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CONSTRUCTION AND DEVELOPMENT OF SEMLIKI FOREST VIRUS VECTORS FOR TRANSIENT GENE THERAPY OF MODEL TUMOURS IN MICE

A thesis submitted to the University of Dublin, Trinity College
For the Degree of Doctor of Philosophy

by

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December - 2010

FOR

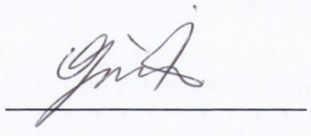
MY LATE GRANDPARENTS,
SEMİHA & MEHMET IRMAK

&

MY PARENTS

Declaration

This thesis is submitted by the undersigned to the University of Dublin, Trinity College for the examination of Doctorate of Philosophy. The work herein is entirely my own work with the exception of the processing and the interpretation of the brain and tumour tissue sections, which have been performed by Mrs. Alexandra Whelan-Buckley and Prof. Brian Sheahan, respectively (Veterinary Pathology Laboratory, University College Dublin). This study has not been submitted as an exercise for a degree to any other university. The librarian of Trinity College Dublin has my permission to lend or copy this thesis upon request.



Güniz İskender

Firstly, I would like to thank my supervisor Prof. Gregory Atkins for taking me as a summer project student in 2004 and then giving me the great opportunity to carry out this Ph.D. project in his group. It was an honour to work with you and to be your last Ph.D. student. Thank you very much for your excellent advices, great support and patience which helped me to come up to this stage. I am also grateful to Mrs. Chris Atkins for all the emotional hospitality and for showing me the beautiful sites of Ireland.

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Summary

Cancer is a molecular and genetic disease that results from multiple alterations of genes, including proto-oncogenes and tumour-suppressor genes. Virotherapy is a branch of cancer gene therapy where oncolytic viruses are used to treat cancerous cells. Semliki Forest virus (SFV) and its derived vectors (recombinant or replication-competent) have been utilised for the treatment of cancer by exploiting their inherent apoptosis-inducing ability or by expressing high amounts of therapeutic proteins.

In this study, the potential of a variety of replicating SFV vectors to induce apoptosis for tumour treatment was assessed. A second 26S subgenomic promoter and a multiple cloning site (MCS) have been cloned into the 5' end of the structural gene region in pSP6-SFV4 (the cDNA clone of SFV), creating RSFV-26SMCS. In an attempt to increase the biosafety of the replicating vector, an attenuating deletion (ΔTN) was introduced into the nsP3 (non-structural protein-3) gene region and the viral structural 6K gene was removed, creating RSFV-ΔTN-Δ6K-26SMCS. The pro-apoptotic *bax* gene was cloned into RSFV-26SMCS and termed RSFV-HABax-26SMCS. CT26 and K-BALB tumour cell lines, which readily form tumours in BALB/c mice, were utilised as rapidly growing poorly immunogenic tumour models.

The *in vitro* growth curve analyses of the replicating SFV vectors showed that the presence of the second 26S subgenomic promoter did not affect the replication rate of the virus as the vectors multiplied to similar titres as SFV4 in BHK-21 (baby hamster kidney-21) cells. However, viral titres were significantly lower in the CT26 and K-BALB cell lines. This was related to the low transduction efficiency of the tumour cell lines compared to the BHK-21 cell line. Whereas BHK-21 cells were completely destroyed following infection with the replicating SFV vectors, the SFV vectors exerted only a transient effect on the viability of CT26 and K-BALB cells and the cells continued to grow by 72 hours post infection (h.p.i.), which was attributed to the lower infection efficiency of the tumour cell lines. The induction of apoptosis in BHK-21, CT26 and K-BALB cells was confirmed by detection of active caspases, and the level of apoptosis was significantly lower in tumour cells compared to BHK-21 cells. RSFV-HABax-26SMCS virus was passaged eight times in the BHK-21 cell line and the stability of the Bax protein was confirmed for each passage by indirect immunofluorescence.

The virulence of the replicating SFV vectors was examined by intramuscular (i.m.) injection of BALB/c mice. Reduced virulence was observed following injections with RSFV-ΔTN-Δ6K-26SMCS virus (90%) or RSFV-HABax-26SMCS virus (70%) compared to SFV4 (40%) and RSFV-26SMCS virus (50%). To confer 100% protection in BALB/c mice, mice were immunized with rSFV VLPs encoding the p62-6K viral structural proteins (rSFV-p62-6K VLPs) prior to i.m. injections with the replicating SFV vectors. Prior immunization conferred protection against SFV, and all mice survived the i.m. injections. Brains of pre-immunized mice injected with SFV vectors were examined histologically, and appeared normal. In contrast, naïve, SFV4-inoculated mice showed brain lesions characteristic of the changes seen previously in SFV4 injected mice surviving to 14 days post infection (p.i.).

Our *in vivo* tumour treatment studies involved the intratumoural (i.t.) treatment of subcutaneous (s.c.) CT26 and K-BALB tumours in immunocompetent BALB/c mice with either TNE buffer alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus. Groups of mice were immunized with rSFV-p62-6K VLPs prior to tumour induction and treatment in an attempt to enhance the anti-SFV immune response and as a safety precaution to reduce mortality. Significant inhibition of CT26 tumour growth was observed in immunocompetent BALB/c mice, and complete and permanent tumour regressions were also found in all treatment groups. Growth of K-BALB tumours was significantly inhibited during the early stages of treatment (at day 15), following i.t. injections with RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus. However, this inhibition was not permanent, and none of the mice showed complete tumour regression. Histological examination of the CT26 and K-BALB tumours showed little evidence of an anti-tumour cellular immune response. The better response of CT26 tumours possibly occurred because the CT26 cells were less aggressive and grew at a slower rate following s.c. induction in BALB/c mice. CT26 cells also showed higher infection efficiency *in vitro* than the K-BALB cells. The significant inhibition of K-BALB tumour growth observed at the early stages of treatment was probably due to the slower growth and lower level of necrosis at that time compared with the later stages of the experiment. The inherent ability of SFV to induce apoptosis is of use in tumour therapy; however, combined therapy with conventional chemo- or radiotherapy may provide better results than gene therapy alone.

Oral Presentation:

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Iskender G, Kelly BJ, Galbraith SE, Sheahan BJ, Atkins GJ.

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'Development of novel and improved Semliki Forest virus vectors for RNA-based transient gene therapy'.

Iskender G, Galbraith SE, Kelly BJ, Callagy SJ, Sheahan BJ, Atkins GJ.

Page Number

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Chapter 5

aa	amino acid
AAV	adeno-associated virus
Ad	adenovirus
AIF	apoptosis inducing factor
Apaf-1	apoptosis protease-activating factor-1
APC	antigen presenting cell
BBB	blood-brain barrier
BH	Bcl-2 homology domain
BHK	baby hamster kidney
BSA	bovine serum albumin
bp	base pair
C	SFV capsid protein
CAD	caspase-activated deoxyribonuclease
CARD	caspase-recruitment domain
CDK	cyclin-dependent kinase
cDNA	copy/complementary DNA
CMV	cytomegalovirus
CNS	central nervous system
CPE	cytopathic effect
CTL	cytotoxic T-lymphocyte
DAPI	4, -6, diamidino-2-phenylindole
DC	dendritic cell
DD	death domain
DED	death effector domain
DISC	death-inducing signalling complex
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
d.p.i.	days post infection
ds	double-stranded

DTT	dithiotreitol
E. coli	*Escherichia coli*
EDTA	ethylenediaminetetra-acetic acid
EGFP	enhanced green fluorescent protein
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbant assay
ER	endoplasmic reticulum
FADD	Fas-associated death domain
FasL	Fas Ligand
FCS	foetal calf serum
FITC	fluorescein isothiocyanate
FLICA	fluorochrome-labelled inhibitors of caspases
FMDV	foot-and-mouth disease virus
G	gauge
g	gram(s)
g	gravitational force
GAP	GTPase activating protein
GCV	gancyclovir
GDP	guanosine diphosphate
GEFs	guanine nucleotide exchange factors
GM-CSF	granulocyte-macrophage colony-stimulating factor
G-MEM	Glasgow minimal essential medium
GTP	guanosine triphosphate
h	hour(s)
HA	hemagglutinin
HAI	hepatic arterial infusion
H & E	Haematoxylin and Eosin
HEPES	N-2-hydroxyethyl-piperazine-N'-2-ethanesulphonic acid
HIV	human immunodeficiency virus
HLA	human leukocyte antigen
h.p.i.	hours post infection
HPV	human papilloma virus
HSV	Herpes simplex virus
HtrA2	high-temperature requirement protein A2

IAP	inhibitor of apoptosis protein
ICAD	inhibitor of caspase-activated deoxyribonuclease
IFN	interferon
Ig	immunoglobulin
IL	interleukin
i.m.	intramuscular(ly)
i.n.	intranasal(ly)
i.p.	intraperitoneal(ly)
i.t.	intratumoural(ly)
IU	infectious units
Kb	kilobases
kbp	kilo basepairs
kDa	kilo Dalton
M	molar
MCS	multiple cloning site
MEFs	mouse embryonic fibroblasts
MFI	medium for infection
MHC	major histocompatibility complex
min	minutes(s)
ml	millilitre(s)
mm	millimetre(s)
mM	millimolar
MOI	multiplicity of infection
mRNA	messenger ribonucleic acid
MS	multiple sclerosis
NDV	Newcastle disease virus
NF-κB	nuclear factor kappa B
NK	natural killer cell
NSCLC	non-small cell lung carcinoma
nm	nanometre(s)
nsP	SFV non-structural protein
nt	nucleotide
NTR	nontranslated region
OMM	outer mitochondrial membrane

ORF	open reading frame
OV	oncolytic virus
PBS	phosphate buffered saline
PBS+	PBS with calcium, magnesium and sodium bicarbonate
PCR	polymerase chain reaction
PFA	paraformaldehyde
PFU	plaque forming units
p.i.	post-inoculation
PKR	interferon-inducible dsRNA-activated protein kinase
PSA	prostate-specific antigen
Rb	retinoblastoma
RNA	ribonucleoic acid
rpm	revolutions per minute
rSFV	recombinant SFV
RSFV	Replicating SFV
R.T	room temperature
RT-PCR	reverse-transcription PCR
s.c.	subcutaneous(ly)
SCID	severe combined immunodeficiency
sec	second(s)
SFV	Semliki Forest virus
SIV	simian immunodeficiency virus
Smac	second mitochondrial activator of caspases
ss	single-stranded
SV	Sindbis virus
TAA	tumour associated antigen
TBE	Tris-Borate-EDTA
TILs	tumour infiltrating lymphocytes
tk	thymidine kinase
TNF	tumour necrosis factor
TRAIL	TNF-related apoptosis-inducing ligand
ts	temperature-sensitive
U	units
VEE	Venezuelan equine encephalitis

VEGF	vascular endothelial growth factor
VLP	virus-like particle
VV	vaccinia virus
VSV	Vesicular stomatitis virus
wt	wild-type
µg	microgram(s)
µl	microlitre(s)
µm	micrometer(s)
µM	micromolar

Chapter 1

GENERAL INTRODUCTION

1 GENERAL INTRODUCTION

Cancer is generally regarded as a molecular disease caused by abnormalities in the genetic material of transformed cells leading to uncontrolled growth, invasion and sometimes metastasis. Most clinical research has focused on the aetiology of tumours and the host response. Currently, surgery is the primary method for treatment of malignant disease but has been shown to be inefficient in complete eradication of cancerous growths and in preventing the development of metastases. Toxicity associated with chemo- and/or radiotherapy, which are the additional treatment regimes, has led to the emergence of cancer gene therapy and cancer immunotherapy as promising candidates. Cancer gene therapy focuses on induction of apoptosis in cancerous cells or on stimulation of immune mechanisms to eliminate cancer cells. The advantages of viral vectors such as retro, adeno, adeno-associated, herpes viruses, poxviruses and alphaviruses in cancer gene therapy have been demonstrated (Lundstrom, 2001; Vähä-Koskela *et al.,* 2007; Atkins *et al.,* 2008).

Alphavirus expression systems are based on infectious clones, which are grown as plasmids in bacteria. These plasmids are transcribed *in vitro* to produce vector RNA that can be transfected into cells (Berglund *et al.,* 1993). Recombinant alphavirus vectors provide transient gene expression and limited penetration of tissue because they undergo only one round of replication (Liljeström *et al.,* 1991). However, replication-competent vectors allow longer term expression of foreign genes and are capable of replicating more than once (Atkins *et al.,* 2008). In a recent study, the potential of immune stimulation in combination with apoptosis induction by Semliki Forest virus (SFV) and its derived vector was investigated in two murine tumour models (Smyth *et al.,* 2005). The inherent ability of the SFV vector to induce apoptosis has already been exploited to treat tumours in BALB/c *nu/nu* mice (Murphy *et al.,* 2000, 2001).

Virulence of SFV is known to be polygenic, with nsP3, one of the non-structural genes in SFV genome, being the main virulent factor (Tuittila *et al.,* 2000; Tuittila & Hinkkänen, 2003). In a recent study, the wild-type replicating SFV (SFV4) containing in-frame deletions (SN and TN) in the nsP3 gene region showed reduced virulence *in vivo*. Deletion mutants also exhibited a slower replication rate when compared to SFV4 and the level of RNA synthesis was also reduced *in vitro* (Galbraith *et al.,* 2006). SFV4 was further modified by addition of a second 26S subgenomic promoter and a multiple cloning site (MCS) into the 5' end of the structural gene open reading frame (ORF), and

the vector was termed RSFV-26SMCS (*Dr. Sareen Galbraith, Pub. No.: WO/2007/102140*). To attenuate RSFV-26SMCS, the TN deletion was incorporated to the genome of the vector creating RSFV-ΔTN-26SMCS (*Dr. Sareen Galbraith, personal communication*). Another replicating SFV vector has also been constructed by complete deletion of the structural 6K gene (Δ6K) and named RSFV-Δ6K-26SMCS (*Dr. Sareen Galbraith, personal communication*).

In this study, the ΔSN mutation was reintroduced into RSFV-26SMCS and the Δ6K mutation was reintroduced into the RSFV-ΔTN-26SMCS mutant generating RSFV-ΔSN-26SMCS and RSFV-ΔTN-Δ6K-26SMCS, respectively. In addition, the pro-apoptotic *bax* gene was cloned into the MCS of RSFV-26SMCS, creating RSFV-HABax-26SMCS (*Ms. Jennifer Mulholland, project student supervised by Güniz Iskender*). The original (SFV4, RSFV-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS) and the newly constructed (RSFV-ΔSN-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, RSFV-HABax-26SMCS) replicating SFV vectors were then tested *in vitro* and utilized in the treatment of CT26 and K-BALB tumours in immunocompetent BALB/c mice. K-BALB cells are murine sarcoma virus transformed mouse fibroblasts that overexpress the K-*ras* oncogene and form aggressive localised syngeneic tumours in immunocompetent BALB/c mice upon subcutaneous (s.c.) injection (Aaronson & Weaver, 1971; Stephenson & Aaronson, 1972). CT26 tumours are murine colon adenocarcinoma cells, which form localised tumours of low immunogenicity in BALB/c mice after s.c. injection (Brattain *et al.,* 1980).

1.1 CANCER

The term "cancer" refers to a large group of diseases in which abnormal cells display uncontrolled growth and invade adjacent tissues, and sometimes spread to other locations in the body via lymph or blood systems. The development of cancer is dependant on a series of genetic changes in somatic cells, which involve the loss of differentiation, deregulation of cell growth/death, genomic instability, and changes in the relationships of cells with surrounding tissues (Michor *et al.,* 2004; Goymer, 2008). The genetic abnormalities can be due to defective genes inherited from parents, environmental poisons, radiation, dietary influences, viruses or a combination of all the

above. Benign tumours are encapsulated, do not grow in an aggressive manner and show limited spread into the surrounding tissue. Malignant tumours have the capability to dedifferentiate, invade the adjacent tissues and metastasise to sites in different parts of the body.

1.1.1 Genesis of a cancer cell

Cancer development is the result of a series of changes in the genetic material that regulates cell growth and differentiation. So far, more than 350 cancer genes have been identified (Futreal *et al.,* 2004; http://www.sanger.ac.uk/genetics/CGP/Census/) which accounts for over 1% of the genes in the human genome. The main genes which contribute to tumourigenesis are referred to as oncogenes and tumour suppressor genes.

Activation of proto-oncogenes to oncogenes, and inactivation of tumour suppressor genes are the two main genetic alterations that lead to cancer. Overexpression or inactivation of these genes can change the resistance/susceptibility of cells to apoptosis induction and the development of cancers (Zörnig *et al.,* 2001). Proto-oncogenes control normal cell division and differentiation, but can be converted into oncogenes as a result of point mutations, gene amplification, chromosomal translocation, or the action of viruses. Tumour suppressor genes control the cell cycle and promote apoptosis of an infected or damaged cell. Following mutation of tumour suppressor genes, the cell can progress to cancer, usually in combination with other genetic changes or environmental factors (Weinberg, 1994). Oncogenes, such as *ras* and *bcl-2* (B-cell lymphoma gene-2), undergo a mutational event leading to domain gain of function, whereas mutations of tumour suppressor genes, such as *p53*, are associated with recessive loss of function (Hanahan & Weinberg, 2000).

1.1.2 *Ras* oncogenes

The *ras* family of genes is frequently found to be mutated in human tumours. This family consists of three functional genes; H-*ras* (homologous to the oncogene of the Harvey murine sarcoma virus), K-*ras* (homologous to oncogene of Kirsten murine sarcoma virus) and N-*ras* (does not have a retroviral homolog and was first isolated from a neuroblastoma cell line), which encode highly similar proteins with molecular

weights of 21 kDa (Barbacid, 1987). Mutations in K-*ras* are most commonly associated with human disease (Bos, 1989).

The *ras* genes encode 21 kDa guanosine triphosphate (GTP) binding proteins (p21), which function as a key element in many signal transduction pathways by transferring information from the extracellular environment to the internal response machinery. *Ras* p21 proteins cycle between an inactive guanosine diphosphate (GDP)-bound and active GTP-bound state at the plasma membrane of normal cells (Satoh *et al.,* 1992; Boguski & McCormick, 1993). This cycle is regulated by guanine nucleotide exchange factors (GEFs) that drive the formation of active GTP-Ras and GTPase activating proteins (GAPs) which enable GTP hydrolysis and subsequently return the protein to its inactive (GDP-bound) state (Figure 1.1) (Ellis & Clark, 2000; Midgley & Kerr, 2002). Cell surface receptors such as granulocyte-macrophage colony-stimulating factor (GM-CSF) receptor, epidermal growth factor receptor (EGFR), platelet derived growth factor receptor (PDGFR), integrins and cytokine receptors such as Interleukin-2 (IL-2) influence the activity of GEFs and GAPs (Satoh *et al.,* 1992). *Ras* proteins also bind and activate several downstream cascades of protein kinases such as: Raf kinases, phosphatidylinoside 3-kinases (PI3K) and certain protein kinase C (PKC) isoforms (Karnoub & Weinberg, 2008), which are known to control a wide variety of cellular processes including growth, differentiation, apoptosis, proliferation, invasion, cytoskeletal organization and membrane trafficking (Shields *et al.,* 2000).

Mutated *ras* genes were first identified by their ability to transform NIH/3T3 cells after DNA transfection and the sites of activating point mutations in *ras* oncogenes were found to be in codon 12, 13, or 61, with those at codon 12 the most commonly found in *ras*-associated human malignant disease (Bos, 1989). These mutations cause the Ras p21 protein to become insensitive to the down-regulatory action of GAPs, hence locking the protein in the active state and thus stimulating growth or differentiation of the cell autonomously (Ellis & Clark, 2000). The incidence of mutated *ras* genes varies among the different tumour types, with pancreas (90%), colon (50%), thyroid (50%), lung (30%) and melanoma (25%) having the highest prevalence (Bos, 1989).

The correlation between uncontrolled cell proliferation and oncogenic *ras* is well defined but its influence on apoptosis is not fully determined (Cox & Der, 2003). Cell-type is believed to play a major role in the influence of oncogenic *ras*, with different cell types showing contradictory effects (Shields *et al.,* 2000; Giehl, 2005).

Oncogenic *ras* proteins have critical roles in mediating the malignant characteristics of cancerous cells and therefore have good potential as targets for tumour therapy (Downward, 2003; Saxena *et al.*, 2008).

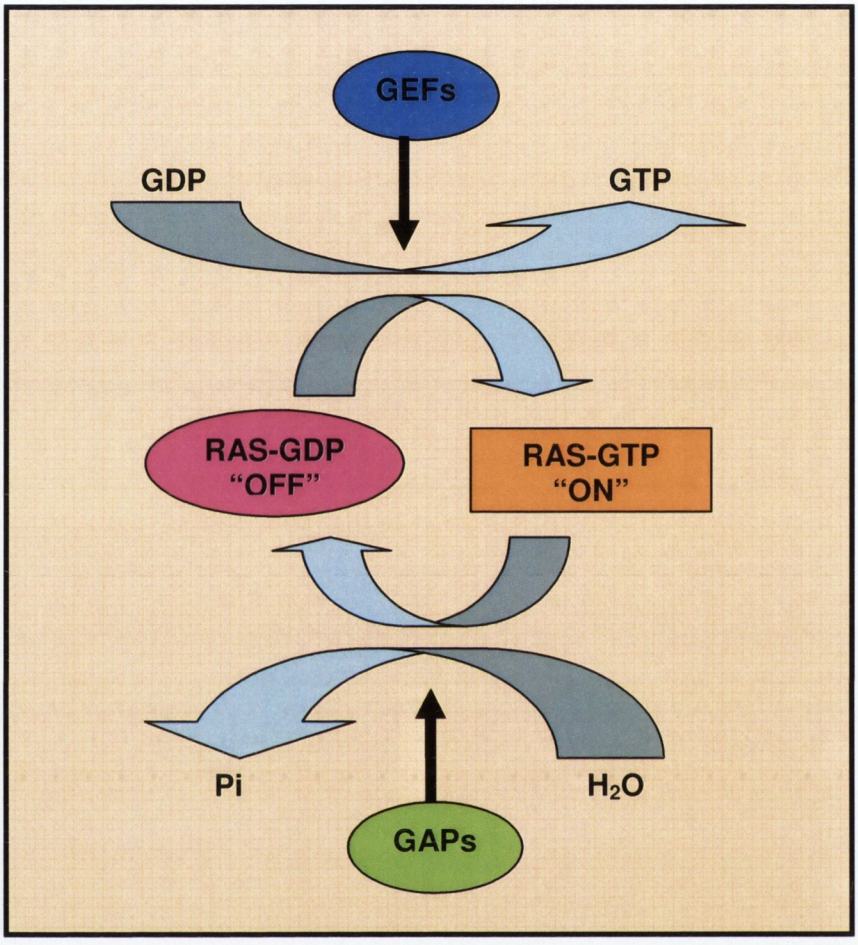


Figure 1.1 The Ras molecular switch

Ras proteins are situated at the inner surface of the plasma membrane and they receive activating signal from ligand stimulated membrane receptors. GEFs drive the exchange of Ras-bound GDP with GTP turning the "on-switch", while GAPs accelerate the rate of GTP hydrolysis to GDP, constituting an off-switch. Ras acts as a binary switch in many signaling pathways and its activation can lead to a variety of cellular responses.

GEF; guanine nucleotide exchange factor, GAP; GTPase avtivating protein, GDP; guanosine diphosphate, GTP; guanosine triphosphate

(Original diagram based on information from Midgley & Kerr, 2002)

1.1.3 Tumour suppressor genes, *p53* and Retinoblastoma (*RB*)

Tumour suppressor genes function to keep the cell under control and their inactivation plays a role in tumourigenesis. The *p53* gene is the first tumour-suppressor gene to be identified and 70% of human tumours contain a functionally inactivated copy of the *p53* gene (Hollstein *et al.,* 1991). The p53 protein has been demonstrated to be a phosphoprotein which functions as a transcription factor for the activation of a variety of cell cycle- and apoptosis-related genes (Levine *et al.,* 1991). Double stranded breaks in the chromosomal DNA such as that induced by ionizing radiation is sufficient to increase the levels of p53. Here the activation of the network depends on ATM (for ataxia telangiectasia mutated, named after a disease in which this enzyme is mutated) and Chk2 kinases, which phosphorylate the p53 protein, therefore prevent its binding with MDM2, the negative regulator of p53 (Carr, 2000). The *mdm2* gene is a transcriptional target of p53, and once synthesized the MDM2 protein can bind to the amino terminus of p53 leading to its degradation by ubiquitination (Oren, 1999). A variety of DNA damaging agents, including chemotherapeutic drugs, UV-radiation and protein kinase inhibitors were shown to activate the *p53* transcription, and post-translation modifications such as phosphorylation and acetylation result in its accumulation in the nucleus (Meek, 1999). However, aberrant growth signals resulting from overexpression of oncogenes, such as *E2F-1*, *myc* or *ras*, results in the accumulation of the $p19^{ARF}$ protein (human homologue is called $p14^{ARF}$), which then binds to MDM2 and inhibits its activity (Palmero *et al.,* 1998; Zindy *et al.,* 1998).

Following cell stress or damage, the p53 protein gets activated and blocks the cell-division cycle by stimulating the expression of $p21^{CIP1/WAF1}$, which in turn inactivates the cyclin-dependent kinases (CDKs) (Harper *et al.,* 1993). Through its negative effects on various CDKs, $p21^{CIP1/WAF1}$ shuts down the multiplication of stressed cells by inhibiting both the G1-to-S (Brugarolas *et al.,* 1995; Deng *et al.,* 1995) and the G2-to-mitosis transitions (Bunz *et al.,* 1998). Cell cycle arrest allows the cell time to repair its DNA. If repair is not possible, p53 induces programmed cell death through upregulation of death receptors such as DR5 and Fas, the pro-apoptotic protein Bax, and through inhibition of anti-apoptotic signals such as NF-κB (Vogelstein *et al.,* 2000).

The retinoblastoma (*RB*) gene, named after the rare childhood eye tumour in which it was discovered is one of the tumour suppressor genes that has been mapped to

chromosome 13q14 (Friend *et al.,* 1986). Mutations in both alleles of the *RB1* gene are a prerequisite for this tumour to develop (Knudson, 1971). Sporadic and familial forms of the disease occur in children under the age of five years where the malignant tumour affects only one eye (unilateral) or both of the eyes (bilateral), respectively. Aside from the retinoblastoma, inactivation of the *RB* gene results in observation of sarcomas, small cell lung carcinomas, bladder and breast carcinomas (Lee *et al.,* 1988; Horowitz *et al.,* 1990).

The *RB* gene encodes a 105 kDa nuclear phosphoprotein (pRB) that is involved in regulating the cell's progression through its growth cycle (Lee *et al.,* 1987). pRB switches between a hyperphosphorylated and hypophosphorylated state in a cell cycle-specific manner, and only the hypophosphorylated form of pRB is capable of binding to the cellular target molecules such as E2F transcription factor (Chellappan *et al.,* 1991; Herwig & Srauss, 1997). Phosphorylation of pRB by CDKs in the late G1 cell cycle phase causes it to lose grip on E2F therefore enabling the latter to bind to a number of host cell promoters and activate a cohort of client genes whose transcription it controls (Sherr, 1994). Studies showed that this pRB inactivation is a key molecular event leading to the S-phase commitment at the G1 restriction point in the cell cycle (Weinberg, 1995). The viral oncoproteins E1A (human adenovirus), large T antigen (Simian Virus (SV) 40), and E7 (human papilloma virus, HPV) were shown to bind to the hypophosphorylated form of pRB, inactivate the protein and therefore deregulate cell growth (Chellappan *et al.,* 1992).

The *RB* gene was also shown to regulate apoptosis by studies undertaken in knock-out mouse models. Cells in the central nervous system, lens and skeletal muscles of *RB*-null mouse embryos were demonstrated to undergo widespread apoptosis (Clarke *et al.,* 1992). Dysfunction of pRB leads to accumulation of p53 and subsequently to p53-induced apoptosis. Higher levels of E2F-1 transactivate expression of $p19^{ARF}$, the accumulation of which blocks MDM2, thus allowing the activation of the p53 network (Zhang *et al.,* 1998; Zhu *et al.,* 1999).

1.1.4 Tumour immunology

The possibility that the immune system could be exploited in cancer therapy was first conceived by Ehrlich (1909) following analysis of the relationship between the immune system and neoplastic disease. Further studies led to the 'immunosurveillance'

theory, that the immune system recognizes and destroys clones of transformed cells before they grow into tumours and kills tumours after they are formed (Thomas, 1959; Burnet 1970). However, there has been a growing recognition that immunosurveillance represents only one dimension of the complex relationship between the immune system and cancer. It has been shown that the immune system not only protects the host against tumour development but also promotes tumour growth by selecting for tumour escape variants with low immunogenicity (Uyttenhove *et al.,* 1983; Shankaran *et al.,* 2001). This has led to the development of the broader term "cancer immunoediting", which encompasses the host-protecting and tumour-sculpting functions of the immune system throughout tumour development (Dunn *et al.,* 2002).

Many normal protein antigens are known to represent human cancer antigens. Stoler et al (1999) estimated that about 11,000 genomic alterations occur in a cancer cell, and such genomic instability provides multiple opportunities for the development of cancer antigens by the overexpression of individual proteins, mutated proteins or proteins abnormally processed (spliced, glycosylated, phosphorylated, or lapidated) post-translationally (Finn, 2008). Tumour antigens can be classified as tumour specific antigens (TSA), when they are expressed only on tumour cells, and tumour associated antigens (TAA) that are also expressed on normal cells. A variety of TAAs have been identified which can be recognised by T-lymphocytes (Boon *et al.,* 1997; Rosenberg, 1999). These antigens form a heterogenous group which can be divided into five categories: (i) mutated antigens; normal proteins that contain a mutation or translocation that give rise to unique epitopes (such as β-catenin, CDK-4, and caspase-8), (ii) cancer-testis antigens; products of genes that are silent in most normal tissues (such as MAGE, BAGE, and GAGE), (iii) differentiation antigens derived from the tissue of origin (such as tyrosinase and carcinoembryonic antigen (CEA), (iv) products of oncogenes and tumour suppressor genes (such as Ras, Her-2/*neu*, and p53), and (v) viral antigens (includes HPV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) antigens) (Bremers & Parmiani, 2000; Rosenberg, 2001). Many tumour antigens are endogenously synthesised and presented as small peptides associated with the major histocompatibility complex (MHC) class I molecules on antigen presenting cells (APCs) to T lymphocytes. Both $CD8^+$ cytotoxic T cells and $CD4^+$ T-helper cells play a role in recognition of the tumour antigens and destruction of the tumour cells. $CD8^+$ cells recognise peptides of 8-10 amino acids (aa) in length that are digested in proteosomes and presented via the endoplasmic reticulum (ER) on cell-surface class I

human leukocyte antigen (HLA; the human analogue of the MHC) molecules. In contrast, $CD4^+$ cells react with peptides of 13-20 aa in length, derived from engulfed extracellular proteins that are digested in intracellular endosomes and presented on cell-surface class II HLA molecules (Toes *et al.,* 1999; Rosenberg, 2001; Wang, 2003, 2009). $CD8^+$ and $CD4^+$ T cells work in synergy to generate an effective cellular immune reaction against the tumour cells (Rosenberg, 2001).

The innate immune system is known to have some ability in the discrimination of tumour cells from normal cells (Diefenbach & Raulet, 2002; Hayakawa & Smyth, 2006). Natural killer (NK) cells, γδT-cells and macrophages induce the innate immune response to tumours through expression of the NKG2B receptor. The interaction of this receptor with a ligand promotes antitumour NK cell response and T-cell antigen receptor (TCR)-dependent T-cell activation (Bauer *et al.,* 1999). Ligands for NKG2B such as MHC class I chain related antigen (MICA), are up-regulated on transformed and virus-infected cells but not on normal cells (Groh *et al.,* 1996). Induction of antitumour adaptive immune responses has also been demonstrated via initial recognition of such ligands by the innate immune system (Diefenbach *et al.,* 2001). NK cells are also cytotoxic towards cells with a deficient expression of MHC class I molecules, and this pathway may be involved in the killing of tumour cells which downregulate MHC class I molecules (Ljunggren & Kärre, 1990; Garrido *et al.,* 1997). Hence, it could be stated that, MHC class I molecules serve as positive indicators for the integrity of cells, protecting against NK cell-mediated cytolysis (Diefenbach & Raulet, 2002). It is also known that NK cells function as regulatory mediators within the adaptive immunity system by secreting a wide variety of cytokines such as interferon-γ (IFN-γ), tumour necrosis factor-α (TNF-α), GM-CSF and IL-2. This cytokine profile activates the macrophages by attracting the helper T lymphocyte response, and thus influences the development of the adaptive immune system (Kos, 1998).

Antibody responses can contribute to target cell killing through complement-dependent cytotoxicity (CDC), and through antibody-dependent cellular cytotoxicity (ADCC), activating NK cells, γδT-cells and macrophages to exert their cytotoxic effects on tumour cells (Herlyn *et al.,* 1985; Mellstedt, 2000; Griggs & Zinkewich-Peotti, 2009).

Despite the presence of specific host immune responses, tumours persist and continue to grow by evading the immune system. This has led to recognition of the phenomenon known as 'tumour escape' (Marincola *et al.,* 2000). The mechanisms by

which tumour cells escape getting killed by the immune system include: (i) induction of tolerance to tumour antigens; (ii) development of tumour cells lacking antigens to which the immune system has responded; (iii) modulation of tumour antigen presentation; (iv) suppression of anti-tumour immunity; (v) poor immunogenicity of the tumour perhaps resulting from lack of expression of MHC class I molecules, and (vi) expression of Fas ligand (FasL) on tumours, which may induce apoptosis of effector cells. The emerging evidence for immune surveillance systems of carcinogenic events is counterbalanced by evidence that the normal immune response to tumour antigens is tolerance induction rather than activation (Rabinovich *et al.*, 2007).

The immune system applies a selective pressure on tumours (which are known to be genetically unstable) resulting in the natural selection of less immunogenic variants. The process of cancer immunoediting has been proposed to have three stages; *elimination*, *equilibrium* and *escape*. *Elimination* represents the recognition and destruction of the developing tumours by the cells of innate and adaptive immune system, which refers to the original immunosurveillance hypothesis. *Equilibrium* represents the process by which the immune system selects and/or promotes the generation of tumour cell variants with increasing capacities to survive immune attack, and *escape* represents a phase where the immunologically sculpted tumour variants expand in an uncontrolled manner in the immunocompetent host (Dunn *et al.*, 2002, 2004). Besides immunoediting, the induction of T-cell anergy and the peripheral location of many tumours should also be considered as the reasons for tumour escape (Pardoll, 2003; Lu & Finn, 2008).

1.2 CANCER THERAPY

The treatment of most malignancies is based on a combined approach involving surgery, radiotherapy and chemotherapy (drug therapy). However some tumours are inoperable or surgery may not be entirely successful. Additionally, the success of drug- and radiotherapy is often unsatisfactory with side effects and toxicities (Eckhardt, 2002). This has resulted in an extensive research for more efficient treatment regimens with less toxic effects.

Gene therapy was originally utilised to replace or correct defective genes in patients with inherited genetic disorders. However, to date, the majority of gene therapy

trials have concentrated on cancer gene therapy (66.5%) as an alternative way for transfer of genes encoding tumour antigens into host cells (Edelstein *et al.,* 2007). Several approaches have been studied by researchers under the context of cancer gene therapy. Expression of various tumour-suppressor genes in cancer cells in which these genes are defective, result in cell death by apoptosis or cell cycle arrest, suppressing the tumour growth (Vattemi & Claudio, 2007). Targeting the signalling pathways that are affected by activated oncogenes, thereby, inhibiting the oncogene expression, also results in cancer cell arrest (Saxena *et al.,* 2008). Suicide gene delivery is another group of cancer gene therapy applications in which viral vectors deliver enzymes that convert prodrugs to toxic products which kill tumour cells and their neighbours (Altaner *et al.,* 2008). The expression of angiogenesis inhibitors (Feldman & Libutti, 2000) and pro-apoptotic factors (Murphy *et al.,* 2001) can also be included in the group of suicide gene delivery. These approaches have had limited success, given the diversity of genetic mutations observed in cancers.

1.2.1 Gene Therapy

Gene therapy involves the manipulation of intracellular DNA to control and destroy malignant cells, and shows considerable promise as a treatment for patients with advanced or resistant carcinoma without systemic toxicity. The current strategies employed in gene therapy of cancer include targeting of oncogenes, tumor suppressor gene replacement, suicide gene therapy, drug resistant gene therapy, and inhibition of angiogenesis.

Oncogene inactivation: The downregulation of an activated oncogene could lead to the destruction of the malignant cell. One of the strategies used to inactivate the function of the activated oncogene includes the design of short single-stranded DNA oligonucleotides that would bind to specific oncogene promoter regions, form triple helix structures, and inhibit the transcription of oncogene into mRNA (Kim *et al.,* 1998). Antisense technology is another method used to abrogate the oncogene translation and involves the introduction into the cell of a gene construct that binds in a complementary manner to the mRNA targeted for inhibition. The advantage of this is the potential to achieve ablation of oncogene activity at the proximal level of mRNA splicing, transport or translation. This strategy has been used effectively for the downregulation of mutant K-*ras* protein in non-small cell lung carcinoma (NSCLC) cell

lines (Mukhopadhyay *et al.,* 1991). Ribozymes are specialized RNA molecules that function as catalysts for other RNAs (Gibson *et al.,* 2000) and are mainly used to inhibit the tumour specific oncogene expression. The advantage of ribozymes is that following degradation of the target mRNA molecule, the molecule becomes free to bind another molecule, thereby increasing the levels of oncogene ablation.

Tumour suppressor gene therapy: Inactivation of tumour suppressor genes lead to formation of cancerous cells with DNA mutations which continue to proliferate, become less differentiated and fail to undergo apoptosis. Viral vectors have the potential to deliver normal tumour suppressor genes in order to replace the defective gene. As *p53* is inactivated in up to 70% of human lung malignancies, a significant number of wild type (wt)-*p53* gene replacement protocols have been applied to lung cancers (Fujiwara *et al.,* 1994). Transfer of wt-*RB* gene by adenoviruses resulted in suppression of human glioma xenografts *in vivo* (Fueyo *et al.,* 1998).

Inhibition of angiogenesis: It is well established that solid tumours need an adequate blood supply with sufficient amount of nutrients and oxygen, and also newly developed blood vessels, in order to sustain their growth and metastasize (Folkman, 2002). Viral vectors have been used for the expression of anti-angiogenic factors such as angiostatin or endostatin (Nguyen *et al.,* 1998) or with genes that inhibit the activity of pro-angiogenic molecules, such as vascular endothelial growth factor (VEGF) (Millauer *et al.,* 1994). Monoclonal antibodies (Mab) specific for VEGF receptor-2 (VEGFR-2) have been designed and used in clinical trials. One of the most successful of these is Bevacizumab (Avastin, Genentech, San Francisco, CA, USA), and was shown to improve survival of patients with metastatic colorectal cancer in a phase III study (Ferrara *et al.,* 2004).

Suicide gene therapy: Suicide genes, the genes that sensitise cells to drugs that are normally non-toxic, are an alternative approach to the development of selective therapy (Moolten *et al.,* 1994). The most commonly used suicide gene is the thymidine kinase (*tk*) gene of herpes simplex virus (HSV-*tk*) (Jia *et al.,* 1994). The HSV-*tk* phosphorylates the systematically delivered antiviral drug, gancyclovir (GCV) into an intermediary metabolite, which is then converted by cellular kinases into a potent inhibitor of DNA polymerase, leading to cell death (Huang *et al.,* 2003). Normal cells remain unaffected by the drug. In a rat glioma model study, marked tumour regression was observed when only a small fraction (10%) of the cells was positively transduced by the vector (Freeman *et al.,* 1993). The transfer of multidrug resistance-1 gene

(MDR-1) into bone marrow cells has been explored as a method to provide increased protection from commonly used myelosuppressive chemotherapeutic agents (Sorrentino *et al.,* 1992).

1.2.2 Immunotherapy

Immunotherapy remains the largest field of cancer gene therapy, and encompasses a variety of strategies to stimulate the induction or potentiation of host immune responses against malignant cells (McCormick, 2001). Cancer immunotherapy primarily aims to restore reactivity of the host's immune system to combat the disease, to prevent metastatic spread and to improve the quality of life of the affected individual. Approaches that are utilised in immunotherapy are based on complementation or stimulation of the immune system via a plethora of compounds, such as cytokines, antibodies, vaccines, and *in vitro*-stimulated effector cells of the immune system (Chaudhuri *et al.,* 2009). Three different approaches have been devised for immunotherapy through the cellular immune system. First, the conditions for the function of the immune system could be improved without actually altering the specific effector cells themselves; for instance by the administration of cytokines like IL-2, IFN-α, IFN-γ, TNF-α and GM-SCF. A second approach involves the administration of a therapeutic vaccine to activate the patient's own immune system and to ensure an active cancer immunotherapy. The third approach, passive cancer immunotherapy, on the other hand, provides a tumour-specific immune response by supplying high levels of *ex-vivo* activated effector molecules, such as cytotoxic T lymphocytes (CTLs) or tumour-specific antibodies (Bremers & Parmiani, 2000; Schuster *et al.,* 2006; Rescigno *et al.,* 2007).

It is more than 100 years since the New York surgeon William B. Coley treated sarcoma patients by vaccination with bacterial toxins (also known as Coley's toxin) and obtained regression of solid tumours (Coley, 1896). This advance gave added significance to the area of immunotherapy and it is now known that the immune system is capable of attacking tumour cells if they are recognized in an immunogenic context.

Vaccination could successfully be achieved following the processing and presentation of TAAs via the host's APCs to the host's T cells. Initially, cancer vaccines were composed of autologous or allogenic whole cancer cells or cancer cell lysates (Ward *et al.,* 2002). Following the discovery of TAAs (Van der Bruggen *et al.,*

1991; Boon *et al.,* 1997), vaccines were based on the genetic identification of cancer antigens. Cancer vaccines can be divided into protein- or polypeptide-containing vaccines, whole cell cancer vaccines or viral vector vaccines (Rosenberg, 2001; Parmiani *et al.,* 2002; Finn, 2003; Rosenberg *et al.,* 2004; Palena *et al.,* 2006). In most approaches the tumour antigens have to be combined with strong adjuvants or cytokines (such as IL-2, IL-12 or GM-CSF) to induce an effective immune response (Pardoll, 1995; Rosenberg, 2001). The requirement for an "antigen presentation" step, which involves the presentation of TAA peptides *in vivo* by cells expressing MHC class II molecules (i.e. APCs), in the induction of strong anti-tumour reactive T cells, has been demonstrated (Huang *et al.,* 1994). Dendritic cells (DCs) are the most potent APCs in the body; these cells have been pulsed *in vitro* with TAAs or cancer cells and further exploited to actively immunize cancer patients, resulting in the formation of DC-based cancer vaccines (Gilboa *et al.,* 2007). However, an early study revealed that the antitumour mechanisms activated by DCs require cooperation between T-cell subsets and the expression of co-stimulatory molecules (such as B7-1), and Th1 cytokines (such as IL-2, IFN-γ, and TNF-α) (Zitvogel *et al.,* 1996). The anti-tumour efficacy of the DC-based cancer vaccines could also be enhanced by combining the injection of TAA-loaded DCs with the administration of cytokines such as IL-12 (Grohmann *et al.,* 1997).

Adoptive cancer immunotherapy, in other words, cellular immunotherapy involves the expansion of lymphoid effector molecules, such as lymphokine-activated killer (LAK) cells and tumour infiltrating lymphocytes (TILs), *in vitro* and infusion into the tumour. The fact that there is a well-documented correlation between the presence of TILs and improved prognosis of disease supports cellular immunotherapy as a viable strategy in the treatment of various cancer types (Clemente *et al.,* 1996; Zhang *et al.,* 2003; Galon *et al.,* 2006; Leffers *et al.,* 2009). Genetically engineered lymphocytes were also used to mediate regression in human patients with metastatic melanoma (Morgan *et al.,* 2006).

Following promising results in animal models, a large number of clinical trials have been completed in human beings. However the results have shown a lack of correlation between immune and clinical response (Ko *et al.,* 2003; Marincola *et al.,* 2003). Genetic variation in humans in comparison to inbred animal strains, a higher tolerance of certain antigens by the human immune system than the murine immune system, immune dysfunction in patients, and the advanced stage of disease in patients

selected for clinical trials have been offered as explanations for these disappointing results (Ko *et al.,* 2003; Rosenberg, 2004).

1.2.3 Viral vectors for cancer therapy

The use of viruses in the treatment of cancer has emerged as a viable approach as the limitations of the conventional anticancer therapies, using radio- and chemo-therapy, have been exposed (Eckhardt, 2002). With the discovery of recombinant DNA technology and the genetic mechanisms leading to cancer, viruses have been genetically engineered to safely target tumours. The initial approach in the gene therapy strategies was to construct replication-incompetent viral vectors where the regions coding for the structural proteins in the virus genome are deleted. Although these mutations eliminate both the possibility and the risk of a spreading infection by the virus, they also affect the efficiency of gene delivery to cancer cells *in vivo*. A variety of viruses have been adapted for gene therapy each with its own advantages/disadvantages depending on the particular application (Vähä-Koskela *et al.,* 2007). Properties of several viral vectors used in gene therapy are outlined in Table 1.1.

Adenovirus (Ad) is a linear, non-enveloped, double-stranded (ds) DNA virus which is capable of infecting dividing and non-dividing cells. Virus replication takes place in the host cell nucleus and no genome integration occurs. The 36 bp genome consists of immediate early (E1A), early (E1-E4), intermediate and late genes (L1-L5). High-titre adenovirus particles (up to 10^{12} PFU/ml) allow local, high efficiency, but transient transgene expression. The most frequently used adenoviral vectors are based on human adenovirus type 5 (Ad5). First generation adenoviral vectors were constructed by deleting the E3 and E1 regions, which enabled the insertion of ~8 kbp of foreign DNA (Danthinne & Imperiale, 2000). Second generation vectors were created by additional deletions in E1-E4 regions, to increase the biosafety and transgene capacity (Schaack, 2005). A study indicated that 55% of adult humans have pre-existing anti-Ad antibodies capable of neutralizing *in vitro* infection by Ad5 (Chirmule *et al.,* 1999). Therefore, adenoviral vectors with most or virtually all residual adenoviral genes deleted, called "gutless" vectors were constructed, which can accommodate inserts up to 30 kb and show reduced immunogenicity with longer-term expression of foreign genes *in vivo* (Schiedner *et al.,* 1998).

	Retrovirus	*Adenovirus*	*Adeno-associated virus (AAV)*	*Herpes simplex virus (HSV)*	*Vaccinia virus (VV)*
Genetic material	ss RNA	ds DNA	ss DNA	ds DNA	ds DNA
Transgene capacity	7 kb	7 – 10 kb	4.5 kb	30 kb	25 kb
Achievable titre	$10^6 - 10^7$ PFU/ml	10^{12} PFU/ml	10^4 PFU/ml	10^{10} PFU/ml	10^8 PFU/ml
Target cells	Dividing	Dividing & Non-dividing	Dividing & Non-dividing	Dividing & Non-dividing	Dividing & Non-dividing
Stability	Low	High	High	High	High
Immunogenicity	High	High	Low	Moderate	High
Expression	Long-term	Transient	Long-term	Transient	Long-term

Table 1.1 Comparison of gene transfer vectors

ds; double-stranded, ss; single-stranded, kb; kilobase, PFU; plaque forming unit.
(Adapted from Walther & Stein, 2000; Aghi & Martuza, 2005)

In 1999, the death of a patient by an inflammatory reaction to high-dose adenovirus based vector prompted researchers to develop strategies aimed at reducing the toxicities associated with viral vectors (Marshall, 1999; Raper *et al.,* 2003). Adenoviral vectors are the most commonly used vector today (24% of all trials worldwide) as cancer gene therapy agents (http://www.wiley.co.uk/genmed/clinical.html.,2009). A series of studies were undertaken and a number of clinical trials have published promising results of NSCL cancer treatment using the Ad-p53 (INGN 201) construct alone, or in combination with chemotherapy or radiation therapy (Swisher *et al.,* 1999; Nemunaitis *et al.,* 2000; Swisher *et al.,* 2003). The first gene therapy product, Gendicine, used for treatment of

head and neck squamous cancers, is based on adenovirus vector carrying the *p53* gene, and was approved for clinical use in China in 2004 (Peng, 2005). The replication-competent adenovirus construct ONYX-015 (described in more detail in section 1.3.2) exhibits marked cytopathic effects in cancer cells with inactive *p53*, but only limited cytotoxicity in normal epithelial and endothelial cells (Heise *et al.,* 1997). Engineered adenoviral vectors have been widely used as oncolytic agents and are explained in section 1.3.2.

Retroviruses are the second most commonly used vector in clinical gene therapy trials (21% of all trials worldwide) (http://www.wiley.co.uk/genmed/clinical.html.,2009). These vectors have high gene transfer efficiency and mediate prolonged gene expression by integrating their reverse transcribed DNA into the host cell genome (Miller *et al.,* 1990; Palù *et al.,* 2000). Despite the development of a leukaemia-like illness, related to a retrovirus vector integrating near the LMO2 proto-oncogene promoter and causing insertional oncogenesis (Hacein-Bey-Abina *et al.,* 2003; Marshall, 2003) in a recent X-linked severe combined immunodeficiency (SCID) gene therapy trial (Cavazzana-Calvo *et al.,* 2000), retroviruses continue to be used in virotherapy. In contrast to Ad vectors, most first generation retroviral vectors do not express residual viral genes and therefore do not elicit a strong immune response, which reduces their cytotoxicity and allows readministration (Mountain, 2000). On the other hand, the limiting factors for most retroviral vectors include their inability to transduce non-dividing cells and the inefficient production in high titres (Walther & Stein, 2000). Retroviral vectors have been shown to induce apoptosis and tumour regression following retrovirus-mediated exogenous wt-*p53* delivery (Fujiwara *et al.,* 1994; Roth *et al.,* 1996). Retroviral vectors have also been demonstrated to mediate an efficient and stable small interfering RNA (siRNA) expression (Barton & Medzhitov, 2002).

Similar to other retroviral vectors, vectors derived from lentiviruses such as HIV and simian immunodeficiency virus (SIV) stably integrate into the genome of transduced target cells to allow long-term expression of the foreign gene. Lentiviral vectors have the ability to infect both dividing and non-dividing cells including quiescent hematopoietic stem cells. Gene therapy studies with lentiviral vectors commenced following the discovery of the first split genome vector system for HIV-1-derived lentiviral vectors by Naldini et al (1996). Replication-deficient vectors based on HIV-1 (Pang *et al.,* 2001; Pellinen *et al.,* 2004) have been exploited for cancer gene

therapy studies. The first clinical trial for cancer utilising lentiviral vectors used a conditionally replicating HIV-1 vector, which expresses antisense RNA against the HIV-1 envelope gene transcript and results demonstrated successful gene delivery to patients´ T cells with good persistence *in vivo* with no evidence of insertional mutagenesis (Levine *et al.,* 2006).

Adeno-associated viruses (AAVs) are small single-stranded (ss) DNA parvoviruses and vectors based on AAV have been shown to have several advantages for gene therapy, including low pathogenicity, broad host range, stable expression of foreign proteins, and ability to replicate in both dividing and non-dividing cells (Monahan & Somulski, 2000; Li *et al.,* 2005). However, viral titres are relatively low (10^4 infectious units (IU)/ml) and production of AAV requires herpes- or adeno- viruses to provide missing structural proteins, which can lead to contamination of preparations with helper virus (Ferrari *et al.,* 1997). The size of AAV also limits the cloning capacity of the vector allowing for insertion of foreign genes ranging only from 4.1 to 4.9 kb (Dong *et al.,* 1996). AAVs possess an integrative capacity for a specific region of chromosome 19 and this enables them to establish latency when helper virus is absent (Samulski *et al.,* 1991). The mostly used AAV vectors in gene therapy area are based on AAV type 2 (AAV2). AAV vectors expressing wt-*p53* have shown great promise in mediating human lung tumour regression both *in vitro* and *in vivo* (Qazilbash *et al.,* 1997), and have also been utilised in various anti-tumour strategies for exogenous expression of anti-angiogenic factors (Nguyen *et al.,* 1998; Ma *et al.,* 2002). Recombinant AAV vectors have also been exploited to deliver immunomodulatory genes to enhance the immune response against cancerous growths. The growth of human colorectal cancers in mice was significantly inhibited following the delivery of AAV2 vectors expressing TNF-related apoptosis-inducing ligand (TRAIL/Apo2L) (Mohr *et al.,* 2004).

Herpesviruses possess a double-stranded linear genome of 150 kb, and can infect non-dividing cells such as neurons. Herpesvirus vectors have a large packaging capacity for insertion of foreign genes of 30 to 50 kb which allows them to be used in the simultaneous delivery of several therapeutic genes in cancer gene therapy (Latchman, 2001). The first replication-competent vector for cancer therapy was based on herpes simplex virus type-1 (HSV-1) F strain and shown to eradicate human glioma xenografts in nude mice (Martuza *et al.,* 1991). HSV replicating vectors used in oncolytic virotherapy have been described in section 1.3.2.

Vaccinia virus (VV) is an enveloped, dsDNA virus with a genome of approximately 200 kb. Their large genome allows the insertion of large genes for virotherapeutic approaches. Their replication is cytoplasmic thus eliminating the chance of genome integration. Vaccinia virus is best known as the first widely used vaccine against smallpox, therefore it has been mostly used in tumour vaccination studies or for transfer of immune-regulatory genes (Qin *et al.,* 2001; Mastrangelo & Lattime, 2002; Arlen *et al.,* 2006). Non-engineered VV has been exploited by intratumoural injection to patients with metastatic melanoma and no serious adverse events were reported (Hunter-Craig *et al.,* 1970; Mastrangelo *et al.,* 1995). Engineered VV vectors have also been constructed and utilised in the area of oncolytic virotherapy and are discussed in section 1.3.2.

Alphaviruses have also recently gained recognition as having strong potential in the field of cancer gene/immunotherapy and are discussed in section 1.6.

1.2.4 Non-viral vectors

Non-viral gene transfer systems were initially developed due to their ease of manufacture and lower toxicity and immunogenicity compared with their viral counterparts. Techniques include direct injection of naked DNA by a physical method, such as gene gun, or transfection across the cell membrane using liposomes, peptide delivery systems, or polymer vectors. Naked DNA vectors result in only very transient gene expression, is rapidly degraded and effects are only limited to the site of injection (Niidome & Huang, 2002). However, immunization using a prostate specific membrane antigen (PSMA) DNA plasmid inserted into a replication-deficient adenoviral expression vector resulted in changes in prostate-specific antigen (PSA) levels, local disease, and distant metastases in prostate cancer patients (Mincheff *et al.,* 2000). In cationic lipids/polymer delivery, the nucleic acid is complexed with molecules that facilitate its delivery to cells by way of electrostatic interactions (Felgner *et al.,* 1987).

1.3 VIRAL ONCOLYSIS

Cancer virotherapy utilises oncolytic viruses (OVs), in other words tumour-specific, self-replicating and lysis-inducing cancer killers, to find and destroy malignant cells. Following the regression of cervical cancers in some patients who had received live attenuated rabies virus vaccines in the early 1920s, the prospect of using replicating viruses in the treatment of cancer was raised (Dock, 1904; De Pace, 1912). It was during the 1950s that cancer virotherapy truly began to gain significance, and a number of human clinical trials were undertaken with viruses such as Newcastle disease virus (NDV) (Flanagan *et al.,* 1955), adenovirus (Huebner *et al.,* 1956), and influenza virus (Sinkovics & Horvath, 1993). Similar experiments were reported up to the early 1980s by which time the oncolytic potential of mumps virus (Asada, 1974; Okuno *et al.,* 1978), measles virus (Bluming & Ziegler, 1971; Taqi *et al.,* 1981) and reovirus (Hashiro *et al.,* 1977) had also been examined.

Developments in recombinant DNA technology allowed for the manipulation of viruses at a molecular level and their exploitation as gene delivery vectors. The initial studies were carried out by replication-deficient vectors. Following the first description of a virus engineered to replicate selectively in dividing cells (Martuza *et al.,* 1991), the field of viral therapy for cancer has expanded dramatically. Several virus types gave promising results in preclinical studies, and different virus species have entered or completed clinical trials (Everts & van der Poel, 2005; Edelstein *et al.,* 2007). Oncolytic viruses can be divided into two groups: (i) naturally occuring oncolytic viruses and (ii) genetically engineered tumour-selective viruses. Irrespective of whether the oncolytic virus has an inherent selectivity for cancer cells or has been genetically modified to replicate selectively in tumour cells, a genetically stable virus is desirable from both safety and manufacturing aspects. Prevention of viral replication in normal tissues is also critical and a secondary mechanism to inactivate the virus should be available. Finally, the virus must be amenable to high-titre production and purification for clinical studies.

1.3.1 Naturally occurring oncolytic viruses

Many oncolytic viruses have an inherent ability to grow in tumour cells as the cellular changes induced by viral infection often resemble the cellular changes acquired

during carcinogenesis. These viruses are mainly dependent on *ras*, interferon-activated protein kinase R (PKR), and interferon activity, and it is through these pathways that their tumour specificity is achieved (Vähä-Koskela *et al.*, 2007).

Reoviruses (Respiratory Enteric Orphan viruses) are non-enveloped, human RNA viruses which were shown to propagate preferentially in tumour cells with an activated *ras* pathway as high levels of *ras* oncogene and its downstream effects can inhibit PKR activity (Coffey *et al.*, 1998). Absence of a method to repress PKR activity restricts the replication of the virus to cells with absent or *ras*-inhibited PKR activity (Strong *et al.*, 1998). As *ras* is the most common oncogene to undergo activating mutations in human cancer (Bos, 1989), the anti-tumour effect of reovirus has been tested in cancer of the human breast, pancreas and lymphoid tissue (Alain *et al.*, 2002; Norman *et al.*, 2002; Etoh *et al.*, 2003; Hata *et al.*, 2008). Encouraging results have been obtained in a number of phase I studies employing Reovirus (Reolysin®; Oncolytics Biotech Inc. Corporation Canada, Calgary, AB) in the treatment of metastases from systemic cancer, prostate cancer, and advanced malignancies, including glioblastoma multiforme (Forsyth *et al.*, 2008; Yap *et al.*, 2008; Gollamudi *et al.*, 2009; http://www.oncolyticsbiotech.com/clinical.html). In a recent study, the combination of reovirus and cisplatin chemotherapy synergistically enhanced cytotoxicity in murine and human melanoma cell lines *in vitro*, and also delayed the melanoma tumour growth *in vivo* and increased the survival time (Pandha *et al.*, 2009).

NDV is an avian paramyxovirus with an ssRNA genome and it exerts oncolytic activity in tumour cells containing defects in the IFN system (Krishnamurthy *et al.*, 2006). The live attenuated 73-T strain of NDV was first used in 1965 as an anti-tumour agent to treat cervical carcinoma (Cassel & Garrett, 1965), and has been shown to induce significant regression in different tumour cell lines and mouse tumour models (Lorence *et al.*, 1994; Phuangsab *et al.*, 2001). 73-T has also been studied as a viral oncolysate, which is the suspension of virus and tumour cells, to target melanoma (Cassel & Murray, 1992; Sinkovics & Horvath, 2000). Another naturally attenuated strain of NDV, PV701 (Wellstat Biologics, Gaithersburg, MD), has been tested in a phase I clinical study in patients with advanced solid tumours (Pecora *et al.*, 2002; Lorence *et al.*, 2007), and phase I/II clinical studies with the NDV-HUJ oncolytic virus commenced for the treatment of recurrent glioblastoma multiforme (Freeman *et al.*, 2006).

Vesicular stomatitis virus (VSV), a rhabdovirus with a negative-stranded RNA genome, shows a viral tropism towards cancer cells that have a malfunctioning IFN-pathway to make replication possible (Stojdl *et al.,* 2000). The oncolytic ability of VSV, either the live attenuated strain or the recombinant vector engineered to express cytokines, has been confirmed in several *in vivo* animal tumour models like hepatocellular carcinoma, metastatic breast carcinoma and melanoma (Balachandran *et al.,* 2001; Fernandez *et al.,* 2002; Ebert *et al.*, 2003, Shinozaki *et al.,* 2004). Point mutations in the matrix (M) protein of VSV have been shown to have an increased ability to induce IFN production which maintains effective virus replication and killing of tumour cells (Stojdl *et al.,* 2003). VSV strains containing mutations in the M protein have been shown to be effective and safe oncolytic agents against invasive malignant gliomas and metastatic breast carcinomas in animal models (Ebert *et al.,* 2005; Lun *et al.,* 2006).

Sindbis virus (SV), and its derived vector, have also been shown to kill several cancer cell lines *in vitro* and to cause regression of *in vivo* animal tumour models (Tseng *et al.,* 2002, 2004). Recently, the replication competent (RC) SV vector modified to express the suicide HSV-*tk* gene, prevented uncontrolled vector propagation, and enhanced tumour killing *in vivo* in combination with the GCV drug (Tseng *et al.,* 2009).

