-V FITC and PI staining using the Apoptosis Detection Kit (Molecular Probes) in accordance with the manufacturers guidelines. Cells were washed in PBS and then resuspended in 100 µl annexin binding buffer. 5 µl Annexin V-FITC antibody was added to the mixture and incubated in the dark for 30 minutes. 1 µl of 1 mg/ml PI solution was added and cells incubated on ice for 10 minutes. Cells were washed twice in PBS. FACS analysis for green and red fluorescence was performed. Live healthy cells showed low levels of fluorescence. Apoptotic cells showed green (Annexin positive) and dead cells showed both red and green fluorescence.

2.2.14 Immunofluorescence

IFA (Immunofluorescence assay) was performed on cells as follows. Cells were fixed for 15 minutes at room temperature in 4% PFA (paraformaldehyde, SERVA) then washed 3x with PBS + 3% FCS. Cells were permeabilised in PBS+ 0.2% TRITON-X (Sigma). Cells were spotted on glass slides and allowed to dry before being kept at -20^{0}c until use. IFA for LANA was performed by incubating cells with LANA antibody (Kellam et al., 1999) diluted 1:120 or negative control (PBS) for 1 hour at 37^{0}c. Cells were washed in PBS+ 3% FCS 3 times. Cells were then incubated with anti-rat FITC antibody (Dako) for 30 minutes at 37^{0}c and then washed in PBS+ 3% FCS 3 times. 50% glycerol was dropped onto the slide and a cover slip added. Cells were then visualised using a Nikon Eclipse E600 inverted microscope with Nikon fluorescent lamp. The aperture was 10, 20, 60 or 80x with total final magnification 100 – 800x. Unless otherwise stated in the figures the magnification used was 100x. Images were taken using a Zeiss Axiocam Camera and software.

2.2.15 FACScalibur software

FACScalibur software (Beckton Dickinson) was used to gate, interpret and correct fluorescence analysis according to the manufacturers guidelines. Fluorescence overlap seen as bleed through to FL2-H of strongly eGFP expressing cells and expression in FL1-H of immature dsRed proteins (Baird et al., 2000) was corrected using samples expressing varying levels of one fluorescent protein and calibrating the software to compensate the readings appropriately. This was repeated with a sample expressing the other fluorescent protein, and the compensation stored and applied to all subsequent samples.

2.2.16 Generation of single cell clones

To make single cell lines, 293t and HeLa cells were infected with pSIN-deGFP at an MOI of 2 to maximise infection rates while minimising multiple insertions. Several 293t and HeLa cell lines were produced from an infected population by serial dilution of cells and plating in 96 well plates in conditioned medium. The mixture was diluted 1 in 20,000 such that each well should contain <1 cell. Conditioned medium was found to improve the speed and success with which colonies were established. The colonies were split into 24 well plates when large enough. When confluent, each colony was screened by FACS and those which displayed a defined eGFP expression assumed to be genuine single cells. These clones were then tested to determine whether they were still amenable to transfection by introducing a LacZ-FLAG plasmid.

2.3 Virus production

2.3.1 293t cell transfection for virus production

Three methods were used to transfect 293t cells. 293t cells were seeded at $4x10^6$ cells in a T-75 flask or 10 cm dish in 10 mls medium the night before transfection. 16 hours later, the medium on the cells was changed prior to transfection for 6 mls of fresh medium.

PEI (polyethyleneimine) transfection was carried out as previously published (Naldini et al., 1996b). 2 eppendorfs were labeled '1' and '2'. Mix '1' contained 10 μg of p8.91 or p8.2 (where experiments called for the HIV-1 accessory genes), 15 μg pHR or pSIN plasmid and 5 μg pVSVg. This mixture was made to a final volume of 1400 μl with 150 mM NaCl. Mix '2' contained 200 μl 10 mM PEI (Sigma) and 1200 μl NaCl. The two mixes were incubated at room temperature for 15 minutes and then added dropwise to the tissue culture medium and placed at 37°c. The medium was changed for 10mls fresh medium after 4 hours.

For calcium phosphate transfection, the quantities of DNA (as for PEI transfection) were diluted in 220 ml in TE buffer (1 mM Tris-HCl, pH 8.0, 0.1 mM EDTA). 250 μl HEPES buffer (280 mM NaCl , 50 mM HEPES, 1.5 mM NaPO4, pH 7.1) was added and the mixture vortexed briefly. 30 μl 2 M $CaCl_2$ was then added slowly over a 30 second windows to the DNA solution. This mixture was incubated at room temperature for 30 minutes. This mixture was then added to the cells and placed at 37°c overnight. Medium was changed in the morning for 10 mls fresh medium.

For FuGene (Roche Applied) transfection the manufacturers guidelines were followed. 1.5 μg pHR or pSIN vector, 1 μg p8.91 or p8.2 and 1 μg pVSVg were added to 200 μl serum free medium. 10 μl FuGene 6 (a FuGene:DNA ratio of 3:1) was added to this mixture without touching the sides of the eppendorf. This mixture was mixed gently and incubated at room temperature for 15 minutes. This mixture was then added dropwise to cells and placed at 37°c. There was no need to change the medium after transfection.

2.3.2 Harvesting virus

Virus was harvested three times after transfection. 48 hours after the medium change (60-65 hours after transfection), supernatant was removed and filtered with a 45 micron (0.45 μm filter). This medium was kept at 4°c if it was

to be used in an experiment within 24 hours, or stored at -80°c in 1 ml aliquots for longer periods. Fresh medium (10 mls) was placed on the cells after harvest. This procedure was repeated 24 and 48 hours later if the cells remained adherent to the plate. If there were large numbers of cells which had detached from the plate that harvest was destroyed. Viral stocks left over after each experiment were discarded once thawed.

2.3.3 Assaying titre

2.3.3.1 p24 ELISA

p24 ELISA was carried out using a kit supplied by Aalto Bio Reagents according to the manufacturers protocol. 24 hours prior to assay, high bind white plates were coated wit 100 ml per well of 5 mg/ml D7320 anti-p24 antibody in 0.1 M $NaHCO_3$. Wells were washed with LBS and blocked with LBS + 2% milk powder w/v for 1hr at room temperature. 20 μl of PBS containing 5% Empigen BB detergent (Surfachem UK Ltd) was added to 80 μl of virus supernatant. This was diluted 1 in 20 or 1 in 200 in PBS + 5% Empigen BB. A dilution series of p24 standard protein (in LBS + 0.05% Empigen was included on the plate. After blocking, plates were washed in LBS and 100 μl of each sample added. Plates were incubated for 4 hours at room temperature and then washed twice with LBS. Secondary anti p24 antibody- alkaline phosphatase (EH12E1-AP) was diluted to 0.5 μg/ml in 4% w/v milk powder + 20% FBS + 0.5% Tween-20 in LBS and incubated on the plates for 1 hour. Plates were washed five times with LBS + 0.5% Tween 20 and then exposed to 100 μl alkaline phosphatase substrate Lumi-Phos Plus (Aureon Biosystems). Plates were incubated in the dark for 30 minutes at 37°c. Plates were read using Stingray software (Dazdaq, UK) and a Lucy 1 Luminometer (Athos-Labtech). The p24 standard was used to calculate the quantity of p24 in each sample and the number of infectious virions.

2.3.3.2 293t infectious units (GFP)

293t cells were seeded at 1 x 10^5 cells per well in a 6 well plate 24 hours before infection. Serial 1:10 dilution of viral supernatant of unknown titre was performed. The final volume of virus that was added to each well was 1 ml. The equivalent amounts of virus added was usually 10 µl, 1 µl, 0.1 µl, and 0.01 µl. If the infection rate in the lowest concentration was above 1%, the experiment was repeated using more dilute concentrations. The infection was assumed to be on 2 x 10^5 cells as these cells double roughly every 24 hours. After 48 hours, the expression of eGFP was assessed by FACS analysis in terms of percent of cells eGFP positive. These values were plotted on a logarithmic graph and the line of best fit through the linear region of infection determined. This region was when infection was below 30% to minimize multiple insertions (see Fig 3.2c for example). The titre could be calculated by finding two points on the graph (points (x1,y1) and (x2,y2) are any two points on the line of best fit through the linear region of a plot of percent of cells infected against MOI) and using the formula

$$293tIu/ml = (\text{No. of cells infected} \times (y2-y1)) / 100 \times (x2-x1)$$

This formula was derived as follows:

$$m = (y2-y1) / (x2-x1)$$

$$(\%\ \text{infection} \times \text{No. of cells infected}) / 100 = \text{Titre} \times \text{volume of virus added}\ *$$

$$\text{volume of virus added} = x2-x1$$

$$\%\ \text{infection} = y2-y1$$

$$\text{Titre} \times \text{volume of virus added} = (\text{No. of cells infected} \times (y2-y1)) / 100$$

$$\text{Titre} = (\text{No. of cells infected} \times (y2-y1)) / 100 \times (x2-x1)$$

Note that for the assumption marked * to be true, infection must be low to minimize multiple insertions (< 30%).

2.3.3.3 TaqMan qPCR estimation of titre

293t cells were seeded and infected as for assaying of titre by 293tIU (GFP). 48 hours after infection, the cells were trypsinised and DNA extracted as

shown in section 2.1.10. This DNA was analysed using TaqMan qPCR (see section 2.1.12) for the presence of both lentiviral insert and for GAPDH (see section 2.6.4 for primer details and optimum reaction conditions).

The number of lentiviral inserts per cell was calculated. Using this information, the number of cells which were infected and the volume of virus added to the cells, the titre of the virus in terms of copy number per 293t cell was estimated.

2.3.4 Infection of cell lines

For infection of cell lines, an appropriate volume of virus was placed on ice or at 4°c until the virus had almost completely thawed. Medium on cells was changed before infection. Virus was diluted (if necessary) and added to the cells dropwise. The vessel was gently agitated to disperse virus and placed at 37°c for 4 hours. After 4 hours, medium was changed for fresh. All medium containing virus was placed in Virkon (or other suitable disinfectant approved to kill HIV-1) before being discarded. Pipettes used to handle medium containing lentivirions were also placed in Virkon for 4 hours before being discarded.

2.3.5 Ultracentrifugation to concentrate virus

Virus was concentrated by ultracentrifugation in a refrigerated BeckMan Coulter ultracentrifuge with a swinging bucket rotor (SW27) at 48,000 g (25,000 RPM), 4°c overnight (12-16 hours). Ultracentrifugation was performed in disposable, sterile tubes rated to 100,000 g (BeckMan Coulter) and the buckets for the rotors were washed thoroughly in Virkon before and after ultracentrifugation. Typically, 20-25 mls supernatant was used in each centrifuge tube. After ultracentrifugation, the supernatant from the tubes was gently decanted into Virkon. A clear or slightly brown (due to the presence of serum) pellet was usually visible in the tube. 1-2 mls of medium appropriate to the target cell (e.g. DMEM, MesenCult or EGM2-MV) was added to this tube and placed on ice for 1 hour. After this time, the medium was pipetted vigorously and the pellet examined to ensure it had completely dissolved.

Further incubation on ice was sometimes necessary for this. The titre of this concentrated stock was determined and it was stored in 100 or 250 µl aliquots at -80°c.

2.3.6 Centricon units to concentrate virus

Centricon 80 or Centricon 20 units (Millipore) with a 100 kDa molecular weight cut-off were used to concentrate virus through a principle of ultrafiltration. The units were used as described by the manufacturer. Centricon 80 units could concentrate 80 mls of supernatant, centricon 20 units up to 20 mls. The centricon units were equilibrated using equilibration buffer (Millipore). Viral supernatant was added to the units and centrifuged at 1200 rpm for 30 minutes. The flow through was discarded into Virkon. The Centricon units were inverted, 2 mls of elution buffer added and the units centrifuged at 1200 rpm for 15 minutes. The titre of this eluate was determined and it was stored at 4°c for immediate use or at -80°c for later use.

2.4 RNA interference

2.4.1 Ambion in-vitro RNA transcription kit

The Ambion Silencer siRNA in vitro transcription kit (Cat # 1620) was used in accordance with the manufacturers guidelines. Briefly, 21 nt target DNA templates were synthesized (MWG Biotech) with the sequence 5'-CCTGTCTC-3' at the end. This sequence is complimentary to the T7 promoter primer sequence provided in the kit. 2 µl of DNA template (100 µM) was added to 6 µl DNA hybridisation buffer and 2 µl T7 promoter primer. One tube for each template oligo (one sense and one antisense) was prepared and incubated at 70°c for 5 minutes and then left to cool to room temperature. 2 µl Klenow buffer, 2 µl dNTP mix, 4 µl nuclease free water and 2 µl Exo-Klenow were added to the reaction (all buffers and enzymes supplied with the

kit). These mixtures were incubated at 37°c for 30 minutes. 2 µl of these solutions were placed in new tubes (one tube for sense, one for antisense) with 4 µl nuclease free water, 10 µl NTP mix, 2 µl 10x Reaction buffer and 2 µl T7 enzyme mix. These mixtures were incubated for 2 hours at 37°c, mixed together and then incubated overnight at 37°c. The resulting mixture was digested with DNAse and filtered using columns provided, quantified by measurement of the A_{260} of the mixture using a spectrophotometer and stored at -80°c.

2.4.2 Transfection of dsRNA into cell lines

dsRNA molecules were either synthesized (section 2.5.1) or purchased prepared from Dharmacon. Cells for transfection were seeded at 70% confluence.

Transfection was performed using FuGene 6 (Roche), Oligofectamine (Invitrogen), TransIt (Mirus), or Effectene (Qiagen). These reagents have been recommended by the manufacturers as being RNAse free and therefore suitable for transfection of dsRNA. They were each used as directed by the manufacturer.

Medium on cells was changed before transfection. FuGene transfection was performed by preparing an eppendorf containing 200 µl of serum free medium containing the DNA or dsRNA. FuGene 6 was added to the mixture at a FuGene:DNA or dsRNA ratio of 3:1. dsRNA was used so as to make a final concentration of 100, 200 or 400 nM. 0.5µg DNA was used per well of a 6 well plate. FuGene was added carefully without touching the sides of the eppendorf. Mixture was incubated for 15-30 minutes at room temperature and then added dropwise to cells.

The Oligofectamine (Invitrogen) protocol involved producing two mixes. For a 6-well plate, one mix contained dsRNA to a final working concentration of 100,200 or 400 nM or 1 µg DNA diluted to 38 µl with serum free DMEM and

the other mix contained 8 μl serum free DMEM and 4 μl oligofectamine. Mix 2 was added to mix 1 and incubated at room temperature for 15 minutes. This mixture was added dropwise to cells which were gently agitated. For larger or smaller wells, the quantities were altered appropriately.

For Transit (Mirus) transfection, 4 μl Transit per μg DNA or RNA was added to 250 μl serum free medium. This medium was incubated for 15 minutes. DNA (1-2 μg per well for a 6 well plate) or dsRNA to a final concentration of 100, 200 or 400 nM was added to the mixture and incubated for 30 minutes. This mixture was added dropwise to cells in tissue culture.

The Effectene (Qiagen) protocol involved preparation of 0.5 μg DNA or dsRNA to a final working concentration of 100, 200 or 400 nM in 50 μl buffer EC (Qiagen). 3 μl enhancer was added, vortexed for 1 second and incubated at room temperature for 2 minutes. 10 μl Effectene was added to the mixture, vortexed for 10 seconds and incubated at room temperature for 5-10 minutes. This mixture was added dropwise to cells in tissue culture.

2.4.3 RNAi target selection

Targets for RNAi were selected based upon the criteria thought to be important for efficient gene knockdown. These criteria were based upon recommendations from Ambion and Dharmacon, and included:

- At least 50 bp from the end of the gene
- At least 19 but no more than 25 bp
- Roughly 50/50 GC content
- Avoid long strings of G's and C's
- Avoid strings of 3-4 A's or T's
- Sequences should start with AA
- Sequences starting with a G may be more efficient for the U6 promoter
- Choose two or three targets from different sections of the gene
- BLAST the sequenced to ensure it has no homology to cellular genes

An alternative was using one of several internet websites for automatic target selection (e.g. www.ambion.com/rnairesource). The target sequence was designed as a short hairpin in the format:
5' – Target sequence – TTCG – complementary sequence – TTTTT – 3' and the complementary oligo with a CTAG placed on the 5' end. For examples of oligos ordered for shRNA see section 2.7.2

2.4.4 Short hairpin oligo annealing conditions
10 µl of a 100 pmol/ml solution of each oligo was placed in 280 µl annealing buffer (100 mM Tris-HCL pH 7.5, 1 M NaCl, 10 mM EDTA). This mixture was heated in a water bath to 95°c for 3 minutes and then allowed to cool to room temperature slowly. Phenol/chloroform extraction was performed (see section 2.1.1) and precipitated DNA resuspended in 50 µl sterile water.

2.4.5 T4 polynucleotide kinase (PNK) treatment of oligos
Unless oligos were ordered 5' phosphorylated, the annealed oligos require T4 PNK treatment before cloning into pGEM-U6. T4 PNK (Promega) was used in accordance with the manufacturers guidelines. 6 µl of 10x T4 PNK buffer, 2 µl of ATP (1 M) and 2 µl of T4 PNK were added to the 50 µl of annealed oligos (section 2.5.4). This mixture was incubated at 37°c for 1 hour and reaction stopped by phenol/chloroform extraction. Precipitated DNA was resuspended in 50 µl sterile water.

2.4.6 Cloning procedure for lentiviral shRNA production
3 mg pGEM-U6M was digested using Sma I (Promega) (section 2.1.1). After 2 hours, Xba I and shrimp alkaline phosphatase were added to the mixture and incubated at 65°c (Xba I is not efficient at 37°c) for 2 hours. This backbone was purified by agarose gel extraction (section 2.1.3). Backbone and oligos were ligated (section 2.1.4). Ligation was usually sufficient for 1 hour at room temperature, although occasionally overnight incubation at 4°c was required.

For oligo insertion into pGEM-U6L, the backbone was digested with Sal I (section 2.1.1) and blunted with Mung Bean nuclease (Promega, section 2.1.7). Phenol/ chloroform extraction was performed and then digestion with Xba I performed. The backbone was extracted and oligos inserted as above.

Successful insertion of the oligonucleotides and accurate sequence was confirmed by DNA sequencing. 1 μg of confirmed pGEM plasmid was digested with EcoR1 (section 2.1.1) and the 300 bp insert extracted after agarose gel analysis (section 2.1.3.). Appropriate lentiviral backbone (pSIN vector) was digested with EcoR1 and shrimp alkaline phosphatase (SAP, Promega). SAP dephosphorylates the vector backbone to prevent self ligation and is added directly to the digest reaction mixture to a final concentration of 1 U/μl. SAP is compatible with almost all Promega 4-core buffers (except D and J).

The insert and lentiviral backbone were ligated (section 2.1.4) and insert confirmed by digestion with Xba I, where the gel reveals a fragment only if insertion was successful. Orientation could be checked by sequencing, although knockdown was found to be independent of orientation and this was not routinely performed.

2.4.7 Formulae for calculating knockdown efficiency

Knockdown efficiency for cells expressing eGFP was calculated based on mean fluorescence intensity (MFI). Two formulae were used to calculate the knockdown efficiency depending on the nature of the experiment performed.

If cells were infected or transiently transfected and the knockdown performed at the same time, the knockdown was assessed in percent as:
100 – ((MFI with sh-eGFP – MFI untreated cells) / (MFI with sh-LacZ – MFI untreated cells) * 100)

If cells were a stable line expressing eGFP, the knockdown was calculated as

100- ((MFI with sh-eGFP) / (MFI with sh-LacZ) *100)

The calculation of knockdown therefore does not include the fact that even with 100% knockdown cells would still have some autofluorescence, and therefore underestimates the knockdown. This effect was assumed to be negligible.

2.5 *in vivo* studies

2.5.1 Immunodeficient mice for *in vivo* PEL model

immunodeficient mice for experiments were bred by Adrian Thrasher, Institute of Child Health (ICH) and used with his permission. Their genotype was RAG (recombination activating genes) -/-, C3 -/- and common gamma chain -/-. Mice were cared for at the ICH by scientific support staff.

Mice were culled in accordance with the ATCC defined criteria for humane conditions. A weight gain of more than 6 grams above the mean in the weight control group was taken as the cut-off for severe ascites and mice were culled. A palpable solid tumour mass exceeding 1 cm was taken as solid tumour development and mice were culled.

2.5.2 Injections of PEL cells and lentiviral vector

PEL cells were injected intraperitoneally into mice in the midline of the abdomen. A short needle and a transverse angle was used and the mice positioned such as to maximize the chances of a peritoneal rather than subcutaneous or bowel injection was performed. Lentiviral vector injections were performed on the lateral side of the abdomen at a different injection site from the PEL cells.

2.5.3 Statistical analysis of *in vivo* experiments

Outliers in experiments were included in any statistical analysis. On bar charts where error bars are indicated the bars indicate the standard error of the mean unless otherwise indicated in the figure legends. The standard error of the mean (σ_M) is calculated by:

$$\sigma_M = \frac{\sigma}{\sqrt{N}}$$

where σ is the standard deviation of the group and N is the number of samples

The standard deviation (σ) was calculated by finding the square root of the variance (σ^2)

$$\sigma^2 = \frac{\Sigma(X - \mu)^2}{N}$$

Comparison of two weight groups was performed using a t test to determine the significant difference between two means. The calculations were performed using GraphPad Prism version 3.1.

2.6 Information regarding methods

2.6.1 Primers used for PCR cloning

Name	Target	Conc F	Conc R	Cycling conditions
eGFP F	5'-CCACAAGTTCAGCGTG-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 50°c for 2 minutes
eGFP R	5'-CAGGACCATGTATC-3'			72°c for 1 minute
dsRED F	5'-GTAATGCAGAAGAAGACT-3'	75ng	75ng	35 cycles of 95°c for 1 minute, 55°c for 2 minutes
dsRed R	5'-ATGTCCAGCTTGGAGTCCACG-3'			72°c for 1 minute
EF1a F	5'-CACAACACCTGAAATGGAAGAA-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 55°c for 1 minute
EF1a R	5'-TTTTATGCGATGGAGTTTCCCCACA-3'			72°c for 1 minute
CMV F	5'-AAGACCCCACCTGTAGGTTTGGC-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 50°c for 1 minute
CMV R	5'-AAATGAAAGACCCCCGCTG-3'			72°c for 1 minute
B actin F	5'-GTTCCATGTCCTTATATGGAC-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 58°c for 2 minutes
B actin R	5'-TTGGACGGGCGGCGGATCGGCAAA-3'			72°c for 1 minute
SFFV F	5'-ATAAAAGATT TTATTTAGT-3'	50ng	50ng	35 cycles of 95°c for 1 minute, 55°c for 1 minute
SFFV R	5'-GCAGAAGCGCGCGAACAGAA-3'			72°c for 1 minute
cPPT F	5'-CGCCAAATGGCAGTATTCATCC-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 50°c for 1 minute
cPPT R	5'TCCAAACTGGATCTCTGC-3'			72°c for 1 minute
WPRE F	5'-GAATGTCAATAAAACAAAATGGTGG-3'	100ng	100ng	35 cycles of 95°c for 1 minute, 55°c for 1 minute
WPRE R	5'-CGGTATCGATCAGGCGGGGAGGC-3'			72°c for 1 minute

Sequences for cloning chosen by personal communication (eGFP, dsRed and CMV - personal communication Dr. Hsei-Wei Wang) or using NetPrimer (http://www.premierbiosoft.com/netprimer/)

2.6.2 RNAi short hairpin primers

Target Name	*Target sequence*
eGFP	GTTCATCTGCACCACCGGCAAGC
LacZ	GCTGTGATTGCGTCTGGGTTTGC
SURVIVIN	GGACCACCGCATCTCTACATTC
dsRed	TAATGCAGAAGAGACCATGG
vCYCLIN C	GTTCCTGCCAACGTCATTC
vCYCLIN N	GTCGCGTTGGCCCTTAATC
vFLIP C	GTGCTCGTCAGGTTCTCC
vFLIP N	GTAACAAGAAGGAAGTTAC
LANA C	GCTAGGCCACAACACATCT
LANA N	GTCCCACAGTGTTCACATC
vFLIP 5	AAGCTTAATAGCTGTCCAGAATG
vFLIP 444	AATAGGCGTGAGGCTTCTGAGCT
vFLIP 967	AAGTGCGGAGCGGCGACGGTGGCT
vCYCLIN 6	AACTGCCAATAACCCGCCCTCGG
vCYCLIN 316	AAGCTCAGAAGCCTCACGCCTAT
vCYCLIN 675	AAGCATATTGGGATGCGATGTTT
LANA 623	AAGGTCCCTCTACACTAAACCCA
LANA 1278	AAGTTCACAACAGCAGCAGGAGC
LANA 2369	AAGTGGATTACCCTGTTGTTAGC

Primer template (eGFP used as example):

S 5' - GTTCATCTGCACCACCGGCAAGC TTCG GCTTGCCGGTGGTGCAGATGAAC TTTTT -3'
(5' - TARGET SEQUENCE HAIRPIN COMPLEMENTARY TARGET POLY-T – 3')

AS 5' – CTAG AAAAA GTTCATCTGCACCACCGGCAAGC CGAA GCTTGCCGGTGGTGCAGATGAAC -3'

The as primer is the complementary sequence to the s primer (to allow annealing) with a 5' Xba I cloning site (CTAG)

2.6.3 PCR primers for analysis

Name	Target	Conc F	Conc R	Cycling conditions
U6 Distal (for confirming sequence of inserted hairpins	5' – CCCATGATTCCTTCATATTTGC – 3'	250nm		Standard PCR (2.1.8), and sequencing
SFFV F	5' –CAAGAACAGATGGTCACCGCA -3'	500nm		30 cycles: 94°C 15s 47°C 1min 72°C 45s
WPRE R (for confirming insertion of genes after SFFV promoter)	5' - CCTGAACTAGTAACACATA – 3'		300nm	

2.6.4 TaqMan qPCR/qRT-PCR primers, probes and conditions

Name	Target / Probe	qPCR/RT-PCR
Lentiviral packaging cDNA	F 5'-ACTTGAAAGCGAAAGGGAAACCA-3' 300pmoles	qPCR
	R 5'-GTGCGCGCTTCAGCAA-3' 300pmoles	
vCyclin	F 5'-CATTGCCCGCCTCTATTATCA-3' 300pmoles	qPCR/ qRT-PCR
	R 5'-ATGACGTTGGCAGGAACCA-3' 300pmoles	
vFLIP	Forward 5'-TTTCCCCTGTTAGCGGAATGT-3' 300pmoles	qPCR/qRT-PCR
	Reverse 5'-CTAAGTGAAGCAGGTCGCGC-3' 300pmoles	
LANA	F 5'-TTGCCACCCACGCAGTCT-3' 500pmoles	qRT-PCR
	R 5'-GGACGCATAGGTGTTGAAGAGTCT-3' 500pmoles	(700pm/ 700pm
	Probe 5'-TCTTCTCAAAGGCCACCGCTTTCAAGTC-3'	used for qPCR)
Human GAPDH	Forward 5'-GGAGTCAACGGATTTGGTCGTA-3' 300pmoles	qRT-PCR
	Reverse 5'-GGCAACAATATCCACTTTACCAGAGT-3' 300pmol	(700pm/ 700pm
	Probe 5'-CGCCTGGTCACCAGGGCTGC-3'	used for qPCR)
Mouse GAPDH	Forward 5'-GGCATGGCCTTCCGTGT-3' 300pmoles	qPCR
	Reverse 5'-GGTTTCTCCAGGCGGCA-3' 900pmoles	

SYBR green used for all reactions except LANA and human GAPDH, where probes significantly improved the accuracy of the assay. Standard TaqMan cycling conditions for an ABI Prism 7000 machine were used throughout.

2.6.5 Antibodies and reaction conditions

Antibody	Manufacturer	Western Blot concentration	Secondary	Antibody	Manufacturer	Staining concentration	Secondary antibody
LN53 (rat anti-LANA)	n/a	1/1000 (1/120 for IFA)	Mouse anti-rat HRP (Immunological Direct), 1/5000	CD31	Research Diagnostics	1/500	(1)
				CD34-FITC	Research Diagnostics	1/120	n/a
Sheep anti-vcyclin	Exalpha biological	1/1000	Goat anti-sheep HRP (Santa Cruz), 1/5000	CD38	Santa Cruz	1/120	(1)
Rat anti-vFLIP	Gift from Mary Collins	1/200	Mouse anti-rat HRP (Immunological Direct), 1/5000	CD45	Research Diagnostics	1/500	(2)
				CD49b	Santa Cruz	1/120	(1)
Mouse anti β-actin	Oncogene	1/5000	Rat anti-mouse (Santa Cruz), 1/10000	CD133	Santa Cruz	1/120	(1)
				SH2-FITC	StemCell Technologies	1/1000	n/a
Mouse anti-eGFP	BD Biosciences	1/8000	Rat anti-mouse (Santa Cruz), 1/10000	SH4-FITC	StemCell Technologies	1/1000	n/a
Mouse anti-FLAG	Santa Cruz		Rat anti-mouse (Santa Cruz), 1/10000	HLA-DR	Research Diagnostics	1/200	(3)
				Thy1 (CD90)	Research Diagnostics	1/500	(3)
Mouse anti-survivin	Cell Signalling Technology		Rat anti-mouse (Santa Cruz), 1/10000	Lin	ZyMed	1/1000	(2)
				Rat anti-murine MHC-PE	StemCell Technologies	1/500	n/a

Secondary antibodies: (1) rat anti-mouse-FITC (Santa Cruz) used 1/120 ; (2) goat anti-rat-FITC (Santa Cruz) 1/120 ; (3) goat anti-rabbit-FITC (Immunologicals Direct) 1/500

2.6.6 Cell line information

Cell line	Cell type	Special Features	ATCC number
293t	Human embryonic kidney	Derived from 293 cells Large T antigen positive	Not in ATCC database
HeLa	Human cervical epithelial adenocarcinoma	HPV positive	ATCC CCL 2
MOLT-4	Human acute T lymphoblastic leukaemia (T cell)		ATCC CRL 1582
Ramos	Human Burkitts lymphoma derivative (B cell)	EBV positive	ATCC CRL 1596
BC-3	B-Cell (see table 1.4.1)	KSHV positive	Not in ATCC database
JSC-1	B-Cell (see table 1.4.1)	KSHV positive	Not in ATCC database
HBL-6	B-Cell (see table 1.4.1)	KSHV positive	Not in ATCC database

Chapter 3:

Lentiviral vectors to infect human primary and stem cells

Chapter aims:

The aims of this chapter were to isolate and culture human primary and adult stem cells and show that a lentiviral system can transfer genes into these cells. Production and quantification of virus was optimized and elements of the viral backbone which may be required for gene transfer were identified. The effect of infection on the ability of these cells to differentiate was also determined.

3.1 Isolation and culture of primary and stem cells

Primary cells are cultured from tissue without direct genetic modification. Cell lines have genes introduced allowing them to replicate indefinitely, or have been derived from cancerous tissue which already contains mutations allowing this. Primary cells have a limited lifespan in culture and eventually reach senescence. This limit varies between cell types. Primary cells are more useful for some *in vitro* studies because of the altered gene expression in cell lines. Primary cells are not as easy to handle as cell lines as they tend to be refractory to both chemical and electrical transfection. Viral vectors can be used to transduce these cells (Akkina et al., 1996; Dilloo et al., 1997; Drexler et al., 2004; Gruber et al., 1985; Lebkowski et al., 1988).

Cell cultures of primary and stem cells were established to allow investigation of growth characteristics and cell surface marker profiles. Human dermal microvascular endothelial cells (DMVEC) were purchased and were maintained in EGM2-MV (both from Clonetics). The manufacturer's guidelines suggested supplementing medium with epidermal growth factor (EGF), vascular endothelial growth factor (VEGF) and basic fibroblastic growth factor (bFGF) (see materials and methods). The cells maintained a 'cobble-stone' appearance (Fig 3.1a) and stained positive (see section 2.2.5) for markers of endothelial lineage such as CD34 and CD31 (Fig 3.1b).

To determine the expansion potential of these cells, the cell doubling time over serial passage was investigated. The cells maintained a cell doubling time of around 3 days until passage 14. The population became completely senescent by passage 16-18 (Fig 3.1c). To avoid potential experimental bias from using senescent cells, all experiments conducted using DMVEC were therefore performed between passage 4 and 10.

Human mesenchymal stem cells (MSC) and haematopoietic stem cells (HSC) were isolated from fresh human bone marrow (supplied by the Molecular

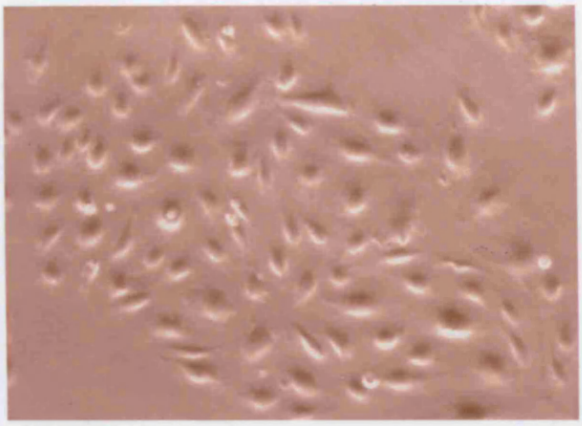
Haematology Unit, UCH). Briefly, bone marrow was diluted in Mesencult (Stem Cell Technologies) and placed in 10 cm dishes. After 48 hours, the supernatant was then removed and fresh medium replaced. After 10-14 days, cells were clearly visible, and by day 28 morphologically resembled MSC (Fig 3.1d) (For details of this protocol, please see materials and methods section 2.2.6). Analysis of these cells showed that they displayed a cell surface marker profile consistent with undifferentiated MSCs (Pittenger et al., 1999) (Fig 3.1e).

There is not currently any known method of culturing HSC without stimulating them to differentiate. Growth in EGM2-MV stimulates HSC to differentiate into endothelial cells (personal communication, Hsei-Wei Wang). The other CD34 +ve cells present in the supernatant are presumed not to proliferate in EGM2-MV and therefore the remaining cells were treated as a pure population of HSC. Staining of supernatant before culture in EGM2-MV did show a large population with a cell surface phenotype consistent with HSC (Fig 3.1f).

As MSCs can spontaneously begin to differentiate, MSCs were stained for SH2 and SH4 before experiments. These markers are associated with the undifferentiated state in MSCs and indicative that these cells have not begun differentiation (Pittenger et al., 2000).

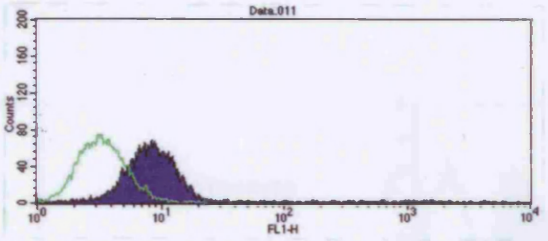
Figure 3.1 Isolation and culture of primary and stem cells

a

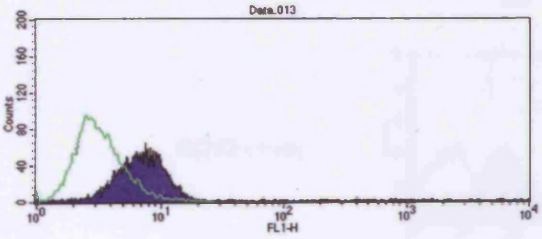


b

CD34 (+ve)



CD31 (+ve)



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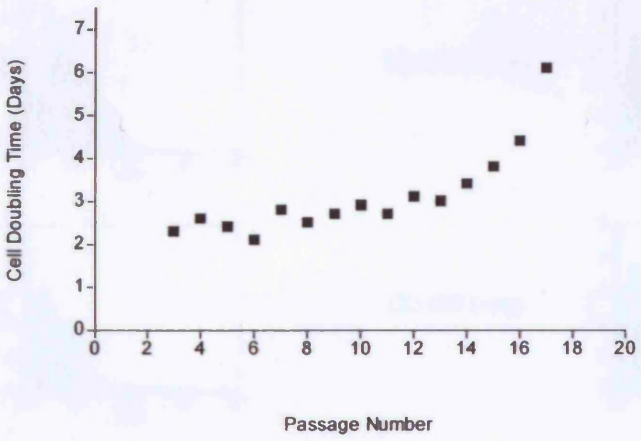
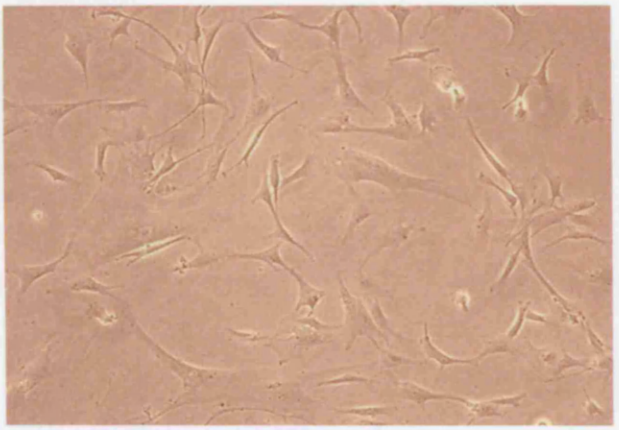


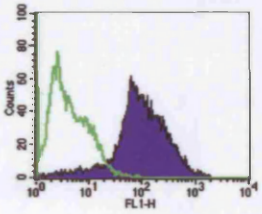
Figure 3.1 Isolation and culture of primary and stem cells

d

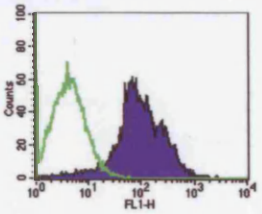


e

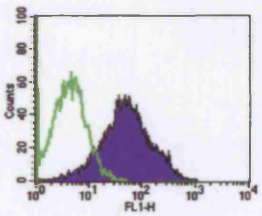
SH2 (+ve)



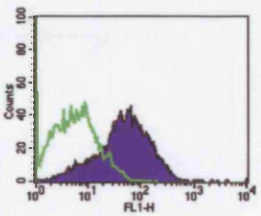
SH4 (+ve)



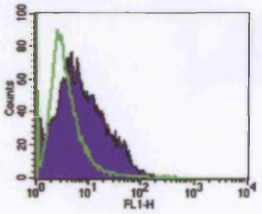
CD49b (+ve)



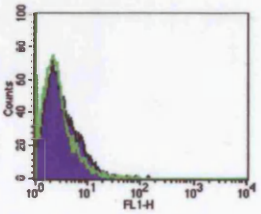
CD13 (+ve)



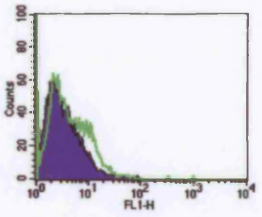
CD45 (low)



HLA-DR (-ve)



CD34 (-ve)



CD133 (-ve)

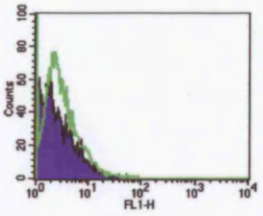


Figure 3.1 Isolation and culture of primary and stem cells

f

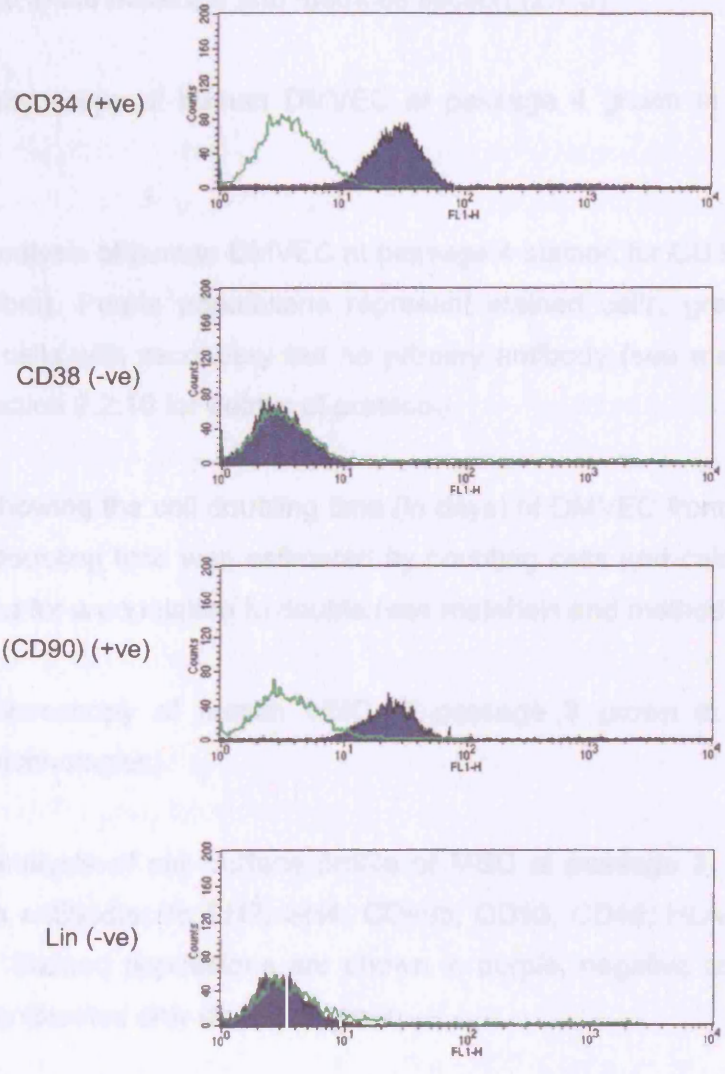


Figure 3.1 Isolation and culture of primary and stem cells

Details of the antibodies and staining concentrations for these experiments can be found in the materials and methods section (2.7.5)

(a) Light microscopy of human DMVEC at passage 4 grown in EGM2-MV (Clonetics)

(b) FACS analysis of human DMVEC at passage 4 stained for CD34 (top) and CD31 (bottom). Purple populations represent stained cells, green overlay represents cells with secondary but no primary antibody (see materials and methods section 2.2.10 for details of protocol).

(c) Graph showing the cell doubling time (in days) of DMVEC from passage 3 – 18. Cell doubling time was estimated by counting cells and calculating the time required for a population to double (see materials and methods 2.2.9).

(d) Light microscopy of human MSC at passage 3 grown in Mesencult (StemCell technologies).

(e) FACS analysis of cell surface profile of MSC at passage 3. Cells were stained with antibodies to SH2, SH4, CD49b, CD13, CD45, HLA-DR, CD34 andCD133. Stained populations are shown in purple, negative controls with secondary antibodies only shown in green.

(f) FACS analysis of cell surface profile of HSC at passage 4. Cells were stained with antibodies to CD34, CD38, Thy 1 and Lin. Stained populations are shown in purple, negative controls with secondary antibodies only shown in green.

3.2 Accurate determination of viral titre

In order to make results of experiments involving lentiviral vectors valid both between experiments and to other reports, accurate determination of viral titre is required.

In the current literature, several methods have been described to assess viral titre. The most common and perhaps easiest method of assessing viral titre involves measuring the quantity of virus required to infect one cell, commonly the 293t HEK cell (the 293t infectious unit, or 293tIU) (Naldini et al., 1996b). Measurement of the quantity of HIV-1 core antigen (p24) is popular for viruses which do not express markers. Recently, TaqMan quantitative PCR (qPCR) on infected cells or qRT-PCR on viral supernatant have been used (Klein et al., 2003).

These methods each hold advantages and disadvantages. Measurement of 293tIU is convenient but difficult when using viruses which do not express a marker such as eGFP or puromycin resistance. Measuring p24 is prone to error as it does not measure infectious virions. The gag protein (p24) measures particle production but not delivery of the transgene. Assessment of titre by qPCR has the advantage of being both accurate and reproducible however is technically much more difficult than the other methods.

To optimize a reliable method of determining titre, a pHR (second generation) vector containing *eGFP* expressed from the CMV promoter (gift from Didier Trono, University of Geneva, Switzerland) and a pSIN (self-inactivating) vector expressing eGFP from the CMV promoter (produced from pCSGW, a gift from Adrian Thrasher, Institute of Child Health, London) were used (Fig 3.2a). pSIN vectors are theoretically safer due to deletions in the LTR. Three batches of virus were produced, and the titre then analysed by measuring p24 concentration and 293tIU by expression and qPCR detection of the lentiviral insert. Titre in 293tIU/ml was determined by choosing a linear region on a logarithmic representation of the infection rates at increasing quantity of

virus (Fig 3.2b). These data indicate that there is considerable variation in titre depending on the method of assessment. (Fig 3.2c).

The considerable variation in the p24 titres between batches with comparable expression levels in terms of 293tIU may be because of the detection of non-infectious virions in the supernatant. Although p24 has the advantage of being fast and easy, it was unreliable and was not a good indicator of infectivity. This method was therefore only used when other methods proved to be unsuitable.

qPCR analysis of infected cells showed some surprising results. These data indicate that several copies of the packaging signal are present in cells to achieve expression. One copy should be sufficient for expression. To determine whether this result was an experimental error, the relationship between viral dose and copy number of the packaging signal was assessed (Fig 3.2d). These data indicate that any error in this method is consistent.

The standard used was prepared by serial dilution of a plasmid. An accurate concentration was determined using a spectrometer and the molecular mass of the plasmid determined by pDRAW32 software. These data were used to calculate the molar concentration of the plasmid, and dilutions to concentrations of 1×10^7 copies per μl made. Serial dilutions of this standard were used to calibrate the TaqMan qPCR machine in accordance with guidelines from ABI.

To test whether my standard was at fault, a second standard was prepared (to rule out error in manufacture). This new standard gave results very similar to the old (data not shown). It was decided that, although the results were high, they were within an order of magnitude of other published results. The results were also reproducible and consistent.

