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DEVELOPMENT OF SFV-BASED PLASMID ELECTROPORATION FOR USE IN TUMOUR TREATMENT AND VACCINE DELIVERY

A thesis submitted to the University of Dublin, Trinity College

For the Degree of Doctor of Philosophy

By

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Declaration

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SUMMARY

Electroporation is the delivery of short and intense electric pulses into living cells (either in tissue or suspension), which results in a transient and reversible increase in permeability of the cell transmembrane. This study is designed to develop electroporation for use as a method of Semliki Forest virus (SFV) - based plasmid delivery for both tumour treatment and vaccine delivery.

The impact of a number of different electroporation conditions have been examined for efficiency of in vitro electroporation in a variety of cell lines. These have shown the influence of the cell type on the pulse conditions applied to obtain high levels of gene expression while minimising the level of cell death. The results have also shown the efficacy of SFV-based plasmids in comparison to those commercially available. Intramuscular electroporation was optimised for the delivery of the SFV-based *LacZ* plasmids. Electroporative delivery of these plasmids was shown to produce an effective immune response comparable to that of current viral vectors.

Optimised intratumoural electroporation was used for the delivery of two SFV-based RNA cancer gene therapies. pSFV-*Bax* was shown to induce apoptosis at over double the rate of the SFV vector when electroporated in vitro and to result in transient growth inhibition of both CT26 and OE19 tumours in vivo following one electroporative treatment. Electroporated pSFV-*IL-12* was shown to induce the production of biologically active IL-12 in vitro and to result in transient growth inhibition of CT26 in vivo following one or two electroporative treatments. The potential of electroporation of SFV-based plasmids for tumour treatment and vaccine delivery has been shown.

For Mum, Dad, Sabrina & Karen

"Today, in some place, a treasure awaits you. It may be a fleeting smile, it may be a great victory -- it doesn't matter. Life is made up of large and small miracles"

Paulo Coelho

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Presentations & Publications

Oral Presentation ;

"Characterisation of electroporated pSFV1-Bax as a cancer genetherapy"

J. M. Piggott, M. N. Fleeton, G. J. Atkins

Joint Meeting Bioelectrochemistry, 19 – 24 June 2005, Coimbra, Portugal.

"Development of SFV RNA based electroporative vaccines"

Piggott J M, Fleeton M N, Doody T, Soden D, Atkins, G J, O' Sullivan G

Bioelectrochemistry Society Annual Meeting, April 2007, Toulouse, France.

Poster presentation ;

"Development of an electroporatively delivered SFV RNA vaccine"

J. M. Piggott,Fleeton M, Sheehan B, Doody T, Collins R, Soden D, Wilke N, Morrisssey A, O'Sullivan G, Atkins G J

2nd FEMS Congress of European Microbiologists, to be held in Madrid, July 4-8 2006

"Development of SFV Bax RNA for electroporative cancer treatment"

Piggott J M, Soden D, Doody T G, Fleeton M N, Sheahan B, O'Sullivan G, Atkins G J.

Irish association for cancer research Annual Meeting, 2-3rd March 2007, Cork, Ireland.

"Development of SFV RNA gene therapies for electroporative cancer treatment"

Piggott J M, Soden D, Doody T G, Fleeton M N, Atkins G J, O'Sullivan G C

American Society of Gene Therapy Annual Meeting, June 2007, Seattle, USA.

"Development of pSFV-Bax RNA for use in electroporative cancer treatment"

Piggott J M, Soden D, Doody T, O'Sullivan G, Atkins G J.

XV Annual Congress of the European Society of Gene and Cell Therapy, October 2007, Rotterdam, The Netherlands.

DEVELOPMENT OF SFV-BASED PLASMID ELECTROPORATION FOR USE IN TUMOUR TREATMENT AND VACCINE DELIVERY

JOHN M. PIGGOTT

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Table of Contents

List of Figures and Tables

Chapter Three

Chapter Four

Chapter Five

Chapter 1

Introduction

1.1 Introduction

In the past number of years improvements in the diagnosis of cancer has led to this disease being detected at an ever-earlier stage. A large number of these early stage tumours are of low malignancy (e.g. renal adenoma, oncocytoma, angiomyolipomas) and while early stage tumours are often treatable these treatments themselves may induce angiogenesis or other serious side effects (Retsky *et al.*, 2007, Peeters *et al.*, 2006).

The first ever all-Ireland report of cancer statistics has found that one in eight women and one in six men will die of cancer before their 74th birthday and that one in three people in Ireland is likely to develop cancer before the age of 74 (Souhami *et al.*, 2002). While methods of cancer detection are continuing to improve, methods of cancer treatment have changed very little in the past years. It has become apparent that more sophisticated methods of cancer treatment are necessary. Surgery is still the most efficient treatment but there are many reasons why a candidate may not be suitable for surgery or why surgery may not be a viable option of cancer treatment. Chemotherapies or radiotherapies are most often administered systemically and so the patient may experience extensive side effects as a result of this treatment (or surgery may only lead to temporary amelioration).