1.3.2 Engineered oncolytic viruses

Apart from manipulating naturally occurring oncolytic viruses, it is now possible to genetically modify viruses which do not normally display oncotropism to selectively infect and kill tumour cells. Viruses can be manipulated at the molecular level to render them less pathogenic, and confer selective replication in cancer cells. Selective replication can be achieved through the deletion of active genes or functional gene regions that are necessary for efficient replication and/or toxicity in normal cells but are dispensable in tumour cells.

Adenoviruses have been extensively used as oncolytic agents (Kirn, 2000). ONYX-015, also known as *dl*1520 is a modified Ad lacking the 55 kDa E1B protein. It was the first engineered replication-selective virus to be used in humans. This genetically engineered virus was believed to replicate only in cells lacking functional *p53* (Bischoff *et al.,* 1996). However, later studies showed that ONYX-015 was

cytopathic even in the presence of *p53* (Goodrum & Ornelles, 1998) and that tumour selectivity of ONYX-015 depends on the late Ad mRNA export from the nucleus (O'Shea *et al.,* 2004). The ONYX-015 engineered adenovirus vector has been used in Phase I and II trials for head and neck, ovarian, pancreatic, hepatocellular and metastatic colorectal carcinomas and malignant gliomas using a variety of injection routes (Alemany, 2007). Treatment of squamous cell carcinomas of the head and neck with ONYX-015 yielded the most encouraging results (Ganly *et al.,* 2000; Kirn, 2001; Nemunaitis *et al.,* 2001). Another Ad mutant, termed Δ24, carrying a 24 bp deletion in the E1A region which is responsible for binding *RB* and driving the infected cell into S-phase, has been shown to replicate efficiently in cells lacking the pRB family (Fueyo *et al.,* 2000). The Δ24 virus mutant shows promise as anti-cancer agents but has not yet been tested in clinical trials. ONYX-015, Δ24, and other modified adenoviruses show an increased rate of oncolysis when combined with conventional therapeutic agents in the treatment of a variety of cancers including pancreatic carcinoma and glioblastoma (Khuri *et al.,* 2000; Lamfers *et al.,* 2002; Reid *et al.,* 2002; Hecht *et al.,* 2003; Nemunaitis *et al.,* 2003; Chiocca *et al.,* 2004). CG7060 and CG7870 (Cell Genesys, South San Francisco, CA, USA) are two transcriptionally targeted adenoviruses for prostate cancer which contain PSA gene promoter-enhancer elements upstream of the E1A gene. CG7870, unlike CG7060 contains a wild-type E3 region, which significantly increased cell killing and oncolytic efficacy (Yu *et al.,* 1999). CG7060 was also shown to preferentially replicate in PSA-producing tissues (Rodriguez *et al.,* 1997), and the virus was tested in patients with locally recurrent prostatic carcinoma by intraprostatic injection following primary radiotherapy (DeWeese *et al.,* 2001). Intravenous (i.v.) CG7870 was also evaluated in a phase I trial for treatment of metastatic prostate carcinoma and transient minor prostate-specific antigen decreases were observed (Small *et al.,* 2006). An oncolytic Ad similar to ONYX-015, termed H101 is the first oncolytic virus vector approved for commercial use in China for treatment of head and neck squamous cell carcinoma in combination with chemotherapy (Xia *et al.,* 2004; Garber, 2006).

HSV-*tk* (*dl*sptk) was the first genetically engineered oncolytic virus in which the *tk* gene was deleted from the genome of HSV-1 (Martuza *et al.,* 1991). This mutant was shown to replicate selectively in glioma cell lines and was used to treat human glioma xenografts in BALB/c *nu/nu* mice (Martuza *et al.,* 1991). To reduce the possibility of reversion to wild-type strains and to restrict the replication of the virus in the adult

central nervous system (CNS), herpes viruses have been engineered to contain mutations in the main neurovirulence gene gamma-34.5 (*γ34.5*). HSV *γ34.5*-deleted mutants have been shown to replicate at a high rate in cells with activated mitogen-activated protein (MAP) kinase or extracellular signal-regulated kinase (ERK), which in turn inhibits the IFN/dsRNA-dependent PKR response mechanism (Smith *et al.,* 2006). Four viruses with deletions in one or both copies of the main virulence *γ34.5* gene have been tested in patients: G207, 1716, NV1020 and OncoVEX$^{GM\text{-}CSF}$ (BioVex Ltd, Abingdon, UK). HSV-1 mutant, G207, which has deletions in both copies of the *γ34.5* gene and contains a *Lac*Z gene insertion in the ICP6 (ribonucleotide reductase subunit) region was found to display antitumour efficacy in both *in vitro* and *in vivo* models of malignant gliomas (Mineta *et al.,* 1995) and was the first HSV vehicle to enter clinical trials (Markert *et al.,* 2000). The HSV-1 1716 virus, a recombinant based on HSV-1 strain 17 and deleted for the *γ34.5* gene was administered to glioblastoma and melanoma patients by intracavitary (post-operative) or intratumoral injections (MacKie *et al.,* 2001; Papanastassiou *et al.,* 2002; Harrow *et al.,* 2004). The NV1020 virus which has a deletion in one of the two copies of the *γ34.5* gene was originally developed as a vaccine. Promising results have been published from a phase I trial of NV1020 delivered by hepatic arterial infusion (HAI) in HSV sero-positive patients with colorectal liver metastases (Kemeny *et al.,* 2006). An ongoing phase I/II trial is evaluating repeat HAI of NV1020 in combination with chemotherapy in patients with colorectal cancer. The oncolytic HSV vector OncoVEX$^{GM\text{-}CSF}$ has been generated with deletions in *γ34.5* (to reduce pathogenicity) and ICP47 (to restore MHC I presentation) in combination with the GM-CSF transgene insertion. A phase I trial of intratumoral injection of OncoVEX$^{GM\text{-}CSF}$ into cutaneous metastases from solid tumors and melanomas showed promising results (Hu *et al.,* 2006). The outcomes of the clinical trials were promising when the HSV-1-derived oncolytic mutants were administered both alone and in combination with chemotherapy or radiotherapy (Prados *et al.,* 2003; Kanai *et al.,* 2010).

Influenza virus, a well-known human pathogen, engineered to lack the virulent NS1 gene, has been shown to have oncolytic specificity in tumour cells with defective IFN pathways (Bergmann *et al.,* 2001). The oncolytic action of the NS1-deleted influenza virus was demonstrated in IFN-resistant tumour cells and in a subcutaneous tumour model in SCID mice (Muster *et al.,* 2004).

The initial vaccinia virus gene deletion mutant, with an inactivated *tk* gene, gave a higher rate of replication in tumour cells compared to normal cells (Puhlmann *et al.*, 2000). The tumour specificity of this vector was enhanced following removal of the vaccinia growth factor (VGF) gene, which enabled the virus to be dependent on cancerous tissues that have large amounts of nucleotides available (McCart *et al.*, 2001). The novel replication-competent VV vector JX-594 (Jennerex Biotherapeutics, San Francisco, CA) is based on strain Wyeth with a deletion in the *tk* gene that expresses GM-CSF. The *tk* deletion was shown to improve the cancer-selectivity in patients with metastatic melanoma, as demonstrated in a phase I trial (Mastrangelo *et al.*, 1999). Recently, JX-594 vector was demonstrated to induce oncolysis of tumours and metastases in syngeneic models in both rats and rabbits following i.v. administration (Kim *et al.*, 2006).

The cytolytic potential of naturally occurring or engineered oncolytic viruses can be further enhanced through the expression of heterologous genes encoding cytotoxic proteins, drug-sensitising factors, or cytokines (Cattaneo, 2008).

1.4 APOPTOSIS

The term "apoptosis" was first used by Kerr et al in 1972 to describe a morphologically distinct form of cell death observed by electron microscopy. It is a naturally occurring process of cell suicide that plays a major role in the development and maintenance of metazoans for eliminating unwanted or superfluous cells (Jacobson *et al.*, 1997; Vaux & Korsmeyer; 1999). Apoptosis is also utilised as a method of cell killing by the immune system, in cytotoxic immune responses, lymphocyte deletion during maturation, and in the resolution of immune responses through the elimination of effector cells (Henkart & Sitkovsky, 1994; Strasser, 1995). Inappropriate regulation of apoptosis can lead to development of a variety of diseases, including cancer, acquired immunodeficiency syndrome (AIDS), autoimmunity, neurodegeneration, and ischaemic stroke (Thompson, 1995).

The morphological changes that occur during apoptosis include cell shrinkage, chromatin condensation, plasma membrane blebbing and separation of cell fragments into intact vesicles (apoptotic bodies) during budding. Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without nuclear fragments that are

maintained within an intact plasma membrane. These vesicles are quickly phagocytosed by monocytes and neighbouring cells, preventing secondary necrosis. The cellular contents of the apoptotic bodies are not released into the surrounding tissue, and this prevents the induction of an inflammatory reaction (Hockenbery, 1995; Savill & Fadok, 2000).

In contrast to apoptosis, necrosis is the passive form of cell death that is not under cellular control and is characterised by cellular swelling and rupture with the release of cellular contents into the surrounding environment, evoking inflammation and damage to the surrounding tissue (Majno & Joris, 1995; Raff, 1998). It is associated with a variety of cell injuries including physical, chemical, thermal and hypoxic insults.

1.4.1 Caspases, the executioners of apoptotic cell death

Caspases are a family of cysteine aspartic-acid specific proteases, which are widely expressed in an inactive proenzyme form (zymogen) in healthy cells. Upon activation, they play a central role in the initiation and execution of apoptosis and in the processing of proinflammatory cytokines (Wolf & Green, 1999; Logue & Martin, 2008). Caspases share many similarities in amino acid sequence, structure, and substrate specificity (Nicholson, 1999). The procaspase is comprised of a large subunit (~ 20 kD), a small subunit (~ 10 kD), and an NH_2-terminal domain which is removed during activation (Thornberry & Lazebnik, 1998). Upon activation, the zymogen is autoproteolytically cleaved at specific aspartate residues. Following this cleavage, the large and small subunits are separated, and the N-terminal prodomain is removed. Cleaved subunits associate to form heterodimers, and two of these heterodimers combine to form a tetramer with the two active catalytic sites residing at opposite ends of the complex and functioning independently (Walker *et al.,* 1994; Wilson *et al.,* 1994; Rotonda *et al.,* 1996).

To date, 14 mammalian caspases have been identified, although the specific function(s) of some individual caspases have not yet been fully discovered. The caspase family can be divided into two subgroups: those that are involved in apoptosis (caspase-2, -3, -6, -7, -8, -9, and -10) and those that are activated during inflammatory responses (caspase-1, -4, -5, and -11). Caspases that are involved in apoptosis can be further sub-divided into upstream (initiator) caspases (2, 8, 9, and 10) and downstream (effector)

caspases (3, 6, and 7). Initiator caspases respond to proapoptotic signals and subsequently induce the activation of effector caspases (Logue & Martin, 2008).

Heterogeneity exists between the initiator and effector caspases, regarding the structure of the prodomain. The long N-terminal prodomains of the initiator caspases contain caspase-recruitment domains (CARDs) (caspases -2 and -9) or death effector domains (DEDs) (caspase -8 and -10), which structurally differentiate them from the effector caspases. These prodomains function as protein-protein interaction motifs to assist the binding of the initiator procaspases to adaptor molecules which promote caspase aggregation and activation through an autoproteolytic process termed "proximity-induced intermolecular processing" (Earnshaw *et al.,* 1999; Kumar, 1999). Apoptosis occurs as the final stage of the 'caspase cascade' where activation of initiator caspases directly induces the activation of effector caspases. Initiator and effector caspases can activate a variety of other caspases (including those upstream of themselves), thus providing several positive feedback loops and as a result, amplifying the death signal (Slee *et al.,* 2001).

In contrast to initiator caspases, effector caspases have short or absent prodomains and get activated by other caspases rather than autoproteolysis. Upon activation, effector caspases direct the formation of apoptosis through disruption of cellular structures and cellular metabolism, inactivation of apoptosis inhibitory proteins (AIPs), and the activation of additional destructive enzymes (Adrain & Martin, 2001; Creagh *et al.,* 2003). So far over 400 substrates have been identified that regulate the function of effector caspases, however the cleavage of only a small subset of these substrates has been shown to be associated with apoptosis (Lüthi & Martin, 2007). Examples of substrates include the anti-apoptotic protein Bcl-2 (Grandgirard *et al.,* 1998), the DNA repair enzyme PARP (polyADP-ribose) (Lazebnik *et al.,* 1994), and ICAD (inhibitor of caspase-activated deoxyribonuclease) (Enari *et al.,* 1998). In healthy living cells, caspase-activated deoxyribonuclease (CAD) resides in an inactive form with ICAD but cleavage of ICAD by caspase-3 enables CAD to function as a nuclease and affect the internucleosomal DNA fragmentation, which is a characteristic of apoptosis (Enari *et al.,* 1998). Caspase-3 is believed to be the dominant effector caspase, which plays a central role in the demolition phase of apoptosis (Slee *et al.,* 2001).

1.4.2 Apoptotic pathways

The three main pathways of apoptosis induction are: the death-receptor (extrinsic) pathway, the mitochondrial (intrinsic) pathway, and the granzyme-B initiated pathway (Logue & Martin, 2008) (Figure 1.2).

1.4.2.1 The death-receptor apoptotic pathway

Fas/Apo1/CD95 and TNF receptor 1 (TNFR1)/p55/CD120a are the best characterised death receptors that belong to the tumour necrosis factor (TNF) receptor superfamily and share similar cysteine-rich extracellular domains and 80 aa long cytoplasmic domains termed death domains (DDs) (Ashkenazi & Dixit, 1998). They are located in the plasma membrane and are involved in the transduction of pro-apoptotic extracellular signals. Each death receptor induces apoptosis using a different pathway but they typically activate the initiator caspase, caspase-8. The Fas death receptor pathway is mainly involved in the deletion of superfluous lymphocytes and in the killing of target cells (such as virally infected or cancer cells) by CTLs and NK cells (Nagata, 1997).

Upon activation and oligomerisation of the Fas receptor following engagement with its extracellular FasL, DDs of the receptor cluster together and recruit adaptor molecules, which also contain DDs. In the case of Fas the adaptor molecule is termed FADD (Fas-associated death domain), and contains a DED motif. Procaspase-8 molecules then bind to the FADD molecules through homotypic interactions between their DEDs and are activated through self-cleavage due to proximity-induced intermolecular processing (Ashkenazi & Dixit, 1998; Muzio *et al.,* 1998). Within seconds of the receptor engagement, the death-inducing signalling complex (DISC) forms (Kischkel *et al.,* 1995).

After caspase-8 gets activated in the DISC, one of two possible mechanisms follows. In many cell types, stimulation of death receptors results in activation of large amounts of caspase-8, followed by direct activation of the downstream caspases, caspase-3 and caspase-7 (Thorburn, 2004). However, certain cell types, such as hepatocytes, fail to activate sufficient amounts of caspase-8 within the DISC to initiate the proteolytic cascade.

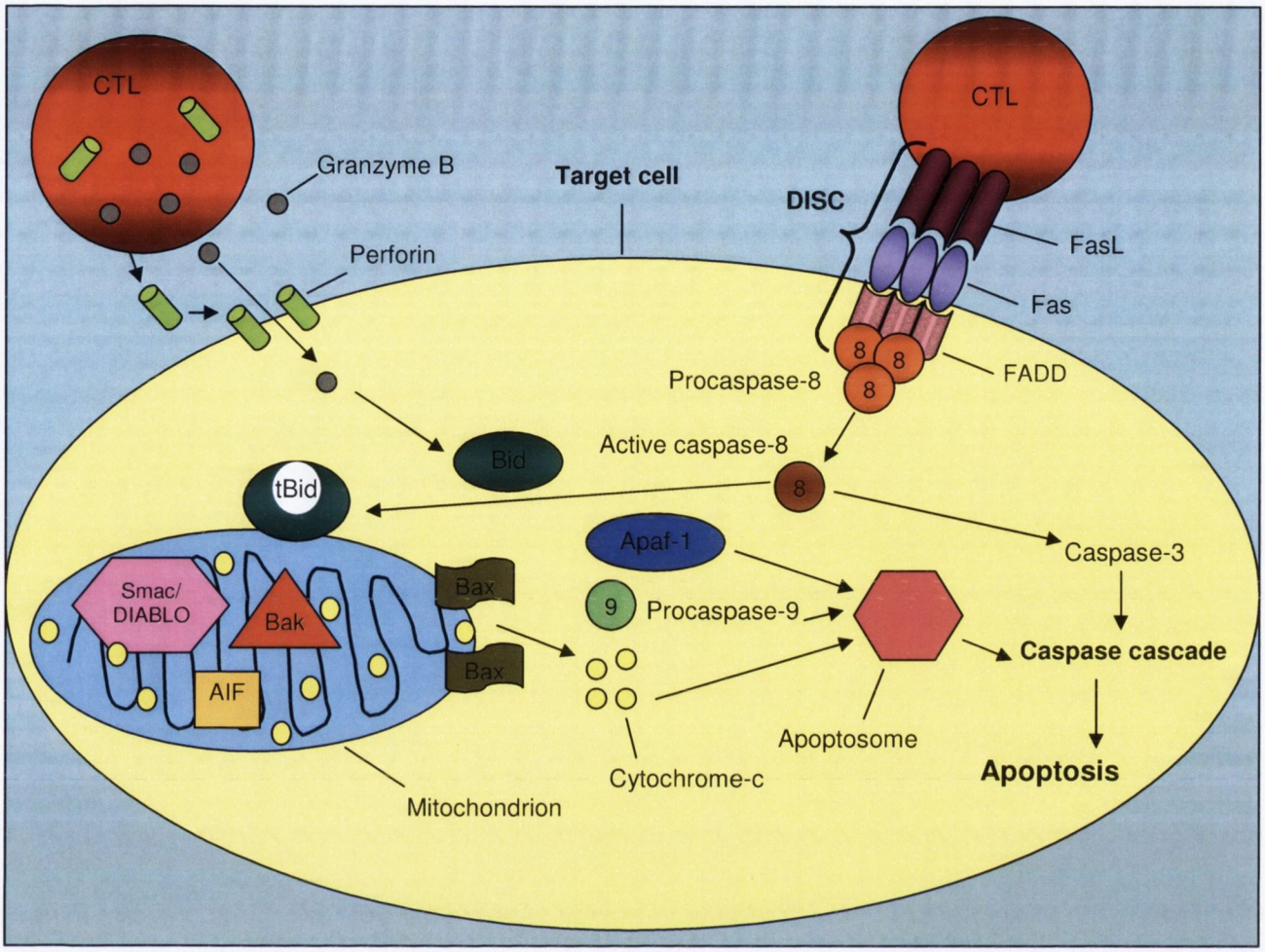


Figure 1.2 The pathways of apoptosis induction

In the death receptor pathway, FasL binds to the Fas receptor which results in trimerisation of the receptor and recruitment of procaspase-8 molecules via an adaptor molecule FADD. Procaspase-8 is activated due to proximity induced intermolecular processing and subsequently activates caspase-3 which activates the caspase cascade and induces formation of apoptosis. Alternatively, caspase-8 can also cleave Bid to tBid which induces the mitochondrial apoptotic pathway. Bid can also be activated through cleavage to gtBid by granzyme B. Translocation of Bax from the cytosol to the mitochondrial outer membrane also leads to induction of the intrinsic apoptotic pathway. Following the loss in mitochondrial membrane potential, cytochrome c is released into the cytosol. Cytochrome c then interacts with Apaf-1 and procaspase-9 to form the apoptosome which activates the caspase cascade. AIF, Bak and Smac/DIABLO comprise the other mitochondrial proteins that are released during apoptosis. Bax oligonmerization is crucial for mitochondrial membrane permeabilization and the anti-apoptotic Bcl-2/Bcl-X_L proteins have been shown to prevent oligomerization of Bax and therefore the induction of apoptosis. AIF; apoptosis inducing factor, Apaf-1; apoptosis protease-activating factor-1, CTL; cytotoxic T-lymphocyte, FADD; Fas-associated death domain protein, tBid; truncated Bid, Smac; second mitochondrial activator of caspases

(Original diagram based on information from: Ashkenazi & Dixit, 1998, Green & Reed, 1998; Lord *et al.,* 2003; Logue & Martin, 2008)

In such circumstances, the Bid protein, a BH3-only member of the Bcl-2 protein family, gets proteolytically cleaved and a truncated form of the molecule (tBid) translocates to mitochondria where it promotes the release of cytochrome c and induces the mitochondrial pathway of apoptosis (see below) (Li *et al.,* 1998; Scaffidi *et al.,* 1998).

1.4.2.2 The mitochondrial apoptotic pathway

Intrinsic signals such as ionising radiation, cytotoxic drugs, toxins, viral infections, and heat-shock which cause cell stress or damage are the main inducers of the intrinsic pathway of apoptosis. These stimuli change the permeability of the inner mitochondrial membrane (IMM), decreasing the mitochondrial membrane potential (MMP) and causing the release of several proteins, most commonly cytochrome c, from the mitochondrial intermembrane space into the cytoplasm (Green & Reed, 1998). The Bcl-2 family of proteins also control the permeability of the outer mitochondrial membrane (OMM) and are discussed in greater detail in section 1.4.3.

Once released, cytoplasmic cytochrome c interacts with the C-terminus of the cytosolic protein apoptosis protease-activating factor-1 (Apaf-1). Apaf-1 together with deoxyadenosine triphosphate (dATP) and procaspase-9, promotes the assembly of apoptosome, a wheel-like structure comprised of seven molecules of Apaf-1 and a similar number of caspase-9 dimers (Acehan *et al.,* 2002). The N-terminal CARD domain of Apaf-1 binds to the CARD domain of procaspase-9, gets activated through proximity-induced intermolecular processing, and as a result procaspase-9 molecules are recruited to the apoptosome (Acehan *et al.,* 2002). The apoptosome propagates the death signal through activation of downstream caspases and subsequent initiation of the caspase cascade (Hill *et al.,* 2004).

Other mitochondrial proteins released during apoptosis include AIF (apoptosis-inducing factor) (Susin *et al.,* 1999), Smac (second mitochondrial activator of caspases) (Du *et al.,* 2000), DIABLO (human ortholog of Smac; direct IAP-binding protein with a low pI) (Verhagen *et al.,* 2000), HtrA2 (high-temperature requirement protein A2) (Suzuki *et al.,* 2001), and endonuclease G (Li *et al.,* 2001). Smac/DIABLO and HtrA2 are believed to inhibit the activity of the IAP (inhibitor of the apoptosis) group of proteins which have an inhibitory effect on caspases and subsequently lower the threshold for the commitment of a cell to apoptosis. AIF and endonuclease G assist the degradation of DNA in the stage of apoptosis where the cell has committed to die.

These proteins operate through a caspase-independent pathway (Garrido & Kroemer, 2004; Saelens *et al.,* 2004; Galluzzi *et al.,* 2008a).

1.4.2.3 The granzyme-B initiated pathway of apoptosis

NK cells and CTLs eliminate pathogenic cells via the death receptor mediated and/or perforin/granzyme-dependent pathways. The latter pathway involves the release of cytolytic molecules, particularly perforin and the serine protease, granzyme B (Chavez-Galan *et al.,* 2009). Perforin is a pore-forming protein that facilitates the formation of pores in the target cell plasma membrane and delivery of other granule components into target cells. Granzyme B enters the cytosol and induces apoptosis through the activation of substrates such as procaspase-3, procaspase-8 and Bid (Lord *et al.,* 2003). Granzyme B cleaves Bid directly at a specific site producing a fragment, gtBid, which translocates to the mitochondria and promotes the release of cytochrome c (Barry *et al.,* 2000). Granzyme B preferentially cleaves Bid and subsequently induces the mitochondrial pathway of apoptosis (Pinkoski *et al.,* 2000).

1.4.3 Apoptosis regulatory genes

Apoptosis has a pivotal role in embryogenesis, maintaining tissue homeostasis and defence against pathogens. Dysregulation of apoptosis can lead to cancer, autoimmunity and degenerative disorders (Thompson, 1995). Overexpression or inhibition of several proteins including IAPs (Liston *et al.,* 1996), FLIPs (Fas-associated death domain-like inhibitory proteins) (Irmler *et al.,* 1997), TRIP (TNFR-associated factor (TRAF)-interacting proteins) (Lee & Choi, 1997), tumour suppressor protein p53 (Levine *et al.,* 1991), and the Bcl-2 family proteins (Reed, 1997) can affect the susceptibility or resistance of cells to apoptosis induction and the development of cancers (Zörnig *et al.,* 2001).

The tumour suppressor p53 protein is one of the most important regulators of apoptosis where it functions as a transcription factor and either induces cell growth arrest or apoptosis (Levine *et al.,* 1991). 70% of human tumours contain a functionally inactivated copy of the *p53* gene (Hollstein *et al.,* 1991). Following DNA damage, levels of p53 increase and cell division is blocked allowing time for DNA repair to occur (Kastan *et al.,* 1991). Following cell cycle arrest, the p53 protein mediates

apoptosis through upregulation of death receptors such as DR5 and Fas, the pro-apoptotic protein Bax, and through inhibition of anti-apoptotic signals such as NF-κB (Vogelstein *et al.,* 2000). Genotoxic stress inducers of apoptosis such as UV- and X-ray radiation, and chemotherapeutic drugs, are also dependent on *p53* induction (Kuerbitz *et al.,* 1992; Lowe *et al.,* 1993).

The Bcl-2 family of proteins maintain the integrity of the OMM, thus regulating the release of apoptosis-regulating factors, such as cytochrome c, from mitochondria into cytoplasm (Green & Reed, 1998). All antiapoptotic members of the Bcl-2 family, including Bcl-2, Bcl-x_L, Bcl-w and Mcl-1, contain four conserved Bcl-2 homology (BH) domains (BH1- BH4), whereas proapoptotic members; Bax, Bak and Bok contain BH1, BH2, and BH3 (but not BH4), and have been termed "multi-domain" proteins (Reed, 2006). The BH3-only proteins contain only the BH3 domain, and act as damage sensors and serve in the promotion of apoptosis. Some BH3-only proteins such as Bid, Bim and Puma have dual functions regulating both the anti-apoptotic and pro-apoptotic Bcl-2 family members (Korsmeyer *et al.,* 2000).

Bax resides in the cytosol or loosely attached to the OMM of healthy cells and translocates in the mitochondria upon induction of apoptosis. Simultaneously with translocation, Bax oligomerization occurs which is crucial for mitochondrial membrane permeabilization (Antonsson *et al.,* 2000). The mechanism by which the Bcl-2 proteins control mitochondrial membrane permeability remains controversial. Bax and structurally related pro-apoptotic proteins (Bak, Bok) are hypothesized to form proteinaceous or lipidic pores upon oligomerization in mitochondrial membranes (Chipuk & Green, 2008), and gene ablation studies in mice showed that either Bax or Bak is necessary for membrane permeabilization (Wei *et al.,* 2001). Truncation of Bid to tBid by caspase-8 results in its translocation from the cytosol to the mitochondria where it synergises with Bax to induce mitochondrial permeability (Li *et al.,* 1998; Wei *et al.,* 2000; Kuwana *et al.,* 2002). Bax has also been hypothesized to interact with and regulate the mitochondrial transition pore (MTP) by inducing opening of the voltage-dependent anion channel (VDAC) in synthetic liposomes (Shimizu *et al.,* 1999). The Bcl-2 protein was defined as unique because it contributed to malignant cell expansion primarily by inhibiting cell death rather than by promoting cell proliferation (Vaux *et al.,* 1988). Bcl-2 is predominantly present in the OMM and does not oligomerize in mitochondrial membranes, but rather prevents oligomerization of Bax and therefore the induction of apoptosis (Kluck *et al.,* 1997; Gross *et al.,* 1998). Overexpression of the

Bcl-2 gene was detected in human B-cell leukaemia and follicular lymphomas where it was found to be localised to chromosome 18, at the site of one of the breakpoints of a reciprocal translocation, also involving chromosome 14 (Tsujimoto *et al.,* 1985). A variety of human malignancies, including carcinomas of the breast, prostate, ovary, colon, and lung, contain high levels of Bcl-2 (Reed, 1995). Studies of the human Bax gene in colon cancers uncovered the first examples of loss of function mutations in any pro-apoptotic Bcl-2-family gene (Rampino *et al.,* 1997).

1.4.4 Viral infection and apoptosis induction

Multicellular organisms have several host defence mechanisms against viral infections. The initial line of defence involves induction of the cellular immune response, leading to secretion of cytokines that activate and recruit macrophages and NK cells. The second line of defence is activation of humoural immune response involving cytotoxic T cells and antibodies generated from B cells. Secretion of interferon following replication of virus can inhibit the spread of infection and is considered as the third line of host defence (Roulston *et al.,* 1999).

Death of the cell before the virus has sufficient time to complete its replication cycle, together with the induction of immunity, results in elimination of the virus. Also, cellular perturbation caused by virus infection can trigger cellular detectors to initiate an innate apoptotic response (Everett & McFadden, 1999). However, many viruses have evolved to use apoptosis to their advantage. Because apoptosis involves the packaging of cellular contents, including progeny virions, into membrane-bound apoptotic bodies that are subsequently taken up by neighbouring cells, the inflammatory response is limited and the viral infection is allowed to spread and go undetected by the host. As the virus particles are enclosed within membrane-bound vesicles, they are protected from inactivation by host antibodies (Roulston *et al.,* 1999). Several viruses are known to encode proteins which serve to inhibit or promote apoptosis, and in that way replication cycle of the virus is completed irrespective of prior cell death, and progeny virions are produced (Hay & Kannourakis, 2002; Li & Stollar, 2004; Galluzzi *et al.,* 2008b).

The mechanism by which alphaviruses induce apoptosis remains to be fully determined. However, recent studies showed that SFV induces apoptosis through the caspase-8 and Bad/Bid-mediated death signalling pathway and requires RNA

replication for apoptosis induction (Urban *et al.,* 2008). SV-induced apoptosis was initiated at the level of viral entry (Jan & Griffin, 1999).

1.5 SEMLIKI FOREST VIRUS

SFV belongs to the genus *Alphavirus*, and is a member of the *Togaviridae* family. SFV was first isolated in the Semliki Forest, Uganda in 1944 by Smithburn & Haddow and its natural vertebrate hosts are wild birds and small rodents. SFV is usually considered avirulent for human beings; however an outbreak of SFV was reported in Bangui, Central African Republic (CAR) in 1987 with patients suffering from severe headache, fever, myalgia and arthralgia (Mathiot *et al.,* 1990). One case of lethal SFV infection occurred in a laboratory worker (Willems *et al.,* 1979) who may have been immunodeficient and was potentially exposed to large amounts of virus. Other alphavirus strains include Eastern equine encephalitis (EEE) virus, Western equine encephalitis (WEE) virus, Venezuelan equine encephalitis (VEE) virus, and Sindbis virus (SV). SFV and SV together with VEE have provided much of the information known today regarding alphaviruses.

1.5.1 The SFV virion

SFV is a small, spherical, enveloped virus with a diameter of approximately 69 nm. The virion is composed of icosahedral protein lattices tightly surrounded by a host-membrane derived lipid bilayer into which 80 hetero-oligomeric spikes (each comprised of E1, E2 and E3 proteins) are inserted (Figure 1.3). The nucleocapsid core is made up of a single-stranded plus-sense RNA genome of approximately 11.4 kb encircled with 240 copies of capsid (C) protein. The C protein, which has a molecular mass of 30 kDa, possesses a protease activity in its conserved C-terminal chymotrypsin-like serine protease COOH terminal domain that results in its autocleavage from the nascent structural polypeptide; RNA binding is mediated by the N-terminal domain (Melancon & Garoff, 1987; Owen & Kuhn, 1996). The SFV nucleocapsid has a diameter of 38 nm and possesses a T = 4 icosahedral symmetry (Paredes *et al.,* 1993). Due to its fenestrated structure, the RNA within the nucleocapsid is sensitive to RNase

degradation and the loss of RNA leads to the shrinkage of the SFV nucleocapsid which also occurs at slightly acidic pH (Söderlund *et al.*, 1979).

The spike proteins of the envelope are composed of a trimer of the E1, E2 and E3 glycoproteins, with three heterotrimers forming each spike (Garoff *et al.*, 1974). The spike proteins are organised in a T = 4 symmetry forming an icosahedral lattice, which is characteristic of alphaviruses (von Bonsdorff & Harrison, 1975).

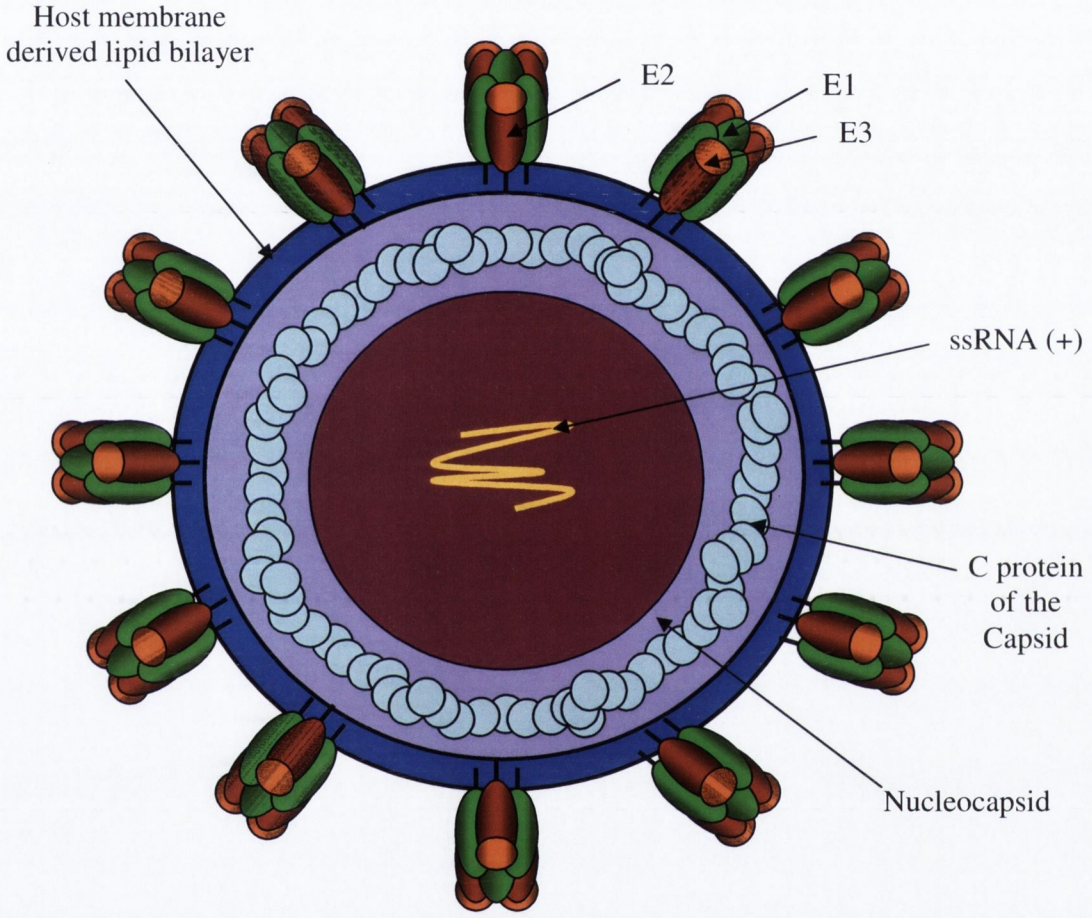


Figure 1.3 Structure of the SFV virion

The SFV virion is 60-65 nm in diameter and consists of a nucleocapsid containing 240 copies of the Capsid protein and a single stranded RNA genome of positive polarity. The host membrane derived lipid bilayer contains 80 spikes, each consisting of three copies of the E1, E2 and E3 glycoproteins.

(Original diagram based on information from Strauss & Strauss, 1994)

The E1 glycoprotein (49 kDa) is responsible for viral fusion and is attached to the lipid bilayer via its C-terminal hydrophobic domain comprising two arginine residues (Garoff & Söderlund, 1978; Garoff *et al.,* 1980). A study by Walhberg et al (1992) has shown that E1-E1 interactions are responsible for maintaining the stability of the spikes. The E2 protein consists of 422 aa and has a molecular mass of 52 kDa. It contains an external hydrophilic domain serving as a receptor binding subunit, a small hydrophobic transmembrane region, and a synthetic peptide of 31 residues corresponding to the C-terminal region which is involved in nucleocapsid formation (Metsikko & Garoff, 1990; Skoging *et al.,* 1996). E2 is formed from a precursor, p62, which also contains a smaller, 10 kDa glycoprotein called E3. E3 remains associated with the mature SFV virion but it gets degraded into the extracellular medium during maturation of the SV particles (Garoff *et al.,* 1974; Welch & Sefton, 1979). The function of E3 remains to be fully determined, although it has been established that it has a central role in the heterodimerization of the spike proteins and transport of the viral structural components to the site of budding (Lobigs *et al.,* 1990).

Submolar quantities (7 to 30 molecules per virion) of an additional viral protein termed 6K, a small hydrophobic peptide (66 aa long) that is produced as a linker between E2 and El, is also present in SFV virions (Loewy *et al.,* 1995). 6K is known to be essential for virus budding (Liljeström *et al.,* 1991; Lusa *et al.,* 1991).

1.5.2 Genome structure and organisation

The structure of the SFV genome is illustrated in Figure 1.4. The SFV genome consists of a single-strand positive-sense RNA molecule of approximately 11.4 kb with a sedimentation coefficient of 42S (Strauss & Strauss, 1994). The genome is 5' capped with a 7-methylguanosine residue, and polyadenylated at the 3' tail. These features allow the genome to function directly as mRNA upon introduction to the cytosol (Clegg & Kennedy, 1974). The non-structural proteins (nsPs) (nsP1, nsP2, nsP3 and nsP4) are translated from the 5' two-thirds of the genome, while the structural proteins (C, P62, 6K and E1) are processed from the remaining 3' one-third of the genome (Strauss & Strauss, 1994).

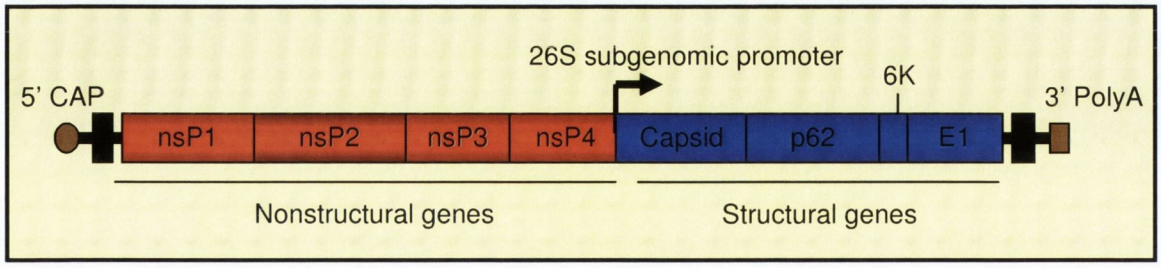


Figure 1.4 Schematic representation of the SFV genome

The 11.2 kb positive-sense RNA genome of Semliki Forest virus is capped at the 5' end (brown circle) and polyadenylated at the 3' end (brown rectangle). Black rectangles represent the 5' and 3' non-translated regions (NTRs). The genome has two open reading frames (ORFs): the first (red) encodes the four non-structural (ns) proteins that form the replicase complex, and the second (blue) codes for the structural proteins (p62 is the precursor of E2 and E3).

1.5.3 The SFV replication cycle

The SFV replication cycle is illustrated in Figure 1.5.

1.5.3.1 Viral entry

Alphaviruses infect cells by receptor-mediated endocytosis, a pathway that normally functions as an uptake mechanism for receptor-ligand complexes. The viral envelope proteins bind to a cell surface protein which acts as a receptor, the virus is then internalised in vesicles and transported to endosomes where the acidic pH causes the viral spikes to mediate fusion between the viral and endosomal membranes and finally the nucleocapsid is released into the cytosol (Helenius *et al.*, 1980; DeTulleo & Kirchhausen, 1998).

Arthropod-borne viruses use a number of distinguishable receptors which differ depending on the host and the tissue. Several proteins have been suggested as possible functional receptors for SFV such as antigens of the MHC; HLA-A and HLA-B in humans or H-2K and H-2D in mice (Helenius *et al.*, 1978).

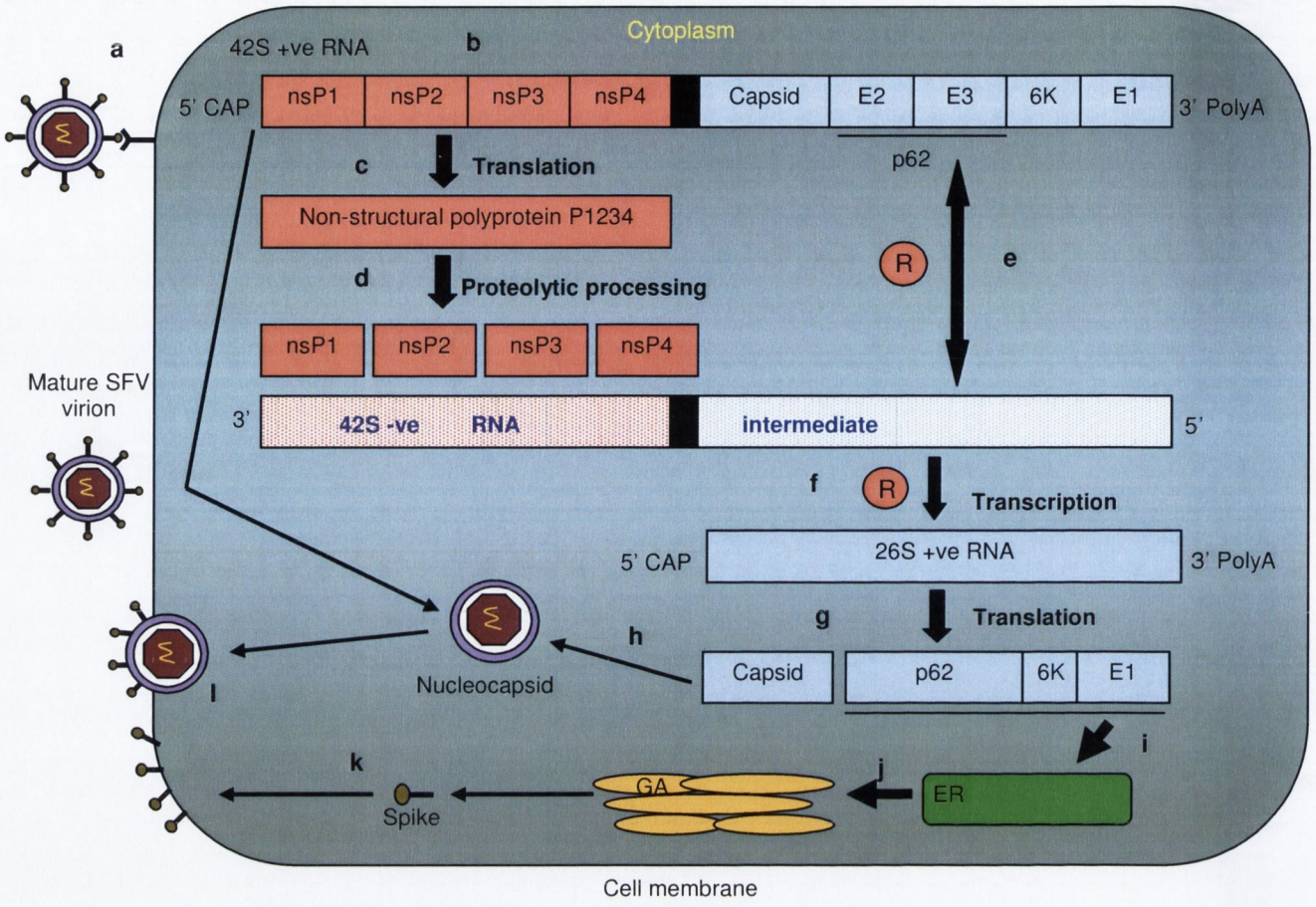


Figure 1.5 SFV replication cycle

(a) Virus entry occurs by receptor-mediated endocytosis. (b) Full-length 42S positive-stranded RNA genome is released into the cytoplasm of the cell. (c) Viral replication starts with translation of the 5' two-thirds of the genome into the polyprotein P1234, which encodes for the non-structural proteins (nsP1-4). (d) P1234 is subsequently processed to non-structural proteins nsP1-nsP4, through the action of the nsP2 and nsP4 autoproteases. (e) Replicase catalyses formation of both a 42S negative-stranded RNA intermediate and a 42S positive-stranded genomic RNA. (f) Replicase also catalyses transcription from an internal 26S promoter to form a subgenomic positive-stranded RNA which is then (g) translated into a structural polyprotein (C, p62, 6K, E1). (h) Capsid protein autoproteolytically cleaves itself from the nascent polypeptide chain. Capsid proteins then complex with newly synthesised genomic RNA to form mature nucleocapsids. (i) The remaining structural polyprotein is cleaved into p62, 6K, and E1 in the ER and (j) p62 is cleaved into E2 and E3 in the trans-Golgi. (k) Glycosylated spike proteins are transported to the host cell plasma membrane. Capsid proteins in mature nucleocapsids interact with the cytoplasmic tail of the E2 proteins in mature spike complexes, and (l) SFV particles are released from the cell by budding.

ER; endoplasmic reticulum, GA; golgi-apparatus

(Original diagram based on information from Strauss & Strauss, 1994)

These findings were disputed by Oldstone et al (1980) who found that MHC antigens are not a specific receptor for SFV. In contrast, for SV, a 67 kDa high-affinity laminin receptor and heparan sulphate have been identified as putative mammalian receptors, while a 63 kDa protein was determined to be the receptor in chicken cells (Wang *et al.*, 1992; Byrnes & Griffin, 1998). Given the broad host range of alphaviruses and their ability to replicate in many different cell types, it is possible that these viruses utilise more than one receptor with varying affinities to gain access to the cells (Strauss & Strauss, 1994). The E2 subunit of the SV spike has been shown to interact with the cell surface and thereby facilitate viral entry (Dubuisson & Rice, 1993).

Following the internalisation of the virus particles in clathrin-coated vesicles and transport to the endosome, a reduction in endosomal pH leads to conformational changes in the viral spike proteins. The E1-E2 heterodimers dissociate leaving E2 as a monomer while E1 forms a homotrimer at the centre of the spike (Wahlberg *et al.*, 1992). The changes in E1 protein lead to exposure of the fusion domain which directly mediates the fusion of the viral membrane with the endosomal membrane (Wahlberg *et al.*, 1992). It was also found that the change in El requires, in addition to acid pH, the presence of cholesterol at the target membrane (Kielian & Helenius, 1985). Following fusion, nucleocapsid is released into the cytoplasm by a mechanism which is believed to be mediated by host cell ribosomes (Singh & Helenius, 1992). This causes the release of viral RNA into the cytoplasm.

1.5.3.2 Viral RNA replication

RNA replication occurs in the cytosol, more specifically, within 0.6-2.0 μm type-I cytoplasmic vacuoles (CPV-1) which are derived from endosomes and lysosomes (Froshauer *et al.*, 1988; Peränen & Kääriäinen, 1991). In the early phase of infection, the 5' two-thirds of the genome is translated to form the non-structural polyprotein, P1234, from which the mature ns proteins, nsP1-nsP4, are released by proteolytic cleavages (Lachmi & Kääriäinen, 1976). The initiation site for the translation is an AUG codon at the 5' NTR of SFV (Kääriäinen *et al.*, 1987). Autoproteolysis of the full-length ns polyprotein P1234 of SFV follows two alternative pathways yielding either P123 (nsP1 + nsP2 + nsP3) and nsP4, or P12 (nsP1 + nsP2) and P34 (nsP3 + nsP4) (Takkinen *et al.*, 1991). The P123 and the P12 precursors are processed by the nsP2 proteinase, whereas P34 is cleaved by nsP4-mediated autoproteolysis (Takkinen *et al.*,

1990). The final cleaved products of the protein precursors, the mature non-structural proteins, have significant functions related with the replication of the virus. Protein nsP1 is required for minus-strand RNA synthesis (Wang *et al.,* 1991). It also possesses methyltransferase and guanyltransferase activities responsible for capping the genomic and subgenomic RNAs during transcription (Mi & Stollar, 1991; Ahola & Kääriäinen, 1995). nsP2 is involved in synthesis of the 26S subgenomic mRNA (Sawicki *et al.,* 1978; Keränen & Kääriäinen, 1979) and cessation of minus-strand RNA synthesis (Sawicki & Sawicki, 1993). The C-terminal domain of nsP2 contains a proteolytic activity which is responsible for processing of the non-structural polyprotein (Ding & Schlesinger, 1989; Hardy & Strauss, 1989; Strauss *et al.,* 1992), while its N-terminal domain is believed to be an RNA helicase (Gorbalenya & Koonin, 1989). nsP3 is a phosphoprotein and the phosphorylation sites have been mapped to be serine and threonine residues (Peränen *et al.,* 1988). The function of nsp3 is not well understood but known to be required in some capacity for RNA synthesis (LaStarza *et al.,* 1994a). Recently, it was observed that different stages of SFV4 multiplication get affected by the presence of nsP3 and its more stable deletion mutants (Varjak *et al.,* 2010). The finding that nsP3 from SFV4 is unstable when expressed alone compared to nsP3 expressed in the form of P123, raises the possibility that components of the viral replicase machinery or, alternatively, alphavirus replication can inhibit nsP3 degradation, and stabilize SFV nsP3 (Varjak *et al.,* 2010). nsP4 is linked with the RNA polymerase activity of SFV (Sawicki *et al.,* 1990). It contains the GDD motif characteristic of viral polymerases (Kamer & Argos, 1984) and temperature-sensitive *(ts)* mutations in nsP4 region are known to halt viral RNA synthesis (Hahn *et al.,* 1989). The replicase is responsible for the synthesis of minus-strand 42S RNA species from the positive-strand (Strauss & Strauss, 1994). This intermediate RNA species serves as the template for the generation of new positive-strand 42S RNA molecules, and for the production of a smaller positive-strand 26S RNA, by virtue of its internal subgenomic promoter (Sawicki *et al.,* 1978). Daughter RNA molecules are capped and polyadenylated by the replicase complex, after which full-length 42S RNAs are predominantly encapsidated into new virions. The subgenomic 26S RNAs serve as mRNA and encode the viral structural proteins (Welch & Sefton, 1980; Welch *et al.,* 1981).

1.5.3.3 Synthesis of viral structural proteins and viral release

The structural polyprotein precursor is translated from the 26S subgenomic RNA and comprises all the viral structural proteins in the order NH_2-C-p62-6K-E1-COOH. The C protein has a serine protease activity at its COOH-terminal domain that cleaves itself co-translationally from the nascent polypeptide chain (Aliperti & Schlesinger, 1978; Hahn *et al.,* 1985; Melancon & Garoff, 1987). Newly made C proteins form multimers and bind rapidly to genomic 42S RNA molecules in the cytoplasm of infected cells, forming nucleocapsids. The NH_2-terminal domain of the SFV capsid protein has been considered to be the RNA-binding domain because it contains clusters of positively charged amino acids (Arg and Lys) which are thought to be necessary for making non-specific interactions with RNA (Garoff *et al.,* 1980; Forsell *et al.,* 1995). Nucleocapsid formation occurs in association with the large ribosomal subunits (Ulmanen *et al.,* 1976). The packaging signal which is located in the nsP2 gene is vital for viral RNA packaging (White *et al.,* 1998).

Cleavage of the C protein from the polypeptide exposes a sequence at the N-terminal region of the p62 polypeptide precursor which facilitates translocation into the lumen of ER (Garoff *et al.,* 1990). The ER signal peptidase cleaves E1 and 6K from the polyprotein leading to the oligomerisation of p62 and E1 into heterodimers following the insertion of E1 into the ER membrane (Barth *et al.,* 1995). This complex, resistant to low pH, interacts with 6K and is transported out to the plasma membrane, via the Golgi apparatus, where 6K dissociates and virus budding takes place (Wahlberg *et al.,* 1989; Lusa *et al.,* 1991). The cleavage of p62 into E2 and E3 occurs post-translationally and most probably by a host cell furin in the trans-Golgi (de Curtis & Simons, 1988; Sariola *et al.,* 1995). The E1-E2-E3 heterotrimers trimerise into homotrimers which constitute mature SFV spike complexes that are transported to the plasma membrane. Budding of virus particles occurs at the plasma membrane, driven by interactions between the capsid proteins of the mature nucleocapsid and the cytoplasmic tail of E2 (Garoff & Simons, 1974; Suomalainen *et al.,* 1992).

1.5.4 Effects of SFV infection in host cells

Alphaviruses have a broad host range as a result of their natural life cycle, which includes transmission through arthropod vectors to wild rodents and birds. Under

laboratory conditions SFV infects cultured mammalian and insect cells, together with avian, amphibian and reptilian cells (Strauss & Strauss, 1994). In alphavirus-infected vertebrate cells, such as BHK-21 (baby hamster kidney) cells, the activity of the host-cell translational machinery is inhibited by the production of viral mRNAs and the infection leads to death of most cells by induction of apoptosis or programmed cell death (Strauss & Strauss, 1994; Glasgow *et al.,* 1997). Following the onset of infection, the amount of viral structural proteins do not peak until about 6 hours post infection, after when the host cell protein synthesis is completely shut down and only viral proteins are produced (Liljeström & Garoff, 1991). During the first 24 h of infection approximately 10^4 viral particles are produced per infected cell and extensive cytopathic effect (CPE) in the infected cells is characterised by cell shrinkage, rounding and cytoplasmic blebbing (Levine *et al.,* 1993). CPE in the majority of vertebrate cells is apparently related with the induction of apoptosis, but fully differentiated macrophages and mature neurons are naturally resistant to apoptosis and undergo necrosis (Balluz *et al.,* 1993; Glasgow *et al.,* 1997; Scallan *et al.,* 1997). In contrast to vertebrate cells, infection of invertebrate cells such as mosquito cells by alphaviruses leads to persistent infection with no visible CPE (Davey & Dalgarno, 1974; Karpf & Brown, 1998). It was observed that the formation of CPE in BHK-21 cells is not influenced by the deletion of the viral structural proteins; however the deletion of most of the nsP2 gene in SFV has been shown to abrogate RNA synthesis and also the induction of apoptosis, suggesting a role for viral RNA replication in apoptosis induction (Glasgow *et al.,* 1998). The structural E2 protein of SV has been demonstrated to enhance the induction of apoptosis in the BHK-21 cell line (Frolov & Schlesinger, 1994).

A number of molecular events have been shown to affect the outcome of alphaviral infection. The expression of the anti-apoptotic *bcl-2* gene has been shown to restrict the replication of SV and SFV as well as delaying the induction of apoptosis in the rat prostate cancer cell line AT3 (AT3-Bcl-2), suggesting the involvement of the intrinsic pathway of apoptosis (Levine *et al.,* 1993; Lundstrom *et al.,* 1997; Scallan *et al.,* 1997; Murphy *et al.,* 2001). In contrast, Grandgirard et al (1998) later demonstrated that SFV and SV induce apoptosis in *bcl-2* overexpressing cells by caspase-3-mediated proteolytic inactivation of *bcl-2*. It was also recently shown that high-level expression of *bcl-2* from bicistronic SFV vectors does not protect BHK-21, AT3-Neo or AT3-Bcl-2 cells from SFV induced death and apoptosis does not follow the intrinsic pathway as no cytochrome c release was observed from the mitochondria upon infection (Kiiver *et*

al., 2008). SFV-induced apoptosis has also been shown to occur in the absence of *p53* expression as the human lung carcinoma cell line H358a which contains a homozygous deletion of the *p53* gene, readily undergoes apoptosis following SFV infection (Glasgow *et al.,* 1998). Evidence exists for the involvement of mitochondrial pathway and the death receptor pathway in the induction of apoptosis due to alphavirus infection (Li & Stollar, 2004) and it was recently discovered that SFV requires apoptosome formation, caspase-3/-7 activation, and mitochondrial Bak activation to induce mitochondrial apoptosis (Urban *et al.,* 2008). Ras (Joe *et al.,* 1996), NF-κB (Lin *et al.,* 1995), and protein kinase C (Zrachia *et al.,* 2002) have also been demonstrated to influence alphavirally-induced apoptosis.

1.5.5 Pathogenesis of SFV in mice

The original isolate of SFV is designated L10 and is neurovirulent for neonatal, suckling and adult mice, causing fatal encephalitis by infection of neurons in the CNS when administered either intraperitoneally (i.p.), intracranially or intranasally (i.n.) (Atkins *et al.,* 1999). Another strain of SFV, A7 was isolated from mosquitoes in Mozambique and is avirulent in adult mice but fatal in neonatal mice (McIntosh *et al.,* 1961). The strain A7(74) was generated by passaging of A7 in primary chick cells for further selection of avirulence (Bradish *et al.,* 1971). The original infectious clone of SFV, derived from the prototype strain, is designated pSP6-SFV4 (Liljeström *et al.,* 1991) and has lost some of its virulence, possibly due to a number of passages in cell culture. The virus produced by transcription of this infectious clone is labelled SFV4 and shows complete virulence in both neonatal and adult mice when administered i.n. However, when the same dose of the virus is administered i.p., only 60-70% of adult mice die (Glasgow *et al.,* 1991).

Route of administration, age of host and the particular strain of SFV all play significant roles in the outcome of infection. Following i.p. infection in adult mice, SFV replication occurs in smooth, skeletal and myocardial muscles and precedes the formation of high-titre plasma viremia and subsequent invasion of the CNS (Pusztai *et al.,* 1971; Fazakerley *et al.,* 1993, Amor *et al.,* 1996). Intranasal infection is a more direct route of infection to the CNS and yields more consistent results than i.p. infection. By this method the initial infection is targeted to the olfactory bulbs, allowing analysis of early events following CNS infection. Macrophages and axonal transport

play a role in the transport of virus from nerve endings in the nasal mucosa (Sheahan *et al.,* 1996; Sammin *et al.,* 1999).

The virulence of SFV strains in mice is determined by their ability to replicate in neurons, and the efficiency of the replication. Early studies have shown that virulent strains caused more neuronal damage than avirulent strains in the CNS as they spread rapidly in the CNS, probably by axonal transport, leading to a lethal threshold before the immune system can intervene (Atkins *et al.,* 1985; Kaluza *et al.,* 1987; Balluz *et al.,* 1993). Intraperitoneal injection with both the A7 and L10 strains of SFV results in similar levels of viremia prior to CNS invasion (Fazakerley *et al.,* 1993). Following i.p. infection of adult (3-4 week old) mice with the A7(74) strain of SFV, the virus crosses the blood-brain barrier (BBB) and initiates perivascular foci of infection by damaging the vascular endothelial cells (Soilu-Hänninen *et al.,* 1994); failure to multiply rapidly in neurons results in clearance of the virus from the CNS by the immune system (Atkins *et al.,* 1985; Gates *et al.,* 1985; Balluz *et al.,* 1993, Fazakerley *et al.,* 1993). In contrast, neonatal mice, (12 days of age or less), inoculated i.p. with SFV A7(74) die rapidly from a fulminant encephalitis. This age-related virulence of SFV is not determined by maturity of the immune system, but by maturity of the neurons (Oliver *et al.,* 1997; Oliver & Fazakerley, 1998). Another crucial difference between virulent and avirulent strains is the rapidity of spread of neuronal damage in the CNS (Balluz *et al.,* 1993). Both the L10 and A7 strains infect neurons and oligodendrocytes, but differ in respect to the kinetics of viral spread and subsequent neuronal damage (Smyth *et al.,* 1990; Fazakerley *et al.,* 1993). Whereas A7(74) infection remains in small, delimited foci surrounding cerebral capillaries, L10 infection also initiates small perivascular foci but subsequently disseminates widely in the CNS (Oliver *et al.,* 1997). With virulent strains, virus titres increase in the brain until death, unlike avirulent strains such as A7, where the titre peaks and then declines (Balluz *et al.,* 1993). The neuronal death caused by all strains of SFV in neonatal mice occurs by apoptosis (Allsopp *et al.,* 1998) and contrasts with that caused by virulent strains of SFV in adult mice which occur by necrosis. In other words, only mature neurons undergo necrosis upon infection with virulent SFV (Allsopp & Fazakerley, 2000; Fazakerley & Allsopp, 2001).

Both virulent and avirulent strains of SFV induce CNS demyelination following destruction of oligodendrocytes by SFV-induced apoptosis and myelin damage by CTLs (Kelly *et al.,* 1982; Atkins *et al.,* 1990). Demyelination occurs as discrete plaques and avirulent SFV infection of mice has been suggested as a model for human

demyelinating disease such as multiple sclerosis (MS) (Atkins *et al.,* 1994). Virulent strains also induce demyelination but this is obscured by death of the host (Atkins *et al.,* 1999). Virulent and avirulent strains of SFV can also be transmitted transplacentally and are teratogenic in mice. Replication of the virus occurs first in the placenta and results in foetal death before intervention by the maternal humoural immune response (Atkins *et al.,* 1999).