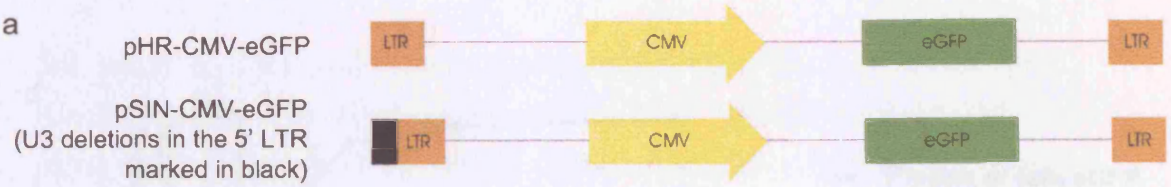
Published literature (Woods et al., 2003) indicate that several integrated copies of the lentiviral vector may be present in transduced cells even when

there are low expression levels, and therefore the 'infectious unit' simply means the average number of virions required for detectable expression. This clearly implies that a single copy is sufficient to express the transgene, if the integration is in an appropriate genomic location however the average number of integrations for an infectious unit may actually be higher.

It was decided to use 293tIU as a method of expressing titre wherever possible. This number can be directly correlated with the percent of cells which will be infected. Where it is not possible to use 293tIU (such as viruses which do not contain a marker), qPCR would be used to assess the integrated copies per cell, and this would be converted into 293tIU by dividing by 4.71. This number was chosen from the line of best fit on figure 3.2d. These data also imply that although MOI is a useful indicator of dosing, it should not be used to accurately indicate copy number.

Figure 3.2 Accurate determination of viral titer

a



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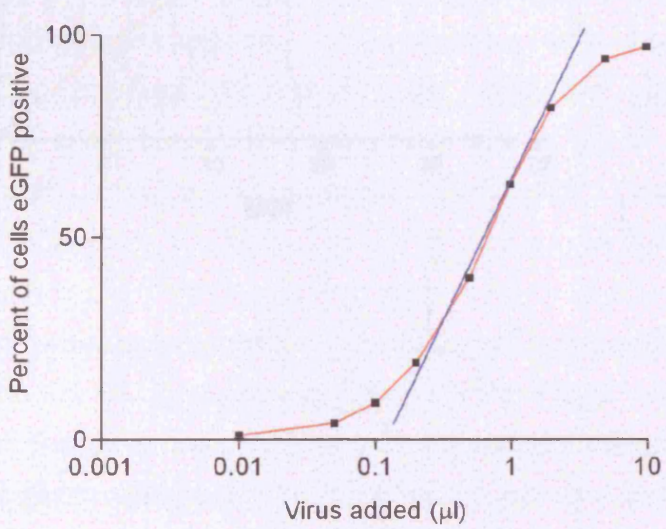


c

		293tIU	p24	qPCR
pHR	1	5 x 10^7	7 x 10^7	3 x 10^8
	2	2 x 10^7	1.2 x 10^8	1 x 10^8
	3	3 x 10^7	1.7 x 10^7	1.2 x 10^8
pSIN	1	1.3 x 10^8	9 x 10^7	5 x 10^8
	2	3 x 10^7	1 x 10^8	1 x 10^8
	3	1 x 10^7	6 x 10^7	4 x 10^7

Figure 3.2 Accurate determination of viral titer

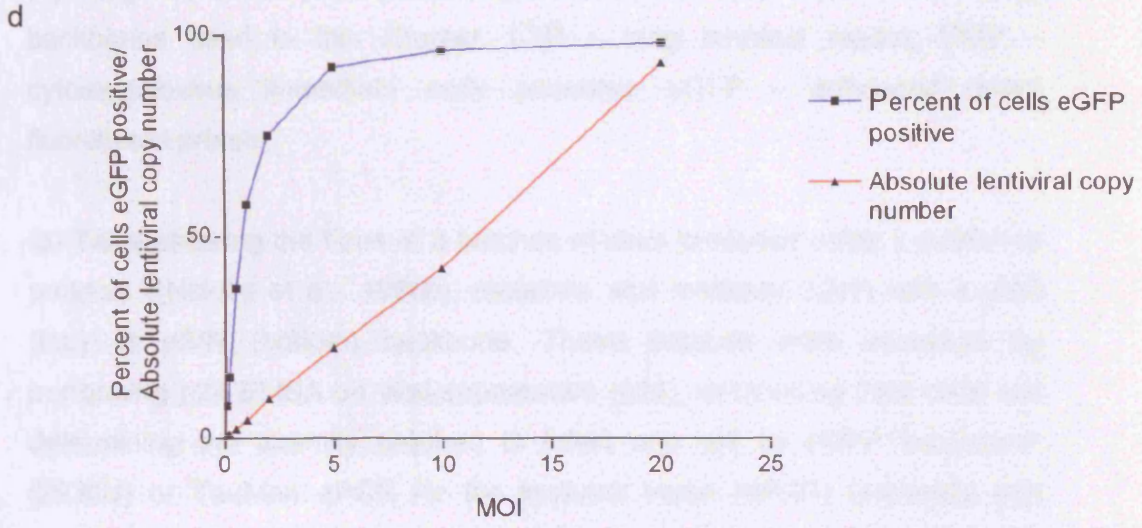


Figure 3.2 Accurate determination of viral titre

(a) Diagram of the pHR (second generation) and pSIN (self inactivating) backbones used in this chapter. LTR – long terminal repeat; CMV – cytomegalovirus immediate early promoter; eGFP – enhanced green fluorescent protein.

(b) Table showing the titres of 3 batches of virus produced using a published protocol ((Naldini et al., 1996b), materials and methods 2.3.1) with a pHR (top) or pSIN (bottom) backbone. These batches were assessed by performing p24 ELISA on viral supernatant (p24), or infecting 293t cells and determining the quantity required to infect one cell by eGFP expression (293tIU) or TaqMan qPCR for the lentiviral insert (qPCR) (materials and methods section 2.3.3).

(c) Graph illustrating the determination of viral titre by expression in 293t cells. The percent of cells eGFP positive (determined by FACS analysis) was measured after infection of 2 x 10^5 293t cells with serial 10-fold dilutions of viral vector. The dilution was performed such that all wells had 1ml of medium added and the equivalent amount of lentiviral vector ranged from 0.01 to 10 μl. The titre was determined by the formula (see materials and methods 2.3.3.2 for derivation)

$$293tIu/ml = (No.\ of\ cells\ infected \times (y2-y1)) / 100 \times (x2-x1)$$

where (x1,y1) and (x2,y2) are any two points on the line of best fit through the linear region of infection (blue line on Fig 3.2c)

(d) Graph representing the absolute copy number of the lentiviral vector per cell determined by qPCR and percent of cells eGFP positive by FACS analysis 48 hours after infection of 1 x 10^5 293t cells with MOI of lentiviral vector ranging from 1 to 20.

3.3 Production of lentiviral vectors

Production of virus involves transfection, production and harvest. To produce the highest titre virus, all 3 factors were optimized. The optimum transfection method, quantity of medium for production and length of time before harvest was investigated.

Calcium phosphate, PEI and lipid based reagents are popular methods of transfecting cells. These methods were assessed in terms of their efficiency at infecting a population of 293t cells. It was found that FuGene gave higher transfection efficiency in 293t cells than calcium phosphate or PEI (Fig 3.3a). FuGene also required less plasmid.

Production and packaging of virions involves production of viral proteins in the appropriate quantities. A single rate limiting step could decrease the optimum titre. Production of virus requires expression of proteins from 3 plasmids. These plasmids encode the HIV-1 genome (p8.91 or p8.2), the VSVG envelope (pVSVG) or the gene of interest (pSIN- or pHR-). The optimum ratio of plasmids for virion production was determined (Fig 3.3b). 2 micrograms p8.91, 2 micrograms pVSVg and 3 micrograms of pSIN vector using a 3:1 ratio of FuGene:DNA was chosen for future experiments.

Virions in the medium are constantly being produced and degraded. The half life for pseudotyped virions at 37°C is low (Strang et al., 2004). The rate of production has been optimized, and the rate of breakdown cannot readily be altered. As with all equilibria, the final concentration will be a balance between synthesis, degradation, and the volume of the medium. Decreasing the volume of the medium while keeping the rate of synthesis and degradation constant should increase the concentration of the virus produced.

Most experiments culture 293t cells in 10 to 20 mls of medium for harvesting. The effect of using smaller volumes for virus production was investigated. It

was found that 8mls of culture medium gave the optimum titre while preserving the health of the 293t cells (Fig 3.3c).

Protocols described in previous reports indicate that the culture should be left for 60 to 96 hours between transfection and harvest. No rationale for this was found, however. The optimum and maximum length of time between transfection and harvest of virus was therefore determined. These data indicate that virus can be harvested as early as 36 hours after transfection, although 48 hours gives the optimum titre. Virus production can remain stable for 96 hours after transfection, and so a harvest anywhere within these time limits (48-96 hours) will give acceptable yields (Fig 3.3d).

These data indicate that optimization of protocols for viral production can yield titres of up to 2×10^8 293tIU per ml. This is significantly higher than many published reports, which require concentration of supernatant to achieve titres of this order of magnitude.

Figure 3.3 Production of lentiviral vectors

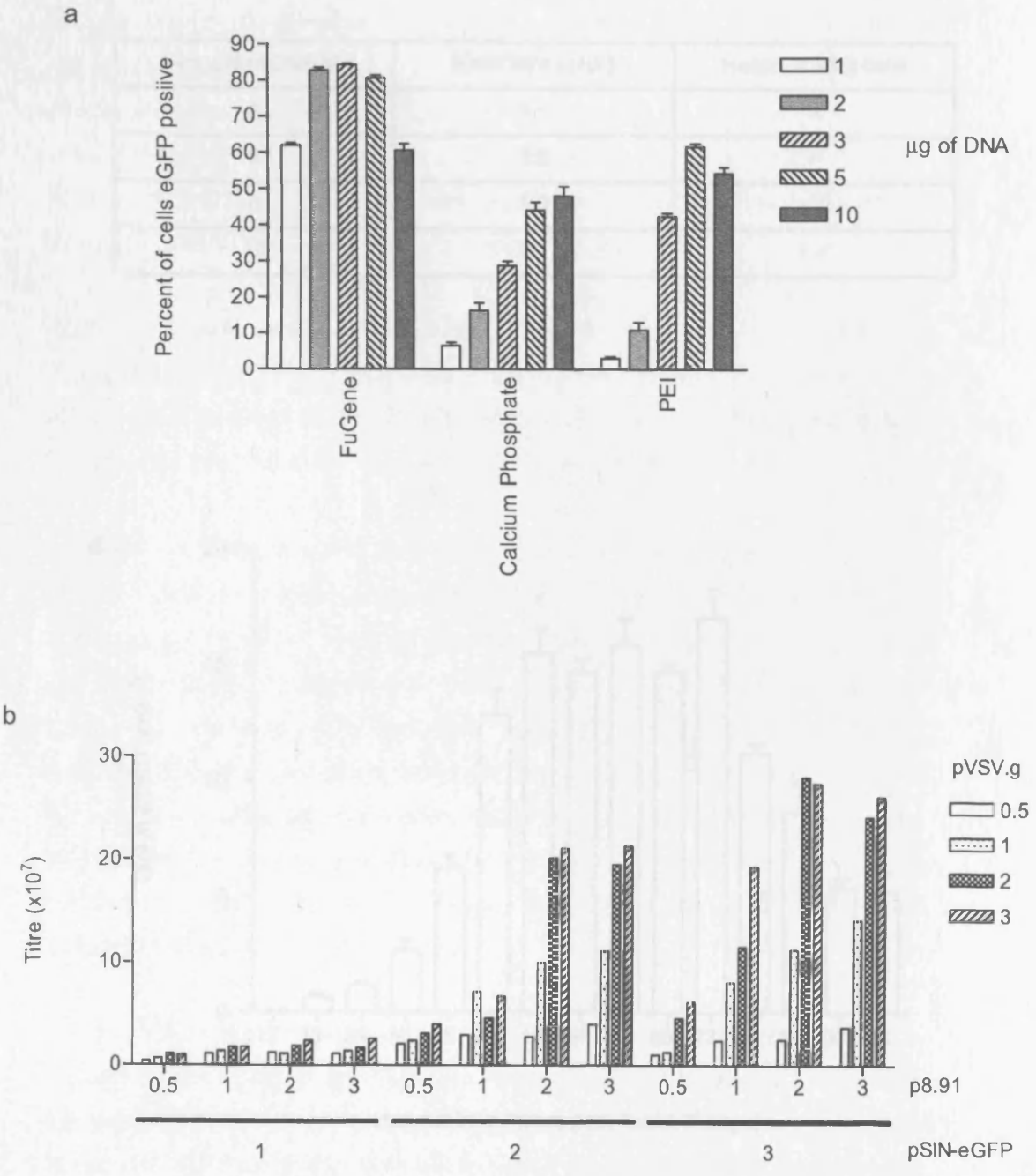


Figure 3.3 Production of lentiviral vectors

c

Amount of DMEM	Final Titre ($x10^7$)	Health of 293t cells
4	1.1	×
6	3.2	✓
8	3.0	✓✓
10	2.1	✓✓

d

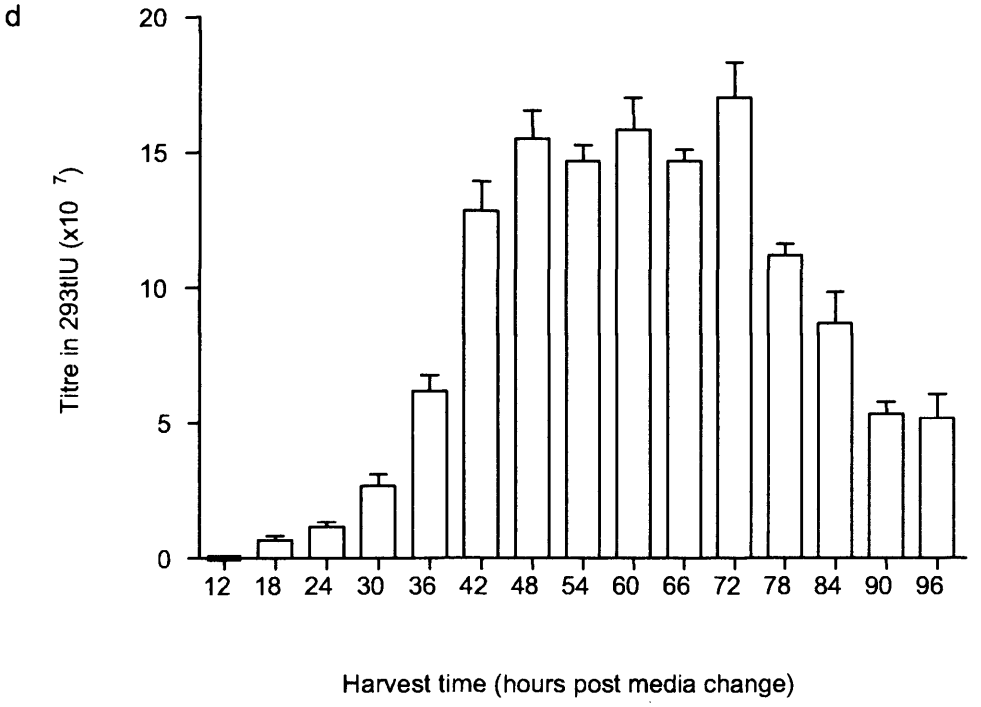


Figure 3.3 Production of lentiviral vectors

(a) Graph assessing transfection efficiency of FuGene, Calcium phosphate and PEI on 293t cells. pSIN-CMV-eGFP was transfected into 5 x 10^6 293t cells in a 10cm plate format in quantities from 1 to 10 μg. The protocols for transfection were in accordance with the manufacturers recommendations (FuGene) or published protocols (see materials and methods 2.3.1). The percent of cells eGFP positive was determined by FACS analysis 24 hours after transfection. Experiments were performed in triplicate and error bars indicate the standard error of the mean of this experiment.

(b) Graph showing the final titre produced by altering the ratio of plasmids for viral production. 5 x 10^6 293t cells were transfected with FuGene, pVSVg, p8.91 and pSIN-CMV-eGFP as in (a). Virus was harvested after 60 hours and the titre estimated by eGFP expression in 293t cells (Chapter 3.2).

(c) Table indicating the final titre of virus (in 293tIU) after transfection of 5 x 10^6 293t cells in a 10cm plate with FuGene, 3 μg pSIN-CMV-eGFP, 2 μg pVSVg and 2 μg p8.91. 4 hours after transfection, the medium was changed and the amount of DMEM (in mls) shown added to the cells. After a further 48 hours, the health of 293t cells was assessed visually according to the presence of cells or cell debris floating in the medium. '×' indicates > 50 cells in medium not adhered to the plate visible in each 200x magnification view. '✓' indicates between ten and fifty cells in medium not adhered to plate visible in each 200x view, and '✓✓ ' indicates less than ten cells in medium not adhered to plate were visible.

(d) Graph showing the final titre (in 293tIU) of lentiviral vector produced as in (c) with 8mls of DMEM. Medium was changed after 4 hours, and virus was harvested after the time indicated on the x axis. After harvesting, virus was frozen at -80°C until the end of the experiment when the titration was

performed. Experiment was performed in duplicate and the error bars indicate the standard error of the mean.

3.4 Elements enhancing transgene expression in primary and stem cells

Many primary and stem cells are refractory to transfection. There have been reports however that these cells can be transduced by viral vectors, and there is evidence that lentiviral vectors may be particularly suited to this task (Demaison et al., 2002). Additional elements may however be required to achieve efficient infection (see section 1.3.2.4).

The ability of lentiviral vectors based on the pHR or pSIN backbones to infect primary and stem cells was tested. Vectors encoding the enhanced green fluorescent protein (eGFP) expressed from the CMV promoter were chosen (Fig 3.2a). An MOI of 5 293tIU/cell was chosen for this experiment as Chapter 3.2 showed this was sufficient to saturate infection in 293t cells. These vectors were capable of transducing primary cells and some adult stem cells (Fig 3.4a and b), with no significant difference between the two vectors. Fig 3.4b indicates that HSC are not easily infected with the lentiviral vectors. Since self inactivating vectors have been proposed to be safer the pSIN backbone was used for further experiments.

The CMV promoter expresses well in adherent cells, but not in suspension cells. The lack of expression in HSC could be due to low infection rates in addition to the inability of the CMV promoter to express.

A more suitable promoter which would express in HSC was required. To investigate this, different promoters were cloned into a pSIN backbone. The EF1α (Ma et al., 2003) and SFFV promoter (Demaison et al., 2002) have both been used in stem cells and so were chosen here.

The expression of these vectors (pSIN-EF1α-eGFP and pSIN-SFFV-eGFP, Fig 3.4c) in HSC, MSC and DMVECs was determined. As these vectors may show promoter dependent eGFP expression in 293t cells, the titres of the samples were equalized by TaqMan qPCR before infection. Expression in

HSC was low with all promoters, although these results are difficult to interpret given the low infection rate of the lentiviral vector (Fig3.4d). The SFFV promoter was the most versatile, showing expression in all tested cells and was therefore used for further experiments.

Several elements to enhance infection or expression have been used in both primary and stem cells (Demaison et al., 2002; Gropp et al., 2003; Ma et al., 2003). To show the benefits of these elements in this lentiviral system, the cPPT or WPRE (cloned by PCR from pCSGW) were added either together or individually to a pSIN-SFFV-eGFP backbone. The infection and expression of these vectors in MSC, HSC and DMVEC was assessed by qPCR (Fig 3.4e) and FACS (Fig 3.4f).

It was found that the cPPT marginally enhanced infection but not expression levels, the WPRE enhanced expression but not infection in primary and stem cells. This confirms previous reports regarding these elements (Demaison et al., 2002; Ma et al., 2003), and for further experiments a pSIN-SFFV vector containing both the cPPT and the WPRE was used.

Figure 3.4 Elements enhancing transgene expression in primary and stem cells

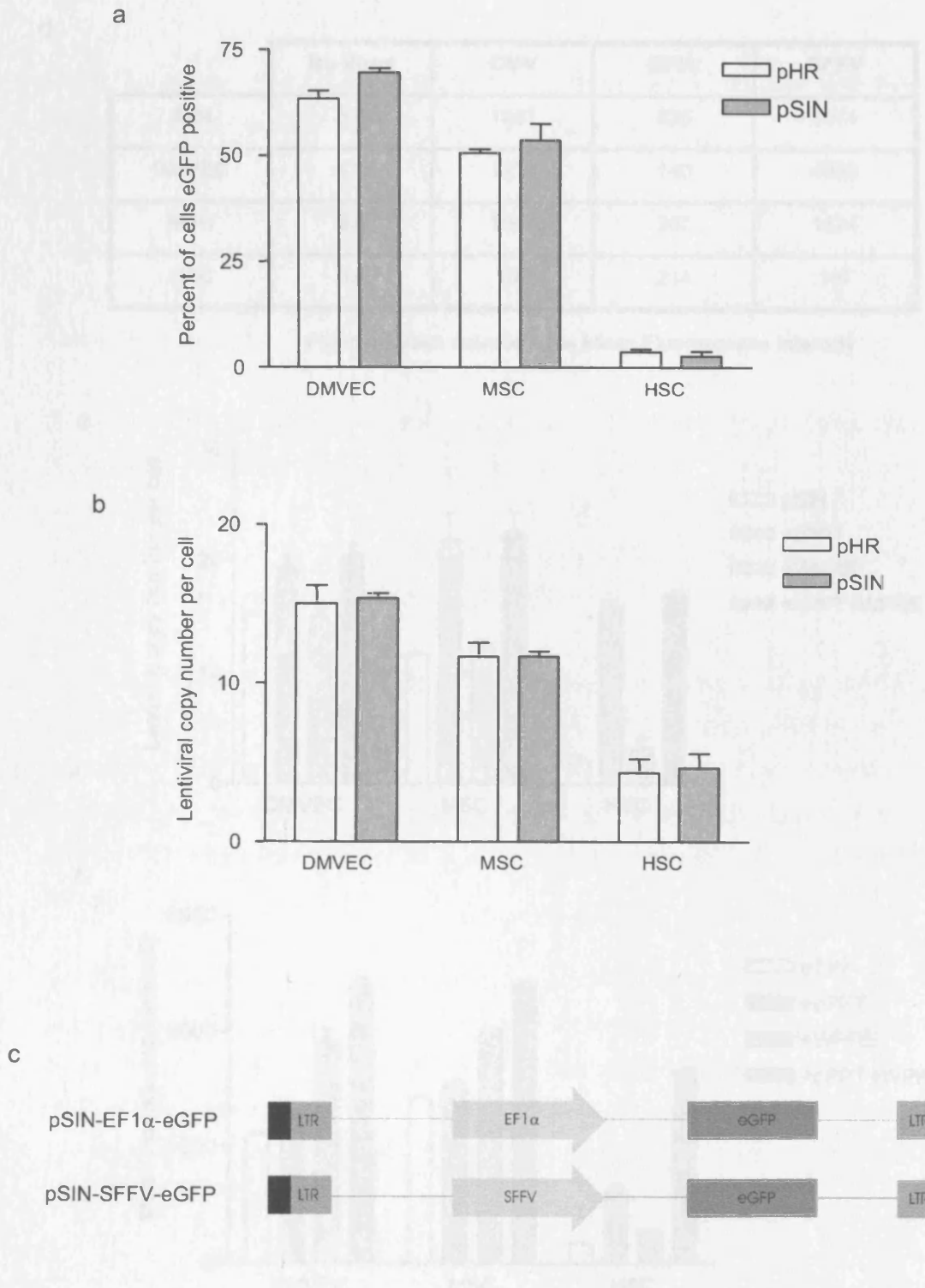


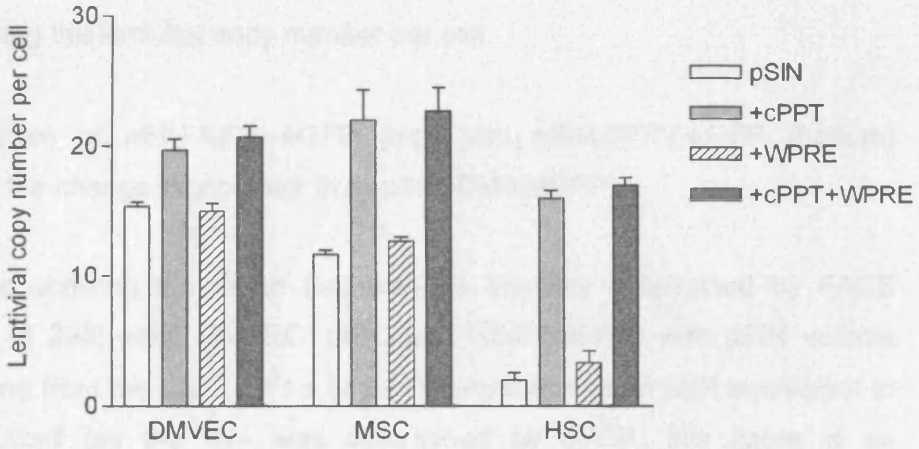
Figure 3.4 Elements enhancing transgene expression in primary and stem cells

d

	No Virus	CMV	EF1α	SFFV
293t	3.8	1857	835	1574
DMVEC	4.1	1214	140	1089
MSC	6.6	1057	257	1524
HSC	6.4	102	214	197

Figures shown represent the Mean Fluorescence Intensity

e



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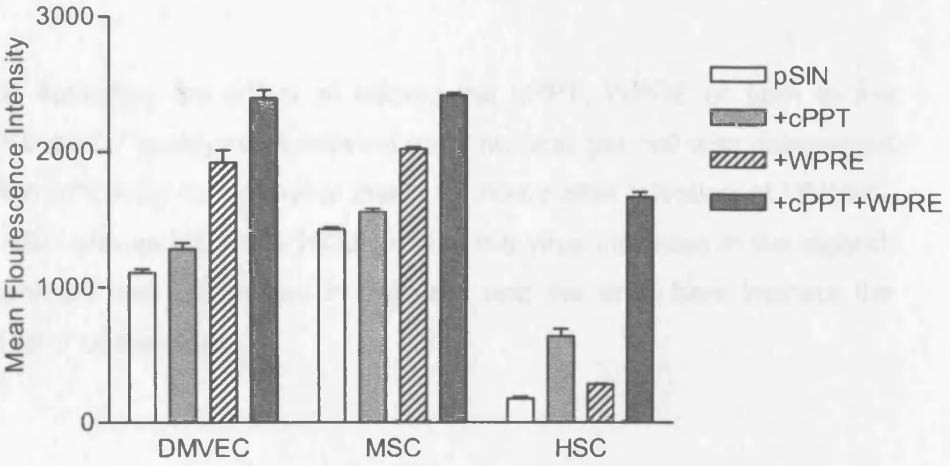


Figure 3.4 Elements enhancing transgene expression in primary and stem cells

Unless otherwise stated in this experiment, all viruses were produced with the FuGene protocol (see section 2.3.1 and Chapter 3.3).

(a) Graph indicating the percent of DMVEC, MSC or HSC eGFP positive by FACS analysis after infection of 1 x 10^4 cells with an MOI of 5 293tIU/cell of pHR-CMV-eGFP or pSIN-CMV-eGFP. FACS analysis was performed 72 hours after infection.

(b) Graph of qPCR analysis for the lentiviral insert of experiment performed in (a) showing the lentiviral copy number per cell.

(c) Diagram of pSIN-EF1α-eGFP (top) and pSIN-SFFV-eGFP (bottom) showing the change in promoter from pSIN-CMV-eGFP

(d) Table showing the mean fluorescence intensity determined by FACS analysis of 293t cells, DMVEC, MSC and HSC infected with pSIN vectors expressing from the CMV, EF1α or SFFV promoters at an MOI equivalent to 5 293tIU/cell (as the titre was determined by qPCR, this figure is an approximate equivalent only). FACS analysis was performed 72 hours after infection.

(e) Graph indicating the effect of adding the cPPT, WPRE or both to the pSIN-SFFV-eGFP backbone. Lentiviral copy number per cell was determined by TaqMan qPCR for the lentiviral insert 72 hours after infection of DMVEC, MSC or HSC with an MOI of 5 293tIU/cell of the virus indicated in the legend. The experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(f) Graph showing the mean fluorescence intensity of the cells from the experiment performed in (e).

3.5 Enhancing infection

Infection rates in MSC and HSC are significantly lower than those achieved with 293t cells. Methods to enhance infection were investigated. In some cells, cell cycle progression is necessary for infection (Korin and Zack, 1998). These cells may benefit from a longer incubation with virus. Infection rates can also be enhanced with polybrene. Infecting cells in 'suspension' has also been tried (Clements et al., 2006). A lower MOI was used here than in the previous experiment to allow the effects of any enhancement to infection to be seen more clearly.

The effects of incubation with polybrene at 1, 4 and 10 μg/ ml on infection rates in DMVEC, MSC and HSC was investigated. Polybrene has a positive effect on infection in HSC, but a more marginal effect on MSC and DMVEC (Fig 3.5a).

To determine whether those cells which do not show a shift in fluorescence are uninfected or are not expressing, the infected populations of HSC, MSC and DMVEC were sorted by FACS and tested for the presence of the lentiviral insert by qPCR. These data indicate that even at high MOI, cells not expressing are not infected (Fig 3.5b).

HSC are slowly dividing and any given population will also contain a proportion of quiescent cells. They might therefore benefit from an incubation longer than 4 hours. The incubation time was determined as the time between addition of virus to medium and changing the medium. 293t, MSC, HSC and DMVEC were incubated for between 1 hour and 24 hours with viral vector. Medium on stem cells needs to be changed every day, so longer incubations were not feasible. The length of incubation was found to be critical in some cells, but not in others. 293t cells could efficiently be infected with incubations as short as 1 hour, whereas primary and stem cells required 8-16 hours for optimum infection. Overnight incubation only enhanced

infection rates for stem cells (Fig 3.5c). All further infection for stem cells were therefore carried out overnight.

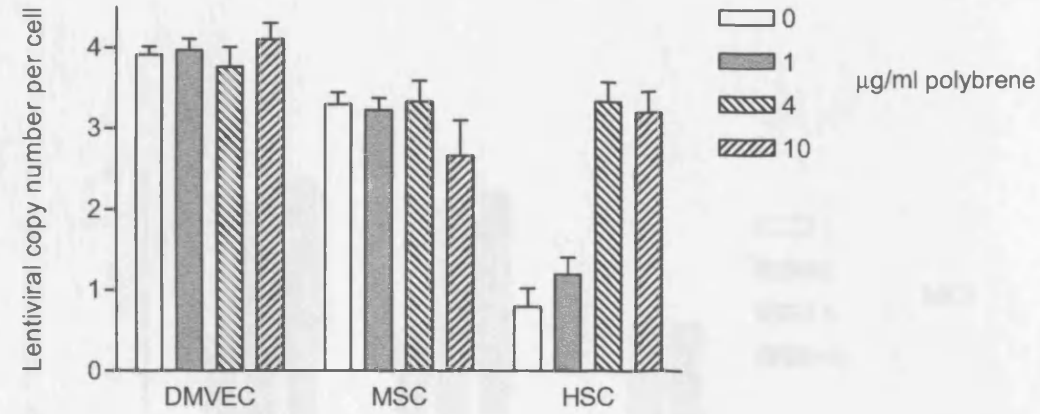
Infecting adherent cells directly after trypsinisation and before plating was found to provide high infection rates. Infections performed in this manner showed that at an MOI of 5, 95% of DMVEC and 92% of MSC could be infected (Fig 3.5d).

It was noted from experiments involving high MOI that cellular morphology changed and cell doubling time increased. The effect on cell doubling time and BrdU uptake after infection was assessed in 293t cells, MSC, HSC and DMVEC. An increase in cell doubling time and decrease in BrdU uptake was seen with MOI above 10 in all cell types (Fig 3.5e-f). These results were not easily explained. Viral infection should not cause such a dramatic increase in cell doubling time after 7 days. It is possible that these cells are showing this decrease in cell doubling time because of the effects of handling and trypsinisation, although this would not explain why there was no effect in control cells (uninfected). These data may also be counting apoptosing or dead cells. RT-PCR for interferon responsive genes or a gene expression microarray would be useful in these cells to determine whether this is an interferon response, an apoptotic process or a previously undescribd effect of viral infection.

These data therefore indicate that care needs to be taken to ensure that the minimum amount of virus required to reach the desired expression level is used.

Figure 3.5 Enhancing infection

a



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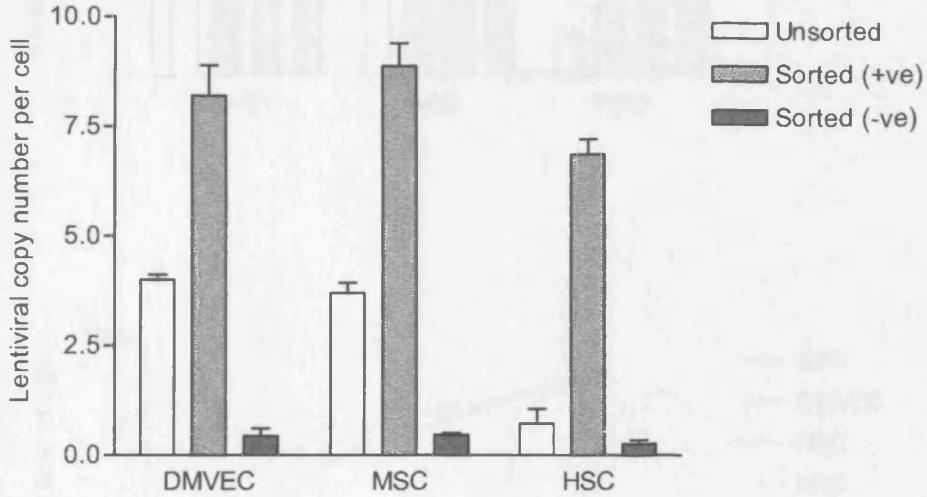

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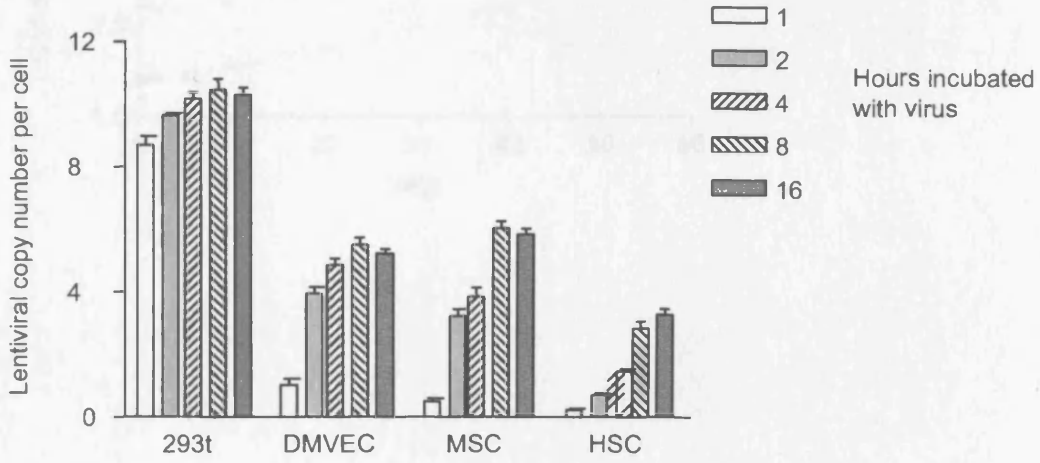
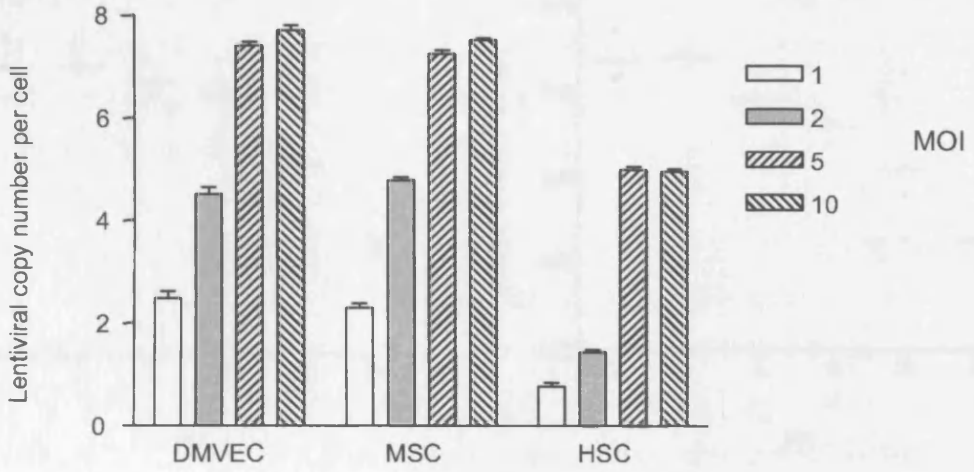


Figure 3.5 Enhancing infection

d



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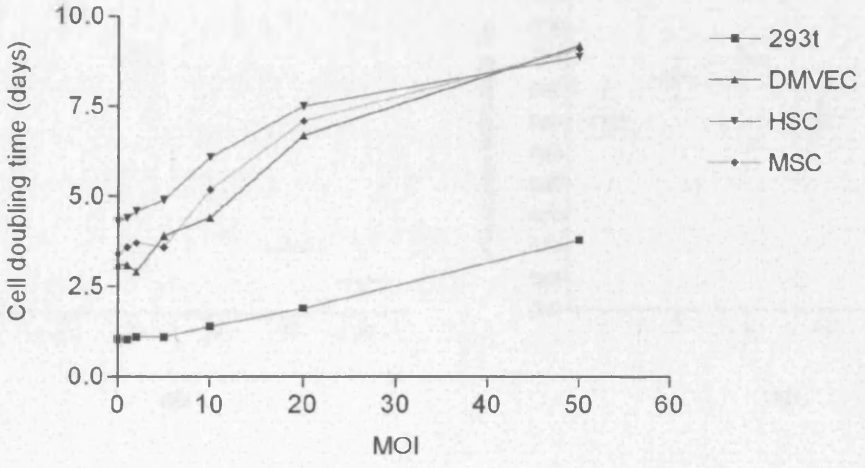

Figure 3.5 Enhancing infection

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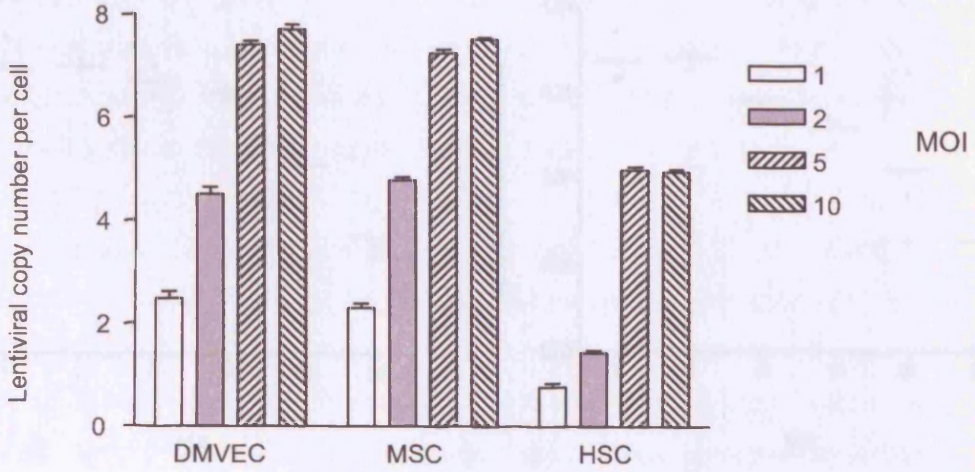

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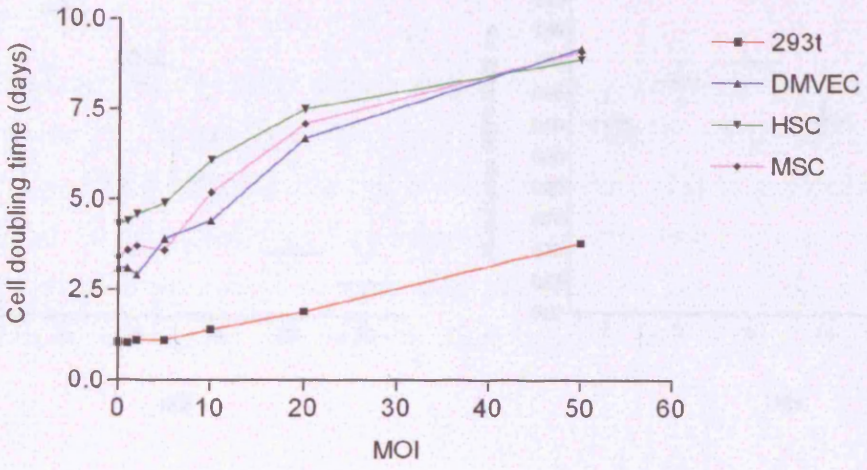


Figure 3.5 Enhancing infection

f

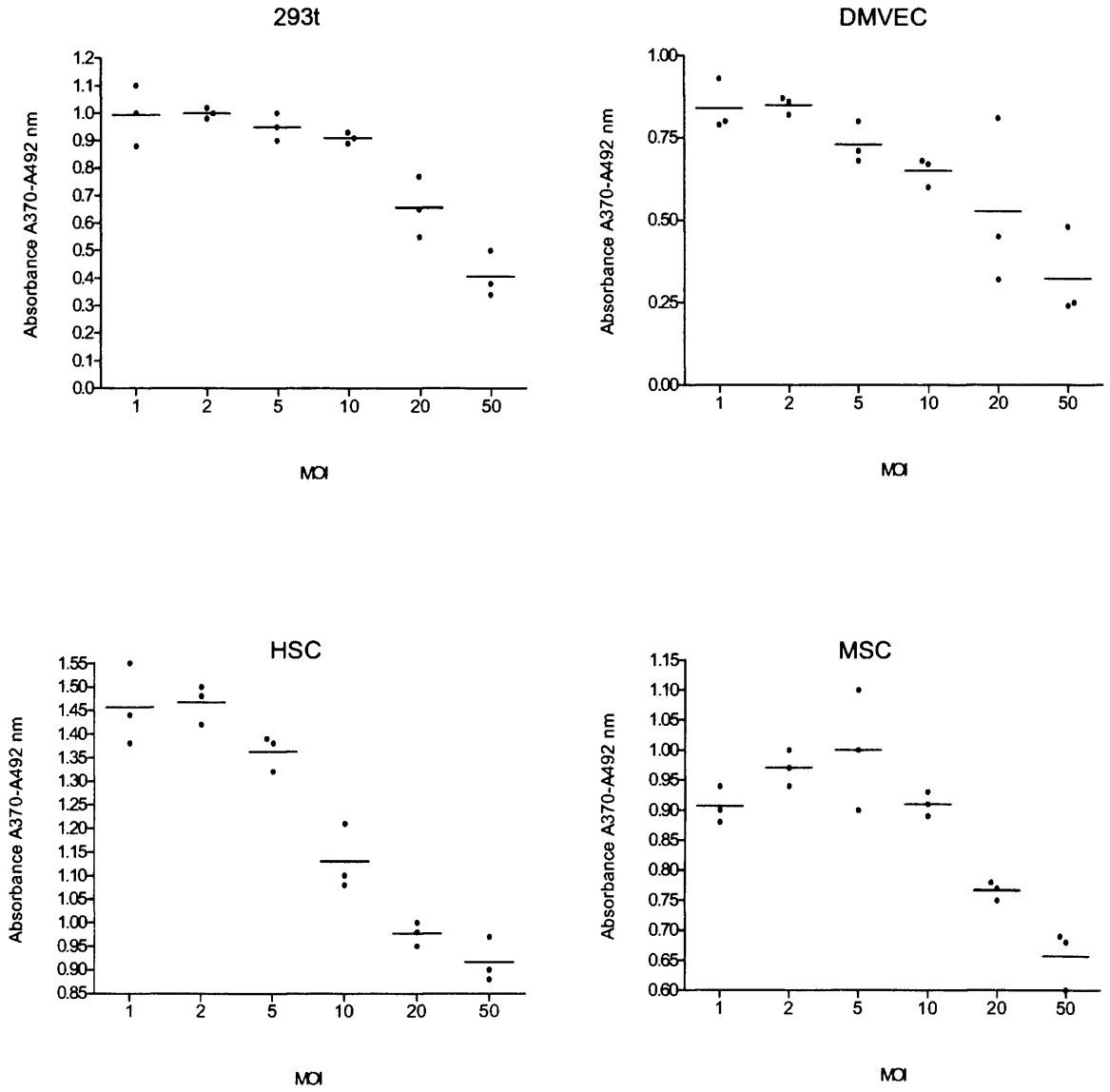


Figure 3.5 Enhancing Infection

(a) Graph showing the lentiviral copy number per cell determined by TaqMan qPCR analysis of 1 x 10^4 DMVEC, MSC or HSC in a 6 well plate incubated with pSIN-SFFV-eGFP at an MOI of 2 293tIU/cell and the concentration of polybrene indicated in the legend. Medium was changed after 4 hours, and analysis performed after 72 hours. Experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(b) Graph showing the lentiviral copy number per cell of 1 x 10^4 DMVEC, MSC and HSC infected with an MOI of 2 293tIU/cell of pSIN-SFFV-eGFP. Cells were infected for 4 hours, the medium changed and after 72 hours were sorted using Mo-Flo FACS for fluorescence intensity. Sorted (+ve) indicates those cells which fluoresced significantly above the background level (determined by uninfected cells). Sorted (-ve) indicates the population which fluoresced at levels comparable to uninfected cells. Experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(c) Graph showing the lentiviral copy number per cell determined by TaqMan qPCR analysis of 1 x 10^5 293t cells, and 1 x 10^4 DMVEC, MSC and HSC infected at an MOI of 2 293tIU/cell of pSIN-SFFV-eGFP and incubated with virus for the time indicated in the legend. After this time, medium was changed and analysis was performed after a further 72 hours. Experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(d) Graph showing the lentiviral copy number per cell determined by TaqMan qPCR analysis of 1 x 10^4 DMVEC, MSC and HSC infected at the MOI indicated in the legend of pSIN-SFFV-eGFP. Cells were incubated with virus for 4 hours, medium was changed and analysis performed after a further 72 hours. Experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(e) Graph showing the cell doubling time in days of 1 x 10^5 293t cells, and 1 x 10^4 DMVEC, HSC and MSC infected with the MOI indicated on x axis of pSIN-SFFV-eGFP. Cells were incubated with virus for 4 hours and then the medium changed. Analysis of cell doubling time (see materials and methods 2.2.9) was performed 7 days after infection.