New non-invasive localized cancer therapies that can be applied to both early and late stage cancers are necessary to improve overall patient care and to reduce side effects often experienced when therapies are applied systemically.

1.2 Cancer

Cancer is considered to be a genetic disease. Alteration to the DNA inside cells can endow it to grow anywhere and divide indefinitely. There are multiple factors involved in the causation of cancer, and both external and intrinsic events play a role in the malignant transformation of cells. An understanding of the biochemical abnormalities in tumour cells and the differences from normal cellular biology can lead to the development of effective, nontoxic, tumour-specific treatments (Macdonald *et al.*, 1997). Cancer cells differ from their normal counterparts in several ways, some of which are related to their capacity for uncontrolled growth. Analysis often shows increased amounts of DNA per cancer cell, indicating an abnormal increase in chromosomal material, and the nuclei carrying this DNA may take on abnormal shapes. An unusually high percentage of cells in a cancerous growth are undergoing an increased rate of mitosis / dereased rate of apoptosis, as compared with an undetectably low rate in normal cells. Cancer cells often lose tissue-specific differentiation and acquire a high degree of malignancy. A malignant tumour consists of fully transformed cells that can invade adjacent tissues and spread (metastasize) to other sites in the body to form secondary tumour growths, though it shoud be noted that not all malignant tumours do metastasize e.g. (King *et al.*, 2000).

1.3 Current management of cancer

For many years, standard cancer therapy has been based on surgery, radiotherapy, and chemotherapy, used alone or in combination. Surgery can effectively remove some tumours, but surgery alone cannot effectively treat cancers that have spread widely

through the body. Radiotherapy and chemotherapy can kill cancer cells and shrink tumours, but at safe doses, they may fail to eradicate all cancer cells while at the same time causing side effects by damaging healthy cells and causing other physiological side effects.

1.3.1 Chemotherapy

Currently available anticancer drugs can be divided into three broad categories:

1. Those that interact with DNA (cytotoxics).
2. Those that associate with membrane receptors (hormonal agents).
3. Those that bind to antigen expressed at the cell surface.

The principal mechanism of action of each of the cytotoxic drugs is uncertain, although all of them produce DNA damage. Alkylating agents induce arrest of DNA transcription/replication. Antimetabolites induce DNA injury by inhibiting thymidine synthetase, purine synthesis, or DNA repair. The anthracyclines damage DNA by intercalating with DNA, generating free radicals, or interacting with DNA-modifying enzyme topoisomerase. The final common pathway of cytotoxic-induced cell death is via apoptosis.

1.3.1.1 Limitations of cancer chemotherapy

Despite the introduction of numerous chemotherapeutic agents, cancer survival rates for clinically advanced cancers have improved little during the past quarter of a century. Although notable successes have been achieved in the treatment of acute leukemias and malignant lymphomas, advanced solid malignancies (with the exception of testicular

cancer and some other rare neoplasms) are usually incurable. The major limitations of chemotherapy are toxicity and multidrug resistance.

All of the commonly used chemotherapy agents are associated with acute toxic effects. These effects are a limiting factor in the dosing levels of these substances. Various supplementary treatments are used to counteract the adverse effects. For example, myelotoxicity is treated with colony growth-stimulating factors such as G-CSF, while other treatments have been developed to counteract nausea and vomiting. One major adverse effect of chemotherapy in long-term survivors is development of secondary malignancies as a result of alkylating agents. Various measures to reduce the incidence of chemotherapy-associated toxicity include combination therapies, development of less toxic chemotherapeutic agents, and improved targeted methods of drug delivery to avoid systemic toxicity.

Several chemotherapy regimens include combinations of cytotoxic agents, each of which has been shown to be effective as a single agent against the cancer type to be treated. The toxicity profiles of the individual drugs are not superimposed to limit the severity of toxicity. Dosage schedules are chosen to optimize tumour response and toxicity profile. Scheduling of myelosuppressive chemotherapy is limited by bone marrow recovery. Regimens for a myelosuppressive therapy alternating with non-myelosuppressive therapy are used in such cases. High-dose chemotherapy has been combined with autologous bone marrow or peripheral stem cell rescue as a salvage therapy.

1.3.2 Radiotherapy

Radiotherapy is an established treatment for certain types of cancer. The role of radiotherapy for a particular tumour is determined by the radiosensitivity of the tumour. Pharmacological agents can interact with radiotherapy in several ways, and attempts are being made to determine how drugs and radiation can be combined in an optimal manner. Radiotherapy has been claimed to have a curative role as a sole treatment for some cancer, as well as being a component of multimodal treatments (including chemotherapy) for breast cancer and soft tissue sarcoma. For cancers with both local control problems and metastatic potential, experts generally agree that a combination of chemotherapy and radiotherapy is more effective than either approach alone.

1.3.3 Surgery

Surgery plays an important role in the multidisciplinary management of cancer. In the absence of metastases, the surgeon can ablate the primary tumour with a reasonable chance of cure. This depends on the location of the tumour and the completeness of the excision, for example glioblastoma of the brain, where it is not possible to remove the tumour completely. Surgical methods are combined with other modalities such as chemotherapy and radiotherapy and innovative methods of drug delivery during surgery are also becoming possible. Surgical procedures may be involved in implantation of anticancer therapeutic agents.