The virulence of individual alphaviruses is affected by a complex interaction between the host and the virally encoded factors. A single amino acid change in the E2 glycoprotein of SV (which is known to be involved in the induction of apoptosis) has been shown to overcome *bcl-2* inhibition of apoptosis and confer increased neurovirulence, further implicating a correlation between alphavirus-induced apoptosis and neurovirulence (Ubol *et al.,* 1994; Lewis *et al.,* 1996). Studies with the chimeric virus CME2, containing the E2 gene of the avirulent A7(74) virus in the SFV4 clone, and specific E2 amino acid mutations in CME2 have been shown to reduce the virulence in adult mice, indicating that the E2 domain is critical for SFV pathogenesis (Glasgow *et al.,* 1994; Santagati *et al.,* 1995, 1998). Studies involving the non-structural region of the SFV genome, have demonstrated a major role for the nsP2 and nsP3 (particularly nsP3) genes in determining the neurovirulence of the virus. Modification of the nuclear targeting signal in the nsP2 gene of SFV4 by site-directed mutagenesis did not affect the multiplication of the virus in cell culture but reduced its virulence for mice (Rikkonen, 1996; Fazakerley *et al.,* 2002; Lundstrom *et al.,* 2003; Tamm *et al.,* 2008). By analysis of chimera viruses between the virulent SFV4 strain and avirulent strains, it was established that the nsP3 gene is a virulence determinant (Tarbatt *et al.,* 1997; Tuittila *et al.,* 2000). *In vivo* studies of nsP3 macro domain mutants of SFV have shown that mutation of the opal termination codon between nsP3 and nsP4 to encode arginine increases neurovirulence (Tuittila *et al.,* 2000; Tuittila & Hinkkanen, 2003). In addition, mutations in the macro domain at nsP3 amino acid 11 (valine to isoleucine), 48 (alanine to glutamate) and 70 (glycine to alanine) cumulatively contribute to increased neurovirulence, but the mechanisms are not known (Tuittila & Hinkkanen, 2003). In a recent study, mutations in the nsP3 macrodomain of SV were shown to significantly reduce virulence in 2 week-old mice and diminish the apoptosis-inducing ability of the virus in mature neurons (Park & Griffin, 2009).

1.5.6 Host immune responses to SFV infection

Prior to mounting specific adaptive immune responses to viral infection, the early innate immune response induces the secretion of the cytokines IFN-α and IFN-β, limiting viral replication and activating NK cell-mediated cytotoxicity (Biron, 1998). Macrophages and DCs in lymphatic tissues are thought to predominantly secrete type-I IFNs. In alphaviral infection, IFN-α/β production has been shown to be dependant on the formation of dsRNA and is proportional to the amount of virus produced (Griffin, 2001).

The adaptive immune response to SFV infection, which is detectable by day 5 post infection, encompasses both neutralising and non-neutralising antibodies, together with T-cell mediated immunity (Amor *et al.,* 1996). The humoral response is believed to be essential in recovery from disease with the appearance of antibody coinciding with the cessation of viremia (Griffin, 2001). An initial neutralising serum immunoglobulin M (IgM) antibody response by the host is followed rapidly by an IgG2a response and a much slower IgG1 response (Parsons & Webb, 1992; Fazakerley *et al.,* 1993). Seropositivity is maintained for long periods in mammals and confers protection from reinfection (Griffin, 2001). $CD8^+$ CTLs play a role in the context of the cellular immune response and are essential in the development of the lesions of demyelination associated with avirulent A7(74) infection (Subak-Sharpe *et al.,* 1993).

It is likely that the humoral immune response is predominantly responsible for recovery from SFV infection, whereas the cellular responses are involved in the eradication of virus from tissues harbouring persistent infections and in the development of demyelinating disease.

1.6 THE SEMLIKI FOREST VIRUS VECTOR SYSTEM

1.6.1 Alphaviruses as expression systems

Infectious clones of SV (Rice *et al.,* 1987), VEE (Davis *et al.,* 1989) and the prototype (Liljeström *et al.,* 1991) and A7(74) strains of SFV can be *in vitro* transcribed to generate full-length infectious viral RNA, as they contain the viral cDNA under the control of a prokaryotic DNA-dependant RNA polymerase promoter, such as SP6 (SV

and SFV) or T7 (VEE). Transfection of cells, usually by electroporation, with these infectious RNA transcripts results in intracellular replication and the propagation of progeny virus particles.

Several characteristics of alphaviruses are considered as advantageous for the development of expression vectors. Firstly, because their genome is small they can easily be manipulated, and because the replication of the virus takes place in the cytosol as their genome functions directly as an mRNA due to its positive polarity, there is no possibility of insertional mutagenesis. Secondly, alphaviral RNA essentially prevents host cell macromolecular synthesis with virtually all proteins synthesised by infected cells encoded by the subgenomic viral RNA. Thirdly, because the gene products of the subgenomic RNA are not involved in intracellular RNA replication, replacement of this region does not affect the replication of the virus.

1.6.2 Development of the SFV vector system

There are three main types of SFV vectors: recombinant virus-like particles (VLPs), layered DNA-RNA vectors and replication-competent vectors (Figure 1.6). The basic "suicide" SFV vector system consists of an expression vector in which the viral structural protein genes are replaced with a polylinker sequence to facilitate the insertion of heterologous genes, and two helper vectors (split-helper system) that supply (*in trans*) the structural protein genes (Smerdou & Liljeström, 1999) (Figure 1.6 b-d). The recombinant vector RNA contains the replicase genes and the packaging signal (which resides in nsP2), the 26S subgenomic promoter followed by a multiple cloning site (MCS) where the gene of interest could be inserted, and the 5' and 3' ends of the genome required for replication (Figure 1.6 b). The two helper plasmids encode the capsid (pSFV-CS219A), and the envelope proteins E1, p62 and 6K (pSFV-HelperS2), respectively, under the control of a 26S subgenomic promoter with the 3' and 5' replication signals, but lack the packaging signal located in the non-structural region of the genome (Figure 1.6 c and d). Co-transfection of all three RNA transcripts into packaging cells (usually BHK-21 cells) results in encapsidation of only the recombinant RNA and production of infectious VLPs coding for the foreign gene (Liljeström & Garoff, 1991; Smerdou & Liljeström, 1999) (Figure 1.7).

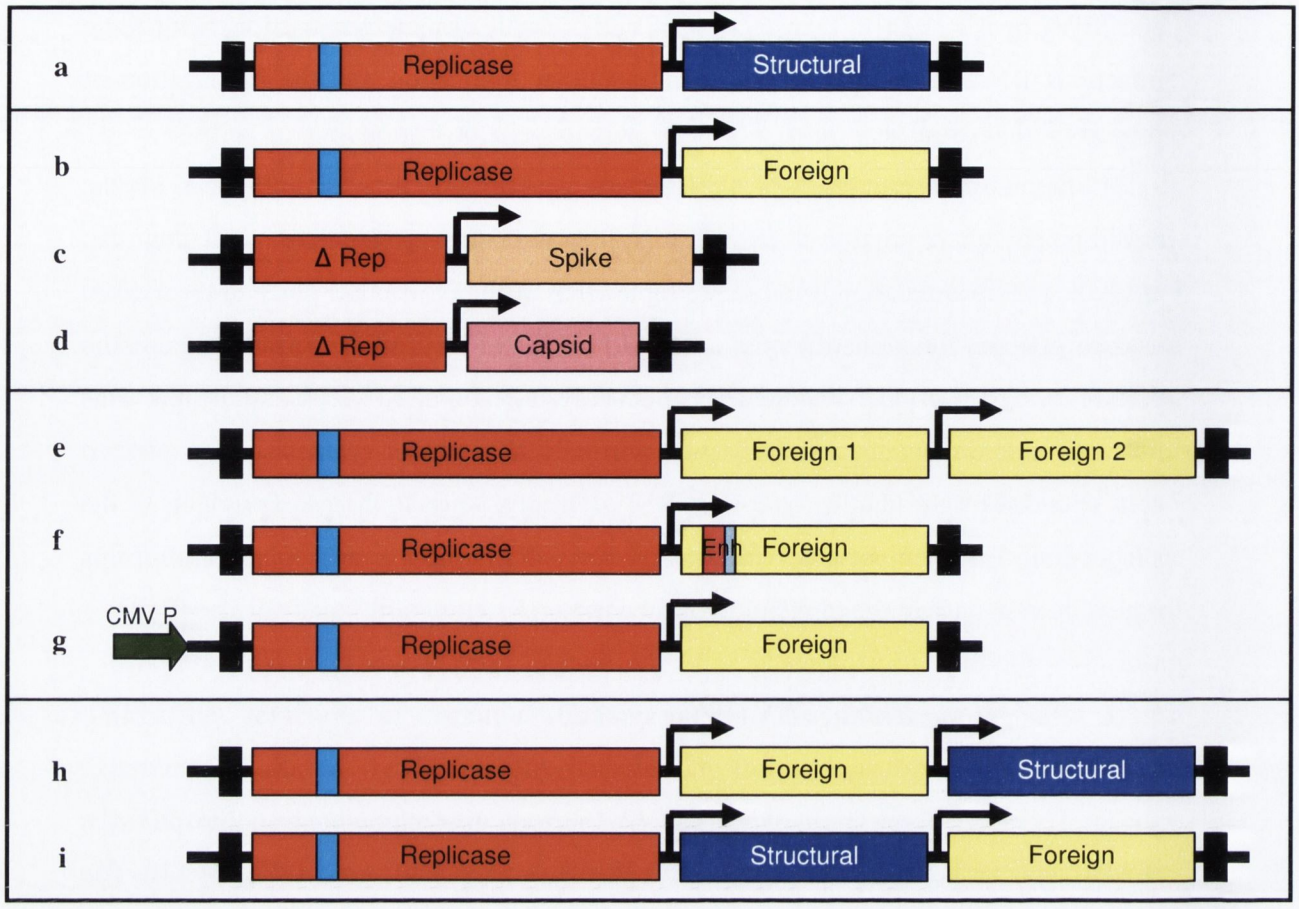


Figure 1.6 Alphaviral expression vectors

(a) The wild-type SFV genome. This vector carries the four non-structural genes coding for the replicase complex (nsP1-4) (red), the packaging signal in nsP2 (turquoise rectangle), and the structural genes (C, p62, 6K, E1) (blue) under the control of subgenomic 26S promoter (black arrow). ***(b) Virus-like particle vector***. The backbone carries the replicase complex but the structural genes are deleted and replaced with a polylinker region (yellow) into which a foreign gene can be inserted. ***(c & d) Spike and capsid helper vectors***. These vectors encode the structural proteins but the region that includes the RNA-packaging signal is deleted. ***(e) Double subgenomic promoter***. This vector contains a second 26S subgenomic promoter which allows the cloning of a second foreign protein. ***(f) SFV10Enh vector***. This contains an SFV "translational enhancer" element (Enh) which consists of the first 34 amino acids of the SFV capsid protein (dark pink) followed by the 17 nucleotide foot and mouth disease virus 2A protease (light blue). ***(g) The layered DNA-RNA vector***. cDNA from the non-structural region of SFV is cloned into a DNA vector under the control of a cytomegalovirus (CMV) immediate-early promoter (green arrow). ***(h & i) Replicating vectors***. A second subgenomic 26S promoter and MCS are cloned either at the 5' (h) or 3' (i) end of the structural gene region.

Abbreviations: CMV P, cytomegalovirus immediate-early promoter; Enh, enhancer element; MCS, multiple cloning site; Δ Rep, deleted replicase.

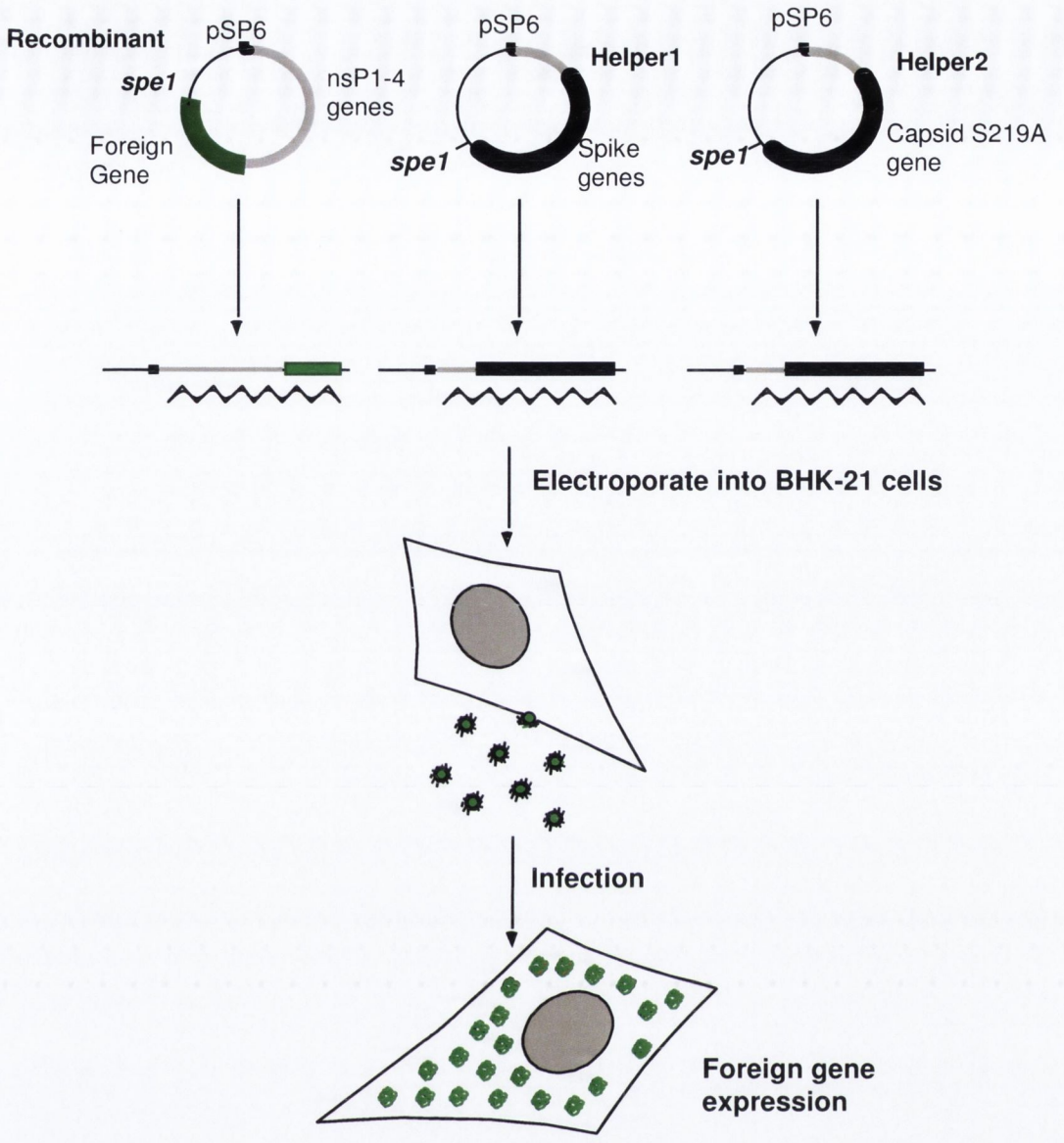


Figure 1.7 Production of recombinant SFV (rSFV)-Virus like particles (VLPs)

rSFV-VLPs are generated by electroporation of cells with the recombinant SFV RNA coding for the replicase-foreign gene, and the two helper RNAs, each coding for the capsid protein and the spike glycoproteins, separately. Following the replication and translation of RNAs, the viral recombinant RNA associates with the capsid protein and gets transported to the cell membrane where it assembles with spike proteins forming the recombinant virus particles. These are identical to SFV particles except for the content of the packaged RNA. In the virus all of these genetic elements are linked together on one RNA molecule. In the virus-like particles, only the replicase-foreign gene RNA transcripts are packaged.

(Adapted from Berglund *et al.*, 1996)

The inability of recombinant particles (rSFV) to undergo more than one round of replication has led to the term "suicide" particles. This system induces high-level, transient, foreign gene expression (Liljeström *et al.,* 1991). Two helper RNAs are used to reduce the possibility of recombination between vector and helper RNAs and the generation of wild-type virus. The split-helper system reduces the recombination frequencies between the three cotransfected RNA molecules, to negligible (approx. 10^{-12}) values. Similar vector systems were also described for SV (Bredenbeek *et al.,* 1993; Frolov *et al.,* 1997) and VEE (Pushko *et al.,* 1997). For SFV, a mutation in the capsid gene, to abolish its self- cleavage ability, is also introduced as a secondary precaution (Smerdou & Liljeström, 1999). A second subgenomic promoter may also be cloned into the backbone vector to accommodate a second foreign protein. This double subgenomic promoter expression vector allows expression of two foreign proteins within the same cell (Figure 1.6 e).

Cells transfected with SFV vectors which contain the N-terminal 34 amino acid sequence of the translational enhancer from the capsid protein have been shown to produce up to 10 times more foreign protein than the original SFV vector. These vectors express the foreign gene as a fusion protein. To obtain high levels of expression of heterologous protein without the additional N-terminal Capsid residues, the 2A autoprotease from foot-and-mouth disease virus (FMDV) has been inserted as a linker between the enhancer and foreign gene sequences (Smerdou & Liljeström, 1999). The capsid translational enhancer sequence was also included upstream of the envelope proteins (together with the 2A autoprotease of FMDV) in the pSFV-HelperS2 construct to ensure comparable levels of expression of the envelope and C proteins (Smerdou & Liljeström, 1999).

A DNA-RNA layered vector has also been constructed with sequences encoding the alphavirus replicon under the control of a cytomegalovirus (CMV) promoter (Berglund *et al.,* 1998) (Figure 1.6 g). In this case, the CMV promoter does not directly drive expression of the heterologous gene, but rather stimulates the transcription of a cDNA copy of the vector RNA after transfection into the nucleus of the cell. The SFV replicase complex which is produced following translation of the RNA leads to amplification of the recombinant RNA and high level production of the foreign gene. When utilised as a vaccine vector, the immune response induced is higher than that induced by a standard vector (Berglund *et al.,* 1998).

Originally based on the SV genome, a replicating vector was created by addition of a second 26S subgenomic promoter and a MCS, either at the 5' or 3' end of the structural gene ORF (Raju & Huang, 1991; Hahn *et al.,* 1992) (Figure 1.6 h and i). Unlike the suicide particle system, the replication-competent alphavirus vectors undergo several rounds of replication when administered to the host and produce high levels of foreign protein (Hahn *et al.,* 1992). However, these vectors give rise to infectious virus, and high levels of structural protein synthesis, they may also potentially generate immune responses against the vector, making the system unsafe for *in vivo* studies. Also, these vectors are relatively unstable and the foreign gene is deleted early during passage, particularly when inserted 3' of the structural protein genes (Pugachev *et al.,* 1995). In an attempt to prevent possible biosafety issues associated with a replicating vector, non-revertible in-frame deletions and mutations have been introduced into the nsP3 gene and into the nuclear localisation signal in the nsP2 gene of the SFV genome, respectively, that resulted in noncytopathic virus (Lundstrom *et al.,* 2003; Galbraith *et al.,* 2006; Casales *et al.,* 2008). A type of replication-competent vector, based on the avirulent A7(74) strain of SFV has been constructed by addition of a second 26S subgenomic promoter and MCS inserted into the 3' end of the structural gene ORF. *In vitro* and *in vivo* testing of this vector expressing a reporter gene was shown to give unstable foreign gene expression (Vähä-Koskela *et al.,* 2003).

1.6.3 Potential applications of the SFV vector system

Initially, the recombinant VLPs and the layered DNA-RNA vector were utilised as vaccine vectors. Since the replication of SFV is cytoplasmic, there is no possibility of nuclear integration or insertional mutagenesis, and due to apoptosis of infected cells, the virus genome does not persist in the tissue. Murine studies employing RT-PCR analysis revealed that rSFV VLPs do not disperse throughout other organs of the body. The detectable rSFV RNA persists only at the site of administration for 7 days and in local lymph nodes for up to 24 h post inoculation (Morris-Downes *et al.,* 2001a). In addition, most humans and animals have no pre-existing immunity against the vector highlighting the potential of the SFV vector system as a prototype vaccine (Zhou *et al.,* 1994, 1995). SFV-based vaccines have shown to induce strong and specific immune responses that protect against challenge following immunisation. Prototype vaccines

studied include those aimed at: HIV (Hanke *et al.*, 2003), respiratory syncytial virus (RSV) (Fleeton *et al.*, 2001; Chen *et al.*, 2002), Hepatitis C virus (HCV) (Brinster *et al.*, 2002), the structural prME proteins of louping-ill virus (LIV) (Fleeton *et al.*, 1999, 2000; Morris-Downes *et al.*, 2001b), the envelope glycoproteins of simian immunodeficiency virus (SIV) (Nilsson *et al.*, 2001), influenza virus (Berglund *et al.*, 1999; Fleeton *et al.*, 2001), Murray Valley virus (Colombage *et al.*, 1998), and rubella virus (Callagy *et al.*, 2007).

SFV vectors have also been investigated as anti-tumour vaccines and oncolytic agents in the field of cancer gene therapy. The inherent ability of the SFV vector system to induce apoptosis in a variety of cell types has allowed its exploitation as a prototype cancer therapy agent. Unlike SV, SFV does not specifically target tumour tissue if given peripherally; however, if given intratumourally (i.t.), the vector remains within the tissue (Rodriguez-Madoz *et al.*, 2007). The cytopathic effect of the vector alone was successfully employed in the treatment of human non-small cell lung carcinoma xenografts (H358a) in BALB/c *nu/nu* mice by direct i.t. injections with rSFV VLPs expressing enhanced green fluorescent protein (EGFP) (Murphy *et al.*, 2000). This treatment was less successful in fast-growing rat AT3 prostate carcinoma (AT3-Neo) cells in nude mice, however, inhibition of tumour growth was observed in AT3-Neo and AT3-Bcl-2 tumours following i.t. injections of rSFV VLPs expressing the pro-apoptotic gene *bax* (Murphy *et al.*, 2001). Recombinant SFV vectors expressing T-cell epitopes and cytokines such as IL-12 and IL-18 have also been effective in the inhibition of tumour growth in immunocompetent mice (Asselin-Paturel *et al.*, 1999; Yamanaka *et al.*, 2000; Chikanna-Gowda *et al.*, 2005, 2006; Rodriguez-Madoz *et al.*, 2005; Smyth *et al.*, 2005). Peripheral immunisation with an SFV vector expressing the murine VEGFR-2 was shown to induce inhibit tumour growth in 4T1 breast carcinoma model by inhibition of angiogenesis or an antivascular effect (Lyons *et al.*, 2007). Tumour regression was also observed in mice treated with the wild-type SFV virus (SFV4) following vaccination with rSFV VLPs encoding the p62-6K structural proteins (Smyth *et al.*, 2005). The replication-competent SFV vector, VA7-EGFP (Vähä-Koskela *et al.*, 2003) has been shown to have oncolytic potential against human melanoma xenografts in SCID mice (Vähä-Koskela *et al.*, 2006). The oncolytic effect of VA7-EGFP has also been studied in various rodent tumour models including glioma and subcutaneous lung carcinoma (Määttä *et al.*, 2007). Recently, intravenously (i.v.)

administered VA7-EGFP was shown to eradicate both subcutaneous and orthotopic human glioma xenografts in nude BALB/c mice (Heikkilä *et al.*, 2010).

The high level of immune stimulation induced by SFV has generated interest in the utilisation of SFV vectors as anti-tumour vaccines. Tumour vaccines based on alphaviruses can be divided into two groups: prophylactic vaccines, which stimulate preventive immunity prior to establishment of tumours, and therapeutic vaccines that are used in the therapy of established tumours, and are designed to stimulate or augment an immune response against existing tumour cells. Initial work, carried out by Colmenero et al (1999), showed that mice vaccinated with rSFV VLPs expressing the P815A TAA (rSFV/E-P1A) were protected against lethal challenge with the P815 tumour by antigen specific cytotoxic lymphocyte responses. In a later study, rSFV VLPs expressing the P815A TAA were administered i.t. to established tumours and were shown to be effective in inhibiting tumour growth, as were VLPs expressing IL-12 (Colmenero *et al.*, 2002). Similarly, VLPs expressing HPV (associated with the development of cervical carcinoma) antigens E6 and E7 have been demonstrated to induce immunity in mice and provide protection against challenge with HPV-transformed cells (Daemen *et al.*, 2003). Furthermore rSFV expressing E6 and E7 were shown to activate E6-/E7 specific T-cell response in immunocompetent transgenic mice (Riezebos-Brilman *et al.*, 2005) and this response was enhanced by addition of IL-12 (Riezebos-Brilman *et al.*, 2009).

Phase I and II clinical trials are being developed to use liposome-encapsulated SFV VLPs expressing IL-12 in the treatment of glioblastoma multiforme (Ren *et al.*, 2003), and one preclinical study utilised the alphavirus-based replicon vaccines for influenza (Hubby *et al.*, 2007).

The neurotropism of SFV has been exploited in the application of rSFV VLPs as a vector for the CNS. Administration of rSFV VLPs expressing the cytokine IL-10 by the non-invasive i.n. route was shown to have a therapeutic effect on experimental autoimmune encephalomyelitis (EAE), a murine model of MS (Jerusalmi *et al.*, 2003). A replication-competent SFV vector termed VA7, derived from the avirulent strain A7(74), induced higher levels of heterologous gene expression in CNS cells of rat hippocampal slices and in CNS of adult mice when compared to the rSFV VLPs, which highlighted the potential of VA7 as a CNS vector (Vähä-Koskela *et al.*, 2003). Further studies are required before the great potential of the SFV vector system in vaccine production, cancer gene therapy, and treatment of CNS diseases can be exploited.

1.7 OBJECTIVES OF THIS STUDY

Recombinant SFV particles have been developed as a vector for foreign gene expression, vaccine delivery and anti-tumour agents. Recently, the replication competent SFV vector based on the avirulent strain of SFV, VA7, has been constructed and its potent cytotoxic effects have been established in cell culture allowing its utilization for treatment of neoplastic disease *in vivo* (Vähä-Koskela *et al.*, 2003, 2006).

This study follows the previous work conducted by Galbraith et al (2006) where the replicating virus, SFV4, was mutated by introducing two in-frame deletions into the nsP3 gene (ΔSN and ΔTN). The deletion mutants SFV4-SN and SFV4-TN were tested *in vitro* and the virulence were also examined (Galbraith *et al.*, 2006). The SFV4 replicating virus was subsequently modified by addition of a second 26S subgenomic promoter and a MCS to the 5' end of the structural gene ORF and termed RSFV-26SMCS (*Dr. Sareen Galbraith, Pub. No.: WO/2007/102140*). The ΔTN mutation was subsequently reintroduced into RSFV-26SMCS creating RSFV-ΔTN-26SMCS (*Dr. Sareen Galbraith, personal communication*). Another replication-competent SFV vector has also been constructed by complete deletion of the structural 6K gene (Δ6K) from the genome of RSFV-26SMCS, and termed RSFV-Δ6K-26SMCS (*Dr. Sareen Galbraith, personal communication*).

Thus, the aims of this study were as follows:

1) To reintroduce the ΔSN mutation into RSFV-26SMCS creating RSFV-ΔSN-26SMCS.
2) To reintroduce the Δ6K mutation into the RSFV-ΔTN-26SMCS mutant generating RSFV-ΔTN-Δ6K-26SMCS.
3) To clone the pro-apoptotic *bax* gene into the MCS of RSFV-26SMCS, creating RSFV-HABax-26SMCS (Ms. Jennifer Mulholland, project student supervised by Guniz Iskender).
4) To assess the growth, infection efficiency and apoptosis-inducing ability of the original (SFV4, RSFV-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS) and the newly constructed (RSFV-ΔSN-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, RSFV-HABax-26SMCS) replicating SFV vectors on CT26

and K-BALB cells *in vitro*. BHK-21 cells were employed as a positive control for SFV-associated CPE and apoptosis induction.

5) To examine the virulence of the original and newly constructed replicating SFV vectors in BALB/c mice.

6) To examine the ability of SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, and RSFV-HABax-26SMCS to inhibit the growth of CT26 and K-BALB tumours *in vivo*.

Chapter 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Cell Lines

The Baby Hamster Kidney 21 (BHK-21), CT26 and K-BALB cell lines were obtained from the American Type Cell Culture (ATCC, USA). The BHK cell line, Swedish BHK (sBHK), was a gift from Prof. P. Liljeström (Microbiology and Tumorbiology Centre, Karolinska Institute, Stockholm, Sweden).

2.1.2 Cell Culture

Glasgow Minimal Essential Medium (G-MEM) (BHK-21), Dulbecco's Modified Eagle Medium (DMEM), Dulbecco's phosphate buffered saline without calcium, magnesium and sodium bicarbonate (PBS), Dulbecco's phosphate buffered saline with calcium, magnesium and sodium bicarbonate (PBS+), newborn calf serum (NCS), foetal calf serum (FCS), tryptose phosphate broth, HEPES, sodium pyruvate and trypsin EDTA were from Invitrogen (UK). Penicillin-streptomycin-L-glutamine solution was from Sigma (UK). 75 cm^3, 150 cm^3 cell culture flasks, 60 mm^2 cell culture dishes, flat-bottomed 96-well, 6-well, and 12-well cell culture plates were from Sarstedt (Germany). The CellTiter 96® AQ_{ueous} One Solution Reagent was from Promega (USA) and the Vybrant FAM Caspase-3/-7 Assay Kit was from Invitrogen (UK).

2.1.3 Expression Vectors

The SFV expression vectors used in this study are highlighted in Appendix 8.1. The wild type SFV expression vector, pSP6-SFV4 was a gift from Prof. P. Liljeström (Appendix 8.1a). pSP6-SFV4 comprising the SN deletion in the nsP3 region of the SFV genome; pSP6-SFV4-ΔSN (Appendix 8.1b), pSP6-SFV4 with a second 26S subgenomic promoter and a MCS; RSFV-26SMCS (Appendix 8.1c), RSFV-26SMCS comprising the TN deletion in the nsP3 region of the genome; RSFV-ΔTN-26SMCS (Appendix 8.1d), RSFV-26SMCS with a complete deletion of the 6K gene; RSFV-Δ6K-26SMCS (Appendix 8.1e), and RSFV-26SMCS expressing the EGFP gene; RSFV-EGFP-26SMCS (Appendix 8.1f), were constructed and kindly provided by Dr. Sareen Galbraith. RSFV-26SMCS expressing the pro-apoptotic *bax* gene; RSFV-

HABax-26SMCS (Appendix 8.1g) was constructed by Ms. Jennifer Mulholland (project student, Dept of Microbiology, Trinity College) under the supervision of Güniz Iskender.

2.1.4 Molecular Biology Reagents

The nsP3-SN, nsP3-TN and the 6K primer pairs were designed outside the deletion areas using the pSP6-SFV4 sequence (Figure 2.1 a – d) whereas the 26S primer pair was designed using the RSFV-26SMCS sequence (Figure 2.1e). All primers were obtained from MWG-Biotech AG (Germany). Primer pairs designed for sequencing and PCR amplification of the deletion areas and the area of the second 26S subgenomic promoter are outlined in Table 2.1.

Taq Polymerase buffer, $MgCl_2$, dNTP mix, Taq Polymerase, RNasin, nuclease-free water, the 1 Kb and Lambda (λ) (*Hind*III) DNA markers were from Promega (UK). The restriction enzymes *Bgl*II, *Bsm*I, and *Spe*I, T4 DNA Ligase, bovine serum albumin (BSA) and quick ligation kit were from New England Biolabs (NEB) (Massacheussets, USA). The gel extraction (for larger DNA fragments), nucleotide extraction and plasmid purification kits for mini-preps were from Qiagen Ltd. (West Sussex, UK). The Wizard gel extraction kit for smaller DNA fragments was from Promega (UK). SP6 RNA Polymerase buffer and CAP were from NEB (UK). Dithiotreitol (DTT) was from Sigma (USA). SP6 RNA Polymerase and $m^7G(5')ppp(5')G$ were from Amersham Pharmacia Biotech (Uppsala, Sweden).

2.1.5 Equipment

A Beckman L8-M ultracentrifuge was used for centrifugation during virus-like particle (VLP) production. Ultracentrifuge tubes, the SW28 rotor and the swing buckets were from Beckman-Coulter Instruments Inc. (CA, USA). A refrigerated tabletop centrifuge for spinning bacterial cultures was from IEC Micromax. Electroporation cuvettes were from BTX (San Diego, USA). A Nikon Eclipse E400 epifluorescence microscope was used for both bright field and fluorescence microscopy. The following Nikon filters were used for fluorescence detection: GFP filter at 460-500 nm, DAPI filter at 340-380 nm, and G2A filter at 510-560 nm. All micropipettes used were from Gilson. The following equipment was used: BOD incubator for cell culture from Revco

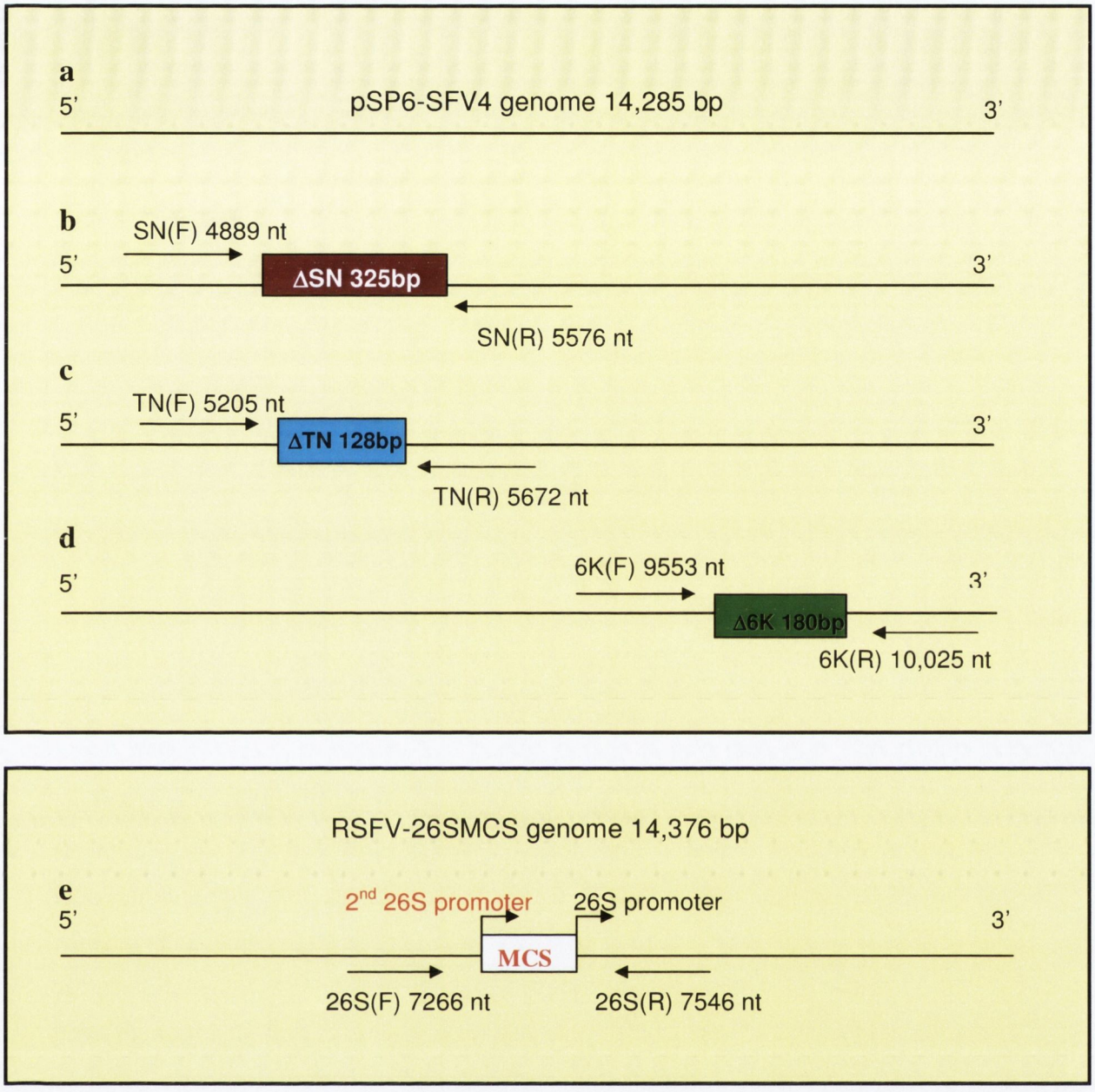


Figure 2.1 Designing of the primer pairs for the deletion areas and for the second 26S subgenomic promoter

Replicating SFV vectors were sequenced to confirm the presence of the nsP3-SN, nsP3-TN, and the 6K deletions, by specific primers (Table 2.1) designed outside the deletion areas (b-d) using the pSP6-SFV4 sequence (a). The presence of the second 26S subgenomic promoter was also confirmed by the specific 26S primer pair (Table 2.1) which was designed using the sequence of the RSFV-26SMCS plasmid (e).

Table 2.1 Primer pairs designed for sequencing and PCR amplification of the deletion areas and the area of the second 26S subgenomic promoter

Primer pair	Sense	Position	Sequence (5'-3')	PCR Product Size (bp)
SN (F) SN (R)	+ -	4889 – 4905 5559 – 5576	GCAATGACAGCAGAACG GTTGTAAATGTCCGCTGC	325
TN (F) TN (R)	+ -	5205 – 5221 5656 – 5672	TGGCTCCCATAGTAGTG TCTCCCTCTCAGTATCC	128
6K (F) 6K (R)	+ -	9553 – 9570 10008 – 10025	ATATCGATCTTCGCGTCG ACCGTCTTGTACTCACAG	180
26S (F) 26S (R)	+ -	7266 – 7283 7529 – 7546	GAGGGCTGCAAGAGTATC CGGCCGTAAAACGTTTGC	280

Ultima, PCR machine (Hybaid), water-bath (Memmert), BioRad Gel Doc 2000 and accompanying Multi-Analist (version 1.1) software, heating block (Grants instruments (Cambridge) Ltd, England), BioRad Gene Pulser XCell, electronic multi-channel pipette (Biohit, Proline), Multiscan RC plate reader (Thermo Labsystems), haemocytometer (Neubauer-Blaubrand, Germany), Vortex-2-Gene (Scientific Industries), Chemical fumehood (Chemical Systems Control Ltd, Ireland), multispeed refrigerated centrifuge (Medical Supply, Ireland), a basic pH meter (Denver Instruments, USA).

2.1.6 Mice

Specific pathogen-free (spf) 40–60-day-old female BALB/c mice (Harlan, UK) were maintained in accordance with principles outlined in S1 17/94 European Communities Regulations 1994, for care and use of laboratory animals. Syringes (1.0 ml and 0.5 ml microfine insulin) and needles (23G) were from Becton Dickenson (UK), halothane from Rhone Merieux, UK.

2.1.7 Antibodies

Anti-HA antibody produced in rabbit was from Sigma (USA). Biotinylated mouse anti-rabbit IgG antibody and streptavidin/FITC were from Dako (Denmark). Normal mouse serum and VECTASHIELD hardest mounting medium with DAPI and VECTASTAIN reagent was from Vector Laboratories Inc. (USA).

2.1.8 Histological and pathological studies

Isopentane, paraformaldehyde (PFA), haematoxylin Harris, eosin aqueous solution, dichromate eosin and DPX solutions were from BDH Ltd (UK).

2.1.9 Miscellaneous

Sucrose was from BDH Ltd (UK). Crystal violet solution was from Clin-Tech (UK). Trypan blue, Tween 20, ampicillin, and 10% dimethylsulfoxide (DMSO) were from Sigma (USA). Tween 80 was from Merk (Germany). Agarose was from Promega (UK). TNE buffer: 50 mM Tris, 0.1 M NaCl and 1 mM EDTA were dissolved in 800 ml distilled water, pH adjusted to 7.4 and volume to 1 litre. Noble agar was from Difco (Detroit, USA). The suppliers of other materials were as follows: Ethidium bromide (Biorad, USA), eppendorf tubes (Axygen), Tris-Borate-EDTA (TBE) (Promega, USA), blue/orange-loading dye (Promega, USA), cryotubes (Nunc, Denmark), SW-28 ultra-centrifuge tubes (Ultra-clear, Beckman).

2.2 METHODS

2.2.1 CELL CULTURE

The BHK-21 cell line was used for the production of virus from replicating SFV vectors and for *in vitro* studies. CT26 and K-BALB tumour cells were used for both *in vitro* and *in vivo* tumour treatment studies. Since it has already been established that BHK-21 cells are susceptible to SFV-induced apoptosis upon infection with SFV and

its derived vector (Glasgow *et al.*, 1998), they were employed as a positive control for *in vitro* assays. sBHK cells were used for the production of high concentration virus.

BHK-21 cells were propagated in BHK-21 medium (G-MEM), supplemented with 5% (v/v) NCS, 5% (v/v) tryptose phosphate broth, 100 U/ml penicillin-streptomycin and 2mM L-glutamine, and maintained at 37°C in a humidified atmosphere of 5% CO_2 in 75 cm^3 cell culture flasks.

sBHK cells were cultured in G-MEM, containing 10% (v/v) FCS, 5% (v/v) tryptose phosphate broth, 2% (v/v) 1M HEPES, 100U/ml penicillin-streptomycin and 2mM L-glutamine, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in 150 cm^3 cell culture flasks.

CT26 tumour cells were grown in DMEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, 2mM L-glutamine, 2% (v/v) 1M HEPES and 1 mM sodium pyruvate, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in 150 cm^3 cell culture flasks.

K-BALB tumour cells were cultured in DMEM supplemented with 10% (v/v) FCS, 100 U/ml penicillin, 100 µg/ml streptomycin and 2mM L-glutamine, and maintained at 37 °C in a humidified atmosphere of 5% CO_2 in 150 cm^3 cell culture flasks.

BHK-21, sBHK, CT26 or K-BALB cells were sub-cultured as follows: confluent cell monolayers were washed twice with PBS. 0.5% Trypsin 5.3mM EDTA was added and monolayers were incubated at 37 °C until detachment was obvious at which point supplemented fresh specific cell culture medium was added to terminate trypsinisation. The resulting cell suspension was then aspirated several times to break up cell clumps and split into fresh 75 cm^3 or 150 cm^3 flasks containing specific cell growth medium. BHK-21 cells used in *in vitro* assays were typically sub-cultured at a ratio of 1:3 but due to the rapidly growing nature of sBHK, CT26 and K-BALB cells, they were sub-cultured at a ratio of 1:4 or higher depending on confluency. Cell lines used in *in vitro* and *in vivo* assays were passaged not more than 12 times.

Medium for infection (MFI) was composed of G-MEM supplemented with 0.2% (v/v) BSA, 2 mM L-glutamine, and 20 mM HEPES.

Stocks of cells were prepared by slow freezing (at the rate of -1°C/min) to -70°C in medium containing 10% DMSO and subsequently stored in liquid nitrogen.

2.2.2 CONSTRUCTION OF REPLICATING SFV VECTORS

2.2.2.1 Construction of RSFV-ΔSN-26SMCS

A strategy (Figure 2.2) was designed using the unique restriction sites in 13.95 kbp pSP6-SFV4-ΔSN (Appendix 8.1b) and 14.37 kbp RSFV-26SMCS (Appendix 8.1c) plasmid vectors to ultimately obtain RSFV-ΔSN-26SMCS, the mutant vector containing the nsP3-SN deletion in combination with a second 26S subgenomic promoter and a MCS (Figure 2.3).

2.2.2.1.1 Preparation of the insert and the vector fragments

The purified stocks of the pSP6-SFV4-ΔSN and the RSFV-26SMCS vector were double digested at the unique *Bgl*II and *Bsm*I restriction sites in several reaction sets. Each of the digestion reaction mixture contained 5 µg of pSP6-SFV4-ΔSN DNA or 10 µg of RSFV-26SMCS DNA, 5 µl of 10x NEBuffer 3 (50 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol (DTT) pH 7.9) and 0.5 µl (5U) *Bgl*II enzyme. The reaction mixtures were incubated at 37°C in a water-bath for 2 hours (h). After 2 h, 1 µl (10U) *Bsm*I enzyme was added into each of the digestion mixtures and the reactions were maintained at 65°C for further 2 h incubation. Following the incubation steps, 5 µl aliquot of the reaction mixtures was loaded on a 1% (w/v) agarose gel stained with ethidium bromide. A 20 ml 1% TBE agarose gel containing 1 µl 200 mg/ml ethidium bromide was prepared and 5 µl of each DNA digest was mixed with 5 µl 1X TBE buffer and 2 µl 6X blue/orange loading dye. Total amount of the samples were loaded and run at 85 mA alongside 12 µl 1 Kb molecular weight marker (1 µl 1 Kb marker, 2 µl loading dye, 9 µl 1X TBE). Gels were run for approximately 45 min at which point they were visualised using a BioRad Gel Doc 2000 and accompanying Multi-Analyst (version 1.1) software (Figure 3.3 a and b). Following analyses of 5 µl aliquot of the digestion mixtures, the remaining of the reactions was loaded onto 1% agarose gel, and the 12,018 bp fragment that includes the nsP3-SN deletion and the 2031 bp fragment that incorporates the second 26S subgenomic promoter were extracted and purified using the *Qiagen Gel Extraction Kit* as per the manufacturer's instructions. The purified samples were eluted in 30 µl of nuclease-free water.

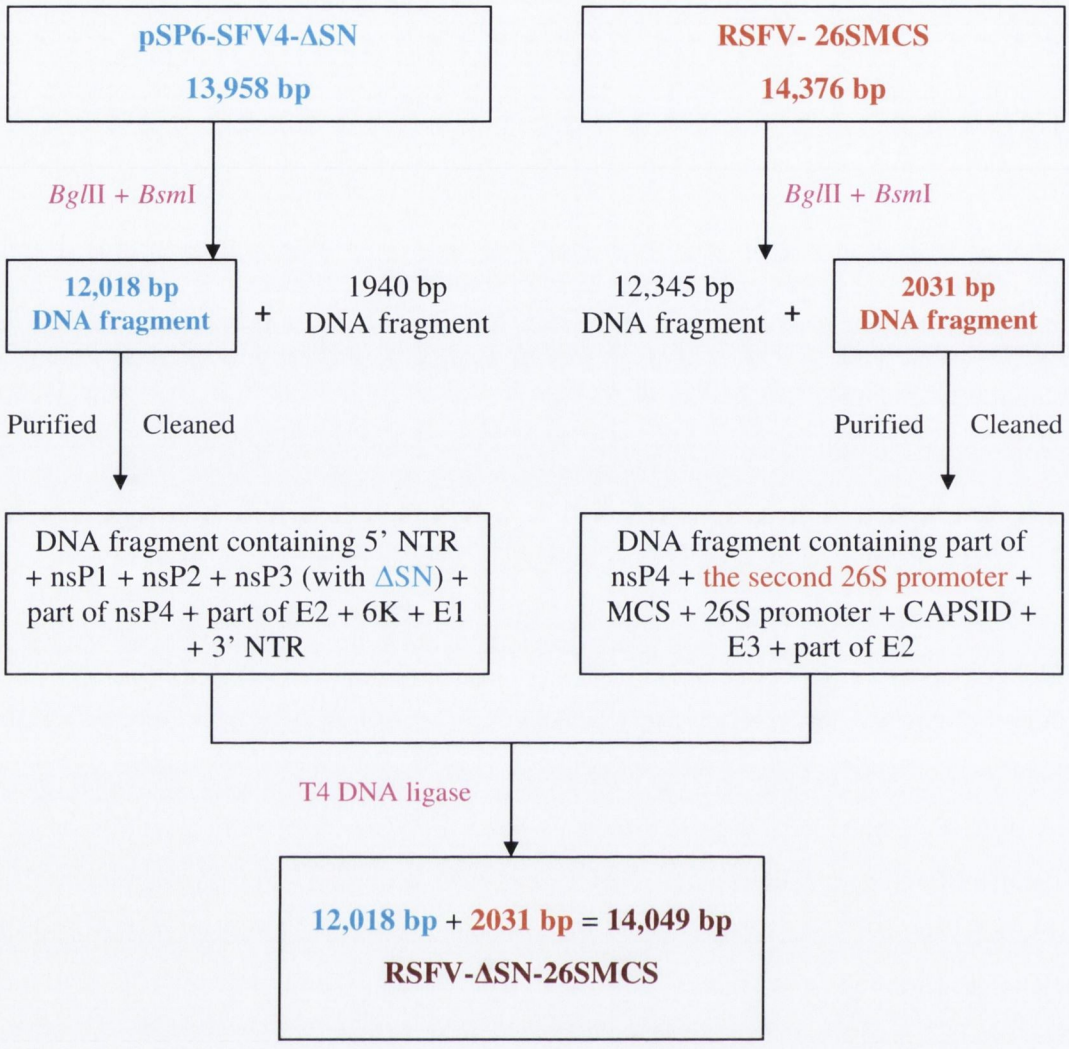


Figure 2.2 Schematic representation of RSFV-ΔSN-26SMCS cloning strategy

The purified stocks of the 13.95 kbp plasmid pSP6-SFV4-ΔSN (Appendix 8.1b) and the 14.37 kbp plasmid RSFV-26SMCS (Appendix 8.1c) vectors were double digested at the unique *Bgl*II and *Bsm*I restriction sites. The plasmid fragments that incorporate the ΔSN region (12,018 bp) or the second 26S subgenomic promoter (2031 bp) were extracted and purified, and the final products were analysed by gel electrophoresis. Following the ligation of the two plasmid fragments, a number of colonies were selected and the plasmid DNA from the transformed colonies was screened for the presence of the insert fragment (2031 bp) by restriction enzyme analysis. The region containing the ΔSN was PCR amplified, and the correct clone was sent for sequencing.

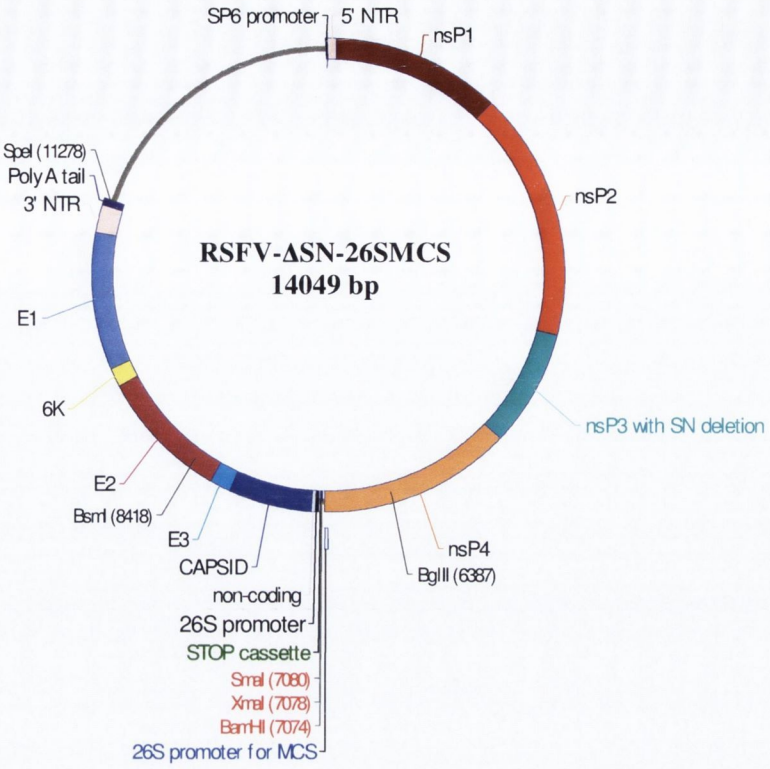


Figure 2.3 Plasmid map of the RSFV-ΔSN-26SMCS vector

RSFV-ΔSN-26SMCS plasmid vector contains the SN deletion in the nsP3 region (turquoise), and the second 26S subgenomic promoter (font colour blue) preceding the MCS which is comprised of *Sma*I, *Xma*I and *BamH*I restriction sites (font colour red).

The final products were analysed and quantified by electrophoresis of a 1 µl aliquot (1 µl DNA sample, 2 µl loading dye, 9 µl 1X TBE) on a 1% (w/v) agarose gel stained with ethidium bromide (Figure 3.3 c and d).

2.2.2.1.2 DNA ligation

Ligation of the 2031 bp fragment (insert) that incorporates the second 26S subgenomic promoter and the 12,018 bp fragment (vector) that includes the nsP3-SN deletion was carried out at a molar ratio of 3:1 insert to vector. The ligation mix contained 8 µl of the insert fragment, 0.5 µl of the vector fragment, 10 µl of 2x Quick ligase buffer (132 mM Tris-HCl, 20 mM $MgCl_2$, 2 mM DTT, 2 mM adenosine triphosphate (ATP) 15% polyethylene glycol (PEG 6000) pH 7.6) and 1 µl (10U) Quick

T4 DNA ligase. The mixture was incubated at 25°C for 5 minutes (min), chilled on ice and stored at -20°C.

2.2.2.1.3 Transformation of *E. coli* XL-10 Gold ultracompetent cells

Escherichia coli (*E. coli*) strain XL-10 Gold ultracompetent cells were used for the transformation of ligated RSFV-ΔSN-26SMCS as these competent cells were created for transformation of large DNA molecules. Following the pre-chilling of the transformation tubes, 2 µl of the β-mercaptoethanol (β-ME) mix provided with the kit was added to 50 µl of competent cells and incubated on ice for 10 min, swirling gently every 2-3 min. 10 µl of the ligation mix was added to the competent cells and incubated on ice for 30 min. The cells were transformed by heat shock at 42°C for 35 seconds (sec) and placed on ice for a further 2 min. Transformed cells were incubated in 900 µl of pre-heated (42°C) NZY^+ broth (1.25 ml of $MgCl_2$, 1.25 ml of $MgSO_4$ and 1 ml of Glucose added to 100 ml of NZY broth) and allowed to grow at 37°C for 1 h with shaking at 200 rpm. Transformed cells were plated onto ampicillin L-agar plates containing 1 mg/ml ampicillin. The plates were incubated inverted overnight at 37°C and observed for colony growth. A negative control transformation mixture was also set up, where the XL-10 Gold ultracompetent cells were incubated containing no DNA but ampicillin.

2.2.2.1.4 Screening of the plasmid DNA from the transformed colonies

A few isolated transformed colonies of recombinant plasmids were picked and grown in 10 ml of L-broth containing 100 µg/ml ampicillin separately. The cell cultures were grown for 18 h shaking at 37°C and used to purify the plasmid, using the *Qiagen Miniprep Plasmid Purification Kit* as per the manufacturer's instructions. Purified DNA was eluted in 50 µl of nuclease free water by centrifugation. 1 µl from each DNA sample was run on 1% agarose gel alongside 1 Kb DNA ladder to check the size of the sample (Figure 3.4a).

Each plasmid DNA from different transformed colonies was screened for the presence of the insert fragment that incorporates the second 26S subgenomic promoter by restriction digestion analysis. To test the presence of the insert 1 µl of each DNA

was double digested with *Bgl*II and *Bsm*I enzymes as described in section 2.2.2.1.1. The digested products for each DNA samples were analysed on 1% agarose gel alongside Lambda marker (Figure 3.4b).

The positive clones which contained the insert fragment were also screened by *Kpn*I restriction digestion analysis where the reaction mixture contained 1 µl DNA, 2 µl of 10x NEBuffer 1 (10 mM Bis-Tris-Propane-HCl, 10 mM $MgCl_2$, 1 mM DTT pH 7.0), 2 µl of 10x BSA and 0.5 µl (5U) *Kpn*I enzyme. 5 µl aliquot of the digested products was analysed on 1% agarose gel alongside 5 µl aliquot of *Kpn*I digested RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS, RSFV-26SMCS and pSP6-SFV4 to compare the size of the fragments and to confirm the presence of the SN deletion. Results were analysed alongside 1 Kb DNA marker (Figure 3.5 a and b).

Following the confirmation of the insert fragment and the nsP3-SN deletion, the region that contains the ΔSN was PCR amplified in a reaction set. A total volume of 50 µl PCR reaction was comprised of 50 ng of RSFV-ΔSN-26SMCS DNA, 10 pmol/µl forward and reverse primers of SN, 10X Taq Polymerase buffer, 25 mM $MgCl_2$, 10 mM dNTP mix, 0.5 U Taq Polymerase and nuclease free water. The positive and negative control reactions were also set with the same volume of reagents containing 50 ng of pSP6-SFV4 DNA and in the absence of DNA, respectively. The reactions were incubated at 94°C for 2 min, followed by 30 cycles of 94°C for 30 sec, 49°C or 50°C for 30 sec (depending on primer pair annealing temperature), 72°C for 45 sec or 1 min and a final extension of 72°C for 5 min.

The regions containing the nsP3-TN deletion and 6K deletion in RSFV-ΔTN-26SMCS and RSFV-Δ6K-26SMCS plasmid vectors (which were constructed and kindly provided by Dr. Sareen Galbraith, Appendix 8.1 d and e), respectively, were also PCR amplified by the specific TN and 6K primers (Table 2.1) as described above.

All PCR products were analysed on 1% agarose gel alongside 1 Kb DNA marker ladder (Figure 3.6 a and b).

2.2.2.1.5 Sequencing of the region incorporating ΔSN in RSFV-ΔSN-26SMCS

One RSFV-ΔSN-26SMCS clone was sent for sequencing to LARK Technologies Inc. (UK) using the forward and reverse primers of SN (Table 2.1). The region incorporating the second 26S subgenomic promoter was also sequenced with the

forward and reverse primers of 26S (Table 2.1). Chromas 2.23 files were analysed with BLAST- bl2seq (NCBI database) (Appendix 8.2).

RSFV-ΔTN-26SMCS and RSFV-Δ6K-26SMCS clones had already been constructed by Dr. Sareen Galbraith. The presence of the ΔTN in RSFV-ΔTN-26SMCS and the presence of the Δ6K in RSFV-Δ6K-26SMCS clone were confirmed by sequencing the genomes with specific primer pairs outlined in Table 2.1. RSFV-ΔTN-26SMCS and RSFV-Δ6K-26SMCS plasmids were sequenced by LARK Technologies and the results were analysed with BLAST- bl2seq (NCBI database) (Appendices 8.3 and 8.4, respectively).

2.2.2.2 Construction of RSFV-ΔTN-Δ6K-26SMCS

A strategy (Figure 2.4) was designed using the unique restriction sites in 14.24 kbp RSFV-ΔTN-26SMCS (Appendix 8.1d) and 14.19 kbp RSFV-Δ6K-26SMCS (Appendix 8.1e) plasmid vectors to ultimately obtain RSFV-ΔTN-Δ6K-26SMCS, the replicating SFV vector containing both the ΔTN and the Δ6K in combination with a second 26S subgenomic promoter and a MCS (Figure 2.5).

2.2.2.2.1 Preparation of the Δ6K insert and the SFV vector containing the ΔTN

The purified stocks of the RSFV-Δ6K-26SMCS and the RSFV-ΔTN-26SMCS vectors were double digested at the unique *Bsm*I and *Spe*I restriction sites in several reaction sets. Each of the digestion reaction mixture contained 10 µg of RSFV-Δ6K-26SMCS DNA or 5 µg of RSFV-ΔTN-26SMCS DNA, 5 µl of 10x NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM DTT pH 7.9), 5 µl of 10x BSA and 0.5 µl (5U) *Spe*I enzyme. The reaction mixtures were incubated at 37°C in a water-bath for 2 h. After 2 h, 1 µl (10U) *Bsm*I enzyme was added into each of the digestion reaction mixtures and the reactions were maintained at 65°C for further 2 h incubation. Following the incubation steps, 1 µl aliquot of the reaction mixtures was analysed by gel electrophoresis (Figure 3.7a) and the remaining of the mixtures was loaded onto 1% agarose gel for extraction and purification steps. The 2680 bp fragment that incorporates the Δ6K region and the 11,387 bp fragment that includes the ΔTN were extracted and purified using the *Qiagen Gel Extraction Kit* as per the manufacturer's instructions. The purified Δ6K or ΔTN samples were pooled and eluted in 30 µl of

nuclease-free water. The final products were analysed and quantified by electrophoresis of a 1 µl aliquot on a 1% agarose gel as described in section 2.2.2.1.1 (Figure 3.7b).