(f) Graphs of BrdU uptake (see materials and methods 2.2.12) of cells from experiment performed in (e).

3.6 Stable transgene expression

MSCs have the ability to differentiate into many cell types, notably adipocytes, chondrocytes and osteocytes. Conditions that stimulate the differentiation of rat, mouse and human MSCs into these lineages have been established. Differentiation of HSC is possible in the laboratory, although little is known about these pathways. It is not currently possible to grow human HSC in an undifferentiated state.

The ability of MSC's cultured in our laboratory to differentiate into adipocytes and osteocytes was assessed. Conditions previously shown to be efficient at inducing differentiation were used (Pittenger et al., 1999). After differentiation, oil red O or alkaline phosphatase staining was performed. Oil red O staining indicates lipid deposition within cells and is considered to be a marker of adipocytic lineage. Alkaline phosphatase activity is considered to be a specific marker of osteocytic lineage. After culture for 28 days in medium stimulating differentiation, MSCs showed efficient differentiation (Fig 3.6a).

HSC derived by CD34 selection from bone marrow (Chapter 3.1) formed adherent cells which morphologically resembled endothelial cells after 14 days in culture in EGM2-MV medium (Fig 3.6b). It was not possible to prove these cells to be endothelial cells as antibodies to markers which are present on endothelial cells but not HSC were not available at the time.

The ability of human MSC to differentiate after infection was determined. After differentiation of MSC into either adipocytes or osteocytes, these cells are difficult to remove from plates in culture for FACS analysis. Osteocytic differentiation was therefore chosen as it is possible to quantify the differentiation through colorimetric assessment of the cells at 405nm. Cells showed impaired ability to differentiate into osteocytes when the infectious MOI was above 20 293tIU/cell (Fig 3.6c). Reduction in adipocytic differentiation potential was also seen, although this effect proved difficult to quantify (data not shown). For further experiments an MOI of 5 293tIU/cell

was chosen as this value has no significant effect on cell doubling time (Fig 3.5e) or BrdU uptake (Fig 3.5f).

HSC infected with a lentiviral vector retained the ability to form cells which were morphologically consistent with endothelial cells. This ability was compromised by infection with high MOI (data not shown), however it is again not possible to assess this quantitatively.

It has been documented that in stem cells (most notably embryonic stem cells) a degree of silencing occurs when these stem cells are stimulated to differentiate after infection with viral vectors. Lentiviral integrations have been reported to be resistant to silencing, even in ES cells (Gropp et al., 2003). To investigate whether this was true of the lentiviral system employed in our hands, MSCs were infected with pSIN-SFFV-eGFP and stimulated to differentiate towards adipocytes and osteocytes. The expression of eGFP was analysed by fluorescence microscopy 28 days after stimulation (Fig 3.6d). Oil Red O and alkaline phosphatase staining confirmed the lineage of these differentiated cells. HSC infected with the lentiviral vector and then cultured in EGM2-MV for 28 days also showed stable expression of eGFP by FACS analysis after differentiation (Fig 3.6e).

Long term expression without differentiation in MSCs was assessed. MSCs were infected with pSIN-eGFP and cultured until the cells entered senescence. The cells continued to express eGFP throughout their entire lifespan (15 passages, Fig 3.6f). HSC cannot be cultured without differentiation, and so this assay could not be performed with HSC.

These data indicate the robustness of expression from this lentiviral system. Stem cells can be efficiently infected, and they maintain this expression both in the undifferentiated state and when stimulated to differentiate. Using optimized infection protocols, over 90% of cells can be infected without compromising differentiation potential.

Figure 3.6 Stable transgene expression

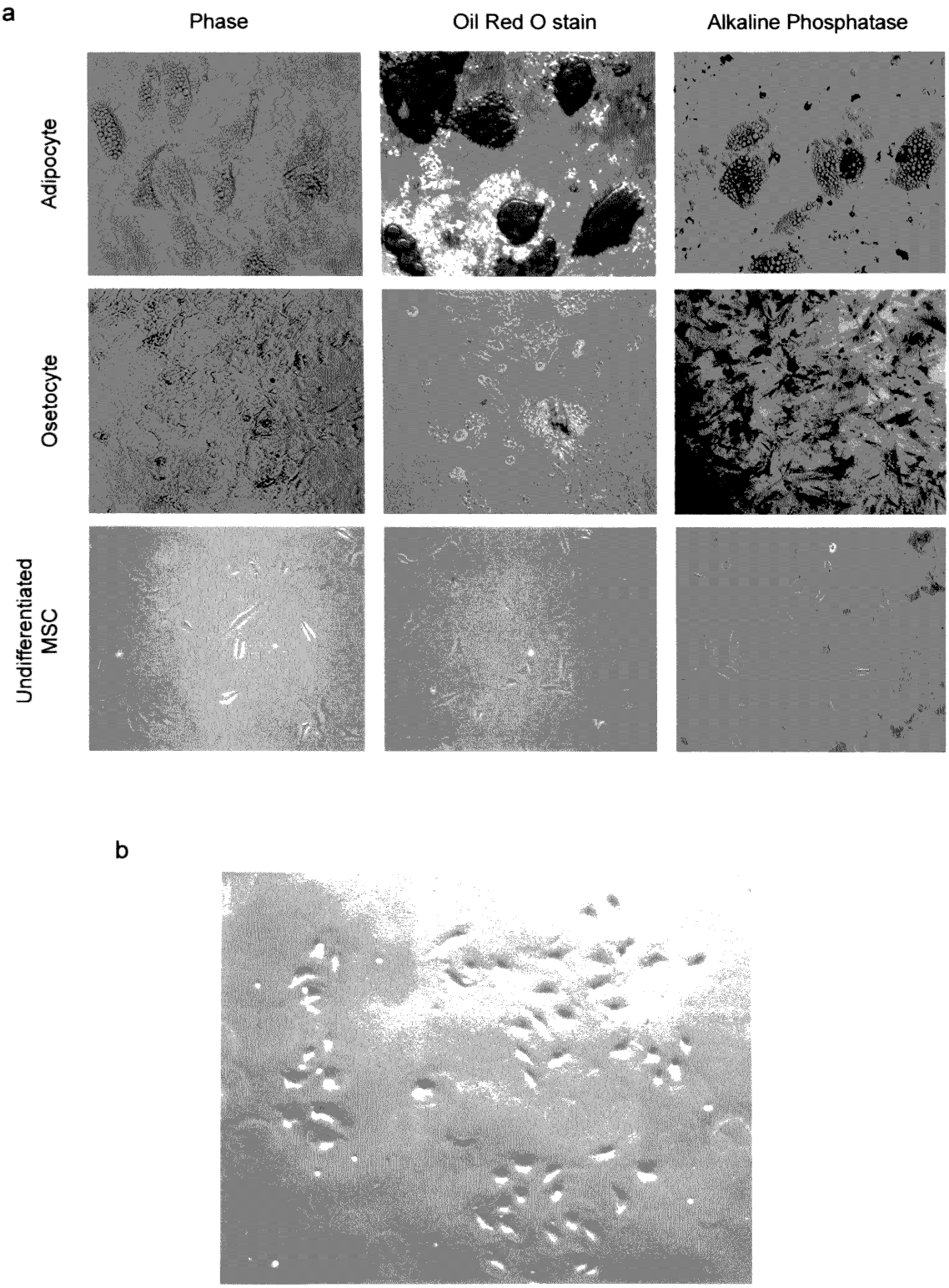
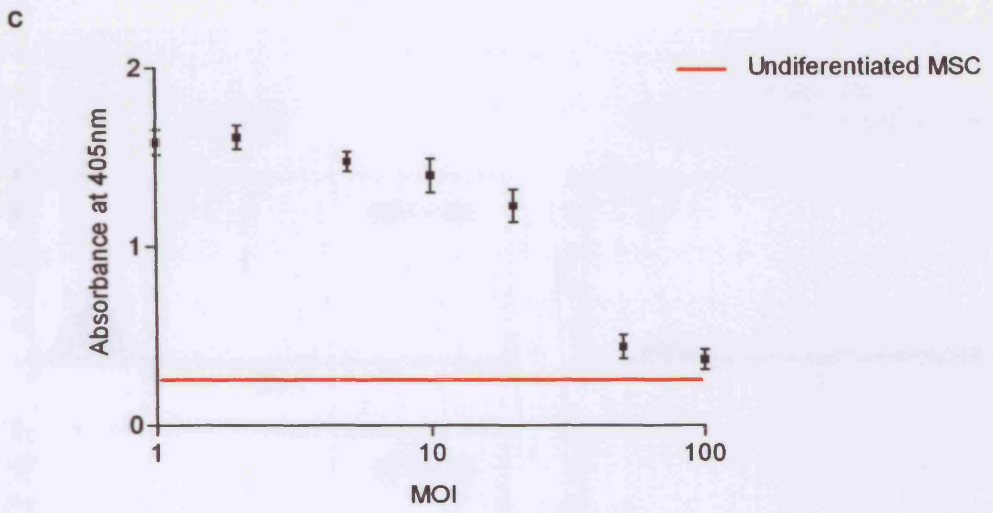


Figure 3.6 Stable transgene expression



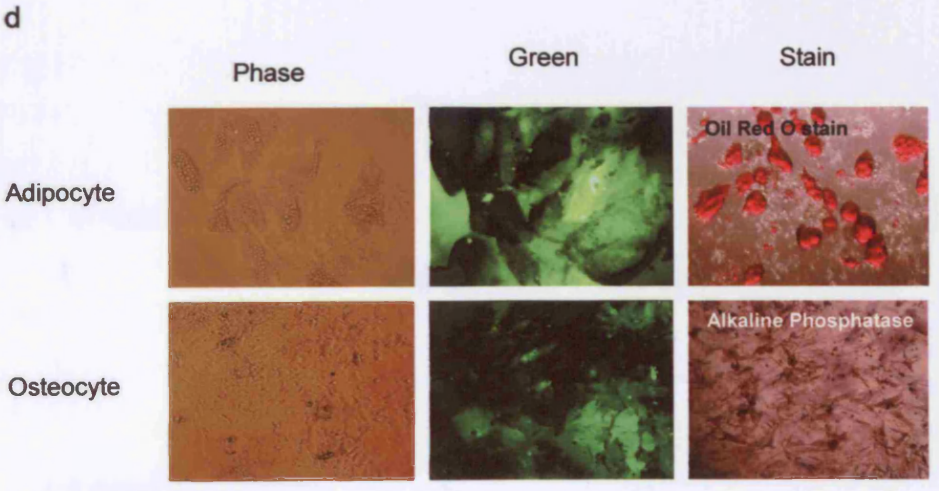
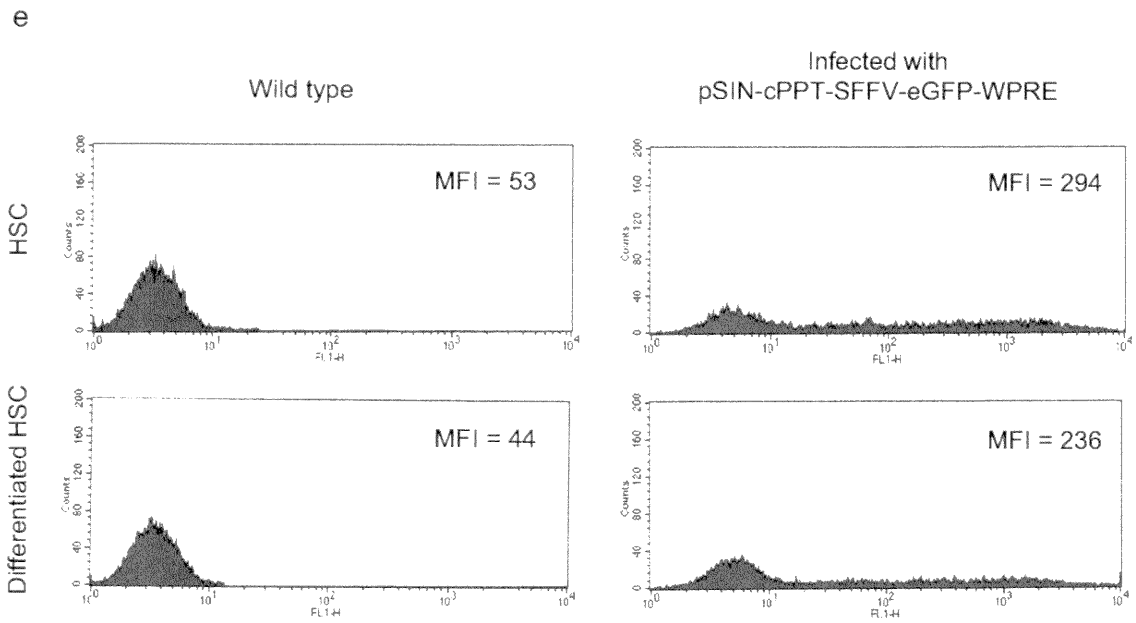


Figure 3.6 Stable transgene expression



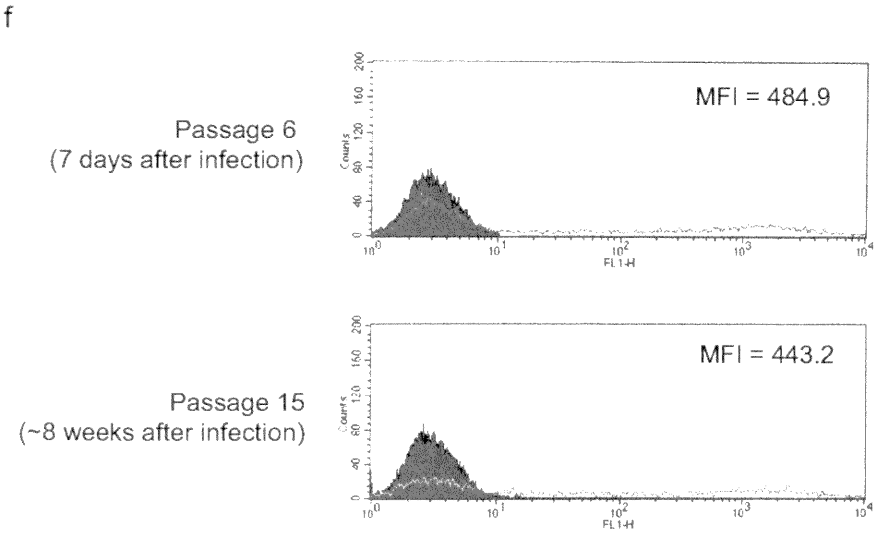


Figure 3.6 Stable transgene expression

Unless otherwise stated, the lentiviral vector for these experiments was pSIN-cPPT-SFFV-eGFP-WPRE and the cells were incubated overnight with the vector.

(a) Light microscopy of MSCs stimulated to differentiate into adipocytes (top), osteocytes (middle) or unstimulated (bottom). No staining was performed in the left hand column, an oil red O stain for lipid droplets (a marker of adipocytic lineage) in the middle column and alkaline phosphatase activity assay (marker for osteocytic lineage) in the right hand column. See materials and methods section 2.2.8 for differentiation conditions.

(b) Light microscopy of HSCs selected from human bone marrow by CD34 expression and grown in EGM2-MV for 14 days. Cells were diluted into EGM2-MV at a density of 1 x 10^5 live CD34 +ve cells per ml in 4 mls of medium in a 6 well plate. Live cells were determined by trypan blue staining (see materials and methods 2.2.1).

(c) Graph indicating the absorbance at 405 nm (A405) determined by colorimetry. The absorbance at 405 nm indicates the expression of alkaline phosphatase and therefore is a quantitative measurement of the amount of osteogenic differentiation. MSC were infected with the MOI shown. Medium was changed the morning after infection for one stimulating differentiation into osteocytes. A405 was measured after 28 days.

(d) Phase (left), green fluorescence (middle) and stain (oil red O, top right and alkaline phosphatase, bottom right) microscopy of MSC infected at an MOI of 5 293tIU/cell and cultured for 28 days in conditions promoting adipocytic (top) or osteocytic (bottom) differentiation.

(e) FACS analysis of human CD34 +ve HSC infected with an MOI of 5 293tIU/cell of lentiviral vector in the presence of 4 μg/ml polybrene 48 hours after infection (top) and after 14 days in culture in EGM-2MV (bottom).

(f) FACS analysis of MSC infected with an MOI of 5 293tIU/cell of lentiviral vector at passage 4 and cultured until cell senescence (around passage 15). FACS analysis was performed at passage 6 (7 days after infection, top) and at passage 15 (8 weeks after infection, bottom).

3.7 Bicistronic expression in stem cells

Many applications for lentiviral systems call for the expression of more than one transgene, or the expression of an additional marker gene. Methods for this include the use of dual promoter and IRES expression systems. My previous results with different promoters in stem cells (Chapter 3.4) imply that it might be difficult to find two different promoters which would reliably express in stem cells. It was therefore decided to investigate the production of both a generic IRES expression system for multiple transgene expression and an IRES expressing an antibiotic resistance cassette for selection.

The EMCV IRES was chosen as this IRES has previously been shown to require fewer cellular cofactors than early IRES (Borman et al., 1994) and offer strong expression (Jang and Wimmer, 1990), however this IRES still requires some cellular factors. Initial assessment was performed with a construct expressing both dsRed and eGFP (Fig 3.7a). The expression levels of both these proteins can be measured by FACS analysis as eGFP represented on FL1-H, and dsRed on FL2-H.

The expression in 293t, DMVEC, HSC and MSC with pSIN-RIG was assessed. Infection at an MOI of 2 showed expression of both dsRed and eGFP in 293t cells and DMVEC, although dsRed expression levels in stem cells was found to be low (Fig 3.7b). An MOI of 2 293tIU/cell was chosen to allow small differences in the expression between 293t and stem cells to be more clearly visible. Strong bleed of FL1-H levels into FL2-H was found during calibration of the FACS machine. All measurements of FL2-H have therefore been compensated to allow for FL1-H bleed. This was performed using a filter plug-in for the FACSCalibur software.

Expression of the 3' gene (eGFP) is controlled by ribosomal entry at the site of the IRES. This is the cofactor dependent step, and so the lack of expression of the 5' gene (dsRed) could not be readily explained by a lack of

these factors. There is little indication in literature as to why this may be, although a report (Baird et al., 2000) suggested that premature termination of translation of dsRed prevents tetramer formation and may yield a non-fluorescent protein. A 50 base pair linker sequence was added to the 3' end of the dsRed gene in case premature termination of transcription was occurring (Fig 3.7c). Infection of stem cells with this construct significantly enhanced expression of dsRed (Fig 3.7d).

To determine whether the expression of the 3' gene (eGFP) could be used as an indicator of the strength of expression of the 5' gene (dsRed) in MSC, FACS analysis was used to link expression after infection with pSIN-RIG+. In an infected population, cells expressing eGFP also expressed dsRed, and these data imply that the strength of expression of the two genes is directly proportional (Fig 3.7e).

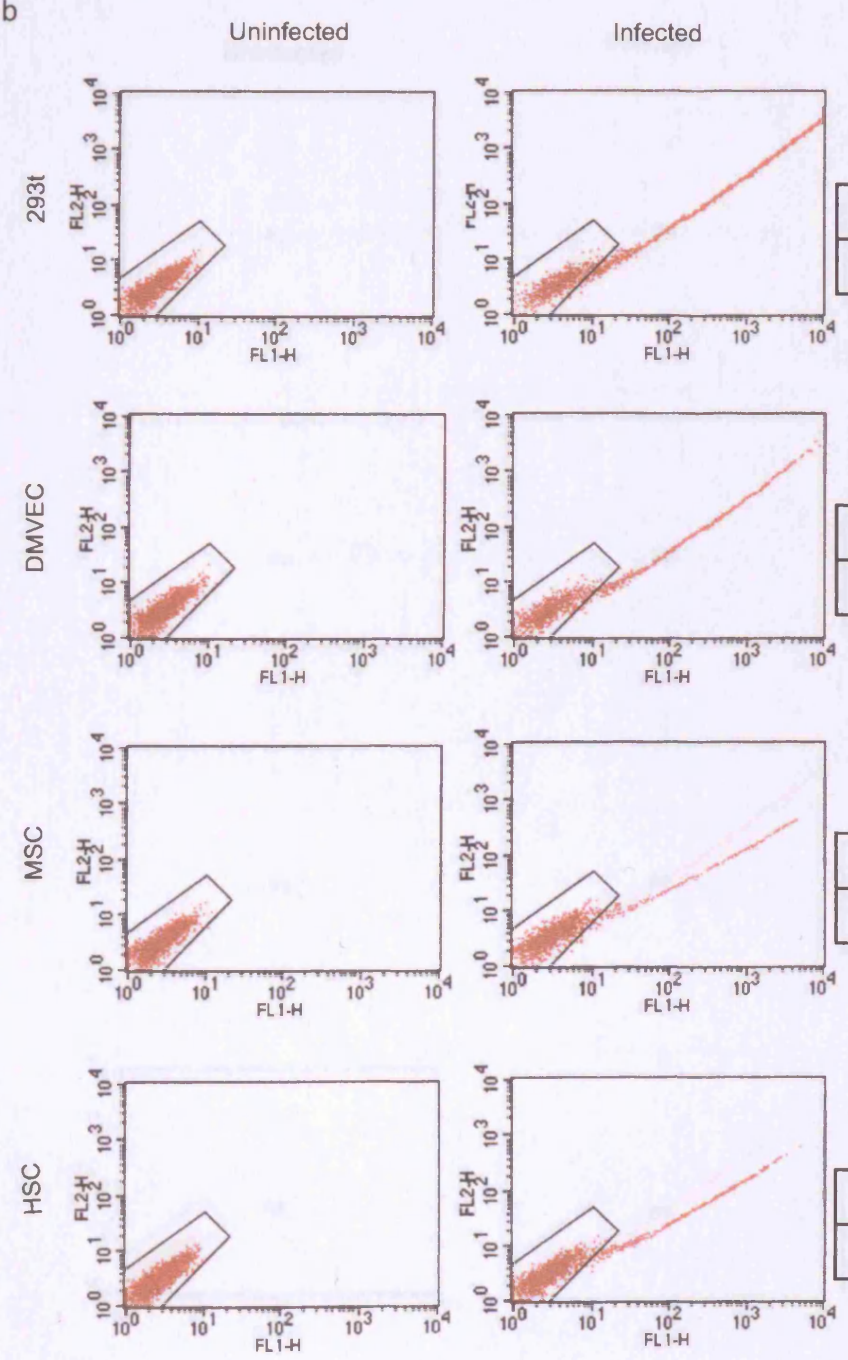
In order to facilitate production of pure populations expressing a protein at low MOI, a resistance cassette can be used. Selection after infection is particularly desirable since adverse effects of infection with high MOI has already been shown (Fig 3.5). A commercially available neomycin resistance cassette (Clontech, California, USA) was cloned by PCR into the 3' transgene location and eGFP into the 5' transgene location to create pSIN-eGFP-IRES-Neo (Fig 3.7f). Infection of both 293t cells and MSC with a low MOI of this vector yielded a population which initially expressed low levels of eGFP, however after 7 days in culture with neomycin showed almost 100% of the cells to be eGFP positive (Fig 3.7g).

These data indicate that IRES systems can offer a simple method of expressing multiple genes from a single lentiviral vector. This can be used for complex gene expression or production of a pure population of cells expressing a transgene through the use of a resistance cassette. These data also indicate that the strength of expression of the 3' gene is an indicator of the strength of expression of the 5' gene.

Figure 3.7 Bicistronic expression in stem cells

a

b



	MFI FL1-H	MFI FL2-H
Before	3.3	4.2
After	334.2	221.6

	MFI FL1-H	MFI FL2-H
Before	2.3	3.5
After	211.5	196.1

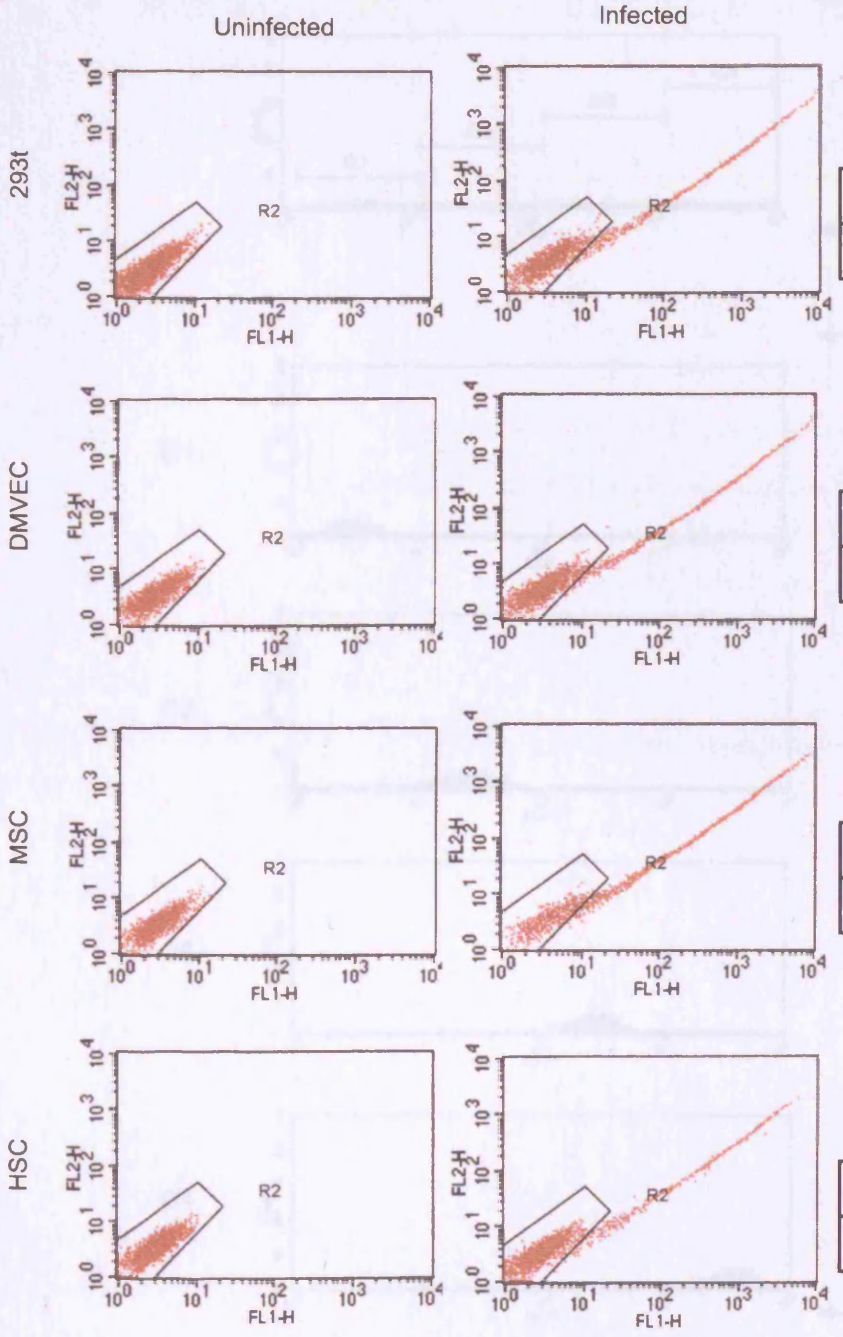
	MFI FL1-H	MFI FL2-H
Before	4.1	3.7
After	81.4	10.1

	MFI FL1-H	MFI FL2-H
Before	3.24	4.16
After	57.2	9.6

Figure 3.7 Bicistronic expression in stem cells

c

d



	MFI FL1-H	MFI FL2-H
Before	4.11	4.27
After	351.3	268.4

	MFI FL1-H	MFI FL2-H
Before	3.41	2.96
After	302.7	254.1

	MFI FL1-H	MFI FL2-H
Before	3.5	4.01
After	195.2	152.8

	MFI FL1-H	MFI FL2-H
Before	4.28	3.88
After	110.1	96.4

Figure 3.7 Bicistronic expression in stem cells

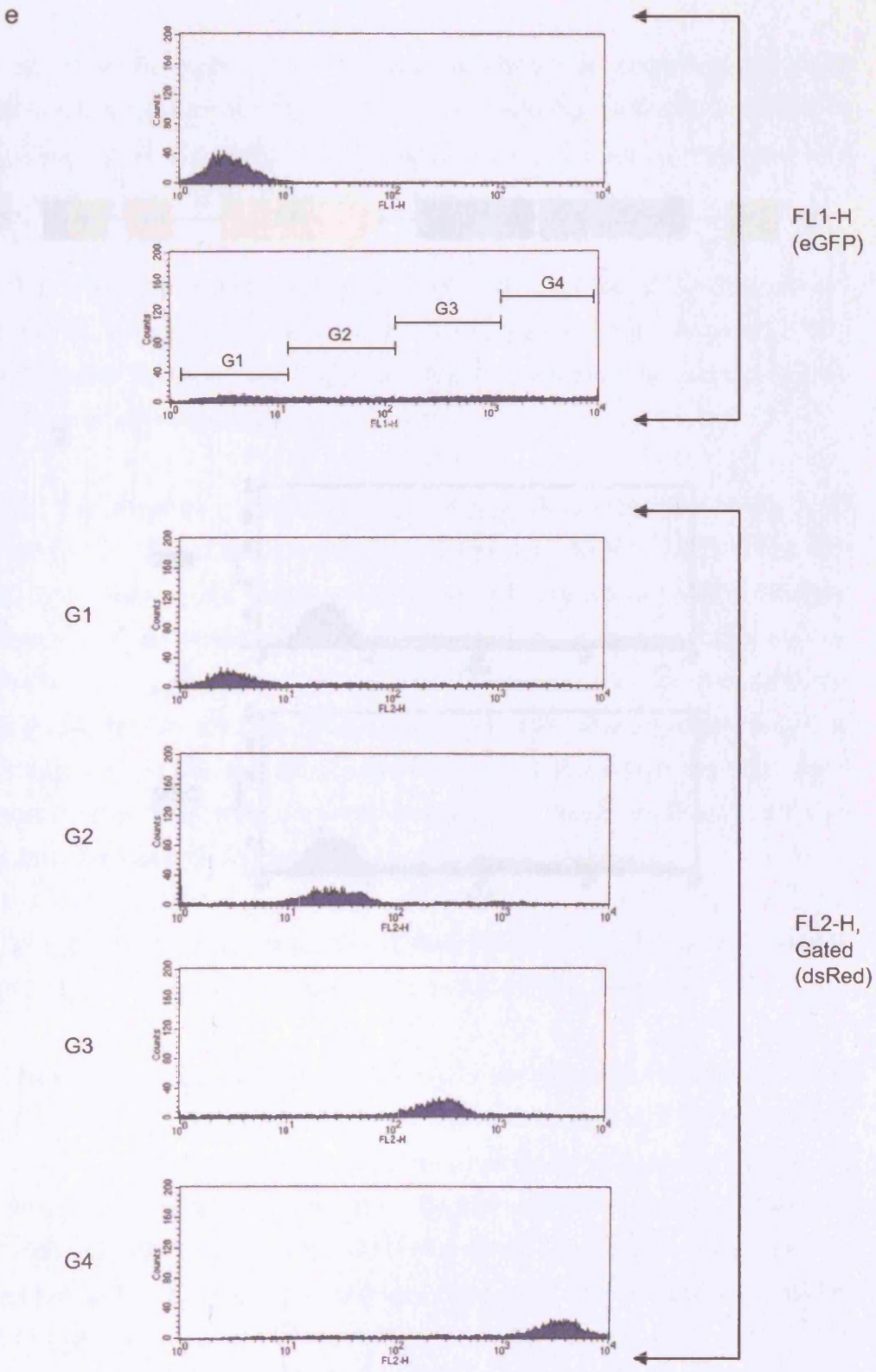
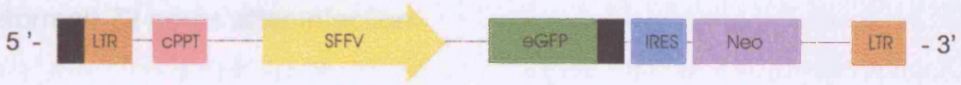


Figure 3.7 Bicistronic expression in stem cells

f



g

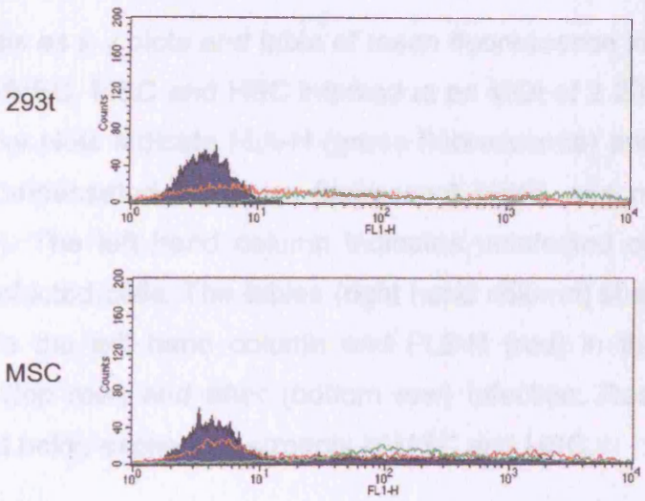


Figure 3.7 Bicistronic expression in stem cells

Unless otherwise stated, infection was performed by incubating cells with virus overnight and changing medium in the morning. HSC were infected in the presence of polybrene at a concentration of 4 μg/ml. Analysis was performed 72 hours after infection.

(a) Diagram of the lentiviral vector pSIN-cPPT-SFFV-Red-IRES-eGFP-WPRE (pSIN-RIG). LTR– long terminal repeat; cPPT- central polypurine tract; SFFV- spleen focus forming virus; IRES- internal ribosomal entry site; eGFP- enhanced green fluorescent protein

(b) FACS analysis as x-y plots and table of mean fluorescence intensity (MFI) of 293t cells, DMVEC, MSC and HSC infected at an MOI of 2 293tIU/cell with pSIN-RIG. The xy plots indicate FL1-H (green fluorescence) and FL2-H (red fluorescence, compensated for green fluorescent bleed, see materials and methods 2.2.15). The left hand column indicates uninfected cells, and the middle column infected cells. The tables (right hand column) show the MFI of FL1-H (green) in the left hand column and FL2-H (red) in the right hand column, before (top row) and after (bottom row) infection. Results indicate that dsRed is not being expressed strongly in MSC and HSC.

(c) Diagram of the lentiviral vector pSIN-cPPT-SFFV-Red-linker-IRES-eGFP-WPRE (pSIN-RIG+). The 50 bp linker is indicated as a black box.

(d) FACS analysis as x-y plots and table of mean fluorescence intensity (MFI) of 293t cells, DMVEC, MSC and HSC infected at an MOI of 2 293tIU/cell with pSIN-RIG+. The xy plots indicate FL1-H (green fluorescence) and FL2-H (red fluorescence, compensated for green fluorescent bleed, see materials and methods 2.2.15). The left hand column indicates uninfected cells, and the middle column infected cells. The tables (right hand column) show the MFI of FL1-H (green) in the left hand column and FL2-H (red) in the right hand column, before (top row) and after (bottom row) infection. The MFI of FL2-H

indicate that fluorescence on both axes is detectable in both 293t and stem cells after inclusion of the 50bp linker.

(e) FACS analysis of MSC after infection with an MOI of 5 293tIU/cell of pSIN-RIG+. The top two plots indicate eGFP expression in these cells (top uninfected, bottom infected) and show the four gates for fluorescence on FL1-H (G1-4). The fluorescence on FL2-H (dsRed expression compensated for eGFP bleed) for cells within gates G1-4 are shown in the bottom four graphs. These data indicate that the strength of expression of eGFP can be used as an indicator of the strength of expression of dsRed, an vice-versa.

(f) Diagram of pSIN-cPPT-SFFV-eGFP-linker-IREs-Neo-WPRE (pSIN-eGFP-Neo)

(g) FACS analysis of 293t cells (top) and MSC (bottom) infected with pSIN-eGFP-Neo at an MOI of 1 293tIU/cell. Cells were then cultured for 7 days either normally, or in medium containing 10 μg/ml neomycin sulphate. FACS analysis after this time indicated that the population of cells grown in neomycin (green line) showed a shift in fluorescence to the right when compared to cells grown in normal medium (red line). This shows a selection pressure favouring eGFP expression through the presence of the neomycin resistance cassette.

3.8 Vector safety for *in vivo* use

Stem cells have wide ranging applications both *in vitro* and *in vivo* (Clements et al., 2006). Lentiviral vectors broaden the scope of these cells, however before these vectors can be used *in vivo*, there needs to be significant advances in both the technology and the availability of data regarding the safety and long term effects of these vectors.

The *in vivo* safety of these vectors involves key issues surrounding the fact that they contain genetic information from HIV-1. The production of replication competent lentiviruses (RCL) would lead to replication in the host. This may potentially cause immunodeficiency, and represents the construction and release of a new human pathogen. Recombination or release of integrated transgenes because of coinfection with wild type virus is a concern. This could allow the production of a transmissible viral progeny containing genetically modified components. The possibility of insertional mutagenesis from the action of reverse transcriptase and integrase in these vectors has recently been raised (Themis et al., 2005). Finally, germ line mutations are of concern. In order to develop a 'cure' for a genetic disease, this is a necessary step however the effects on future generations is not well understood.

It is possible to test some of the concerns regarding the safety of these vectors. The experiments here are based upon previous investigations into lentiviral safety (Bukovsky et al., 1999).

The presence of RCL can be determined by infecting large numbers of cells with virus and determining their ability to pass on the expression of transgene. RCL would make cells produce new virions, so the medium in which cells infected with RCL are cultured in would also contain virions.

100 plates of 293t cells were infected at an MOI of 10 with pSIN-cPPT-SFFV-eGFP-WPRE (a total of 10^{10} 293tIU). After 48 hours, the medium was changed to remove any residual virus and then after a further 48 hours the

medium harvested and placed in culture with uninfected 293t cells. Any RCL in the first batch of virus would result in virions in the medium, which would give expression in the second batch of cells.

FACS analysis of these cells after 48 hours showed no expression of eGFP (Fig 3.8a). These data indicate that there was no production of RCL, and therefore that the rate of RCL production is < 1 per 1 x 10^{10} 293tIU.

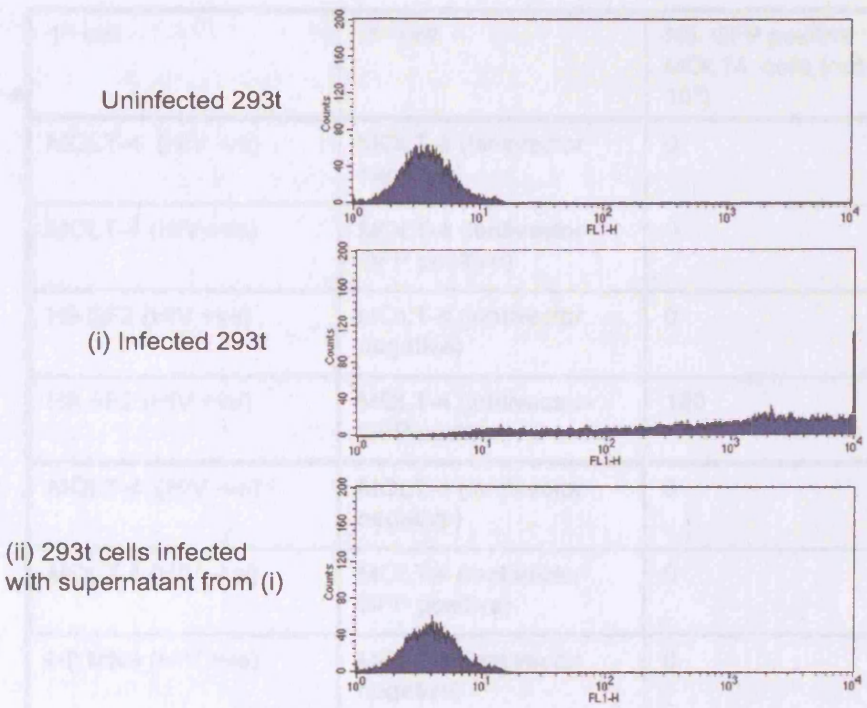
The release of transgenes from both the pHR' and pSIN vectors was also assessed by coinfection of MOLT-4 cells with wild type HIV-1 (Fig 3.8b).

The results imply that transgenes delivered by the pHR vector are capable of mobilization by wild type HIV-1. The pHR backbone was much more subject to recombination than the pSIN backbone as determined by the mobilization of infectious virions encoding eGFP. Much larger scale experiments would be required to assess this accurately (Fig 3.8c). The release of a single GFP positive event from the pSIN backbone could be a lentivirion remaining in culture after medium change.

These data indicate that 3-plasmid production systems produce safe vectors with very low RCL rates. This would be expected due to the absence of much of the HIV-1 genome from the plasmids. It is also shown here that lentiviral vectors can be mobilized after infection, but that deletions in the LTRs of pSIN vectors make them safer than pHR vectors in the presence of wild type HIV-1.

Figure 3.8 Vector safety for in vivo use

a



b

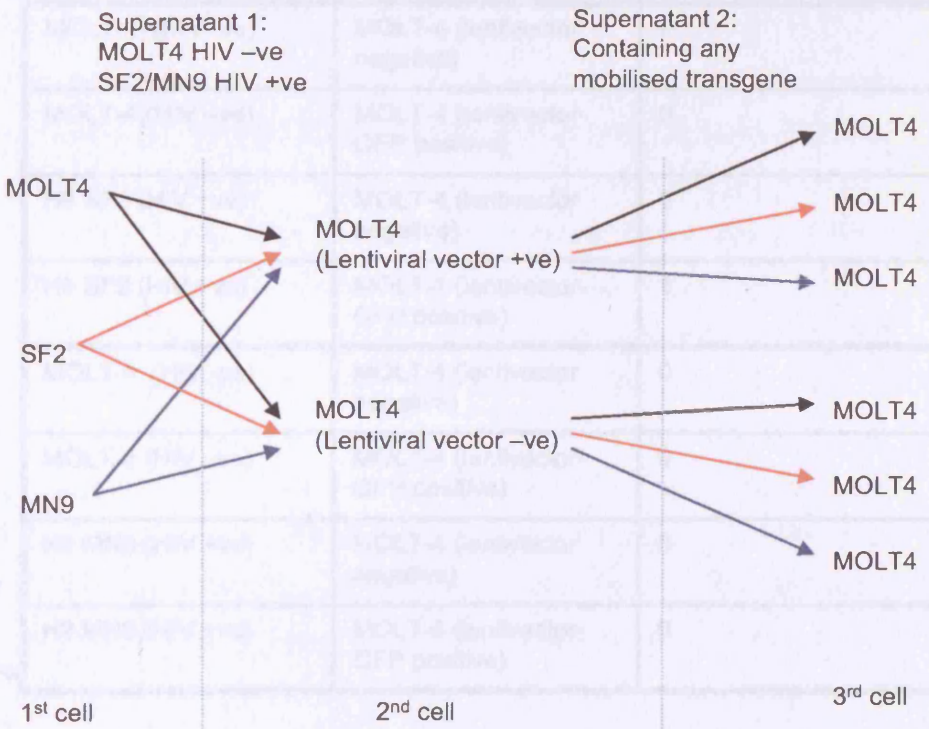


Figure 3.8 Vector safety for in vivo use

c

1st cell	2nd cell	No. GFP positive MOLT4 cells (out of 10^8)
MOLT-4 (HIV -ve)	MOLT-4 (lentivector negative)	0
MOLT-4 (HIV –ve)	MOLT-4 (lentivector-GFP positive)	0
H9 SF2 (HIV +ve)	MOLT-4 (lentivector negative)	0
H9 SF2 (HIV +ve)	MOLT-4 (lentivector-GFP positive)	120
MOLT-4 (HIV -ve)	MOLT-4 (lentivector negative)	0
MOLT-4 (HIV –ve)	MOLT-4 (lentivector-GFP positive)	0
H9 MN9 (HIV +ve)	MOLT-4 (lentivector negative)	0
H9 MN9 (HIV +ve)	MOLT-4 (lentivector-GFP positive)	810
MOLT-4 (HIV -ve)	MOLT-4 (lentivector negative)	0
MOLT-4 (HIV –ve)	MOLT-4 (lentivector-GFP positive)	0
H9 SF2 (HIV +ve)	MOLT-4 (lentivector negative)	0
H9 SF2 (HIV +ve)	MOLT-4 (lentivector-GFP positive)	1
MOLT-4 (HIV -ve)	MOLT-4 (lentivector negative)	0
MOLT-4 (HIV –ve)	MOLT-4 (lentivector-GFP positive)	0
H9 MN9 (HIV +ve)	MOLT-4 (lentivector negative)	0
H9 MN9 (HIV +ve)	MOLT-4 (lentivector-GFP positive)	0

pHR

pSIN

Figure 3.8 Vector safety for *in vivo* use

(a) Experiment to demonstrate that pSIN vectors in 3-plasmid transfection systems produce undetectable quantities of RCL. 100 plates of 1 x 10^7 293t cells were infected at an MOI of 10 of pSIN-SFFV-eGFP (a total of 10^{10} 293tIU). After 48 hours, the medium was changed to remove any residual virus and then after a further 48 hours the medium harvested and placed in culture on 100 plates of uninfected 293t cells (1 x 10^7 cells/plate). After 72 hours, FACS analysis of uninfected 293t cells (top), 293t cells from the first infection (infected with vector) (middle) and of 293t cells infected with supernatant harvested from infected cells (bottom) showed that infected 293t cells produced no further infectious virion. FACS analysis was performed by trypsinising all 100 plates and analyzing 10^7 cells from each batch.