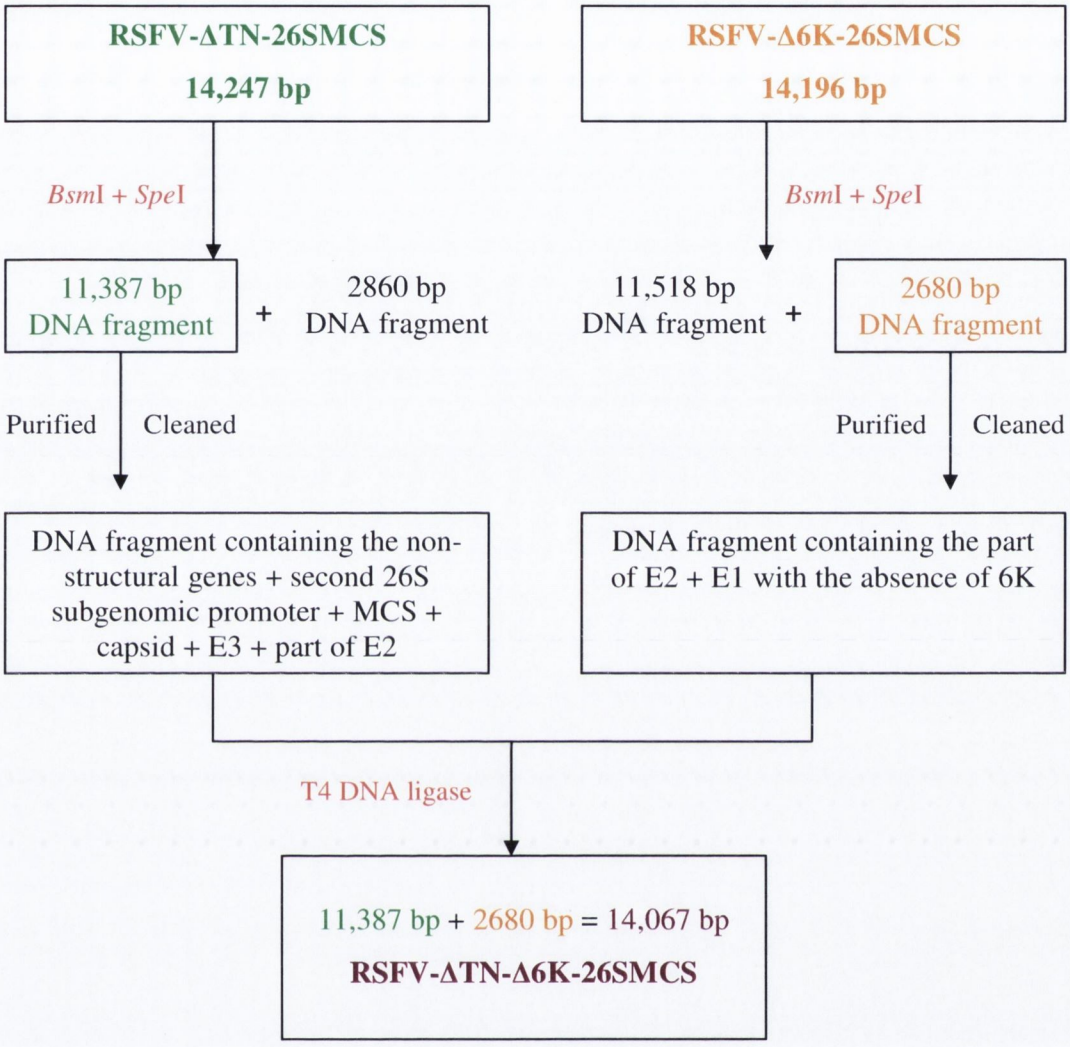


Figure 2.4 Schematic representation of RSFV-ΔTN-Δ6K-26SMCS cloning strategy

The purified stocks of the 14.24 kbp plasmid RSFV-ΔTN-26SMCS (Appendix 8.1d) and the 14.19 kbp plasmid RSFV-Δ6K-26SMCS (Appendix 8.1e) vectors were double digested at the unique *Bsm*I and *Spe*I restriction sites. The plasmid fragments that incorporate the ΔTN (11,387 bp) or the Δ6K (2680 bp) were extracted and purified, and the final products were analysed by gel electrophoresis. Following the ligation of the two plasmid fragments, few colonies were selected and the plasmid DNA from the transformed colonies was screened for the presence of the insert fragment (2680 bp) by restriction enzyme analysis. The regions containing the Δ6K or the ΔTN were also PCR amplified separately, and the correct clones sent for sequencing.

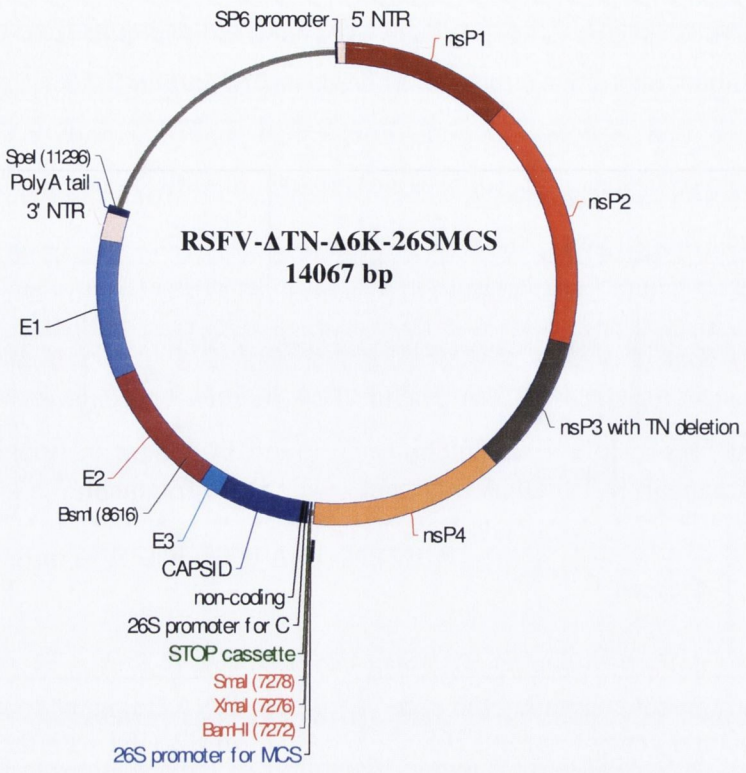


Figure 2.5 Plasmid map of the RSFV-ΔTN-Δ6K-26SMCS vector

RSFV-ΔTN-Δ6K-26SMCS plasmid vector contains the TN deletion in the nsP3 region (grey), and the second 26S subgenomic promoter (font colour blue) preceding the MCS which is comprised of *Sma*I, *Xma*I and *BamH*I restriction sites (font colour red).

2.2.2.2.2 DNA ligation

Ligation of the 2680 bp fragment (insert) that incorporates the Δ6K region to the 11,387 bp fragment (vector) containing the nsP3-TN deletion was carried out at a molar ratio of 3:1 insert to vector as described in section 2.2.2.1.2.

2.2.2.2.3 Transformation of *E. coli* XL-10 Gold ultracompetent cells

Ligated RSFV-ΔTN-Δ6K-26SMCS plasmid was transformed into *E. coli* strain XL-10 Gold ultracompetent cells as described in section 2.2.2.1.3. The plates were incubated inverted overnight at 37°C and observed for colony growth.

2.2.2.2.4 Screening of the plasmid DNA from the transformed colonies

A few isolated transformed colonies of recombinant plasmids were picked, grown in L-broth and the plasmid was purified as described in section 2.2.2.1.4. 1 µl from each DNA sample was run on 1% agarose gel alongside 1 Kb DNA ladder to check the size of the sample (Figure not shown).

Each plasmid DNA from different transformed colonies was screened for the presence of the insert fragment that incorporates the Δ6K by restriction digestion analysis. To test the presence of the insert 1 µl of each DNA was double digested with *Spe*I and *Bsm*I enzymes as described in section 2.2.2.2.1. The digested products for each DNA samples were analysed on 1% agarose gel alongside 1 Kb ladder (Figure 3.8).

Following the confirmation of the insert fragment, the region that contains the Δ6K and the region that contains the ΔTN were PCR amplified in separate reaction sets with the 6K or TN primer pairs, respectively, as described in section 2.2.2.1.4. The positive and negative control reactions were also set with the same volume of reagents containing 50 ng of pSP6-SFV4 DNA and in the absence of DNA, respectively. The reactions were incubated as described in section 2.2.2.1.4, and all PCR products were analysed on 1% agarose gel alongside 1 Kb DNA marker ladder (Figure 3.9 a and b).

2.2.2.2.5 Sequencing of the regions incorporating the ΔTN or the Δ6K in RSFV-ΔTN-Δ6K-26SMCS

Two clones with both the ΔTN and the Δ6K were sent for sequencing to LARK Technologies Inc. using the forward and reverse primers of TN and 6K separately (Table 2.1). Region incorporating the second 26S subgenomic promoter was also sequenced in the clones with the forward and reverse primers of 26S. Chromas 2.23 files were analysed with BLAST- bl2seq (NCBI database) (Appendix 8.5).

2.2.3 PRODUCTION OF VIRUS FROM REPLICATING SFV VECTORS

2.2.3.1 Expression Vectors

Virus was produced from the original (pSP6-SFV4, RSFV-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS, and RSFV-EGFP-26SMCS) and the newly constructed replicating SFV vectors (RSFV-ΔSN-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, and RSFV-HABax-26SMCS) for the *in vitro* and *in vivo* assays.

2.2.3.2 Preparation of the SFV plasmids

Ultracompetent XL-10 Gold cells were transformed with pSP6-SFV4, RSFV-26SMCS, RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, RSFV-HABax-26SMCS, or RSFV-EGFP-26SMCS separately as outlined in section 2.2.2.1.3. Transformed pure single colonies were then grown in L-broth separately for each plasmid DNA and each of the plasmid DNAs was purified from *E. coli* XL-10 Gold cells using the *Qiagen Miniprep Plasmid Purification Kit* as described in section 2.2.2.1.4. Plasmid concentrations were estimated using a DNA/RNA spectrophotometer and visualised by gel electrophoresis as described in section 2.2.2.1.1.

2.2.3.3 Linearisation of plasmid DNA with *Spe*I

The unique *Spe*I restriction site preceding the non-structural protein genes and SP6 promoter in each of the SFV plasmids was exploited to linearise the plasmids prior to *in vitro* RNA transcription. A total of 10 µg DNA was linearised in a 30 µl volume containing 3 µl *Spe*I buffer, 3 µl 10x BSA and 10 U *Spe*I. Following 2 h incubation at 37°C in a water-bath, cut and uncut plasmids were visualised by gel electrophoresis alongside 1 Kb DNA ladder as described in section 2.2.2.1.1. Linearised plasmids were then cleaned using the *Qiagen Nucleotide Removal Clean-up Kit* and the plasmid DNA was resuspended in a final volume of 50 µl nuclease free water. A 1 µl aliquot of the cleaned DNA was loaded and run on 1% agarose gel alongside 1 Kb DNA molecular weight ladder to quantify the amount of DNA by comparing band intensities to those of

the 1 Kb marker bands (which were of known concentrations) as described in section 2.2.2.1.1.

2.2.3.4 *In vitro* SP6 RNA transcription

1.5 µg of each linearised and purified plasmid DNA (section 2.2.3.3) was used as a template in 50 µl SP6 *in vitro* RNA transcription reactions containing 10x SP6 buffer (40 mM Hepes-KOH pH 7.4, 6 mM MgOAc, 2 mM spermidine-HCl), 1 mM $m^7G(5')ppp(5')G$ (CAP), 5 mM DTT, 1 mM each rATP, rCTP, rUTP, and 500 µM dGTP, 60 U RNasin and 34 U SP6 RNA polymerase in nuclease free water. This reaction was incubated for 1 h 50 min at 37°C and placed on ice. A 1 µl aliquot of each of the RNA transcripts of SP6-SFV4, RSFV-26SMCS, RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, RSFV-HABax-26SMCS or RSFV-EGFP-26SMCS was run on 0.6% agarose gel in the same manner as described in section 2.2.2.1.1 for quantification of the RNA and rest of the transcribed RNAs was stored at -70°C for further use.

2.2.3.5 Electroporation of RNA

In order to produce virus from the transcribed RNAs, two 75 cm^3 flasks of 80% confluent BHK-21 cells were propagated, washed and trypsinised as described in section 2.2.1 and resuspended in 10 ml of fresh BHK medium. Cells were centrifuged at 400 *x g* for 10 min at room temperature (R.T) and the pellets were gently resuspended in 10 ml PBS. The cell suspensions were centrifuged again as above and the pellets were resuspended gently in 700 µl PBS. Each of the 50 µl of *in vitro* transcribed RNAs of SFV vectors (section 2.2.3.4) was thawed, added into one of the cell suspensions and mixed gently. The other cell suspension was utilized as the negative control. Both suspensions were added to 0.4 µm gap electroporation cuvettes separately and electroporation was then performed in single pulse at 0.85 kV and 25 µF capacitance, using a BioRad Gene Pulser XCell. Cells were then immediately and gently resuspended in 20 ml of fresh, preheated (37°C) BHK medium, added to 75 cm^3 flasks and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h.

2.2.3.6 Harvesting the virus-containing supernatant

After the observation of a significant amount of cytopathic effect (CPE), typically within 24 hours post infection (h.p.i.), in the BHK-21 cells electroporated separately with each of the *in vitro* transcribed RNAs, the virus-containing supernatant was decanted into fresh 20 ml centrifuge tubes and clarified by centrifugation at 400 *x g* for 10 min at 4°C. The supernatant was then filter sterilized into a fresh tube through a 0.2 µm filter before being aliquoted into 1 ml amounts. The samples were kept at -70°C.

2.2.3.7 Production of virus from BHK-21 cells

The replicating SFV vectors used in this study are based on the infectious clone of SFV, pSP6-SFV4, which contains the full coding and non-coding genomic cDNA sequence of virulent SFV (Liljeström *et al.,* 1991). The virus-containing supernatant from each of the infectious clones (section 2.2.3.6) was allowed to adsorb onto subconfluent BHK-21 cells in a 75 cm^3 flask for 1 h at 37°C in a humidified atmosphere of 5% CO_2 with rocking every 15 min. One subconfluent 75 cm^3 flask of BHK-21 cells was also incubated with PBS for 1 h at 37°C as the negative control. After 1 h the inoculum was removed and 20 ml fresh BHK-21 medium was added prior to incubation at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. The supernatant was then decanted, centrifuged at 3,000 *x g* for 10 min to remove cellular debris and filtered through a 0.2 µm filter. The virus stocks, now termed SFV4, RSFV-26SMCS virus, RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus, RSFV-Δ6K-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, RSFV-HABax-26SMCS virus or RSFV-EGFP-26SMCS virus, were stored at -70°C in 1 ml aliquots.

2.2.3.8 Titration of the virus by plaque assay

BHK-21 cells were seeded at a concentration of 10^5cells/well in a total volume of 3 ml per 60 mm^2 cell culture dish and incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h, until they reached subconfluency. The cells were inoculated in duplicate with 0.5 ml of each serial dilution of virus in PBS from 10^{-2} to 10^{-10}. Incubation was carried out for 1 h at 37°C in a humidified atmosphere of 5% CO_2 with

rocking every 15 min. One dish was mock-infected with 500 µl PBS and acted as the negative control.

On removal of the inoculum, equal volumes of 1.8% noble agar heated to 45°C were mixed with overlay medium (10X Minimal Essential Medium (MEM) supplemented with 10% NCS, 10% tryptose phosphate broth, 200 U/ml penicillin, 200 µg/ml streptomycin, 4 mM L-glutamine and distilled water) at 37°C and added to the dishes at a final volume of 3 ml per dish. When the agar had solidified, typically within 5 min at room temperature, the plates were incubated for 48 h at 37°C in a humidified atmosphere of 5% CO_2. After 48 h, 2 ml of 10% formalin was added to each dish and allowed to fix the cells for 10 min at RT in a fume hood. The formalin and agar were then removed under running tap water. The cells were stained with 2 ml of crystal violet for 3 min and then rinsed under running tap water. The dishes were left to dry at R.T, the plaques counted and the viral titres expressed as plaque forming units (PFU)/ml.

2.2.4 *IN VITRO* ASSAYS

2.2.4.1 Growth curve analysis of the replicating SFV vectors

In order to assess the multiplication efficiency of viruses produced from SFV vectors *in vitro*, viral growth curves were generated over a 24 h time period. BHK-21 cells were seeded into 6-well cell culture dishes at a concentration of 8 x 10^5 cells/well in the appropriate growth medium, and incubated at 37°C overnight. The medium was removed and the cells were infected with SFV4, RSFV-26SMCS virus, RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus or RSFV-Δ6K-26SMCS virus at a multiplicity of infection (MOI) of 10 or 0.1 PFU/cell in a total volume of 250 µl MFI for 1 h at 37°C with rocking every 15 min. Two additional wells were used as the mock-infection negative controls and incubated with MFI only. After 1 h, 2.5 ml of fully supplemented growth medium was added on top of the inoculums and the plates were incubated at 37°C in a humidified atmosphere of 5% CO_2. The cell culture supernatant was then harvested in duplicate at 2, 4, 6, 8, 10, 12, and 24 h.p.i., aliquoted, and stored at -70°C. The amount of virus produced at each time was quantified by plaque assay in two separate experiments as described in section 2.2.3.8 and the viral growth curves were plotted as the average of the two plaque assays.

The multiplication efficiency of RSFV-ΔTN-Δ6K-26SMCS virus and RSFV-HABax-26SMCS virus was also analysed in BHK-21 cells, and the results were compared to the infection efficiencies observed in the CT26 and K-BALB tumour cell lines. BHK-21, CT26 or K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 8 x 10^5 cells/well and infection was carried out with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a MOI 0.1 PFU/cell as described above. Viral growth curves were plotted as the average of the two plaque assays performed on the cell culture supernatant which was harvested in duplicate at 4, 8, 12 and 24 h.p.i.

2.2.4.2 *In vitro* stability of the Bax protein expressed by RSFV-HABax-26SMCS

2.2.4.2.1 Passaging the RSFV-HABax-26SMCS virus

The RSFV-HABax-26SMCS virus produced in section 2.2.3.7 was utilised to infect one 75 cm^3 flask of ~90% confluent BHK-21 cells. The medium was removed, 1 ml RSFV-HABax-26SMCS virus was added and the cells were incubated for 1 h at 37ºC with rocking every 15 min. After 1 h the inoculum was removed, 20 ml of fresh BHK-21 medium was added and the cells were incubated for 24 h at 37°C in a humidified atmosphere of 5% CO_2. RSFV-HABax-26SMCS virus passage 2 (P2) was harvested by centrifuging the supernatant at 400 *x g* for 10 min 4°C, aliquoted into 1 ml amounts and stored at -70°C. The RSFV-HABax-26SMCS virus was passaged eight times in BHK-21 cells as described above and the HA-Bax protein expression was analysed for each passage by indirect immunofluorescence in BHK-21 cells, to confirm the stability of the *bax* gene.

2.2.4.2.2 Immunofluorescence analysis of the Bax protein

A 75 cm^3 flask of ~90% confluent BHK-21 cells was propagated, washed and trypsinised as described in section 2.2.1. After gentle aspiration, cells were seeded onto 22 mm^2 glass cover slips in a 6-well plate in a final volume of 3 ml/well. The plate was incubated at 37°C in a humidified atmosphere of 5% CO_2 for 24 h. Medium was removed from the wells and the monolayers were washed twice with 2 ml PBS+. Duplicate wells of the confluent BHK-21 cells were infected with the RSFV-HABax-

26SMCS virus P1, P2 or P3 and MFI only (negative control) in a total volume of 500 µl and incubated for 1 h at 37°C with rocking every 15 min. 2.5 ml of fresh BHK-21 medium was added to each well and incubated at 37°C in a humidified atmosphere of 5% CO_2.

After 16-18 h, immunofluorescence was carried out. Medium was removed from the infected and mock-infected wells and the monolayers were washed twice for 5 min with PBS+ in the 6-well plate. After washing, cells were fixed with 4% PFA in PBS- for 20-30 min. After two 5 min PBS washes the PFA was removed and the cells were incubated in ethanol: acetic acid (2:1) at -20°C for 5-10 min. The PBS washes were repeated as above prior to blocking of the cells with 5% mouse serum in PBS for 1 h at a final volume of 2ml/well. The coverslips were incubated monolayer-down with 50 µl drops of primary (Rabbit anti-HA) antibody (1:25 in PBS containing 5% mouse serum) on slides covered with parafilm in a humidified chamber at 37°C for 1 h. Coverslips were then removed, placed in a holder and washed twice for 10 min in PBS containing 0.05% Tween 80 (PBS-T) and twice for 5 min in PBS. After washing, cells were incubated as above with PBS containing 5% mouse serum and biotinylated mouse anti-rabbit IgG (1:1000) for 30 min. After washing twice for 10 min in PBS-T and twice for 5 min in PBS, the cells were incubated with streptavidin/FITC (1:150 in HEPES-NaCl buffer (0.5 M NaCl, 10 mM HEPES)) for 30 min. Following the incubation, the cells were washed twice for 15 min in high-salt HEPES-NaCl buffer. After rinsing in distilled water, the cells on the coverslips were mounted on slides with hardest vectashield mounting medium (DAPI). The cells were examined using a fluorescent microscope and images made using Act1 software in conjunction with a Nikon camera attached to the fluorescent microscope set at 20x or 40x magnification.

2.2.4.3 Infection efficiency of the cell lines with RSFV-EGFP-26SMCS virus

To determine the infection efficiency of BHK-21 and tumour cells, with the replicating SFV vector, RSFV-EGFP-26SMCS; BHK-21, CT26 or K-BALB cells were seeded in 12-well cell culture plates at a concentration of 8 x 10^5 cells/well in 2 ml maintenance medium and allowed to adhere for ~ 24 h. Cells were then washed once with 2 ml PBS and infected with RSFV-EGFP-26SMCS virus (section 2.2.3.7) in duplicate using a range of different MOIs (0.1, 1, 10, and 100) in 250 µl MFI for 1 h at 37°C with rocking every 15 min. Virus inoculum was then removed, 2 ml maintenance

medium at 37°C was added to each well and cells were maintained for 18 h at 37 °C in a humidified atmosphere of 5% CO_2. Supernatants were removed into 15 ml falcon tubes, cells were trypsinized with 250 µl 1x trypsin and aspirated in 250 µl maintanence medium, and the cell suspension was transferred into the falcon. Tubes were filled with 12 ml PBS and centrifuged at 400 *x g* for 10 min at 4°C. Supernatant was removed, the pellet at the bottom of the tube was flicked after which the cells were resuspended in 9 ml PBS and the tubes were centrifuged at 400 *x g* for 10 min at 4°C. Supernatant was removed, cells were resuspended in 400 µl of 2% PFA in PBS, and the number of EGFP expressing cells was determined by flow cytometer. Fixed cells could be maintained at 4°C for 3-4 days until the analysis could be performed.

2.2.4.4 Cell viability assay

In order to assess the effect of replicating SFV vectors on viability of cultured cells *in vitro* at 24 h intervals over a 5 day period, BHK-21, CT26 or K-BALB cells were seeded at a concentration of 1.5 x 10^4 cells/well in 96-well cell culture dishes in 200 µl growth medium and allowed to adhere for ~ 12 h. Monolayers were washed once with 200 µl PBS and infected in triplicate with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a MOI of 100 in a final volume of 25 µl MFI. After 1 h incubation at 37 °C, the inocula were removed, 200 µl of fully supplemented growth medium at 37 °C was added and the cells maintained at 37 °C in a humidified atmosphere of 5% CO_2. At 24, 48, 72, 96, and 120 h.p.i., 20 µl of the *CellTiter 96® AQ_{ueous} One Solution Reagent* was added directly to culture wells, incubated for 1 h at 37 °C in a humidified atmosphere of 5% CO_2, and then the absorbance was recorded at 490 nm with a 96-well plate reader. The quantity of formazan product as measured by the amount of 490 nm absorbance shows the number of living cells in culture. Results were converted and displayed as the percentage of viable cells following infection with SFV.

2.2.4.5 Caspase activation assay

In order to detect the apoptosis induction in cells following infection with replicating SFV vectors; BHK-21, CT26 or K-BALB cells were seeded at a concentration of 10^6 cells/well in 6-well cell culture dishes in 3 ml maintenance medium

and allowed to adhere for ~ 24 h. Monolayers were washed twice with 2 ml PBS and infected in duplicate with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a MOI of 100 in a final volume of 500 µl MFI. After 1 h incubation at 37 °C with rocking every 15 min, the inocula were removed, 3 ml of fully supplemented growth medium at 37 °C was added and the cells maintained at 37 °C in a humidified atmosphere of 5% CO_2. After incubation for 18 and 48 h, cells were sampled using the *Vybrant FAM Caspase-3 and -7 Assay Kit* as per the manufacturer's instructions. Following fixation, cells were analysed and the percentage of FLICA (Fluorescent-Labeled Inhibitor of CAspases) which is an indicator of the active caspases, was measured by flow cytometre.

2.2.5 PRODUCTION OF CONCENTRATED VIRUS FOR USE IN *IN VIVO* STUDIES

It was necessary to concentrate the virus to titres of ~10^{10} infectious units (IU)/ml, in order to obtain a more significant response to treatment of CT26 and K-BALB tumours *in vivo*.

Four 150 cm^3 flasks of sBHK cells were grown to ~80% confluence as described in section 2.2.1 and infected with 2 ml of the supernatant containing virus (SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, or RSFV-HABax-26SMCS) as described in section 2.2.3.7 with a total volume of 25 ml medium per flask.

2.2.5.1 Concentration of virus by ultracentrifugation

Supernatants were pooled (100 ml) and and centrifuged at 6,000 *x g* for 15 min at 4°C in a Sorvall Rc 5C Plus centrifuge. Supernatants were decanted into fresh centrifuge tubes and re-centrifuged under the same conditions twice. Clarified supernatants were then divided into three SW-28 ultracentrifuge tubes and 5 ml of 20% sucrose solution (in TNE buffer) was slowly added to the base of each tube to form a sucrose cushion. Tubes were then filled to within 2-3 mm from the top with sBHK growth medium, placed into SW28 ultracentrifuge buckets and balanced by using another mimic tube. The buckets were fixed to the SW28 rotor and centrifuged at 100,000 *x g* for 2 h at 4°C in the Beckman ultracentrifuge. After 2 h supernatants were

removed, the sides of the tubes dried with a sterile cotton swab and the tubes left standing upside down on a sterile tissue paper. Once the tubes were completely dry (within 2-3 min), the viral pellets were resuspended in 200 µl TNE buffer. Tubes were then covered with parafilm and incubated on ice for 2 h or overnight at 4°C. The pellets were gently resuspended, removed and pooled on ice. A further 200 µl of TNE buffer was used to wash each tube in succession by aspiration before being collected into the previously harvested TNE to a final volume of ~800 µl. The resuspended virus was aliquoted in 50-100 µl volumes into cryotubes on ice and stored at -70°C.

2.2.5.2 Plaque assay of the concentrated virus

The titre of the concentrated virus was calculated using plaque assay as described in section 2.2.3.8. In order to accommodate the substantially higher viral titres generated, duplicate serial dilutions were prepared from 10^{-6} to 10^{-12}.

2.2.6 *IN VIVO* WORK

2.2.6.1 *In vivo* analyses of the replicating SFV vectors

BALB/c mice were maintained as outlined in section 2.1.6 and used for *in vivo* experimentation. Groups of 10 female BALB/c mice were inoculated intramuscularly (i.m.) with 10^6 PFU of RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus, RSFV-Δ6K-26SMCS virus or RSFV-26SMCS virus in 50 µl TNE buffer in the right *tibialis anterior* leg muscle. Groups of 10 female BALB/c mice were also injected i.m. with 10^6 PFU of SFV4 in 50 µl TNE and with 50 µl TNE buffer alone as the positive and negative controls, respectively. All mice were checked daily for 14 days, to observe any clinical signs following i.m. injection. Immunity of surviving mice was tested by intraperitoneal (i.p.) challenge with 10^6 PFU of virulent SFV L10 in 500 µl TNE buffer, which is lethal for unprotected mice. Mice were observed daily for a further 14 days and at the experiment end surviving mice (if there is any) were sacrificed.

In a separate experiment, virulence of RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS was tested in BALB/c mice as described above and the results were compared to the groups of mice injected with RSFV-26SMCS virus and SFV4 which

were considered as the positive controls. Group of 10 female BALB/c mice injected i.m. with 50 µl TNE buffer was considered to be the negative control group. After checking each mouse for clinical signs for 14 days, surviving mice were challenged i.p. with L10 as described above.

2.2.6.2 Virulence analyses of the replicating SFV vectors in BALB/c mice pre-immunised with rSFV-p62-6K VLPs

For immunisation, rSFV-p62-6K VLPs were produced as described by Smyth et al (2005) and the titre was adjusted to 2 x 10^7 IU/ml in TNE buffer. Mice to be immunised received a 50 µl i.m. injection of the rSFV-p62-6K VLPs in TNE at a concentration of 10^6 IU in the left *tibialis anterior* leg muscle and were boosted with the same injection in the right *tibialis anterior* leg muscle 14 days post-inoculation (p.i.).

Two weeks post-immunisation, groups of 10 female BALB/c mice were injected i.m. in the right *tibialis anterior* leg muscle with 10^6 PFU of SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus in 50 µl TNE, and with 50 µl TNE buffer only as the negative control group. Two groups of 10 female BALB/c mice were injected i.m. with 10^6 PFU of SFV4 in 50 µl TNE or with 50 µl TNE buffer only, without prior administration of rSFV-p62-6K VLPs, as the control groups.

Following 14 days of injection, three mice from each group were sampled for testing the presence of pathological lesions of SFV4 infection (refer to section 2.2.7.1.1) and rest of the group were challenged i.p. with 10^6 PFU of L10. Surviving mice were sacrificed 14 days post-challenge.

2.2.6.3 *In vivo* tumour treatment studies

BALB/c mice used for the tumour experiments were maintained under pathogen-free conditions as described in section 2.1.6. Fifteen female BALB/c mice of 40-60 day old were used for each treatment group throughout the tumour treatment experiments. Prior to tumour induction, BALB/c mice were immunised with rSFV-p62-6K VLPs as described in section 2.2.6.2 and 28-30 days post-boost the right flank of

each BALB/c mouse was shaved in order to facilitate tumour induction and measurement of tumour growth.

2.2.6.3.1 Induction of CT26 or K-BALB tumours *in vivo*

Tumour cells (CT26 or K-BALB) were grown in 150 cm^3 cell culture flasks as described in section 2.2.1 and harvested by trypsinisation during their exponential phase of growth. Cells were centrifuged at 400 *x g* at 4°C and resuspended gently in non-supplemented DMEM. Cells were centrifuged and resuspended as before, adjusted to a final concentration of 10^7 cells/ml and injected subcutaneously (s.c.) (10^6 / mouse in 100 µl) into the right flank of each pre-immunised mouse using a 1 ml syringe and 23G needle. Mice were examined daily for signs of s.c. tumour growth and when detected, tumour diameters were measured using a digital calliper in 2 perpendicular diameters with the average tumour diameter calculated as the square-root of the product cross-sectional diameters, assuming spherical shape. Treatment commenced once tumours had reached an average diameter of 4 mm (6-13 days post injection in case of both CT26 and K-BALB tumours).

2.2.6.3.2 Treatment of CT26 and K-BALB tumours *in vivo* using the replicating SFV vectors

Concentrated virus (SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, and RSFV-HABax-26SMCS) was prepared, titrated as described in section 2.2.5 and adjusted to a concentration of 10^{10} PFU/ml in TNE buffer. Mice received a total of 6 intratumoural (i.t.) injections each on every alternate day over an eleven-day period, of 50 µl TNE buffer alone (control groups) or the TNE buffer containing concentrated virus using a 0.5 ml insulin microsyringe. Injections were directed towards the centre of each tumour and administered slowly to facilitate the absorption of the fluid by the tumour and to avoid the surrounding normal tissue. Tumours were measured daily as described in section 2.2.6.3.1 and mice were euthanized by isofluorane overdose and cervical dislocation when the average tumour diameter approached 15 mm. The schematic representation of treatment strategy is given in Figure 2.6.

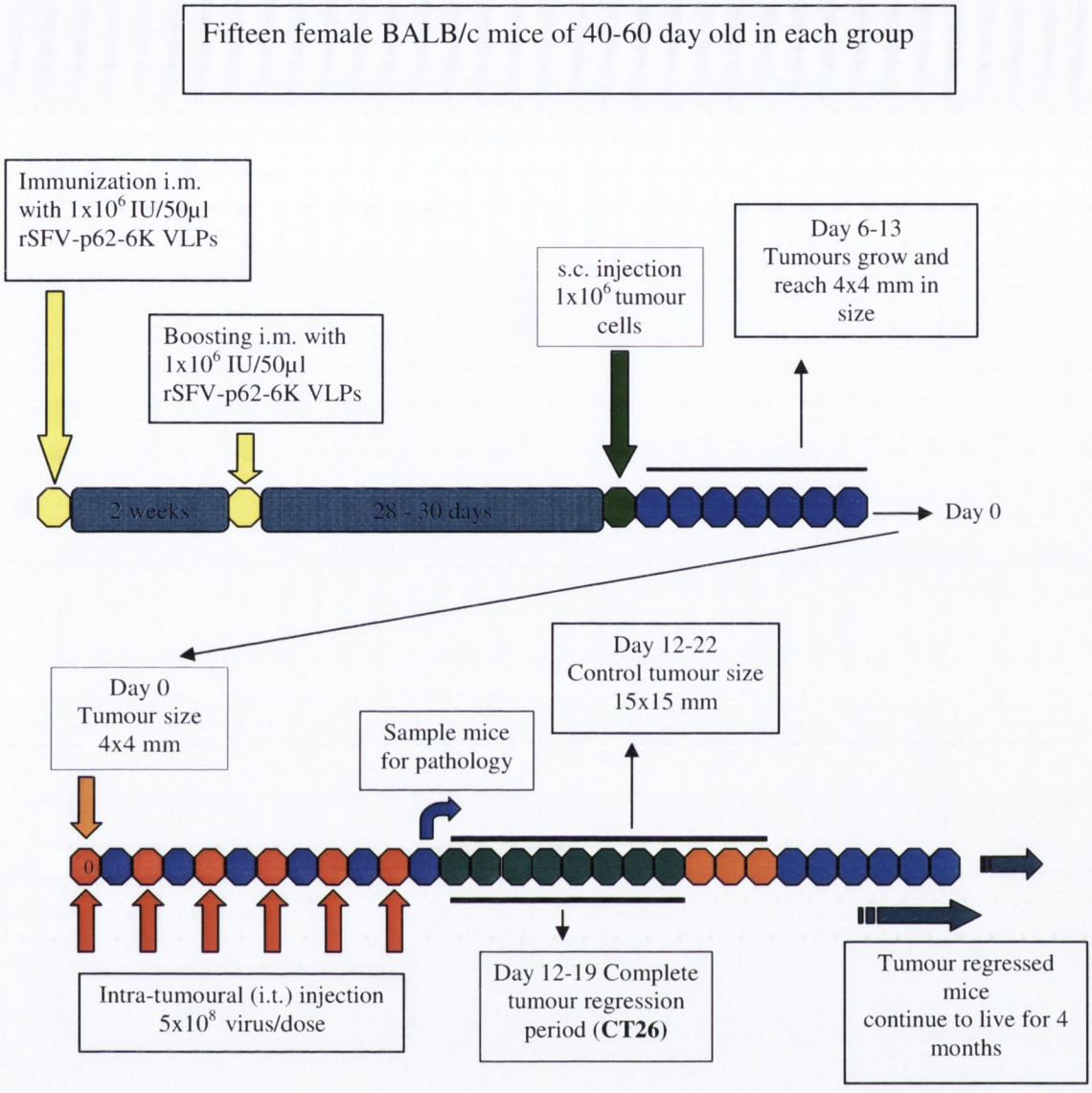


Figure 2.6 General strategy for *in vivo* treatment of CT26 or K-BALB tumours in immunocompetent BALB/c mice

2.2.7 HISTOPATHOLOGY

2.2.7.1 Sampling of mice for histopathology

2.2.7.1.1 Perfusion of mice, processing, paraffin embedding and sectioning of tissue

Following the virulence analyses of the replicating SFV vectors in naïve or pre-immunised BALB/c mice, brains were sampled in triplicate from different groups of mice for histopathology (section 2.2.6.2). These groups include:
Group 1: TNE inoculated mice without prior immunisation with rSFV-p62-6K VLPs,
Group 2: TNE inoculated mice with prior immunisation with rSFV-p62-6K VLPs,
Group 3: SFV4 injected mice without prior immunisation with rSFV-p62-6K VLPs,
Group 4: SFV4 inoculated mice which were pre-immunised with rSFV-p62-6K VLPs,
Groups 5, 6, and 7: Mice pre-immunised with rSFV-p62-6K VLPs and inoculated with RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus respectively.

The mice were heavily anaesthetised with halothane and perfused through the left ventricle with PBS followed by 10% formal saline for 5 min and allowed to fix overnight at 4°C. Brains were excised, placed in 5% formal saline and processed for paraffin embedding.

Following the tumour treatment experiments, mice were sampled in triplicate on the day following the 6^{th} treatment (11 dyas post-treatment). Mice were euthanized by isofluorane overdose and cervical dislocation, and tumours were excised, placed in 5% formal saline and processed for paraffin embedding. Processing of tissues, paraffin embedding and sectioning of sampled brains and tumours was performed by Mrs. Alex Whelan-Buckley (Veterinary Pathology Laboratory, University College Dublin). Fixed brains were divided coronally at the levels of the olfactory cortex, optic nerves and pons/cerebellum whereas tumours were processed whole. Samples were dehydrated through a series of graded alcohol washes as follows; 50% (v/v) alcohol for 1 h, 70% (v/v) alcohol for 1 h and 90% (v/v) alcohol for 1 h, before 2 x 40 min absolute alcohol washes. Samples were then immersed for 1 h in an absolute alcohol and xylene (1:1) solution before 3 x 40 min xylene washes. This was followed by 4 x 40 min immersions

in paraffin wax and subsequent mounting onto blocks. For routine histology, 3 μm sections were cut using a microtome and dried overnight at 37°C in an oven.

2.2.7.2 Histopathology

2.2.7.2.1 Haematoxylin and eosin (H & E) staining for routine histology

Sections of paraffin embedded brains and tumours were examined routinely using the H&E staining method. Dried sections were dewaxed with 3 x 10 min washes in 100% xylene and subsequently rehydrated through 100%, 95%, 70% ethanol for 5 min each followed by distilled water. Sections were then placed in Harri's haematoxylin for 10 min, rinsed under running tap water until cleared, differentiated in 1% acid alcohol and 'blued-up' by dipping three times in 3% ammonia water. After a subsequent wash under running tap water, sections were counterstained in 1% dichromate eosin for 2 min and washed under running tap water for a further 5 min or until cleared. Stained sections were then dehydrated and mounted using DPX by covering the section with a coverslip in order to prevent the occurrence of air bubbles. H&E stained slides were prepared from two separate sections of each tumour to provide representative pathological observations.

2.2.7.2.2 Routine histology

Coded H&E stained brain section were examined blind for the presence of lesions characteristic of SFV4 infection.

Coded H&E stained tumour sections without any pre-knowledge of the treatment given were examined by light microscopy and relevant histological details were noted such as: tumour cell morphology, mitotic index, invasion of surrounding tissues by tumour cells, development of areas of necrosis within tumours, thromboses and haemorrages, tumour ulceration, presence of tumour infiltrating lymphocytes (TILs) and their location in the tumour microenvironment.

2.2.8 STATISTICAL ANALYSIS OF THE DATA

Statistical comparisons for the cell viability and the caspase activation experiments were performed using the two-way ANOVA with Bonferroni post-tests. Results were considered statistically significant when probability (P) < 0.05. Statistical analysis of the survival curves in the virulence experiments was performed by using the Logrank Test. A P-value smaller than 0.05 was considered to be statistically significant. The statistical significance of difference in the tumour volume among the different experimental treatment groups was analysed by one-way ANOVA. Tukey's multiple comparisons post- test was used to compare the P value of each group with the control TNE treated group. Kaplan-Meier survival curves were used to analyse the overall median survival of mice and survival proportions in each treatment group.

Chapter 3

CONSTRUCTION AND *IN VITRO* CHARACTERISATION OF REPLICATING SFV VECTORS

3.1 INTRODUCTION

SFV has been shown to induce apoptosis in a variety of cell lines (Glasgow *et al.,* 1997; Scallan *et al.,* 1997; Smyth *et al.,* 2005). The SFV vector and rSFV particles have also been demonstrated to induce apoptosis independently of viral structural proteins and the status of cellular p53 (Glasgow *et al.,* 1998; Murphy *et al.,* 2001). Unlike the VLP vector, the replication competent vectors allow longer term gene expression and undergo several rounds of replication (Atkins *et al.,* 2008). Originally based on the full-length SV genome, the replicating vector has two 26S subgenomic promoters; one encoding cloned genes from the MCS, and the other encoding the viral structural proteins. The second 26S subgenomic promoter and the MCS could be cloned either to the 5' or 3' end of the structural gene ORF (Raju & Huang, 1991; Hahn *et al.,* 1992). Replicating vectors with a second 26S subgenomic promoter and a MCS at the 5' end of the structural gene coding region have been shown to give more stable foreign gene expression than those with the subgenomic promoter and MCS at the 3' end of the structural gene coding region (Pugachev *et al.,* 1995; Vähä-Koskela *et al.,* 2003).

One of the non-structural proteins of alphaviruses, nsP3, is a phosphoprotein (Li *et al.,* 1990; Vihinen *et al.,* 2001) and it was shown to play a role in subgenomic 26S and negative-strand RNA synthesis (LaStarza *et al.,* 1994a). The phosphorylation of the hypervariable domain of nsP3 could indicate an accessory function in negative-strand RNA synthesis (De *et al.,* 2003). Studies with other alphaviruses such as SV also showed reduced virus yield and reduced minus strand and RNA synthesis following early *in vitro* infection with mutants generated from random insertion and large deletions in the hypervariable region of the nsP3 gene (LaStarza *et al.,* 1994b).

In an attempt to prevent possible biosafety issues associated with a replicating vector, attenuating deletions named ΔSN and ΔTN have been introduced into the nsP3 region of the pSP6-SFV4 genome, creating the deletion mutants SFV4-SN and SFV4-TN (Galbraith *et al.,* 2006). ΔSN comprises a 325 bp deletion area between nucleotides 5056 (***Sac***II restriction site) and 5381 (***Nae***I) whereas ΔTN is a 128 bp deletion between nucleotides 5381 (***Nae***I) and 5509 (***Tth***111I) in the nsP3 region of the SFV genome (Galbraith *et al.,* 2006). The nucleotide sequences which constitute the SN and TN deletion areas are illustrated in Figure 3.1. The effects of ΔSN and ΔTN on RNA

```
SFV4 951    AGTGGTTAGT CCGCGGAAGT ATGCCGCATC TACGACGGAC CACTCAGATC
                       SacII
SFV4 1001   GGTCGTTACG AGGGTTTGAC TTGGACTGGA CCACCGACTC GTCTTCCACT

SFV4 1051   GCCAGCGATA CCATGTCGCT ACCCAGTTTG CAGTCGTGTG ACATCGACTC

SFV4 1101   GATCTACGAG CCAATGGCTC CCATAGTAGT GACGGCTGAC GTACACCCTG

SFV4 1151   AACCCGCAGG CATCGCGGAC CTGGCGGCAG ATGTGCACCC TGAACCCGCA

SFV4 1201   GACCATGTGG ACCTCGAGAA CCCGATTCCT CCACCGCGCC CGAAGAGAGC

SFV4 1251   TGCATACCTT GCCTCCCGCG CGGCGGAGCG ACCGGTGCCG GCGCCGAGAA
                                                 NaeI
SFV4 1301   AGCCGACGCC TGCCCCAAGG ACTGCGTTTA GGAACAAGCT GCCTTTGACG

SFV4 1351   TTCGGCGACT TTGACGAGCA CGAGGTCGAT GCGTTGGCCT CCGGGATTAC

SFV4 1401   TTTCGGAGAC TTCGACGACG TCCTGCGACT AGGCCGCGCG GGTGCA
                       Tth111I
```

Figure 3.1 Nucleotide sequences of the regions comprising the SN and TN deletions

The sequence of the nsP3 region in pSP6-SFV4 was obtained from the GenBank database (http://www.ncbi.nlm.nih.gov/nuccore/16767845). The nucleotides comprising the SN and TN deletion areas are highlighted in red and blue, respectively. The SN deletion stretches from the *Sac*II restriction site to the *Nae*I restriction site, and the TN deletion stretches from the *Nae*I restriction site to the *Tth111*I restriction site.
Adapted from Galbraith et al (2006).

synthesis and multiplication of the virus *in vitro* have been analysed. Results showed that viruses containing these deletions produced lower titres of virus than SFV4 (virus produced from pSP6-SFV4) in BHK-21 cells and in mouse BALB/3T3 cells. This was also reflected in the lower viral RNA synthesis found in the BHK-21 cell line (Galbraith *et al.,* 2006). The replicating virus SFV4 was subsequently modified by addition of a second 26S subgenomic promoter and a MCS to the 5' end of the structural gene ORF and termed RSFV-26SMCS where the initial "R" means "replicating" and "26SMCS" denotes the presence of a second 26S subgenomic promoter and a MCS in the SFV vector (*constructed by Dr. Sareen Galbraith, Pub. No.: WO/2007/102140*) (Figure 3.2b). To attenuate this vector, the TN deletion was reintroduced into the genome of RSFV-26SMCS generating the RSFV-ΔTN-26SMCS vector (*constructed by Dr. Sareen Galbraith, personal communication*) (Figures 3.2c).

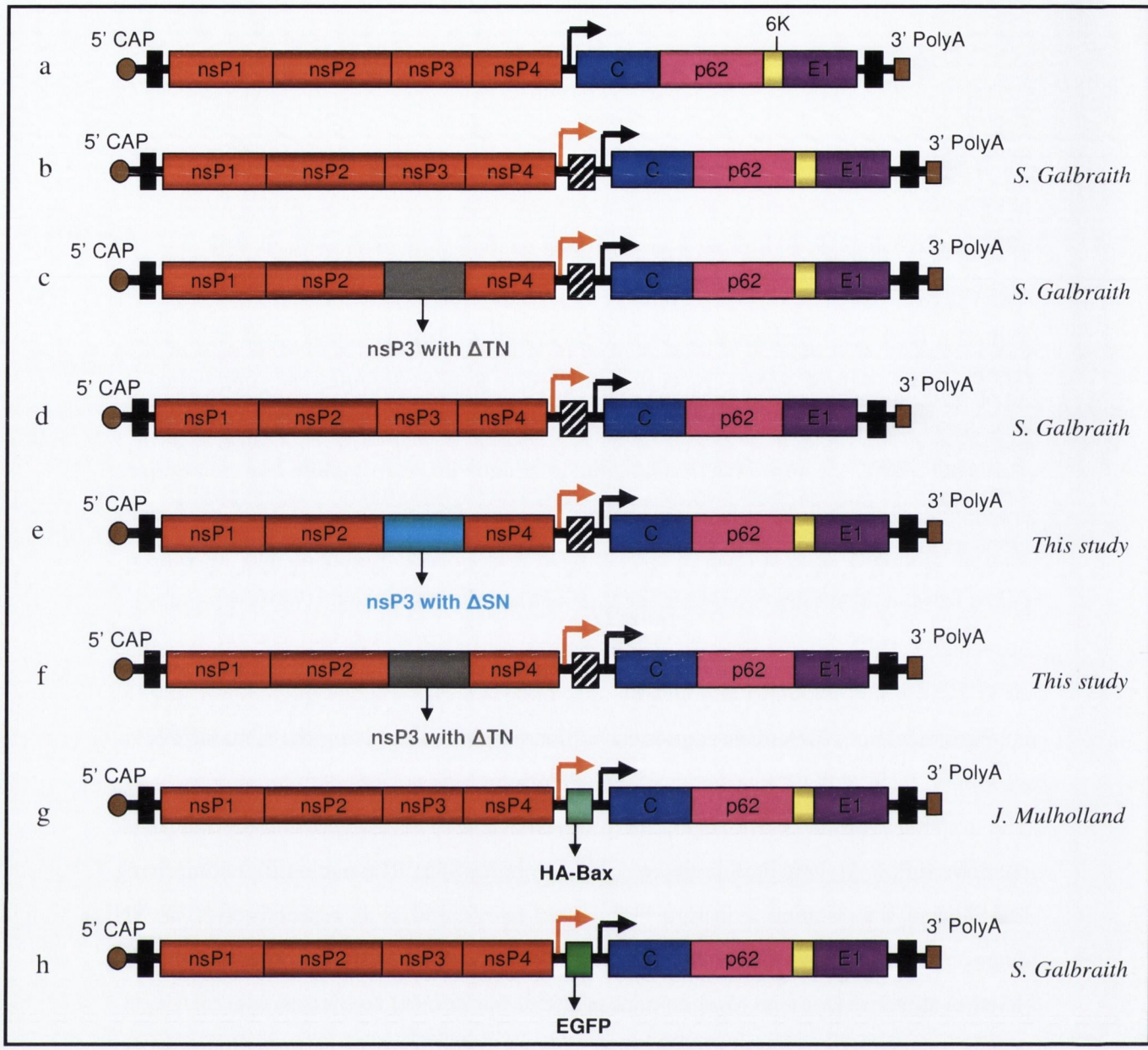


Figure 3.2 Schematic representation of replicating SFV vectors used in this study

(a) pSP6-SFV4; Infectious clone of SFV which is used to produce SFV4 virus.

(b) RSFV-26SMCS; Replicating SFV vector containing a second 26S subgenomic promoter and a MCS at the 5' end of the structural-gene coding region. This vector is based on the genome of pSP6-SFV4 (constructed by Dr. Sareen Galbraith).

(c) RSFV-ΔTN-26SMCS; RSFV-26SMCS containing the TN deletion in the nsP3 gene (constructed by Dr. Sareen Galbraith).

(d) RSFV-Δ6K-26SMCS; RSFV-26SMCS with the complete deletion of the 6K gene (constructed by Dr. Sareen Galbraith).

(e) RSFV-ΔSN-26SMCS; RSFV-26SMCS containing the SN deletion in the nsP3 gene.

(f) RSFV-ΔTN-Δ6K-26SMCS; RSFV-26SMCS containing the TN deletion in the nsP3 gene with the complete deletion of the 6K gene.

(g) RSFV-HABax-26SMCS; RSFV-26SMCS containing the *bax* gene with a HA tag cloned into the MCS (constructed by Ms. Jennifer Mulholland)

(h) RSFV-EGFP-26SMCS; RSFV-26SMCS expressing the EGFP gene from the MCS (constructed by Dr. Sareen Galbraith)

C; The Capsid structural gene, Black shaded box; MCS, Black arrow; 26S subgenomic promoter, Orange arrow; The second 26S subgenomic promoter

Deletion of the 6K gene in the infectious clone of SFV, pSP6-SFV4, has been demonstrated to reduce the rate of virion production in the early stages of infection without greatly affecting the total virus yield in cell culture (Liljeström *et al.,* 1991). Mutations in 6K have also been shown to result in deformed multicored virions. However, interestingly, 6K deletion mutants are still viable (Schlesinger *et al.,* 1993; Ivanova *et al.,* 1995; McInerney *et al.,* 2004). A major defect in the final assembly and budding of the new virus has also been observed following deletion of the 6K gene (Loewy *et al.,* 1995). Based on these results, the structural 6K gene was removed from the genome of RSFV-26SMCS, creating the RSFV-Δ6K-26SMCS mutant (*constructed by Dr. Sareen Galbraith, personal communication*) (Figure 3.2d).

The Ras family of proteins plays a key role in signal transduction pathways and is involved in cellular differentiation, proliferation and survival. *Ras* genes mutate and act as oncogenes in approximately 30% of all human malignancies, and of these, K-*ras* is the most common (Bos, 1989). One of the murine tumour models used in this study, the Kirsten murine sarcoma transformed BALB/3T3 cell line, K-BALB, overexpresses the K-*ras* oncogene and forms aggressive localized syngeneic tumours in immunocompetent BALB/c mice upon s.c. injection (Aaron & Weaver, 1971; Stephenson & Aaronson, 1972). The other murine tumour model used in this project the murine colon adenocarcinoma cell line CT26, forms localized tumours of low immunogenicity in BALB/c mice following s.c. injection (Brattain *et al.,* 1980). In a recent study, it was observed that the CT26 and K-BALB murine tumour models were both prone to SFV4-induced apoptosis, and that wild-type SFV inhibits tumour growth *in vivo* (Smyth *et al.,* 2005).

Following the findings observed by Smyth et al (2005) and Galbraith et al (2006), the aim of the project was to construct a replication-competent SFV vector which replicates well *in vitro* producing high-titre virus but with reduced virulence, so that it could be utilised to inhibit the growth of CT26 and K-BALB tumours *in vivo* with increased biosafety. Therefore in this study, the ΔSN mutation was incorporated into RSFV-26SMCS and the Δ6K mutation was reintroduced into the RSFV-ΔTN-26SMCS mutant generating RSFV-ΔSN-26SMCS and RSFV-ΔTN-Δ6K-26SMCS, respectively (Figure 3.2 e and f).

A number of mammalian genes encoding antagonists of programmed cell death have been identified. Among the first discovered, *bcl-2* oncogene family made the

contributions as regulators of apoptosis. The *bcl-2* family of proteins can be anti-apoptotic (including *bcl-2* itself and *bcl-X_L*) or pro-apoptotic (*bax*, *bad*, *bak* and *bid*). *bax* is encoded by a gene whose transcription is known to be activated by *p53* (Weinberg, 2007). In a recent study (Murphy *et al.*, 2001), the pro-apoptotic *bax* gene with a hemagglutinin (HA) tag was cloned into the MCS of rSFV to enhance cell killing in tumour tissues. BHK-21 cells were shown to express endogenous *bax* therefore the HA tag was utilised to detect the Bax protein in cell culture expressed by the SFV vector (Murphy *et al.*, 2001). *In vitro* infections with VLPs expressing the *bax* gene (rSFV-HABax) were shown to enhance apoptosis in standard BHK cells, rat prostate cancer (AT3-Neo) cells and AT3 cells expressing the *bcl-2* oncogene (AT3-Bcl-2 cells), which normally inhibits apoptosis (Murphy *et al.*, 2001). It was also previously shown that three cell types overexpressing the functional *bcl-2* displayed caspase-3 activation and underwent apoptosis in response to infection with SFV, which indicates that SFV can overcome *bcl-2*-mediated apoptosis inhibition (Grandgirard *et al.*, 1998). However in the study conducted by Murphy et al (2001), *bax* expressing particles could be produced only at low titres as the apoptosis was enhanced by the pro-apoptotic *bax* gene and as a result the cells transfected with rSFV-HABax particles were not allowed sufficient time to produce and accumulate high levels of the recombinant virus. As the replicating SFV vector (RSFV-26SMCS) comprises the structural genes, upon infection of the cell with the virus, more than one round of replication leads to the production of several progeny virions. Therefore, in this study, the pro-apoptotic *bax* gene was cloned into the MCS of RSFV-26SMCS; creating RSFV-HABax-26SMCS (Student project conducted by Ms. Jennifer Mulholland, supervised by Güniz Iskender) (Figure 3.2g).

This chapter describes how RSFV-ΔSN-26SMCS and RSFV-ΔTN-Δ6K-26SMCS vectors were constructed and the experiments that were undertaken to *in vitro* characterise the original (SFV4, RSFV-26SMCS, RSFV-ΔTN-26SMCS, RSFV-Δ6K-26SMCS) and the newly constructed (RSFV-ΔSN-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, RSFV-HABax-26SMCS) replicating SFV vectors following infection of BHK-21, CT26 and K-BALB cell lines. In addition, in this chapter, the stability of the *bax* gene was examined by indirect immunofluorescence and a full-length replicating SFV vector which expresses the EGFP gene (RSFV-EGFP-26SMCS, *constructed by Dr. Sareen Galbraith*) (Figure 3.2h) was utilised to examine the infection efficiency of different cell lines, BHK-21, CT26 and K-BALB. pSP6-SFV4 and RSFV-26SMCS

plasmid vectors were used as the positive controls during the *in vitro* experiments. It has been previously demonstrated that rSFV VLPs and SFV4 infect BHK-21 cells efficiently and rapidly induce apoptosis upon infection (Glasgow *et al.*, 1997). Therefore, the BHK-21 cell line was chosen to serve as a positive control for the viability and the caspase activation studies, and the growth curve analyses.

3.2 RESULTS

3.2.1 Construction of the plasmid RSFV-ΔSN-26SMCS vector

The purified stocks of the 13.95 kbp plasmid pSP6-SFV4-ΔSN (Appendix 8.1b) and the 14.37 kbp plasmid RSFV-26SMCS (Appendix 8.1c) vectors (which were constructed and kindly provided by Dr. Sareen Galbraith) were double digested at the unique *Bgl*II and *Bsm*I restriction sites in several reaction sets (Figure 3.3 a and b). The 2031 bp fragment (insert) that incorporates the second 26S subgenomic promoter and the 12,018 bp fragment (vector) that includes the nsP3-SN deletion were extracted and purified, and the final products were analysed by gel electrophoresis (Figure 3.3 c and d). A 1:3 molar ratio of vector to insert was used for the ligation reaction. Following the ligation of the two plasmid fragments, ligation mixture was transformed into ultracompetent cells and the mixture was plated onto bacterial plates to follow the colony growth. Following incubation of the plates at 37°C o/n, a number of colonies (six) were selected, the plasmid DNA was purified and 1 µl from each of the colonies was analysed by gel electrophoresis (Figure 3.4a). The purified DNA from the transformed colonies was screened for the presence of the insert fragment (2031 bp) by *Bgl*II and *Bsm*I restriction enzyme digestion and it was observed that four out of six colonies produced the right size fragments (Figure 3.4b). Following confirmation of the presence of the insert fragment, two out of these four positive RSFV-ΔSN-26SMCS clones were digested with *Kpn*I restriction enzyme to check the presence of the SN deletion in the genome. Digestions were analysed by gel electrophoresis alongside *Kpn*I digested RSFV-26SMCS, pSP6-SFV4, RSFV-ΔTN-26SMCS, and RSFV-Δ6K-26SMCS, to compare the size of the bands produced. RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, and RSFV-Δ6K-26SMCS produced the right size fragments following digestion with *Kpn*I showing that the plasmids comprised the SN, TN or the 6K deletions respectively (Figure 3.5 a and b). The region containing the ΔSN was PCR amplified using primers located at position 4889 nt and 5576 nt (as shown in Figure 2.2) in the positive RSFV-ΔSN-26SMCS clone and analysed on 1% agarose gel confirming the presence of the deletion (Figure 3.6a).

Figure 3.3 Gel analysis of the construction of the RSFV-ΔSN-26SMCS vector

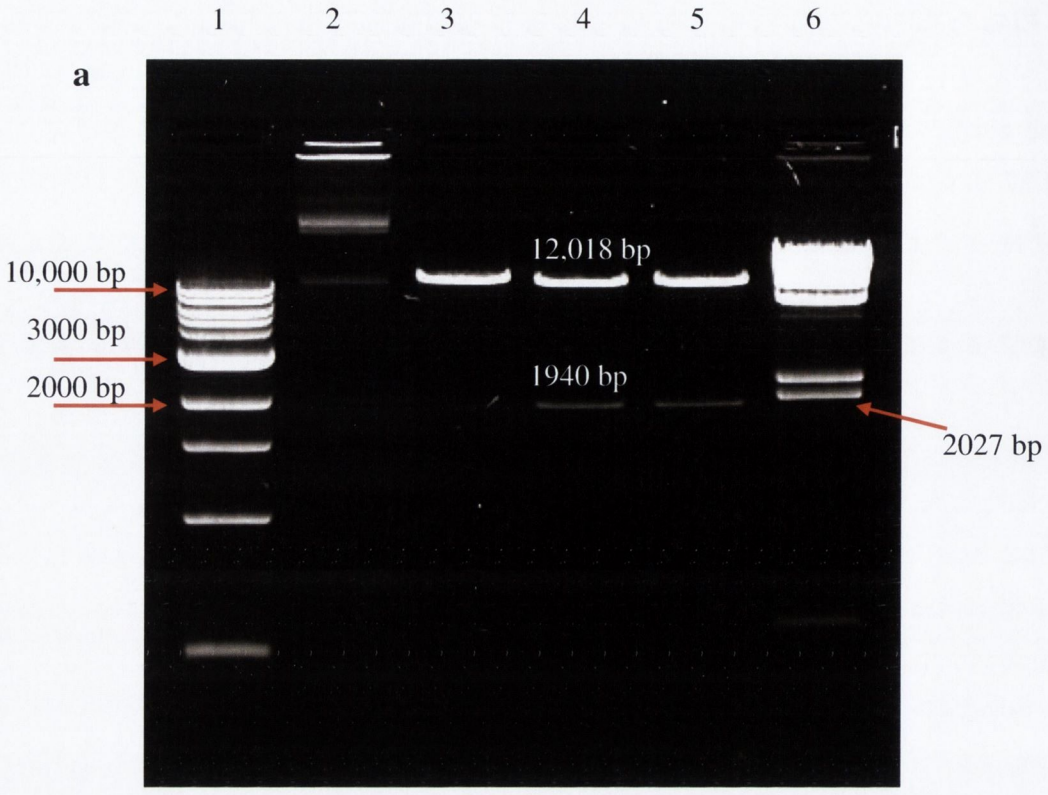
The purified stocks of the 13.95 kbp plasmid pSP6-SFV4-ΔSN (Appendix 8.1b) and 14.37 kbp plasmid RSFV-26SMCS (Appendix 8.1c) vectors were double digested at the unique *Bgl*II and *Bsm*I restriction sites in three reaction sets, and 5 µl aliquot was analysed on 1% (w/v) agarose gel, (a) and (b) respectively. The remaining of the digestion mixtures was then loaded into 1% agarose gel and the plasmid fragments that incorporate the ΔSN region (12,018 bp) or the fragment that contains the second 26S subgenomic promoter and MCS (2031 bp) were extracted and purified. Following gel electrophoresis of 3 µl aliquot from each extracted and purified samples, concentration of the final products was calculated by comparing the band intensities of the vector and the insert fragments to the intensity of the 1 Kb DNA ladder, (c) and (d) respectively. Lambda (*Hind*III) DNA marker was also utilised to compare the size of the bands.

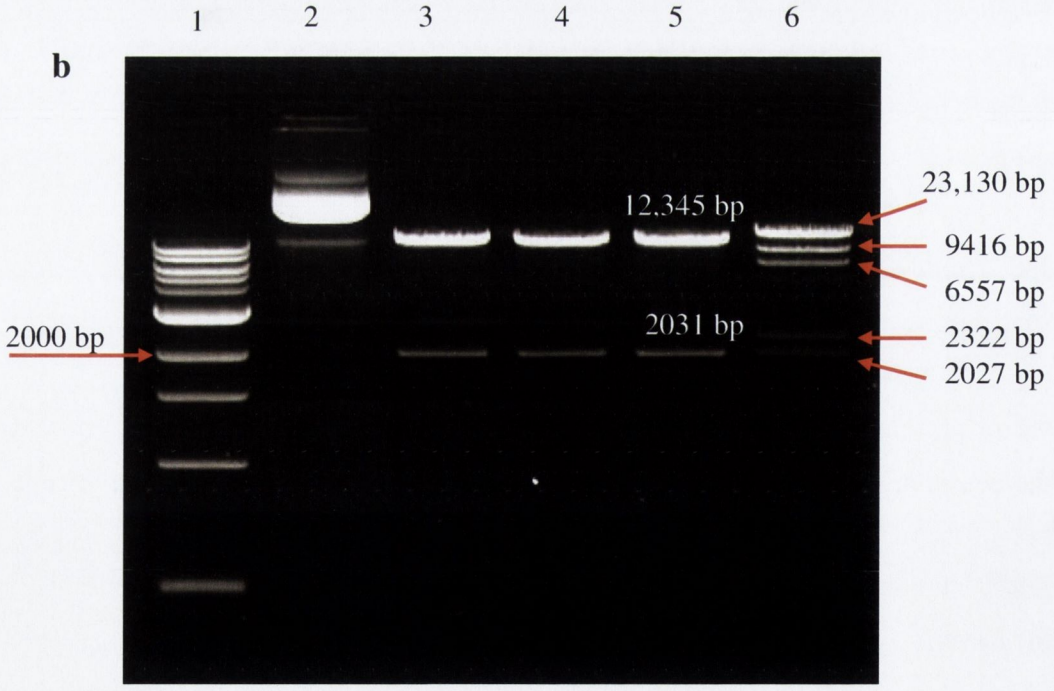
(a) *Lane 1*: 1 µg of 1 Kb DNA molecular weight marker. *Lane 2*: 3 µl uncut plasmid pSP6-SFV4-ΔSN DNA. *Lanes 3, 4, and 5*: pSP6-SFV4-ΔSN plasmid vector double digested with *Bgl*II and *Bsm*I restriction enzymes (5 µl aliquot). The 12,018 bp fragment incorporates the SN deletion in the nsP3 region of the genome. *Lane 6*: Lambda DNA marker (1 µl). Digest number 1 (lane 3) did not produce the expected sized fragments so it was discarded.

(b) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: 1 µl uncut plasmid RSFV-26SMCS DNA. *Lanes 3, 4, and 5*: 5 µl aliquot of RSFV-26SMCS plasmid vector double digested with *Bgl*II and *Bsm*I restriction enzymes. The 2031 bp fragment contains the second 26S subgenomic promoter and the MCS. *Lane 6*: Lambda DNA marker (1 µl).

(c) *Lane 1*: 1 Kb DNA ladder. *Lanes 2 and 3*: 3 µl aliquot of gel extracted and purified vector plasmid fragment (12,018 bp) that contains the SN deletion region from both of the digestion mixtures. As digest number 1 did not produce the right size bands (a) only two of the reaction mixtures were utilised to extract and purify the 12,018 bp fragments. *Lane 4*: Lambda DNA marker (1 µl).

(d) *Lane 1*: 1 µg of 1 Kb DNA molecular weight marker. *Lanes 2, 3, and 4*: 1 µl aliquot of gel extracted and purified insert plasmid fragment (2031 bp) that incorporates the second 26S subgenomic promoter.


1
2
3
4
5
6
a
10,000 bp
3000 bp
2000 bp
12,018 bp
1940 bp
2027 bp


1
2
3
4
5
6
b
2000 bp
12,345 bp
2031 bp
23,130 bp
9416 bp
6557 bp
2322 bp
2027 bp

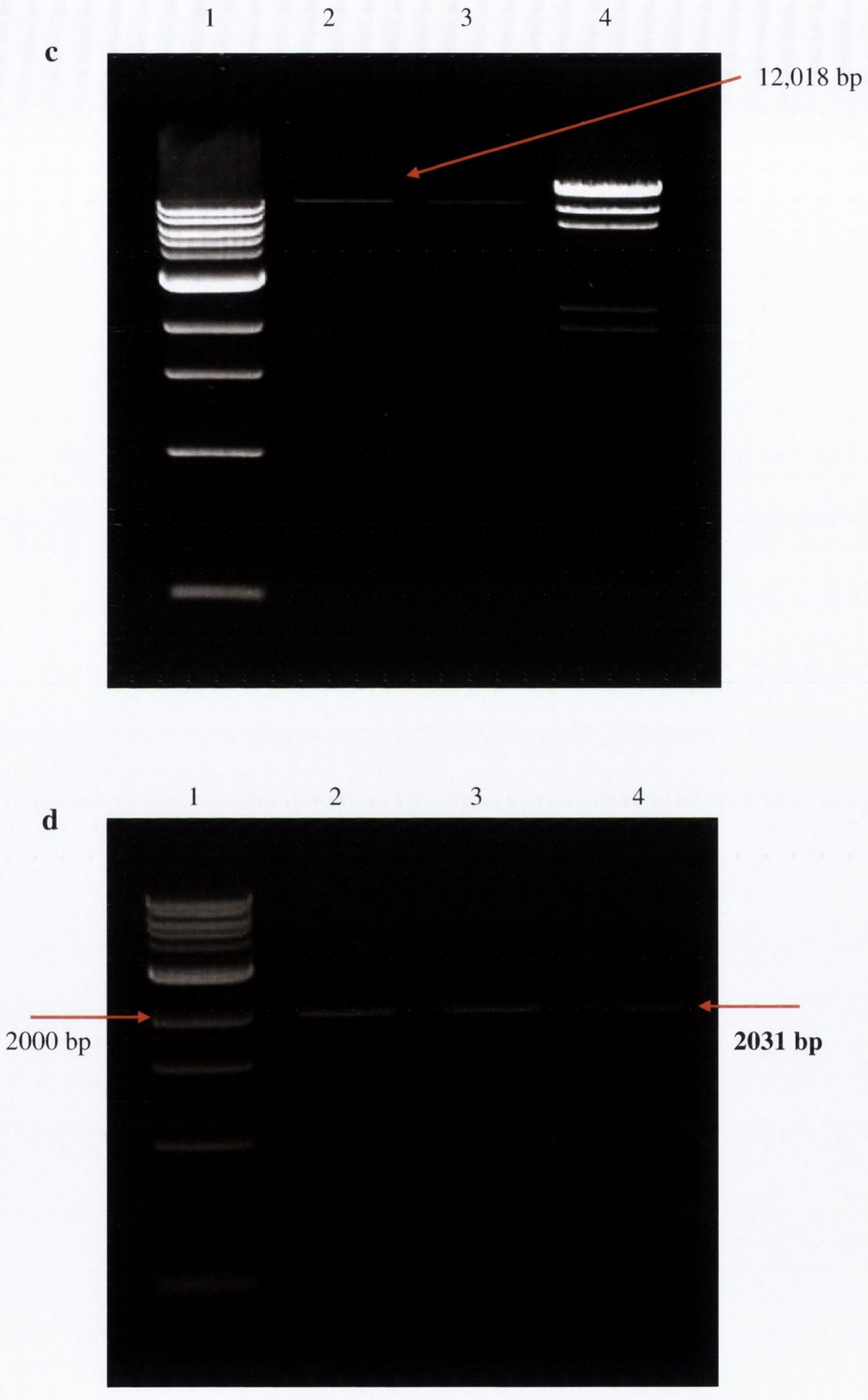

1
2
3
4
c
12,018 bp
1
2
3
4
d
2000 bp
2031 bp

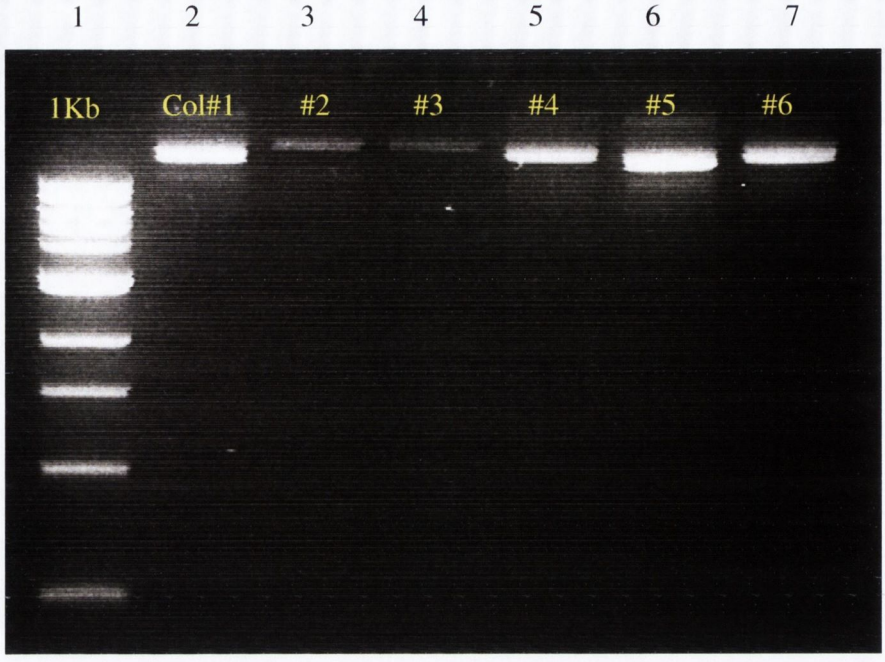
Figure 3.4 Screening for RSFV-ΔSN-26SMCS clones by restriction analysis

Following ligation of the vector and the insert fragments, the ligation mix was transformed into XL-10 Gold ultracompetent cells, the transformation mixture was plated onto bacterial plates and the plates were incubated at 37°C for colony growth. Six different colonies (Col#1-6) were picked and the plasmid was purified using the *Qiagen Miniprep Plasmid Purification Kit* as per the manufacturer's instructions. Concentration of the purified DNA was analysed by gel electrophoresis of 1µl aliquot from each sample (a). 1.0 µg of plasmid DNA from each colony was screened for the presence of the insert fragment that incorporates the second 26S subgenomic promoter using restriction enzymes *Bgl*II and *Bsm*I (as described in section 2.2.2.1.1). 5 µl aliquot of the digested product was analysed on 1% agarose gel and observed for specific size of fragments (b). If the ligation has worked, *Bgl*II and *Bsm*I cut the vector RSFV-ΔSN-26SMCS at 6387 bp and 8418 bp respectively (refer to Figure 2.3), producing two plasmid fragments of size of 2031 bp (8418 – 6387 = 2031 bp) and 12,018 bp (14,049 (size of the vector) – 2031 = 12,018 bp).

(a) *Lane 1*: 1 Kb DNA marker ladder (1 µl). *Lanes 2-7*: 1 µl aliquot of the purified DNA from six different colonies was analysed by gel electrophoresis.

(b) *Lane 1*: Lambda DNA marker (1 µl). *Lanes 2-7*: Six different colonies double digested with *Bgl*II and *Bsm*I restriction enzymes. Colonies #1, #4, #5, and #6 produced the right sized fragments possibly being the positive clone for RSFV-ΔSN-26SMCS. *Lane 8*: Lambda DNA marker (1 µl).

a


1
2
3
4
5
6
7
1Kb
Col#1
#2
#3
#4
#5
#6

b

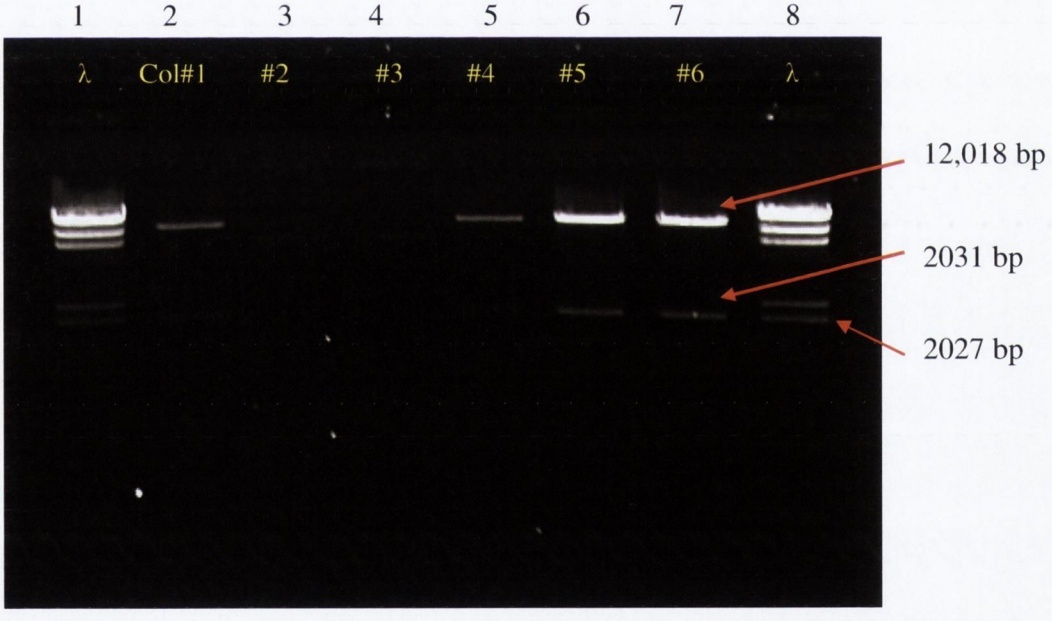

1
2
3
4
5
6
7
8
λ
Col#1
#2
#3
#4
#5
#6
λ
12,018 bp
2031 bp
2027 bp

Figure 3.5 Restriction digestion analyses of the RSFV-ΔSN-26SMCS vector

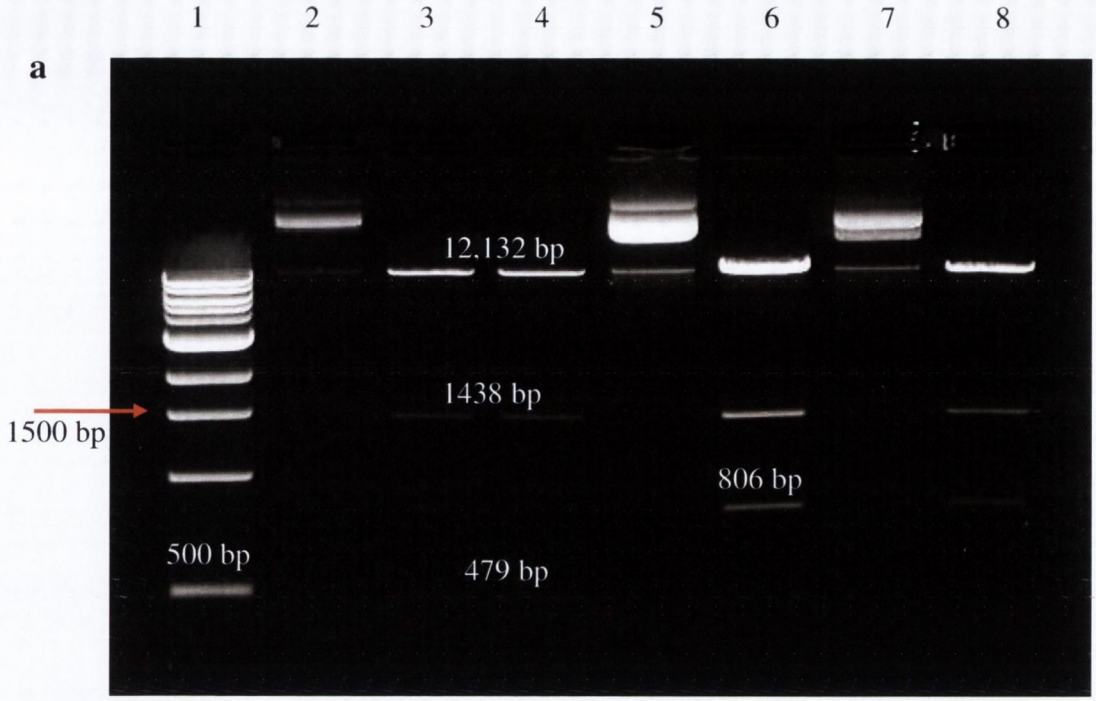
Following confirmation of the presence of the insert fragment, two positive RSFV-ΔSN-26SMCS clones were digested with *Kpn*I restriction enzyme and 5 µl aliquot of the digestion mixture was analysed on 1% agarose gel alongside 5 µl aliquot of *Kpn*I digested RSFV-26SMCS and pSP6-SFV4 (a). 5 µl aliquot of *Kpn*I digested RSFV-ΔSN-26SMCS was also analysed alongside RSFV-ΔTN-26SMCS and RSFV-Δ6K-26SMCS digested with *Kpn*I restriction enzyme (5 µl aliquot) by gel electrophoresis (b).

See the table below for the position of *Kpn*I in each of the vectors and the size of the fragments produced following digestion with *Kpn*I. 1 Kb DNA ladder was used to compare the size of the DNA fragments produced by the restriction enzyme.

Vector	Size of the vector (bp)	Position of *Kpn*I (nt)	Size of the fragments (bp)
RSFV-ΔSN-26SMCS	14,049	3600, 5038, 5517	479, 1438, 12132
RSFV-ΔTN-26SMCS	14,247	3600, 5038, 5715	677, 1438, 12132
RSFV-Δ6K-26SMCS	14,196	3600, 5038, 5844	806, 1438, 11952
RSFV-26SMCS	14,376	3600, 5038, 5844	806, 1438, 12132
pSP6-SFV4	14,285	3600, 5038, 5844	806, 1438, 12132

(a) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: Undigested RSFV-ΔSN-26SMCS plasmid DNA (1 µl). *Lanes 3 and 4*: *Kpn*I digested positive RSFV-ΔSN-26SMCS clones 1 and 2, respectively (5 µl). *Lane 5*: Undigested RSFV-26SMCS plasmid DNA (1 µl). *Lane 6*: *Kpn*I digested RSFV-26SMCS plasmid (5 µl). *Lane 7*: Undigested pSP6-SFV4 plasmid DNA (1 µl). *Lane 8*: *Kpn*I digested pSP6-SFV4 plasmid (5 µl).

(b) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: 1 µl uncut RSFV-ΔSN-26SMCS plasmid DNA. *Lanes 3 and 4*: 5 µl aliquot of *Kpn*I cut positive RSFV-ΔSN-26SMCS clones 1 and 2, respectively. *Lane 5*: 1 µl uncut RSFV-ΔTN-26SMCS plasmid DNA. *Lane 6*: 5 µl aliquot of *Kpn*I cut RSFV-ΔTN-26SMCS DNA. *Lane 7*: 1 µl uncut RSFV-Δ6K-26SMCS plasmid DNA. *Lane 8*: 5 µl aliquot of *Kpn*I cut RSFV-Δ6K-26SMCS DNA.


1
2
3
4
5
6
7
8
a
12,132 bp
1438 bp
1500 bp
806 bp
500 bp
479 bp

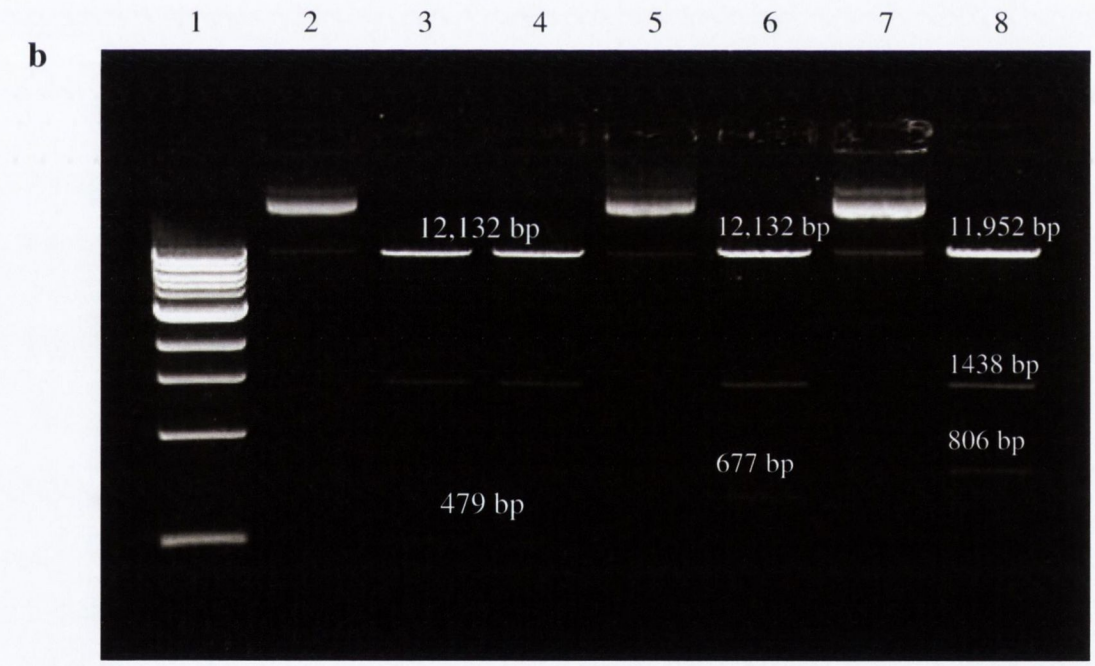

1
2
3
4
5
6
7
8
b
12,132 bp
12,132 bp
11,952 bp
1438 bp
806 bp
677 bp
479 bp

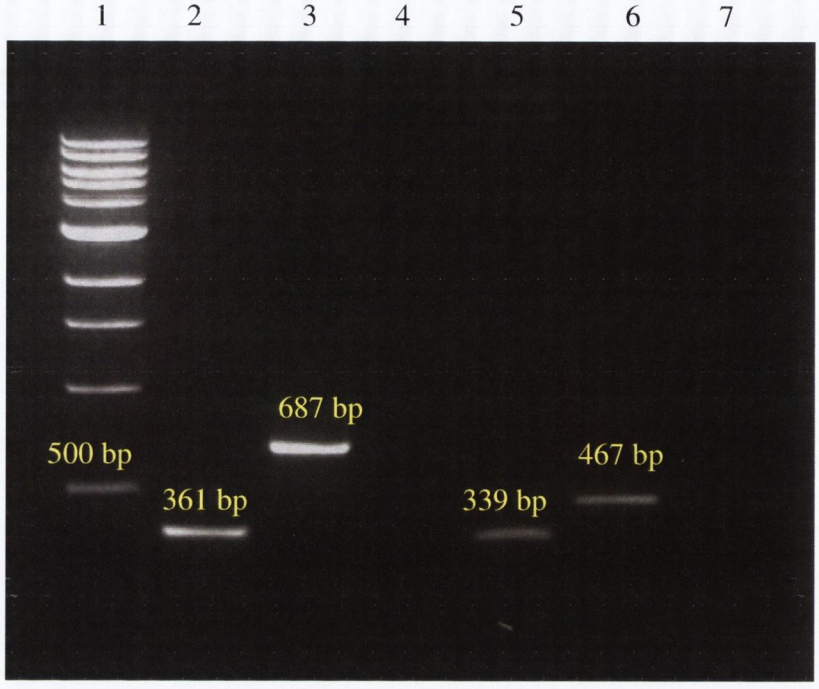
Figure 3.6 Gel analyses of the PCR amplified regions incorporating the ΔSN, ΔTN, or the Δ6K in RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, or RSFV-Δ6K-26SMCS respectively

The regions containing the ΔSN (325 bp), ΔTN (128 bp), or the Δ6K (180 bp) in RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, or RSFV-Δ6K-26SMCS respectively were PCR amplified in separate reaction sets (as described in section 2.2.2.1.4), and the products were analysed by electrophoresis of a 5 µl aliquot on 1% agarose gel, (a) and (b). Primers specific for ΔSN, ΔTN, and Δ6K were outlined in Table 2.1. Also refer to Figure 2.2 for design of the primers.

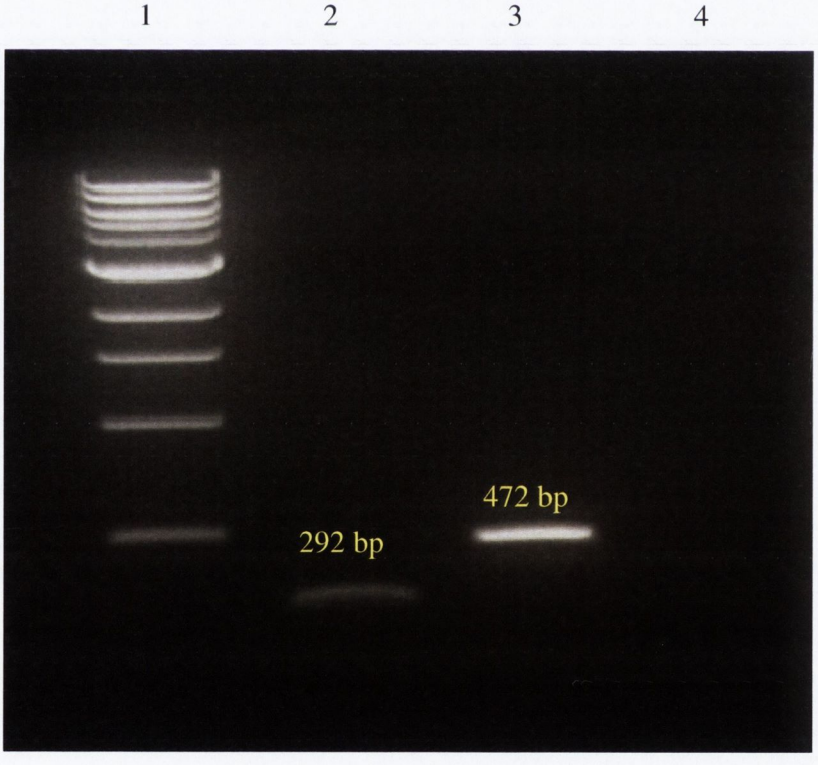
(a) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: The PCR reaction containing RSFV-ΔSN-26SMCS DNA. The PCR amplified region by the SN primers is 361 bp (687 bp - 325 bp (size of the SN deletion) = 361 bp). *Lane 3*: The positive control reaction containing pSP6-SFV4 DNA. The size of the region amplified by the SN primers is 687 bp as the forward and reverse primers start at 4889 nt and 5576 nt in the pSP6-SFV4 genome, respectively (5576 - 4889 = 687 bp) (refer to Figure 2.2a). *Lane 4*: The negative control reaction lacking DNA. *Lane 5*: The PCR reaction containing RSFV-ΔTN-26SMCS DNA. The PCR amplified region by the TN primers is 339 bp (467 bp - 128 bp (size of the TN deletion) = 339 bp). *Lane 6*: The positive control reaction containing pSP6-SFV4 DNA. The size of the region amplified by the TN primers is 467 bp as the forward and reverse primers start at 5205 nt and 5672 nt in the pSP6-SFV4 genome, respectively (5672 - 5205 = 467 bp) (refer to Figure 2.2b). *Lane 7*: The negative control reaction with no DNA.

(b) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: The PCR reaction containing RSFV-Δ6K-26SMCS DNA. The PCR amplified region by the 6K primers is 292 bp (472 bp - 180 bp (size of the 6K deletion) = 292 bp). *Lane 3*: The positive control reaction containing pSP6-SFV4 DNA. The size of the region amplified by the 6K primers is 472 bp as the forward and reverse primers start at 9553 nt and 10,025 nt in the pSP6-SFV4 genome, respectively (10,025 - 9553 = 472 bp) (refer to Figure 2.2c) *Lane 4*: The negative control reaction lacking DNA.

a



b



The presence of the ΔTN or Δ6K was also checked by PCR amplification of the regions in RSFV-ΔTN-26SMCS or RSFV-Δ6K-26SMCS using the specific TN or 6K primers, respectively (Figure 3.6 a and b).

One RSFV-ΔSN-26SMCS clone was sent for sequencing to LARK Technologies Inc. using the forward and reverse primers of SN (refer to Table 2.1). The region incorporating the second 26S subgenomic promoter was also sequenced in the clone with the primer pair of 26S (Table 2.1). Chromas 2.23 files were analysed with BLAST- bl2seq (NCBI database) and the presence of the SN deletion and the second 26S subgenomic promoter was confirmed for the RSFV-ΔSN-26SMCS clone (Appendix 8.2).

The presence of the TN deletion in RSFV-ΔTN-26SMCS clone and the 6K deletion in RSFV-Δ6K-26SMCS clone was also confirmed by sequencing. The presence of 26S promoter was also verified for each clone following analyses of the sequencing results (Appendices 8.3 and 8.4, respectively).

3.2.2 Construction of the plasmid RSFV-ΔTN-Δ6K-26SMCS vector

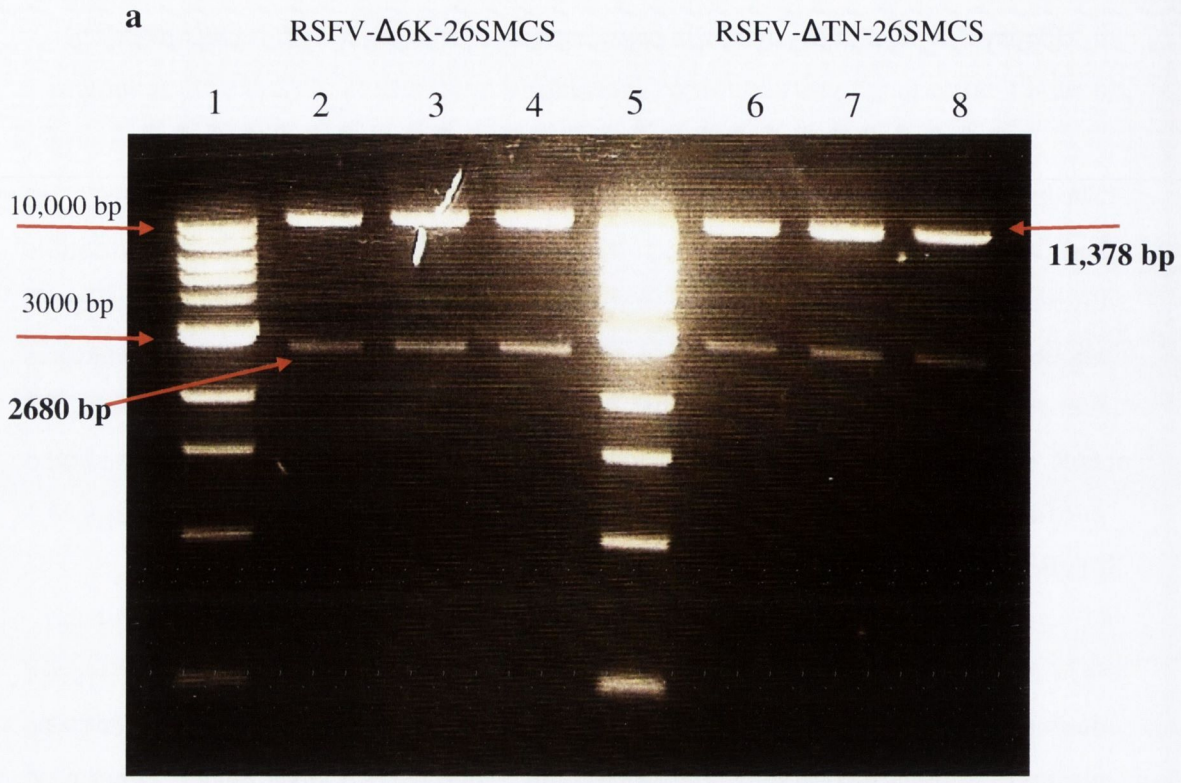
The purified stocks of the 14.27 kbp plasmid RSFV-ΔTN-26SMCS (Appendix 8.1d) and the 14.19 kbp plasmid RSFV-Δ6K-26SMCS (Appendix 8.1e) vectors (which were constructed and kindly provided by Dr. Sareen Galbraith) were double digested at the unique *Bsm*I and *Spe*I restriction sites in several reaction sets (Figure 3.7a). The plasmid fragments that incorporate the Δ6K region (2680 bp) or the TN deletion (11,387 bp) were extracted and purified, and the final products were analysed by gel electrophoresis (Figure 3.7b). The intensity of the band for the insert fragment was very low compared to the backbone fragment, therefore a 1:3 molar ratio of vector to insert was used for the ligation. Following the ligation of the two plasmid fragments, a number of colonies were selected and the plasmid DNA from the transformed colonies was screened for the presence of the insert fragment (2680 bp) by *Spe*I and *Bsm*I digestion. It was observed that all of the five clones contained the insert fragment (Figure 3.8). The regions containing the Δ6K and the ΔTN were also PCR amplified separately in the first four clones and analysed on 1% agarose gel. The presence of the 6K deletion was confirmed for all the clones (Figure 3.9a) while only the first three clones comprised the TN deletion (Figure 3.9b).

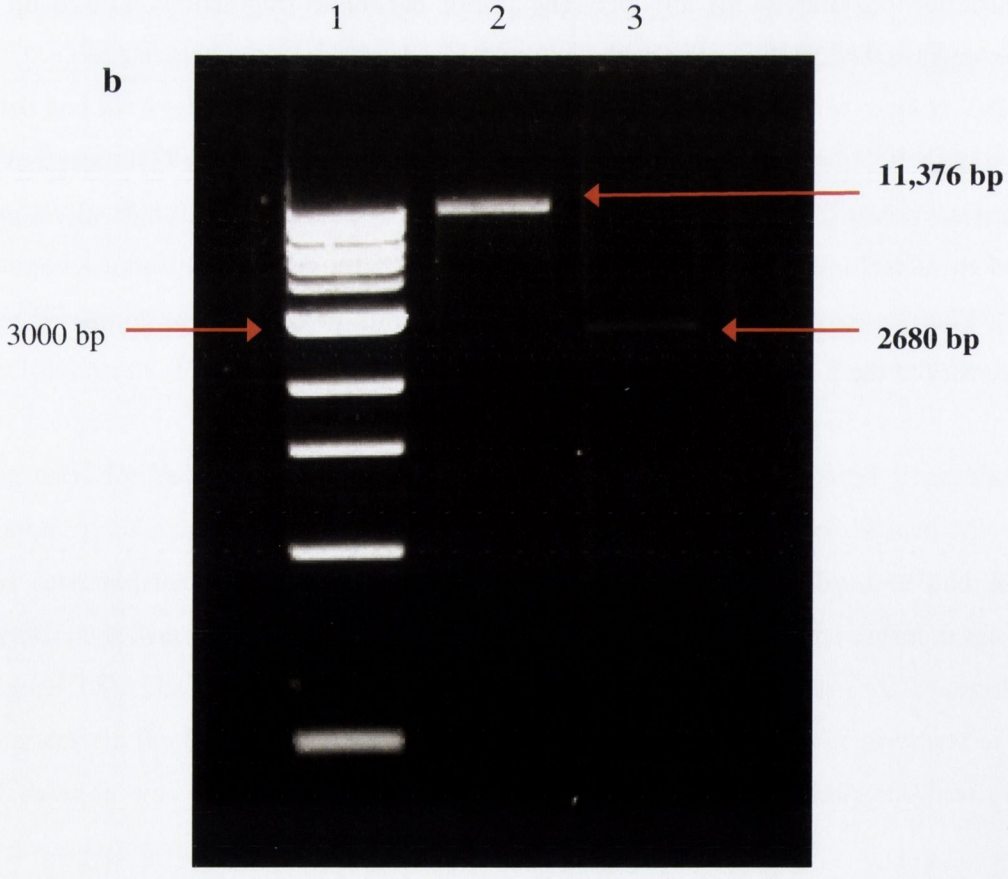
Figure 3.7 Gel analysis of the construction of the RSFV-ΔTN-Δ6K-26SMCS vector

The purified stocks of the 14.19 kbp plasmid RSFV-Δ6K-26SMCS (Appendix 8.1e) and the 14.27 kbp plasmid RSFV-ΔTN-26SMCS (Appendix 8.1d) vectors were double digested at the unique *Bsm*I and *Spe*I restriction sites in several reaction sets and 1 µl aliquot was analysed on 1% agarose gel (a). The remaining of the digestion mixtures was then loaded into 1% agarose gel and the plasmid fragments that incorporate the Δ6K region (2680 bp) or the TN deletion region (11,378 bp) were extracted and purified. Concentration of the final products was calculated by gel electrophoresis of 1 µl aliquots based on the band intensity (b).

(a) *Lane 1*: 1 Kb DNA ladder. *Lanes 2, 3, and 4*: RSFV-Δ6K-26SMCS plasmid vector double digested with the *Bsm*I and *Spe*I restriction enzymes (1 µl aliquot). The 2680 bp plasmid fragment incorporates the Δ6K region. *Lane 5*: 1 Kb DNA ladder. *Lanes 6, 7, and 8*: RSFV-ΔTN-26SMCS plasmid vector double digested with the *Bsm*I and *Spe*I restriction enzymes (1 µl aliquot). The vector backbone fragment is 11,378 bp and contains the TN deletion area.

(b) *Lane 1*: 1 µg of 1 Kb DNA molecular weight marker. *Lane 2*: Gel extracted and purified vector plasmid fragment that contains the TN deletion region (1 µl). *Lane 3*: Gel extracted and purified insert plasmid fragment that incorporates the Δ6K region (1 µl). The intensity of the vector and the insert plasmid fragments was compared to the intensity of the 1 Kb DNA ladder bands.


a
RSFV-Δ6K-26SMCS
RSFV-ΔTN-26SMCS
1
2
3
4
5
6
7
8
10,000 bp
3000 bp
2680 bp
11,378 bp


1
2
3
b
11,376 bp
3000 bp
2680 bp

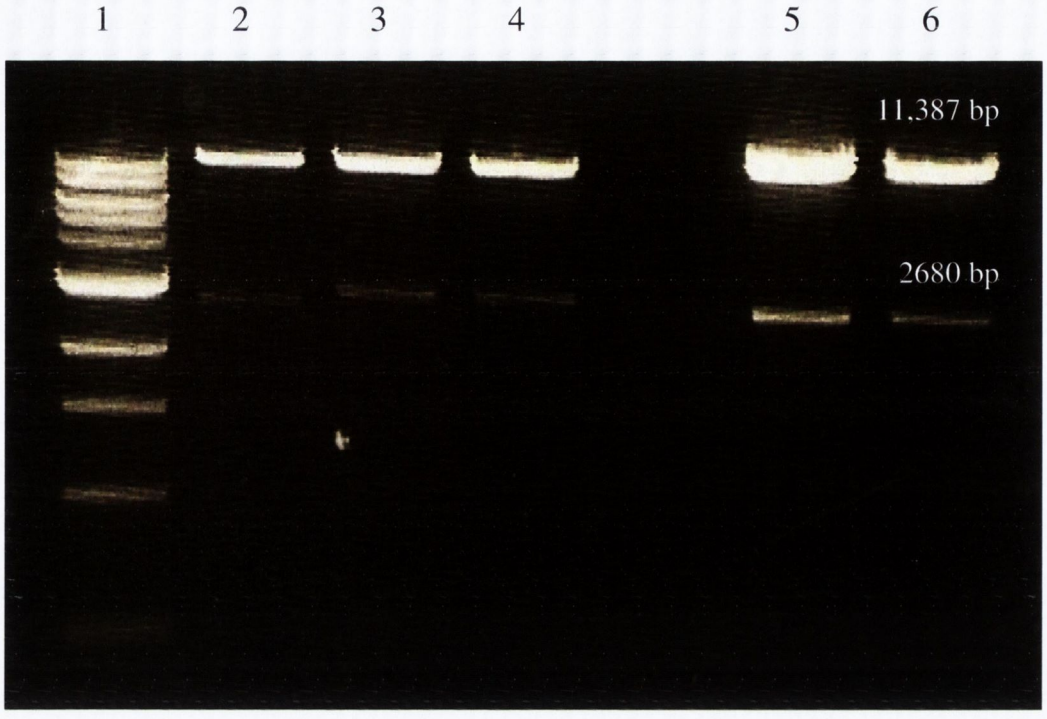


Figure 3.8 Screening for RSFV-ΔTN-Δ6K-26SMCS clones by restriction digestion analysis

Five different colonies were picked and the plasmid was purified using the *Qiagen Miniprep Plasmid Purification Kit* as per the manufacturer's instructions. 1.0 µg of each plasmid DNA was screened for the presence of the insert fragment that incorporates the Δ6K using the restriction enzymes *Spe*I and *Bsm*I (as described in section 2.2.2.2.1). 1.0 µl-digested product was analysed on 1% agarose gel and observed for specific size of fragments.

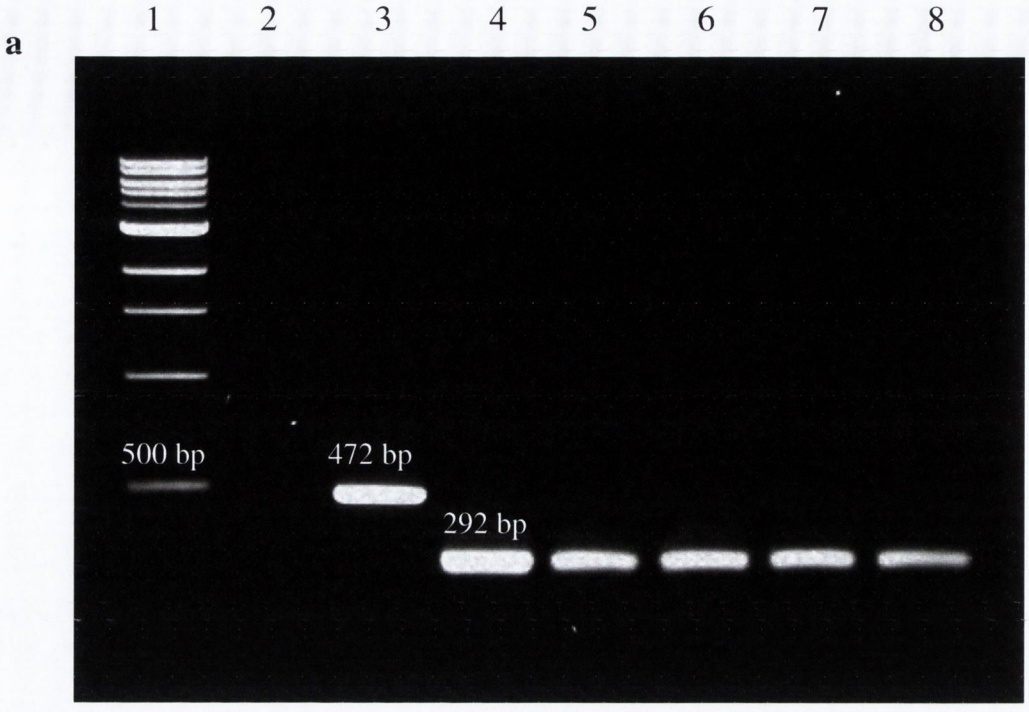
Lane 1: 1 Kb DNA marker ladder. *Lanes 2-4* and *Lanes 5-6*: *Spe*I and *Bsm*I digested RSFV-ΔTN-Δ6K-26SMCS (five different colonies with the insert in the correct orientation). *Spe*I and *Bsm*I cut the vector RSFV-ΔTN-Δ6K-26SMCS at 11,296 bp and 8616 bp, respectively. The double digest gives the size of the insert fragment as, 11,296 bp – 8616 bp = 2680 bp. Size of the backbone of the vector is 14,067 bp (size of the RSFV-ΔTN-Δ6K-26SMCS vector) - 2680 bp (size of the insert) = 11,387 bp (refer to Figure 2.5).

Figure 3.9 Gel analyses of the PCR amplified regions incorporating the Δ6K or ΔTN in RSFV-ΔTN-Δ6K-26SMCS

Following the confirmation of the insert fragment by restriction digestion analysis, the region that contains the Δ6K and the region that contains the ΔTN were PCR amplified in separate reaction sets (as described in section 2.2.2.2.4), and the products were analysed by electrophoresis of a 5 µl aliquot on a 1% agarose gel, respectively (a) and (b). Primers specific for 6K and TN were used as outlined in Table 2.1.

(a) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: The negative control reaction with no DNA. *Lane 3*: The positive control reaction containing pSP6-SFV4 DNA. The size of the region amplified by the 6K primers is 472 bp as the start nucleotide of the forward and the reverse primer is 9553 and 10,025 respectively (10,025 - 9553 = 472 bp). *Lane 4*: The PCR reaction containing RSFV-Δ6K-26SMCS DNA. The PCR amplified region by the 6K primers is 292 bp (472 bp - 180 bp (size of the 6K deletion) = 292 bp) (refer to Figure 2.2c). *Lanes 5-8*: Four different RSFV-ΔTN-Δ6K-26SMCS clones in which the region containing the Δ6K was PCR amplified to check the presence or the absence of the deletion. The presence of the 6K deletion was confirmed for all the clones as the size of the PCR amplified region was 292 bp.

(b) *Lane 1*: 1 Kb DNA ladder. *Lane 2*: The negative control reaction with no DNA. *Lane 3*: The positive control reaction containing pSP6-SFV4 DNA. The size of the region amplified by the TN primers is 467 bp as the start nucleotide of the forward and the reverse primer is 5205 and 5672 respectively (5672-5205 = 467 bp). *Lane 4*: The PCR reaction containing RSFV-ΔTN-26SMCS DNA. The PCR amplified region by the TN primers is 339 bp (467 bp - 128 bp (size of the TN deletion) = 339 bp) (refer to Figure 2.2b). *Lanes 5-8*: Four different RSFV-ΔTN-Δ6K-26SMCS clones in which the region containing the ΔTN was PCR amplified to check the presence or the absence of the TN deletion. The presence of the TN deletion was confirmed for the first three clones as the size of the PCR amplified region was 339 bp. The last sample was discarded.


a
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472 bp
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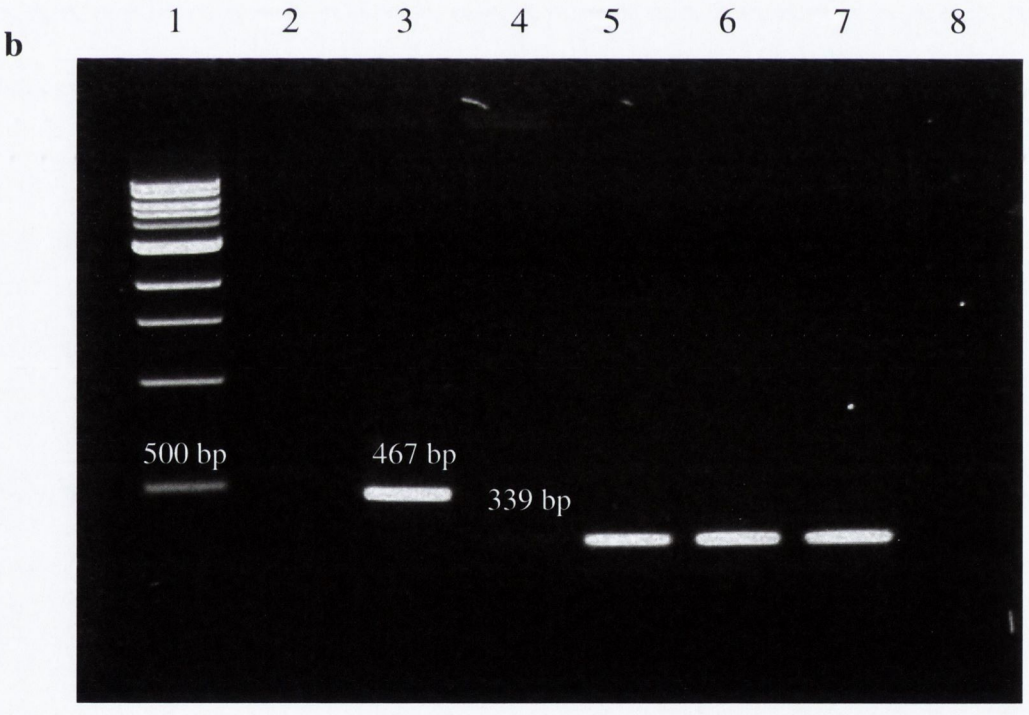

b
1 2 3 4 5 6 7 8
500 bp
467 bp
339 bp

One RSFV-ΔTN-Δ6K-26SMCS clone was sent for sequencing using the primer pairs designed for TN and 6K (primer pairs mentioned in Table 2.1) separately. The presence of the TN and 6K deletions was verified by analysing the Chromas 2.23 files with BLAST- bl2seq (Appendix 8.5 a and b). The region containing the second 26S subgenomic promoter was also analysed and its presence was confirmed in the RSFV-ΔTN-Δ6K-26SMCS clone (Appendix 8.5c).

3.2.3 Construction of the plasmid RSFV-HABax-26SMCS vector

Cloning of the HA-Bax gene into the RSFV-26SMCS vector was achieved by Ms. Jennifer Mulholland under the supervision of Güniz Iskender. Orientation of the insert was analysed by restriction enzyme digestions and the vector comprising the HA-Bax gene (RSFV-HABax-26SMCS) (Appendix 8.1f) was sent for sequencing. Following confirmation of the gene, virus was produced as described in section 2.2.3.

3.2.4 Growth of the replicating SFV vectors in cell culture

Virus from each replicating vector was produced and titrated as described in section 2.2.3. BHK-21 cells were seeded into 6-well cell culture dishes at a concentration of 8 x 10^5 cells/well and infected with RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus, or RSFV-Δ6K-26SMCS virus at a MOI of 10 or 0.1 PFU/cell. SFV4 and RSFV-26SMCS virus were utilised to infect different sets of BHK-21 cells and they were employed as the positive controls whereas PBS was used as the negative control. Plates were incubated and supernatants were harvested at 2, 4, 6, 8, 10, 12 and 24 h.p.i (see section 2.2.4.1). The amount of virus produced by the constructs at each time point was quantified by plaque assay as described in section 2.2.3.8 and viral growth curves plotted accordingly.

Following infection of BHK-21 cells at MOI=10, RSFV-ΔTN-26SMCS had the highest replication rate compared to the other vectors with viral titres increasing in a linear fashion over time and reaching a peak value of 4 x 10^9 PFU/ml by 12 h.p.i. which decreased to 2 x 10^9 PFU/ml by 24 h.p.i. as expected. RSFV-ΔSN-26SMCS and RSFV-Δ6K-26SMCS had a similar but slightly slower replication rate than RSFV-ΔTN-26SMCS where the viral titres of both of the constructs increased over time and reached

a peak of 2.5 x 10^9 PFU/ml and 1.55 x 10^9 PFU/ml by 10 h.p.i., respectively but then decreased at 12 h.p.i. and reached a steady state by 24 h.p.i. RSFV-26SMCS replicated at a higher rate compared to SFV4 where the viral titres followed a linear increasing fashion with a peak value of 1.36 x 10^9 PFU/ml at the experiment end. Surprisingly SFV4 replicated at the slowest rate when compared to the other replicating SFV vectors. Viral titres of pSP6-SFV4 increased in a linear fashion and reached the peak value of 10^9 PFU/ml by 8 h.p.i., and then decreased until the experiment end (Figure 3.10a).

Infection of BHK-21 cells with the replicating SFV vectors was also studied at a MOI of 0.1 PFU/cell. The growth kinetics of SFV4 followed a linear fashion where the viral titres reached a peak value of 7.2 x 10^8 PFU/ml by 12 h.p.i. and decreased to 9.2 x 10^7 PFU/ml by 24 h.p.i. RSFV-26SMCS had a similar rate of replication to SFV4 where it replicated in an increasing manner over time and reached its highest value at 24 h.p.i. RSFV-ΔSN-26SMCS and RSFV-Δ6K-26SMCS grew at a similar rate where both of the vectors replicated to high titres at the early stages of infection, decelerated and increased back towards the experiment end. Replication rate of RSFV-ΔTN-26SMCS followed an increasing linear fashion over time where the viral titres reached a peak of 4.4 x 10^8 PFU/ml by 12 h.p.i. and decreased to 3 x 10^8 PFU/ml by 24 h.p.i. (Figure 3.10b).

Viral growth curves were also determined for RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS. BHK-21, CT26, or K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 8 x 10^5 cells/well and infected with RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a MOI of 0.1 PFU/cell. Different sets of BHK-21, K-BALB and CT26 cells were also infected with SFV4 and RSFV-26SMCS virus as the positive controls, and PBS was used as the negative control. Plates were incubated and supernatants were harvested at 4, 8, 12 and 24 h.p.i (see section 2.2.4.1). The amount of virus produced by the constructs at each time point was quantified by plaque assay as described in section 2.2.3.8 and viral growth curves plotted accordingly.

BHK-21 cells are routinely used in the laboratory to propagate SFV4 therefore the virus replicated at the greatest speed and to the highest levels in this cell line. The viral titres increased in a linear fashion to a peak of 5.6 x 10^8 PFU/ml at 8 h.p.i. before reaching a constant state by 12 h.p.i. and 24 h.p.i.

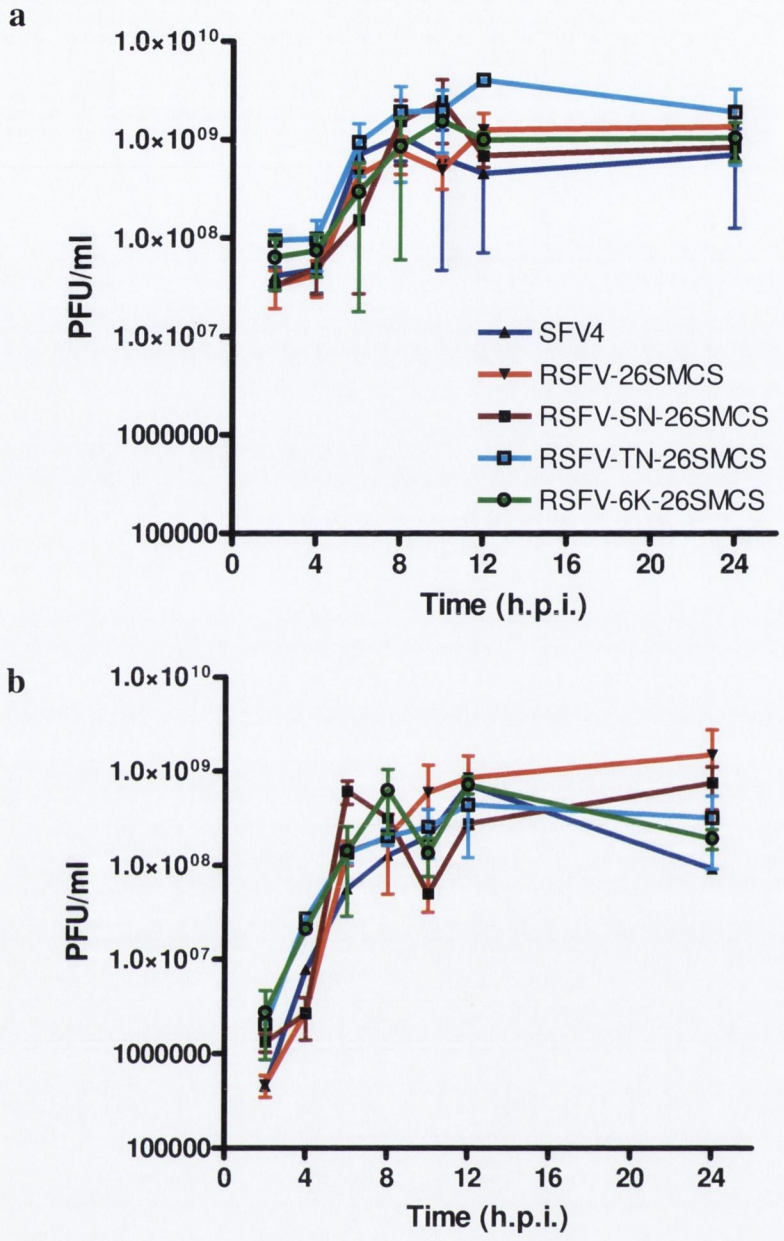


Figure 3.10 Growth of the replicating SFV vectors containing the single deletions (SN, TN, 6K) *in vitro*

BHK-21 cells were seeded into 6-well cell culture dishes at a concentration of 10^6 cells/well and infected with RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus, RSFV-Δ6K-26SMCS virus, RSFV-26SMCS virus or SFV4 at a MOI of 10 PFU/cell (a) and 0.1 PFU/cell (b). Supernatants were sampled over time (2, 4, 6, 8, 10, 12 and 24 h) and viral titres calculated by plaque assay. *Points;* mean of two replicates, *bars;* +/- SEM. Results are representative of two independent experiments.

As RSFV-26SMCS is based on the virulent strain of SFV, its replication rate was similar to SFV4 with viral titres of 1.2 x 10^8 PFU/ml and 4 x 10^8 PFU/ml at the early stages of infection but reaching a peak of 8.9 x 10^8 PFU/ml at 24 h.p.i. The replication rate of RSFV-ΔTN-Δ6K-26SMCS virus was lower than the replication rates of SFV4 and RSFV-26SMCS virus. The viral titres increased over time and reached a peak of 2 x 10^8 PFU/ml by 12 h.p.i. before reaching a slightly higher value of 3.35 x 10^8 PFU/ml at 24 h.p.i. Viral titres of RFV-HABax-26SMCS increased in a linear fashion over time reaching a peak value of 4 x 10^8 PFU/ml at 24 h.p.i. (Figure 3.11a).

The growth kinetics of the viral constructs in CT26 cell line was significantly slower when compared to BHK-21 cells. Viral titres produced by SFV4 were 5000-fold less than the amount produced in BHK-21 cells by 8 h.p.i. Viral titres then slightly increased at 12 h.p.i. but then decreased to 6.4 x 10^4 PFU/ml at 24 h.p.i. RSFV-26SMCS had a descending replication rate at the early stages of infection, which accelerated by 12 h.p.i. and reached a peak value of 2.4 x 10^5 PFU/ml at 24 h.p.i. An earlier and higher peak, compared to SFV4, in viral growth was observed when cells were infected with RSFV-ΔTN-Δ6K-26SMCS virus which reached 1.6 x 10^5 PFU/ml by 4 h.p.i., and was followed by a drop in viral titres to 6 x 10^4 PFU/ml at 24 h.p.i. RSFV-HABax-26SMCS produced the least amount of virus in CT26 cells when compared to the other viral vectors with titres of 5 x 10^4 PFU/ml by 4 and 12 h.p.i., and then reached a peak of 8.5 x 10^4 PFU/ml at 24 h.p.i. (Figure 3.11b).

The growth kinetics of the viral constructs in the K-BALB cell line was similar to the growth rates observed in the CT26 cell line which were significantly slower than in the BHK-21 cells. The highest replication rate was observed by RSFV-26SMCS with viral titres increasing in a linear fashion over time and reaching a peak of 1.2 x 10^6 PFU/ml at 24 h.p.i. SFV4 produced nearly 10-fold less amount of virus at 8 h.p.i. when compared to RSFV-26SMCS, which then increased to 1.5 x 10^5 PFU/ml by 12 h.p.i. and reached the peak value of 2 x 10^5 PFU/ml at 24 h.p.i. RSFV-ΔTN-Δ6K-26SMCS virus grew at a steady rate until 12 h.p.i. with titres ranging between 6 x 10^4 PFU/ml and 8.4 x 10^4 PFU/ml and then reaching a peak of 3 x 10^5 PFU/ml by 24 h.p.i. RSFV-HABax-26SMCS produced less amount of virus in the K-BALB cells than in the CT26 cells with titres increasing in a linear fashion after 8 h.p.i. and reaching a peak value of 7.7 x 10^4 PFU/ml at 24 h.p.i. (Figure 3.11c). There was no statistically significant difference between the replication rate of the replicating SFV vectors in BHK-21, CT26 or K-BALB cells.