(b) Diagram of the protocol employed to demonstrate the ability of wild type virus to mobilize the lentiviral vector. SF2 and MN9 cells are HIV-1 positive T cell lines which produce infectious HIV-1 in the medium. MOLT4 cells are a HIV negative T cell line.

(c) Results for experiment detailed in (b). Medium from MOLT4, SF2 or MN9 cells was harvested (1st cell). MOLT4 cells were either infected with an MOI of 5 293tIU/cell of pHR-CMV-eGFP, pSIN-CMV-eGFP or left untreated (2nd cell). 72 hours after the 2nd cells were infected with lentiviral vector, medium from the 1st cell (HIV-1 +ve SF2, HIV-1 +ve MN9 or HIV-) was added to uninfected or infected MOLT4 cells. After a further 72 hours, medium from these second cells was harvested and used to infect new MOLT4 cells (3rd cell). After 72 hours, these cells were fixed and analysed for eGFP expression by FACS analysis (right hand column). These results indicate that superinfection of wild type HIV-1 on cells already infected with a lentiviral vector can result in mobilization of the transgene. These data also show that the pSIN backbone is far more difficult to mobilize than the pHR backbone.

3.9 Discussion and conclusions

In this chapter, I show that human adult stem cells can be isolated from human bone marrow using a simple protocol and that MSC can remain undifferentiated in culture. Conditions which maintain HSC in culture without differentiating are not currently known, however cells which match the immunophenotype of human HSC could be isolated from bone marrow samples.

Quantification of virions was a major concern in this chapter. Although the 293tIU is an established method for quantification of virus, this cannot be used for viruses which do not express a marker. Viruses which express eGFP under the strength of different promoters can yield different figures based on the strength of expression. There is also the possibility that the vector passively transfers eGFP protein, resulting in a high measured titre (Nash and Lever, 2004). Waiting 96 hours instead of 48 hours after infection to measure eGFP titre would minimize this effect.

The use of p24 concentration to determine titre is also widespread, however this method was shown to be unreliable in my hands. The p24 assay is reliant on a standard curve to estimate the number of virions. It does not indicate the infectious ability of the virions, just the presence of p24. TaqMan qPCR is a relatively novel technology for quantification of copy number of DNA. This makes the technology ideal for the purposes of assessing viral titre. Unfortunately, my results indicated that several copies of the viral DNA were present in every expressing cell.

One possible explanation for overestimation of the titre is the measurement of non-integrated DNA present after the RT step but not integrated into the host. This would be detected by qPCR but not by p24 or by measuring eGFP expression. This effect could be reduced by leaving cells for longer than 72 hours after infection, by which time free DNA would be degraded. Although

this is a possible source of error, I do not think that this effect would be significant enough to account for my results.

Although it is possible that not every copy of the virus expresses, that several copies are required to represent 1 293tIU, or that non-integrated DNA is being measured it is more likely that my standards were incorrect or that my PCR reaction conditions were not fully optimized.

As I was chiefly using this method to convert viral titres into equivalent 293tIU, it was not necessary for me to determine the absolute number of viral copies per cell. Controls within my experiments indicated that my results were reproducible and that my standard appeared to be correct. I would like to have investigated this further, perhaps by producing a cell line which was known by fluorescence in-situ hybridization (FISH) or genomic Southern blot to contain 1 or 2 copies of the lentiviral vector and recalibrating my assay using this cell line as a standard.

The production of high titre lentiviral vectors remains an obstacle to overcome. The protocol for producing vector is still based upon early references (Naldini et al., 1996b). These publications do not indicate the rationale for the protocols chosen. These conditions were therefore investigated, and it was found that optimization of harvest time, quantity of medium and ratio of packaging plasmids would regularly yield high titre ($>10^8$ 293tIU/ml) virus. This is considerably higher than most publications using lentiviral vectors, and demonstrates the necessity for optimization of systems in individual hands rather than blind use of conditions from previous publications.

Both adult stem and primary endothelial cells could be infected with a lentiviral vector. Chapter 3.4 shows evidence for the use of additional elements such as the cPPT and WPRE to enhance infection and expression in primary and stem cells.

An important observation of the effect of lentiviral infection on stem cells was made. The disturbance of the growth rate of these cells from infection even with a low MOI of virus was marked. This effect is not often reported in publications involving lentiviral vectors, and most experiments using these vectors do not consider the potential for the vectors to disturb cell growth.

Although the disturbance at low MOI is slight in cell lines, it is greater in stem cells and therefore the possibility of greater implications for experiments using these methods needs further investigation. Microarray analysis of infected cells may yield more information about which genes and cellular pathways may be responsible for this. It is possible that this effect is either due to overexpression of a foreign gene or a transient antiviral response, or it may be a longer lasting effect of integration of foreign genetic material.

The data presented here indicate stem cells can continue to differentiate normally after infection with low MOI. This would seem to support a more transient response to infection, although the detrimental effects on differentiation after infection with MOI above 20 293tIU/cell would indicate permanent damage from the infection process. These data warrant much more detailed investigation of the pathways which are disrupted, however this is beyond the scope of this thesis. It was sufficient for me to demonstrate that at low MOI these cells can behave essentially normal.

Investigation of bicistronic expression shows that the lentiviral system can be used with an IRES to express two genes driven by the same promoter in stem cells. Although the data presented here show that stem cells can coexpress two fluorescent proteins, the requirement for compensation of eGFP bleed into FL2-H means that these data cannot be regarded as conclusive. It was very difficult, even with the assistance of the FACScalibur software, to separate these variables and this represents a weakness in my experiment. These data could be strengthened through the use of an alternative fluorescent protein such as the newer dsRed derivatives which have more defined excitation-emission spectra. Alternatively, quantitative analysis of

protein expression through ELISA or quantitative Western blot analysis could have been performed. It was sufficient for my purposes to demonstrate that a resistance cassette could be used to establish a pure population expressing a transgene, however further investigation and conclusive evidence that the expression levels of these proteins are linked is something I would like to have performed as it adds to the value and utility of the vector.

The last results presented here show that the pSIN vector is much safer than the pHR based vectors. The pSIN vector is much less likely to recombine with and be mobilized by wild type HIV-1, although these vectors are equally unlikely to form RCL. This result was expected from the deletions in the LTR which make the vectors self-inactivating and confirm previous reports (Bukovsky et al., 1999).

Lentiviral vectors offer considerable advantages over other *in vitro* and *in vivo* systems. For expression systems in *in vitro* experiments, they offer transduction of cells which are difficult or impossible to transfect, and offer stable expression. Lentiviral vectors are more robust than retroviruses, especially in stem cells, and are as easy to produce as both adenoviral and retroviral vectors.

These data support the use of lentiviral vectors for future *in vitro* and *in vivo* work in our hands. We show that we can use these vectors with minimal disturbance to target cells. Recent gene therapy trials with retroviruses have revealed potential preferential integration sites for MoMLV *in vivo*, which raises the potential for lentiviral vectors to suffer from the same problems. I could have determined the frequency and sites of integration with the vectors I had produced using FISH and linear amplification-mediated PCR (LAM-PCR). This would have been a more complete extension of this work. I could also have confirmed via measure of the interferon responsive genes whether the interferon response was permanently upregulated in these cells. These data would be useful for clinical applications with these vectors.

Despite the demonstration in published literature of the efficacy and ease of use that lentiviral systems allow, their use *in vivo* is underrepresented in scientific research. It is possible that lentiviral systems based on other viruses such as the feline immunodeficiency virus (FIV) or equine infectious anemia virus (EIAV) may be considered more suitable for *in vivo* applications since they are non pathogenic to humans, however these vectors show many of the same problems as HIV-1 based vectors. HIV-1 based vectors can be made more appealing for therapeutic applications by more thorough characterization of their effects in cells and more mainstream acceptance that HIV-1-based vectors are *not* HIV-1.

Chapter 4:
Lentiviral delivery of RNA Interference

Chapter Aims:

The aims of this chapter were to knockdown gene expression of endogenous and exogenous genes by expressing short hairpin RNA from a polymerase III promoter in a self inactivating lentiviral backbone (pSIN). It was also shown that this lentiviral expression system can deliver effective RNAi to cell types refractory to transfection and therefore not amenable to synthesized dsRNA.

4.1 Evaluation of RNAi in cell lines using dsRNA

To assess a lentiviral system for delivery of shRNA, a validated target sequence and a cell line in which RNAi functions well is required. First, the efficacy of commercially available transfection reagents to transduce both adherent and suspension cell lines with a plasmid expressing eGFP was evaluated. The manufacturer's guidelines were used (see materials and methods 2.4.2) and the percentage of cells which were eGFP positive 16 hours after transfection was assessed by FACS analysis. All four tested reagents performed well in 293t and HeLa cells, however they all performed significantly worse in suspension and primary cells (Fig 4.1a). Oligofectamine was chosen for future transfections with dsRNA, as it transfected both 293t and HeLa cells and showed some ability to transfect human umbilical vein endothelial cells (HUVEC) and dermal microvascular endothelial cells (DMVEC).

A target sequence for eGFP was chosen to test the efficacy of RNAi based on criteria available from www.ambion.com (5'-GTTCATCTGCACCACCGGCAAGC-3'). dsRNA targeting this sequence was synthesised by Dharmacon. One of the problems with knocking down the expression of eGFP is the long protein half life in cells. To avoid this, a commercially available destabilized form of eGFP (deGFP) which has a significantly reduced half life in cells (< 6 hours, http://www.clontech.com/clontech/techinfo/vectors_dis/pd2EGFP.shtml) when compared to wild type eGFP (half life of 26 hours (Corish and Tyler-Smith, 1999)) was used to create a lentiviral construct (pSIN-deGFP). The sequence of deGFP was analysed to ensure the target sequence was present. Accurate assessment of the efficacy of RNAi is difficult to perform when infected cells demonstrate a broad range of expression levels, as happens during lentiviral infection. To avoid this, and hopefully detect a more readily visible shift, a cell population with a uniform expression level of deGFP was required. This could be achieved by cell sorting, or single cell cloning to develop a cell line. Cell sorting proved unreliable, and because of problems such as contamination of sorted cells and impure populations, it was decided that the best standard

would be to generate a cell line expressing a uniform level of eGFP by a limiting dilution method.

293t and HeLa cells were infected with pSIN-deGFP at an MOI of 2. Several 293t and HeLa cell lines were produced from an infected population by serial dilution of cells and plating in 96 well plates in conditioned medium. Conditioned medium was used as it was found that single 293t cells grew into colonies much faster in conditioned medium than when in DMEM (data not shown). The mixture was diluted such that each well should contain ≤1 cell. The colonies were split into 24 well plates when confluent. After a further 7 days each colony was screened by FACS and those which displayed a defined homogenous eGFP expression were assumed to have originated from single cells. These clones were then tested to determine whether they were still amenable to transfection by introducing a LacZ-FLAG plasmid. Only 2 293t lines (293t-D1 and 293t-D12) and 1 HeLa line (HeLa-D7) showed suitable FACS profiles and maintained transfection ability (Fig 4.1b) and were used for further investigation.

To determine the copy number of the lentiviral vector in these cells each clone was analysed using TaqMan qPCR for both the lentiviral insert and GAPDH (see materials and methods). This allowed the estimation of the number of copies of the insert per cell (Fig 4.1c). These data indicate that despite the low MOI, several copies of the lentiviral vector are present in each cell, however the number is sufficiently low it is unlikely to have any adverse affect on the cell.

Initial transfection of commercially synthesised dsRNA targeting eGFP into D12 293t cells, under conditions shown above to be effective, showed no significant silencing effect. The eGFP dsRNA did function in D7 HeLa cells producing a measurable silencing effect after 48 hours (Fig 4.1d).

To determine whether eGFP dsRNA was failing to enter the 293t cells or whether the cells were not processing the dsRNA, FACS analysis of 293t

cells after transfection with fluorescein tagged dsRNA molecules with the same sequence (gift from Sam Wilson, Windeyer Institute) showed that oligofectamine was able to deliver the dsRNA into 293t-D12 cells and therefore that delivery was not the cause of lack of function (Fig 4.1e). It was therefore concluded that in our hands 293t cells do not process dsRNA well for RNAi.

Several other groups have used 293t cells for research involving dsRNA and have found it to be functional. Other members of my laboratory confirm my results regarding 293t cells (Heike Laman, personal communication). I can offer no explanation of why this may be. For all further investigation of dsRNA, HeLa-D7 cells were chosen as they are both easy to culture and amenable to the effects of dsRNA.

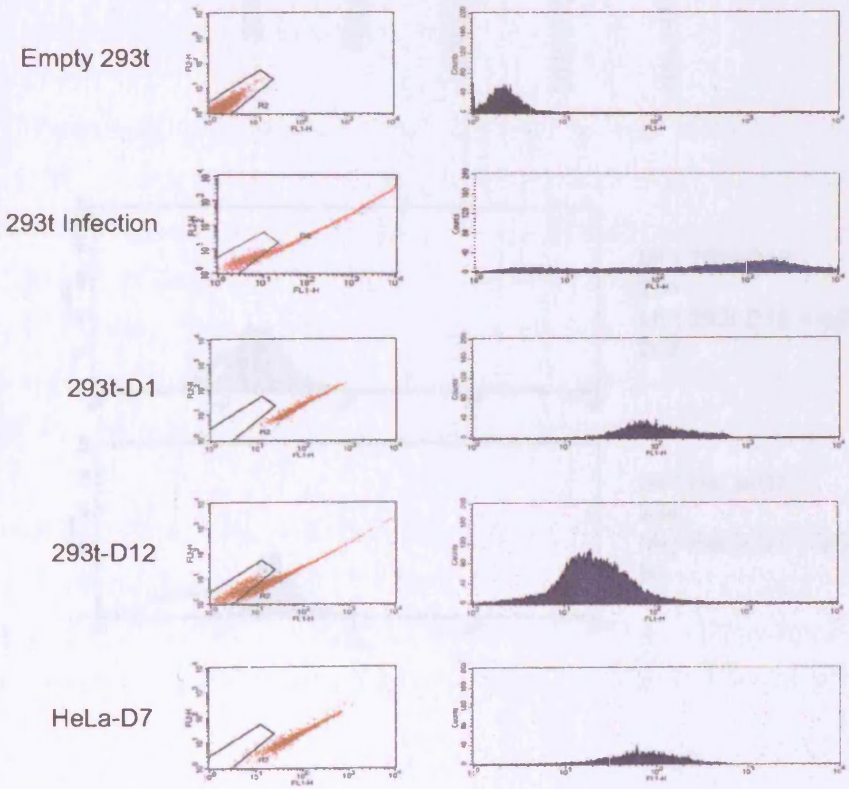
Figure 4.1 Evaluation of RNAi in cell lines using dsRNA

a

		Fugene		Oligofectamine		TransIT		Effectene	
Adherent	293t	68	73	64	61	52	58	68	71
	HeLa	64	69	59	67	45	51	62	69
	HUVEC	13	9	17	19	8	4	9	10
	DMVEC	10	12	21	15	6	7	6	2
Suspension	MSC	3	1	2	6	2	7	2	6
	Ramos	4	2	6	1	2	1	1	1
	BC-3	55	67	2	4	1	6	21	17
	MOLT-4	2	1	0	0	1	0	1	2

% cells eGFP positive by FACS analysis 16 hours after transfection

b



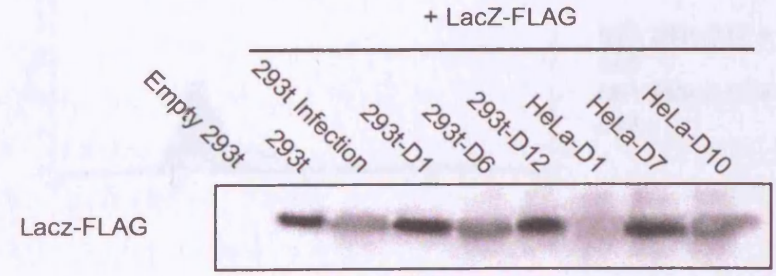
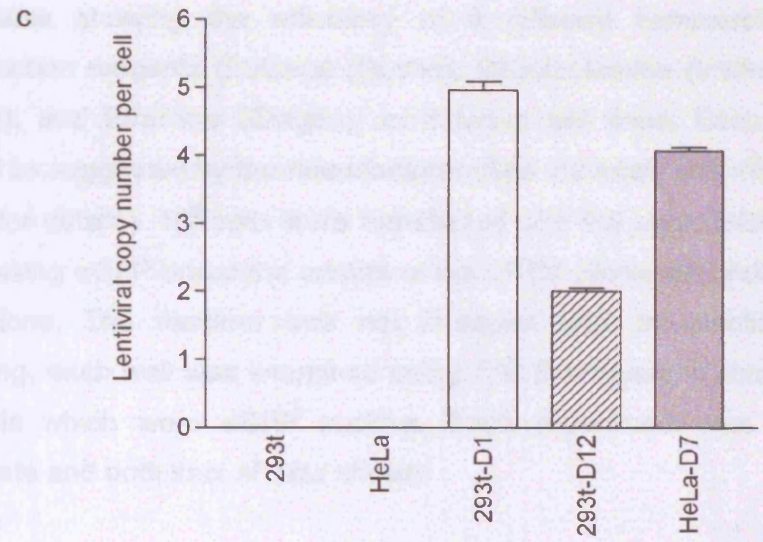
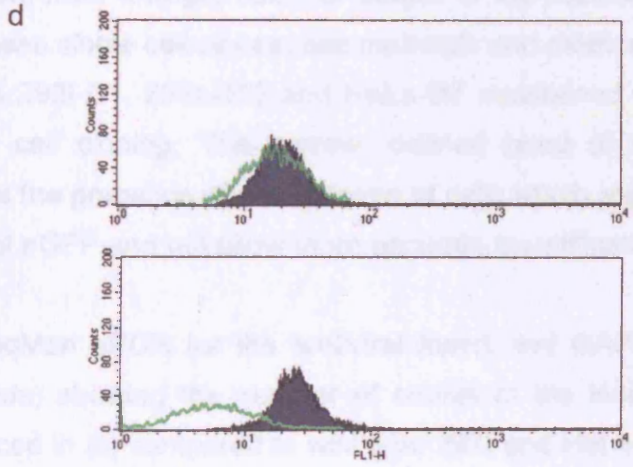


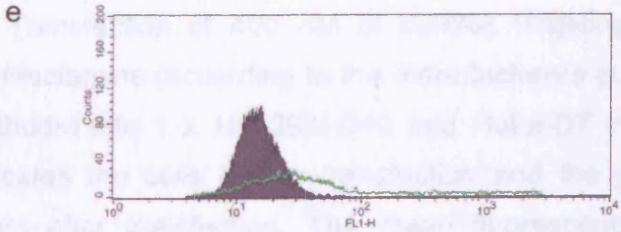
Figure 4.1 Evaluation of RNAi in cell lines using dsRNA





MFI 293t-D12:
254
MFI 293t-D12 + eGFP knockdown:
242

MFI HeLa-D7:
284
MFI HeLa-D7 + eGFP knockdown:
53



MFI 293t-D12 + dsRNA:
248
MFI 293t-D12 + tagged dsRNA:
613

Figure 4.1 Evaluation of RNAi in cell lines using dsRNA

(a) Table showing the efficiency of 4 different commercially available transfection reagents (FuGene (Roche), Oligofectamine (Invitrogen), TransIt (Mirus), and Effectine (Qiagen)) in different cell lines. Each reagent was tested as suggested by the manufacturer (See materials and methods section 2.4.2 for details). 10^5 cells were transfected with 0.5 μg pCSGW (a plasmid expressing eGFP under the control of the SFFV promoter) under appropriate conditions. The medium was not changed after transfection. The next morning, each well was examined using FACS analysis to show the percent of cells which were eGFP positive. Each experiment was performed in duplicate and both sets of data shown.

(b) FACS analysis and Western blot analysis of cell lines assumed to originate from a single cell. For details of the method used to generate and test these single cell clones, see materials and methods section 2.2.16. Three clones 293t-D1, 293t-D12 and HeLa-D7 maintained transfection ability after single cell cloning. The narrow, defined band of eGFP expression (top) implies the presence of a population of cells which expressed a roughly equal level of eGFP and will allow more accurate quantification of knockdown.

(c) TaqMan qPCR for the lentiviral insert and GAPDH (see materials and methods) showing the number of copies of the insert per cell in cell lines produced in (b) compared to wild type 293t and HeLa cells. The analysis was performed in triplicate and the error bars indicate the standard error of the mean.

(d) Transfection of 400 nM of dsRNA targeting eGFP (Dharmacon) with oligofectamine (according to the manufacturers guidelines, see materials and methods) into 1 x 10^5 293t-D12 and HeLa-D7 cells. The purple population indicates the cells before transfection and the green overlay the cells 48 hours after transfection. The mean fluorescence intensity (MFI) of each population is marked on the side of the FACS plot and indicates no significant

knockdown in 293t-D12 cells and a significant knockdown of eGFP expression in HeLa-D7 cells.

(e) FACS analysis 24 hours after transfection of 400 nM of fluorescein tagged dsRNA targeting eGFP into 293t-D12 cells using oligofectamine in accordance with the manufacturers guidelines. The purple population indicate 293t-D12 cells transfected with 400 nM of dsRNA targeting eGFP and no fluorescent tag. The green overlay represents 293t cells transfected with dsRNA with the fluorescein tag, and show a significant increase in mean fluorescent intensity (MFI).

4.2 Comparison of polymerase III promoters for RNAi

Short hairpin designs transcribed from a polymerase III promoter have been shown to induce functional RNAi (Brummelkamp et al., 2002b; Sui et al., 2002). These promoters have different cell type specificities and silencing requirements. As with all polymerase III promoters, they have motif dependent initiation of transcription. An example of this is the human U6 promoter which begins RNA transcription faithfully after the sequence "GACCG". The final guanine base pair is said to be the +1 base, and it is believed that altering this base pair abolishes RNA transcription. Polymerase II promoters generally lack such faithful initiation of transcription and because of the highly sequence dependent nature of RNAi were thought until recently to be unsuitable for RNAi (Zhou et al., 2005).

Several polymerase III promoters have been considered for use in RNAi. To determine the most functional promoter in human B cell lines and primary cells, I compared a human U6 promoter (cloned by PCR from BC-3 cells), a mouse U6 promoter (from the pSILENCER vector, Ambion) and the H1 promoter (from the pSUPER vector, gift from Bryan Cullen). The human U6 and murine U6 promoters have a similar size, around 300 base pairs, although the human H1 promoter is smaller, around 99 base pairs. The three promoters have little sequence homology. All three promoters were cloned by PCR into a pGEM backbone with a short hairpin based on the target sequence previously shown to be effective in reducing eGFP expression (Fig 4.1d). These vectors were called pGEM-hU6-sheGFP (**h**uman **U6** promoter, with **s**hort **h**airpin targeting **eGFP**), pGEM-mU6-sheGFP (**m**urine **U6** promoter) or pGEM-H1-sheGFP (**H1** promoter). A short hairpin targeting a section of LacZ was chosen as a negative control and cloned into each vector. The naming convention was used again for this hairpin, e.g. pGEM-hU6-shLacZ (**h**uman **U6** promoter, with **s**hort **h**airpin targeting **LacZ**) (Fig 4.2a).

After transfection into 293t-D12 and HeLa-D7 cells, all three pGEM vectors expressing a hairpin targeting eGFP induced a dose dependent silencing effect when compared to a LacZ hairpin. (Fig 4.2b).

A comparison of these vectors in a wider range of cell lines was performed. A low concentration of DNA (0.5 μg) was used to highlight small differences in promoter function. The results showed that the hU6 promoter was generally stronger than the mU6 promoter but was more limited in which cells it was functional in. The H1 promoter showed less cell type specificity but was broadly less efficient in terms of silencing efficiency (Fig 4.2c).

The human U6 promoter was chosen for further RNAi studies and for lentiviral short hairpin RNA expression, as it functions strongest in human cells such as BC-3 and primary endothelial cells.

Figure 4.2 Comparison of polymerase III promoters for RNAi

a

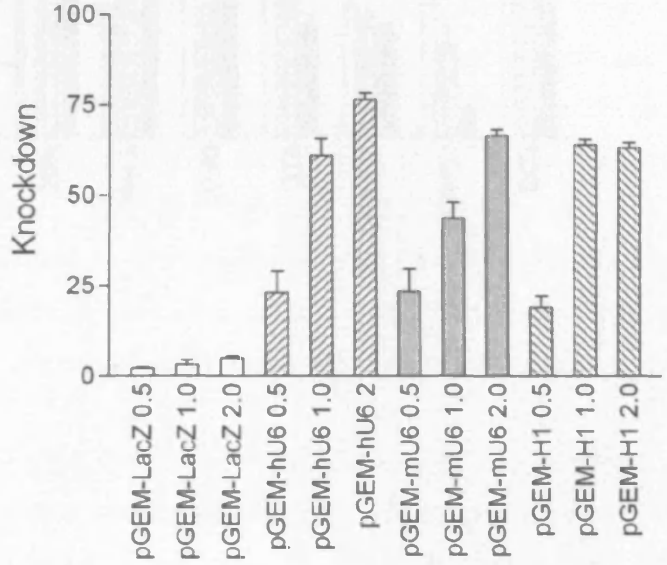
Anti - eGFP target sequence: GTTCATCTGCACCACCGGCAAGC

Anti - eGFP hairpin structure:

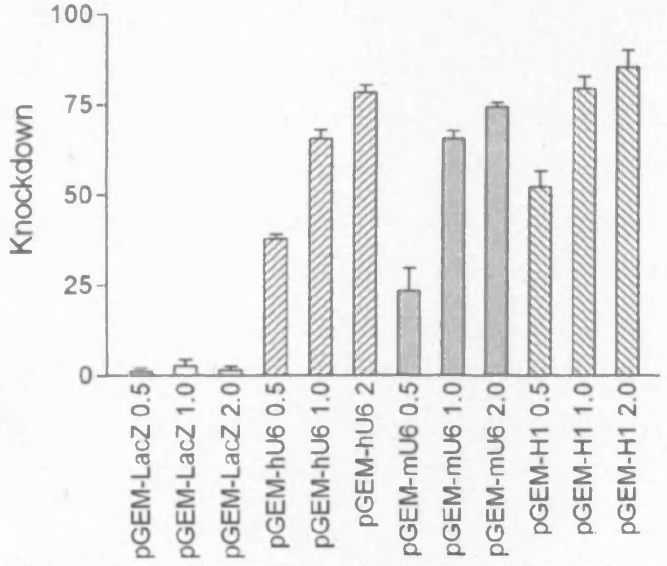
Anti - LacZ target sequence: GCTGTGATTGCGTCTGGGTTTGC

Anti - LacZ hairpin structure:

b



293t-D12



HeLa-D7

Figure 4.2 Comparison of polymerase III promoters for RNAi

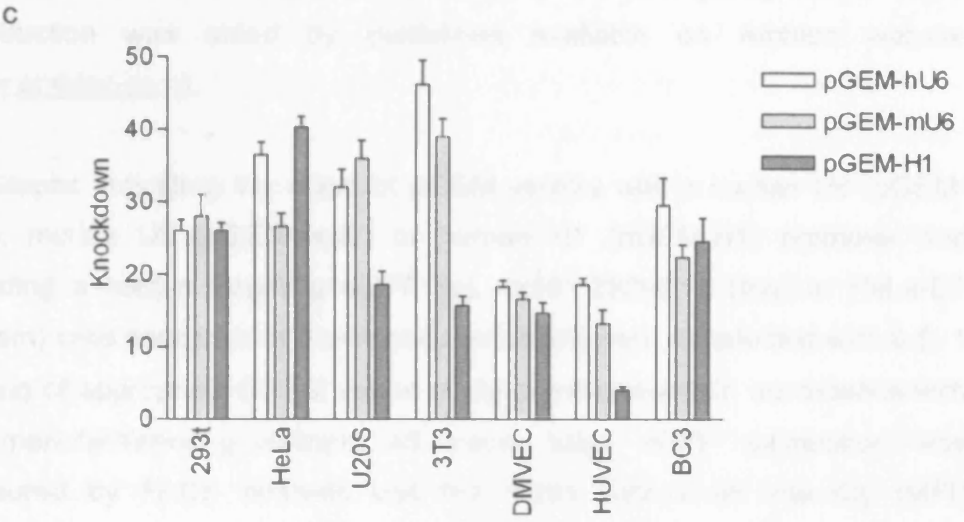


Figure 4.2 Comparison of polymerase III promoters for RNAi

(a) Top; target sequence against eGFP (see section 4.1) and hairpin structure targeting eGFP. Bottom; target and hairpin structure targeting LacZ. Hairpin construction was aided by guidelines available on Ambion website (www.ambion.com).

(b) Graphs indicating the effect of pGEM vectors with a human U6 (pGEM-hU6), murine U6 (pGEM-mU6) or human H1 (pGEM-H1) promoter and encoding a hairpin targeting eGFP (a). $1x10^5$ 293t-D12 (top) or HeLa-D7 (bottom) cells seeded in a 6 well plate overnight were transfected with 0.5, 1 or 2 μg of appropriate pGEM vector using oligofectamine in accordance with the manufacturers guidelines. 48 hours later, eGFP expression was measured by FACS analysis and the mean fluorescent intensity (MFI) assessed. Knockdown is expressed compared to infection with sh-LacZ (see materials and methods 2.4.7 for formula). Each experiment was performed in triplicate and the error bars indicate the standard error of the mean.

(c) Assessment of the function of the human U6 (pGEM-hU6), murine U6 (pGEM-mU6) and human H1 (pGEM-H1) promoters in a wider variety of cell types. 1 x 10^5 293t, HeLa, U20S, and 3T3 cells and 1 x 10^4 HMVEC and DMVEC cells were transfected with oligofectamine according to the manufacturers guidelines. 1 x 10^5 BC-3 Cells were transfected with FuGene according to the manufacturer's guidelines, as they were not transfectable with oligofectamine (Fig 4.1a). The DNA mixture included 0.5 μg of pd2-eGFP and 2 μg of pGEM vector and one control for each cell line transfected with 0.5 μg pd2-eGFP and 2 μg pGEM vector containing a hairpin targeting LacZ. FACS analysis was performed 48 hours after transfection and the knockdown is expressed compared to infection with shLacZ. Each experiment was performed in triplicate and the error bars indicate the standard error of the mean.

4.3 Requirements for effective silencing

Several factors were identified which might affect silencing efficiency. These include target sequence length, GC content, length and GC content of hairpin, potential secondary structure and specificity. This list was compiled from knowledge of the mechanism of RNAi and reports indicating that these factors may be important using dsRNA[1]. It was decided to investigate which of these, if any, were important in this system. For all experiments, the hairpins were transfected into HeLa-D7 cells and the knockdown effect observed as percent decrease in mean fluorescent intensity (MFI, see materials and methods section 2.4.7). A high knockdown therefore indicates a successful hairpin. All investigations were performed by synthesising oligonucleotides and creating pGEM-hU6 vectors containing hairpin structures (see materials and methods for cloning procedures). The eGFP hairpin (Fig 4.2a) was used as a template and variables investigated sequentially.

The size of the stem loop was determined to be largely irrelevant to silencing effect, although a 4-7 base pair length found to be optimum (Fig 4.3a). Four base pair stem loops were used for further studies as longer loops had little effect on silencing and cost was kept minimum by using shorter sequences. The GC content of the stem loop was also found to have little effect on silencing (Fig 4.3b). For further investigation, a 4 bp stem loop with a 50% GC content was used (TTCG).

The minimum length for silencing was investigated by progressively truncating or extending the sequence previously used. A target less than 19 base pairs long was found to be non-functional, whilst there was no significant silencing difference between targets 21 and 29 base pairs long (Fig 4.3c). Sequences longer than 29 base pairs have been reported to

[1] http://www.ambion.com/techlib/resources/RNAi/index.html

induce a non specific silencing effect (Grant et al., 1995) and so were not investigated.

Point mutations were introduced into the hairpins to determine the specificity for the target sequence. It is important for the use of RNAi for us to be sure that any knockdown effects seen are as a result of the knockdown of the protein targeted, and not as a result of the dsRNA. It was found that a 1 bp mutation at the terminal ends of the sequence inhibited but not abolished knockdown. A 1 bp mutation in the middle or a 2 bp mutation at the terminal ends abolished the knockdown effect (Fig 4.3d). It was concluded therefore that shRNA is sequence specific, with the chances of off target knockdown small.

A 21 or 23 base pair target with a 4 base pair stem loop with 50% GC content was therefore shown to be both economical and effective for silencing.

Figure 4.3 Requirements for effective silencing

a

Hairpin:	Knockdown:
3 bp - TTC	28.2 ± 3.1%
4 bp - TTCG	77.1 ± 2.1%
5 bp - TATCG	74.5 ± 1.9%
6 bp - TATCAG	76.4 ± 4.2%
7 bp - TATACAG	78.0 ± 0.3%
8 bp - TATACACG	71.9 ± 3.2%
9 bp - TGATACACG	70.5 ± 2.2%
10 bp - TGACTACACG	64.8 ± 3.2%
11 bp - TGACGTACACG	52.2 ± 4.2%
12 bp - TGACAGTACACG	22.0 ± 1.1%

b

GC content		Knockdown
25	AGAT	72.6 ± 4.8 %
25	TACT	71.1 ± 3.9%
50	TTCG	77.5 ± 7.2%
50	ACTG	75.3 ± 5.1%
75	CGAG	70.8 ± 2.2%
75	GACG	73.2 ± 6.7%
100	CGCG	75.6 ± 1.4%
100	GCCG	71.3 ± 4.2%

c

Target Sequence Length	Target Sequence	Knockdown
15	ATCTGCACCACCGGC	0 ±0
17	CATCTGCACCACCGGCA	8.3 ± 7.1 %
19	TCATCTGCACCACCGGCAA	58.7 ± 5.1 %
21	TTCATCTGCACCACCGGCAAG	75.4 ± 6.0 %
23	GTTCATCTGCACCACCGGCAAGC	77.9 ± 4.8 %
25	AGTTCATCTGCACCACCGGCAAGCT	71.3 ± 2.2 %
27	AAGTTCATCTGCACCACCGGCAAGCTG	73.8 ± 5.2 %
29	GAAGTTCATCTGCACCACCGGCAAGCTGC	78.6 ± 4.1 %

Figure 4.3 Requirements for effective silencing

d

Mutant Number	Mutation
1	GTTCAT[illegible]GCACCACCGGCAAGC
2	G[illegible]TCA[illegible]CT[illegible]CACCACCGGCAAGC
3	G[illegible]TCA[illegible]CTGCACCACCGGCAAGC
4	G[illegible]TCATCTGCACCACCGGCAAGC
Original Target	GTTCATCTGCACCACCGGCAAGC
5	GTTCATCTGCACCACCGGCAA[illegible]C
6	GTTCATCTGCACCACCG[illegible]CAA[illegible]C
7	GTTCATCTGCAC[illegible]ACCG[illegible]CAA[illegible]C
8	GTTCATCTGCACCAC[illegible]AGCAAGC
9	GTTCATCTGC[illegible][illegible]ACCGGCAAGC

Mutant Number	Knockdown
1	2 ± 0.9 %
2	1 ± 0.4 %
3	12 ± 1.2 %
4	71 ± 3.1 %
Original Target	74 ± 0.8 %
5	72 ± 2.1 %
6	9 ± 1.0 %
7	1 ± 0.1 %
8	3 ± 0.3 %
9	0.02 ± 0.02 %

Figure 4.3 Requirements for effective silencing

Tables showing the effect of target and hairpin variation on knockdown effect. pGEM-hU6 vectors targeting eGFP were created utilising the hairpin structure in Figure 4.2a as a template and the indicated component altered. All oligonucleotides were synthesised by Dharmacon and cloning performed as detailed in the materials and methods section. Throughout, knockdown is estimated by calculating the percent knockdown in MFI compared to a control hairpin (see materials and methods 2.4.7). MFI was determined by FACS analysis 48 hours after transfection. For transfection, 1 x 10^5 HeLa-D7 cells were transfected with 2 μg of pGEM-hU6 plasmid. Transfection was performed using oligofectamine in accordance with the manufacturer's guidelines. Figures indicate the mean value (1st number) and range (2nd number) of experiments performed in duplicate. Rows highlighted in red signify the most efficient silencing conditions for each variable.

(a) Table showing the effect of altering hairpin length on knockdown in HeLa-D7 cells. Hairpins of 3-12 base pairs in length were cloned into pGEM vectors (left hand column) and knockdown assessed (right hand column).

(b) Table showing the effect of hairpin GC content on knockdown. The GC content (left column) and sequence (middle column) of hairpins cloned into the pGEM-U6 vector were assessed for knockdown efficiency (right hand column)

(c) Table showing the effect of length of target sequence on knockdown. Target sequences 15-29 base pairs long were introduced as hairpins into pGEM-U6 (left hand column) and the knockdown assessed (right hand column).

(d) The effect of point mutations on silencing effect. Nine oligonucleotide sequences containing 1,2 or 3 single base pair mutations were introduced as hairpins into pGEM-hU6 vectors to determine the ability of these mutations to

abolish silencing effect (top). Characters highlighted in red show the base pairs which vary from that of original target. 48 hours after transfection of 2 μg of each mutant construct into 1 x 10^5 HeLa-D7 cells, the knockdown effect was measured (bottom).

4.4 Cloning U6 promoter-driven shRNA into a lentiviral vector

To create a lentiviral vector expressing short hairpin RNA, a suitable cloning process was investigated. The only restriction site available for use to enable cloning of oligos into pGEM-hU6 is Sal I. A single mutation in the +1 base pair would allow the use of Sma I (Fig 4.4a). A mutation in the +1 base pair is believed to inhibit the efficiency of the U6 promoter (Alberts et al., 1994). Sma I would be a much more convenient enzyme to use for cloning oligos, so to test the extent of the inhibition, a point mutation was introduced into pGEM-hU6 to create pGEM-hU6M. This vector did show a slight reduction of silencing effect due to alteration of the +1 base pair, but this could be overcome by increasing the transfected quantity of vector (Fig 4.4b). This implies the decrease in efficacy is due to the U6 promoter function rather than alteration of the RNAi machinery.

Unfortunately both Sal I and Sma I are present within the pSIN backbone, so creation of a one-step cloning vector was prevented. pGEM-hU6M was used as a shuttle vector for future cloning since the Sma I site meant that cloning could be performed more easily. EcoRI sites in the pGEM-hU6 vector allowed easy removal of the promoter-hairpin sequence for insertion into the lentiviral vector (see materials and methods section 2.4.6).

The optimal location within the pSIN backbone of the U6 promoter (without the +1bp mutation) was assessed by the creation of 6 lentiviral vectors using the same short hairpin targeting deGFP (Fig 4.4c). The promoter was found to be most functional when present near the 5' LTR. The knockdown effect was also found to be orientation independent (Fig 4.4d).

Lentiviral vectors based on pGEM-hU6 and pGEM-hU6M and encoding a hairpin against eGFP or LacZ were created. These vectors were called pSIN-U6-sheGFP or pSIN-U6M-shLacZ, and were tested in HeLa-D7 cells. hU6 vectors proved to be slightly more efficient than hU6M vectors in terms of efficiency, however the saturation of the silencing effect was equal (Fig 4.4e).

Since the knockdown level which could be achieved using the U6M promoter was so similar to that using hU6, and the cloning procedure involving hU6 was considerably less efficient (see materials and methods), it was decided that U6M vectors would be used for further RNAi studies. The U6 promoter would be cloned into the 5' position between the cPPT and the SFFV promoter. The orientation would be checked and documented in case further studies showed this to be relevant, although it was assumed at this stage not to affect silencing. The vector names were simplified to pSIN-sh(target). These vectors also express a puromycin resistance gene driven by the SFFV promoter (Fig 4.4c).

Figure 4.4 Cloning U6 promoter driven shRNA into a lentiviral vector

a

U6 Sequence:

TGTACGATAGAAAGNTAATAATTGNCGTNNGAGGTTAGTTNNGCAGNTTATANANAAAN
TTATGTTATNNANAGANATGGACTATCTATATGCTTACCGTAACATTGNAAGTATATCGA
TTGCGTTGGCTTTATATATCTGTGATGGAAAGGACGAAACACCGTCGACGCCCTTCTC
TAGATTTTTTATTG

Sal I – GCCCTTC – Xba I

U6 promoter AAACACC - +1 bp G

Proposed U6M Sequence:

TGTACGATAGAAAGNTAATAATTGNCGTNNGAGGTTAGTTNNGCAGNTTATANANAAAN
TTATGTTATNNANAGANATGGACTATCTATATGCTTACCGTAACATTGNAAGTATATCGA
TTGCGTTGGCTTTATATATCTGTGATGGAAAGGACGAAACACCCGGGGCTTCTCTAGA
TTTTTTATTG

Sma I – GCTTC – Xba I

U6 promoter AAACACC - + 1 bp C

b

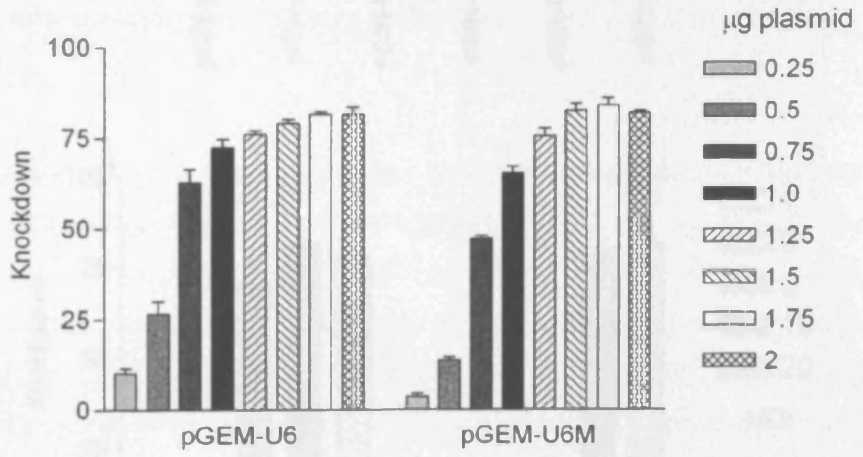
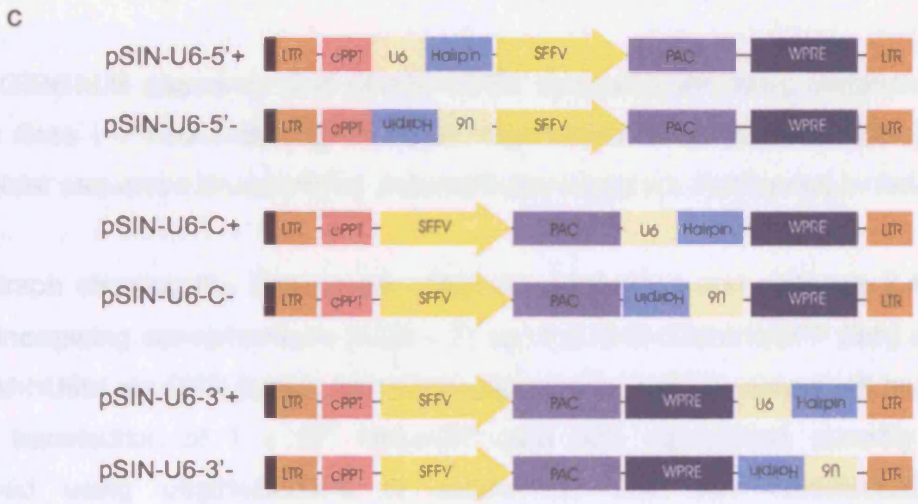
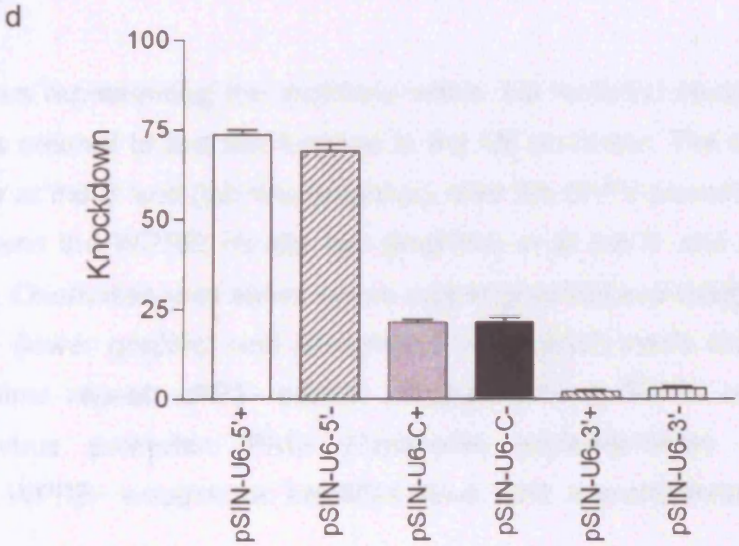


Figure 4.4 Cloning U6 promoter driven shRNA into a lentiviral vector





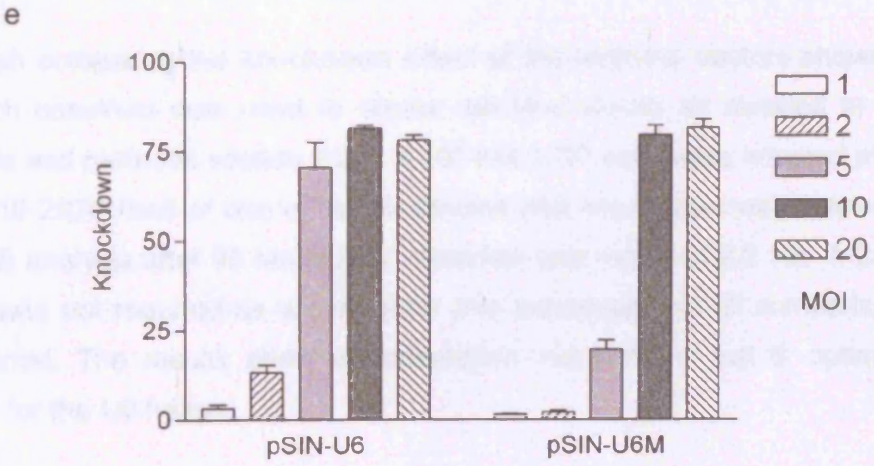


Figure 4.4 Cloning the U6 promoter into the lentiviral backbone

(a) pGEM-hU6 sequence and pGEM-hU6M sequence including mutation to allow Sma I – Xba I cloning to insert oligonucleotide sequences. The U6 promoter sequence is underlined and restriction sites are highlighted in red.