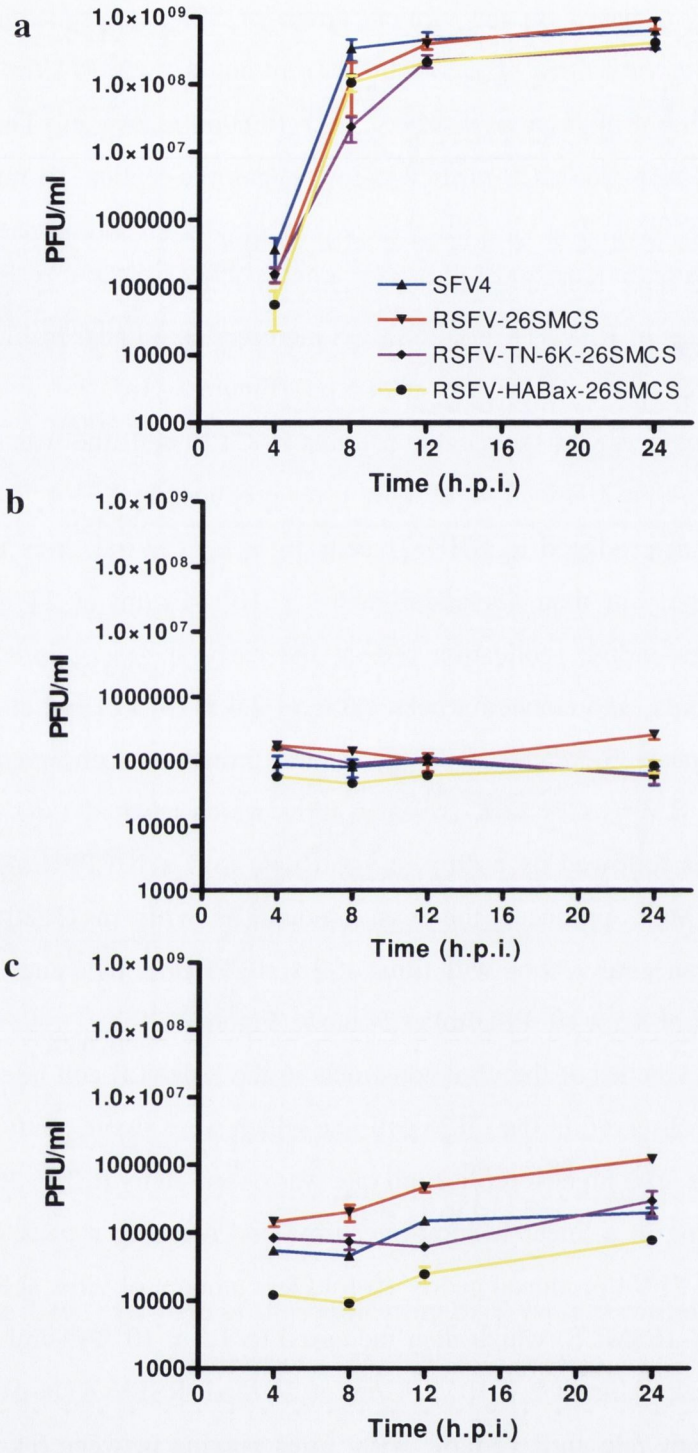


Figure 3.11 Growth of RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS *in vitro*

BHK-21 (a), CT26 (b), or K-BALB (c) cells were seeded into 6-well cell culture dishes at a concentration of 10^6 cells/well and infected with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a MOI of 0.1 PFU/cell. Supernatants were sampled over time (4, 8, 12 and 24 h) and viral titres calculated by plaque assay. *Points*: mean of two replicates, *bars*; +/- SEM. Results are representative of two independent experiments

3.2.5 *In vitro* stability of the Bax protein expressed by RSFV-HABax-26SMCS

Following electroporation of BHK-21 cells with RSFV-HABax-26SMCS RNA, electroporated cells were seeded onto 22 mm^2 glass cover slips in a 6-well cell culture plate and after 16-18 h incubation at 37°C in a humidified atmosphere of 5% CO_2, immunofluorescence was carried out as described in section 2.2.4.2.2 to detect the HA-Bax protein expression. Cytoplasmic staining of the HA-Bax protein was observed in the cells electroporated with RSFV-HABax-26SMCS RNA (Figure 3.12d), while there was no evidence for HA-Bax staining in PBS-electroporated cells (Figure 3.12b).

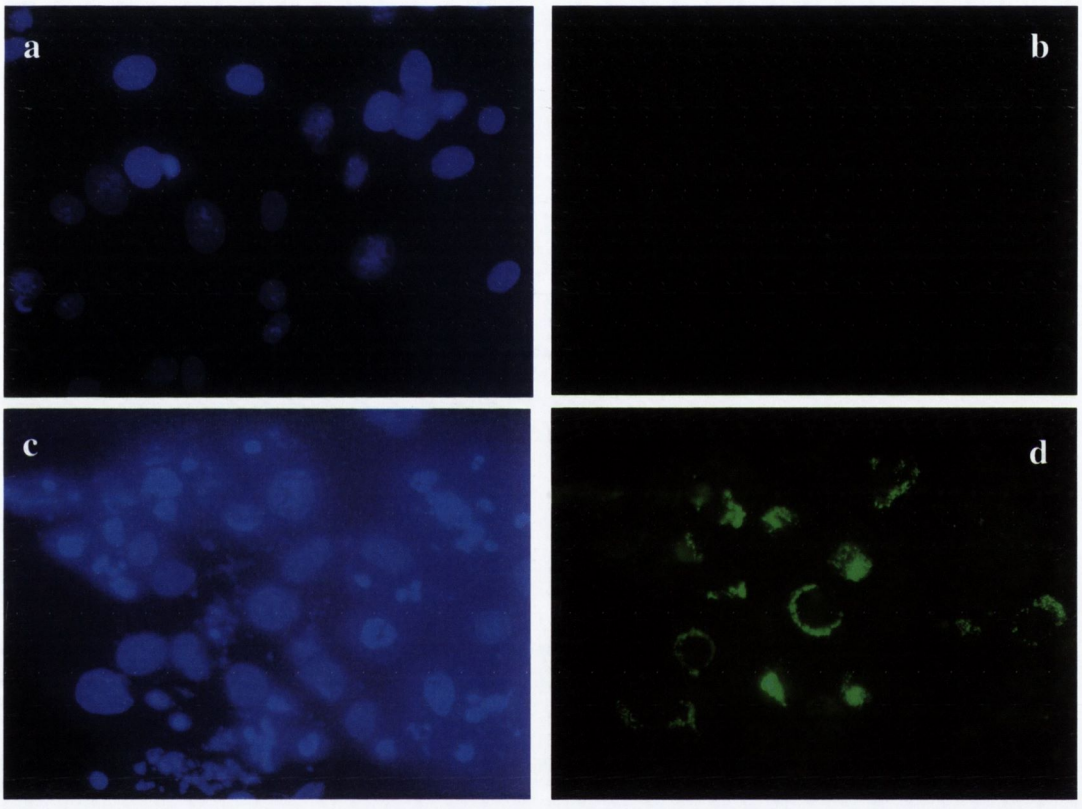


Figure 3.12 Immunofluorescence analysis of the Bax protein

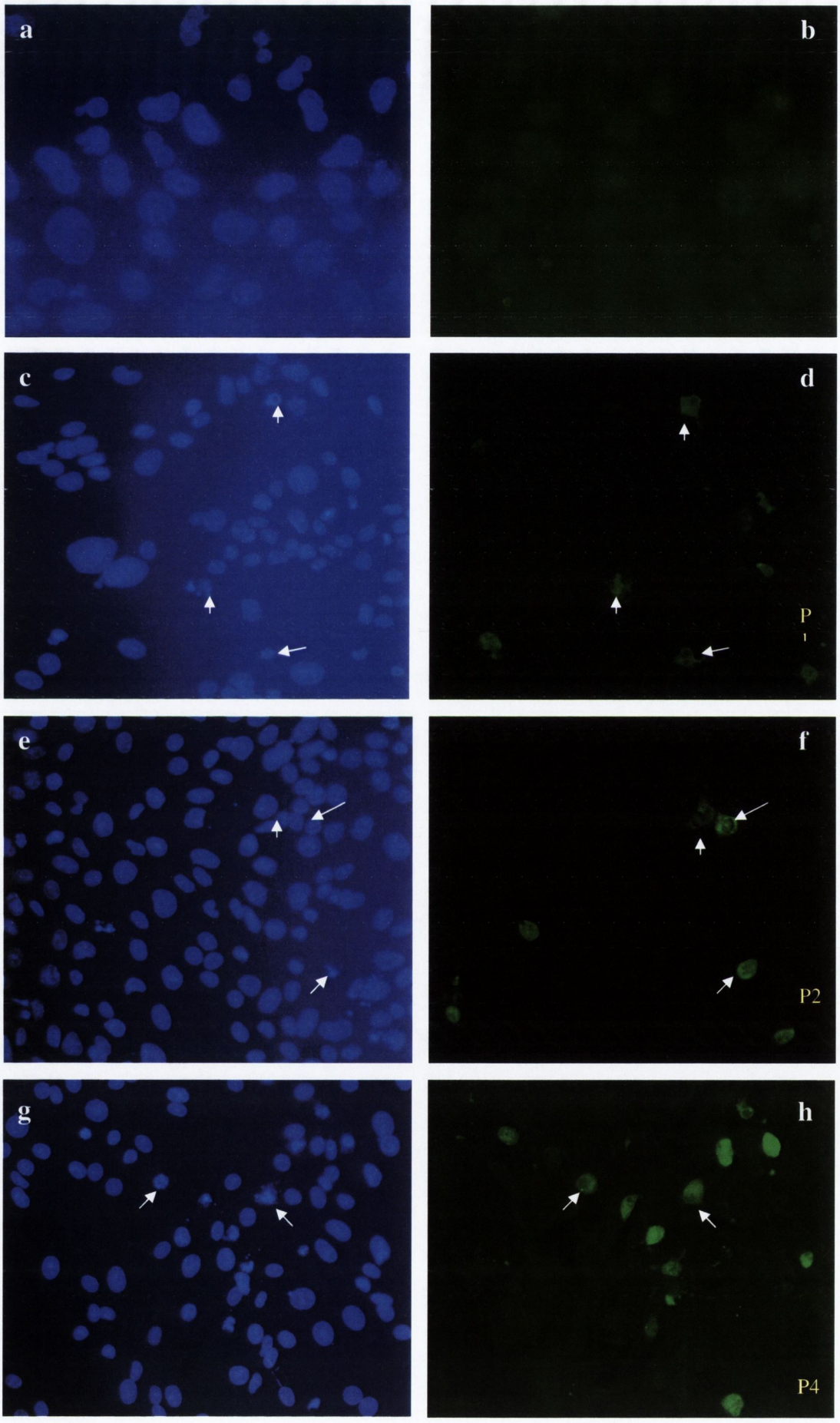
Immunofluorescence was carried out on BHK-21 cells electroporated with PBS control (a and b) or RSFV-HABax-26SMCS RNA (c and d), and cells were examined under the fluorescence microscope at a magnification of 40x. The DAPI stained nuclei of PBS electroporated control cells (a) did not show any evidence of Bax expression when examined under the FITC filter of the miscroscope (b). However, apoptotic bodies were observed following electroporation of the BHK-21 cells with RSFV-HABax-26SMCS RNA (c), and staining of the FITC labeled Bax protein expressed from the RSFVHABax-26SMCS vector was visible (d).

To check the stability of the Bax protein expressed by RSFV-HABax-26SMCS *in vitro*, RSFV-HABax-26SMCS virus was passaged eight times in BHK-21 cells and the HA-Bax protein expression was analysed for each passage by indirect immunofluorescence in BHK-21 cells as described in sections 2.2.4.2.1 and 2.2.4.2.2 respectively. The HA-Bax protein expression was observed in cells infected with RSFV-HABax-26SMCS virus after the 1st passage (P1) (Figure 3.13d), 2nd passage (Figure 3.13f), 4th passage (Figure 3.13h), 6th passage (Figure 3.13j), and the 8th passage (Figure 3.13l). The HA-Bax staining was observed in highly apoptotic cells which are designated by arrows in Figure 3.13 c-d, e-f, g-h, and i-j. HA-Bax protein expression was also confirmed for the 3rd, 5th, and the 7th passage of RSFV-HABax-26SMCS virus (data not shown). There was no evidence for HA-Bax staining in the cells incubated with infection medium alone (Figure 3.13 b).

3.2.6 Infection efficiency of the replicating SFV vectors in BHK-21, CT26 and K-BALB cells

Different cell lines display varying infection efficiencies to viral vectors, therefore the infection efficiencies of the CT26 and K-BALB tumour cell lines was assessed in comparison to the easily infectable BHK-21 cell line. BHK-21, CT26 or K-BALB cells were seeded in 12-well cell culture dishes at a concentration of 8 x 10^5 cells/well, infected with RSFV-EGFP-26SMCS virus at increasing MOIs and fixed for flow cytometer analysis as described in section 2.2.4.3. The percentage of positive cells (cells expressing the EGFP gene) for each MOI was determined by flow cytometre in a population of 2 x 10^4 cells.

There was a linear relationship between the percentage of positive BHK-21 cells and the MOI, up to an MOI = 10 where BHK-21 cells were infected most efficiently with 93% of cells expressing EGFP. At MOI = 100 this percentage remained constant. Transfection efficiencies of the CT26 cells were significantly lower than those observed with the BHK-21 cells. Again, a linear relationship existed between the number of infected CT26 cells and the MOI of virus used but only 12% of cells expressed the reporter gene at MOI = 10, and 28% of CT26 cells were positive for the vector at MOI= 100.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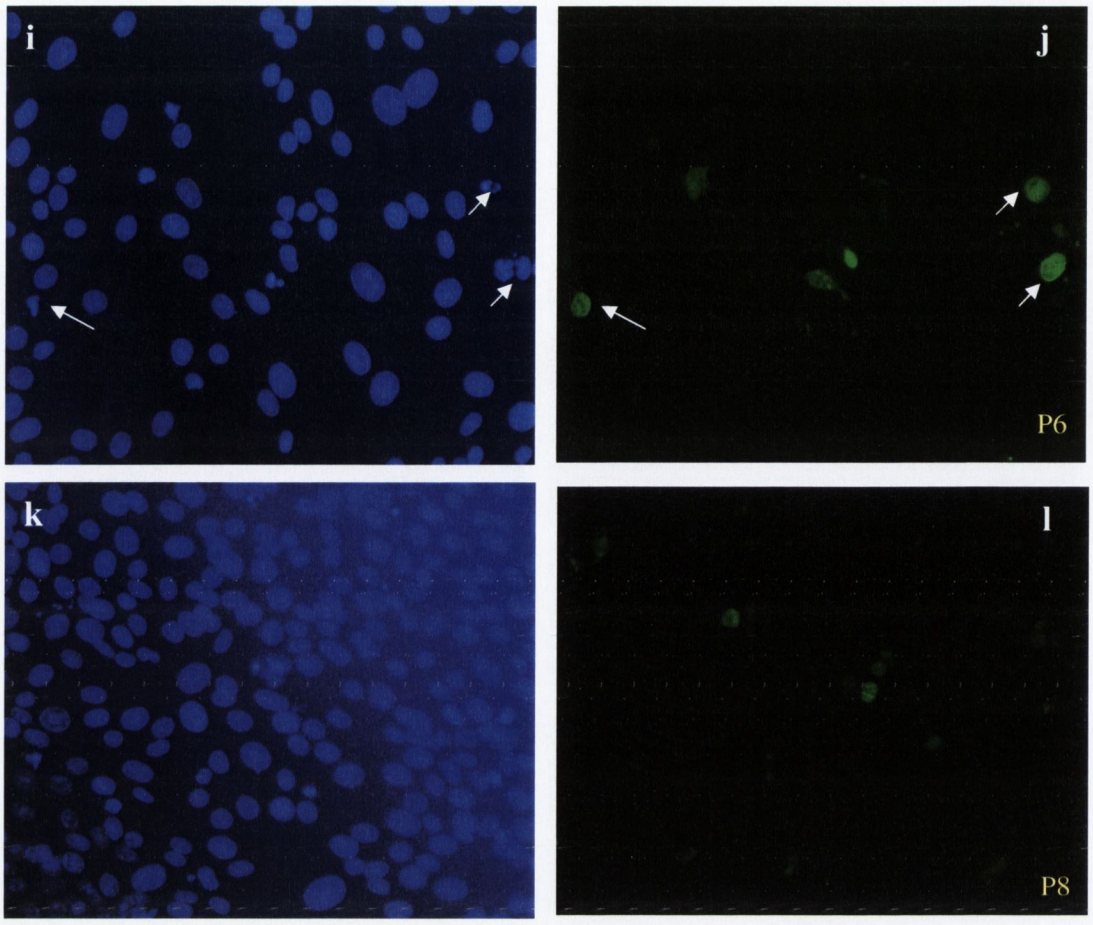


Figure 3.13 Stability check of the Bax protein by indirect immunofluorescence

RSFV-HABax-26SMCS virus was passaged eight times in BHK-21 cells and the HA-Bax protein expression was analysed for each passage by indirect immunofluorescence in BHK-21 cells under the fluorescence microscope at a magnification of 40x and 20x. Only the results for the 1st, 2nd, 4th, 6th, and the 8th passages are shown.

(a) The DAPI stained nuclei of cells incubated with infection medium as the negative control. (b) BHK-21 cells incubated with infection medium did not show any evidence of Bax expression following examination under the FITC filter of the microscope.

DAPI stained nuclei of cells infected with RSFV-HABax-26SMCS virus after the (c) 1st passage (P1), (e) 2nd passage (P2), (g) 4th passage (P4), (i) 6th passage (P6), and (k) 8th passage (P8)

Staining of the FITC labelled HA-Bax protein in BHK-21 cells, post infection with RSFV-HABax-26SMCS virus (d) P1, (f) P2, (h) P4, (j) P6, and (l) P8

Images a – b were taken under 40x magnification, whereas the others were taken under 20x magnification.

The number of EGFP-positive K-BALB cells was significantly lower than the number of EGFP-positive BHK-21 cells at each MOI ($P < 0.001$), and CT26 cells at MOI = 10 and MOI = 100 ($P < 0.001$). Infection of K-BALB cells with RSFV-EGFP-26SMCS virus even at the maximum MOI (MOI =100), resulted in production of very low numbers of EGFP-positive cells (only 8%) (Figure 3.14a).

Infection efficiency of the BHK-21, CT26 and K-BALB cells with RSFV-EGFP-26SMCS was also analysed at 24 h intervals over a three-day period. BHK-21, CT26 and K-BALB cells were seeded in 12-well cell culture dishes at a concentration of 8×10^5 cells/well and infected with RSFV-EGFP-26SMCS virus at MOI = 100 as described in section 2.2.4.3. At each time point (24 h, 48 h, and 72 h), cells were fixed for flow cytometer analysis as described in section 2.2.4.3, and the percentage of positive cells (cells expressing the EGFP gene) was determined by examination of a population of 2×10^4 cells. A two-way ANOVA with Bonferroni post-tests was used to compare the infection efficiency of different cell lines at different time points.

Infection efficiency of BHK-21 cells peaked by 24 h.p.i. with 97% of cells being positive for the reporter gene, which then decreased to 84% and 58% in a linear fashion by 48 h.p.i. and 72 h.p.i., respectively. CT26 cells displayed significantly lower infection efficiencies when compared to BHK-21 cells at 24 and 72 h.p.i. ($P < 0.001$). The percentage of CT26 cells expressing the EGFP gene was at 77% at 24 h.p.i. and remained constant at 48 h.p.i. Number of EGFP-positive CT26 cells then decreased to 37% at 72 h.p.i. Infection efficiency of the K-BALB cell line was significantly lower than the BHK-21 and the CT26 cell lines at each time point ($P < 0.001$) with the number of positive cells reaching a peak value of 11% at 24 h.p.i. and dropping to 4% in a linear fashion by 72 h.p.i. (Figure 3.14b).

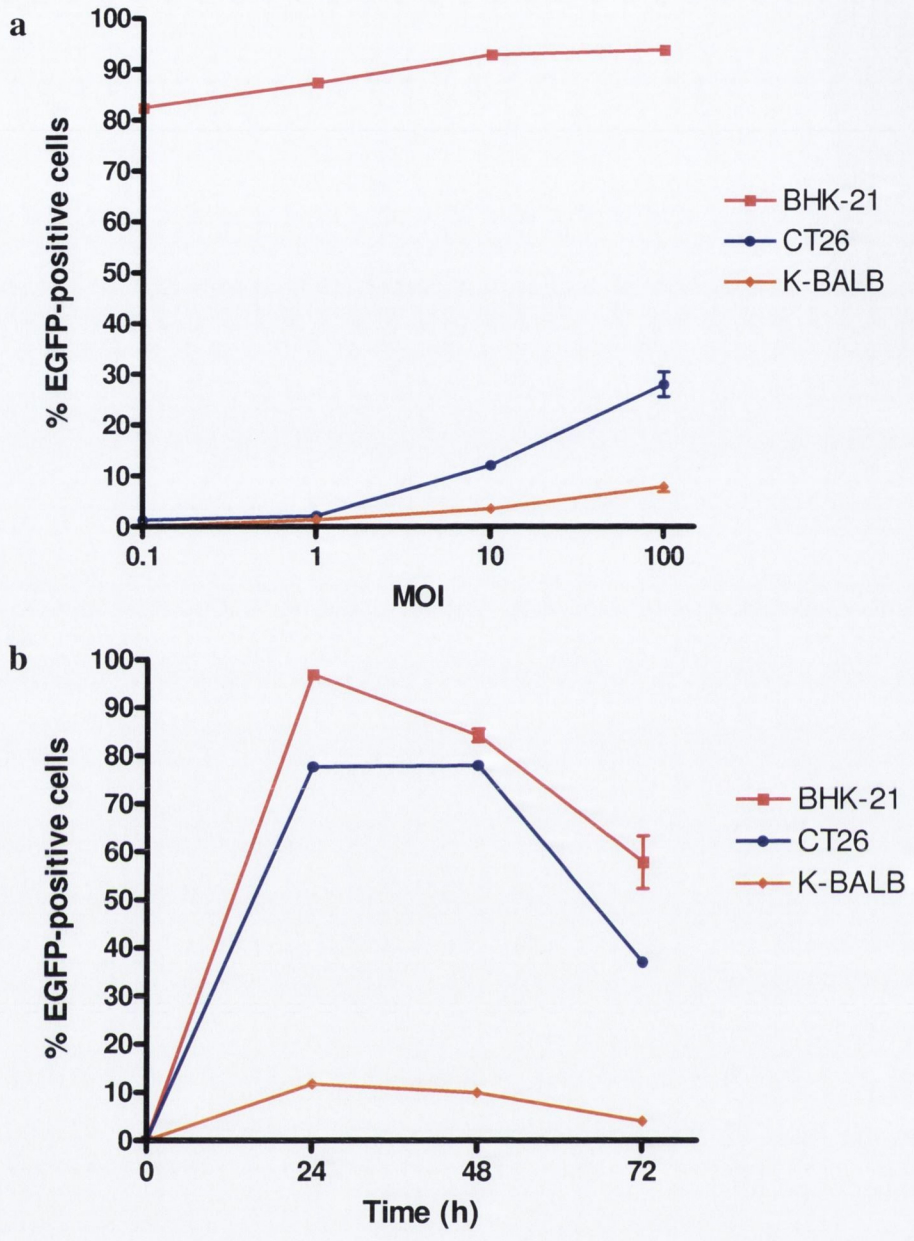


Figure 3.14 Infection efficiency of BHK-21, CT26 and K-BALB cells with RSFV-EGFP-26SMCS

Cells were seeded in 12-well cell culture dishes at a concentration of 8 x 10^5 cells/well and infected with RSFV-EGFP-26SMCS virus at increasing MOIs (a) and at MOI = 100 (b). After incubation for 18 h, cells were prepared and fixed with 2% PFA. The percentage of positive cells (cells expressing the EGFP gene) for each MOI (a) and for each time point (b) was determined by flow cytometre count following examination of a population of 2 x 10^4 cells. *Points:* mean of two replicates, *bars:* +/- SEM

3.2.7 Growth of BHK-21, CT26 and K-BALB cell lines following infection with the replicating SFV vectors

BHK-21, CT26, or K-BALB cells were seeded at a concentration of 1.5 x 10^4 cells/well in 96-well cell culture dishes and infected with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus (MOI = 100) as described in section 2.2.4.4. Following the addition of the *CellTiter 96*® *AQ_{ueous} One Solution Reagent* directly into the culture wells, viability was assessed by the absorbance recorded at 490 nm with a 96-well plate reader which measures the quantity of formazan product that is directly proportional to the number of living cells in culture. Viability was measured at 24 h intervals over a five-day period. Statistical comparisons were performed using a two-way ANOVA with Bonferroni post-tests to compare the virus groups to the negative control TNE group. Results were considered statistically significant when $P < 0.05$.

Viability of the control BHK-21 cells remained constant for the initial 72 h.p.i., before declining in number due to overconfluence in spent medium. The average percentage of viable cells was 73% of controls by 24 h.p.i. following infection with SFV4 and the replicating SFV vectors. Viability was observed to drop steadily and reach an average percentage ranging between 20% and 26% of the controls from 48 h.p.i. onwards showing that BHK-21 cells were killed efficiently with SFV4 and the replicating SFV vectors, respectively ($P < 0.001$) (Figure 3.15a).

Mock-infected CT26 cells grew rapidly and reached the peak at 48 h.p.i. after which the viable cell numbers remained somewhat constant until the experiment end. Growth of CT26 cells was severely inhibited with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus by 48 h.p.i. ($P < 0.001$). By 72 h.p.i. the percentage of viable CT26 monolayers infected with RSFV-ΔTN-Δ6K-26SMCS virus and RSFV-HABax-26SMCS virus had increased to 52% and 42% respectively ($P < 0.001$) while the percentage of viable cells following infection with SFV4 and RSFV-26SMCS remained around 30% for the following 72 h ($P < 0.001$). After incubation for 72 h, viability of CT26 cells infected with RSFV-ΔTN-Δ6K-26SMCS virus and RSFV-HABax-26SMCS virus increased in a linear fashion and monolayers recovered with cell numbers approaching 96% and 88% of the control wells by 120 h.p.i, respectively ($P > 0.05$).

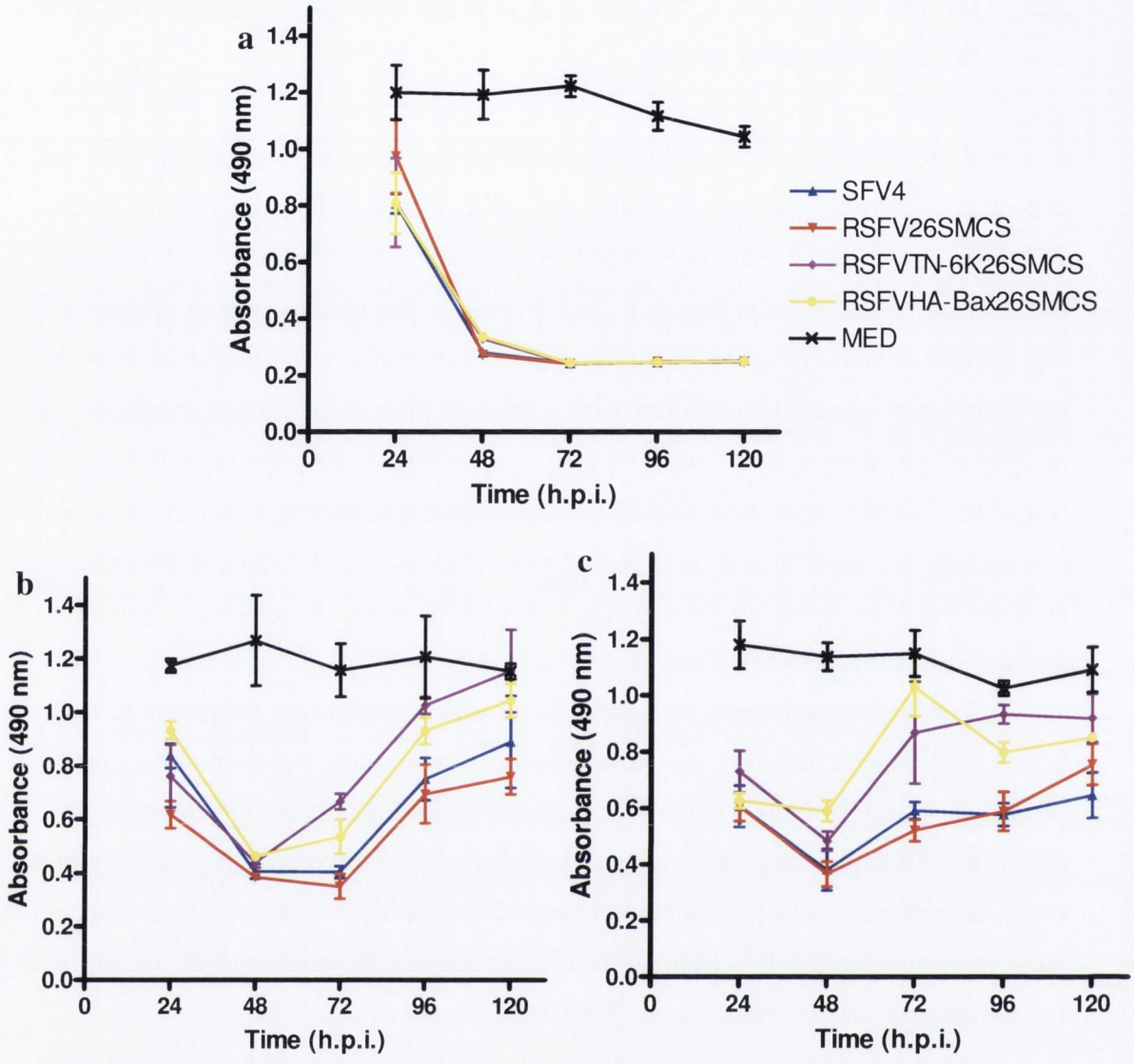


Figure 3.15 Viability of BHK-21, CT-26 and K-BALB cells following infection with the replicating SFV vectors

BHK-21 (a), CT26 (b), or K-BALB (c) cells were seeded at a concentration of 1.5 x 10^4 cells/well in 96-well cell culture dishes and infected with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus (MOI = 100). Following addition of the *CellTiter 96® AQ_{ueous} One Solution Reagent* directly into the culture wells, viability was measured by the absorbance recorded at 490 nm with a 96-well plate reader which shows the quantity of formazan product that is directly proportional to the number of living cells in culture. Viability was measured at 24 h intervals over a five-day period.

Points; mean of three replicates, *bars*; +/- SEM. Results are representative of two independent experiments. Statistical analysis was performed by two-way ANOVA with Bonferroni post-tests to compare the differences between the SFV vectors and the control group.

SFV4 and RSFV-26SMCS virus infected monolayers displayed recovery at a steady rate between 72 and 120 h.p.i. with percentage of viable cells reaching to 74% and 63.5% of controls respectively at the experiment end ($P < 0.05$ and $P < 0.01$ respectively) (Figure 3.15b).

Control K-BALB cells grew in a similar manner to CT26 cells over the five-day period, where the cell numbers remained constant for the initial 72 h.p.i. before declining by 96 h.p.i. and recovering by 120 h.p.i. Viability of SFV4 and RSFV-26SMCS virus infected K-BALB cells was reduced by 30% compared to the mock-infected controls by 48 h.p.i., after which monolayers continued to grow in a linear fashion and reached 54% and 63% of the controls respectively at 120 h.p.i. ($P < 0.001$). K-BALB cells infected with RSFV-HABax-26SMCS virus exhibited 52% reduced viability at 48 h.p.i., compared to the negative control cells ($P < 0.001$), before starting to recover by 72 h.p.i. and reaching 89.5% of the control wells ($P > 0.05$). Viability of the K-BALB cells reduced to 78% of the control cells by 96 h.p.i. and 120 h.p.i. following infection with RSFV-HABAx-26SMCS virus ($P > 0.05$). RSFV-ΔTN-Δ6K-26SMCS virus infection resulted in a drop in viability to 62% of the controls at 24 h.p.i. ($P < 0.001$) which then continued to drop steadily to 42% by 48 h.p.i. ($P < 0.001$) after which a significant recovery at viability was observed by 96 h.p.i. with 91% of the controls ($P > 0.05$) (Figure 3.15c).

3.2.8 Induction of apoptosis in BHK-21, CT26, and K-BALB cells following infection with the replicating SFV vectors

To detect the activation of caspases which is the indicator of apoptosis induction; BHK-21, CT26 or K-BALB cells were seeded into 6-well cell culture dishes at a concentration of 10^6 cells/well and infected with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at MOI = 100. Following incubation for 18 h and 48 h, cells were sampled and fixed for analysis by flow cytometer as described in section 2.2.4.5. Amount of FLICA measured was the representative of the percentage of caspase-3/-7 positive cells.

Upon infection with SFV4, BHK-21 cells showed only a slight rise with 8% of cells positive for active caspase at 18 h.p.i. ($P < 0.001$, compared to the negative control group), however the number of positive cells increased to 93% at 48 h.p.i. ($P < 0.001$). Infection with RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-

HABax-26SMCS virus resulted in low number of active-caspase-positive cells at 18 h.p.i. However, apoptosis induction then increased at 48 h.p.i. with 93.5%, 86% and 89% of cells being positive respectively ($P < 0.001$, when compared to MFI group). SFV4 and RSFV-26SMCS produced significantly higher numbers of caspase-positive cells when compared to RSFV-ΔTN-Δ6K-26SMCS at 48 h.p.i. ($P < 0.01$) (Figure 3.16a).

The number of CT26 cells positive for caspases was significantly lower than the active-caspase positive BHK-21 cells at 48 h.p.i. ($P < 0.01$), following infection with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus. SFV4 infection resulted in detection of caspase positive cells at 6% and 9% at 18 h.p.i. and 48 h.p.i., respectively. Following infection of CT26 cells with RSFV-26SMCS virus, active-caspase-positive cells reached 17% by 48 h.p.i. RSFV-ΔTN-Δ6K-26SMCS virus and RSFV-HABax-26SMCS virus resulted in production of caspase positive cells at 12% and 9%, respectively at 48 h.p.i. There was no statistically significant difference between the various virus groups at different time points (Figure 3.16b).

K-BALB cells were shown to be susceptible to apoptosis induction when infected with the replicating SFV vectors but the effect was observed at a much lower extent than in BHK-21 and CT26 cell lines. Infection of K-BALB cells with SFV4 resulted in production of the highest number of caspase-positive cells at 10% at 48 h.p.i.; however the result was not statistically significant when compared to the negative control MFI group and the other virus groups. RSFV-26SMCS and RSFV-HABax-26SMCS virus induced a low level of apoptosis with 3% and 5% of cells positive at 48 h.p.i. ($P > 0.05$). Apoptosis induction was detectable in RSFV-ΔTN-Δ6K-26SMCS virus infected K-BALB cells at 18 h.p.i. (4% of cells positive), and only increased to 5% at 48 h.p.i. ($P > 0.05$). The difference between the virus groups and the negative MFI control group, and the difference between each virus group was found to be insignificant at both 18 h.p.i. and 48 h.p.i. (Figure 3.16c).

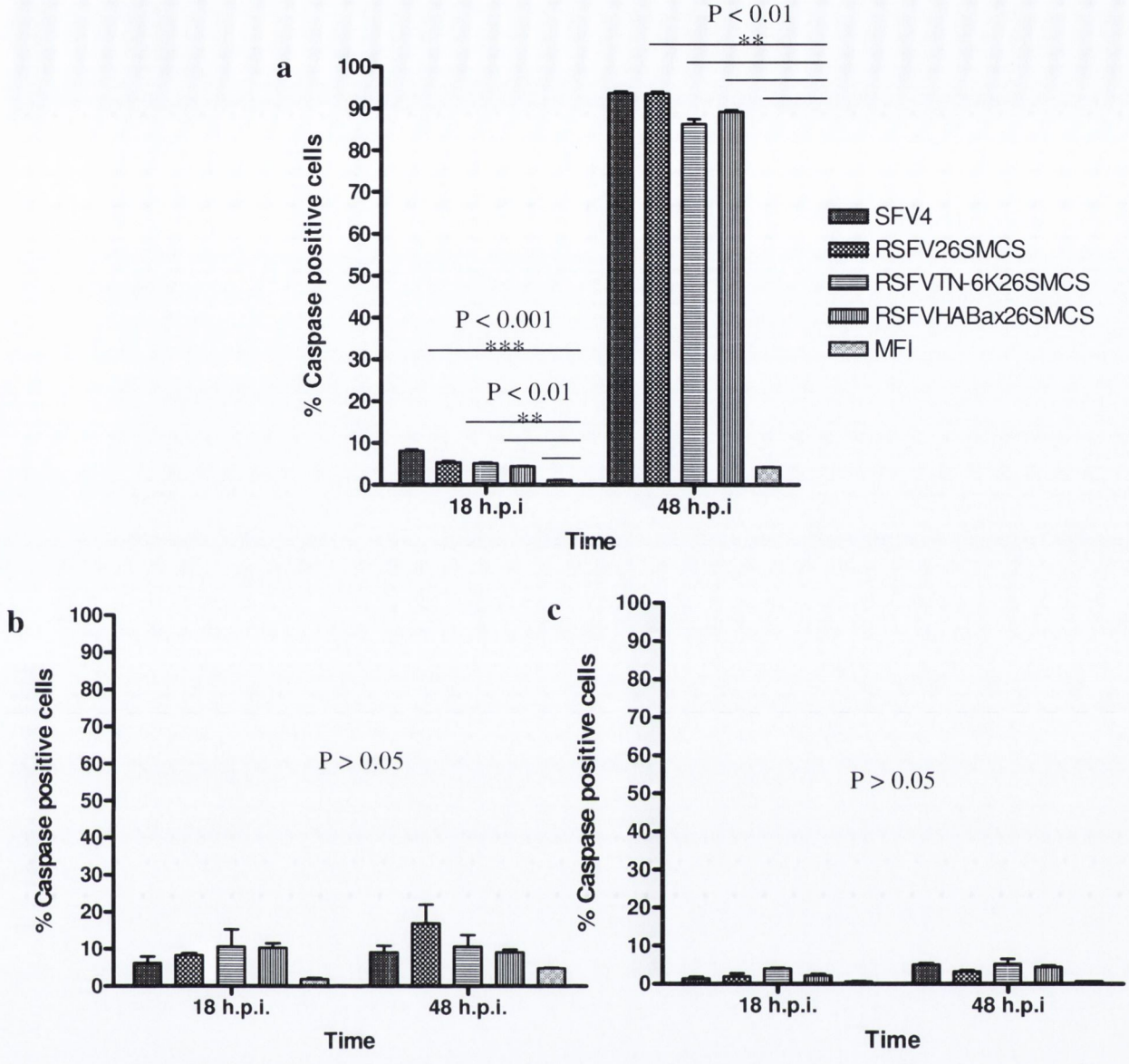


Figure 3.16 Apoptosis induction in BHK-21, CT26 and K-BALB cells infected with the replicating SFV vectors, as determined by FLICA

BHK-21 (a), CT26 (b), or K-BALB (c) cells were seeded into 6-well cell culture dishes at a concentration of 1 x 10^6 cells/well and infected with MFI alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at MOI = 100. After incubation for 18 and 48 h, cells were sampled and fixed for analysis by flow cytometer as described in section 2.2.4.5. Amount of FLICA measured was the representative of percentage caspase-3/-7 positive cells.

Points; mean of two replicates.

Statistical analysis was performed by two-way ANOVA with Bonferroni post-tests to compare the differences between the SFV vectors and the control group.

3.3 DISCUSSION

Alphaviruses have the ability to induce apoptosis in mammalian cell cultures by producing viral mRNAs and inhibiting the activity of host-cell translational machinery (Strauss & Strauss, 1994; Glasgow *et al.,* 1997). Upon infection of vertebrate cells with SFV, extensive CPE is observed by 24 h.p.i., which is an indicator of programmed cell death (Glasgow *et al.,* 1997). It was observed that the non-structural region of SFV rather than the structural proteins, affects the induction of apoptosis (Glasgow *et al.,* 1998; Murphy *et al.,* 2000), whereas SV requires the structural proteins to mediate the apoptosis induction (Joe *et al.,* 1998).

The capability of SFV to induce apoptosis in different cell lines has made the SFV vector system attractive as a tumour therapy agent. Recombinant SFV particles, alone and in combination with therapeutic genes or T-cell epitopes, have been shown to induce apoptosis in various tumour cell lines (Murphy *et al.,* 2000; Yamanaka *et al.,* 2000; Murphy *et al.,* 2001; Chikanna-Gowda *et al.,* 2005; Rodriguez-Madoz *et al.,* 2005; Smyth *et al.,* 2005; Chikanna-Gowda *et al.,* 2006). The replication-competent vector based on the avirulent A7(74) SFV strain, VA7, has also been used to induce viral oncolysis in several tumour models (Vähä-Koskela *et al.,* 2006; Määttä *et al.,* 2007).

One of the mouse tumour models utilised in this study are K-BALB cells, which express oncogenic K-*ras*, and form rapidly growing syngenic tumours in immunocompetent BALB/c mice upon s.c. injection (Aaronson & Weaver, 1971; Stephenson & Aaronson, 1972). The *ras* genes are well known to cause deregulation in cell cycle progression and uncontrolled cellular proliferation. However, the ability of *ras* to further influence cells to undergo apoptosis is less definitive. Studies have indicated that oncogenic Ras promotes apoptosis in fibroblasts and lymphocytes but acts in an anti-apoptotic way on epithelial cells and myeloid cells (Downward, 1998; Arber, 1999; Cox & Der, 2003), showing that cell type plays a major role in the effects of Ras on apoptosis. CT26 tumour cells constituted the second murine tumour model used in this project. CT26 cells are derived from a colon adenocarcinoma cell line and form localized tumours of low immunogenicity in BALB/c mice following s.c. injection (Brattain *et al.,* 1980; Wang *et al.,* 1995).

In this study, the replication-competent SFV vector based on the infectious cDNA clone of SFV (pSP6-SFV4) (RSFV-26SMCS) has been exploited as a tumour

therapy agent (RSFV-26SMCS). Following establishment that the nsP3 gene is a virulence determinant for SFV (Tarbatt *et al.,* 1997; Tuittila *et al.,* 2000), the replicating SFV vector containing a non-revertible in-frame deletion (SN) in the nsP3 region was constructed and sequenced to confirm the presence of the deletion and the second 26S subgenomic promoter. Subsequently, the amount of virus produced by the SFV vectors containing the single deletions (nsP3-SN, nsP3-TN (Galbraith *et al.,* 2006), and 6K) was studied in BHK-21 cells.

To increase the biosafety of the replicating SFV vector, the nsP3-TN deletion was combined with the complete deletion of the 6K structural gene, creating RSFV-ΔTN-Δ6K-26SMCS. In an attempt to increase apoptosis in infected cells (Murphy *et al.,* 2001), the pro-apoptotic *bax* gene was also cloned into the RSFV-26SMCS creating RSFV-HABax-26SMCS, by the student Ms. Jennifer Mulholland (supervised by Güniz Iskender). Following confirmation of the presence of the deletions and the foreign gene by sequencing, the growth of RSFV-ΔTN-Δ6K-26SMCS viru and RSFV-HABax-26SMCS virus was analysed in BHK-21, CT26 and K-BALB cells. The ability of replication-competent SFV vectors to infect the tumour cell lines and the effect of infection on cellular viability was also assessed. As it is known that activated K-*ras* increases the resistance/susceptibility to apoptosis, the induction of apoptosis by the SFV vectors was also examined. BHK-21 cells are known to be highly susceptible to SFV infection and the associated induction of apoptosis (Glasgow *et al.,* 1997), and were employed as a positive control in the assays. Combination of the nsP3-SN and the nsP3-TN deletions, or the nsP3-SN and the 6K deletions produced non-viable virus, therefore studies were undertaken using RSFV-ΔTN-Δ6K-26SMCS virus.

The growth of replicating vectors containing the single SN (RSFV-ΔSN-26SMCS) and TN deletions (RSFV-ΔTN-26SMCS) in the nsP3 region of the genome separately and that of the replicating vector with the 6K deletion (RSFV-Δ6K-26SMCS) was examined in the BHK-21 cell line over a 24 h period with 2 h intervals. The growth of SFV4 and RSFV-26SMCS was also analysed and the results were compared. Following infection of BHK-21 cells at a MOI of 10 and 0.1 PFU/cell, it was observed that vectors comprising the single deletions produced large amounts of virus similar to SFV4 and RSFV-26SMCS virus. The presence of the second 26S subgenomic promoter did not affect the replication rate of RSFV-26SMCS or the vectors containing the single deletions, virus replicated to high titres in BHK-21 cells at a MOI of 10 and 0.1 PFU/cell.

Growth of the replicating vector containing the TN and 6K deletions (RSFV-ΔTN-Δ6K-26SMCS), and the SFV vector expressing the *bax* gene (RSFV-HABax-26SMCS) was also examined in BHK-21, CT26 and K-BALB cells. Growth rates were compared with that of pSP6-SFV4 and RSFV-26SMCS. As expected, SFV4 grew to the highest titres in BHK-21 cells and to significantly lower levels in the CT26 and K-BALB cell lines. This was due to a lower infection efficiency of these cell lines (section 3.2.6) which would increase the time taken for the cell population to succumb to infection. As expected, RSFV-26SMCS and SFV4 grew at a similar rate in BHK-21 cells. RSFV-26SMCS is based on the wild-type genome therefore it was expected to act like the wild-type virus. RSFV-ΔTN-Δ6K-26SMCS produced significantly less virus than pSP6-SFV4 at 8 h.p.i. in BHK-21 cells. This could be due to the inhibitory effects of the deletions on the replication of the virus as the vector accommodates two deletions in one genome. It is also known that incorporation of a second subgenomic promoter can compete with the native subgenomic promoter for viral transcriptase complexes and, as a result, virus replication is slowed (Raju & Huang, 1991). However, the presence of the second subgenomic promoter did not affect the replication rate of SFV vectors containing the single deletions. The presence of the two deletions however exerted no apparent effect on virus growth after 8 h until the end of the experiment. Replication rates of SFV vectors were slower in the tumour cell lines compared to the BHK-21 cell line, as expected. This may reflect the variability in infection with different cell lines. There was no significant difference between the replication rates of SFV vectors in the BHK-21 or the tumour cells. The replication-competent SFV vector expressing the *bax* gene (RSFV-HABax-26SMCS) had a high replication rate similar to that of pSP6-SFV4 and RSFV-26SMCS in the BHK-21 cell line. This was surprising, as in a previous study, *bax*-expressing particles could be produced only at low titres compared to EGFP-expressing particles under standard conditions for particle production (Murphy *et al.*, 2001). The increased growth of virus observed in this study possibly occurred because the *bax* gene was cloned into the replicating vector instead of the VLPs.

To check the stability of the Bax protein *in vitro*, RSFV-HABax-26SMCS virus was passaged eight times in BHK-21 cells and the expression of Bax protein was checked for each passage by indirect immunofluorescence. Analysing the stability of the Bax protein was crucial to see if the expression of the *bax* gene is transient during the replication of RSFV-HABax-26SMCS virus. The HA-Bax protein expression was

observed to be nuclear in highly apoptotic cells (which are designated by arrows in Figure 3.13) and the protein expression was confirmed to be stable for each passage in the BHK-21 cell line.

Infection efficiencies of BHK-21, CT26 and K-BALB cells with RSFV-EGFP-26SMCS virus were assessed at a variety of MOIs. Cells expressing the EGFP gene were counted by flow cytometre and were considered as positive. Over 90% of BHK-21 cells were positive at a MOI of 10 PFU/cell and at the maximum MOI (MOI = 100) whereas CT26 and K-BALB cells were not as easily transduced with just 28% and 8% of cells positive, respectively, when infected at a MOI of 100 PFU/cell. This low infection efficiency correlated with the results obtained from the SFV growth curves discussed above and indicated that the replicating SFV vectors had limited ability to infect these tumour cell lines. There is another possibility that SFV vectors induced only one round infection in tumour cell lines at MOI = 100, therefore only 28% and 8% of CT26 and K-BALB cells got transfected with the virus, respectively.

Infection with the replication-competent SFV vectors resulted in a rapid inhibition in cellular viability in the BHK-21 cell line as determined by the *CellTiter 96® AQ_{ueous} One Solution* assay. BHK-21 cells were completely obliterated by SFV replicating vectors underlining the susceptibility of this cell line to SFV infection and its high infection efficiency. Despite their lower infection efficiencies compared to BHK-21 cells, CT26 and K-BALB cells were susceptible to SFV-induced cytopathic effect. Infection with replicating SFV vectors resulted in suppression of the growth of CT26 and K-BALB cells between 24 and 48 h.p.i, after which the infected cells died off and the remaining uninfected cells proliferated showing that the effects of the SFV vectors on the viability of CT26 and K-BALB cells were transient. RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS were shown not to induce efficient cell killing in CT26 and K-BALB cells, when compared to SFV4 and RSFV-26SMCS. The presence of the double deletions in one genome could have caused pressure on the virus, and the low infection efficiency of the tumour cell lines could have hindered efficient replication of RSFV-ΔTN-Δ6K-26SMCS in the tumour cells.

The pro-apoptotic *bax* gene was shown not to enhance the cell killing and the level of apoptosis in the CT26 and K-BALB cells. In a previous study, Murphy et al (2001) found that rSFV VLPs expressing the pro-apoptotic *bax* gene increased apoptosis in AT3-Neo and AT3-Bcl-2 cells. Given that the response of a cell to a death signal is determined by the ratio of Bcl-2 to Bax (Oltvai *et al.,* 1993), Bax probably

counteracted the Bcl-2-mediated suppression of cell death by Bcl-2-Bax heterodimerization. The finding by Oltvai et al (1993) that *bax* RNA is present in normal tissues also indicates that Bax in itself does not cause cell death and overexpressed Bax accelerates apoptotic cell death only following a death signal. In a recent study Bax-/- mouse embryonic fibroblasts (MEFs) were shown to be more sensitive to caspase-3 activation, cytochrome c release and apoptosis than wild type (wt) cells (Urban *et al.,* 2008). The molecular basis of this anti-apoptotic action of Bax remains unknown. The differing results obtained in our study and that of Murphy et al (2001) may be due to the use of different host cell lines. Instead of the *bax* gene the TRAIL type II transmembrane protein, a member of the TNF family could have been cloned to RSFV-26SMCS. The cell-surface expressed TRAIL has been shown to induce apoptosis in a wide variety of transformed cell lines (Wiley *et al.,* 1995).

To investigate the mechanism of cell death in BHK-21, CT26, and K-BALB cells following infection with SFV, activation of caspase-3/ -7 was examined in all three cell lines (see section 2.2.4.5). Following examination of caspase activation it was found that BHK-21 cells were more susceptible than the other cell lines to SFV-induced apoptosis and so were effective in their role as positive control for these experiments. A similar pattern was observed to that in the viability and infection efficiency assays discussed above with BHK-21 cells showing a sustained effect in SFV infection. Both CT26 and K-BALB cells displayed significantly lower numbers of positive caspase-3/-7 cells compared to BHK-21 cells. The relatively low level of caspase positive cells displayed following infection of K-BALB cells could be related to activated K-*ras*. It was also recently shown that although caspase-3 and caspase-7 are dominant mediators of SFV-induced apoptosis, the virus also induces apoptosis through a caspase independent death program (Urban *et al.,* 2008).

In conclusion, it was shown that replication-proficient SFV vectors were able to infect CT26 and K-BALB tumour cells and induce apoptosis at a relatively low level (compared to BHK-21 cells) *in vitro*. The overall findings were encouraging and indicated that the potential of replication-competent SFV vectors as tumour therapy agents in CT26 and K-BALB mouse tumour models should be assessed.

Chapter 4

VIRULENCE ANALYSES OF REPLICATING SFV VECTORS

4.1 INTRODUCTION

Virulence of SFV is known to be polygenic with determinants distributed through most of the genome (Glasgow *et al.,* 1991, 1994; Santagati *et al.,* 1995; 1998; Rikkonen, 1996; Tarbatt *et al.,* 1997; Ahola *et al.,* 2000; Tuittila *et al.,* 2000; Vihinen *et al.,* 2001; Fazakerley *et al.,* 2002; Tuittila & Hinkkanen, 2003; Logue *et al.,* 2008). Analysis of chimeras between the virulent and avirulent strains of SFV showed that the major virulence determinants of SFV lie within the non-structural genes that form the replicase complex proteins (Tarbatt *et al.,* 1997; Tuittila *et al.,* 2000). The nsP3 gene which is one of the non-structural proteins in the SFV genome, and which has been shown to affect virulence of SFV, has three domains; the first is conserved among alphaviruses, coronaviruses, Hepatitis E virus and Rubella virus (Koonin & Dolja, 1993), the second is conserved among alphaviruses, and the third C-terminal domain is hypervariable (Strauss & Strauss, 1994). It is also known that nsP3 is a phosphoprotein and the phosphorylation sites have been mapped to serine and threonine residues, which are located mainly in the hypervariable domain (Vihinen *et al.,* 2001). It has been shown that individual amino acids in the conserved domain of nsP3 cumulatively determine virulence of SFV (Tuittila & Hinkkanen, 2003) and that the nonphosphorylated nsP3 gene reduces virulence in mice after peripheral inoculation (Vihinen *et al.,* 2001). Considering these findings, the wild-type virus containing two large in-frame deletions in the hypervariable domain of nsP3 region, named ΔSN and ΔTN have been constructed (SFV4-SN, SFV4-TN) by Galbraith et al (2006). Examination of these constructs *in vivo* showed that all mice survived the i.m. inoculation with the deletion mutants and showed no clinical signs of disease; whereas 70% of mice inoculated i.m. with SFV4 died (Galbraith *et al.,* 2006). It is also known that the nsP3 macro domain affects SV replication in neurons and specific mutations in this macro domain reduce the virulence of SV in 2-week-old mice (Park & Griffin, 2009).

The structural protein region of the SFV genome has also been shown to influence the neurovirulence of the virus (Glasgow *et al.,* 1991; Santagati *et al.,* 1995; Tarbatt *et al.,* 1997), including, the small 6K protein which is involved in virus maturation (Liljeström *et al.,* 1991). Initial experiments in mice using SFV4 with an absent 6K gene (SFV4-6K) indicate that, following peripheral inoculation, the virus

multiplies in the mice and stimulates protective immunity, but does not infect the CNS. All mice survive the infection (*Dr. Sareen Galbraith, personal communication*).

In this chapter, replicating SFV vectors comprising in-frame deletions in the nsP3 region of the genome (RSFV-ΔSN-26SMCS and RSFV-ΔTN-26SMCS), and the RSFV-26SMCS vector with the complete deletion of the 6K gene (RSFV-Δ6K-26SMCS) were tested for virulence by inoculating groups of female BALB/c mice i.m. The results showed that virulence of the constructs was not reduced by the deletions in the nsP3 region of the SFV genome. Following these results, a replicating SFV vector comprising the nsP3-TN and the 6K deletions was created, tested *in vitro* (*chapter 3*), and the virulence examined in BALB/c mice. After i.m. inoculation, the percentage survival of mice inoculated i.m. with RSFV-ΔTN-Δ6K-26SMCS virus was higher (90%) than that of mice injected i.m. with the vectors containing the single deletions. Virulence of RSFV-HABax-26SMCS was also examined in mice and results were not statistically significant when compared to the controls.

In summary, none of the replicating SFV vectors mentioned above showed reduced virulence *in vivo*. It was necessary to obtain full immune protection from SFV of mice in order to prevent possible complications that would occur by pathogenic properties of SFV, during tumour treatment studies. Therefore the strategy used by Smyth et al (2005) was adopted and utilized to vaccinate mice prior to administration of the virus. By vaccination, it was aimed to enhance any anti-SFV immune response generated and protect mice from neurotropic properties of SFV. To detect differences in mice inoculated with virus without prior immunisation, negative and positive control groups were also designed where mice were mock-immunised with TNE (refer to section 2.2.6.2). Groups of mice which were pre-immunized i.m. with rSFV-p62-6K VLPs prior to i.m. inoculation with the deletion mutants were protected against the virulent SFV. Prior administration of rSFV-p62-6K VLPs recruited the host immune system as a result of the expression of the SFV antigen E2 which is a highly antigenic envelope protein of SFV. The E2 protein is known to contain both B- and T-cell epitopes and is encoded by the p62 gene of SFV along with the E3 protein (Snijders *et al.,* 1992; Strauss & Strauss, 1994). Several plasmids with specific deletions have been produced from the pSP6-SFV4 infectious clone of SFV (Barth and Garoff, 1997). pSFV-p62-6K contains deletions of the capsid and E1 genes, and is used in this study to express the E2 protein and hence stimulate immunity.

It is already known that SFV4 is a neurotropic virus and kills 60-70% of BALB/c mice when administered i.p. (Glasgow *et al.,* 1991). Therefore it was of interest to monitor the appearance of any clinical or pathological changes associated with SFV4 infection. To assess pathological changes associated with SFV4 infection in immunised and non-immunised mice, brains from triplicate mice were processed for H&E staining and neuropathological examination. H&E staining of paraffin-embedded formalin fixed tissue sections is the most widely used stain for routine histopathology. Haematoxylin is a basic stain which preferentially reacts with nucleic acids and acidic proteins and eosin is an acidic counterstain which provides pinkish red cytoplasmic colouration. The results showed that immunisation conferred protection against challenge with SFV4 with no immunised mice presenting clinical signs or pathological lesions of SFV4 infection.

4.2 RESULTS

4.2.1 Intramuscular inoculation of BALB/c mice with the replicating SFV vectors containing the single ΔSN, ΔTN or the Δ6K

Groups of 10 female BALB/c mice were inoculated i.m. with SFV4, RSFV-26SMCS virus, RSFV-ΔSN-26SMCS virus, RSFV-ΔTN-26SMCS virus, RSFV-Δ6K-26SMCS virus or TNE as described in section 2.2.6.1. Mice were checked daily for 14 days and deaths were recorded. Immunity of surviving mice was tested by i.p. challenge with virulent SFV L10 and the mice observed daily for a further 14 days (see section 2.2.6.1).

Mice injected with RSFV-ΔSN-26SMCS virus, showed a 60% survival rate which was significantly different when compared to the groups of mice injected with RSFV-26SMCS virus or SFV4 using the Logrank statistical test ($P = 0.028$) (Figure 4.1a). Injection of mice i.m. with RSFV-ΔTN-26SMCS virus or RSFV-Δ6K-26SMCS virus resulted in 30% and 50% survival rates, respectively. These values were not significantly different compared to RSFV-26SMCS and SFV4 which were considered as the positive control groups ($P > 0.05$) (Figure 4.1 b and c). 90% of mice inoculated i.m. with SFV4 or RSFV-26SMCS virus died by 10 days post-infection (p.i.) as a result of encephalitis following rapid replication of the virus and passage across the BBB (Fazakerley 2002, 2004). It is known that SFV4 shows virulence in 60-70% of adult mice when administered i.p. (Glasgow *et al.*, 1991).

All mice in the negative control group survived the injection with TNE buffer. Mice inoculated with SFV were protected against the lethal L10 challenge whereas mice injected with TNE buffer died post-challenge (data not shown).

4.2.2 *In vivo* analyses of the replicating SFV vectors in naïve BALB/c mice or in BALB/c mice pre-immunised with rSFV-p62-6K VLPs

In a separate experiment, the virulence of the replicating SFV vector containing the ΔTN and Δ6K was examined by inoculating groups of BALB/c mice i.m. with 10^6 PFU/injection of virus and monitoring them for clinical signs daily for 14 days, as described in section 2.2.6.1.

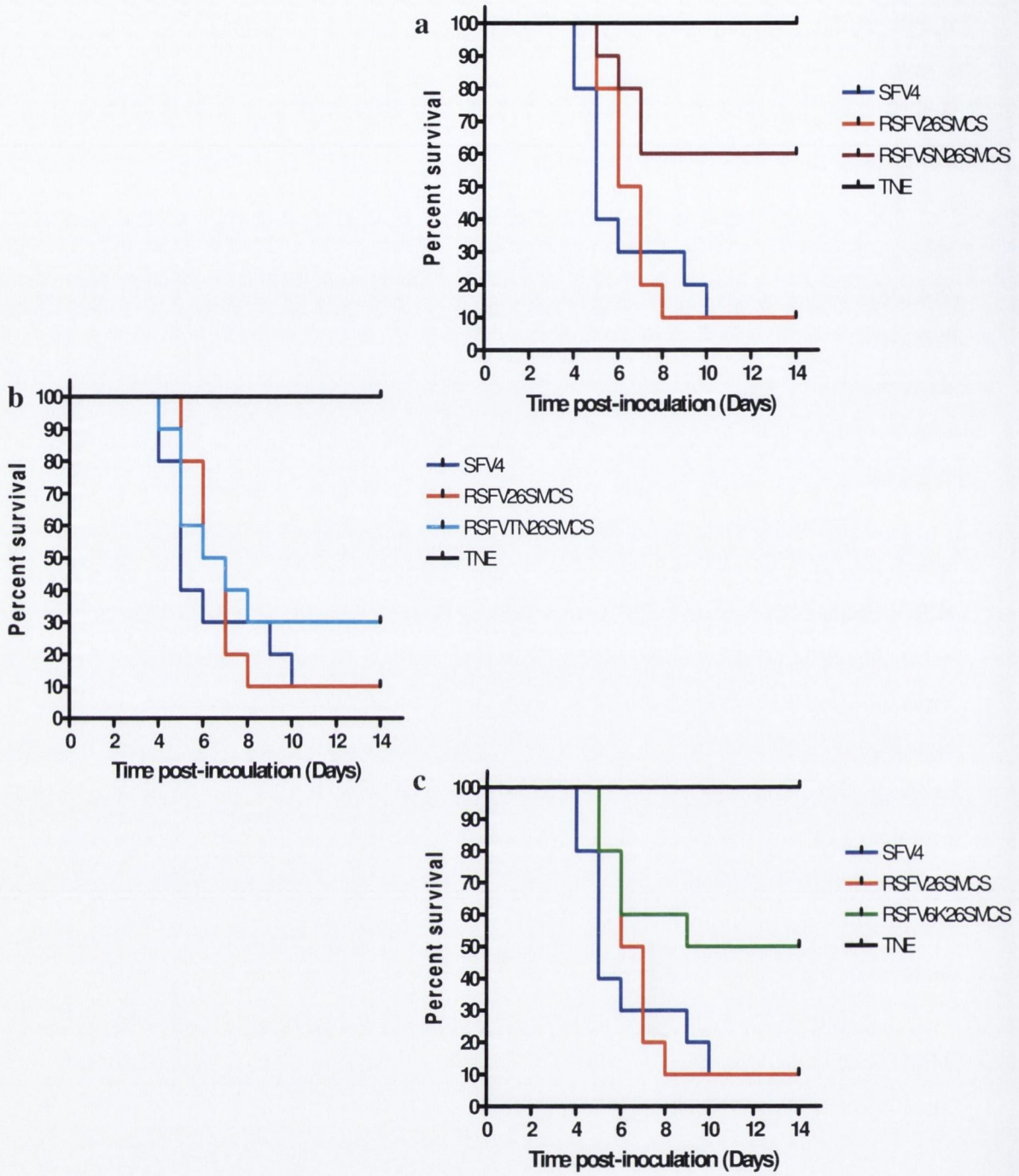


Figure 4.1 Virulence analyses of the replicating SFV vectors

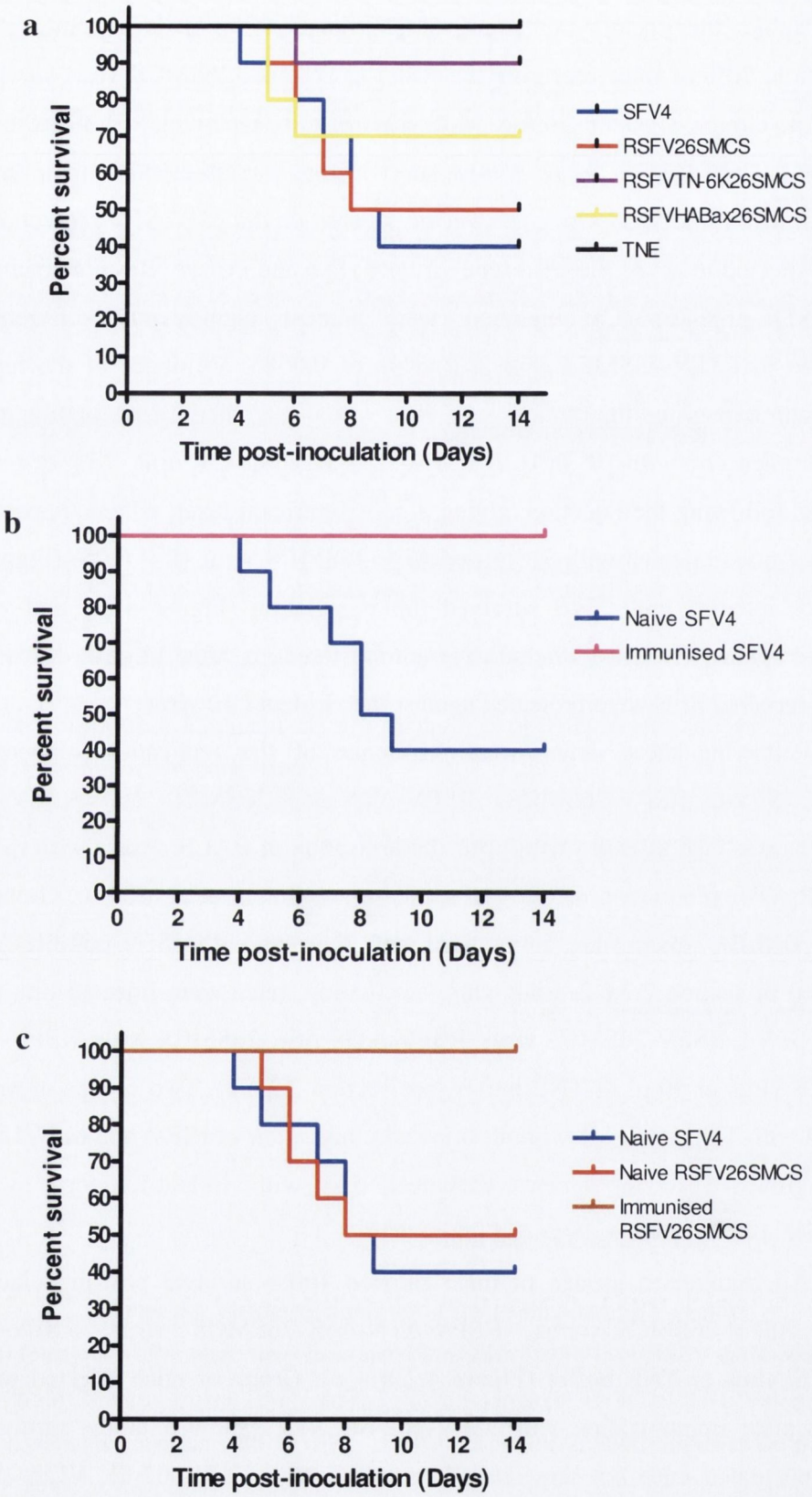
Groups of 10 female BALB/c mice were inoculated i.m. with 10^6 PFU of RSFV-ΔSN-26SMCS virus (a), RSFV-ΔTN-26SMCS virus (b) or RSFV-Δ6K-26SMCS virus (c) in 50 µl TNE buffer. Groups of 10 BALB/c mice were also injected with 10^6 PFU of SFV4 or RSFV-26SMCS virus as the positive controls, or 50 µl TNE alone as the negative control. All mice were checked daily for clinical signs following i.m. injection. Fourteen days post inoculation; all surviving mice were challenged i.p. with 10^6 PFU of virulent SFV L10 in 500 µl TNE. Fourteen days post challenge, all surviving mice were sacrificed.

Statistical analysis of the survival curves was performed by using the Logrank Test. A P-value smaller than 0.05 was considered to be statistically significant.

Unlike the vectors containing single deletions in their genome, after i.m. inoculation, 90% of mice receiving the RSFV-ΔTN-Δ6K-26SMCS virus survived and showed no clinical signs of disease, while 60% of mice receiving SFV4 died by 9 days p.i. (P = 0.0265) (Figure 4.2a). A 50% survival rate was observed in mice inoculated i.m. with RSFV-26SMCS virus; this vector is based on the pSP6-SFV4 infectious clone and is expected to act as the wild-type virus *in vitro* and *in vivo*. Results obtained from RSFV-ΔTN-Δ6K-26SMCS injection were almost significantly different when compared to RSFV-26SMCS with a P-value of 0.0583. Virulence of the replicating SFV vector expressing the foreign gene, *bax*, was also studied by inoculating groups of BALB/c mice i.m. with 10^6 PFU of RSFV-HABax-26SMCS virus. 70% of mice were protected following the injection, giving a non-significant result when compared to the groups of mice injected with SFV4 or RSFV-26SMCS virus (P > 0.05) (Figure 4.2a). All mice injected with TNE survived the inoculation (Figure 4.2a) and were not protected against virulent L10 challenge administered i.p. after 14 days. Mice that had already received SFV were protected against the virulent L10 virus.

Following these experiments, virulence of the replication-competent SFV vectors (SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS) was also assessed with prior immunisation of BALB/c mice with rSFV-p62-6K VLPs (For production of the VLPs, please see Smyth et al (2005)). Groups of 10 female BALB/c mice were immunised and boosted with rSFV-p62-6K VLPs as described in section 2.2.6.2. Following vaccination, mice were injected i.m. with 10^6 PFU of SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, RSFV-HABax-26SMCS virus or 50 µl of TNE buffer (see section 2.2.6.2). Two groups of mice were injected with TNE or SFV4 without prior administration of rSFV-p62-6K VLPs as the control groups. Surviving mice were challenged i.p. with virulent L10 virus to test their immunity, 14 days p.i., as described previously.

All immunised groups of mice showed 100% survival post-inoculation with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, RSFV-HABax-26SMCS virus or TNE buffer (Figure 4.2 b – e). Group of mice injected with TNE without prior immunization with rSFV-p62-6K VLPs showed 100% survival while SFV4-inoculated mice not previously immunised with rSFV-p62-6K VLPs showed a survival rate of 40%.


a
Percent survival
Time post-inoculation (Days)
SFV4
RSFV26SMCS
RSFVTN-6K26SMCS
RSFVHABax26SMCS
TNE
b
Percent survival
Time post-inoculation (Days)
Naive SFV4
Immunised SFV4
c
Percent survival
Time post-inoculation (Days)
Naive SFV4
Naive RSFV26SMCS
Immunised RSFV26SMCS

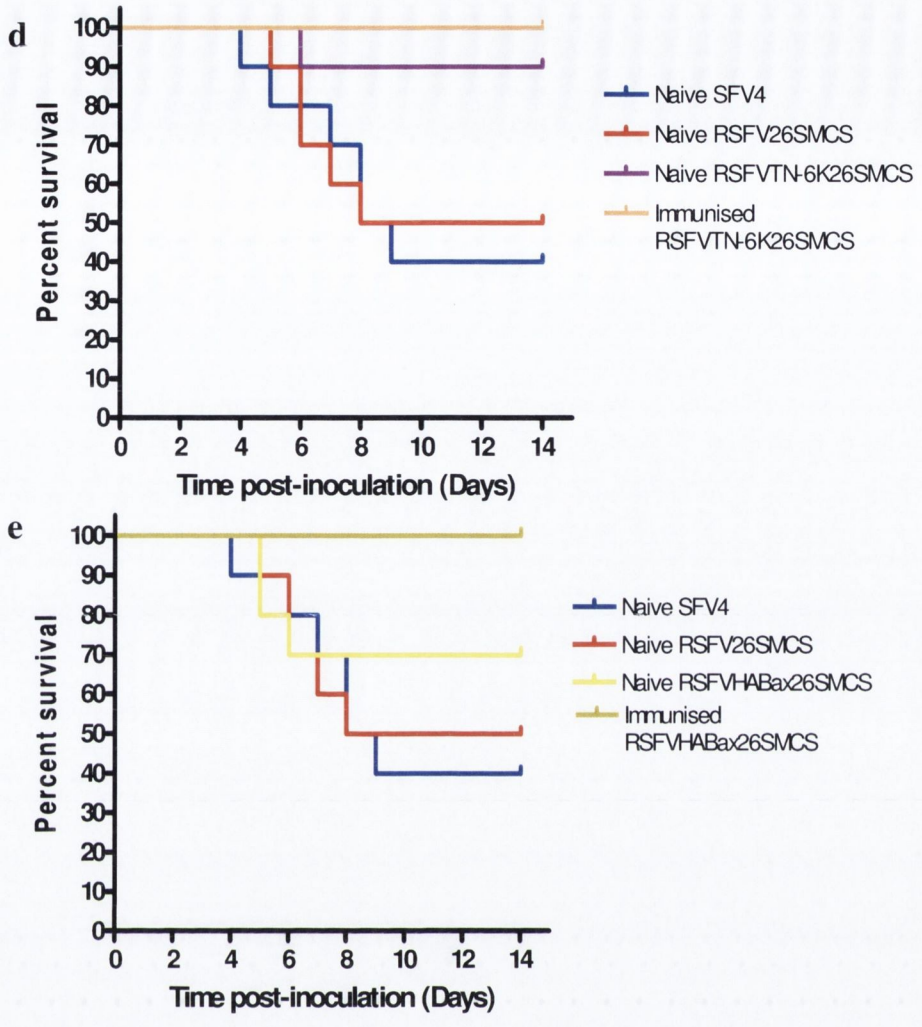


Figure 4.2 Virulence analyses of the replicating SFV vectors in naïve BALB/c mice or in BALB/c mice pre-immunized with rSFV-p62-6K VLPs

(a) Groups of 10 female BALB/c mice were inoculated i.m. with 10^6 PFU of SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus in 50 µl TNE buffer. A group of 10 BALB/c mice was also injected with 50 µl TNE alone as the negative control. All mice were checked daily for clinical signs following i.m. injection. Fourteen days post inoculation; all surviving mice were challenged i.p. with 10^6 PFU of virulent SFV L10 in 500 µl TNE buffer. Fourteen days post challenge, all surviving mice were sacrificed.

(b) – (e) Concurrently, mice to be immunised received a 50 µl i.m. injection of the rSFV-p62-6K VLPs in TNE at a concentration of 10^6 IU and were boosted two weeks later with the same injection. Two weeks post-immunisation, groups of 10 BALB/c mice were injected i.m. with 10^6 PFU of the virus stocks listed above.

Statistical analysis of the survival curves was performed by using the Logrank Test. $P < 0.05$ was considered to be statistically significant.

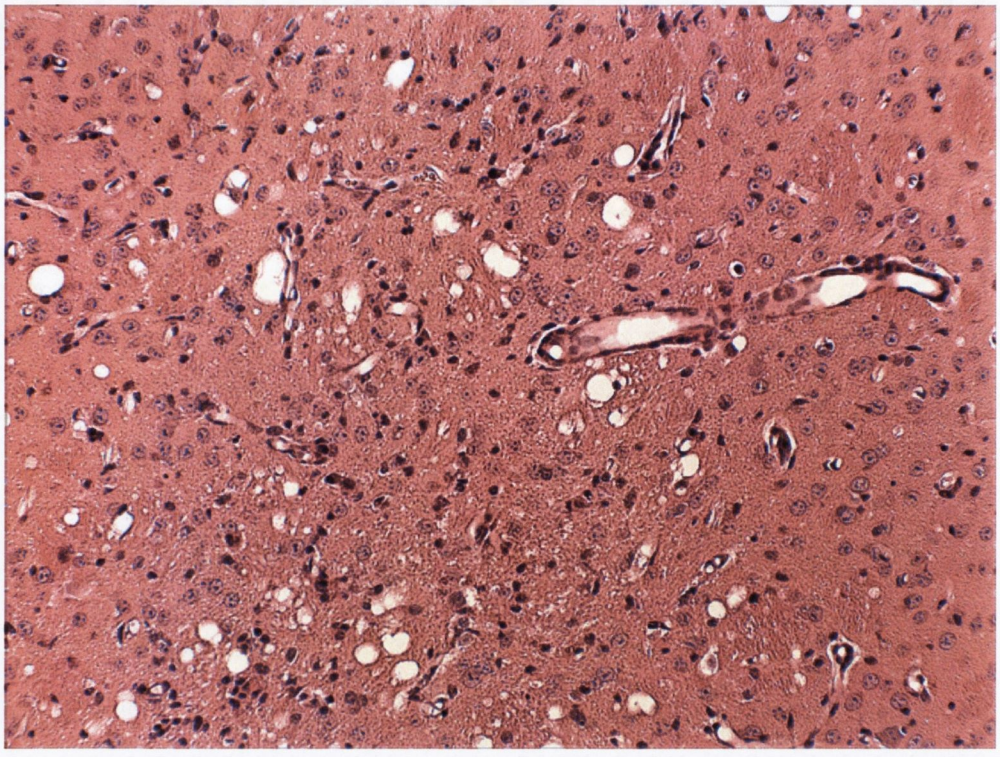
Using the virulent L10 virus, TNE buffer mock-immunised BALB/c mice died following challenge and mice pre-immunized with rSFV-p62-6K VLPs survived because of stimulated immunity (previously described by Smyth et al (2005)).

4.2.3 Pathological examination of brains from mice injected with replicating SFV vectors

Following *in vivo* analyses of various replication-competent SFV vectors, it was observed that the virulence of the vectors was not reduced and neither the vectors with single deletions in their genome (Figure 4.1) nor the replicating vector with a double deletion (Figure 4.2a) had resulted in 100% survival of mice. Therefore, it was decided to immunise groups of mice with rSFV-p62-6K VLPs prior to inoculation with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus (section 2.2.6.2). Two control groups of mice were injected with either TNE or SFV4 without prior immunisation with rSFV-p62-6K VLPs (Groups 1 and 3; section 2.2.7.1.1). Fourteen days post-inoculation, brains of three mice from each group were sampled for histological examination and detection of lesions characteristic of SFV infection as described in section 2.2.7.1.1.

Whereas only 4 of ten naïve mice survived inoculation with SFV4, immunised mice remained clinically normal following challenge. Histological examination of brains from the naïve SFV4 inoculated mice showed multiple foci of malacia, vacuolar degeneration and gliosis randomly distributed in the neutropil. Malacic areas were infiltrated with macrophages and were frequently calcified. The morphology of the brain lesions was characteristic of the changes seen previously in SFV4 inoculated mice surviving to 14 days after infection (Figures 4.3 a and b). Brains from mice immunised with rSFV-p62-6K VLPs and challenged with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus all appeared normal (Figure 4.4).

a



b

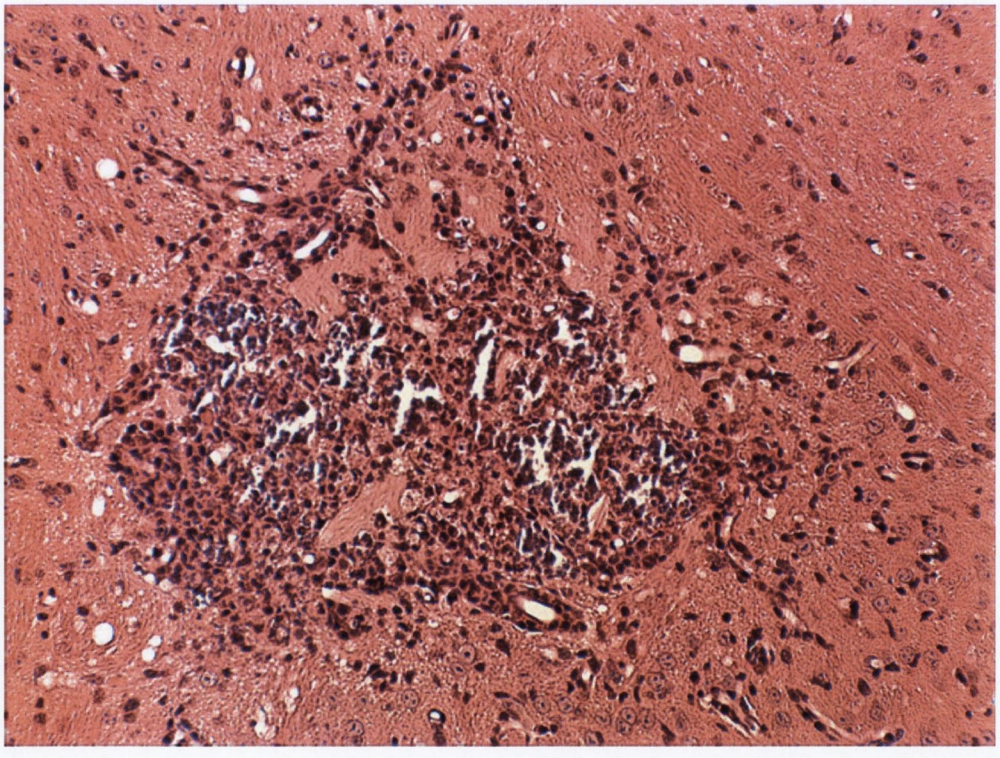


Figure 4.3 Sections of brain from a naïve BALB/c mouse 14 days following challenge with SFV4

(a) Area of vacuolar degeneration and gliosis in the thalamus. H&E, 200x.

(b) Focal area of malacia, macrophage accumulation and calcification (dark blue granules) in the mid-brain. H&E, 200x.

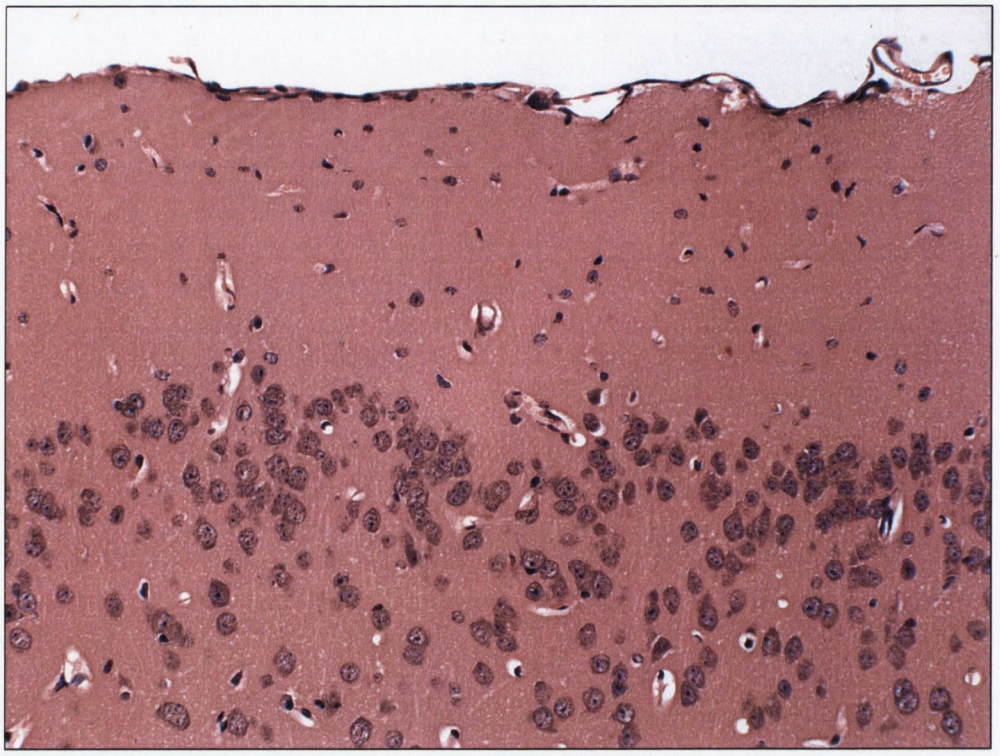


Figure 4.4 Brain from a BALB/c mouse immunised with rSFV-p62-6K VLPs prior to SFV4 injection

The normal appearance of neurons and neutrophil in the cerebral cortex. H&E, 200x

4.3 DISCUSSION

Identification of the virulence determinants of alphaviruses is essential to understanding the pathogenesis of alphavirus-induced neurologic disease. Previous neurovirulence studies with Sindbis-group viruses have identified the structural gene E2 and the 5' non-coding region as the virulence determinants (Lustig *et al.,* 1988; Tucker & Griffin, 1991; Dubuisson *et al.,* 1997). The non-structural protein nsP1, the nsP3 position 537 and an 18-aa deletion from nsP3 at position 386-403 have been shown to affect the neurovirulence of the SV strain AR86 (Heise *et al.,* 2000; Suthar *et al.,* 2005). The wild-type SFV, SFV4 and the avirulent strains, A7 and A7(74) were sequenced and multiple changes were identified throughout the genome (Tuittila *et al.,* 2000). The E2 gene, the 5' NTR, and the non-structural nsP3 gene appeared to be the major virulence determinants between these particular strains (Santagati *et al.,* 1995, 1998; Tarbatt *et al.,* 1997; Tuittila *et al.,* 2000). Mutations in the nuclear localisation signal (NLS) in the nsP2 gene were shown to reduce the cytopathogenicity of SFV *in vitro* and the neurovirulence *in vivo* (Fazakerley *et al.,* 2002; Lundstrom *et al.,* 2003; Tamm *et al.,* 2008). Sindbis viruses containing mutations in the nsP2 gene also exhibited reduced cytopathogenicity (Dryga *et al.,* 1997).

In this chapter we analysed the effect of two in-frame deletions in the nsP3 gene; ΔSN and ΔTN, and also the effect of 6K deletion on the virulence of SFV. It was previously shown by Tuittila & Hinkkanen (2003) that three amino acid mutations, at positions 435, 442 and 469, within the non-conserved domain of nsP3 cumulatively affect neurovirulence of SFV. Another study showed that SFV containing a non-phosphorylated nsP3 gene was less virulent after i.p. inoculation (Vihinen *et al.,* 2001). Previously it was also observed that wild type SFV containing SN and TN deletions in the hypervariable domain of nsP3 separately (SFV4-SN and SFV4-TN) showed reduced virulence in BALB/c mice and all mice survived following i.m. and i.p. inoculations with the deletion mutants (Galbraith *et al.,* 2006). In our study, intramuscular testing of RSFV-ΔSN-26SMCS or RSFV-ΔTN-26SMCS in female BALB/c mice resulted in 60% and 30% survival, respectively. This compared with a survival rate of 10% for mice inoculated with SFV4 and RSFV-26SMCS. Mice inoculated with RSFV-ΔSN-26SMCS virus were the only group that were significantly different from mice inoculated with SFV4 or RSFV-26SMCS ($P = 0.03$). All surviving mice resisted challenge with the virulent SFV L10. Our studies showed that the nsP3-SN and the nsP3-TN single

deletions failed to reduce the virulence of the replicating SFV vectors *in vivo*. These results conflicted with those of Galbraith et al (2006). Personal communication with Dr. Galbraith revealed that the dose of virus used in her experiments was lower than that stated in the paper, 10^4 PFU/injection instead of 10^6 PFU/injection, and that the 100-fold lower dose of virus used protected all mice from the neurovirulent properties of SFV. In adult mice, SFV4 is virulent by i.n. or intracerebral (i.c.) inoculation (Glasgow *et al.,* 1991; Fazakerley, 2002), however is avirulent when inoculated i.p., at least at 5 x 10^3 PFU and no infectious virus was detected in the brains of mice (Fragkoudis *et al.,* 2007). Our findings also correlated with the *in vitro* growth curve assays where the deletion mutants grew at least as well as the wild-type viruses in BHK-21 cells at all time points tested (*chapter 3*).

The RSFV-Δ6K-26SMCS vector was constructed by complete deletion of the 6K gene from the full-length replicating vector, RSFV-26SMCS. *In vivo* analysis of the vector in BALB/c mice by i.m. injection resulted in 50% survival which was not statistically significant when compared to the groups of mice injected with RSFV-26SMCS virus or SFV4, showing that the removal of the 6K gene did not attenuate the SFV vector.

To enhance the biosafety of the replicating vectors, the ΔTN and Δ6K were incorporated into the genome of RSFV-26SMCS, creating RSFV-ΔTN-Δ6K-26SMCS (*chapter 3*). A 90% survival was observed in BALB/c mice following i.m. injection with 10^6 PFU of RSFV-ΔTN-Δ6K-26SMCS virus, which was significantly different than SFV4-injected mice. It was concluded that the accommodation of two deletions in one genome probably produced pressure on the vector and reduced the virulence *in vivo*.

The pro-apoptotic *bax* gene was cloned into RSFV-26SMCS, creating RSFV-HABax-26SMCS and *in vivo* testing of this vector showed 70% survival in BALB/c mice. Statistical analysis showed that this level of survival was nearly significant when compared with SFV4 inoculated mice ($P = 0.0595$). Lewis et al (1999) showed that a recombinant SV vector expressing the *bax* gene (SV-HABax) inhibits neuronal apoptosis in newborn CD-1 mice by inducing anti-apoptotic activity and results in 80% survival of mice. However, in the same study, deletion of the N-terminus of the *bax* gene converted Bax into a pro-apoptotic factor *in vivo*. The presence of the N-terminal 33 aa residues does not affect the pro-apoptotic activity of the gene *in vitro*. It was also shown that the *bax* gene can have a pro-apoptotic or anti-apoptotic function that is

neuron type-specific demonstrating the importance of cellular factors in determining the function of Bax (Lewis *et al.,* 1999). The anti-apoptotic proteins Bcl-2 and Bcl-xL are converted into pro-apoptotic proteins when their N termini are removed with cleavage by caspases (Cheng *et al.,* 1997; Clem *et al.,* 1998). Structural data indicate that caspase-8 cleaves Bid, a Bcl-2-related protein, and induces a conformational change that may facilitate exposure of its BH3 domain, which may be involved in triggering death (Chou *et al.,* 1999; McDonnell *et al.,* 1999). This BH3 domain is conserved in Bax, Bcl-2 and Bcl-xL. Like Bcl-2 and Bcl-xL, there is a possibility that Bax could be cleaved during apoptosis triggered by alphavirus infection and chemotherapeutic agents (Thomas *et al.,* 1996; Grandgirard *et al.,* 1998). The cleavage site in Bax was mapped to a position between BH3 and BH4, a position similar to Bcl-2 (Grandgirard *et al.,* 1998; Wood *et al.,* 1998), and proteases rather than caspases could convert the pro-apoptotic protein Bax into an anti-apoptotic protein by proteolytic cleavage, relocalization or other mechanisms (Kirsch *et al.,* 1999).

To obtain 100% survival in BALB/c mice following injections with replicating SFV vectors, mice were immunised with rSFV-p62-6K VLPs prior to i.m. inoculation with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus. This strategy resulted in an immune reaction against SFV which fully protected mice from subsequent injections with replication-competent SFV vectors (as previously described by Smyth et al (2005)). Meanwhile, mock-immunised BALB/c mice which were utilised as the control groups, showed only 40% and 50% survival following injections with SFV4 and RSFV-26SMCS virus, respectively. It is known that, after inoculation, SFV replicates well in the periphery and causes a high titre viraemia, which enables virus passage across the BBB and the induction of brain lesions (Pathak & Webb, 1974; Soilu-Hänninen *et al.,* 1994; Fazakerley, 2002, 2004). Therefore, to investigate the possibility of neuropathological changes characteristic of SFV infection, brains of three mice from each group were sampled for histological examination, 14 days post-inoculation. In naïve, SFV4-inoculated mice, brain lesions characteristic of the changes seen previously in SFV4 injected mice surviving to 14 days p.i. were observed. Brains from mice immunised with rSFV-p62-6K VLPs and challenged with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus all appeared normal, confirming that the rSFV-p62-6K VLPs had induced anti-SFV immunity.

Chapter 5

TREATMENT OF CT26 AND K-BALB TUMOURS *IN VIVO* USING REPLICATING SFV VECTORS

5.1 INTRODUCTION

The use of recombinant viral particles as cancer therapy agents have produced promising results; however these systems have some limitations like induction of transient gene expression, poor transduction of cells and therefore inadequate tumour cell killing. Therefore the therapeutic potential of replication-proficient viruses has gained significance. Replicating viruses allow amplification of the input dose at the tumour site with an increase in the expression of the heterologous protein and cell-killing. An oncolytic virus preferentially replicates and lyses cancer cells. Several oncolytic viruses have been described, some displaying a natural tropism for tumour tissues and some genetically engineered with increased oncolytic capacities (Everts & van der Poel, 2005; Vähä-Koskela *et al.,* 2007).

SFV vectors have already been used as oncolytic agents and as anti-tumour vaccines (Lundstrom, 2000). In a previous study, H358a tumours, which are human NSCLC cells deleted in *p53*, in BALB/c *nu/nu* mice were treated with the inherent p53-independent apoptosis-inducing ability of rSFV VLPs and complete tumour regression was observed in some cases (Murphy *et al.,* 2000). The growth of the rat prostate cancer AT3-Bcl-2 cells was also shown to be significantly inhibited using the SFV replicons expressing the pro-apoptotic *bax* gene. However, the treatment was less effective than that observed in the slower growing human NSCLC cell line, H358a xenografts (Murphy *et al.,* 2001). The anti-tumour effect of the replication-proficient SFV4 and the rSFV-p62-6K VLPs was tested in the rapidly growing CT26 and K-BALB murine tumour models, and the results showed that prior immunisation of mice with rSFV-p62-6K VLPs in combination with apoptosis induction by pSP6-SFV4 and SFV replicons significantly inhibited the growth of the tumours in immunocompetent BALB/c mice (Smyth *et al.,* 2005). It is not yet known if SFV replicates preferentially in tumour tissue but recent studies with SV vectors have shown that the virus has a tropism for a variety of tumour models *in vivo*, as tumour cells express excess, unoccupied 67 KDa laminin receptors on their surface which attracts SV (Tseng *et al.,* 2002, 2004). Recently, the antitumor capacity of replication-competent SFV vector, VA7-EGFP (Vähä-Koskela *et al.,* 2003) was investigated in an immunodeficient SCID mouse model, and shown that vector induces regression of subcutaneous human melanomas, regardless of the injection route (Vähä-Koskela *et al.,* 2006).

The precedence of activating mutations in *ras* genes, which are involved in approximately 30% of human malignancies, has stimulated anti-cancer research targeted either at Ras directly, or at the various signaling pathways in which it plays a major role (Adjei, 2001). Ras proteins are known to be involved in cellular proliferation, differentiation, survival, and apoptosis which can also differ depending on the cell type (Downward, 1998; Shields *et al.,* 2000). The susceptibility of the murine tumourigenic cell lines, K-BALB (which overexpresses the K-*ras* oncogene) and CT26 to SFV-induced apoptosis was examined *in vitro* earlier in chapter 3. Virulence of the replicating SFV vectors was also tested in BALB/c mice prior to tumour treatment experiments (*chapter 4*).

In this chapter, the capability of replication-proficient SFV vectors (SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, and RSFV-HABax-26SMCS) to inhibit the growth of s.c. CT26 and K-BALB tumours was tested in immunocompetent BALB/c mice. Groups of mice were vaccinated prior to tumour induction with rSFV-p62-6K VLPs in an attempt to enhance any anti-SFV immune response generated and as a safety precaution to reduce mortality. Significant inhibition of CT26 tumour growth was observed following i.t. injections with replicating SFV vectors when compared to TNE treated groups, while treatment of K-BALB tumours with replication-competent SFV vectors was transient. Complete CT26 tumour regression was also observed in some mice whereas none of the replicating SFV vectors was capable of eradicating the s.c. K-BALB tumours completely. This result correlated with the *in vitro* experiments where infection of the CT26 cell line with replicating SFV vectors was more efficient and more sustained compared with the K-BALB cell line.

In this study, the tumours were examined routinely using H&E staining of formalin fixed, paraffin-embedded sections. Histological studies showed that areas of necrosis were more prominent in K-BALB than in CT26 tumours and that tumours infected with replicating SFV vectors appeared similar to controls. No evidence of a significant cellular immune response against the tumour cells was detected.

5.2 RESULTS

5.2.1 Intratumoural treatment of CT26 and K-BALB tumours in immunocompetent BALB/c mice using replicating SFV vectors

Groups of 15 female BALB/c mice were immunised with rSFV-p62-6K VLPs as described in section 2.2.6.2; 28-30 days post boost, using the murine colon carcinoma cell line, CT26, or the Kirsten sarcoma virus-transformed cell line, K-BALB, tumours were induced by s.c. injection as described in section 2.2.6.3.1. Treatment commenced once tumours had reached an average diameter of 4 mm whereby they received a total of 6 i.t. injections of TNE medium containing concentrated SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus (10^{10} PFU/ml, for production of concentrated virus please refer to section 2.2.5), and TNE buffer alone as the negative control, on every second day over an 11 day period as described in section 2.2.6.3.2. The size of the tumour was measured in two dimensions and the average tumour volume calculated as follows: ((length x width^2)/2). Measurements were taken daily using a digital calliper before injection and mice were euthanized when average tumour diameter reached 15 mm (1687.5 mm^3 volume).

5.2.1.1 Treatment of CT26 tumours

All mice developed tumours with an average diameter of 4 mm by 7-10 days post induction.

Treatment with the RSFV-ΔTN-Δ6K-26SMCS vector resulted in significant inhibition of CT26 tumours when compared to control tumours with an average tumour volume at 38.4% of control tumours at day 15 of treatment ($P < 0.01$). An average tumour volume of 48.2% of control tumours was observed in mice treated with RSFV-HABax-26SMCS virus at day 15 following initiation of treatment ($P < 0.05$). In SFV4 and RSFV-26SMCS treated groups, average tumour volumes of 43% and 37.3% of controls were observed, respectively, at day 15 of treatment, which was statistically significant ($P < 0.01$, $P < 0.01$) (Table 5.1).

Table 5.1 Overall survival and tumour size at day 15 after commencement of treatment

Tumour model	*Treatment groups*[a]	*Median survival (days)*[b] *(no. of survivors)*[c]	*Average tumour volume (mm^3)*[d]	*% of control*[e]	*Probability*[f]
CT-26	TNE	19 (0)	822.1 (± 113.2)	—	—
	SFV4	28 (4)	353.1 (± 95.15)	43	< 0.01
	RSFV26SMCS	27 (2)	307.2 (± 59)	37.3	< 0.01
	RSFVΔTNΔ6k26SMCS	36 (4)	315.8 (± 98.36)	38.4	< 0.01
	RSFVHA-Bax26SMCS	23 (3)	396.7 (± 71.98)	48.2	< 0.05
KBALB	TNE	14 (0)	1554 (± 53.79)	—	—
	SFV4	16 (0)	1220 (± 146.7)	78.5	> 0.05
	RSFV26SMCS	18 (0)	1171 (± 130.5)	75.3	> 0.05
	RSFVΔTNΔ6k26SMCS	23 (0)	746.7 (± 149.9)	48	< 0.001
	RSFVHA-Bax26SMCS	19 (0)	1015 (± 131.9)	63.3	< 0.01

[a]Fifteen BALB/c mice were used per treatment group.

[b]Mice were culled when the CT26 and K-BALB tumours reached 15 mm in diameter (1687.5 mm^3 in volume. Tumour volume was measured in two dimensions and calculated as follows: (length x $width^2$)/2). Overall survival is based on the time taken to reach this size. The median survival time of mice from treatment groups was determined by Kaplan-Meier survival curves.

[c]Numbers in parantheses indicate animals whose tumours regressed.

[d]Average tumour volume for each group was calculated at day 15 after commencement of treatment.

[e]The percent of control was calculated by dividing the average tumour volume of each treatment group by the average tumour volume of TNE-treated group.

[f]The probability value is based on the average tumour volume at day 15 of treatment compared with that of the TNE treated (control) groups.

Upon statistical analysis using a one-way ANOVA with Tukey's multiple comparison post test over the total duration of the experiment, inhibition of CT26 tumour growth following treatment with replication-competent SFV vectors was found to be significant in comparison to control tumours and no significant difference was noted between the treatment groups. CT26 tumours treated with RSFV-ΔTN-Δ6K-26SMCS virus showed the most significant inhibition compared to controls with a P value less than 0.001. SFV4 and RSFV-26SMCS virus also significantly inhibited the

growth of CT26 tumours when compared to TNE-treated group ($P < 0.01$, $P < 0.01$). At the experiment end inhibition of CT26 tumours following RSFV-HABax-26SMCS virus treatment was not significantly different compared with the TNE (control) group ($P > 0.05$) (Figure 5.1a).

5.2.1.2 Treatment of K-BALB tumours

Approximately one third of mice developed tumours with an average diameter of 4 mm 6 days post induction and by 12 days post induction all mice showed tumours of this size.

On day 15, RSFV-ΔTN-Δ6K-26SMCS treated K-BALB tumours had an average tumour volume of 48% of the control tumours which was statistically significant ($P < 0.001$) (Table 5.1). Over the total duration of the experiment this difference was not statistically significant following comparison with control tumours using a one-way ANOVA with Tukey's multiple comparison post test ($P > 0.05$) (Figure 5.1b). RSFV-HABax-26SMCS virus significantly inhibited the growth of K-BALB tumours at day 15 of treatment resulting in an average tumour volume of 63.3% of the controls ($P < 0.01$) (Table 5.1). However this inhibition was not consistent and significant when compared to TNE treated tumours over the total duration of the experiment ($P > 0.05$) (Figure 5.1b). Statistical analysis showed that inhibition of K-BALB tumour growth with SFV4 and RSFV-26SMCS virus was insignificant when compared to the control tumours over the total duration of the experiment ($P > 0.05$) (Figure 5.1b). There was no statistical difference between the treatment groups on the growth of K-BALB tumours (Figure 5.1b).

5.2.2 Effect of SFV treatment on survival

Survival was defined as the time taken for the CT26 and K-BALB tumours to reach 15 mm in diameter and subsequent euthanasia.

For CT26 tumours, control (TNE treated) tumours grew rapidly and reached an average diameter of 15 mm between days 12 and 26, when mice were euthanized accordingly. Mice in SFV4 treated groups survived to between days 18 and 28 with two mice surviving to days 31 and 36. RSFV-26SMCS treated mice were euthanized between days 17 and 29 and two out of 15 mice survived to days 33 and 37.

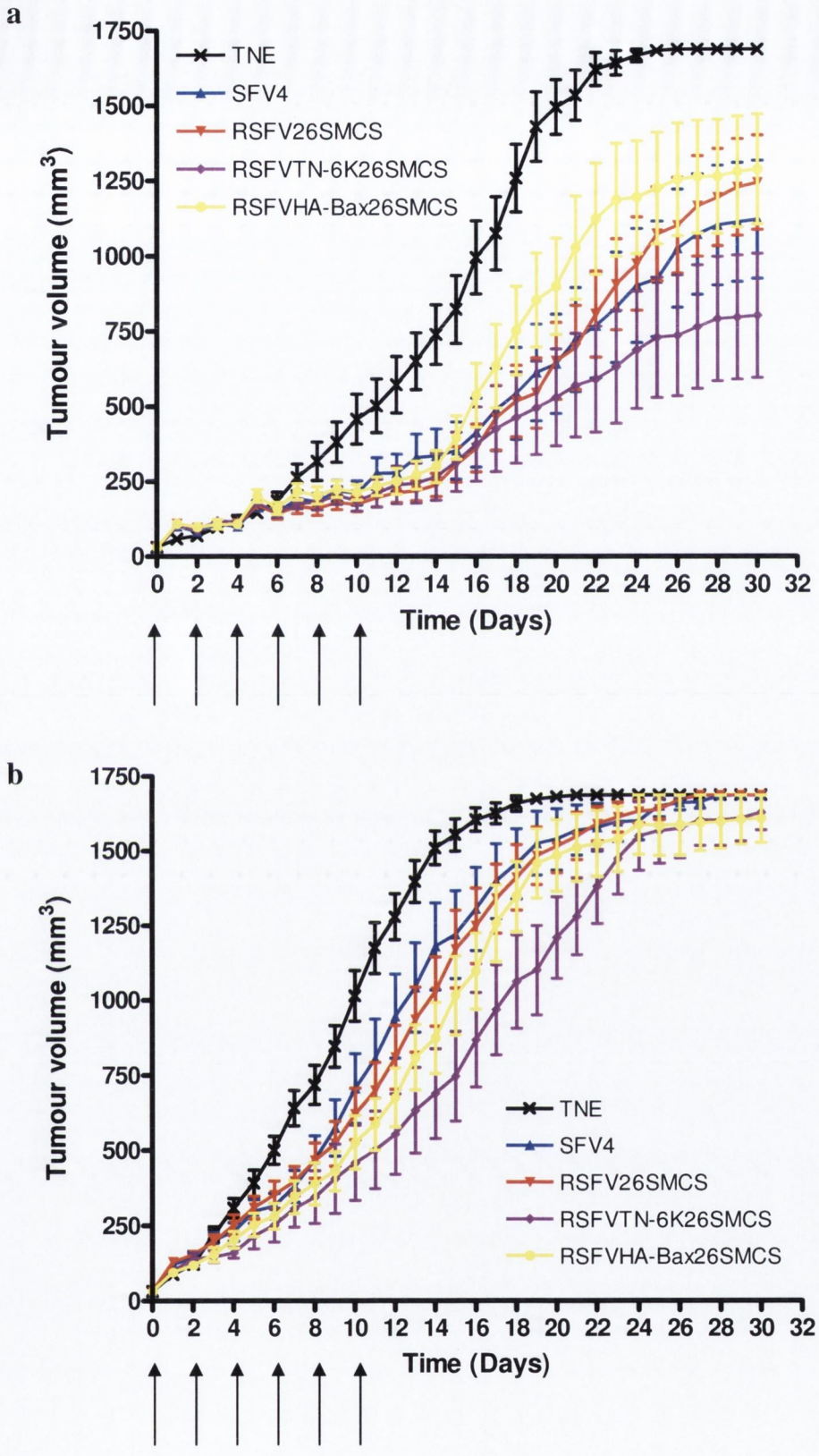
Figure 5.1 Treatment of CT26 and K-BALB tumours in immunocompetent BALB/c mice using replicating SFV vectors

CT26 (a) or K-BALB (b) tumours were induced in rSFV-p62-6K VLP immunised female BALB/c mice by s.c. injection of 100 µl of CT26 or K-BALB cell suspension in non-supplemented DMEM at a concentration of 10^7 cells/ml. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and a group of fifteen mice was used per treatment group. Animals received six i.t. injections of TNE alone or TNE containing SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus at a concentration of 5 x 10^8 PFU/dose which were administered every other day over an eleven day period. Size of the tumour was measured in two dimensions and the average tumour volume calculated as follows: (length x width^2)/2). Mice were euthanized when average tumour diameter reached 15 mm (1687.5 mm^3 in volume).

(a) Inhibition of CT26 tumour growth in SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, or RSFV-HABax-26SMCS treated mice was compared to TNE treated group (controls). The tumour growth inhibition was found to be statistically significant in SFV4, RSFV-26SMCS and RSFV-ΔTN-Δ6K-26SMCS treated groups ($P < 0.01$, $P < 0.01$, $P < 0.001$) when analysed using Tukey's multiple comparison post test by one-way ANOVA.

(b) Inhibition of K-BALB tumour growth in SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS, or RSFV-HABax-26SMCS treated mice was compared to TNE treated tumours. None of the treatment groups resulted in significant inhibition of K-BALB tumour growth ($P > 0.05$) when analysed using Tukey's multiple comparison post test by one-way ANOVA.

Points; mean of fifteen replicates, *bars*; +/- SEM, *arrows*; days of treatment. Results are representative of a single experiment.


a
1750
1500
1250
1000
750
500
250
0
Tumour volume (mm3)
TNE
SFV4
RSFV26SMCS
RSFVTN-6K26SMCS
RSFVHA-Bax26SMCS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (Days)
b
1750
1500
1250
1000
750
500
250
0
Tumour volume (mm3)
TNE
SFV4
RSFV26SMCS
RSFVTN-6K26SMCS
RSFVHA-Bax26SMCS
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32
Time (Days)

RSFV-ΔTN-Δ6K-26SMCS treated group survived the longest with a median survival time of 36 days; most mice were euthanized between days 23 and 36 and three mice survived to days 53, 58 and 60. Mice treated with RSFV-HABax-26SMCS virus were euthanized between days 17 and 36.

The effect of treatments on survival of CT26 tumour bearing mice was significant, with several mice showing complete tumour regression following treatments with replicating SFV vectors. Following treatment with SFV4, four tumours regressed completely (26.6%) between days 10 and 19 of treatment, while two tumours showed complete regression following treatment with RSFV-26SMCS virus (13.3%) at days 10 and 12 following initiation of treatment. Complete tumour regression was also observed in four (26.6%) out of 15 mice following injection with RSFV-ΔTN-Δ6K-26SMCS virus (between 12 to 17 days of treatment). In RSFV-HABax-26SMCS virus treated group, three out of 15 mice showed complete tumour regression (20%) initiating at 12, 17, and 23 days of treatment (Figure 5.2a). The CT26 tumour regressed mice were monitored for 4 months to ensure no tumour re-growth, indicating complete regression (Figure 5.3). The median survival time increased in all groups of mice treated with replicating SFV vectors compared to the control group (TNE) (Figure 5.2a). The percent survival for each treatment group was significantly different when compared to the TNE treated group ($P < 0.0001$), however there was no statistically significant difference between the treatment groups ($P > 0.05$) (Figure 5.2a).

For K-BALB tumours, control mice were euthanized between days 10 and 22. The control (TNE) group and the groups used for the K-BALB tumour treatment experiments had almost similar tumour growth patterns with no survival proportions at the end of the treatment study (Figure 5.4). The RSFV-ΔTN-Δ6K-26SMCS treated group survived the longest with a median survival time of 23 days; most mice were euthanized between days 16 and 27 of treatment and one mouse survived to day 36 (Figure 5.2b).

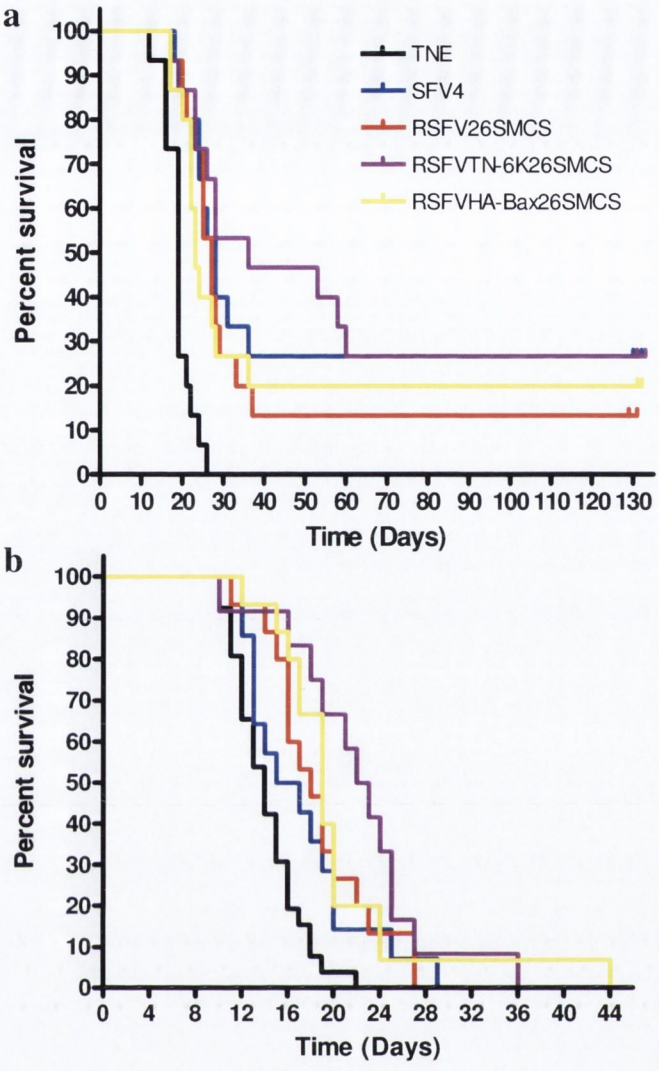


Figure 5.2 Survival proportions of the mice bearing CT26 or K-BALB tumours following treatment with replicating SFV vectors

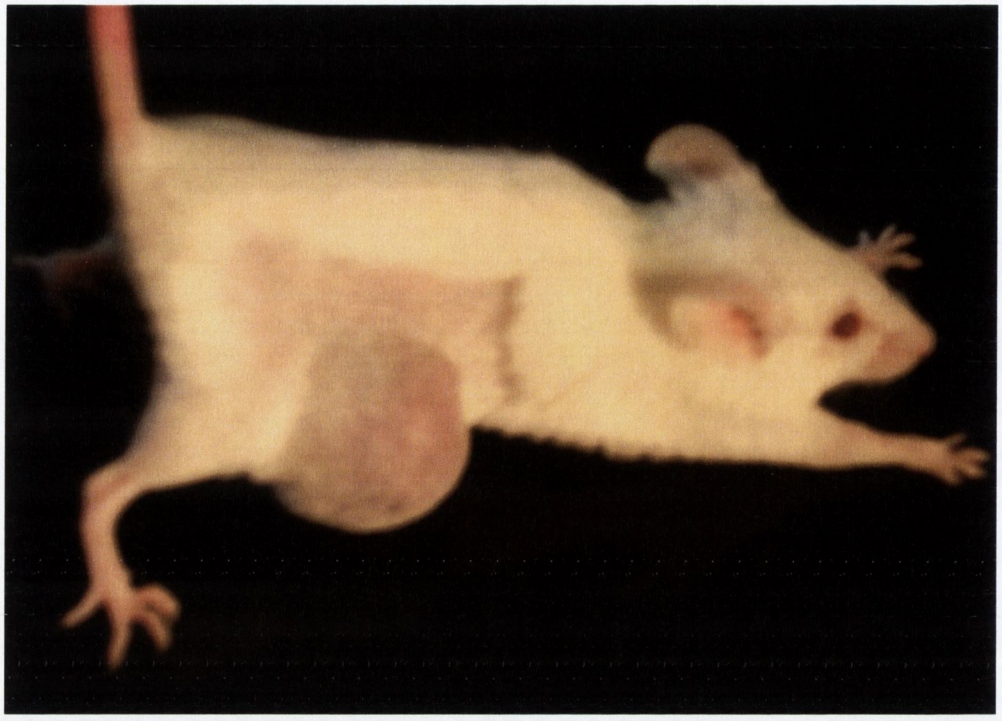
Mice were sacrificed when the tumour size reached 15 mm in diameter. The average time taken to reach this size for a group of mice is the survival time. The total number of mice, which had complete and permanent tumour regression, is considered as number of survivors.

(a) The survival proportions of the mice bearing CT26 tumour was 13.3% when treated with a total of six i.t. injections of SFV4 at a titre of 5 x 10^8 PFU/dose. The survival percentage increased to 20% when tumours were treated with RSFV-HABax-26SMCS virus and complete regression of the CT26 tumour was observed in 26.6% of the mice treated with SFV4 and RSFV-ΔTN-Δ6K-26SMCS virus. The tumour regressions were complete and permanent. TNE treated groups had no survival proportions at the end of the treatment experiment.

(b) Control (TNE) or the treatment groups (SFV4, RSFV-26SMCS, RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS) used for the K-BALB tumour treatment experiments had almost similar tumour growth pattern with 0% survival proportions at the end of the treatment study.

Statistical analysis of survival curves was performed by using the Logrank Test and $P < 0.05$ was considered to be significant.

a



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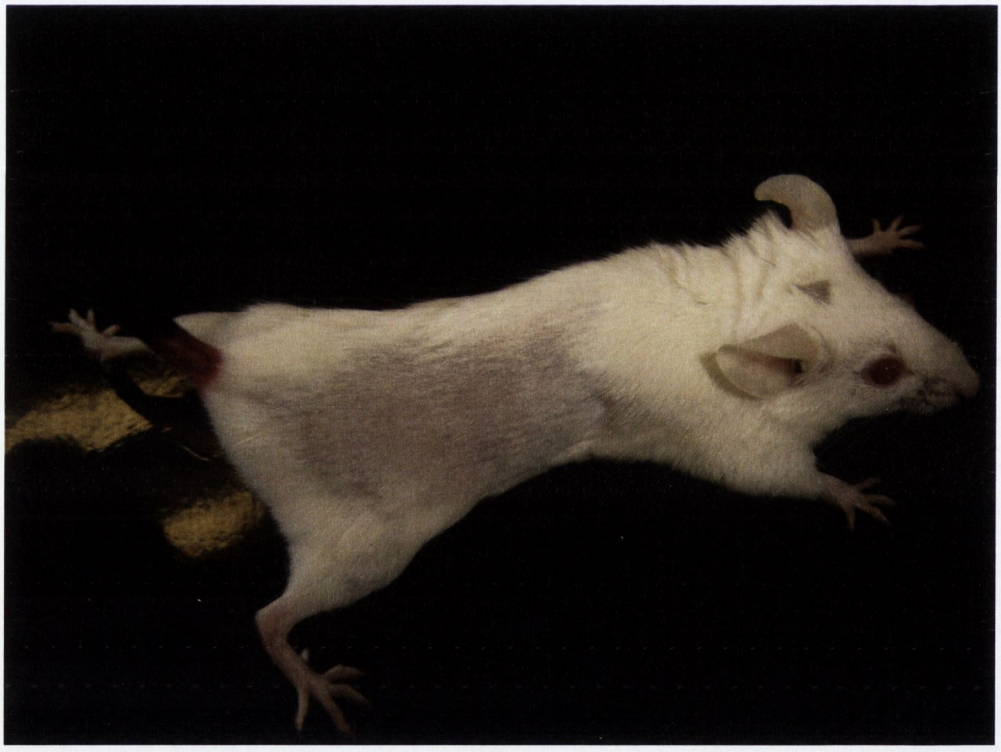


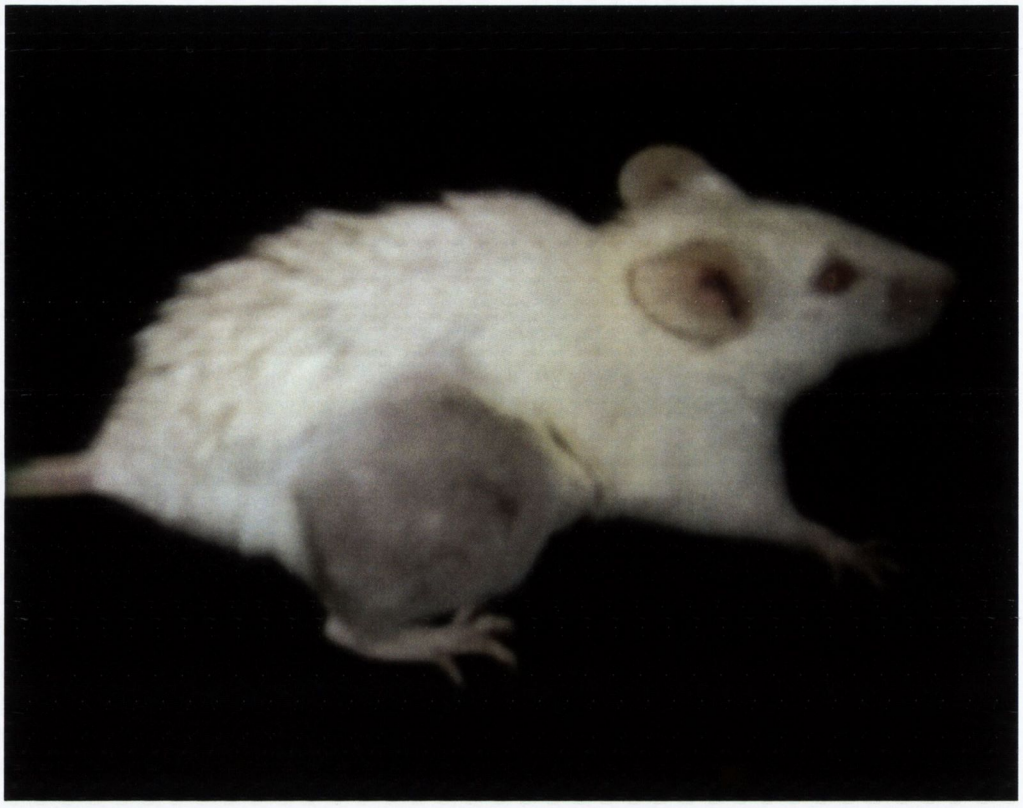
Figure 5.3 CT26 tumour treatments in immunocompetent BALB/c mice

(a) TNE treated CT26 tumour (15 mm diameter) after six i.t. injections.

(b) RSFV-HABax-26SMCS virus treated CT26 tumour. No tumour visible, 30 days post tumour induction.

No tumour re-growth was observed 100 days post-induction indicating permanent tumour regressions (figure not shown).

a



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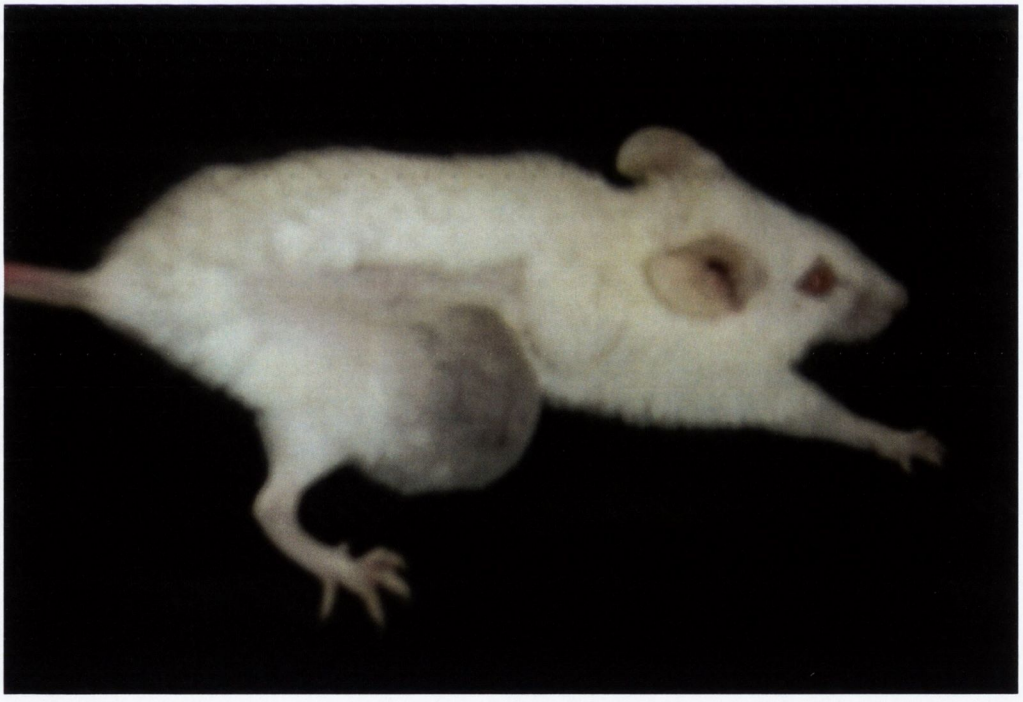


Figure 5.4 BALB/c mice bearing K-BALB tumours, 19 days post treatment

(a) K-BALB tumour treated with TNE (15 mm diameter) after six i.t. injections.