(b) Graph showing the knockdown effect (see materials and methods 2.4.7) with increasing concentrations (0.25 – 2) μg of pGEM-hU6-sheGFP (left) and pGEM-hU6M-sheGFP (right). MFI was assessed by FACS analysis 48 hours after transfection of 1 x 10^5 HeLa-D7 cells with appropriate quantity of plasmid using oligofectamine in accordance with the manufacturer's guidelines.

(c) Diagram representing the locations within the lentiviral backbone of the constructs created to test the function of the U6 promoter. The U6 promoter was either at the 5' end (top two graphics), after the SFFV promoter (between the PAC and the WPRE; middle two graphics) or at the 3' end (bottom two graphics). Orientation was either sense (upper graphic) and designated '+' or antisense (lower graphic) and designated '-' for each insert location. LTR- long terminal repeat; cPPT- central polypurine tract; SFFV- spleen focus forming virus promoter; PAC- Puromycin acyltransferase (resistance) cassette; WPRE- woodchuck hepatitis virus post transcriptional regulatory element.

(d) Graph comparing the knockdown effect of the lentiviral vectors shown in (c). Each construct was used to create lentiviral stocks as detailed in the materials and methods section 2.3. 1 x 10^5 HeLa-D7 cells were infected at an MOI of 10 293tIU/cell of one of the six viruses and knockdown was assessed by FACS analysis after 96 hours (see materials and methods 2.2.15). A LacZ hairpin was not required as a control for this experiment as all comparisons are internal. The results show an orientation independent but 5' optimum location for the U6 hairpin.

(e) Graph to determine the MOI required for an efficient knockdown. Lentiviral stocks of pSIN-U6-sheGFP and pSIN-U6M-sheGFP were created as detailed in the materials and methods section 2.3. These vectors contained the U6 hairpin in the 5' location in the sense orientation. 1 x 10^5 HeLa-D7 cells were infected with the virus at increasing MOIs from 1 to 20 293tIU/cell (see legend) and knockdown was assessed by FACS analysis 96 hours after infection. Knockdown was determined as outlined in the materials and methods section 2.2.15. A LacZ hairpin was not required as a control for this experiment as all comparisons are internal.

4.5 Evaluation of lentiviral RNA interference in cell lines

Knockdown of gene expression was first confirmed in 293t-D12 cells. The MOI and timecourse required for an effective knockdown was established (Fig 4.5a). These data indicate that an MOI of 5 will reach optimum knockdown within 72 hours, and an MOI of 20 will reach optimum knockdown in 48 hours.

To show that the knockdown effect was related to the number of copies of the lentiviral inhibitor, 24 single cell clones were established from 293t-D12 cells infected with the lentiviral inhibitor at an MOI of 1. These populations had knockdowns of between 0 and 70%. It was found that there was a general correlation between the copy number of the lentiviral vector by qPCR and the efficiency of the silencing effect, although the relationship was not absolute (Fig 4.5b). This implies, as expected, that not all integrations of the lentiviral vector express equally, and that there may be several 'silent' integrations which do not express at all.

The expression of the hairpin caused a strong downregulation in eGFP at the mRNA level within 48 hours (Fig 4.5c, left). To ensure that this effect was not unique to 293t-D12 cells, the experiment was repeated in Hela-D7 and 293t-D1 cells (Fig 4.5c, right). Finally, to confirm in 293t-D12 cells that this knockdown resulted in decrease at the protein level, Western blots for eGFP showed that the protein level decreased after 48 hours, with maximum knockdown after 72 and 96 hours. This correlates with the 6 hour half life of destabilized eGFP(Fig 4.5d).

Since dsRNA did not function in 293t cells, it was theorised that the lentiviral vector may not be specifically targeting the eGFP mRNA. To confirm the RNAi was working in a sequence specific manner in 293t cells, the effect of silencing on bicistronic and coinfected monocistronic genes was established.

293t cells were infected with pSIN-RIG+ (construct expressing both dsRed and eGFP, see Chapter 3.7) or pSIN-dsRed and pSIN-deGFP and then

infected 72 hours later with a short hairpin targeting eGFP or dsRed. In cells infected with the bicistronic vector, the shRNA both caused a strong downregulation of both genes. For the dual infection the protein knockdown was only the targeted gene (Fig 4.5e).

These data indicate that a target sequence leads to the entire mRNA strand being degraded. This result is both consistent with and adds experimental evidence to a sequence specific mRNA knockdown effect with RNAi but the evidence of 293t-D12 cells being resistant to dsRNA raises questions regarding the processing of shRNA to dsRNA .

Figure 4.5 Evaluation of lentiviral RNA interference in cell lines

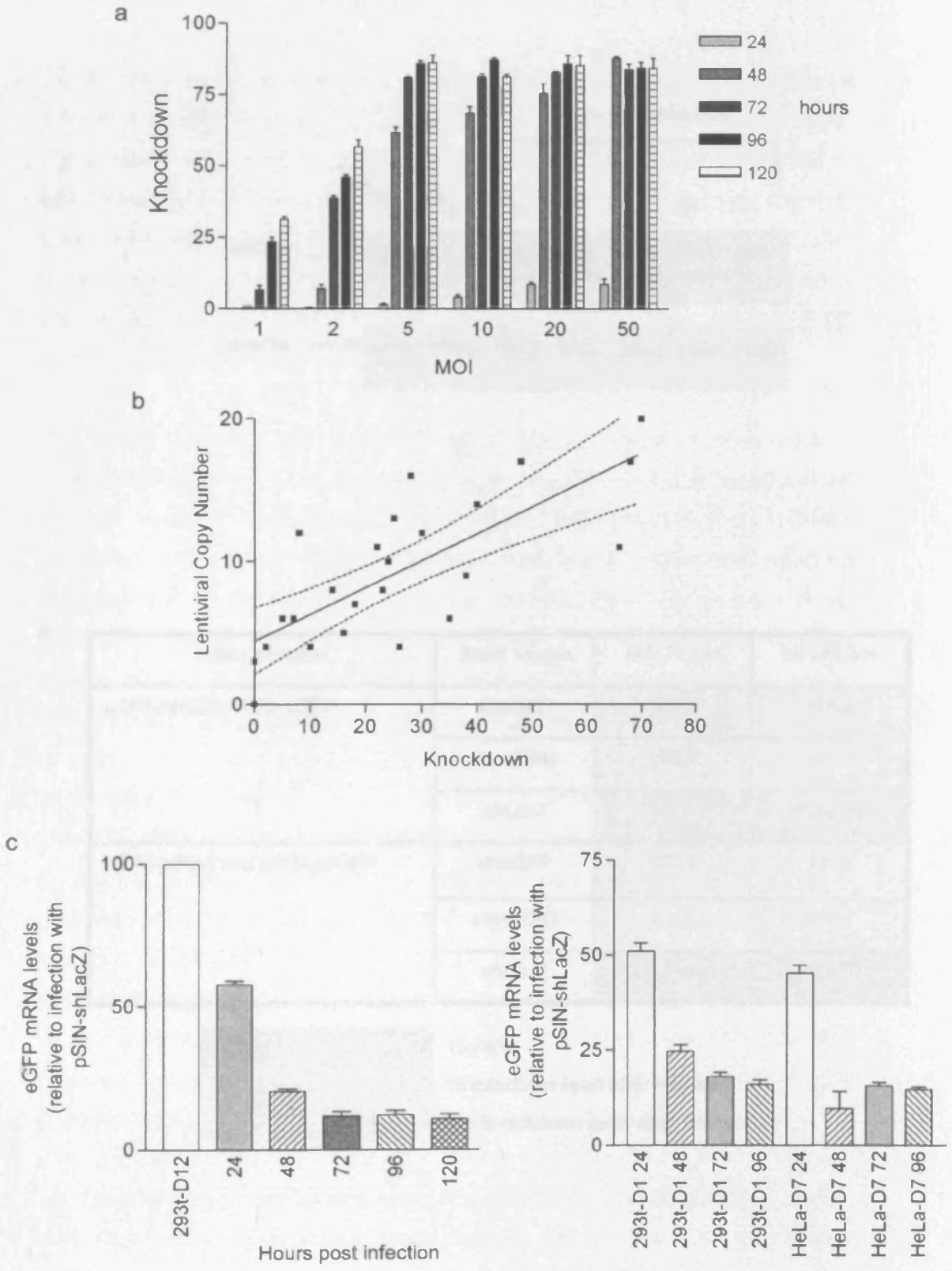
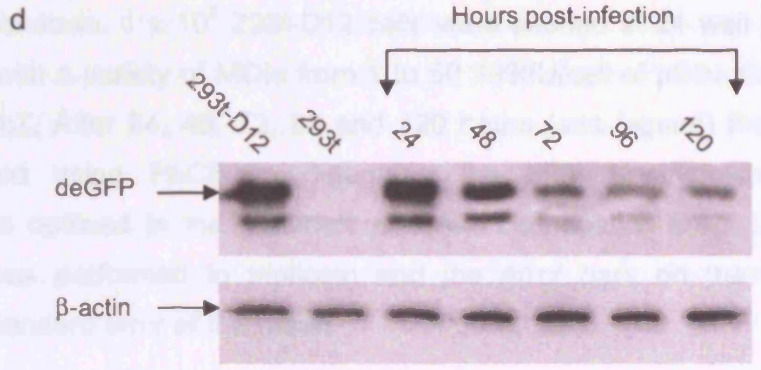


Figure 4.5 Evaluation of lentiviral RNA interference in cell lines

d



e

Initial infection	Short hairpin	MFI FL1-H	MFI FL2-H
pSIN-dsRED-IRES-eGFP	sheGFP	322.1	215.3
	shdsRED	299.2	168.3
	shLacZ	1306	963.5
pSIN-dsRed and pSIN-deGFP	sheGFP	352.1	1716
	shdsRED	1318	381.5
	shLacZ	1410	1972

Control

Knockdown seen after infection

No knockdown seen after infection

Figure 4.5 Evaluation of lentiviral RNA interference in cell lines

(a) Graph showing investigation of the MOI and time required for lentiviral mediated knockdown. 1 x 10^5 293t-D12 cells were seeded in 24 well plates and infected with a variety of MOIs from 1 to 50 293tIU/cell of pSIN-sheGFP or pSIN-shLacZ. After 24, 48, 72, 96 and 120 hours (see legend) the cells were analysed using FACS to determine the MFI. Knockdown was determined as outlined in the materials and methods section 2.4.7 . Each experiment was performed in triplicate and the error bars on the graph indicate the standard error of the mean.

(b) Graph showing the lentiviral copy number (x-axis) compared to knockdown (y- axis) of 24 single cell clones (see section 4.1 or materials and methods section 2.2.16) from 1 x 10^5 293t-D12 cells infected at an MOI of 5 293tIU/cell with pSIN-sheGFP. Knockdown was determined as outlined in the materials and methods section 2.4.7. The lentiviral copy number per cell has been corrected for the presence of pSIN-deGFP by reducing the apparent copy number by 2 (Figure 4.1c). The solid line is the best fit linear regression and the dotted lines represent the 95% confidence intervals for that line.

(c) Graph to show mRNA levels for deGFP assessed by TaqMan qRT-PCR at intervals after infection. 1 x 10^5 293t-D12 cells were infected at an MOI of 5 293tIU/cell with pSIN-sheGFP or pSIN-shLacZ and samples taken for analysis at 24, 48, 72, 96 and 120 hours (left). TaqMan qPCR of experiment repeated with 293t-D1 and HeLa-D7 cells analysed at 24, 48, 72 or 96 hours post infection (right). Results expressed as percent of mRNA relative to LacZ hairpin control. Each analysis was performed in triplicate and the error bars indicate the standard error of the mean. Primers sequences and reaction conditions can be found in the materials and methods section.

(d) Western blot of samples taken for (c). Samples were lysed and protein content equalised by Bradford assay. Results indicate the gradual reduction in eGFP protein levels consistent with the decrease in MFI (a).

(e) Table showing knockdown of bicistronic and monocistronic genes in 293t cells. 293t cells were infected with an MOI of 10 293tIU/cell of pSIN-dsRed-IRES-eGFP or of pSIN-deGFP and pSIN-dsRed. After 72 hours cells were then infected with an MOI of 10 293tIU/cell of pSIN-shLacz, pSIN-sheGFP or pSIN-shdsRed. FACS analysis was performed 96 hours after infection and the mean fluorescence intensity (MFI) on FL1-H (green) and FL2-H (red) determined. Blue shading indicates control experiments, red shading indicates a detectable knockdown, and green shading indicates no significant knockdown.

4.6 Functional inhibition of gene expression in primary and stem cells

The ability of RNAi to knockdown genes in primary cells which are refractory to transfection with dsRNA was assessed. DMVEC and MSCs have relevance to my thesis as models of KSHV infection, and so they were used as examples of primary cells. There have also been limited reports of the function of RNAi in these cells because they are not amenable to transfection.

Because of the limited life span of these cells, it was not possible to clone a DMVEC or MSC line expressing eGFP by limiting dilution. A reduction in average mean fluorescence intensity of the cells after infection with pSIN-deGFP was therefore used as a measure of the efficacy of RNAi. Cells were infected with pSIN-deGFP at an MOI of 10 293tIU/cell and then with either pSIN-sheGFP or pSIN-shLacZ 72 hours later. It was found that RNAi functions well in both DMVECs and MSCs (Fig 4.6a).

One application of RNAi is to produce knockout effects in stem cells both for *in vitro* and *in vivo* applications, however for this to be effective the U6 promoter needs to be resistant to silencing. Many promoters and retroviral delivery systems experience inactivation (silencing) when stem cells differentiate (see Chapter 1.1 and Chapter 3).

The effects of silencing in MSC through differentiation was assessed. MSCs were infected with pSIN-deGFP and then pSIN-U6M-sheGFP or pSIN-U6M-shLacZ and placed in medium stimulating differentiation. The MSCs differentiated completely into both osteocytic and adipocytic lineages (Fig 4.6b). These cells were therefore unaffected by the double infection and demonstrated stable reduction of eGFP expression throughout differentiation.

Vectors expressing lentiviral shRNA were used in hES cells in the laboratory and also demonstrated effective knockdown through differentiation into all three germ cell layers (Clements et al., 2006).

The ability of these vectors to knockdown an endogenous gene rather than an artificially introduced one has not been shown. There is no reason to believe RNAi would behave differently for endogenous genes.

To show that these vectors are capable of both knocking down an endogenous gene and introducing a foreign gene throughout differentiation, it was decided to knockdown survivin expression and introduce eGFP, which is easier to detect and quantify than puromycin resistance. Survivin is an endogenous anti-apoptotic protein present in most cells and is not involved in differentiation (Caldas et al., 2005).

A vector based on pSIN-U6M which encoded a short hairpin targeting survivin and expressing eGFP instead of puromycin resistance was constructed - pSINeGFP-shsurvivin (Fig 4.6c). As a control, a vector expressing a short hairpin to LacZ- pSINeGFP-shLacZ- was also created. MSC were infected with these vectors, and then cultured in conditions designed to promote differentiation. The differentiated cells were checked to ensure appropriate expression of Oil Red O or alkaline phosphatase to confirm their adipocytic or osteocytic lineage (Fig 4.6d). A Western blot of the differentiated cells indicated that survivin levels were still knocked down (Fig 4.6e).

The results indicate that endogenous and exogenous genes are equally amenable to knockdown in these cells, both foreign gene expression and knockdown are stable throughout differentiation and that this does not affect the capability of these cells to differentiate.

Figure 4.6 Functional inhibition of gene expression in primary and stem cells

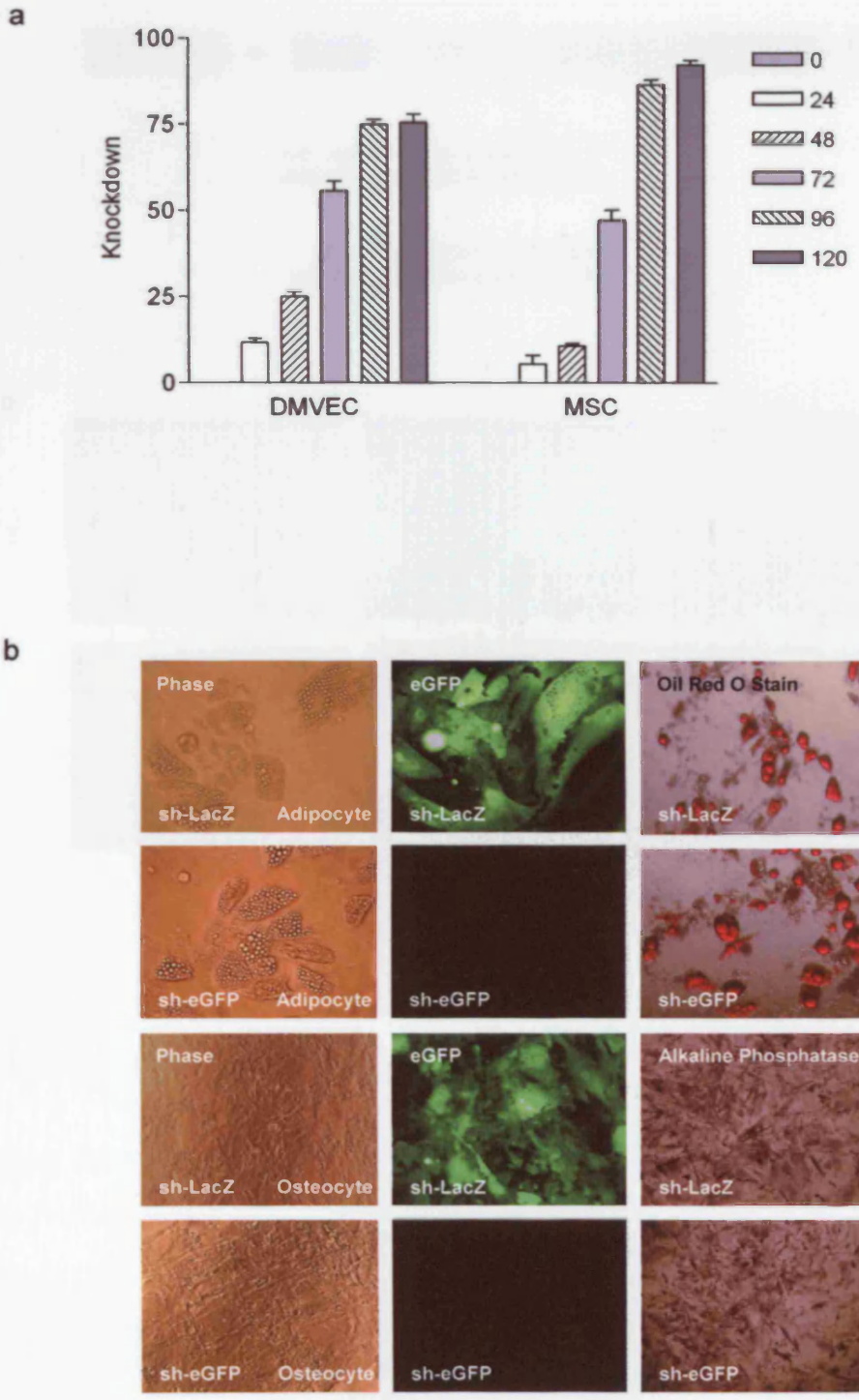


Figure 4.6 Functional inhibition of gene expression in primary and stem cells

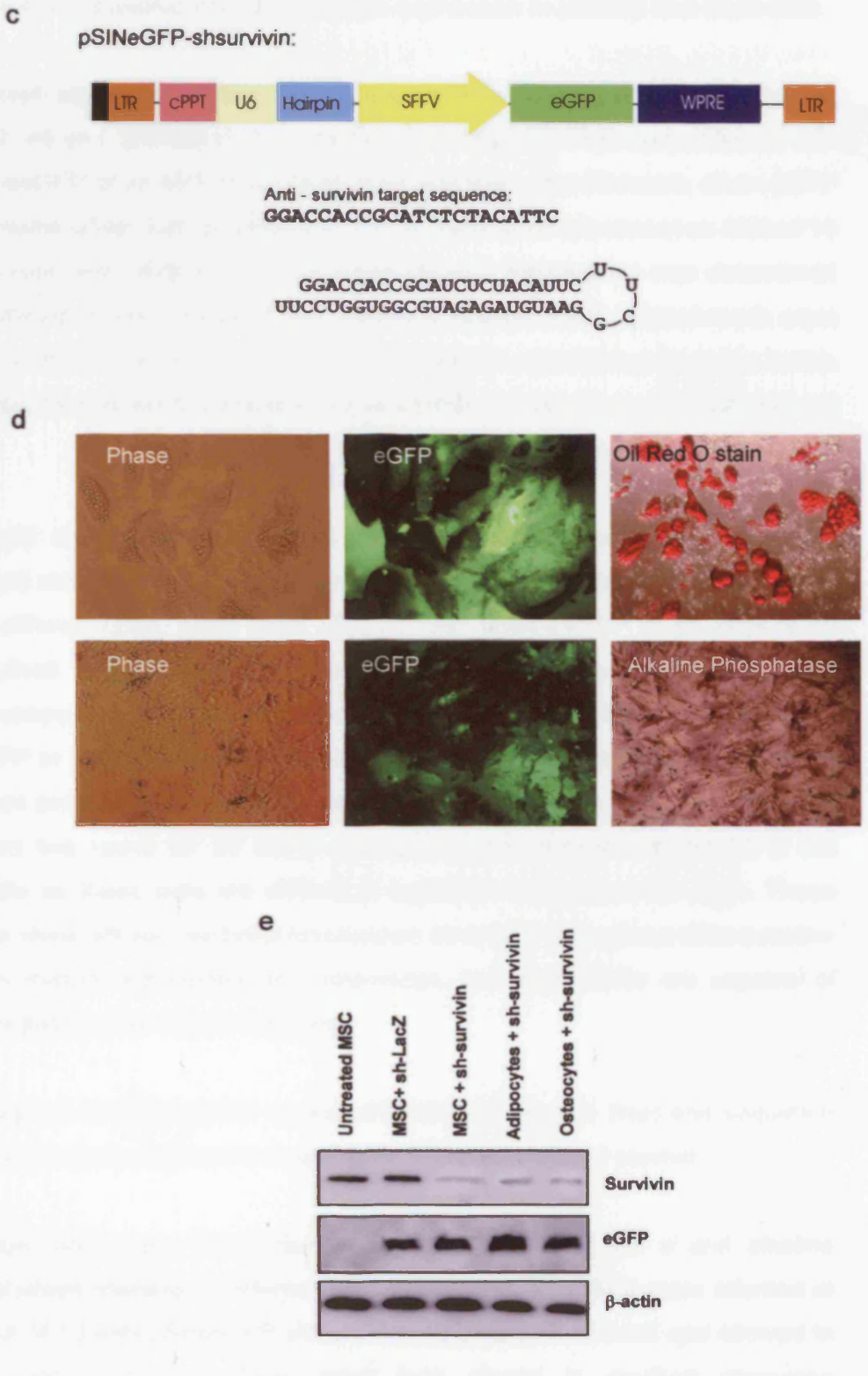


Figure 4.6 Functional inhibition of gene expression in primary and stem cells

(a) Graph showing functional knockdown in DMVEC (left) and MSC (right) 24, 48, 72, 96 and 120 hours after infection (see legend). Cells were infected with pSIN-deGFP at an MOI of 10 293tIU/cell and then after 72 hours, when eGFP was visible under light microscopy, 1 x 10^4 cells were infected at an MOI of 10 293tIU/cell with pSIN-sheGFP or pSIN-shLacZ. Knockdown was determined as outlined in the materials and methods section 2.4.7. Experiments were performed in triplicate and error bars indicate the standard error of the mean. The results indicate functional lentiviral shRNA in both primary endothelial cell types.

(b) Light and green fluorescence microscopy and oil red o (for adipocytic lineage) and alkaline phosphatase (for osteocytic lineage) staining of MSCs after differentiation. Cells were infected with pSIN-deGFP at an MOI of 10 293tIU/cell and then after 72 hours, when eGFP was visible under light microscopy, 1 x 10^4 cells was infected at an MOI of 10 293tIU/cell with pSIN-sheGFP or pSIN-shLacZ. These cells were grown to confluence and placed in medium promoting differentiation into adipocytes (top two rows) or osteocytes (bottom two rows) for 28 days. Determining knockdown with FACS is not possible as these cells are difficult to trypsinise away from the plate. These results show efficient lentiviral knockdown of deGFP throughout differentiation and in mature adipocytes and osteocytes, and that MSCs are capable of differentiating after double infection.

(c) Diagram of the lentiviral vector pSINeGFP-shsurvivin (top) and sequence and hairpin (bottom) used to knockdown the expression of survivin.

(d) Light and green fluorescence microscopy and oil red o and alkaline phosphatase staining of differentiated MSCs. 1 x 10^4 MSCs were infected at an MOI of 10 with pSINeGFP-shsurvivin or pSINeGFP-shLacZ and allowed to grow until confluence. They were then placed in medium promoting differentiation into adipocytes or osteocytes and after 28 days were examined

under light (left column) and fluorescence microscopy (middle column) and stained for oil red o (indicating adipocytic lineage) or alkaline phosphatase (indicating osteocytic lineage) (right column).

(e) Western blot of cells created in (d). Cells were placed in RIPA buffer in tissue culture plates for 5 minutes and then physically removed. Samples were equalised for protein concentration using the Bradford assay and SDS-PAGE performed using anti-survivin, anti-eGFP and anti-βactin antibodies. The results indicate that survivin inhibition and eGFP expression is stable throughout differentiation.

4.7 Discussion and conclusions

In this chapter I confirm several published reports that lentiviral vectors are capable of delivering short hairpin RNA interference to cell lines and primary cells (Brummelkamp et al., 2002a; Abbas-Terki et al., 2002), and show for the first time that this effect can be delivered to MSC.

The experiments performed in this chapter provided data which, at the time, was unknown. Lentiviral shRNA was functional in our laboratory at around the time the first reports were published (Abbas-Terki et al., 2002) and the data available regarding optimum knockdown conditions and requirements was limited. This knowledge was however essential for us to knockdown genes reliably using this system. Literature reviews and systematic studies are now available which go into far more detail and provide valuable information for those involved in RNAi research (Overhoff et al., 2005).

Much new information regarding the mechanism of RNAi is also now known. Components of the RNA-induced silencing complex have been identified and characterised (Sontheimer, 2005). Interestingly, Argonaute 2, a component strongly implicated in RISC formation, is localised to mRNA decay centres known as cytoplasmic bodies (Sen and Blau, 2005). Studies of RNAi in *c.elegans* have identified over 90 genes which have a role in dsRNA mediated silencing (Kim et al., 2005). This further information does not currently offer any explanation for why 293t-D12 cells should be unable to reduce deGFP expression after transfection with dsRNA, but are able to do so with shRNA.

shRNA also allows stable silencing of gene expression. This ability increases the potential of the technology to include new approaches for investigation. Several reports of stability of expression with lentiviral vectors are confirmed here (Naldini et al., 1996a) including their stability during differentiation (Karlsson et al., 2002; Leurs et al., 2003). This has application to fields as far ranging as cell sources of metastatic disease to tissue engineering. Stable

RNAi throughout differentiation is already yielding data regarding development in animal embryos (Chesnutt and Niswander, 2004).

Stable shRNA is dependent upon the choice of promoter. My investigation of promoter function (Figure 4.2 and Figure 4.4) shows that polymerase III promoters seem to function very broadly. This contrasts to their polymerase II counterparts which do not normally function so broadly across suspension, adherent and stem cells. This may in part be to their fairly conserved sequences and their essential roles within RNA production. Recent use of polymerase II promoters for RNAi (Zhou et al., 2005) and targeting polymerase II promoters specifically to certain tissues (De Palma et al., 2003) raises interesting possibilities for targeting this technology. Reports of inducible systems based around Cre-Lox (Ventura et al., 2004) or tetracycline (Lin et al., 2004) regulated U6 promoters show the true versatility of shRNA based systems.

My findings with regards to the +1 base pair of the hU6 promoter when attempting to create a vector more suitable for cloning are surprising. I was expecting the U6 promoter to have stricter initiation requirements. Since the polymerase III promoters function well in many cell lines, I would liked to have investigated the use of the other promoters and more variants of the U6 promoter to create a more efficient and convenient system for general use. It was disappointing to find that RNA interference using these promoters cannot completely abolish expression of a protein. The most efficient knockdown I established reduced expression levels to around 10% of the levels in the control experiment. This concurs with current literature regarding RNAi, and is believed to be a result of the mechanism of RNAi. The dependence of RNAi on cellular factors which may not be abundant enough for a complete knockdown and the fundamental process of degrading an existing mRNA strand which may already have been translated means the knockdown cannot be 100%. Some studies may require complete silencing of gene expression, and as is shown in Chapter 5, low levels of transgene expression can cloud experimental results. My findings that with the lentiviral vector the

U6 promoter functions well when placed near to the 5' LTR but not elsewhere cannot be readily explained. My findings of orientation independence agree with published reports (Abbas-Terki et al., 2002).

A reflection on the novelty and variability of systems for RNA interference is shown here. This system proved less versatile for shorter and longer sequences than some reported vectors (Brummelkamp et al., 2002a), whilst is equally efficient when optimised. The ability of the vector to function with virtually any length of stem loop, and with no significant GC requirement was surprising but not contrary to published reports.

One important factor for the application of RNAi is the confirmation that only a single base pair mutation is required to diminish or abolish knockdown effect. It was perhaps predictable that mutations in the end of a 23 base pair target would diminish but not abolish effect given the results indicating a 19bp sequence is a requirement. The implications of this for RNAi based therapies has already been seen with the ability of rapidly mutating viruses such as HIV-1 to escape the effects of even multiple target RNA interference (Das et al., 2004). Related to this issue is off-target cellular effects of RNAi. It is possible that RNAi induces changes in non-targeted cellular proteins (Scacheri et al., 2004). Some groups have implicated the interferon response in this (Kim et al., 2004) or the effect of transitive RNAi. Transitive RNAi involves the extension of the target sequence intracellularly through an enzyme RdRP (RNA dependent RNA polymerase) leading to short fragments of off target dsRNA in the cell. There is evidence that this cannot occur in human cells, as they lack the appropriate RdRP (Chi et al., 2003). There is evidence also that no interferon response is invoked by RNAi (Heidel et al., 2004). There is no good conclusion regarding the specificity or off target activity of RNAi. Microarray technology has yielded high profile publications with data showing both no and significant off target gene activity (Chi et al., 2003; Jackson et al., 2003).

One component of RNA interference which was too large to investigate within the scope of this thesis was the exact sequence requirements for targeting. Most major dsRNA synthesis companies now offer on-line utilities for sequence selection (www.dharmacon.com, www.ambion.com/techlib, http://www1.qiagen.com/Products/GeneSilencing/). The overall 'hit' rate for successful knockdown with this lentiviral system was around 50%. It would be interesting to know why some sequences silence much better than others, and more specifically when attempting to knockdown gene expression which targets will be effective.

This chapter describes the use of stable RNA interference knocking down expression of exogenous and endogenous gene expression in MSC and contributes to the study of RNAi in hES (Clements et al., 2006). A vector such as the one demonstrated here which can express and inhibit gene expression may be important for fields such as tissue engineering, where guiding the fate of these stem cells may require more complex manipulation of cellular pathways than offered using other expression systems. The demonstration that these vectors can function without affecting the ability of these cells to differentiate is important. It may be that retro- and lenti- viral vectors prove too dangerous to be ever used in a clinical setting but these findings regarding the polymerase III promoter apply to the use of other viral vectors, such as AAV, which have more defined integration patterns and may offer more of a hope in a clinical setting.

Overall, this chapter demonstrates stable RNA interference across a range of cell lines, primary and stem cells and investigates some of the requirements for optimum silencing whilst highlighting the novelty of this technology and the contradictions present across much of the literature. I show that human stem cells behave normally when transduced, and discuss the implications for this in both *in vitro* research and *in vivo* research.

Chapter 5:

In vitro knockdown of KSHV latent genes

Chapter Aims:

The aim of this chapter was to utilise the lentiviral RNAi system (chapter 4) to knockdown genes within the KSHV oncogenic cluster. In order to achieve this, effective targets for each of the latent genes were isolated. The effects of the knockdown on latent models of KSHV infection (see Chapter 1.4) were determined in order to identify targets which might be suitable for potential therapeutics.

5.1 Knockdown of the genes in the oncogenic cluster in 293t cells

In order to investigate further the potential of lentiviral shRNA (Chapter 4), KSHV was chosen as a target. The ultimate goal of these experiments was to investigate targets for potential therapeutics. As discussed in Chapter 1.4, the oncogenic cluster (*LANA*, *vFLIP* and *vcyclin*) are genes which are expressed in all latent KSHV infections. These genes offer appealing targets for knockdown, since they are known to dysregulate cell cycle components and have key roles in the latent life cycle (Chapter 1.4; Guasparri et al., 2004; Fujimuro and Hayward, 2003b). The knockdown of genes within the oncogenic cluster was therefore predicted to have adverse effects in PEL cells, which are models for the KSHV latent life cycle. We theorised that efficient knockdown of vFLIP and vcyclin would result in cell death, and that knockdown of LANA might have several effects. We expected to see the KSHV genome expelled from the cell as LANA was no longer present to tether the episome. We predict also that the reduction of inhibition of Orf-50/ Rta would cause activation of the lytic life cycle. The removal of LANA may also disrupt the p53/ pRb pathways and therefore result in cell death. To investigate the potential for a therapeutic involving knockdown of these latent genes, an *in vitro* knockdown was firstly demonstrated.

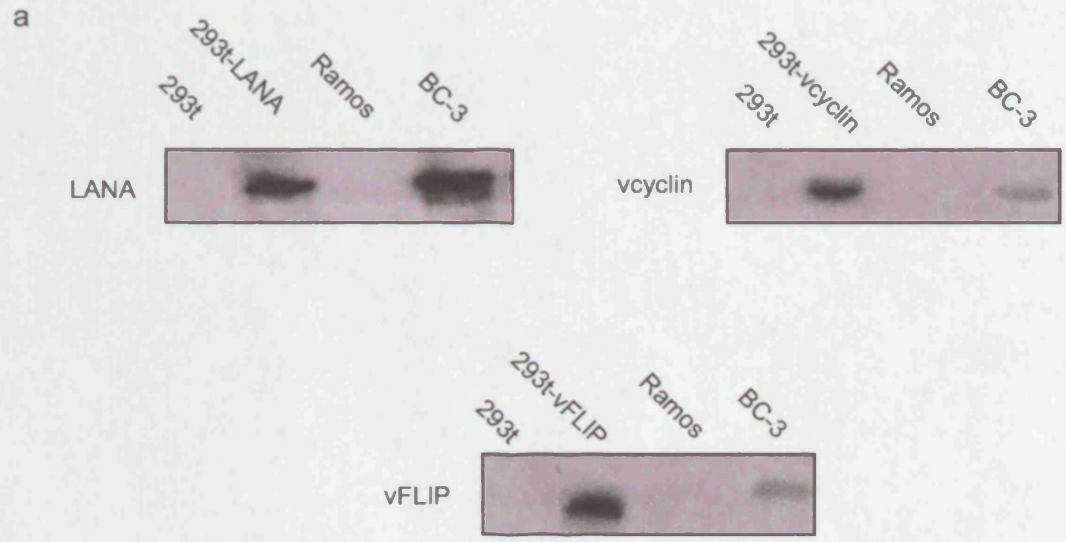
Since we predicted adverse effects in PEL cells from latent gene knockdown, it was thought that this might affect our ability to assess the knockdown effect accurately. 293t cell lines expressing one of the three genes in the oncogenic cluster were therefore created. These cells lines, 293t-LANA, 293t-vcyclin and 293t-vFLIP were created using lentiviral vectors (pHR-CMV-LANA, created by Dan Hollyman; pHR-CMV-vFLIP-IRES-eGFP, created by Nigel Fields; pHR-CMV-vcyclin, see materials and methods). These cell lines continued to grow in culture and expressed the introduced genes 4 weeks after infection (Fig 5.1a). BC-3 cells are PEL cells expressing all 3 KSHV genes from the oncogenic cluster and were used as a positive control. Ramos cells are a KSHV negative B cell line used as a negative control throughout these experiments.

LANA was chosen first as there are published dsRNA sequences targeting *LANA* which are effective (Fujimuro and Hayward, 2003). Two targets within *LANA* were chosen, one at the N terminus and one at the C terminus (Fig 5.1b). Short hairpins were designed according to the criteria found to be optimum in Chapter 4, and these hairpins were subcloned into lentiviral vectors expressing puromycin resistance as well as the short hairpin (Fig 5.1b). The targets were assessed in terms of efficiency of knockdown in 293t-LANA cells. An MOI of 10 293tIU/cell was chosen based on previous experiments indicating that this MOI is sufficient and effective to produce a knockdown. Only one target (LANA-n) was found to be effective. Knockdown with a lentiviral vector was found to be dose dependent (Fig 5.1c), although because of the relatively long half life of LANA the knockdown took almost two weeks to reach maximum effect (Fig 5.1d).

To assess the efficacy of *vFLIP* and *vcyclin* knockdown, 2 short hairpins targeting each of the genes were chosen based on criteria from the Ambion (www.ambion.com) website and cloned into lentiviral vectors expressing the short hairpin and a puromycin resistance cassette (Fig 5.1b). It was found that all four targets induced some degree of silencing of the target gene (Fig 5.1e).

The N terminus of all three latent genes were chosen for further studies based on silencing efficacy. These constructs were named pSIN-shLANA, pSIN-shvcyclin and pSIN-shvFLIP.

Figure 5.1 Knockdown of the genes in the oncogenic cluster in 293t cells



b

vCYCLIN C	**G**ttcctgccaacgtcattc	**vFLIP C**	**G**tgctctgtcaggttctcc
vCYCLIN N	**G**tcgcgttggcccttaatc	**vFLIP N**	**G**taacaagaaggaagttac

LANA C **G**ctaggccacaacacatct

LANA N **G**tcccacagtgttcacatc

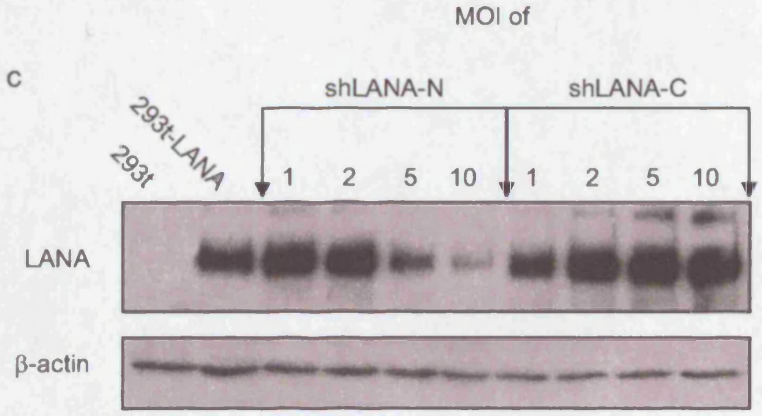


Figure 5.1 Knockdown of the genes in the oncogenic cluster in 293t cells

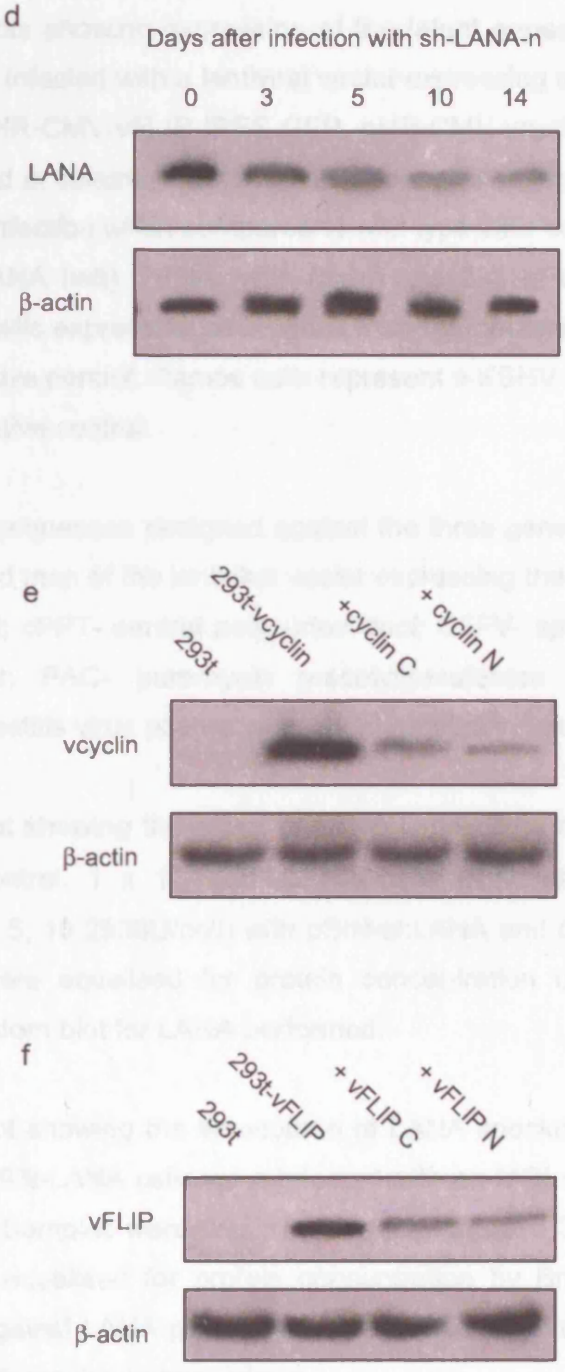


Figure 5.1 Knockdown of the genes in the oncogenic cluster in 293t cells

(a) Western blots showing expression of the latent genes in 293t cell lines. 293t cells were infected with a lentiviral vector expressing a latent gene (pHR-CMV-LANA, pHR-CMV-vFLIP-IRES-GFP, pHR-CMV-vcyclin) at an MOI of 5 and maintained in culture. Western blots show expression of the latent gene 4 weeks after infection when compared to wild type 293t cells. Cell lines were named 293t-LANA (left), 293t-vcyclin (right) and 293t-vFLIP (bottom). BC-3 cells are PEL cells expressing all 3 genes from the oncogenic cluster and are used as a positive control. Ramos cells represent a KSHV negative B cell line used as a negative control.

(b) The target sequences designed against the three genes in the oncogenic cluster (top) and map of the lentiviral vector expressing the shRNA. LTR- long terminal repeat; cPPT- central polypurine tract; SFFV- spleen focus forming virus promoter; PAC- puromycin n-acetyltransferase cassette; WPRE- woodchuck hepatitis virus posttranscriptional regulatory element.

(c) Western blot showing the effect of LANA knockdown on 293t-LANA cells and β-actin control. 1 x 10^5 293t-LANA cells were infected at the MOI indicated (1, 2, 5, 10 293tIU/cell) with pSIN-shLANA and cultured for 7 days. Cell lysates were equalised for protein concentration using the Bradford assay and Western blot for LANA performed.

(d) Western blot showing the timecourse of LANA knockdown in 293t-LANA cells. 1 x 10^5 293t-LANA cells were infected with an MOI of 10 293tIU/cell of pSIN-shLANA. Samples were taken for analysis on days 3, 5, 10 and 14. All samples were equalised for protein concentration by Bradford assay, and Western blot against LANA performed. The results show that LANA takes 5-10 days to reach maximum knockdown

(e) Western blot against vcyclin showing effective knockdown. 1 x 10^5 293t-vcyclin cells were infected with an MOI of 10 293tIU/cell of pSIN-shvcyclin-C

and pSIN-shvcyclin-N and the knockdown effect assessed 7 days post infection. Protein samples were equalised using the Bradford assay and Western blot against vcyclin performed. Results indicate both the N and C terminus targets are effective at knocking down vcyclin expression.

(f) Western blot against vFLIP showing effective knockdown. 1 x 10^5 293t-vFLIP cells were infected with an MOI of 10 293tIU/cell of pSIN-shvFLIP-C and pSIN-shvFLIP-N and the knockdown effect assessed 7 days post infection. Protein samples were equalised using the Bradford assay and Western blot against vFLIP performed. Results indicate both the N and C terminus targets are effective at knocking down vFLIP expression.

5.2 Knockdown of genes in the oncogenic cluster in PEL lines

The efficacy of latent gene knockdown was established in BC-3 cells. BC-3 cells are B cells latently infected with KSHV (Arvanitakis et al., 1996). All 3 targets showed a knockdown effect in BC-3 cells (Fig 5.2a). Due to the long half life of the protein, the knockdown efficiency of LANA was confirmed by qRT-PCR 96 hours post infection (Fig 5.2b).