(b) K-BALB tumour treated with RSFV-26SMCS virus. The tumour is of similar size to the TNE-treated tumours.

5.2.3 Pathology of formalin fixed tumour tissue

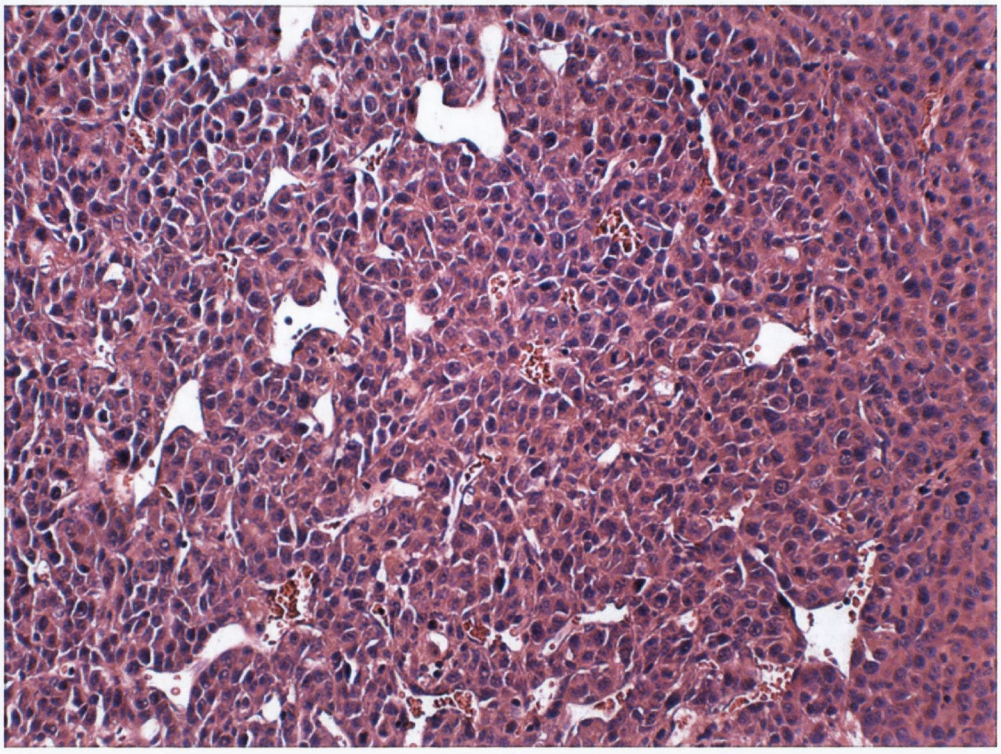
For pathological examination of the CT26 and K-BALB tumours, groups of three BALB/c mice were immunised with rSFV-p62-6K VLPs as described in section 2.2.6.2. Tumours were induced by s.c. injection of CT26 or K-BALB cell lines as described in section 2.2.6.3.1. Treatment commenced once tumours had reached an average diameter of 4 mm when groups of mice received a total of 6 i.t. injections containing SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, RSFV-HABax-26SMCS virus or TNE buffer alone as the negative control. On the day following the 6th treatment (11 days post-treatment) the mice were euthanized by isofluorane overdose and cervical dislocation. Tumours were excised, placed in 5% formal saline and processed for histological examination. The formalin fixed paraffin embedded tumour sections were stained using H & E and examined histologically.

5.2.3.1 CT26 tumours

CT-26 tumours treated with SFV4 were compact, densely cellular and poorly encapsulated. Polyhedral tumour cells were arranged in cords and irregular lobular patterns around thin-walled blood vessels (Figure 5.5a). Bands of panniculus muscle fibers were infiltrated by tumour cells containing frequent and sometimes bizarre mitotic forms (Figure 5.5b). Necrosis was confined to isolated focal areas (Figure 5.5c) and was never massive. Low-grade infiltrates of lymphocytes were confined to the interstitial stroma at the periphery of the tumours and tumour nodules (Figure 5.5d). Similar changes were seen in CT26 tumours treated with TNE, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus.

No evidence of tumour cell proliferation was detected at sites of total tumour regression following treatment. Histological changes were confined to areas of scarring containing perivascular and perineural accumulations of plasma cells (Figure 5.6a). Some areas of scarring contained haemosiderin deposits, indicating previous local haemorrhage at the sites (Figure 5.6b).

a



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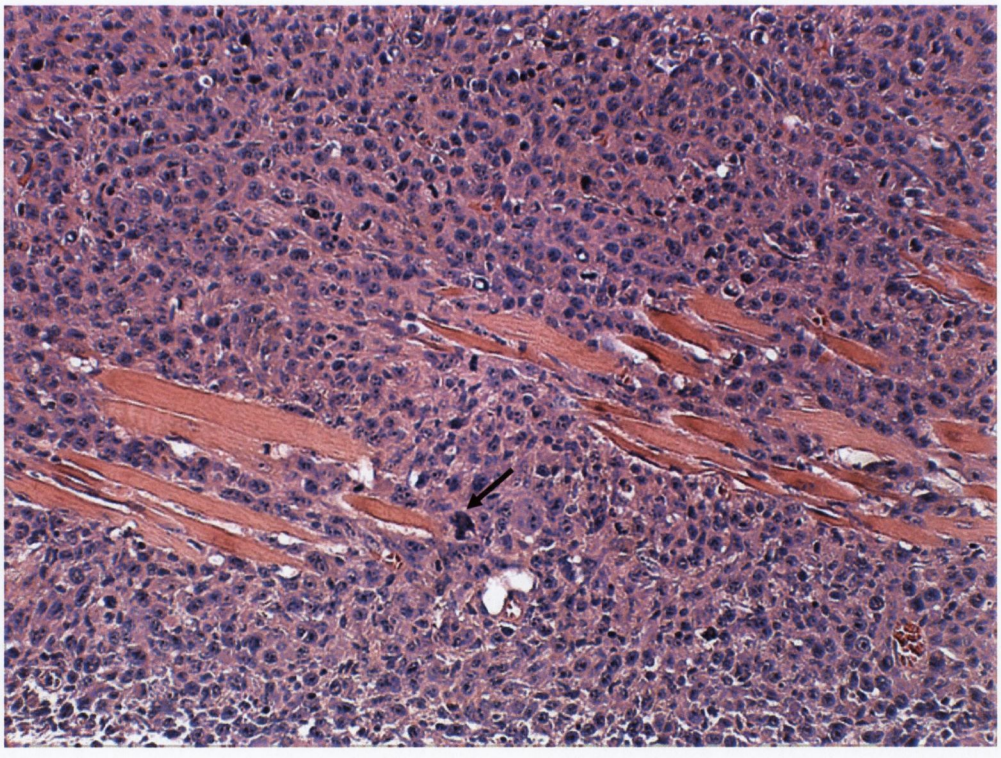
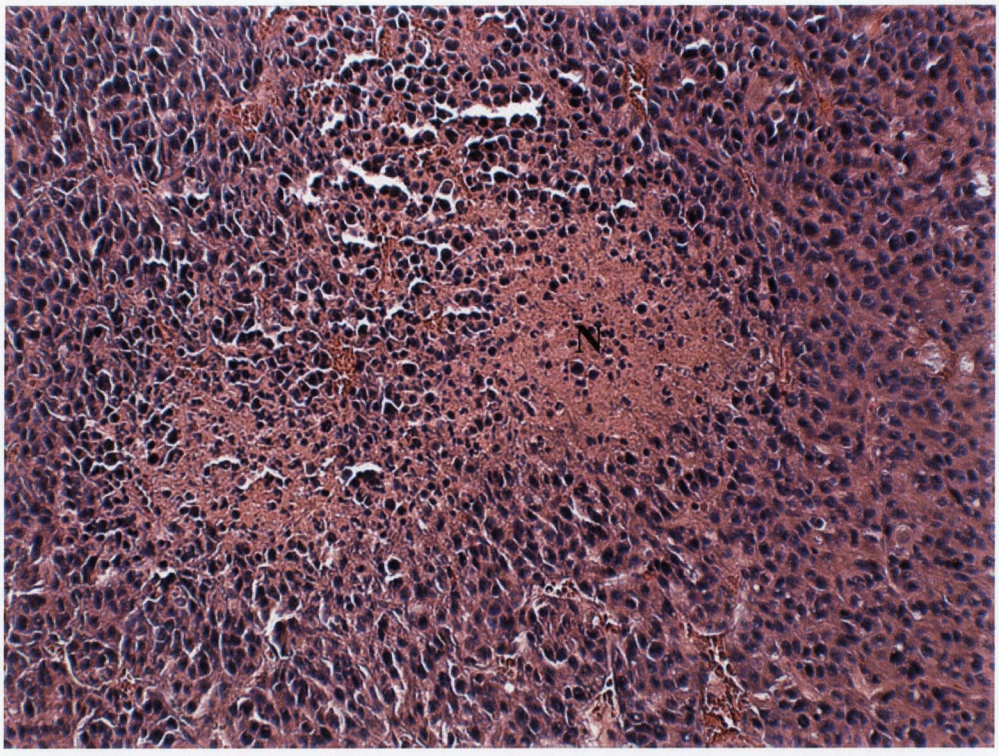


Figure 5.5 CT26 tumours, 11 days post-treatment with SFV4

(a) Cords of polyhedral tumour cells closely associated with thin-walled blood vessels. H&E, 200x.

(b) Tumour cell infiltration between panniculus muscle fibers. Mitotic figures are common and sometimes bizarre (arrow). H&E, 400x.

c



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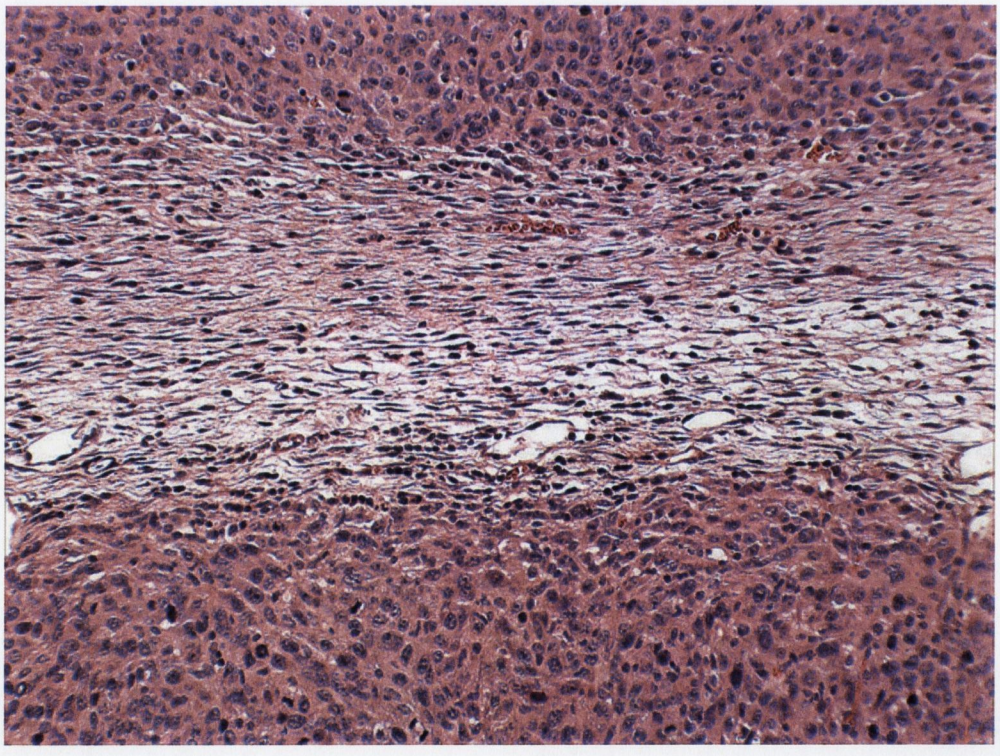
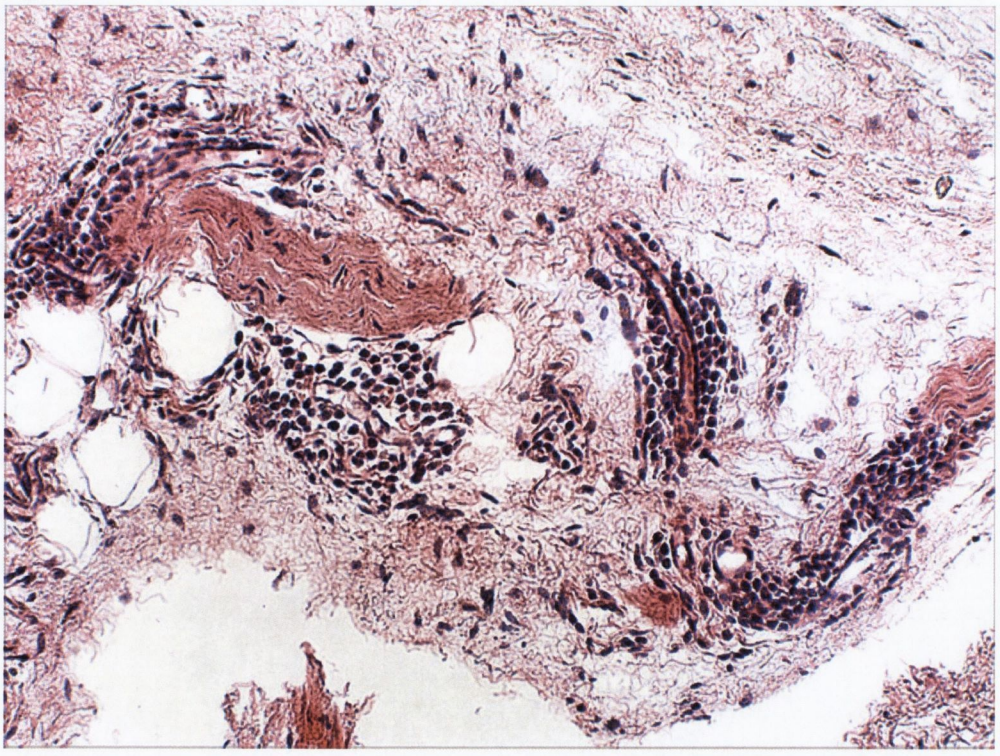


Figure 5.5 CT26 tumours, 11 days post-treatment with SFV4

(c) Focal area of necrosis (N) in a densely cellular tumour. H&E, 200x.

(d) Low-grade infiltrate of lymphocytes in the peritumoural stroma. H&E, 200x.

a



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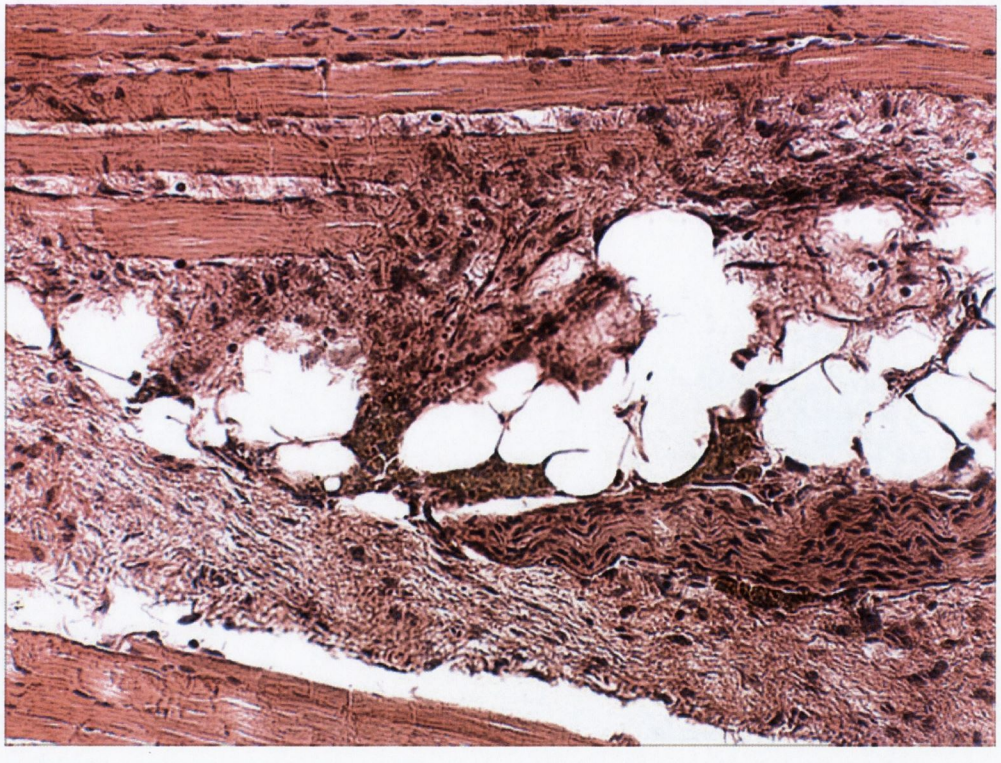


Figure 5.6 Site of regression of a CT26 tumour treated with SFV4

(a) Scar tissue containing perivascular and perineural accumulations of plasma cells. H&E, 200x.

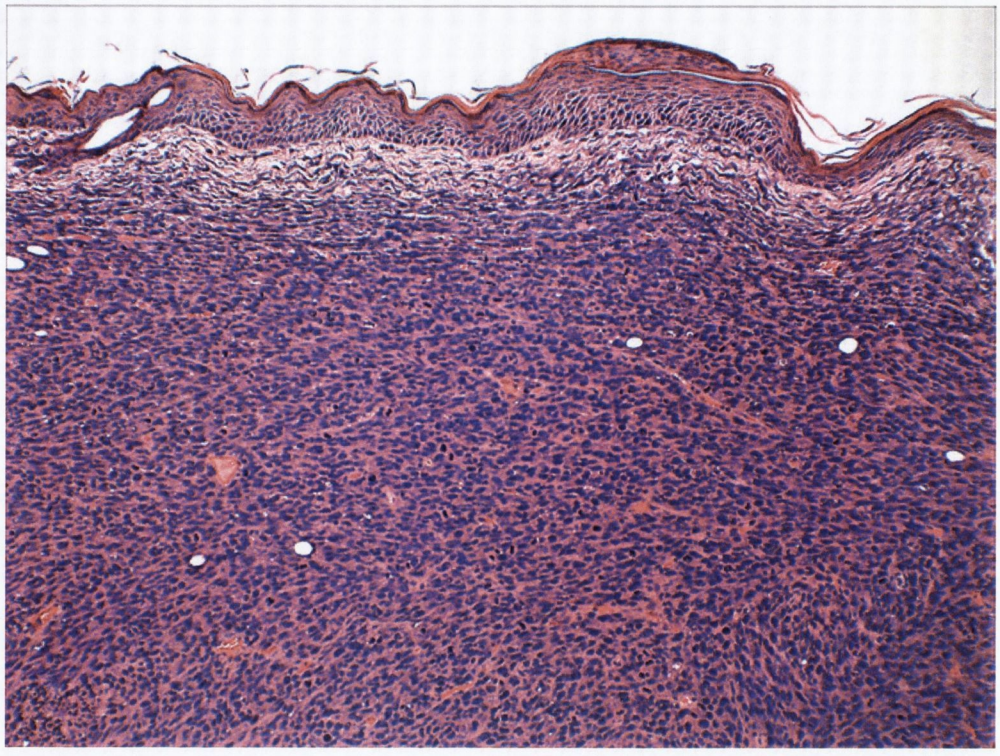
(b) Scar tissue, adipocytes and haemosiderin deposits (brown) between panniculus muscle fibers. H&E, 200x.

5.2.3.2 K-BALB tumours

At low magnification, SFV4-treated K-BALB tumours were circular and un-encapsulated with massive central necrosis. Sheets of polyhedral and spindle shaped cells extended from the epidermal-dermal interface into the dermis and subcutis (Figure 5.7a). Areas of ulceration and necrosis were accompanied by haemorrhage and infiltrates of neutrophils (Figure 5.7b). Islands of tumour cells with well-defined cell borders and frequent mitotic forms were surrounded by areas of necrosis; associated blood vessels were congested and frequently thrombosed (Figures 5.7 c and d).

Similar changes were seen in K-BALB tumours treated with TNE, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus.

a



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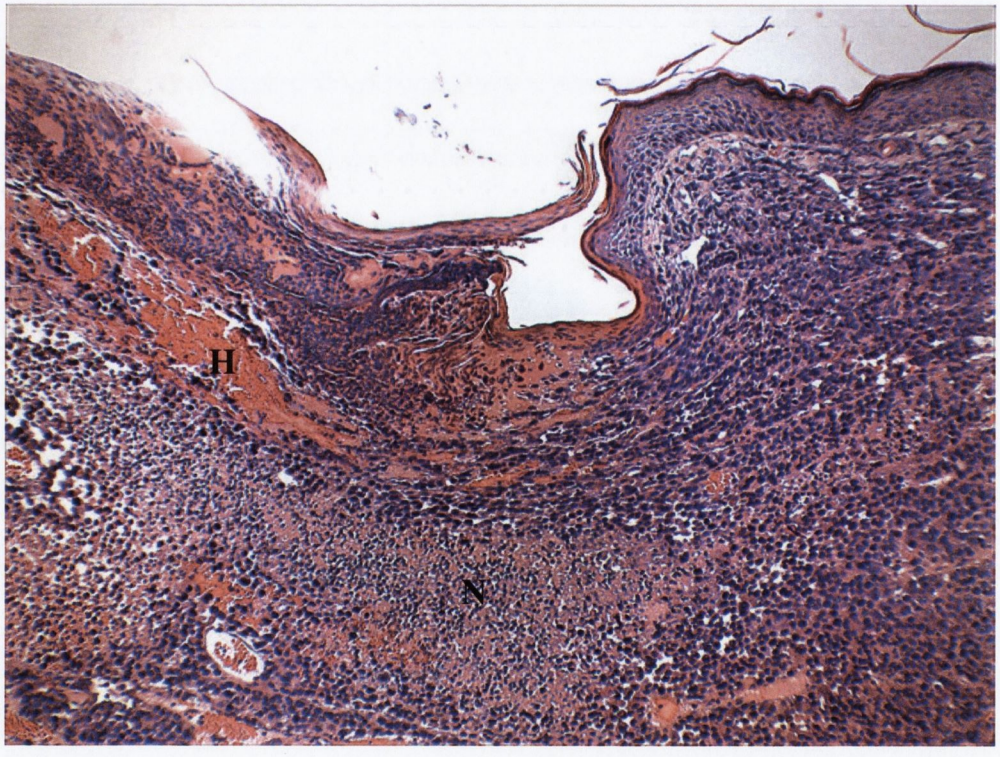
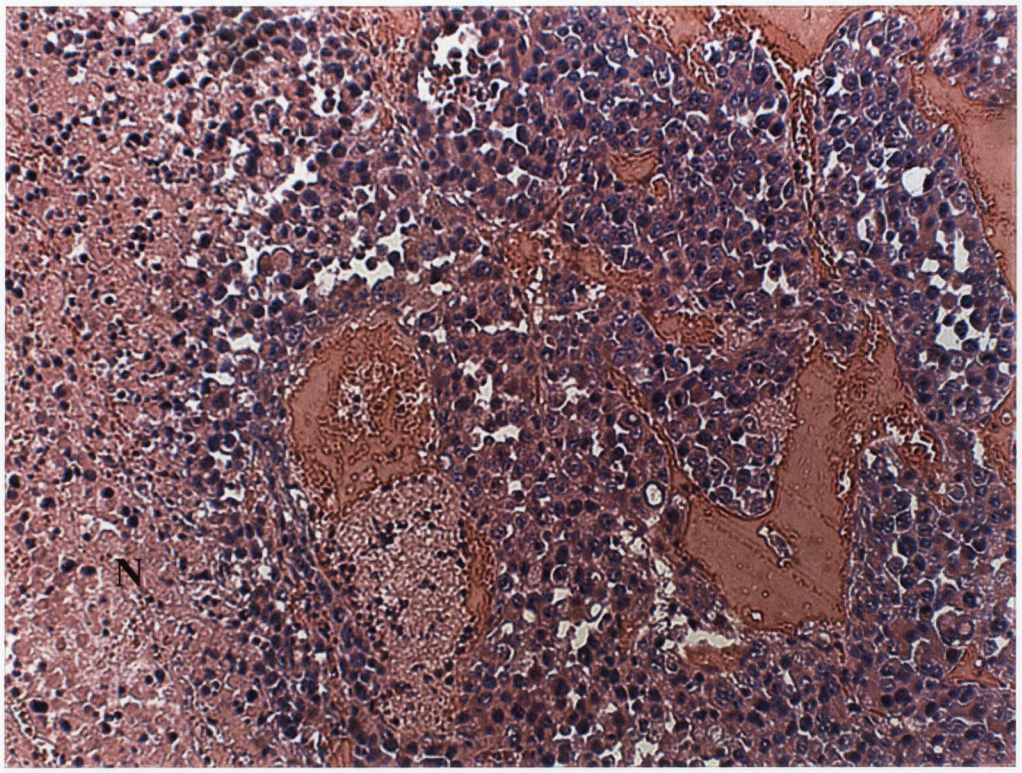


Figure 5.7 K-BALB tumours, 11 days post-treatment with SFV4

(a) Sheets of tumour cells extend to the epidermal-dermal interface with obliteration of the dermal adnexae. H&E, 100x.

(b) Ulceration with haemorrhage (H) and necrosis (N) in the superficial dermis. H&E, 100x.

c



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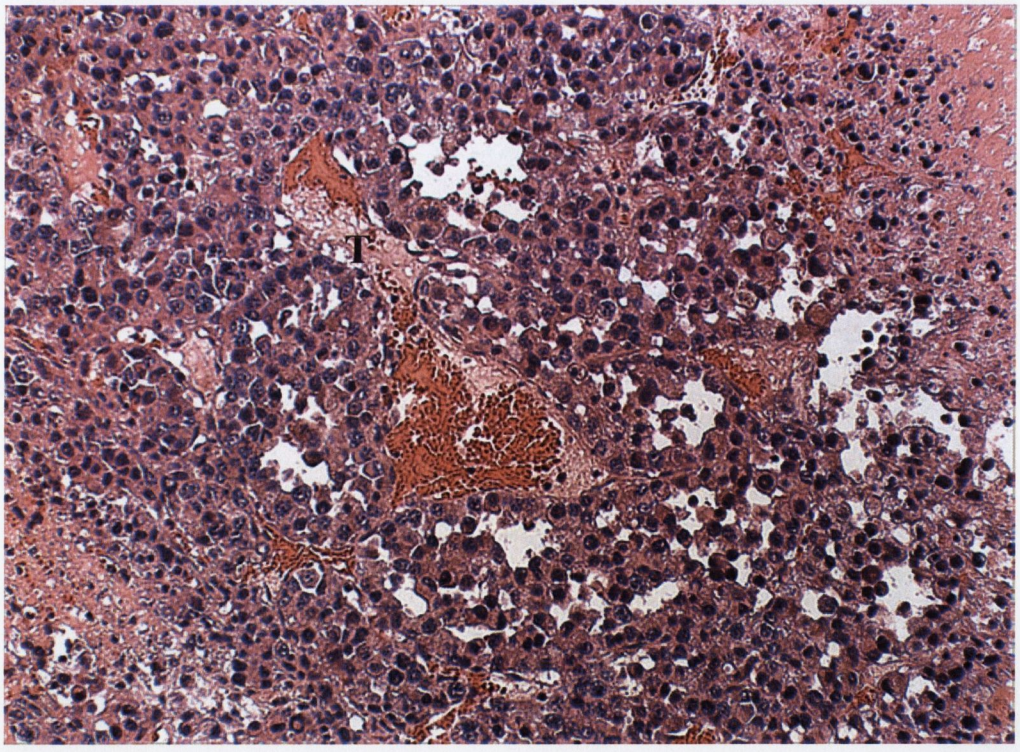

Figure 5.7 K-BALB tumours, 11 days post-treatment with SFV4

(c) Polyhedral tumour cells and thin-walled blood vessels surrounded by necrotic cell debris (N). H&E, 200x.

(d) Congestion and thrombosis (T) of tumour blood vessels. H&E, 200x.

5.3 DISCUSSION

The concept of using viruses in cancer treatment is relatively old, first described in the early 1900s (Kelly & Russel, 2007). The term 'virotherapy' gained significance during the 1950's and a number of human clinical trials were undertaken. However, results did not meet the expectations of either safety and/or efficacy.

Several viruses with natural oncolytic activity, such as NDV, reovirus and more recently VSV have been described and used for cancer gene therapy studies. Developments in recombinant DNA technology and an increasing understanding of the molecular mechanisms involved in carcinogenesis and viral replication has allowed the manipulation of replicating viruses as gene delivery vectors. Initial strategies included deletion of specific viral genes, however, to date; the most common method is to enhance the oncolytic activity of viruses through the genetic addition of immunomodulatory genes, or co-stimulatory molecules (Ring, 2002; Aghi & Martuza, 2005). Adenoviruses, such as *dl*1520 (a.k.a. ONYX-15) have been the major engineered/mutated oncolytic vectors that are utilised in clinical trials and have progressed to phase III trials. HSV, measles, and vaccinia viruses have also been engineered for oncolytic applications (Liu *et al.*, 2007).

Recent studies illustrating the *in vivo* antitumour activity of SV vectors provide encouraging data for the use of SFV vectors, which belong to the same family of viruses (Tseng *et al.*, 2002, 2004). Our *in vivo* studies involved the intratumoural treatment of fast growing, poorly immunogenic localised CT26 and K-BALB tumours in immunocompetent female BALB/c mice with replicating SFV vectors based on the infectious clone pSP6-SFV4. It was already observed that pre-immunisation with rSFV-p62-6K VLPs protected mice from i.m. challenge with SFV4 (*chapter 4*). Therefore the method was utilised to immunize mice prior to tumour induction and i.t. injections.

Treatment of CT26 tumours with replicating SFV vectors resulted in a significant inhibition of tumour growth compared to the negative control tumours. Tumours treated with RSFV-ΔTN-Δ6K-26SMCS virus showed the most significant growth inhibition when compared with tumours treated with TNE. SFV4 and RSFV-26SMCS virus also significantly inhibited the growth of the CT26 tumours. The pro-apoptotic activity of the *bax* gene had no apparent effect on the inhibition of the growth of the CT26 tumours as treatment with RSFV-HABax-26SMCS virus failed to produce significant results over the total duration of the experiment. Our findings contradict a

previous report which showed that the *bax* gene expressed from rSFV VLPs increased apoptosis in the rat prostate cancer cell line AT3-Bcl-2 and BHK-21 cells, and inhibited the growth of AT3-Neo and AT3-Bcl-2 tumours in nude mice (Murphy *et al.,* 2001). This disparity may be related to the utilisation of different cell lines (CT26 and K-BALB) in our experiments. Lewis et al (1999) also observed that cell-specific factors determine the pro-apoptotic versus anti-apoptotic function of Bax. Complete and permanent CT26 tumour regressions were found in all the treatment groups except the negative control group. Four out of 15 mice showed complete tumour regression following 6 i.t. injections with SFV4 (26.6%) and RSFV-ΔTN-Δ6K-26SMCS virus (26.6%); while two out of 15 mice treated with RSFV-26SMCS virus (13.3%) and three out of 15 mice treated with RSFV-HABax-26SMCS virus (20%), had complete CT26 tumour regression.

During early stages of treatment (at day 15) inhibition of K-BALB tumour growth was significant following i.t. treatment with RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus when compared to the negative controls. However, over the total duration of the experiment, growth of K-BALB tumours was not significantly inhibited by any of the treatments. It was concluded that replicating SFV vectors were capable of inhibiting the growth of K-BALB tumours at the early stages when the tumours were small, but then the cells became aggressive and multiplied rapidly to such an extent that SFV was not sufficient to inhibit their growth. K-BALB tumours treated with the various replicating SFV vectors and the negative control group showed almost similar tumour growth patterns with no survival proportions at the end of the treatment study. It is likely that the lower infection efficiency of the K-BALB cell line compared to the CT26 cell line, together with changes in the tumour microenvironment, prevented the replication and spread of the virus.

CT26 cells responded better to SFV vector treatments when compared to K-BALB cells possibly because the CT26 cell line was less aggressive and grew at a slower rate following s.c. induction in BALB/c mice. The CT26 cell line also had higher infection efficiency *in vitro* (*chapter 3*) than the K-BALB cell line, which was consistent with the results obtained by Smyth et al (2005). In our study, prior immunisation of mice with rSFV-p62-6K VLPs possibly had a more substantial effect in the CT26 study compared to K-BALB model as it was previously observed that pre-immunisation of mice enhanced the antitumoural response against CT26 tumours (Smyth *et al.,* 2005). Similar findings were reported by Zhu et al (2007) who showed

that the efficacy of oncolytic HSV was enhanced following i.t. administration in mice pre-immunized with HSV compared to HSV naïve mice.

The histological findings in CT26 and K-BALB tumours indicated that inhibition of tumour growth was related to destruction of tumour cells by oncotic necrosis, a term used to describe the degradative process and cell swelling that occurs after cell death (Majno & Joris, 1995). These results were consistent with the findings of Murphy et al (2000) where inhibition of tumours with recombinant SFV particles was due to oncotic cell death. In our experiments, the low levels of necrosis in CT26 tumours contrasted with the massive necrosis seen in K-BALB tumours, and levels of necrosis in control tumours and tumours treated with replicating SFV vectors were similar in both tumour models. The massive necrosis in K-BALB tumours appeared to be related to the rapid growth of the tumour cells following s.c. induction and the known susceptibility of tumour blood vessels to thrombosis. Ischemic necrosis resulting from thrombosis is characterised by karyolysis and cell swelling and was originally referred to as oncosis by von Recklinghausen in 1910 (Majno & Joris, 1995). Our findings suggest that high levels of ischemic necrosis prevented the virus from completing its replication cycle and inhibited spread of the virus within the tumour. Hindered spread of virus would represent a barrier to destruction and lysis of K-BALB tumour cells by SFV vectors.

Recent studies aimed at understanding the interactions between viruses and tumour tissue have indicated that components of the tumour microenvironment create a barrier to rapid expansion of oncolytic viruses within tumours (De Silva *et al.,* 2010; Stanford *et al.,* 2010). Köpf-Maier and Kestenbach (1990) have shown that after an initial phase of cell death, host-derived mouse fibroblasts rapidly form strands of connective tissue into tumours, provide substrate for the formation of blood-vessels into and around the tumours, and thereby sustain the growth of tumour tissue. These tumour resident and host-derived normal cells also limit virus replication and act as a passive barrier to virus spread. In addition to providing a physical barrier to virus spread as the tumours expand, the fibrous extracellular matrix (ECM) restricts virus binding, adsorption of the viral particles and infection of the tumour cells (Bilbao *et al.,* 2000). There are heterogeneous cell populations within tumours that mimic the phenotype of normal cells and cause partial or complete resistance to OV infection (Valyi-Nagy *et al.,* 2010). The term *dynamic equilibrium* between infected dying cells and live tumour cells describes the growth kinetics of the virus and the tumour cells, and was proposed

to determine the outcome of the treatment. If the tumour cells grow as fast or faster than the virus they are not destroyed and once the virus is cleared they have a chance to regrow.

In addition to the physical barriers described above, other factors may also influence the efficiency of OVs. Hypoxia, acidosis and enhanced proteolytic activity occurring in areas of necrosis and calcification as a consequence of tumour growth *in vivo*, may affect entry, replication or spread of the virus particles, and also the cell surface of nearby live tumour cells in an unfavourable manner (Fukumura & Jain, 2007). Elevated interstitial pressure may also inhibit viruses from entering cells and spreading within the tumours.

Defects in the DNA repair machinery cause the genome of a cancer cell to be prone to mutations during clonal expansion *in vivo* or passage *in vitro*. As cancer cells grow, they acquire the ability to become resistant to both chemo- and radiation therapy, as well as to virotherapy, a term called *multiresistance*. The generation of virus-resistant cells, which is probably due to the selective pressure exerted by the virus or due to adaptive mutations in tumour cells, could reduce the efficacy of OVs (Vähä-Koskela *et al.*, 2006).

Immunogenicity has a pivotal role in OV effectiveness. An ideal oncolytic virus would replicate well and persist within the tumour tissue long enough to destroy the tumours and stimulate an anti-tumour immune response. However the intensity of the immune response is important as too vigorous an immune response would clear the virus prematurely and an ineffective immune response would not result in adequate tumour killing and sustained tumour control. In a recent study, it has been shown that neutrophils play a major role in the shutdown of blood flow within the tumor in response to VSV infection, showing the immediate response of tumours to viral infection (Breitbach *et al.*, 2007). In another study, HSV gene expression was seen to rapidly decrease after 72 h following treatment of a glioma rat model, in association with a rapid infiltration of NK cells, and macrophages/microglia (Fulci *et al.*, 2006). The ability of the innate immune response to limit virus replication has also been demonstrated in several clinical studies (Pecora *et al.*, 2002; Chiocca *et al.*, 2004). The possibility that the immunised mice in our study mounted a vigorous immune response and that early influx of antibodies against SFV left insufficient time for the replicating vectors to multiply and spread within the tumour tissue cannot be discounted.

In our case, the inhibition of CT26 and K-BALB tumour growth was probably due to apoptosis and subsequent necrosis exhibited by the replicating SFV vectors. However, the necrosis resulted from the rapid growth of tumour cells, the too vigorous host immune response generated against SFV and the elements of the tumour microenvironment like ECM, hypoxia or interstitial fluid pressure described above, probably inhibited the efficient dissemination of SFV within the tumour tissue and resulted in a low inhibition of tumour growth. Ischemia and hypoxia appear to cause the massive necrosis seen in K-BALB cells; secondary to rapid growth of tumours. The significant inhibition of K-BALB tumour growth observed at the early stages of treatment (on day 15) may be related to low levels of necrosis at that time as the tumours were growing at a slower rate when compared to later stages of experiment. At the early stages of K-BALB treatment study, higher percentages of tumour cells could have been infected with SFV when compared to the later stages of the experiment. The percentage of SFV-positive K-BALB cells could have been detected by sampling the tumours at an earlier time point during the treatment and by staining the tumour sections with an anti-SFV antibody. The same strategy could have been applied to CT26 tumours.

The anti-SFV immune response induced by prior immunization of mice with rSFV-p62-6K VLPs may have hindered the spread of the virus, but at the same time SFV replicons probably enhanced the inhibition of CT26 and K-BALB tumour growth. In our studies, histological examination of CT26 and K-BALB tumour sections, which were sampled at day 11 after SFV treatment, showed little evidence of an anti-tumour cellular immune response. Leucocytic infiltrates were mainly confined to neutrophils and were concentrated in areas of necrosis and ulceration. Low-grade infiltrates of lymphocytes were located in the interstitial stroma at the periphery of the tumours and tumour nodules, and did not extend between the tumour cells. Earlier sampling at day 1 or day 5 post-initiation of treatment might have provided more precise information in this regard.

The results obtained from this study provide evidence for the potential of a replicating SFV vector as a cancer therapy agent. However, the antitumoural effect of SFV vectors could be enhanced by the expression of cytotoxic factors or the immune responses could be modulated against the vector by expression of the cytokines.

Chapter 6

GENERAL DISCUSSION

6.1 GENERAL DISCUSSION

The use of viruses for therapeutic means ('virotherapy'), including cancer gene therapy, has increased greatly in recent years. Naturally occurring viruses, and viruses that have been engineered to selectively replicate in transformed cells, have been described (Everts & van der Poel, 2005).

Since their development, alphavirus vectors have been utilized as cancer therapy agents and prototype vaccines (Atkins *et al.,* 2008). Alphaviruses have several characteristics that could be advantageous in tumour therapy. They have a broad host range and can infect a variety of cell lines. Alphavirus vectors provide transient high level gene expression which is particularly suited to cytotoxic and immunostimulatory cancer gene therapy. The alphavirus genome is small so it can easily be manipulated and because RNA replication occurs within the cytosol, there is no possibility of insertional mutagenesis which can occur with some DNA virus vectors; this would be beneficial in terms of biosafety. Recombinant SFV particles also can be produced at high titres (> 1 x 10^{10} IU/ml) by using the split-helper vector system (Smerdou & Liljeström, 1999) and the rSFV vector RNA persists at the site of injection for up to 7 days and for only 24 h in lymphoid organs (Morris-Downes *et al.,* 2001a).

Most of the viruses used in gene therapy area are replication-deficient, so-called replicons, and lack the ability to disseminate from cell to cell. This in turn limits their usefulness, especially in the treatment of metastatic tumours. As replication-competent vectors have the capacity to produce new virions and spread readily from cell to cell, increasing attention has been directed towards them. A replication-competent SFV vector, based on the avirulent strain of SFV, A7(74), was created and termed VA7. This vector contains a second 26S subgenomic promoter and a MCS at the 3'end of the structural gene ORF (Vähä-Koskela *et al.,* 2003). Because this vector gave unstable gene expression, in our study, the second 26S subgenomic promoter and the MCS were cloned to the 5' end of the structural gene ORF by Dr. Sareen Galbraith and the vector was designated RSFV-26SMCS as it was based on the infectious clone of SFV, pSP6-SFV4 (*personal communication*). As replication-competent vectors have a higher replication rate than VLPs, they could enter the central nervous system and cause encephalitis. In an attempt to increase the biosafety of replicating SFV vectors, and because nsP3 is recognised as one of the major virulence determinants of SFV (Tuittila *et al.,* 2000; Vihinen *et al.,* 2001; Tuittila & Hinkkanen, 2003), two in-frame deletions

(ΔSN and ΔTN) have been introduced into the hypervariable domain of the nsP3 gene of the pSP6-SFV4 genome, using specific restriction sites. The resulting vectors were designated SFV4-SN and SFV4-TN (Galbraith *et al.,* 2006). Studies with these vectors showed that SN and TN deletions reduce the rate of replication of SFV4 and also the rate of RNA synthesis *in vitro*; all mice survived i.m. inoculation with the deletion mutants and showed no clinical signs of disease (Galbraith *et al.,* 2006). Based on these results, the nsP3-TN deletion was incorporated into RSFV-26SMCS, creating RSFV-ΔTN-26SMCS (*constructed and kindly provided by Dr. Sareen Galbraith*). In this study, a novel replicating SFV vector was constructed by reintroducing the nsP3-SN deletion into RSFV-26SMCS creating RSFV-ΔSN-26SMCS. Deletion of the 6K gene in the infectious clone of SFV, pSP6-SFV4, has been demonstrated to reduce the rate of virion production in the early stages of infection without greatly affecting the total virus yield in cell culture (Liljeström *et al.,* 1991). Initial experiments in mice using SFV4 with the 6K gene deleted (SFV4-6K) indicated that, following peripheral inoculation, the virus multiplies and stimulates protective immunity, does not infect the CNS and all mice survive (*Dr. Sareen Galbraith, personal communication*). Based on these findings, the structural 6K gene was removed from the genome of RSFV-26SMCS creating RFV-Δ6K-26SMCS (*constructed and kindly provided by Dr. Sareen Galbraith*). To further enhance the biosafety of the replicating SFV vector, the TN deletion was combined with the complete deletion of the structural 6K gene (Δ6K) resulting in the formation of RSFV-ΔTN-Δ6K-26SMCS. Following earlier studies which showed that rSFV VLPs expressing the pro-apoptotic *bax* gene enhanced apoptosis *in vitro* and reduced the growth of AT3-Neo and AT3-Bcl-2 tumours *in vivo* (Murphy *et al.,* 2001), the pro-apoptotic *bax* gene was cloned into the MCS of RSFV-26SMCS, creating RSFV-HABax-26SMCS (*constructed by Ms. Jennifer Mulholland, project student under supervision of Güniz Iskender*).

In our study, the growth rate of the replication-competent SFV vectors containing single deletions in their genome; RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS, and RSFV-Δ6K-26SMCS, were analysed in BHK-21 cells by performing plaque assays on supernatants from infected monolayers over a 24 h period at 2 h intervals (*chapter 3*). The growth of SFV4 and RSFV-26SMCS was also analysed and the results were compared. Infection at a MOI of 10 and 0.1 PFU/cell resulted in production of large amounts of virus from the vectors comprising the single deletions, similar to SFV4 and RSFV-26SMCS virus. It was concluded that the presence of SN

and TN deletions had little effect on the binding rate of the replicase complexes to cellular membranes and therefore the virus yield was not reduced. The presence of the second 26S subgenomic promoter did not affect the replication rate of the virus, and RSFV-26SMCS replicated to the highest titres in BHK-21 cells at a MOI of 0.1 PFU/cell. Contrary to the results observed by Galbraith et al (2006), our studies showed that the deletion mutants grew at least as well as the wild-type viruses at all time points tested (in BHK-21 cells). This discrepancy was attributed to the higher dose of virus (10^6 PFU/ml) used in our experiments and that the 100-fold lower dose of virus used by Galbraith et al (2006).

It is well known that cell type affects viral infection efficiency and can be a limiting factor in the effectiveness of viral vectors. Also, because tumour cells can present altered and unstable surface proteins, transformed cells can display differing infection efficiencies compared to non-cancerous cells. The infection efficiency of the RSFV-EGFP-26SMCS vector was tested in the highly-infectable BHK-21 cell line and in the CT26 and K-BALB tumour cell lines (*chapter 3*). The BHK-21 cell line showed the highest transduction efficiency whereas relatively low infection efficiency was observed in CT26 and K-BALB tumour cells, indicating that the replicating SFV vectors had limited ability to infect these cells *in vitro*.

Viral growth curves were generated for RSFV-ΔTN-Δ6K-26SMCS and RSFV-HABax-26SMCS, and were compared with those of SFV4 and RSFV-26SMCS in BHK-21, CT26 and K-BALB cells at a MOI of 0.1 PFU/cell. Results showed that vectors listed above replicate to high titres in BHK-21 cells, however produce significantly lower amounts of virus in CT26 and K-BALB cells indicating lower infection efficiency of the tumour cell lines. No significant difference in the amounts of virus was observed between different SFV vectors in BHK-21, CT26 and K-BALB cell lines.

The effects of infection with the replicating SFV vectors on the viability of BHK-21, CT26 and K-BALB cells were examined using the *CellTiter 96® AQ_{ueous} One Solution* assay (*chapter 3*). Whereas BHK-21 cells were completely destroyed following infection with SFV replicating vectors, the SFV vectors exerted only a transient effect on the viability of tumour cells. The recovery of CT26 and K-BALB cells was attributed to their lower infection efficiency compared to BHK-21 cells.

The effects of infection with the replicating SFV vectors on the induction of apoptosis were also examined *in vitro* using the activation of caspase-3/-7 assay and the

percentage of positive cells was determined by flow cytometry (*chapter 3*). Oncogenic K-*ras* plays a role in apoptosis induction/inhibition and often influences cell survival. Therefore activation of caspase-3/-7 which is an indicator of programmed cell death was examined in BHK-21, CT26 and K-BALB cells following infection with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus. The positive control BHK-21 cell line was found to be more susceptible to SFV-induced apoptosis and both CT26 and K-BALB cells displayed significantly lower numbers of caspase positive cells when compared to BHK-21 cells. Activated K-*ras* could have inhibited SFV-induced apoptosis in K-BALB cells. In a previous study, the effect of Ras on SV-induced apoptosis in a rat pheochromocytoma cell line, PC12, was studied, and shown that Ras caused a marked delay in SV-induced apoptosis; this delay was not, however, associated with any decrease in viral titre (Joe *et al.,* 1996). Recent studies have also shown that although caspase-3 and caspase-7 are dominant mediators of SFV-induced apoptosis, the virus also induces apoptosis through a caspase independent death program (Urban *et al.,* 2008). This suggests that caspase-3 and -7 may not necessarily be activated during induction of apoptosis in the tumour cells. The low levels of apoptosis observed in the tumour cell lines could also be explained by the low infection efficiency of CT26 and K-BALB cells.

It is known that the response of a cell to a death signal is determined by the ratio of Bcl-2 to Bax and that the pro-apoptotic *bax* gene counteracts the Bcl-2 mediated suppression of cell death by Bcl-2-Bax heterodimerization (Oltvai *et al.,* 1993). Whereas rSFV VLPs expressing *bax* have been shown to increase apoptosis in AT3-Neo and more aggressive AT3-Bcl-2 cells (Murphy *et al.,* 2001), in our studies *bax* gene expression did not enhance the killing of BHK-21, CT26, or K-BALB cells. Stability of the *bax* gene expressed from RSFV-HABax-26SMCS vector was confirmed by passaging the RSFV-HABax-26SMCS virus eight times in BHK-21 cells and checking the HA-Bax protein expression by indirect immunofluorescence. In a recent study Bax-/- MEFs were shown to be more sensitive to caspase-3 activation, cytochrome-c release and apoptosis than wt cells (Urban *et al.,* 2008). Lewis et al (1999) also observed that Bax-/- mice are more susceptible to SIN-induced neuronal disease and mortality than normal mice. The molecular basis of this anti-apoptotic action of Bax is still unknown and the possibility remains that the differing results obtained in our study and those of Murphy et al (2001) may be due to the use of different host cell lines.

Prior to tumour treatment studies, it was necessary to examine the virulence of the replicating SFV vectors in immunocompetent BALB/c mice (*chapter 4*). It was observed that none of the SFV vectors with single deletions (RSFV-ΔSN-26SMCS, RSFV-ΔTN-26SMCS and RSFV-Δ6K-26SMCS) showed reduced virulence *in vivo*. The major virulence determinants between the virulent strain SFV4 and the avirulent strain A7(74) of SFV have been shown to be the E2 gene, the 5' NTR, and the non-structural nsP3 gene (Santagati *et al.,* 1995, 1998; Tarbatt *et al.,* 1997; Tuittila *et al.,* 2000). In a previous study, SFV4 deletion mutants (SFV4-SN and SFV4-TN) were shown to reduce virulence in BALB/c mice after peripheral inoculations (i.m. and i.p.) (Galbraith *et al.,* 2006). However, it was concluded that the 100-fold lower dose of virus used by Galbraith et al (2006) afforded protection from the neurovirulent properties of SFV. It is known that in adult mice, SFV4 is virulent by i.n. or intracerebral inoculation (Glasgow *et al.,* 1991; Fazakerley, 2002) but is avirulent when inoculated i.p. at least with a dose of 5000 PFU as low doses fail to establish high-titre viremia and virus fails to enter the CNS (Fragkoudis *et al.,* 2007).

To increase the biosafety of the replicating vector, RSFV-26SMCS containing the ΔTN and Δ6K was created, and tested in BALB/c mice by i.m. injections. A 90% survival rate was observed in mice injected with RSFV-ΔTN-Δ6K-26SMCS virus, which was significantly higher than SFV4-injected mice. The presence of two deletions in one genome probably exerted pressure on the vector, and reduced the virulence of the vector *in vivo*. Intramuscular injection of RSFV-HABax-26SMCS vector protected 70% of BALB/c mice and the results were nearly significantly different from those obtained in mice injected with SFV4. A study by Kerr et al (2002) showed that a neuroadaptive strain of SV (NSV) expressing Bcl-2 and Bax (dsNSV-BCL-2 and dsNSV-BAX, respectively) protected mice from death whereas dsNSV-GFP did not confer protection. Although the precise mechanism for the anti-apoptotic function of the *bax* gene is unknown, it is suggested that caspases convert the pro-apoptotic protein Bax into an anti-apoptotic protein by proteolytic cleavage, relocalization or other mechanisms (Kirsch *et al.,* 1999) as the anti-apoptotic proteins Bcl-2 and Bcl-xL are converted into pro-apoptotic proteins when their N termini are removed with cleavage by caspases (Cheng *et al.,* 1997; Clem *et al.,* 1998).

The inherent ability of rSFV VLPs to elicit strong immune responses was utilised in immunotherapy of cancer (Berglund *et al.,* 1999; Fleeton *et al.,* 1999). As the replication-competent SFV vectors did not confer 100% protection in BALB/c mice,

mice were immunized with rSFV VLPs encoding the SFV structural protein E2 (as well as E3 and 6K) (rSFV-p62-6K VLPs) prior to i.m. injections with SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus or RSFV-HABax-26SMCS virus. By pre-immunisation, it was aimed to enhance the anti-SFV immune response which would protect mice from subsequent injections of SFV. This strategy conferred protection against SFV and all mice survived the i.m. injections (Atkins *et al.,* 1999). Upon histological examination, the brains of pre-immunized mice injected with SFV appeared normal. In contrast, naïve, SFV4-inoculated mice showed brain lesions characteristic of the changes seen previously in SFV4 injected mice surviving to 14 days p.i. (*chapter 4*).

The apoptosis inducing ability of the recombinant SFV vectors and the wild-type SFV have been examined in treatment of various tumour models including rat prostate cancer cells, NSCLC cell lines and murine colon carcinomas (Murphy *et al.,* 2000, 2001; Smyth *et al.,* 2005). The oncolytic effect of the replication-competent SFV vector, VA7-EGFP was also studied for the treatment of several tumour models in nude mice or immunocompetent rats (Määttä *et al.,* 2007), and human glioma xenografts in nude mice (Heikkilä *et al.,* 2010).

Notwithstanding the lower infection efficiency of the CT26 and K-BALB tumour cell lines, our *in vivo* tumour treatment studies involved the intratumoural treatment of fast growing, poorly immunogenic localised CT26 and K-BALB tumours in immunocompetent BALB/c mice. CT26 and K-BALB tumours were treated with either TNE buffer alone, SFV4, RSFV-26SMCS virus, RSFV-ΔTN-Δ6K-26SMCS virus, or RSFV-HABax-26SMCS virus. Groups of mice were vaccinated with rSFV-p62-6K VLPs prior to tumour induction and treatment in an attempt to enhance the anti-SFV immune response and as a safety precaution to reduce mortality (Smyth *et al.,* 2005). Significant inhibition of CT26 tumour growth was observed in immunocompetent BALB/c mice, and complete and permanent tumour regressions were also found in all treatment groups (*chapter 5*). Growth of K-BALB tumours was significantly inhibited during the early stages of treatment (at day 15), however this inhibition was not permanent, and none of the mice showed complete tumour regression (*chapter 5*).

Recent research has shown that components of the tumour microenvironment create a barrier to rapid expansion of OVs within tumours (De Silva *et al.,* 2010; Stanford *et al.,* 2010). For instance, tumour resident and host-derived cells create a physical barrier to OV spread together with the ECM, which restricts virus binding,

adsorption of the viral particles and infection of the tumour cells (Bilbao *et al.*, 2000). Tumour size has also been shown to affect the transduction efficacy of viral particles, as small tumours were transduced more efficiently than large tumours (Cordaro *et al.*, 2000). In our case, the larger size of the K-BALB tumours could have restricted spread of SFV and reduced the transduction efficiency of the replicating SFV vectors. Our histological studies showed that rapid growth of the K-BALB tumours resulted in hypoxic damage characterized by massive necrosis, ulceration, thrombosis and haemorrhage. It was concluded that the necrotic microenvironment inhibited spread of the virus within the tumour and therefore the destruction and lysis of the tumour cells by the SFV vectors. Further inhibition of virus spread probably resulted from disruption of the tumour vasculature through endothelial cell killing and the generation of thromboses following treatment. Levels of necrosis were much lower in the slower growing CT26 tumours and this appeared to be an important factor in explaining the improved response shown by these tumours to treatment with the SFV vectors. The efficacy of the treatments could have been further reduced by the occurrence of virus-resistant cells due to the selective pressure exerted by the virus or to adaptive mutations in tumour cells (Vähä-Koskela *et al.*, 2006).

In our case, the early inhibition of CT26 and K-BALB tumour growth was probably due to cell death initiated by the replicating SFV vectors. Induction of apoptosis in BHK-21, CT26 and K-BALB cells infected with SFV was confirmed by the *in vitro* experiments, even though the level of apoptosis was relatively lower in tumour cells compared to BHK-21 cells. The development of necrosis due to the rapid growth rate of the tumour cells was demonstrated by histological examination of the tumour tissue samples. Besides the necrosis, components of the tumour microenvironment mentioned above probably restricted spread of SFV in the tumour tissue and therefore reduced the transduction efficiency of the replicating SFV vectors.

Smyth et al (2005) described the detection of a strong anti-SFV T_H1 cellular immune response in *ex vivo* splenocyte stimulation assays by quantification of secreted IFN-γ. The anti-SFV humoural immune response was also measured in the same study using ELISA, and was predominantly of the IgG2a isotype. This is typical of the rSFV VLP-induced T_H1-mediated immunity described by previous workers (Zhou *et al.*, 1995; Berglund *et al.*, 1999). The possibility that the immunised mice in our study mounted a vigorous immune response and that early influx of antibodies against SFV left insufficient time for the replicating vectors to multiply and spread within the

tumour tissue cannot be discounted. Alternatively, this anti-SFV immune response could have enhanced the anti-tumour response induced by SFV and caused the inhibition and regression of CT26 tumours. Toda et al (1999) have also shown that the induction of antiviral immune response following treatment of murine tumours with G207 HSV-1 mutant can in turn induce an antitumour immune response which can inhibit non-treated tumours. Given that the K-BALB tumours regrew at the same rate as control tumours following treatment, it is unlikely that specific immune responses were generated against this lowly immunogenic cell line (Stephenson & Aaronson, 1972). Histological examination of the CT26 and K-BALB tumour sections sampled at day 11 post-initiation of treatment showed little evidence of an anti-SFV cellular immune response (*chapter 5*). Earlier sampling at day 1 or day 5 post-initiation of treatment might have provided more precise information in this regard.

In conclusion, we have shown that replication-competent SFV vectors were capable of significantly inhibiting the growth of CT26 and K-BALB tumours at the early stages of treatment and of conferring complete regression of some CT26 tumours. The better response of CT26 tumours possibly occurred because the CT26 cells were less aggressive and grew at a slower rate following s.c. induction in BALB/c mice (*chapter 5*). The infection efficiency of the CT26 cell line was also higher *in vitro* than the K-BALB cell line (*chapter 3*). Prior vaccination with rSFV-p62-6K VLPs also appeared to have a more substantial effect in the CT26 study compared to the K-BALB model. The significant inhibition of K-BALB tumour growth observed at the early stages of treatment (on day 15) was probably due to the slower growth and lower level of necrosis at that time compared with the later stages of the experiment. At the early stages of K-BALB treatment study, higher percentages of tumour cells could have been infected with SFV when compared to the later stages of the experiment.

The results of our experiments were encouraging and indicated that replicating SFV vectors are good candidates to be used as tumour therapy agents, especially for less aggressive tumours. The inherent ability of SFV to induce apoptosis is advantageous in the effective eradication of tumours and this ability could be enhanced by engineering SFV to produce cytotoxic proteins such as Fas-ligand, TRAIL or p53 (Shinoura *et al.,* 2000; Zhao *et al.,* 2006). The anti-tumour effect of the SFV vector could also be enhanced by administering anti-angiogenic or therapeutic factors into the vector which would be secreted by infected cells and diffuse through the tumour (Asselin-Paturel *et al.,* 1999; Colmenero *et al.,* 2002; Chikkanna Gowda *et al.,* 2005;

Lyons *et al.*, 2007). Recently there has been an increasing body of evidence indicating the importance of the host immune response to the efficacy of oncolytic virotherapy as tumour-infiltrating immune cells in response to virus infection have significant antitumour activity either by killing tumour cells directly or recruiting further antitumour immune effectors into the tumour (Prestwich *et al.*, 2008). In our case, the anti-SFV immune response induced by prior immunization of mice with rSFV-p62-6K VLPs may have hindered the spread of the virus, but at the same time SFV replicons probably enhanced the inhibition of tumour growth. As examined in this study, novel replication-competent SFV vectors could further be constructed by deleting certain parts in the viral genome that would attenuate the vector but show efficient replication in transformed cells *in vitro* and induce significant inhibition/elimination of tumour growth *in vivo*. As mentioned above, tumour microenvironment creates a barrier to efficient spread of virus within the tumour tissue. To avoid this complication, oncolytic viruses could be engineered to express extracellular matrix metalloproteinases (MMPs), which are a family of proteolytic enzymes that degrade the extracellular matrix and are essential for tumor spread and neovascularization. In that way, the tumour growth and angiogenesis can be delayed (McNally *et al.*, 2009). Combined therapy with conventional chemo- or radiotherapy may also provide better results than gene therapy alone (Khuri *et al.*, 2000). Intratumoural injection of the adenovirus mutant, H101, in combination with chemotherapy was studied in patients with squamous cell cancer of the head and neck or of the oesophagus, and the results showed that the response rate was significantly higher (78%) in patients who received the combination of viral therapy and chemotherapy than in patients who were treated with chemotherapy alone (39%) (Xia *et al.*, 2004).

The number of clinical trials based on virotherapy is increasing each year. Phase I and II clinical trials for liposomally encapsulated rSFV VLPs expressing IL-12 were recently proposed for the treatment of glioblastoma multiforme, but have yet to be undertaken (Ren *et al.*, 2003). However, the oncolytic potential of a replication-proficient SFV vector in gene therapy remains to be explored by improving the efficacy and ensuring the biosafety of the vector system.

Chapter 7

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7.1 REFERENCES

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Chapter 8

APPENDICES