The structure of the mRNA responsible for LANA, vcyclin and vFLIP production predicts that multiple genes should be silenced since they are tricistronic or bicistronic. We know from Chapter 4.5 that concomitant silencing occurs when multiple genes are present on one transcript.

To investigate this, the effect of knockdown of one latent gene on the others was determined. Some reduction in all three genes from the knockdown of any single gene was expected. The results show surprisingly that despite coexisting on a tricistronic transcript, *LANA* targeted hairpins are independent in their knockdown effect, however hairpins targeting either the *vFLIP* or the *vcyclin* silence both genes simultaneously in BC-3 cells (Fig 5.2c).

The mechanism of RNAi predicts that the secondary structure of the mRNA is responsible for the degree of silencing seen. Further targets were chosen within the mRNA strand in order to determine if *vcyclin* and *vFLIP* could be silenced independently or whether all three genes could be knocked down with a single target (Fig 5.2d). The effect of these targets on all three members of the oncogenic cluster of BC-3 cells was assessed by Western blot, however no target which silenced the *vcyclin* or *vFLIP* individually or which silenced all three genes together was found (Fig 5.2e). Based on the proposed mechanism of RNAi, where the secondary structure of the mRNA is critical for the binding of the RISC, this structure may be responsible for lack of silencing.

These data indicate that we can successfully target the latent genes of KSHV and that *vFLIP* and *vcyclin* silencing is concomitant. The next step is to determine the efficacy of these knockdowns in KSHV models.

Figure 5.2 Knockdown of genes in the oncogenic cluster in PEL lines

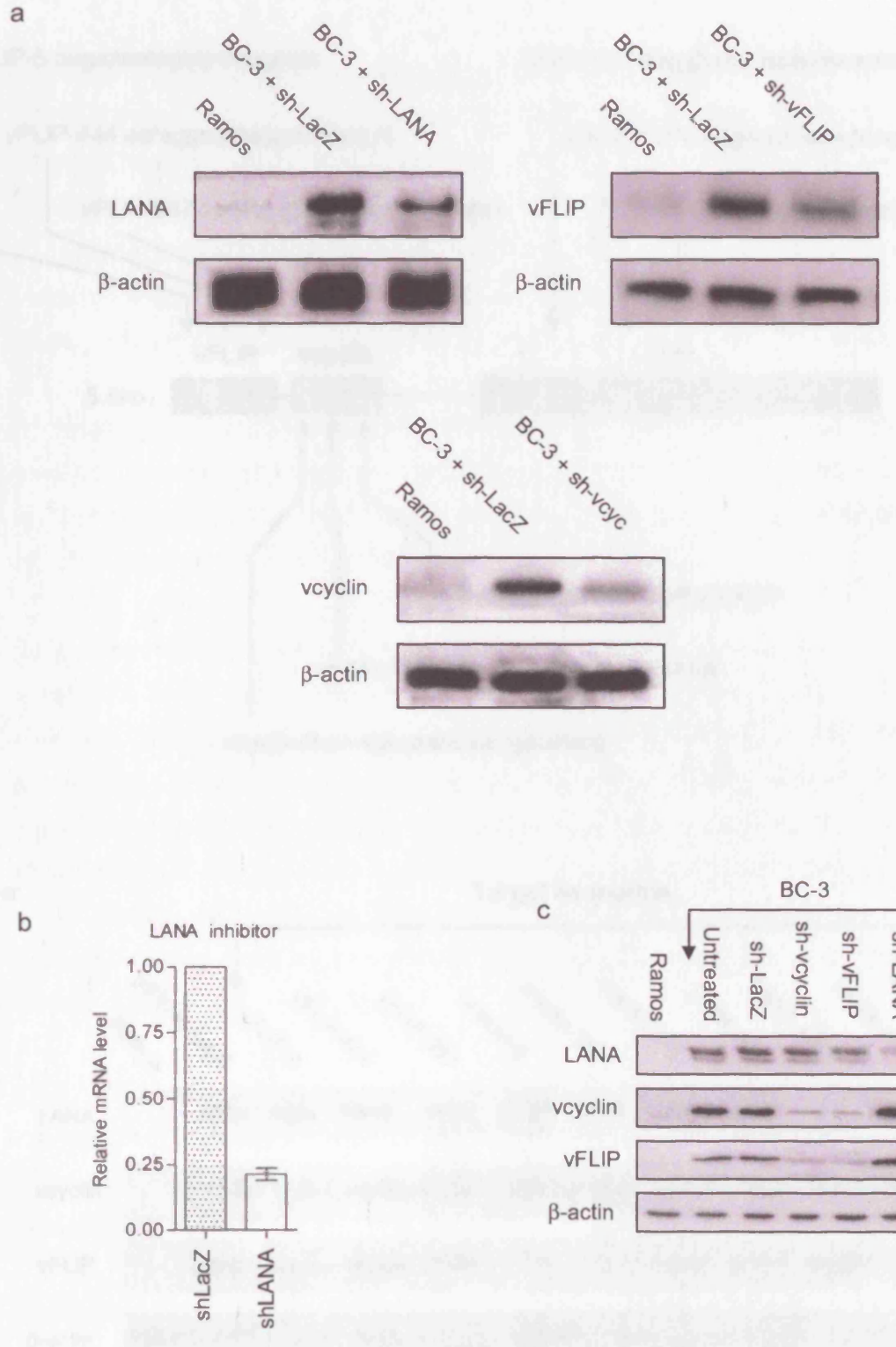
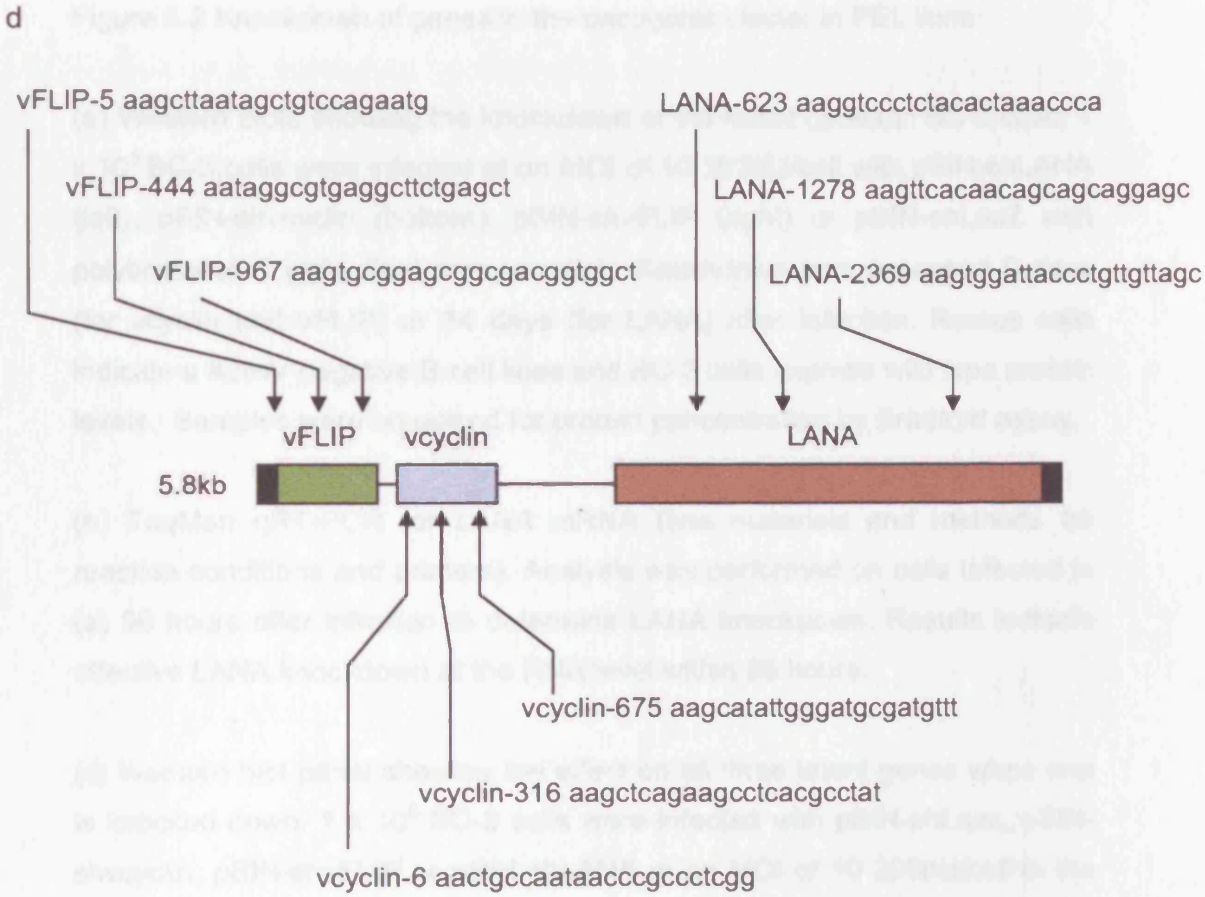


Figure 5.2 Knockdown of genes in the oncogenic cluster in PEL lines



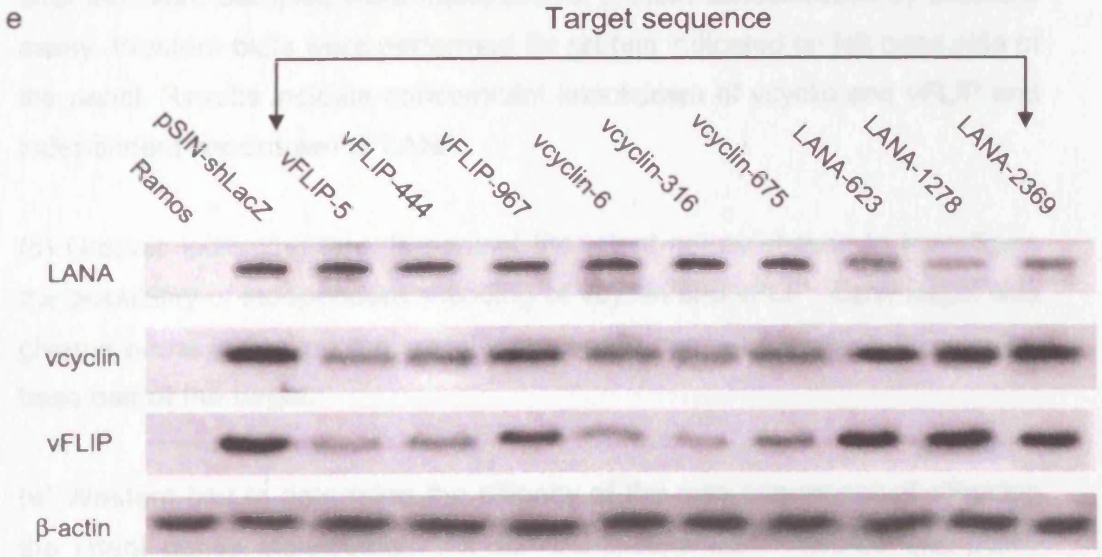


Figure 5.2 Knockdown of genes in the oncogenic cluster in PEL lines

(a) Western blots showing the knockdown of the latent genes in BC-3 cells. 1 x 10^5 BC-3 cells were infected at an MOI of 10 293tIU/cell with pSIN-shLANA (left), pSIN-shvcyclin (bottom), pSIN-shvFLIP (right) or pSIN-shLacZ with polybrene at 1 μg/μl final concentration. Knockdown was assessed 5 days (for vcyclin and vFLIP) or 14 days (for LANA) after infection. Ramos cells indicate a KSHV negative B cell lines and BC-3 cells express wild type protein levels. Samples were equalised for protein concentration by Bradford assay.

(b) TaqMan qRT-PCR for *LANA* mRNA (see materials and methods for reaction conditions and primers). Analysis was performed on cells infected in (a) 96 hours after infection to determine LANA knockdown. Results indicate effective LANA knockdown at the RNA level within 96 hours.

(c) Western blot panel showing the effect on all three latent genes when one is knocked down. 1 x 10^5 BC-3 cells were infected with pSIN-shLacz, pSIN-shvcyclin, pSIN-shvFLIP or pSIN-shLANA at an MOI of 10 293tIU/cell in the presence of 1 μg/μl polybrene. vcyclin and vFLIP protein levels were measured 5 days after infection, and LANA levels were measured 14 days after infection. Samples were equalised for protein concentration by Bradford assay. Western blots were performed for protein indicated on left hand side of the panel. Results indicate concomitant knockdown of vcyclin and vFLIP and independent knockdown of LANA.

(d) Graphic indicating targets against the latent genes chosen to investigate the possibility of independent silencing of vcyclin and vFLIP. Each target was given a name indicating the target gene and a number indicating the starting base pair of the target.

(e) Western blot to determine the efficacy of the nine sequences at silencing the latent genes individually. 1 x 10^5 BC-3 cells were infected with pSIN-

shLacZ or virus produced against each of the nine targets indicated on the top row at an MOI of 10 293tIU/cell in the presence of 1 μg/μl polybrene. vcyclin and vFLIP protein levels were measured 5 days after infection, and LANA levels were measured 14 days after infection. Samples were equalised for protein concentration by Bradford assay. Western blots were performed against protein indicated on left hand side of the panel. No target was found which yielded silencing of the latent genes individually or which silenced all three genes using one target.

5.3 Adverse effects of latent gene knockdown in PEL lines

To determine whether knockdown of the latent genes caused apoptosis in PEL lines, 4 PEL lines (JSC-1, BC-3, BCP-1 and HBL-6) were infected with pSIN-shLANA, pSIN-shvFLIP or pSIN-shvcyclin. Ramos cells (KSHV negative B cells) were used as a control to show that infection with the vector or expression of the shRNA do not have non specific adverse cellular effects. Annexin V staining used to assess cell viability by FACS (see materials and methods section 2.2.13).

After 14 days, all three knockdowns induced some apoptosis. LANA knockdown seemed to have a variable effect in PEL lines. With LANA knocked down, BC-3 and JSC-1 cells did not enter apoptosis as quickly as BCP-1 and HBL-6 cells. No PEL line was found to be resistant to apoptosis after vcyclin/vFLIP knockdown although some (JSC-1) responded faster than others (HBL-6) (Fig 5.3a).

To ensure that this effect was not due to differences in the infection rates within these cells, TaqMan qPCR for the lentiviral insert was used to determine the copy number present after infection. The results indicate that 24 hours after infection the number of copies of the lentiviral insert was slightly less in HBL-6 than in other cells, although the presence of the vector was still significant (Fig 5.3b). Future infections with HBL-6 cells were performed with a higher MOI (1.6x) to compensate for this.

To show that the knockdown in different PEL lines is similar, TaqMan qRT-PCR was used 48 hours after infection to determine *vcyclin*, *vFLIP* and *LANA* mRNA levels. The results indicate a comparable, but not equal, knockdown of these genes in these cells (Fig 5.3c). The lower efficiency of RNAi in BC-3 and JSC-1 cells does not seem to correlate with rates of apoptosis and therefore I propose that the knockdown is still sufficient to induce apoptosis.

To determine whether vcyclin or vFLIP were more critical for cell survival, lentiviral vectors encoding *vcyclin* or *vFLIP* were used to rescue the expression levels of these genes after knockdown. These data indicate that inhibition of the viral FLIP is sufficient to induce apoptosis in both JSC-1 and BC-3 cells, as rescue of vcyclin proved insufficient to prevent cell death (Fig 5.3d). Knockdown of vcyclin and restoration of vFLIP induces apoptosis but at a reduced rate in BC-3 and JSC-1 cells (Fig 5.3e). This suggests that although the knockdown of vcyclin has a role in inducing apoptosis, the key factor in the death of these cells is the loss of vFLIP.

These data show that RNAi is functional in these cells, and also confirms that different cells respond differently to RNAi. It was decided at this point that detailed investigation of the levels of mRNA, infection and knockdown required to cause apoptosis would yield little extra information in terms of potential therapeutics. It seems sufficient that knocking down vFLIP is enough to cause apoptosis, even if the knockdown is less than expected, and that knocking down LANA cannot reliably drive PEL cells into apoptosis. The resistance of PEL cells to LANA knockdown was of interest to us, as this does not fit with our predictions of the cellular role of LANA. This effect was therefore further investigated in the next section.

Figure 5.3 Adverse effects of latent gene knockdown in PEL cells

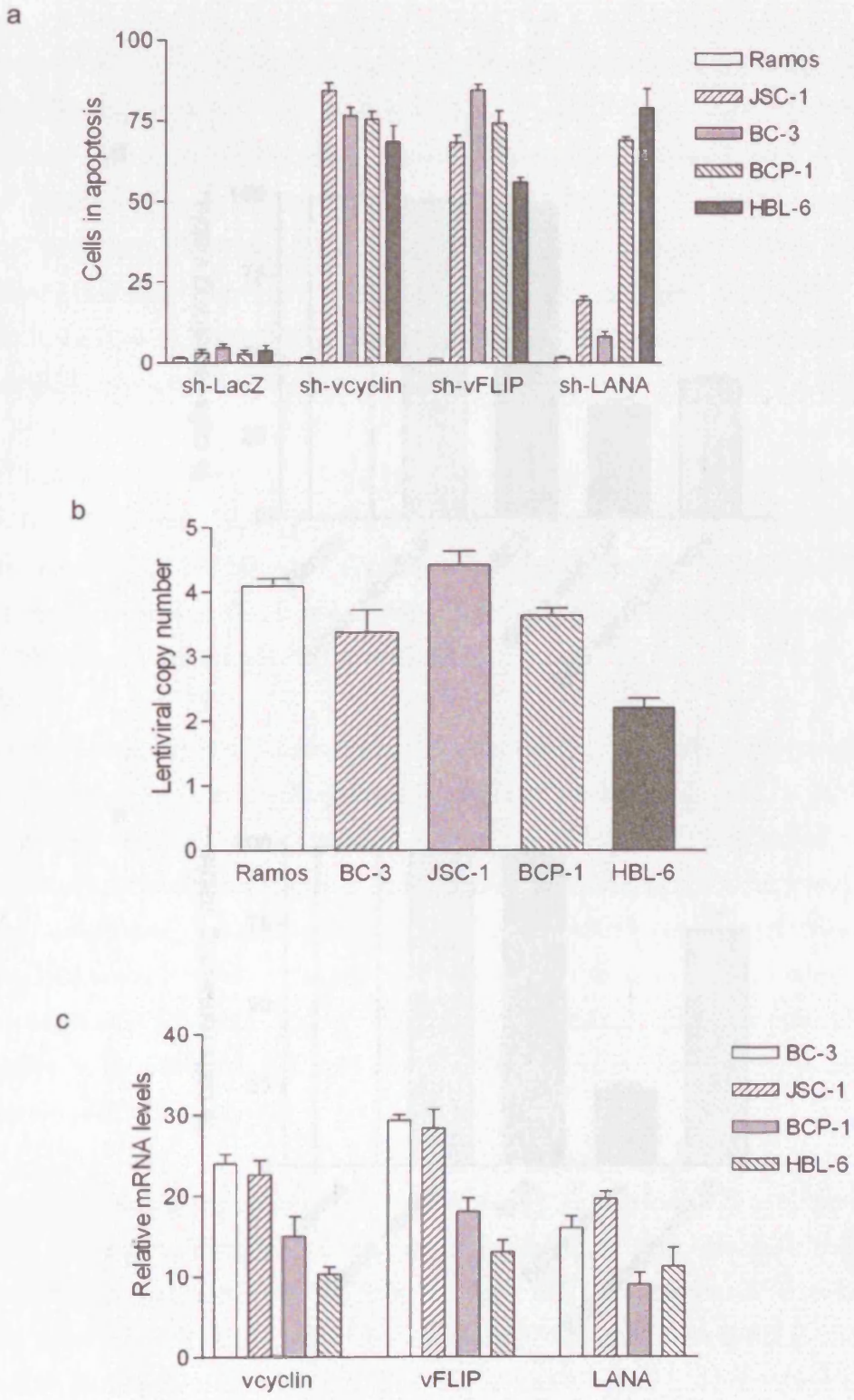
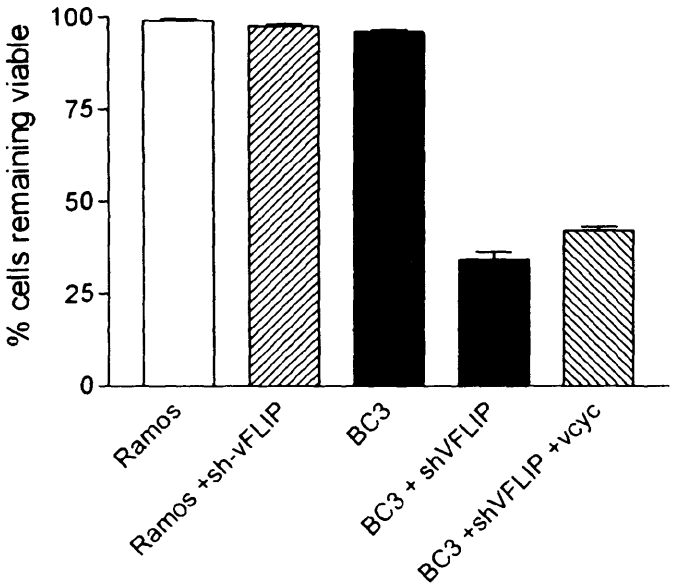


Figure 5.3 Adverse effects of latent gene knockdown in PEL cells

d



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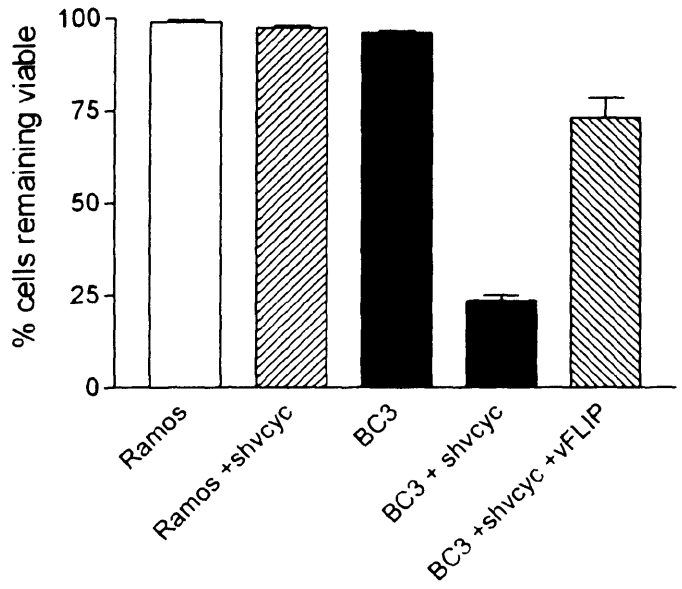


Figure 5.3 Adverse effects of latent gene knockdown in PEL lines

(a) Graph showing percent of cells in apoptosis determined by Annexin-V FITC staining (see materials and methods). 1 x 10^5 Ramos, JSC-1, BC-3, BCP-1 or HBL-6 cells were infected with pSIN-shLacz, pSIN-shvcyclin, pSIN-shvFLIP or pSIN-shLANA at an MOI of 10 293tIU/cell in the presence of 1 μg/μl polybrene. 7 days later, cells were stained with Annexin-V FITC antibody and the number of FITC positive cells measured and assumed to be cells in early or late apoptosis. Each analysis was performed in triplicate and error bars represent the standard error of the mean.

(b) TaqMan qPCR for the lentiviral packaging signal of 1 x 10^5 Ramos, BC-3, JSC-1, BCP-1 and HBL-6 cells infected with at an MOI of 1 293tIU/cell in the presence of 1 μg/μl polybrene. Each analysis was performed in triplicate and error bars represent the standard error of the mean. Results indicate that HBL-6 cells are more refractory to infection.

(c) Graph showing the relative *vcyclin*, *vFLIP* and *LANA* mRNA levels after knockdown determined by TaqMan qRT-PCR of 1 x 10^5 BC-3, JSC-1, BCP-1 and HBL-6 cells 48 hours after infection with pSIN-shLacZ, pSIN-shvcyclin, pSIN-shvFLIP or pSIN-shLANA at an MOI of 10 293tIU/cell in the presence of 1 μg/μl polybrene. 'vcyclin' represents the *vcyclin* mRNA levels after infection with pSIN-shvcyclin, 'vFLIP' represents the *vFLIP* mRNA levels after infection with pSIN-shvFLIP and 'LANA' represents the *LANA* mRNA levels after infection with pSIN-shLANA. All values are relative to that cell type after infection with pSIN-shLacZ.

(d) Graph showing the rescue from apoptosis of PEL cells after knockdown of vFLIP and vcyclin. Results are expressed as percent of cells remaining viable (determined by Annexin-V FITC staining) of 1 x 10^5 Ramos or BC-3 cells 7 days after infection with pSIN-shLacZ (labels 'Ramos' and 'BC-3'), pSIN-shvFLIP (labels 'Ramos+shvFLIP' and 'BC-3+shvFLIP') or pSIN-shvFLIP and

pHR-vcyclin ('BC-3+shvFLIP+vcyc) all at an MOI of 10 293tIU/cell in the presence of polybrene at 1 μg/μl. Results indicate that reintroduction of the viral cyclin is not sufficient to prevent apoptosis in cells where vFLIP is knocked down.

(e) Graph showing the rescue from apoptosis of PEL cells after knockdown of vFLIP and vcyclin. Results are expressed as percent of cells remaining viable (determined by Annexin-V FITC staining) of 1 x 10^5 Ramos or BC-3 cells 7 days after infection with pSIN-shLacZ (labels 'Ramos' and 'BC-3'), pSIN-shvcyclin (labels 'Ramos+shvcyc' and 'BC-3+shvcyc') or pSIN-shvcyc and pHR-vFLIP-IRES-GFP ('BC-3+shvcyc+vFLIP) all at an MOI of 10 293tIU/cell in the presence of polybrene at 1 μg/μl. Results indicate that reintroduction of vFLIP is sufficient to protect cells from apoptosis in cells where vcyclin is knocked down.

5.4 Long term culture of LANA knockdown cell lines

Since lentiviral vector infection is not uniform and some PEL cells could skew results by remaining uninfected, a pure population of LANA negative, KSHV positive cells were created for further investigation. pSIN-shLANA was used to infect BC-3 cells at an MOI of 10 293tIU/cell. This vector (Fig 5.1b) also encodes a puromycin resistance cassette, and after infection these cells (BC3-LN) were cultured in puromycin for 6 months. Analysis performed during and after this time in culture showed that these cells demonstrated a stable knockdown to around 10% of normal LANA levels by Western blot (Fig 5.4a) and 20% by qRT-PCR (Fig 5.4b). To determine whether this residual level of LANA was due to low levels of expression in all cells or some cells escaping knockdown, IFA was performed (Fig 5.4c). Cells were incubated with LN53 antibody and then with an α-rat-FITC antibody. These cells were subsequently sorted, and the fluorescent (i.e. LANA expressing) and negative fraction compared for presence of lentiviral inserts by qPCR (5.4d). These data indicate that some cells, despite adequate infection with the vector continue to express high levels of LANA. Because of the mechanism of RNAi and of puromycin selection, it is likely that all cells have a low level of LANA which may be below the threshold for detection with this method.

The possibility of creating other PEL lines containing little or no LANA was assessed. Only BC-3 cells appeared to be completely resistant to LANA knockdown. Other cell lines such as JSC-1, BCP-1 and HBL-6 cells all show marked apoptosis after infection (Fig 5.4e).

Using a low MOI of shRNA vector, JSC-1 cells seemed to be able to survive with a LANA knockdown (Fig 5.4f). A JSC-1 cell line (JSC-1-LN) with low levels of LANA was created using puromycin selection. These cells showed a detectable decrease in LANA levels by Western blot 1 month after infection (Fig 5.4g). None of the other PEL lines tested could be propagated with a detectable LANA knockdown.

Figure 5.4 Long term culture of LANA knockdown cell lines

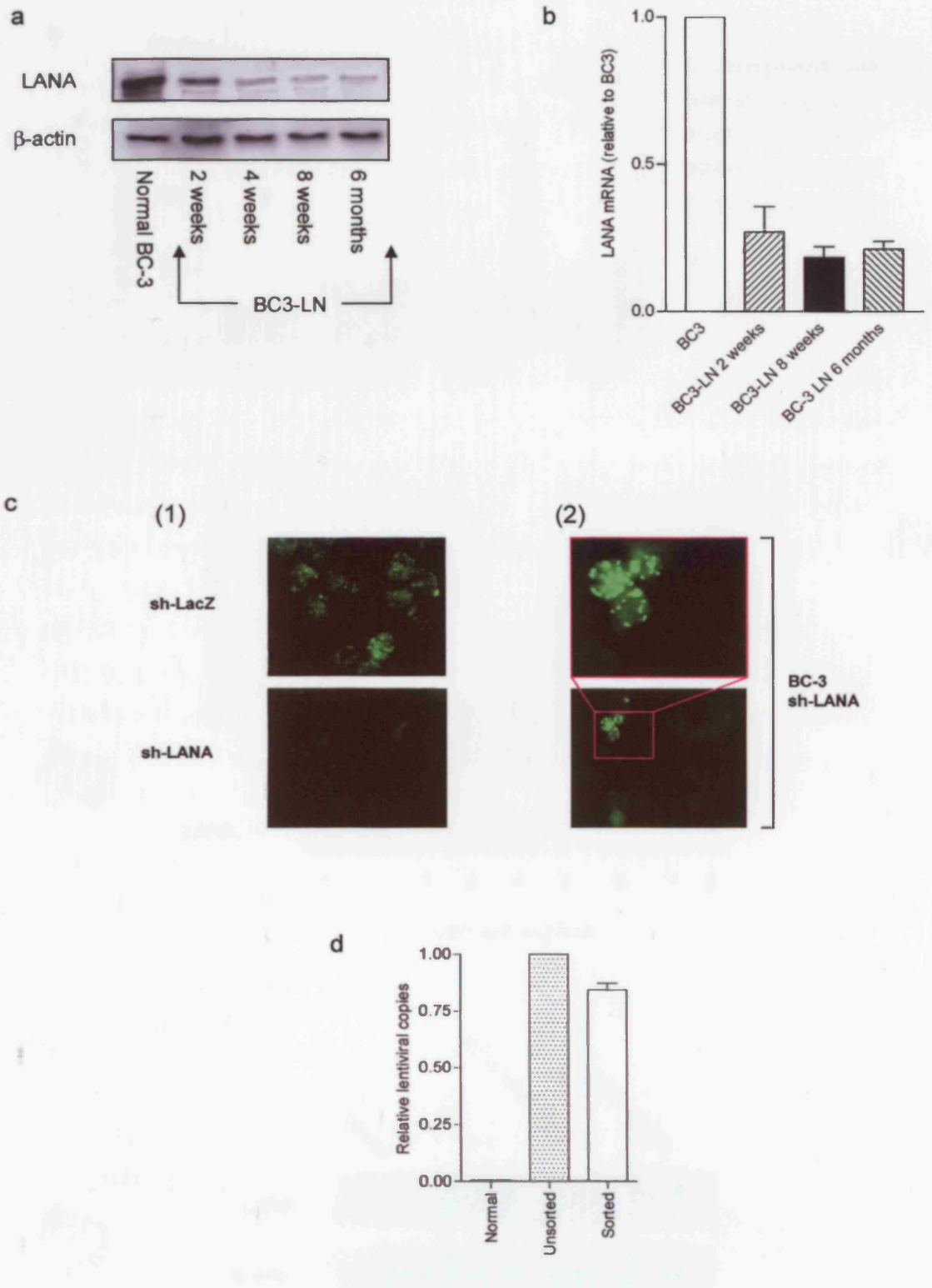


Figure 5.4 Long term culture of LANA knockdown cell lines

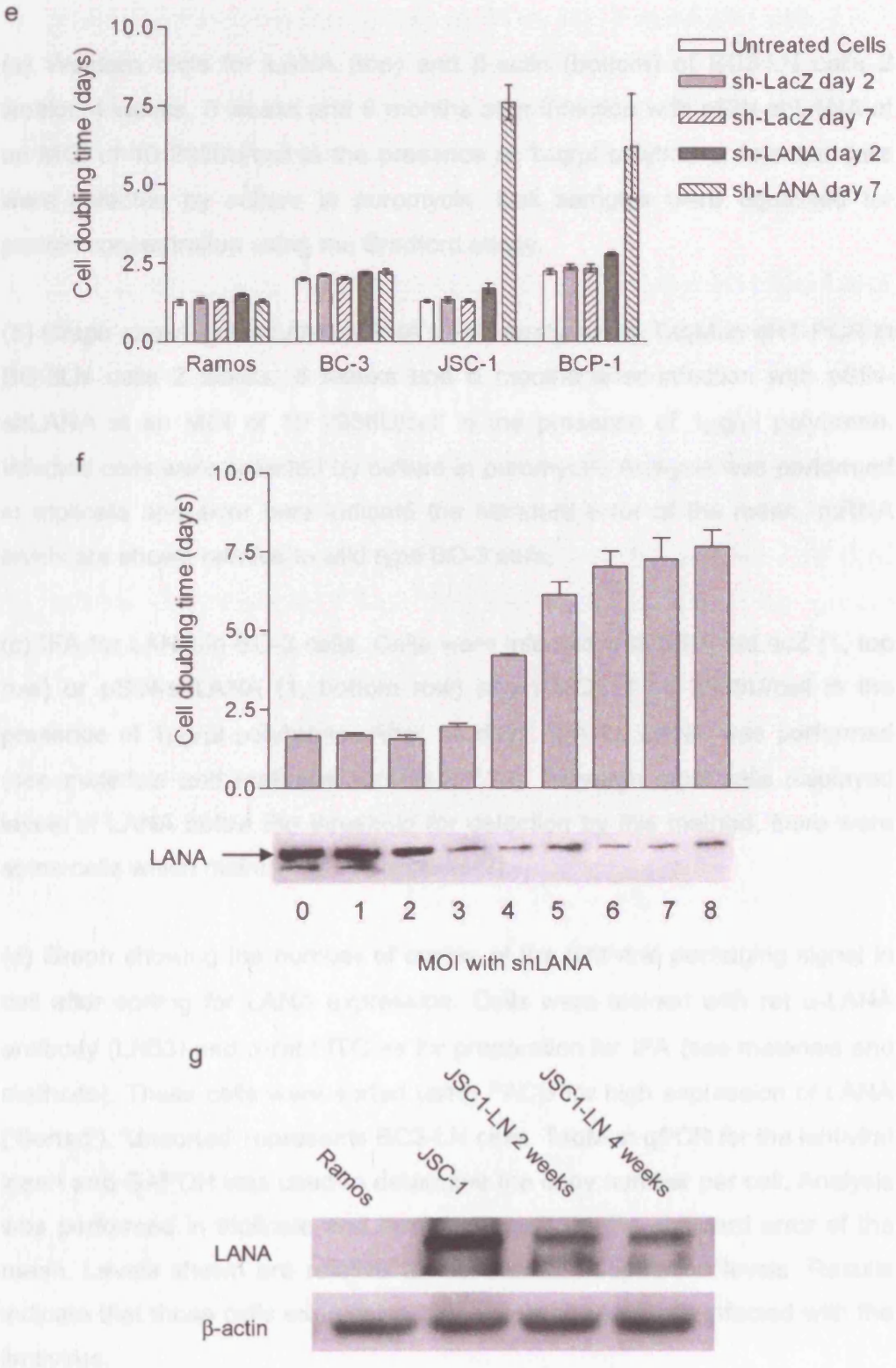


Figure 5.4 Long term culture of LANA knockdown cell lines

(a) Western blots for LANA (top) and β-actin (bottom) of BC3-LN cells 2 weeks, 4 weeks, 8 weeks and 6 months after infection with pSIN-shLANA at an MOI of 10 293tIU/cell in the presence of 1μg/μl polybrene. Infected cells were selected by culture in puromycin. Cell samples were equalised for protein concentration using the Bradford assay.

(b) Graph showing the *LANA* mRNA levels analyzed by TaqMan qRT-PCR in BC-3LN cells 2 weeks, 8 weeks and 6 months after infection with pSIN-shLANA at an MOI of 10 293tIU/cell in the presence of 1μg/μl polybrene. Infected cells were selected by culture in puromycin. Analysis was performed in triplicate and error bars indicate the standard error of the mean. mRNA levels are shown relative to wild type BC-3 cells.

(c) IFA for LANA in BC-3 cells. Cells were infected with pSIN-shLacZ (1, top row) or pSIN-shLANA (1, bottom row) at an MOI of 10 293tIU/cell in the presence of 1μg/μl polybrene. After 14 days, IFA for LANA was performed (see materials and methods section 2.2.14). Although most cells displayed levels of LANA below the threshold for detection by this method, there were some cells which maintained expression (2).

(d) Graph showing the number of copies of the lentiviral packaging signal in cell after sorting for LANA expression. Cells were stained with rat α-LANA antibody (LN53) and α-rat-FITC as for preparation for IFA (see materials and methods). These cells were sorted using FACS for high expression of LANA ('Sorted'). 'Unsorted' represents BC3-LN cells. TaqMan qPCR for the lentiviral insert and GAPDH was used to determine the copy number per cell. Analysis was performed in triplicate and error bars indicate the standard error of the mean. Levels shown are relative to the unsorted population levels. Results indicate that those cells expressing high levels of LANA are infected with the lentivirus.

(e) Graph of cell doubling time of Ramos, BC-3, JSC-1 and BCP-1 cells 2 or 7 days after infection with pSIN-shLacZ or pSIN-shLANA. Infection was performed on 1 x 10^5 cells at an MOI of 10 293tIU/cell in the presence of 1 μg/μl polybrene. Experiment was performed in duplicate and error bars indicate the range.

(f) Graph showing the cell doubling time (in days) and Western blot for LANA of JSC-1 cells 7 days after infection with pSIN-shLANA at increasing MOIs (indicated at bottom) in the presence of 1 μg/μl polybrene.

(g) Western blots for LANA (top) and β-actin (bottom) of JSC1-LN cells 2 weeks and 4 weeks after infection with pSIN-shLANA at an MOI of 4 293tIU/cell in the presence of 1 μg/μl polybrene. Infected cells were selected by culture in puromycin. Cell samples were equalised for protein concentration using the Bradford assay.

5.5 Cellular effects of LANA knockdown

To determine the effect of the LANA knockdown on both BC3-LN and JSC1-LN cells, measurements of life cycle, the copy number of KSHV and ability to enter lytic replication were performed as these are key areas in which LANA is believed to have a role.

BC3-LN cells had no significant difference in BrdU uptake and MTT incorporation (Fig 5.5a) or cell cycle FACS (Fig 5.5b) compared to wild type BC-3 cells. These assays signify the rate of metabolism of these cells (BrdU and MTT) and the populations which are in each stage of the cell cycle. JSC1-LN cells had lower levels of BrdU uptake and MTT incorporation (Fig 5.5c). The cell cycle FACS indicated also that the cells spent longer in phase G1 than wild type JSC1 cells (Fig 5.5d). This analysis fits with the difficulty in creating JSC-1 cell lines which are resistant to LANA knockdown.

Over 6 months in culture, BC3-LN and JSC1-LN cells did show a gradual decrease in the copy number of KSHV present. This is consistent with loss of viral episomes. A residual copy number remained despite the knockdown and no further decrease was seen after 2 months (Fig 5.5e).

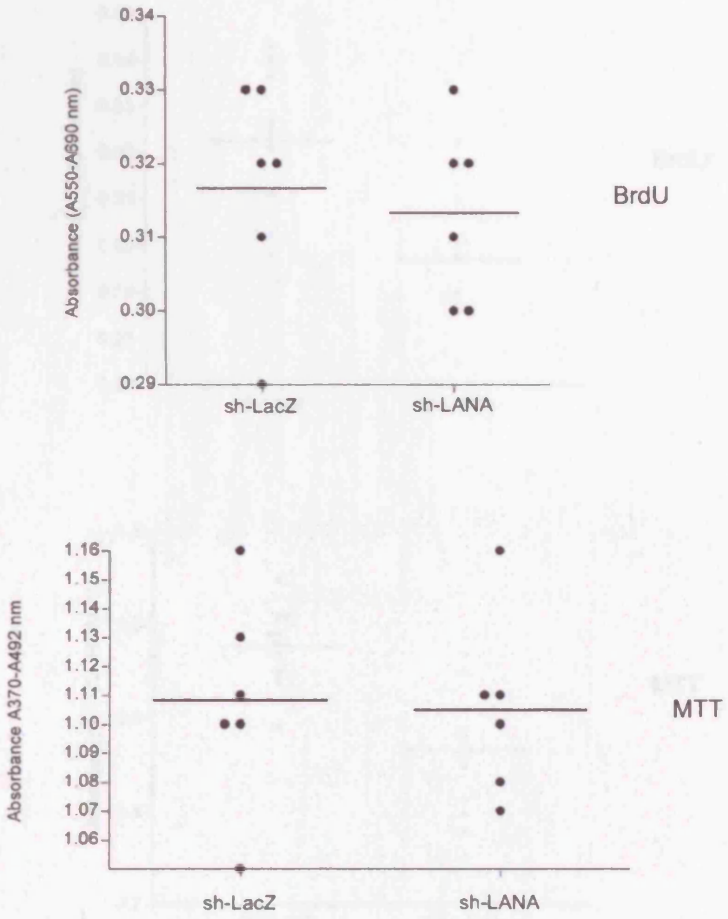
The activation of the lytic life cycle on BC-3-LN and JSC-1-LN cells was assessed in terms of ORF-50 mRNA levels during normal culture and after stimulation with TPA (Fig 5.5f). This represents a standard method of inducing lytic cycle reactivation in PEL cells. These data indicate that BC-3-LN cells are almost completely uninducible, with no significant upregulation of ORF-50 even in the presence of TPA however JSC-1-LN cells proved to be hyperinducible, with significantly raised levels of ORF-50 mRNA (Fig 5.5f).

As the *vcyclin* and *vFLIP* are believed to be transcribed from the same tricistronic mRNA species as *LANA*, the levels of *vFLIP* and *vcyclin* mRNA in BC-3-LN cells 2 weeks, 1 month and 6 months after knockdown was assessed by qRT-PCR. The results indicate that there is a corresponding

decrease in the production of vcyclin and vFLIP, although it is not in proportion to the knockdown and may only be due to the decrease in copy number of KSHV (Fig 5.5g).

Figure 5.5 Cellular effects of LANA knockdown

a



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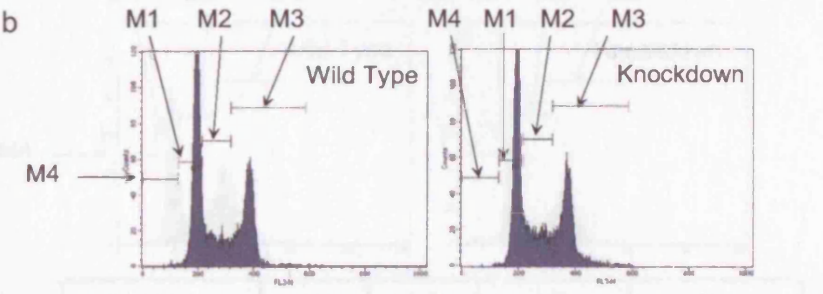
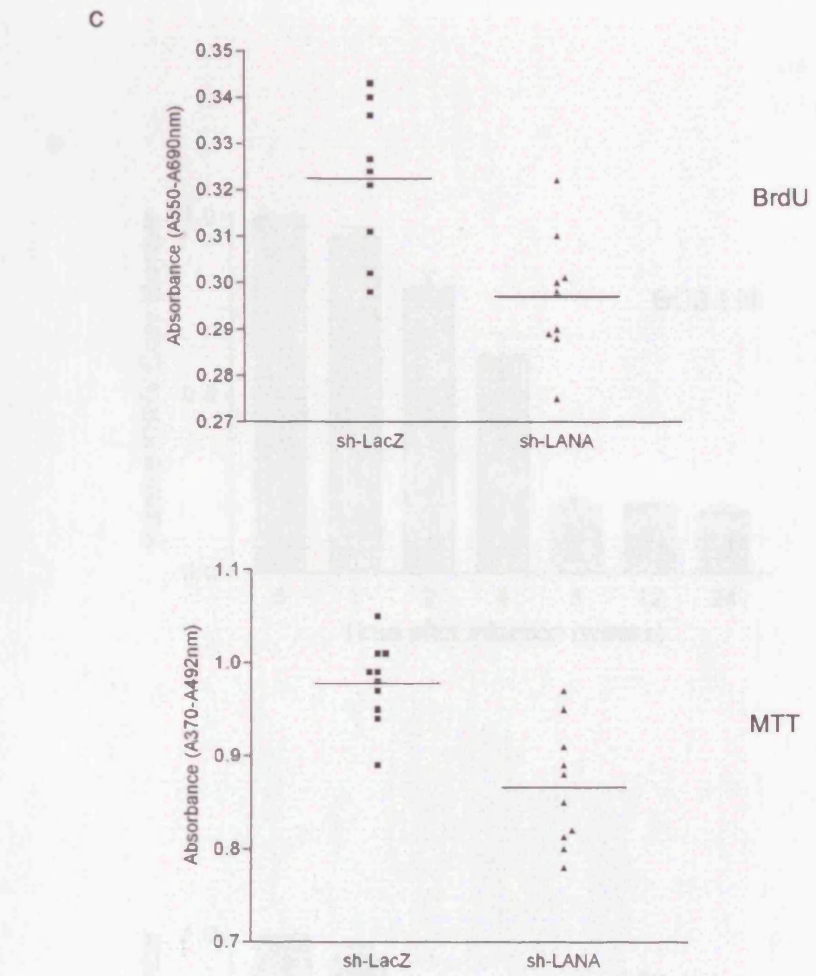
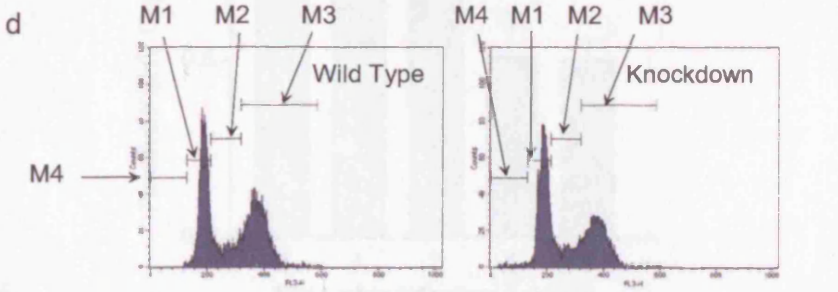


Sample	M1 (G1)	M2 (S)	M3 (G2/M)	M4 (Apoptotic)
Wild Type	43%	21%	32%	4%
Knockdown	43%	18%	35%	6%

Figure 5.5 Cellular effects of LANA knockdown

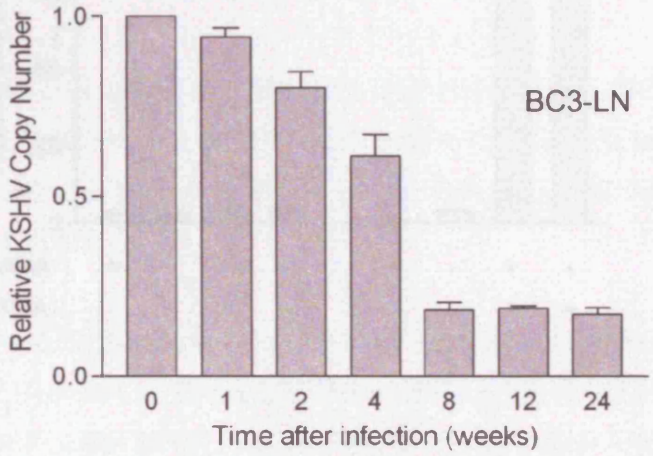




Sample	M1 (G1)	M2 (S)	M3 (G2/M)	M4 (Apoptotic)
Wild Type	44%	19%	31%	6%
Knockdown	53%	22%	15%	10%

Figure 5.5 Cellular effects of LANA knockdown

e



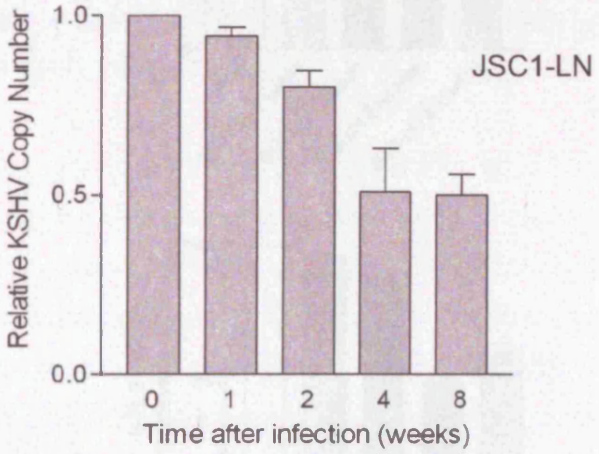


Figure 5.5 Cellular effects of LANA knockdown

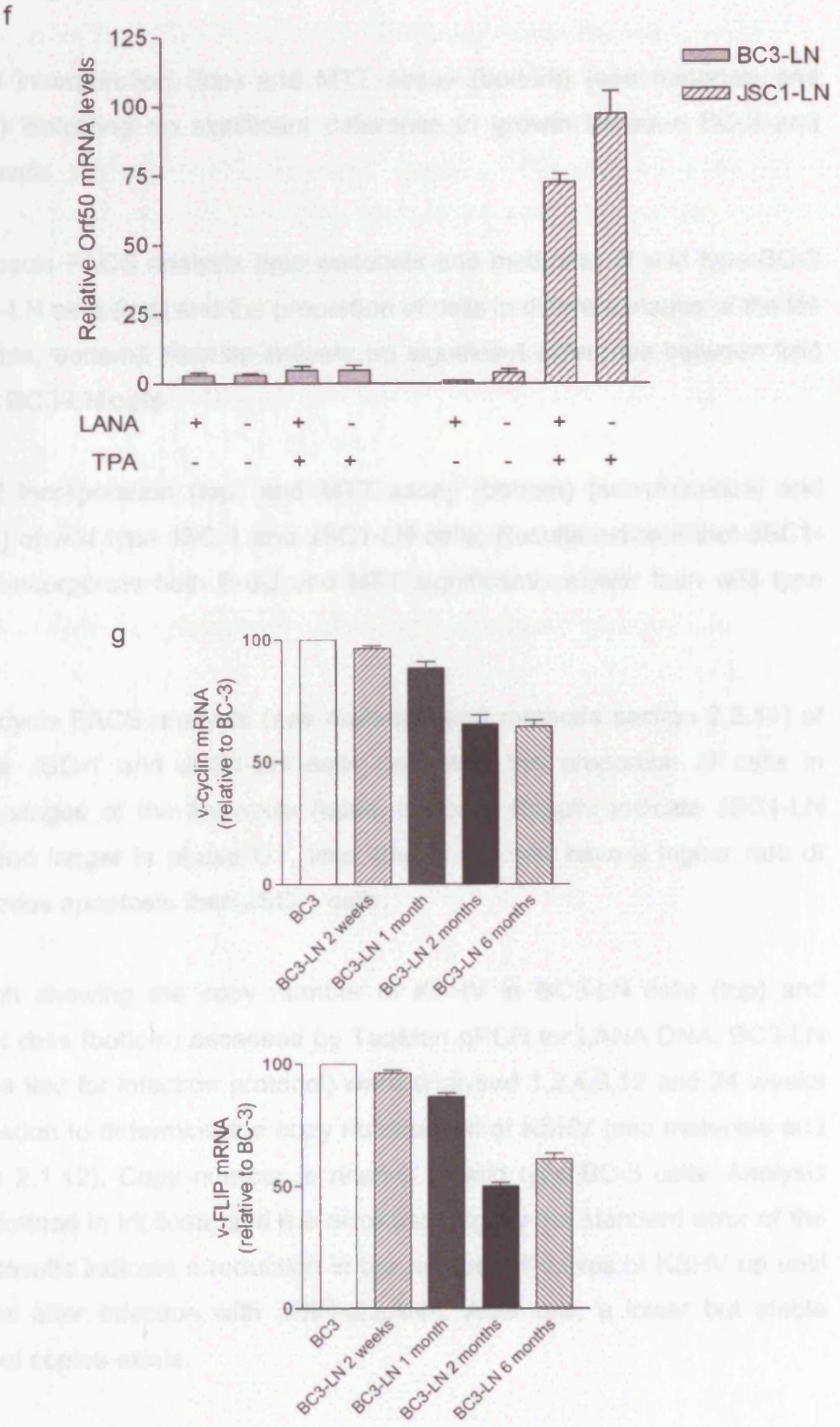


Figure 5.5 Cellular effects of LANA knockdown

(a) BrdU incorporation (top) and MTT assay (bottom) (see materials and methods) indicating no significant difference in growth between BC-3 and BC3-LN cells.

(b) Cell cycle FACS analysis (see materials and methods) of wild type BC-3 and BC3-LN cells (top) and the proportion of cells in different stages of the life cycle (table, bottom). Results indicate no significant difference between wild type and BC3-LN cells.

(c) BrdU incorporation (top) and MTT assay (bottom) (see materials and methods) of wild type JSC-1 and JSC1-LN cells. Results indicate that JSC1-LN cells incorporate both BrdU and MTT significantly slower than wild type cells.

(d) Cell cycle FACS analysis (see materials and methods section 2.2.11) of wild type JSC-1 and JSC1-LN cells (top) and the proportion of cells in different stages of the life cycle (table, bottom). Results indicate JSC1-LN cells spend longer in phase G1, less time in G2 and have a higher rate of spontaneous apoptosis than JSC-1 cells.

(e) Graph showing the copy number of KSHV in BC3-LN cells (top) and JSC1-LN cells (bottom) assessed by TaqMan qPCR for LANA DNA. BC3-LN cells (see text for infection protocol) were analysed 1,2,4,8,12 and 24 weeks after creation to determine the copy number/cell of KSHV (see materials and methods 2.1.12). Copy number is relative to wild type BC-3 cells. Analysis was performed in triplicate and the error bars signify the standard error of the mean. Results indicate a reduction in the number of copies of KSHV up until 2 months after infection with pSIN-shLANA. After this, a lower but stable number of copies exists.

(f) TaqMan qRT-PCR for Orf50 levels in BC3-LN (left) and JSC1-LN cells (right) with or without TPA stimulation. Wild type cells (designated LANA+) or LANA knockdown cells (designated LANA-) were cultured with (TPA+) or without (TPA-) TPA at a concentration of 20 ng/ml for 48 hours and the cells subsequently analyzed for Orf50 mRNA levels. mRNA levels are relative to wild type BC3-LN or JSC1-LN cells with no TPA. Analysis was performed in triplicate and the error bars signify the standard error of the mean. These data indicate that the knockdown of LANA (columns 2 and 4) leads to higher Orf50 mRNA levels both with (first two columns) and without (last two columns) lytic life cycle induction with TPA.

(g) TaqMan qRT-PCR for *vcyclin* (left) and *vFLIP* (right) mRNA levels in BC3-LN cells 2 weeks, 1 month, 2 months and 6 months after creation (see text for infection protocol). mRNA levels are relative to wild type BC-3 cells. Analysis was performed in triplicate and the error bars signify the standard error of the mean.

5.6 Discussion and conclusions

The *in vitro* knockdown of the members of the oncogenic cluster demonstrates the efficacy RNAi both as a tool for research and potential therapeutic.

The varied response of PEL cell lines to LANA knockdown was completely unexpected. LANA is involved in tethering the episome, binding to both pRb and p53 and suppressing the lytic life cycle. We were expecting LANA knockdown to induce apoptosis, cell cycle arrest, and possibly activation of the lytic life cycle. Considering its presence on a tricistronic transcript, we were expecting a *LANA* knockdown to reduce expression of *vFLIP* and *vcyclin* as well. It seemed almost impossible that cells could be relatively unaffected by its knockdown, as our results in BC-3 cells indicate.

Several possible explanations for this lack of effect in BC-3 and JSC-1 cells were considered. Given the varied response of these cells during culture in the laboratory with the selection pressure of a LANA knockdown, these cells may have acquired a mutation in cellular gene expression which allowed them to survive with decreased LANA levels. This same pressure could also have cause the KSHV episome to integrate into the cells, therefore reducing their requirement for LANA to tether the episome.

LANA could also be so redundant in these cells that only a tiny proportion is required for its cellular effects- the knockdown is only around 80% efficient, so around 20% of the mRNA and therefore presumably the protein is still present in the cell. Lastly, several other KSHV proteins have been shown to be present during some programmes of latency, like LANA-2 (vIRF3), and novel functions for these proteins could compensate for the functions of LANA in its absence.

The residual number of episomes of KSHV DNA in BC3-LN and JSC1-LN cells after the knockdown (Fig 5.5e) combined support episomal integration or

redundancy of function, while the fact that only BC-3 and JSC-1 cells seem to be able to survive reduction in LANA levels support mutations in host cells compensating for LANA functions. The limited change in vcyclin and vFLIP levels (Fig 5.5f) also support redundancy in LANA levels, implying that if the knockdown is sufficient to reduce LANA levels below a certain threshold, the cells go into apoptosis. Reduction of LANA expression to the threshold level still allows it to perform cellular functions and the cells are maintainable in culture. I would like to have performed a complete analysis of the effects of LANA and reduction in LANA levels. This could be accomplished by comparing wild type and BC-3-LN and JSC-1-LN cells using a gene expression microarray to determine what, if any, pathways are deregulated and affected by LANA knockdown.

We can theorise why LANA knockdown cells can maintain a low but stable copy number of KSHV DNA (Fig 5.5e). The low level of LANA may be sufficient to maintain some (but not 80-150) copies of the episome, therefore the copy number reduces, or as some publications have implied for other gammaherpesviruses, although never for KSHV, the DNA could have integrated. This would explain also the inability of these cells to produce functional virus when induced- the normal effect of a reduction in LANA is activation of the lytic life cycle, or the hyperinducibility we see with JSC-1-LN cells. I would like to have investigated this further, perhaps by inverse PCR, FISH (fluorescence in-situ hybridisation) or gardella gel analysis to determine whether or not the KSHV DNA had integrated.

The potential for knockdown of members of the oncogenic cluster for inhibiting the virus and therefore as a potential therapeutic was increased by the discovery that all PEL cell models of latent infection were inhibited by reducing the expression of the viral FLIP and vcyclin (Fig 5.3a). This proved extremely effective – it was impossible to maintain cells even with a partial knockdown of vcyclin and vFLIP in culture. The importance of both of these proteins was demonstrated with attempts to rescue the expression of one after knockdown of both – knockdown of the vFLIP provided the fastest and

most complete apoptosis, however vcyclin was shown here to have a role in survival of these PEL models (Fig 5.3d+e).

The interesting nature of the knockdown with regards to its effect on the bicistronic but not tricistronic forms of the mRNA species is unexplained by my results. vFLIP and vcyclin were always knocked down together, however LANA knockdown was always independent. I was unable to isolate a target specific for *vFLIP* or *vcyclin*, or find a target which would knockdown all three genes (Fig 5.2d+e).

Both from the data in chapter 4 and the demonstration here of concomitant *vFLIP* and *vcyclin* knockdown there is evidence that knockdown of genes on a polycistronic strand is sufficient to reduce the expression of both genes. Why this should be different for *LANA* is unknown. From the current literature regarding the mechanism of RNAi it can be theorised that the secondary structure of the tricistronic strand may hide the targets. This is unlikely due to Figs 5.2d and 5.2e, where several separate targets all failed to knockdown *vFLIP* and *vcyclin* independently or all three latent genes together. Hiding all of these targets through modification of structure seems unlikely. Perhaps the 5.1kb mRNA strand containing all three messages is too large to bind with the RISC and be targeted for degradation, however there is no confirmed mechanism by which mRNA is targeted in RNAi and I have no way to prove this. Northern blots on BC-3-LN cells to determine the relative quantity of the three different species of mRNA present would be interesting - although this will not answer the question of how LANA is regulated separately it may provide more insight into the mechanisms involved.

Chapter 6:

In vivo inhibition of PEL

Chapter aims:

The aims of this chapter were to show that the pSIN lentiviral vector can be used as an *in vivo* therapeutic targeting KSHV-driven primary effusion lymphoma (PEL). The biodistribution of the lentiviral vector is shown to be limited when injected intraperitoneally and that therapeutic potential will be limited by transduction efficiency.

6.1 *in vivo* model of primary effusion lymphoma

A study of the effect of latent gene knockdown on an *in vivo* model of KSHV associated morbidity could lead to a better understanding of whether this technique has therapeutic potential. There have been many advances regarding the use of RNAi as an *in vivo* therapeutic. As there are increasing numbers of effective targets one of the main barriers has been the delivery of the knockdown effect. Research has been directed towards modifying dsRNA molecules to increase their stability in the body (Layzer et al., 2004), allowing them to enter cells more easily by attaching them to proteins and therefore decreasing the dose required (Song et al., 2005) or chemically modifying the dsRNA themselves to make them more efficient (Hoshika et al., 2005).

Lentiviral delivery of shRNA is a possible method of inducing an *in vivo* knockdown. Although currently there are barriers to the *in vivo* use of lentiviral vectors (see Chapter 1.1), this should not be the case forever. In order to demonstrate the potential of lentiviral vectors for an *in vivo* therapeutic, the pSIN-shvcyclin vector was chosen. This vector has been shown to be effective and rapid in killing PEL cells (Chapter 5). The vector was modified to include an eGFP marker instead of a puromycin resistance cassette as this would allow easier visualization of infected tissues upon histology. This vector was called pSINeGFP-shvcyclin. The control vector for all infections was an empty vector expressing eGFP (pSIN-eGFP). Experiments throughout Chapters 4 and 5 have demonstrated no undesirable effects from infection with a hairpin targeting LacZ, and no significant difference between these vectors and one expressing no hairpin so it was decided that a pSIN-eGFP control would be sufficient.

First, an *in vivo* model of primary effusion lymphoma was assessed. Primary effusion lymphoma was chosen over the other two KSHV associated cancers (KS and MCD) as there are established models available and the route of administration for treatment would be simpler than for KS and MCD. Previous studies have shown that injecting PEL cells subcutaneously, intravenously or

intraperitoneally can produce murine models of KSHV associated disease (Boshoff et al., 1998; Staudt et al., 2004; Staudt et al., 2004). We chose JSC-1 cells for the generation of our murine model because these cells were easy to infect with our lentiviral vector and showed suitable sensitivity to the knockdown of latent genes (Chapter 5).

Animal handling was performed at the Institute for Child Health, London, by John Anderson and Antigoni Papanastasiou. Immunodeficient mice were selected for the experiment. For this, and all subsequent experiments, mice were age matched (to within 4 weeks) and an equal number of male and female mice selected for each group. The mice chosen were an immunodeficient strain bred at the Institute of Child Health by Adrian Thrasher and John Anderson and had a genotype of RAG-/-, C3 -/- and common gamma chain -/-.

For the initial experiment to develop the model, two groups of 6 treated and 6 untreated (weight control) mice were chosen. These data show that injection of $1x10^7$ JSC-1 cells intraperitoneally caused rapid and reliable development of ascites, gaining as much as 50% of their bodyweight (Fig 6.1a). Girth measurements for these mice showed that this measurement, although an indicator of development of ascites was not a useful indicator of severity. This may be due to operator error, a factor which is difficult to remove from the experiment. For later experiments, it was therefore decided that using weight gain as an indicator of development of ascites and that culling these mice after a weight gain of 6 grams or the presence of a grossly enlarged abdomen inhibiting movement or function gave a more objective measure of suffering due to ascites development. These mice were easy to distinguish from normal mice (Fig 6.1b). These criteria also kept the experiment within ATCC guidelines for the humane treatment of animals.

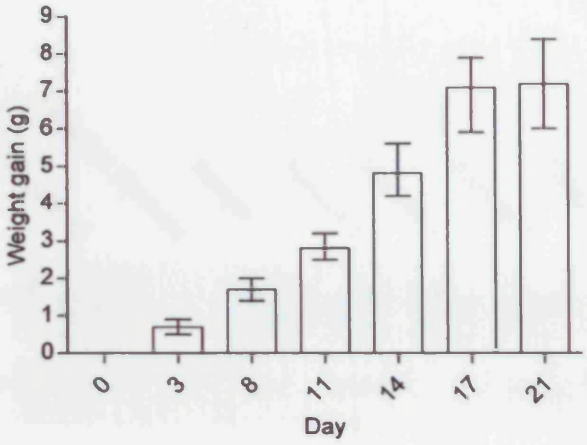
Samples were taken from the 6 mice, all of whom had developed ascites. Western blots for KSHV latent genes were performed to show that the ascites contained KSHV positive PEL cells and the cellular density determined. The

average value was $5x10^7$ cells per ml (Fig 6.1c). The expression of LANA and vcyclin was checked, as ensuring this was a latent infection was critical to the efficacy of our therapeutic. Some variation in the expression of these genes was seen, especially in viral cyclin levels (Fig 6.1d). Although the samples were equalized for protein content, variation in the numbers of murine cells in the samples may account for this. IFA for LANA confirmed that the cellular ascites contained LANA-positive cells presumed to be JSC-1 cells and small LANA-negative cells presumed to be murine (Fig 6.1e). To confirm the origin of these cells, the samples were stained with a PE conjugated antibody specific to murine MHC. FACS showed that most of these smaller cells (low FSC-H) stained positive for murine MHC (high FL2-H) (Fig 6.1f). The other (non-staining) smaller components were likely to be cellular debris, however for this representative sample 15% of the cells present were shown to be murine in origin.

These results combined together led us to believe that the ascites these mice developed was a genuine model of latent KSHV infection containing a mixture of KSHV-positive human cells and KSHV(LANA)-negative murine cells. The latent genes were expressed and this model was therefore suitable for us to test our potential therapeutic.

Figure 6.1 *in vivo* model of primary effusion lymphoma

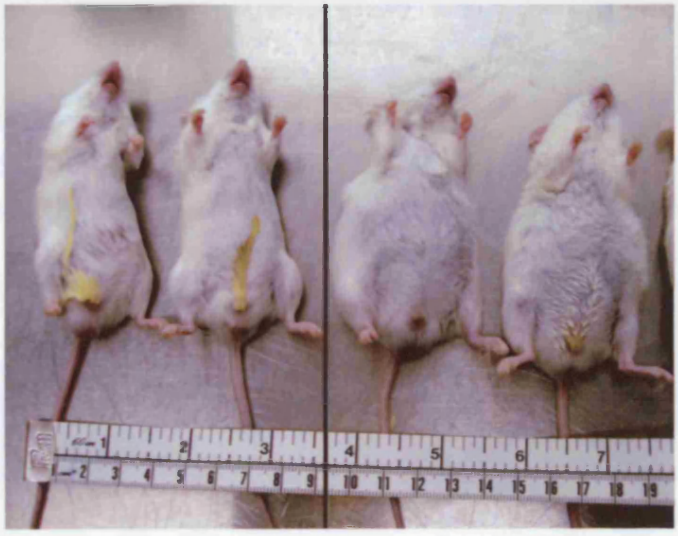
a



Mouse	Change in Girth (mm)	Change in Weight (g)	Mouse	Change in Girth (mm)	Change in Weight (g)
1	8	4.1	4	7	5.2
2	5	5.1	5	11	5.8
3	17	4.3	6	2	4.4
Control 1	1	0.1	Control 2	0	-0.2

b

Normal Mice

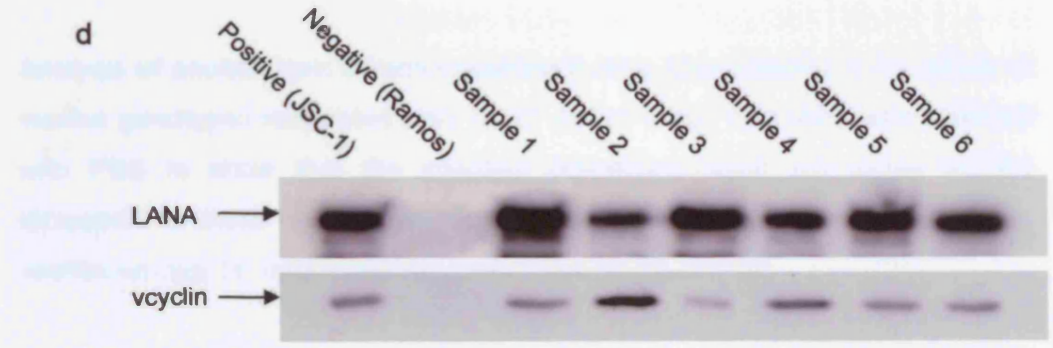


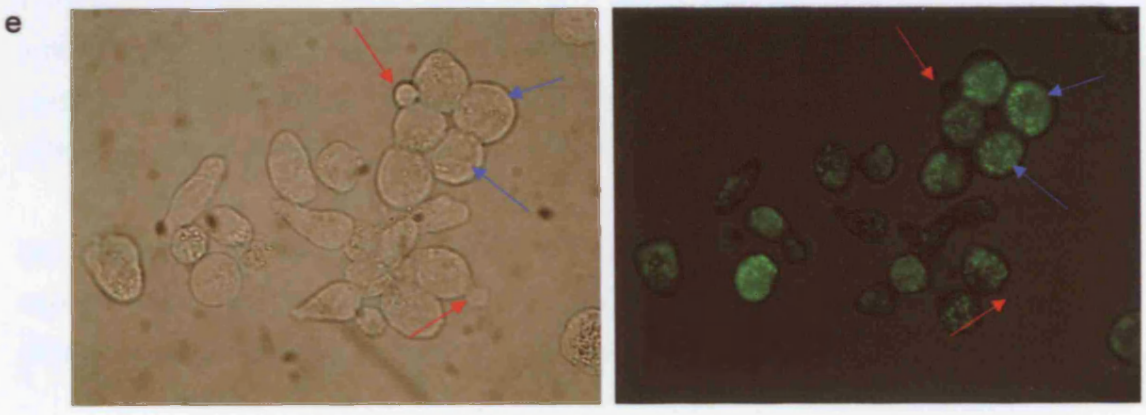
Mice with developed ascites

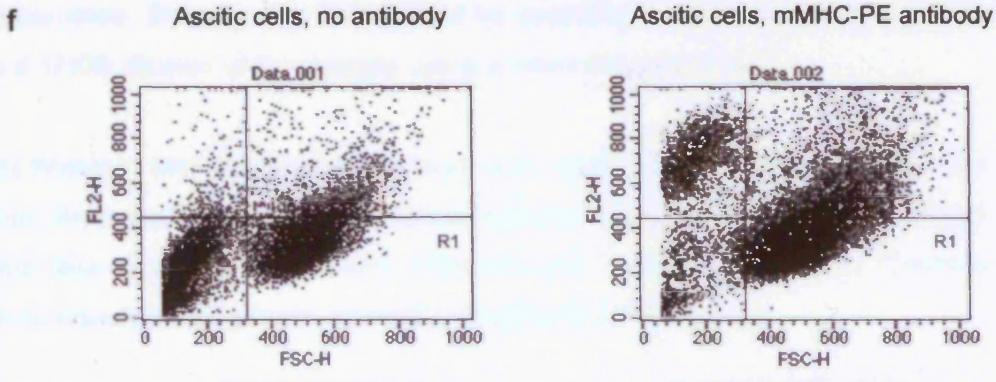
c

Mouse	Density of ascites (x10^7 cells/ml)	Mouse	Density of ascites (x10^7 cells/ml)
1	3.2	4	5
2	4.1	5	5.2
3	4.8	6	7.7

Figure 6.1 *in vivo* model of primary effusion lymphoma







1.2	14.1
20.3	64.4

% in quadrant

16.2	18.3
2.8	62.7

% in quadrant

Figure 6.1 *in vivo* model of primary effusion lymphoma

Analysis of ascites from 6 immunodeficient mice (see Chapter 2 for details of murine genotype) inoculated with $1x10^7$ JSC-1 cells. Two mice were injected with PBS to show that the injection procedure could not cause ascites formation in these mice. One sample was taken from each mouse with ascites on day 14, and these samples used for (c) and (d).

(a) Graph (top) showing the weight gain (in grams, to the nearest 0.1g) of the 6 mice inoculated with JSC-1 cells up to 3 weeks after injection. Error bars indicate the standard error of the mean of the group. Table (bottom) documenting the individual weight change (in grams, to the nearest 0.1g) and girth measurements (in mm) of all eight mice 14 days after inoculation.

(b) Photograph of mice injected with 1×10^7 JSC-1 cells (right) and mice injected with PBS (left) culled after 21 days. The mice injected with JSC-1 cells showed obviously distended abdomens.

(c) Table indicating the individual density (in cells/ml) of ascitic samples from these mice. Density was determined by counting the number of cells present in a 1/100 dilution of the sample using a haematocytometer.

(d) Western blot against LANA (top) and vcyclin (bottom) of ascitic samples from these mice. Lane 1 is a positive control (JSC-1 cells from tissue culture) and lane 2 a negative control (Ramos cells from tissue culture). Samples were equalized for protein content using the Bradford assay.

(e) IFA for LANA of Sample 1. IFA was performed (see Materials and Methods). Blue arrows indicate large, LANA positive cells presumed to be JSC-1 cells, and red arrows small LANA negative cells presumed to be uninfected murine cells. Result is representative of all 6 samples.

(f) FACS analysis of Sample 1 before and after staining with rat anti-mouse MHC-PE conjugated antibody (see materials and methods). Tables indicate the percent of cells present in each quadrant.

6.2 Lentiviral distribution after intraperitoneal injection

The biodistribution and availability of the lentiviral vector are important to determine the optimum dose and best administration route. The distribution of lentiviral vectors injected intraperitoneally both into mice with ascites and control mice was assessed in terms of lentiviral inserts using qPCR. Determination of the presence of lentiviral vector in a range of tissues from the mice allowed determination of the suitability of this route of administration for other purposes.

Three normal mice injected with $1x10^9$ 293tIU of pSIN-eGFP showed a distribution demonstrating that the vector remained broadly limited to the peritoneal cavity (Fig 6.2a). Some vector leaked out, possibly through the injection site and the only tissue in the mouse where no vector was detected was the brain.

Mice with established ascites were also injected and showed a similar but more limited distribution (Fig 6.2b). This was presumably due to the presence of PEL cells in the peritoneal cavity which acted to reduce the amount of virus leaking out of the cavity.

These data indicate that intraperitoneal injection of lentiviral vector is a good route of administration for therapy of ascites. It also shows that the vector is limited to the peritoneal cavity, and is therefore likely to be safe.

Figure 6.2 Lentiviral distribution after intraperitoneal injection

a Untreated mice

Organ	Viral inserts (relative to peritoneum)
Peritoneum	1
Liver	0.03 ± 0.01
Spleen	0.18 ± 0.02
Tail	0.09 ± 0.01
Heart	0.0051 ± 0.0011
Brain	Not Detected
Flank muscle	0.09 ± 0.017

b Mice with ascites

Organ	Viral inserts (relative to peritoneum)
Peritoneum	1
Liver	0.01 ± 0.003
Spleen	0.3
Tail	0.019 ± 0.002
Heart	0.0032 ± 0.0017
Brain	Not Detected
Flank muscle	0.03 ± 0.004

Figure 6.2 Lentiviral distribution after intraperitoneal injection

(a) Table indicating the number of lentiviral inserts per cell of samples taken from 3 mice, 3 days after intraperitoneal injection of $1x10^9$ 293tIU of lentiviral vector. Copies per cell was determined using TaqMan qPCR. Numbers indicate the mean and range of the three samples. Cell number was estimated using primers reacting to both human and murine GAPDH.

(b) Table indicating the number of lentiviral inserts per cell of samples taken from 3 mice inoculated with $1x10^7$ JSC-1 cells, and then $1x10^9$ 293tIU of lentiviral vector 4 days later. Samples were taken on day 7 (3 days after injection with viral vector). Copies per cell was determined using TaqMan qPCR. Numbers indicate the mean and range of the three samples. Cell number was estimated using primers reacting to both human and murine GAPDH.

6.3 Inhibiting primary effusion lymphoma engraftment

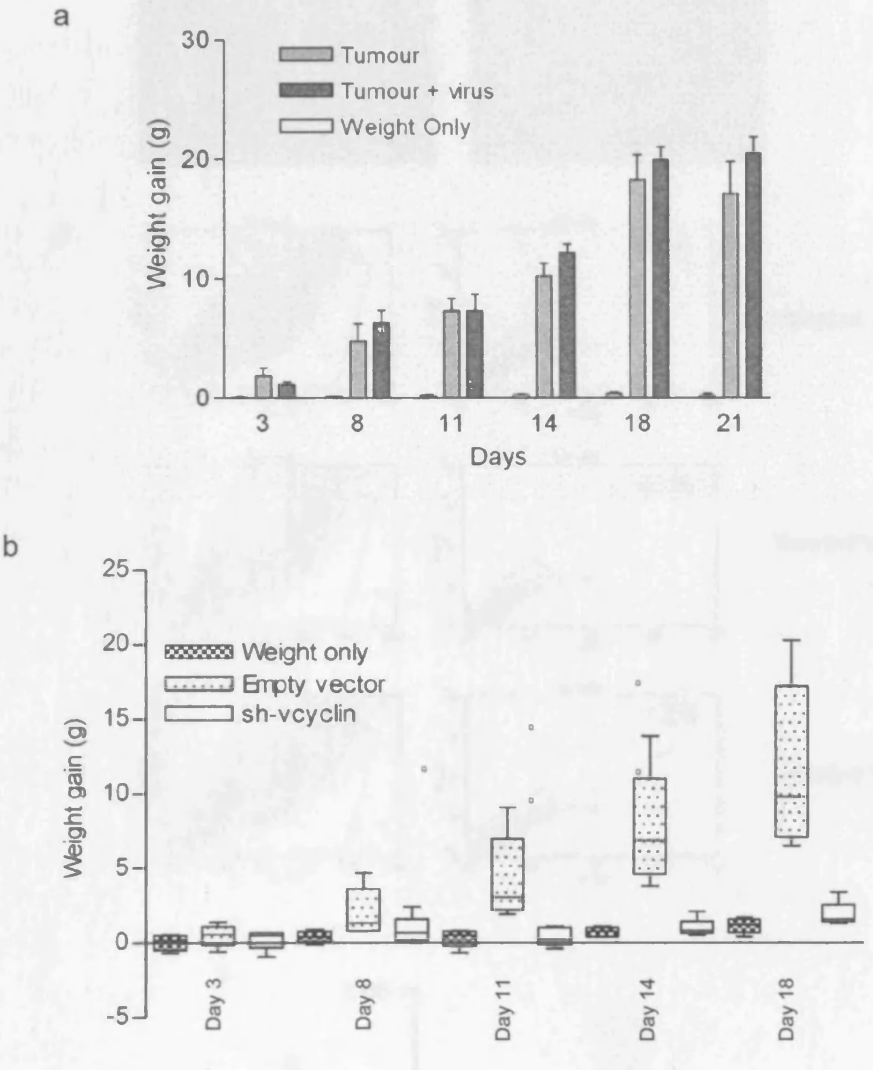
The effect of infection on the development of ascites was established. Six mice were inoculated with 1 x 10^7 JSC-1 cells (tumour), six mice inoculated with 1 x 10^7 JSC-1 cells and 1 x 10^8 293tIU of pSIN-eGFP (tumour + virus) and a further six mice left untreated (weight only). Infection with the lentiviral vector showed no significant effect on either the rate or amount of ascites developed (Fig 6.3a).

To demonstrate the potential of vectors expressing a short hairpin against vcyclin to inhibit the engraftment of primary effusion lymphoma in this model, 1x10^8 293tIU of virions expressing the short hairpin and eGFP (pSINeGFP-shvcyclin) were coinjected with 1x10^7 JSC-1 cells intraperitoneally into 6 mice ('sh-vcyclin'). This represents an MOI of 10 293tIU/cell. One control group, 'weight only' (6 mice) was used only as weight controls to determine normal growth velocity. Another control group 'empty vector' (6 mice) was injected with JSC-1 cells and a lentiviral vector expressing eGFP but no short hairpin (pSIN-eGFP). Both control groups were necessary to demonstrate that the virus itself is incapable of inhibiting engraftment. A repeat administration of 1x10^8 293tIU of appropriate vector (with or without a short hairpin) was given 3 days after the start of the experiment. The weight gain of these mice was charted over the course of 5 weeks (Fig 6.3b). On this graph, mice who gained more than 2x mean weight gain of the weight only control group were considered to have failed treatment, and are marked on the graph as outliers (circles). Outliers are excluded from the ranges on the box and whisker plot but included in all statistical calculations.

These data indicate that 4 of 6 mice in the treated group showed no significant weight gain at the end of the experiment. The remaining mice did develop ascites, however this was at a reduced rate (statistical difference empty vector vs sh-vcyclin $p<0.01$). All 6 mice in the control group developed ascites in a similar fashion to the first experiment.

Samples of ascites from the two affected mice were taken after the mice were culled. These ascites were positive for the viral cyclin by Western blot (Fig 6.3c) and LANA by IFA (Fig 6.3d). A small number of cells expressed eGFP (Fig 6.3e), implying some infection with the lentivirus, but this was at a level sufficiently low that the viral cyclin knockdown did not kill the cells. The lentiviral insert was detected by qPCR (Fig 6.3f) but again at a low level. This would imply that enough uninfected cells survived in these mice to form an effusion despite the administration of treatment.

Figure 6.3 Inhibiting primary effusion lymphoma engraftment



Statistical difference between empty vector and sh-cyclin group; $p<0.01$

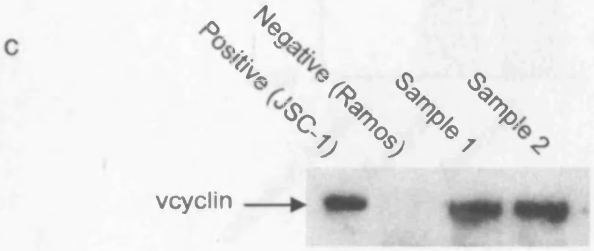


Figure 6.3 Inhibiting primary effusion lymphoma engraftment

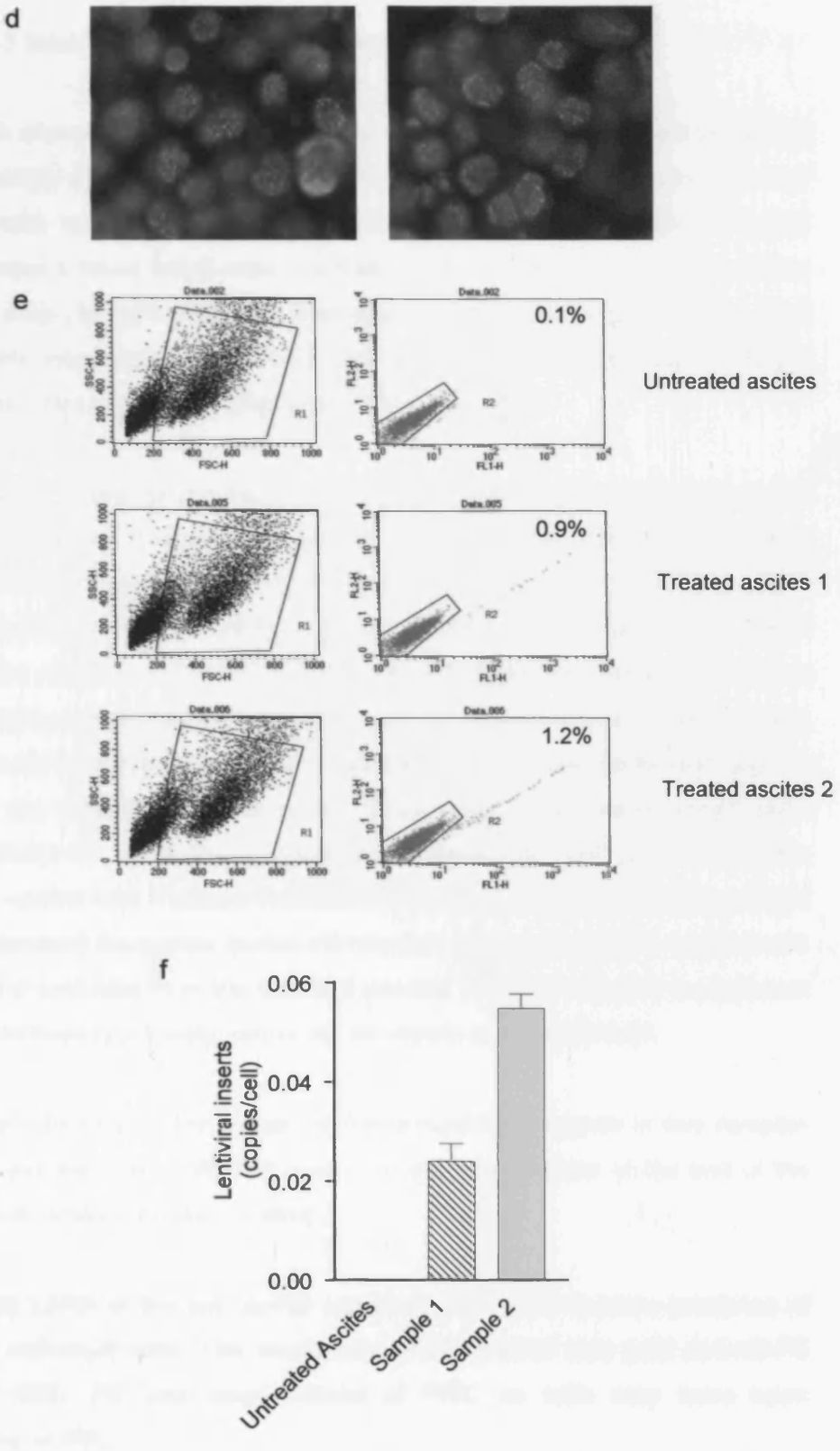


Figure 6.3 Inhibiting primary effusion lymphoma engraftment

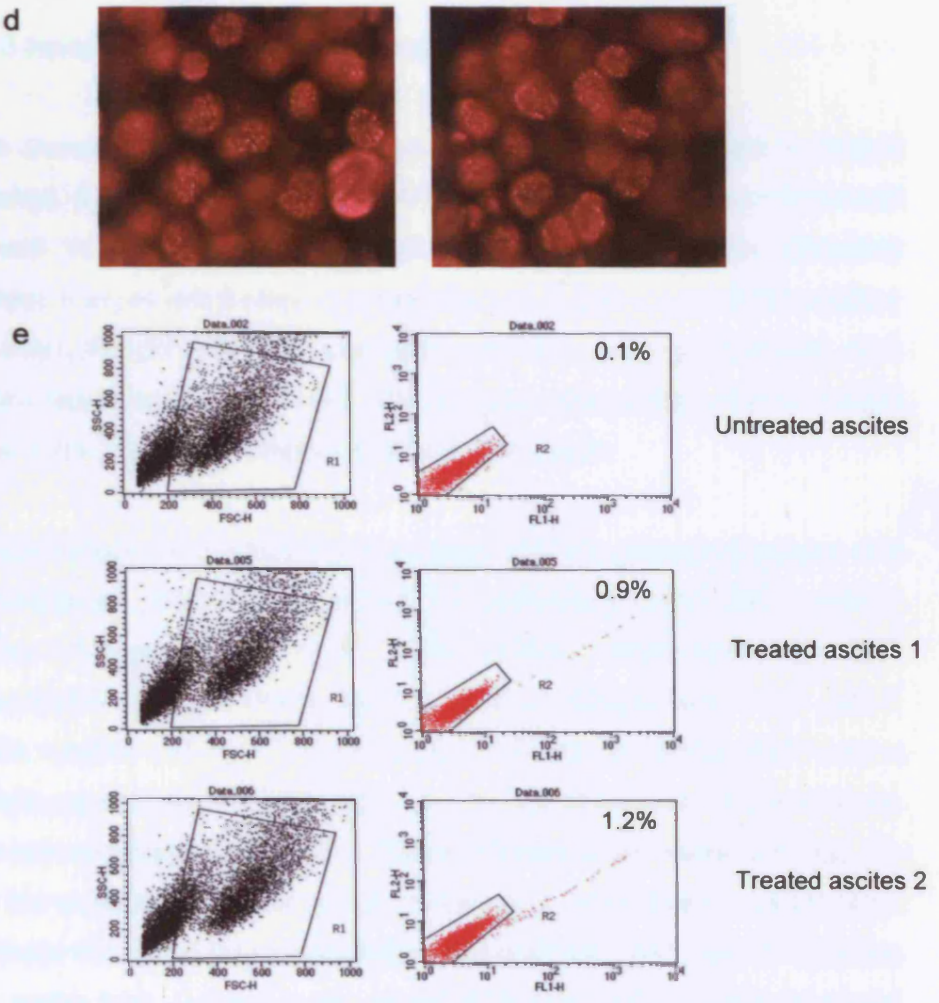


Figure 6.3 Inhibiting primary effusion lymphoma engraftment

(a) Graph showing the weight gain in grams of groups of 6 mice left untreated (weight only), 6 mice inoculated with 1 x 10^7 JSC- cells in the midline and injected with 1x10^8 293tIU lentiviral vector encoding eGFP on the left lateral side (tumour + virus) and 6 mice injected with 1x10^7 JSC-1 cells in the midline (tumour only). Weight gain was assessed over the course of 3 weeks. At 3 weeks, the experiment was ended and all mice were culled due to weight gain. Error bars indicate the standard error of the mean.

(b) Box and whisker plot indicating the weight gain (in grams) of groups of 6 mice left untreated (Weight only), 6 mice inoculated with 1x10^7 JSC-1 cells in the midline and injected with 1x10^8 293tIU lentiviral vector encoding eGFP (Empty vector) on the left lateral side and 6 mice injected with 1x10^7 JSC-1 cells in the midline and 1x10^8 293tIU lentiviral vector encoding eGFP and a short hairpin against vcyclin (sh-vcyclin) on the left lateral side. Repeat doses of virus were administered on day 3. Measurements were made until day 18, when all the mice in the empty vector group were culled due to weight gain. Lines indicate the range (top and bottom whiskers); 25^{th}, 50^{th} and 75^{th} centiles (box and centre line). Outliers in the sh-vcyclin group (distinguished as weight gain 2 standard deviations above the median of the group) are marked with circles and excluded from the box and whisker plot, but included in statistical analysis indicating p(Empty vector vs. sh-vcyclin at day 18)<0.01.

(c) Western blot for vcyclin indicating the presence of v-cyclin in two samples from the ascites of two different mice. Samples were taken at the end of the experiment, when mice were culled.

(d) IFA for LANA of the two ascitic samples used in (b) to show presence of LANA in individual cells. The secondary antibody used was goat anti-rat-PE (Dako, 1:500). PE was used instead of FITC as cells may have been expressing eGFP.

(e) FACS analysis of ascitic samples used in (b) and (c) showing the small proportion of cells expressing eGFP (0.9 and 1.2%)

(f) TaqMan qPCR for the lentiviral insert to assess quantitatively the level of infection in the ascitic samples used for (b)-(d). Cell number was assessed using primers to GAPDH and data represented as lentiviral copies/cell.

6.4 Inhibiting established PEL

Prevention of engraftment does not represent an effective therapeutic, which is the aim of this chapter. Treating established ascites containing many more cells is required to demonstrate the potential of this technology.

The expected requirement from *in vitro* experiments of 10 days for cells to enter apoptosis and the data from *in vivo* experiments that mice begin to die from day 14 after inoculation imply that treatment would have to begin early in order to be successful.

Twelve mice were injected with $1x10^7$ JSC-1 cells (T), and a further 6 mice taken as weight controls (W). The weight gain of the inoculated mice after 3 days was established as greater than 2x the mean weight gain of the control group (Fig 6.4a). These mice were considered to have established ascites.

The mean weight gain of inoculated mice (corrected for the weight gain in the control group) was 1.1g, so this implied the presence of up to $8.5x10^7$ cells (based on 6.1c). To determine the treatment dose required, this figure was taken to be $1x10^8$, and an MOI of 10 293tIU/cell (required for efficient knockdown) was treated as $1x10^9$ 293tIU of vector. The assumption that all virus would remain effectively limited to the peritoneal cavity was made. The tumour cell inoculated mice were divided into two groups. 6 were 'Treated', receiving $1x10^9$ 293tIU of lentiviral vectors expressing eGFP and a short hairpin against viral cyclin (pSINeGFP-shvcyclin) , and the other 6 were 'Untreated', receiving a virus expressing only eGFP (pSIN-eGFP). After 4 days, a repeat dose of $1x10^9$ 293tIU of lentivirus was administered.

These results indicate that the injection of the lentivirus did not significantly alter the development of ascites in the treated mice (Fig 6.4b). Although the gain in weight seemed slower in the Treated group, the p value for this was 0.76 (not significant). Two of the tumour challenged mice failed to develop ascites (1 in each group), and several were noted when culled to have formed

solid tumours. Samples from these tumours were positive for LANA by Western blot (Fig 6.4c).

These results indicate both that in this particular experiment the development of ascites was not as reliable as in previous experiments and that the dose of administered lentivirus was insufficient. Samples from the peritoneum and the ascitic fluid of 2 mice from the untreated group (injected with pSIN-eGFP only) were tested by qPCR for the lentiviral insert (Fig 6.4d). It was found that the peritoneum contained almost 5 times the number of copies per cell of the lentiviral vector than the ascitic sample. It is difficult to estimate the number of cells in the murine peritoneum and also the dynamics of injection of human PEL cells within this environment, so we revised the experimental plan to involve treatment with $1x10^{10}$ 293tIU of lentiviral vector and to increase the group size to 10 mice to allow for the possibility of failed engraftments.

Figure 6.4 Inhibiting established PEL

a

Mouse	Weight (g)	Change (g)	Mouse	Weight (g)	Change (g)
W1	23.2	+0.5	T1	24.7	+1.6
W2	21.1	+0.2	T2	25.1	+1.4
W3	22.3	+0.4	T3	23.1	+1.7
W4	22.4	+0.9	T4	22.9	+1.1
W5	20.9	-0.1	T5	26.1	+2.4
W6	26.7	+1.1	T6	27.1	+1.9
			T7	22.1	+1.7
			T8	26.4	+1.6
			T9	25.2	+1.1
			T10	24.1	+1.9
			T11	23.4	+1.7
			T12	21.1	+1.3

Mean weight change: +0.5g (+2.2%) Mean weight change: +1.61g (+7.4%)

b

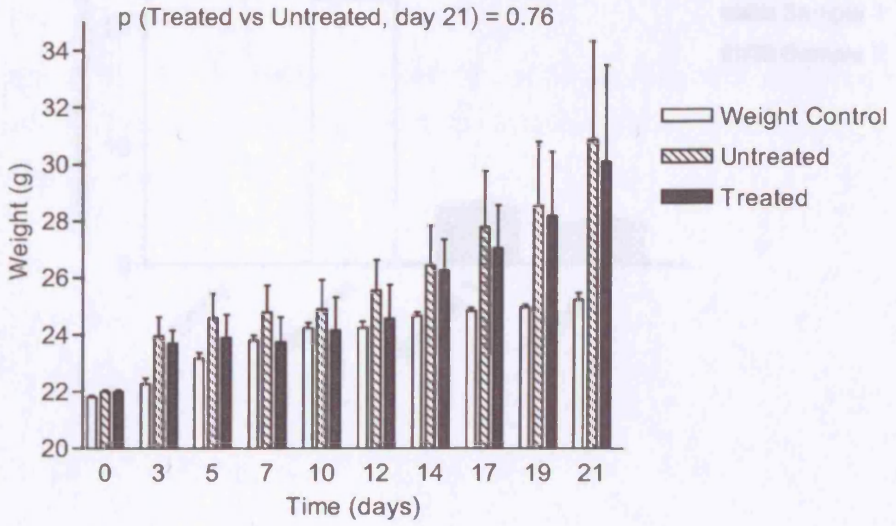


Figure 6.4 Inhibiting established PEL

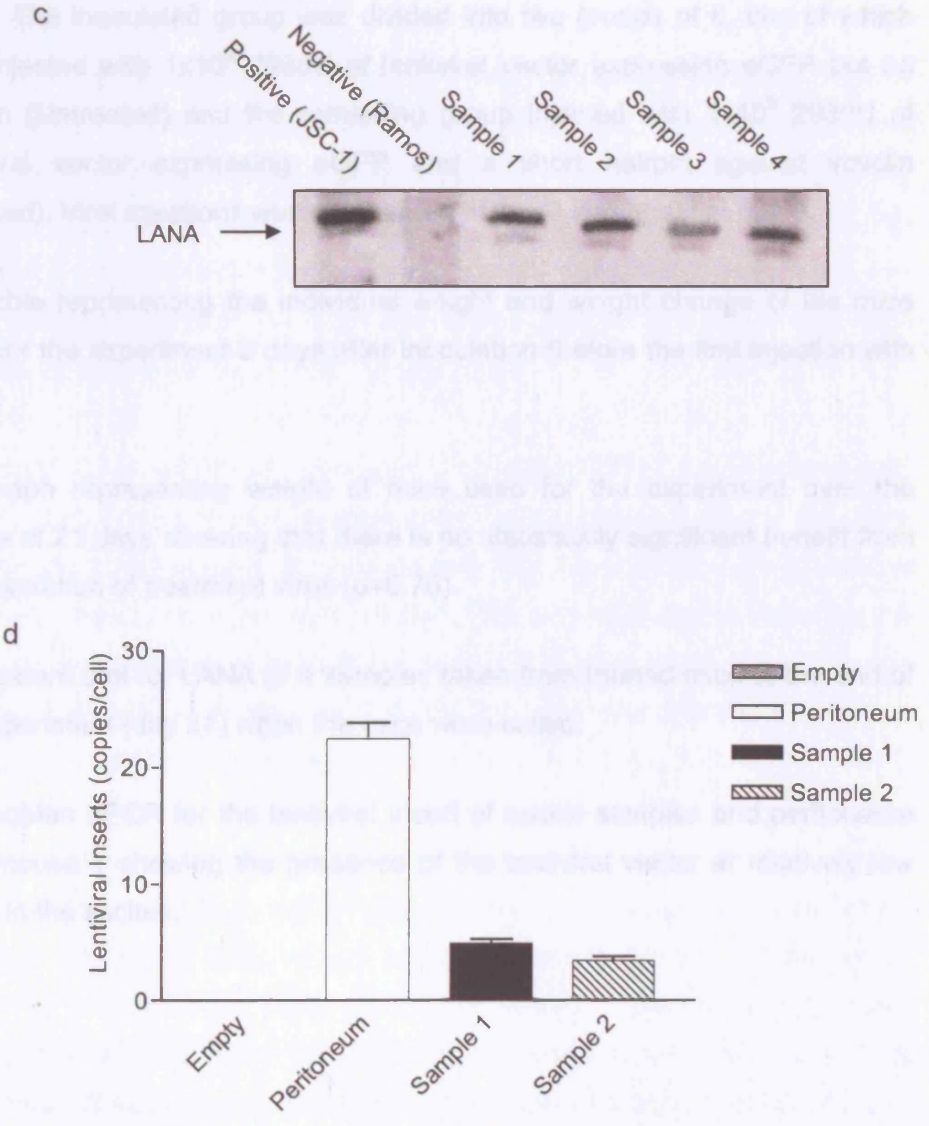


Figure 6.4 Inhibiting established PEL

Experiment to assess the ability of lentiviral vector to treat established ascites. One group (W) are weight only controls, and were not tumour challenged. The other group (T) were challenged with tumour ($1x10^7$ JSC-1 cells). The inoculated group was divided into two groups of 6, one of which was injected with $1x10^9$ 293tIU of lentiviral vector expressing eGFP but no hairpin (Untreated) and the remaining group injected with $1x10^9$ 293tIU of lentiviral vector expressing eGFP and a short hairpin against vcyclin (Treated). Viral injections were performed at day 3 and again at day 5.

(a) Table representing the individual weight and weight change of the mice used for the experiment 3 days after inoculation (before the first injection with virus).

(b) Graph representing weight of mice used for the experiment over the course of 21 days showing that there is no statistically significant benefit from administration of treatment virus ($p=0.76$).

(c) Western blot for LANA of 4 samples taken from treated mice at the end of the experiment (day 21) when the mice were culled.

(d) TaqMan qPCR for the lentiviral insert of ascitic samples and peritoneum from mouse 1 showing the presence of the lentiviral vector at relatively low levels in the ascites.

6.5 Producing large quantities of high titre lentivirus

Aliquots of $1x10^{10}$ 293tIU of lentiviral vector suspended in 300 µl for IP injection represents a considerable challenge both in terms of the concentrations and the overall yield of virus.

Optimisation of the length and the speed were an obvious first step. Current yields with ultracentrifugation are in the order of 10%. Optimisation showed that only 30 minutes at more than 20,000 RPM was required for recovery of lentivirus (Fig 6.5a). The yield was still low (typically around 25-30%) however, so the efficiency of resuspension was examined (Fig 6.5b). It was found that resuspension overnight with a small volume (200 µl) of PBS at 4 degrees with agitation gave yields around 80%.

The use of gel ultrafiltration (Centricon) units with a 100 kDa molecular weight cut-off on the concentrated solution gave further increases in the final titre. This mixture, however was noted to be very viscous and had a proteinaceous component which emerged from solution. This was attributed to the presence of serum in the medium. This highly concentrated virus stock was also found to be toxic to 293t cells (data not shown). This precluded the use of centricon units at this stage of the process.

In order to allow the use of gel ultrafiltration, the ability of 293t cells to produce virus in low serum of serum free conditions was established. OptiMEM (Gibco) is designed to allow the growth of cells in low serum conditions through the addition of growth factors. I therefore investigated whether OptiMEM could be used instead of DMEM supplemented with 10% FCS either alone or as the manufacturer's guidelines indicate with 2% FCS.

It was determined that the use of OptiMEM with 2% serum could produce viral titres equal to that of DMEM with 10% serum. OptiMEM with no FCS did

facilitate production of virus, however the 293t cells adhered to the plate for only 24 hours after transfection in this medium.

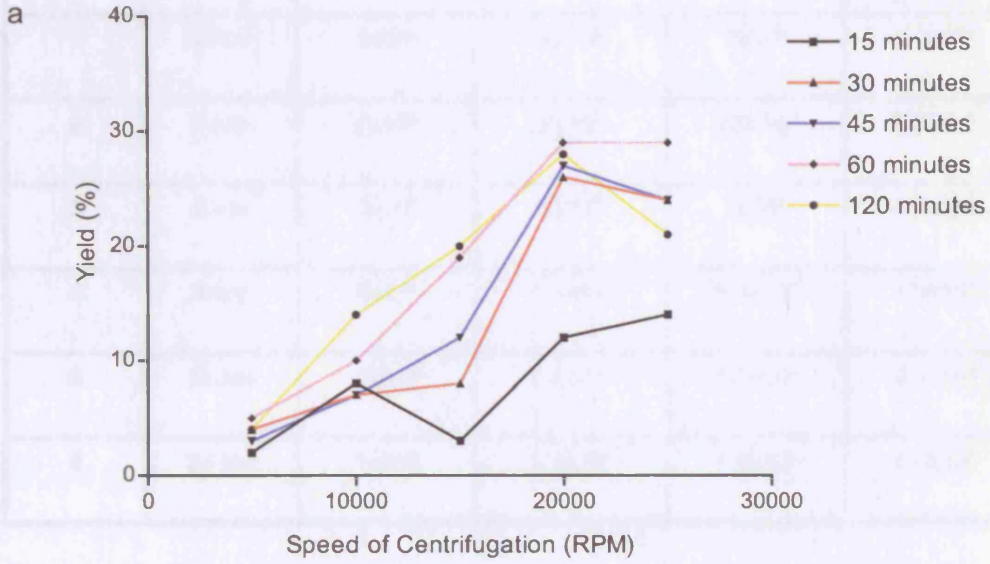
The effective titre of the virus in the supernatant if the harvest period was shorter than 24 hours and more than 2 harvests were performed were assessed. This method has not been documented for lentiviral vectors. It was determined using several plates and medium changes at varying time periods that incubation in fresh medium for 8 hours gave both maximal titre and yield (Fig 6.5c). Harvesting in this manner also maintained the 293t cells for longer in culture, allowing for harvests up to 6 days after the initial medium change.

Using this low serum medium the possibility for using gel ultrafiltration (Centricon) units was reassessed. It was found that the overall viral volume could be reduced around 20-fold, and the titre increased correspondingly when used as described (materials and methods section 3.2.6) although the overall yield was variable between 50 and 80% (Fig 6.5d). This virus was also found not to be toxic to 293t cells (data not shown).

Ultracentrifugation and resuspension with agitation in small volume of the ultrafiltrate allowed the consistent production of high titre ($>10^{10}$ 293tIU/ml) virus required for the treatment of established ascites in mice. The use of multiple harvests from 293t cells also allowed the production of this quantity of virus using roughly 1/8 of the number of plates which would have been required using production methods previous to this optimization.

Figure 6.5 Producing large quantities of high titre lentivirus

a



b

Resuspension Method	Yield 1 (%)	Yield 2 (%)
Agitation in 1ml PBS	27	25
Agitation in 200µl PBS	22	20
Overnight in 200µl PBS	40	49
Overnight with agitation in 200µl PBS	78	83

Figure 6.5 Producing large quantities of high titre lentivirus

c

Plate No.	Harvested every...	Average 1st 24 hours (293tIU/ml)	Average 2nd 24 hours (293tIU/ml)	Average 3rd 24 hours (293tIU/ml)	Cumulative yield over 72 hours (293tIU)
1	2 hrs	$1x10^6$	$4x10^6$	$7x10^6$	$1.7x10^8$
2	4 hrs	$7x10^6$	$2x10^7$	$1.5x10^7$	$3.2x10^8$
3	6 hrs	$2x10^7$	$5x10^7$	$7x10^7$	$4.7x10^8$
4	8 hrs	$8x10^7$	$1.5x10^8$	$1.4x10^8$	$1.1x10^9$
5	12 hrs	$1x10^8$	$2.2x10^8$	$1.7x10^8$	$9.7x10^8$
6	24 hrs	$1x10^8$	$2.1x10^8$	$1.9x10^8$	$4.8x10^8$

d

Before filtration		After filtration		
Volume (ml)	Titre (293tIU)	Volume (ml)	Titre (293tIU)	Yield (%)
80	$1x10^8$	3.9	$1.3x10^9$	65
80	$1.3x10^8$	4.2	$2.1x10^9$	81
80	$7.5x10^7$	4.2	$7.7x10^8$	51

Figure 6.5 Producing large quantities of high titre lentivirus

(a) Graph indicating the yield (%) of 20mls of lentiviral vector (pSIN-eGFP at $1x10^8$ 293tIU/ml) ultracentrifuged at 5,000, 10,000, 15,000, 20,000 or 25,000 RPM in a Beckman SW41 rotor for 15, 30, 45, 60 or 120 minutes. After centrifugation the supernatant was discarded and pellets were resuspended by agitation using a pipette in 1 ml PBS and titre assessed again. Yield is expressed as (total virions in final sample/ total virions in starting sample)*100.

(b) Table comparing yield of lentiviral vector (20 mls of pSIN-eGFP at $1x10^8$ 293tIU/ml) using different resuspension methods after ultracentrifugation at 20,000RPM for 60 minutes with a Beckman SW41 rotor. 'Agitation' involved vigorous pipetting of volume of PBS, 'overnight' involves leaving the viral pellet overnight at 4°c in specified volume, 'overnight with agitation' involves resuspension overnight in specified volume at 4°c on a gyrating platform set to 30 rpm. Yield is expressed as (total virions in final sample/ total virions in starting sample)*100.

(c) Table showing the average titre of pSIN-eGFP virus produced by plates transfected under optimal conditions as describe in Chapter 3. Each plate was harvested every 2, 4, 6, 8, 12 or 24 hours for 72 hours. All harvests were stored at 4°c until the end of the experiment, where the average titre (in 293tIU/ml) in each 24 hours period (average of 12 8ml samples for 2 hour harvests, 2 8ml samples for 12 hour harvests). The cumulative yield (in 293tIU) is the total number of virions produced by that plate over the 72 hour period assessed by combining all harvests and measuring yield. This measure takes into account the volume of virus produced as well as the concentration in assessing yield.

(d) Table showing the use of Centricon-80 units (with 100kDa molecular weight cut-off) to gel filtrate 80ml samples of lentiviral vector (pSIN-eGFP) at stated titre using the manufacturers recommended protocol (see materials

and methods). Titre and volume before and after the filtration step were used to calculate yield as (total virions in starting sample/ total virions in final sample)*100.

6.6 Increased dosage for potential therapeutic

Three groups of 10 mice were allocated into 'sh-vcyclin', 'Empty Vector' and 'Tumour Only' groups. One group of 6 mice was used as a weight control. All mice except the weight control group were inoculated with $1x10^7$ JSC-1 cells and their weight after 3 days was assessed. The mice showed a significant weight gain, with a mean weight gain in each group 10% above the mean of the control group (data not shown).

$1x10^{10}$ 293tIU of lentiviral vector encoding eGFP and a short hairpin against vcyclin (pSINeGFP-shvcyclin) was administered to the sh-vcyclin group, $1x10^{10}$ 293tIU of vector expressing only eGFP (pSIN-eGFP) given to each mouse in the Empty Vector group and the Tumour Only and Weight Control groups were not treated further.

The revised treatment regime was based upon the available data from the previous experiment. It consisted of two large bolus doses of virus in order to increase infection in those cells which were escaping, and a maintenance dose thereafter in case any cells did escape. We assumed the majority of the cells in the ascites are infected with the two bolus doses, and then the remaining task of preventing further development of a much smaller number of PEL cells involves a smaller dose.

The weight change of the mice over the experiment showed a positive benefit from the treatment (Fig 6.6a) and the Kaplan-Meyer survival curve for the mice throughout the course of the experiment showed a large statistical benefit ($p=0.004$) from the administration of the treatment virus (Fig 6.6b).

It was observed that mice in the treated group were not developing ascites but dying of solid tumours. Since we know our treatment is effectively limited to the peritoneal cavity, and is unlikely to have an effect on extra cavity solid tumours, these data can be represented in terms of proportion of mice ascites

free (Fig 6.6c), which shows this treatment has an even stronger effect in terms of specifically inhibiting established ascites.

As the mice in the sh-vcyclin group seemed to develop solid tumours at an increased rate it was proposed that the inhibition of vcyclin may simply alter the phenotype of the tumour rather than treat it.

To determine whether the rate of solid tumour development was actually higher, in all culled mice in the Empty Vector and sh-vcyclin groups full autopsies were performed looking for a solid tumour focus. Several of the untreated group developed both ascites and solid tumours (Fig 6.6d). Inclusion of these data suggests no significant difference in the phenotype of the tumour from the administration of the lentiviral vector.

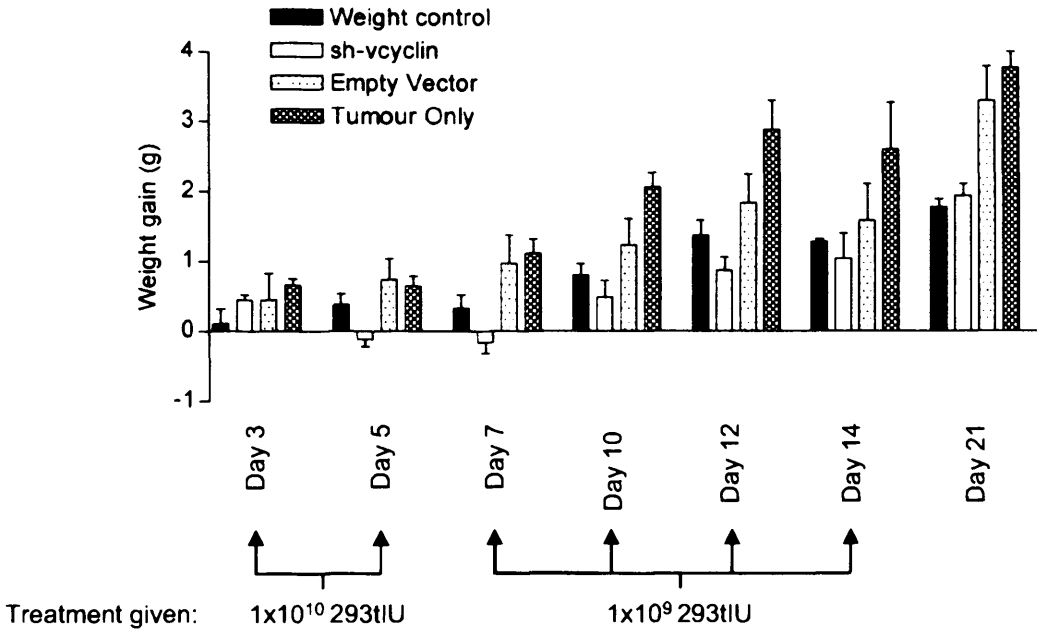
In order to determine whether development of solid tumours was due to failure of treatment or failure of administration, and to prove that the inhibition was not altering the phenotype of the cancer, solid tumours from both sh-vcyclin and Empty Vector mice were sampled and analysed.

Four solid tumours were checked for the lentiviral insert by qPCR (Fig 6.6e). One was found to contain small number of lentiviral inserts (0.03 copies/cell), however the other three were negative. This implies the vector did not reach the solid tumours. Samples of the tumours were all positive for LANA on Western blot (Fig 6.6f), implying that they are PEL cell derived, rather than a coincidental murine tumour or one driven by insertional mutagenesis from the lentivirus.

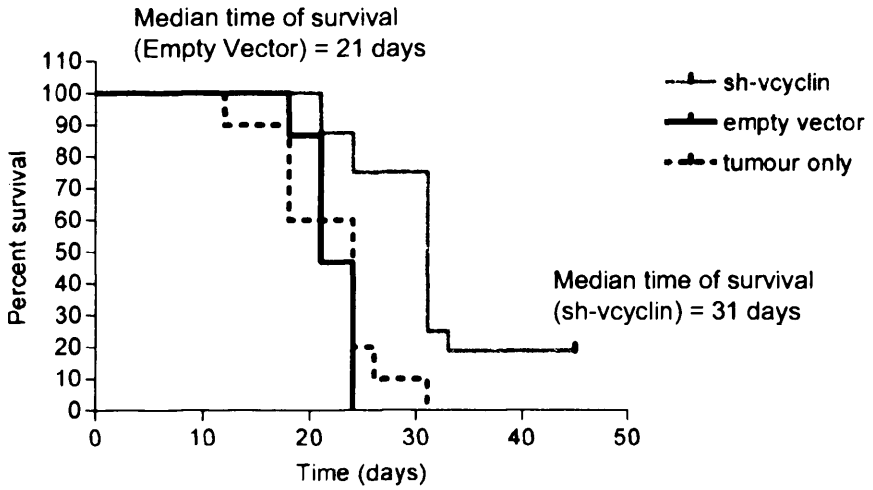
Cells from these solid tumours did not grow well in culture. Apoptosis was clearly seen in all 4 samples when infected with pSINeGFP-shvcyclin at an MOI of 10 293tIU/cell (Fig 6.6g). This demonstrates that these cells had probably never been exposed to vector, and may be amenable to treatment. Importantly, they do not represent an altered phenotype for the cancer.

Figure 6.6 Increased dosage for potential therapeutic

a



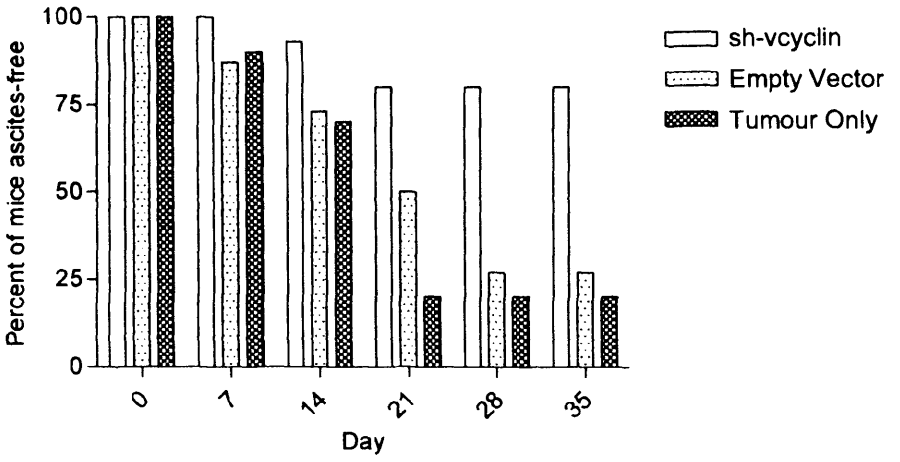
b



Significant difference sh-vcyclin vs empty vector $p<0.004$

Figure 6.6 Increased dosage for potential therapeutic

c



d

	Ascites	Solid tumour	Alive	Median time of survival (days)
sh-vcyclin	2	10	3	31
Empty vector	9	6	0	21

e

Sample	Lentiviral copies/cell
Sample 1	Negative
Sample 2	0.03
Sample 3	Negative
Sample 4	Negative
Peritoneum	27.1

Figure 6.6 Increased dosage for potential therapeutic

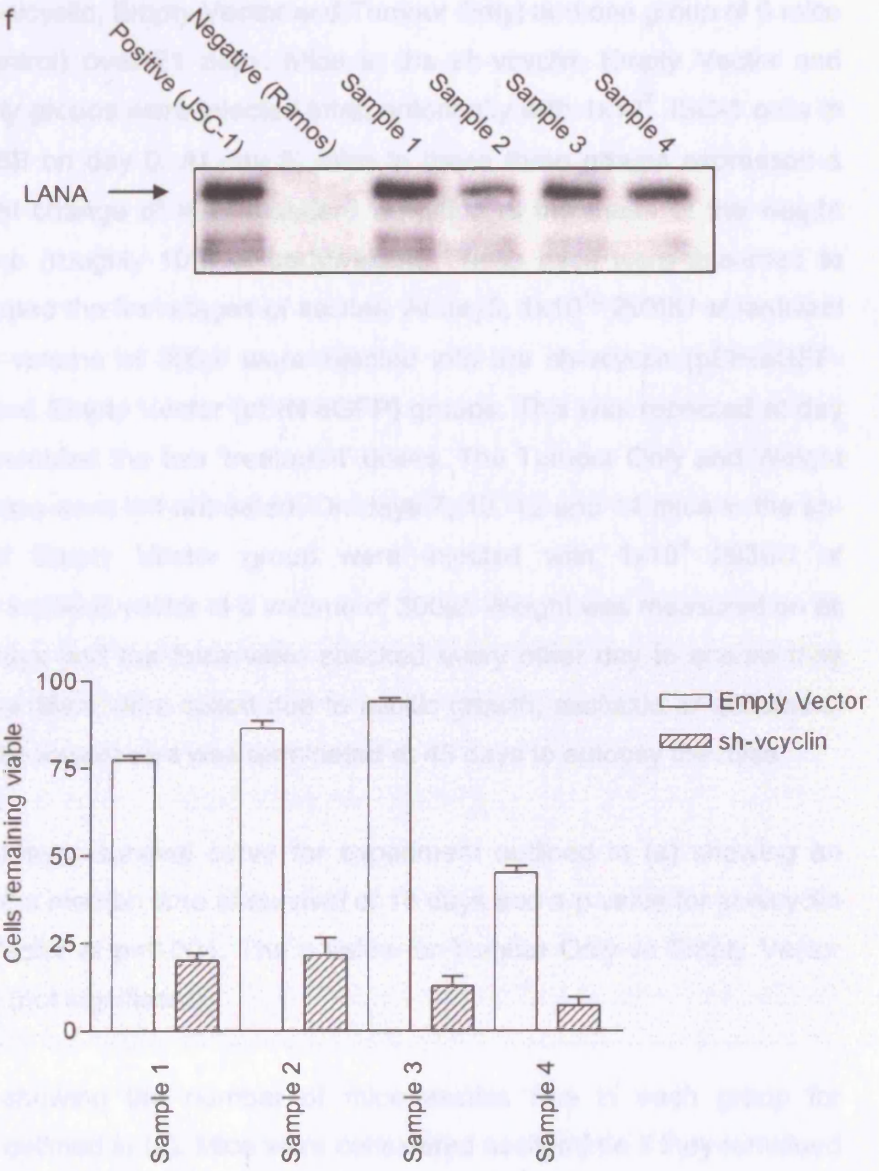


Figure 6.6 Increased dosage for potential therapeutic

(a) Graph showing the weight gain of mice (in grams) divided into 3 groups of 10 mice (sh-vcyclin, Empty Vector and Tumour Only) and one group of 6 mice (Weight Control) over 21 days. Mice in the sh-vcyclin, Empty Vector and Tumour Only groups were injected intraperitoneally with $1x10^7$ JSC-1 cells in 300μl of PBS on day 0. At day 3, mice in these three groups expressed a mean weight change of > 2x standard deviation of the mean of the weight control group (roughly 10% of bodyweight). These mice were assumed to have developed the first stages of ascites. At day3, $1x10^{10}$ 293tIU of lentiviral vector in a volume of 300μl were injected into the sh-vcyclin (pSINeGFP-shvcyclin) and Empty Vector (pSIN-eGFP) groups. This was repeated at day 5, and represented the two 'treatment' doses. The Tumour Only and Weight Control groups were left untreated. On days 7, 10, 12 and 14 mice in the sh-vcyclin and Empty Vector group were injected with $1x10^9$ 293tIU of appropriate lentiviral vector in a volume of 300μl. Weight was measured on all treatment days and the mice were checked every other day to ensure they were healthy. Mice were culled due to ascitic growth, cachexia or abdominal prolapse. The experiment was terminated at 46 days to autopsy the mice.

(b) Kaplan-Meyer survival curve for experiment outlined in (a) showing an increase in the median time of survival of 10 days and a p value for sh-vcyclin vs Empty Vector of p=0.004. The p value for Tumour Only vs Empty Vector was p=0.43 (not significant)

(c) Graph showing the number of mice ascites free in each group for experiment outlined in (a). Mice were considered ascites free if they remained asymptomatic with no significant weight gain (significant = > 2x standard deviation of the mean weight gain in weight control group) or mice who were culled for other reasons during the experiment (e.g. solid tumour). The graph represents the efficacy of the lentiviral vector at treating specifically ascites.

(d) Table showing the development of solid tumour and ascites of the mice in experiment (a) determined by autopsy. 'Ascites' indicates mouse had significant ascites or was culled due to ascites during the experiment. Solid tumour indicates a solid tumour was found outside the peritoneal cavity. Several mice had both ascites and a solid tumour, and are therefore recorded in both columns. The number of mice in each group was 10. There were 3 mice in the sh-vcyclin group alive at the end of the experiment, although all were found to have small extraperitoneal tumours.

(e) Table of analysis of four tumour samples taken from mice with solid tumours after autopsy. Samples 1 and 2 were taken from mice in the Empty Vector group, samples 3 and 4 from mice in the sh-vcyclin group. Analysis indicates the number of lentiviral inserts per cell found in the samples as determined by TaqMan qPCR using standard conditions (see section 2.1.12).

(f) Western blot of the samples taken in (e) for LANA. 10μg of cell lysates was equalized for protein concentration using the Bradford assay and Western blot for LANA performed as detailed in the materials and methods section. Results indicate that all four samples were positive for LANA by Western blot at levels comparable to the wild type cell (JSC-1).

(g) Graph showing the effect on cultures derived from the 4 tumour samples of vcyclin knockdown. The 4 samples were transported from the autopsy to a tissue culture facility. Two sections from each sample were they were agitated for 5 minutes in a trypsin/collagenase mixture. These cells were then placed in serum rich (20%) RPMI in an incubator at 5% CO_2 and at 37°c. Cells were placed both on adherent dishes and in suspension. Only the suspension cell cultures grew. The most viable culture for each sample was selected and then cultured as normal for suspension cells outlined in the materials and methods section. A sample of 10^5 cells from each sample was then exposed to an MOI of 10 293tIU of pSINeGFP-shvcyclin (sh-vcyclin) or pSIN-eGFP (empty vector) and the percent of cells remaining viable (determined by AnnexinV-FITC staining) at day 7 was assessed.

6.7 Discussion and conclusions

It is shown here that short hairpin RNA delivered using lentiviral vectors has potential to inhibit the growth of PEL in a murine model. All PEL are associated with latent gene expression (Dupin et al., 1999). These lymphomas are difficult to treat, being resistant to cytotoxic drugs and there are few therapeutic options. Unlike other KSHV associated morbidities, an animal model has been demonstrated for PEL (Boshoff et al., 1998; Staudt et al., 2004).

Reliable development of ascites using JSC-1 cells was relatively easy to achieve (Fig 6.1a). JSC-1 cells were chosen because they are amenable to infection with a lentiviral vector and are novel in this model. The use of girth measurement as an indicator of ascites proved unreliable.

The cellular component of the ascites was much denser than expected. Human effusions are mostly liquid, with a very limited cellular component driving the effusion by excreting inflammatory peptides whereas in this murine model the ascites are almost exclusively cells. This may represent an altered behaviour of these cells in the murine peritoneal cavity, however in both the human disease and the murine model it is these cells which are driving the effusion. The cellular density may also be a reflection of the immunodeficient nature of the mice, as it would be active immune cells in an immunocompetent host which inhibit cellular growth in the effusion. This is both a weakness in the model and a strength in the therapy- since the cellular component is targetted, an increase in cellular density represents more of a challenge. In culled mice with pure ascites, no intraperitoneal focus was found which might indicate the cells were shed from a primary site (although some had extraperitoneal solid tumours, see below). This implied the model is faithfully mimicking an effusion.

Lentiviral vectors are shown here to be efficient intraperitoneal gene delivery tools. Although this route has been shown effective for systemic gene

expression (Kootstra et al., 2003) there has been little quantitative documentation of the distribution of virus delivered in this fashion. Of concern for RNAi, where ~5-10 293tIU/cell are required to gain an efficient knockdown in gene expression, is the amount of virus which would be required. Production of this quantity of virus is difficult (Zhang et al., 2001; Pham et al., 2001; Ikeda et al., 2003) and the injection of large amounts of viral vector can be problematic for the patient (Marshall, 2002; Marshall, 1999). My results indicate that although lentiviral vectors do distribute throughout the animal, the majority is limited to the peritoneal cavity. This is ideal for our purposes, but can indicate that this administration route is less suitable for other applications. The use of tissue-specific promoters in lentiviral vectors (De Palma et al., 2003) combined with accurate knowledge of vector distribution from this and other studies (MacKenzie et al., 2002; Peng et al., 2001) would allow calculation of optimum dose and administration route for a wider range of applications. This study also therefore adds to the growing knowledge regarding the use of these vectors *in vivo*.

Unfortunately, there may be other hurdles with the use of lentiviral vectors for *in vivo* studies. VSVG pseudotyped lentiviral vectors are inactivated by complement (DePolo et al., 2000; Strang et al., 2004), and the immune response to transgene products in transduced cells (Ghazizadeh et al., 1999) will act when these vectors are used in immunocompetent subjects. Recently, there has been progress with both modifications of the VSVG envelope (Croyle et al., 2004) and the use of alternative viral envelopes (Strang et al., 2004; Strang et al., 2005; Guibinga and Friedmann, 2005) although these approaches have yet to be used *in vivo*.

These results indicate an overall benefit for tumour-bearing mice of treatment with a lentiviral vector encoding short hairpin RNA. RNAi has been used to target human viruses and human cancers, however this is the first demonstration that disruption of a single essential viral gene with shRNA has an *in vivo* survival benefit for virally-driven malignancy (Godfrey et al., 2004).

Co-inoculation of the vector and PEL cells prevented formation of ascites in four of six mice, and slowed the development in the other two. We injected JSC-1 cells in the midline and the virus on one side of the peritoneal cavity to show this as a genuine *in vivo* interaction, and a second injection to infect any cells which escape the first treatment. The delayed development in two mice implies that some cells may either escape infection or escape apoptosis. Analysis of the ascites of these two mice (Fig 6.3) indicate that these cells were escaping infection rather than developing a mutation in the vcyclin gene which made them immune to the RNAi. A higher initial dose of treatment or a longer course of treatment may be required to overcome this effect.

The vector was assessed as a therapeutic for established PEL. As a weight cut-of, three criteria were assessed. A weight gain of 10% of body weight, an absolute weight gain of 1 g or a gain of > 2 times the mean weight gain in the control group were looked at. In practice, all three criteria gave very similar working groups and 2x the mean weight gain in the control group was chosen. These criteria should ensure that ascites had developed and allow early treatment. The data regarding cell density (Fig 6.1c) showed that even this represented a large number of cells. Although the accuracy of determining engraftment based on a 1 g weight gain over 3 days may not be ideal, the necessity for early treatment to see the benefit forced this assumption. Even for the first experiment the treatment was shown to be insufficient, and this would have been compounded by waiting for a more significant weight gain.

During the course of the first experiment, the developing ascites did not behave as anticipated and although the treatment was insufficient for an effect, at least part of the apparent failure could have been due to misinjection of the PEL cells. This highlighted the need for accurate injection and for the use of healthy PEL cells to establish the model. One of the largest problems with the data set as a whole here is that there seems to be very variable growth velocity of PEL derived ascites. In Fig 6.1a, mice had gained approximately 5 g in 14 days. In Figure 6.3a, some mice had gained almost

triple that (14 g) in the same time frame and in 6.4b the mice had gained 25 g in 14 days. In our final figure, 6.4a, the mice gained 3-4 g in 14 days. These figures are hugely discrepant. The only explanation is that variations within the age of the mice (which were matched for each experiment, but not between experiments and were significantly older for that experiment) and the health of the cells injected may affect the outcome. It is also possible that unhealthy cells injected drive a much more fluid (exudate) based ascites, rather than a cellular one and therefore gave misleadingly large numbers. In retrospect, samples from the ascites of these mice would have been useful in determining this.

Another weakness in the model was several mice who developed solid tumours in the treated group. This was felt to be due to tracking of the JSC-1 cells out of the peritoneal cavity along the injection site. This happens for some human tumours in clinical settings, where biopsy sites can become routes of metastasis. The evidence from the later analysis of the solid tumours from the final experiment (Fig 6.6e,f) are indicative that the lentiviral vector may also have leaked out in this fashion, but it did not represent a fraction which was significant enough to inhibit the establishment of PEL. In the second experiment (Fig 6.6a) all injections were performed in the midline, and it was found that most (3 out of 4) of the sampled solid tumours occured along the midline.

Another question which was raised with the results of the first experiment was whether an autocrine function of the viral cyclin or viral FLIP meant that knockdown of the expression of this gene resulted in an alteration of the phenotype of the disease from an effusion to a solid tumour. This was ruled out with the second experiment where solid tumour development was shown to occur independently of the treatment received, but was masked by the rapid development of the ascites (Fig 6.6d).

The failure of the first experiment due to insufficient titre led to the investigation of high scale production of lentiviral vector. Previous reports

(Pham et al., 2001; Zhang et al., 2001; Kumar et al., 2003) all documented 'large-scale- production methods' which still seemed insufficient for the requirements of the experiment. Performing multiple harvests every few hours allowed these quantities of viral vector to be collected (Fig 6.5d,e).

Despite this, however, virion production still remains a serious barrier to the use of this vector as a treatment. Even if it is assumed as stated earlier that the human manifestation of this condition is much less cellular, the volumes involved are measured in litres, not mls, and therefore it can reasonably be assumed that all of the virus harvested across all of the experiments performed over 12 months would still be insufficient to treat one human case.

The decision to use a revised treatment plan was based upon many chemotherapy regimes, which involve large bolus treatments followed by maintenance doses. The rapid reversal in the observed weight gain was surprising. After injection with $1x10^{10}$ 293tIU of pSINeGFP-shvcyclin mice in this group dropped below their starting weight. There was some concern about the wellbeing of the mice because of this- it was presumed that the weight loss was due to a cachectic or metabolic effect in the treated mice. In support of this being a virus related (rather than cell death) issue, a similar but less pronounced effect was seen in the mice injected with empty vector. The mice did not seem to be affected in the long term, however, since by day 14 their weights had normalised to relevant controls (treated to weight only, untreated to tumour only). This may be an artifact of harvesting the virus in serum containing medium. The weight loss may also have been an effect induced by the injection of a concentrated serum containing mixture.

This may represent a serious and as yet undocumented problem for the use of lentiviral vectors for gene therapy. The quantities of vector that would be used in an *in vivo* therapeutic in patients are being approached in this study. Concerns of limitations of lentiviral vectors centre around immune responses, insertional mutagenesis, or recombination with wild type virus but little mention is made of the potential side effects such as this one.

Interestingly, even accounting for this effect, the virus acted much faster *in vivo* than it did *in vitro*. Some development of ascites and then resolution if the cells were unaffected until day 10 after infection was expected, whereas a complete absence of development of ascites was seen in this experiment. It is possible that the virus caused cellular arrest as well as inducing apoptosis. The conditions within the murine peritoneum in terms of growth factors and equivalent serum concentration are unknown, and it is possible that JSC-1 cells growing in this environment are much more susceptible to the effects of the knockdown. These data could be further investigated by examining the knockdown of vcyclin and vFLIP under different serum conditions *in vitro*.

After treatment was stopped, the mice were followed-up and three long term survivors were still apparently tumour free after 45 days. It was disappointing to find so many of the mice develop solid tumours, however this may reflect a failure of the administration of the tumour load not of the treatment itself. This does not mean this would have been less of a problem in a human disease setting. Treatment of the solid tumours was not attempted for two reasons. The first was a lack of viral vector – the requirements to generate a statistically significant result were beyond the means available and secondly it would be difficult to successfully distribute this virus across the tumour mass itself. Extra cavity solid tumours do represent an extremely rare complication of PEL (Chadburn et al., 2004) and may therefore warrant a further study involving this as a potential therapeutic, however this fell outside of the range of this study.

In summary, this chapter represents a novel approach to treating an animal model of primary effusion lymphoma using both novel technology and development of established technologies to successfully reach a goal. Several hurdles were overcome during this process, and several problems both within this study and for further studies were highlighted.

Chapter 7:
Conclusions

This thesis outlines the accomplishment of the goals set out both at the beginning and during the course of my PhD. The major accomplishments included:

- Demonstrating that a pSIN lentiviral vector is able to infect MSC and HSC with a high efficiency and does not affect their ability to differentiate *in vitro*.
- Develop a system for lentivirally delivered shRNA based around a human U6 promoter and show that this system functions in DMVEC, MSC and HSC (Clements et al., 2006).
- Choosing and evaluating targets for RNAi within the KSHV oncogenic cluster which have therapeutic potential (Godfrey et al., 2003) and demonstrating their efficacy *in vitro*.
- Showing the distribution of lentiviral vectors after *in vivo* injection in immunodeficient mice.
- Determining the quantity of lentiviral vector required to form a therapeutic and optimizing both production and concentration of vector to achieve this.
- Using lentivirally delivered RNAi as a therapeutic in a murine model of PEL (Godfrey et al., 2004).

Although there are now several commercial options for virally delivered RNAi, there were none when this work was initiated.

The lentiviral system I developed can be improved upon further. The requirement for a subcloning step to produce the lentiviral vectors is something which I would have liked to have resolved to produce a simpler vector which is easier to use. Unfortunately, there are no sites within the lentiviral backbone which would allow this without modification. Furthermore,

a lentiviral system where RNAi can be conditionally controlled, e.g. with tetracycline, will further expand the use of these vectors.

I have identified shRNA sequences against *vcyclin* and *vFLIP* of KSHV that prove very useful to induce apoptosis of primary effusion lymphoma (PEL) cell lines. This work indicates that targeting these viral transcripts could have significant therapeutic potential to treat PEL, and perhaps also other KSHV-induced tumours, e.g. KS and MCD. A more puzzling conundrum is the observation that targeting LANA does not induce apoptosis, and in fact PEL cells continue to proliferate when small amounts of LANA are present. As LANA is essential to maintain the viral episome, and also to tether KSHV episomes to chromatin during mitosis, this observation could indicate that the amount of LANA in PEL cells are redundant, and only a small amount of LANA is necessary for its essential functions, or it could indicate that KSHV has integrated into the host genome, as can happen in certain EBV cell lines. I did not resolve this issue during this PhD. It is also of great interest that the knockdown of LANA did not have the same effect in all PEL cell lines. LANA is known to suppress the activity of ORF50, the viral protein involved in initiating KSHV replication. A system that only partly suppresses LANA (as shown here with RNAi) may result in an increase viral load, by suppressing the inhibition of ORF50, and promoting lytic replication. This would imply that LANA might not be a suitable therapeutic target.

I collaborated with John Anderson at the Institute for Child Health to establish a murine model for PEL and to test the therapeutic potential in this model of lentiviral-delivered RNAi. These experiments proved encouraging, showing that the vector has potential as an *in vivo* therapeutic

Lastly, it should be noted that this work is being pursued by others: the lentiviral system for RNAi is now being used for several projects. There are plans to investigate further why there are differences between BC3 and JSC1 cell lines in their responses to LANA knock-down, and the effects of concurrent EBV infection on LANA knockdown.

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