1.4 Cancer gene therapy

1.4.1 Cancer immunotherapy

Immune approaches to cancer treatment constitute a significant proportion of new drug development in oncology. Important methods of immunotherapy include cytokines, monoclonal antibodies (MAbs), vaccines, and immune-gene therapy; all of which involve the recruitment of lymphocytes in the treatment of cancer.

1.4.1.1 Lymphocytes

Lymphocytes are one of the five kinds leukocytes found circulating in the blood. While mature lymphocytes are all very similar in appearance, they are extremely diverse in their functions. The most abundant lymphocytes are B lymphocytes (B cells), which are responsible for making antibodies and T lymphocytes (T cells) of which there are a number of subsets including inflammatory T cells (responsible for recruitment of macrophages and neutrophils to the site of infection or tissue damage), cytotoxic T lymphocytes or CTLs (that kill virus-infected tumour cells) and helper T cells (that enhance the production of antibodies by B cells). B cells are not only produced in the bone marrow but also mature there while, the precursors of T cells leave the bone marrow and mature in the thymus.

1.4.1.1.1 T cells

Most of the T cells in the body belong to one of two subsets. These are distinguished by the presence on their surface of either the CD4 or CD8 glycoproteins. The glycoprotein

that is present determines the type of cell the T cell can bind to. $CD8^+$ T cells bind epitopes that are part of class I histocompatibility molecules (almost all the cells of the body express class I molecules), while $CD4^+$ T cells bind epitopes that are part of class II histocompatibility molecules (only specific antigen-presenting cells express class II molecules for example dendritic cells, phagocytic cells like macrophages and B cells). The main role of $CD8^+$ T cells is to monitor all the cells of the body, ready to destroy any that express foreign antigen fragments in their class I molecules for example cytotoxic T lymphocytes (CTLs) are $CD8^+$ T cells that secrete molecules to destroy the cell to which they have bound.

$CD4^+$ T cells bind an epitope consisting of an antigen fragment lying in the groove of a class II histocompatibility molecule. $CD4^+$ T cells are essential for both the cell-mediated and antibody-mediated branches of the immune system. $CD4^+$ cells involved in cell mediated immunity bind to antigen presented by antigen-presenting cells (APCs) like phagocytic macrophages and dendritic cells. The T cells then release lymphokines that attract other cells to the area. The result is inflammation: the accumulation of cells and molecules that attempt to wall off and destroy the antigenic material. The $CD4^+$ cells involved in antibody-mediated immunity (helper T cells) bind to antigens that are presented by B cells. The result is the development of clones of plasma cells secreting antibodies against the antigenic material.

1.4.1.1.2 TH1 and TH2 type immune response

T lymphocytes expressing CD4 (helper T cells) are regarded as being the most prolific cytokine producers. This subset can be further subdivided into Th1 and Th2, and the cytokines they produce are known as Th1-type cytokines and Th2-type cytokines.

Th1-type cytokines tend to produce the proinflammatory responses responsible for killing intracellular parasites and for perpetuating autoimmune responses. Interferon gamma is the main Th1 cytokine. Excessive proinflammatory responses can lead to uncontrolled tissue damage, so there needs to be a mechanism to counteract this. The Th2-type cytokines include interleukins 4, 5, and 13, which are associated with the promotion of IgE and eosinophilic responses in atopy, and also interleukin-10, which has more of an anti-inflammatory response. In excess, Th2 responses will counteract the Th1 mediated microbicidal action. The optimal scenario would therefore seem to be that humans should produce a well balanced Th1 and Th2 response, suited to the immune challenge.

1.4.1.2 Cytokines

Cytokines are proteins that are manufactured by cells of various lineages and when secreted will drive specific responses (e.g. proliferation, growth or maturation) in other susceptible cells. This definition excludes the hormones produced by various endocrine organs and usually implies molecules produced by the cells of the immune system. Responses to cytokines include increasing or decreasing expression of membrane proteins (including cytokine receptors), proliferation, and secretion of effector

molecules. There are a variety of different cytokines based on the cell that they are produced by and the specific response they induce. These include lymphokines (produced by lymphocytes), monokines (produced by monocytes), chemokines (cytokines that drive chemotactic responses), and interleukins (cytokines produced by one leukocyte that drives specific responses in other leukocytes). The largest group of cytokines stimulates immune cell proliferation and differentiation. This group includes Interleukin 1 (IL-1), which activates T cells; IL-2, which stimulates proliferation of antigen-activated T and B cells; IL-4, IL-5, and IL-6, which stimulate proliferation and differentiation of B cells; Interferon gamma (IFN-θ), which activates macrophages; IL-3, IL-7, Granulocyte Monocyte Colony-Stimulating Factor (GM-CSF), which stimulate hematopoiesis and IL-12, which stimulates the growth and function of T cells and alters the normal cycle of apoptotic cell death.

1.4.1.2.1 Interleukin 12

Interleukin 12 (IL-12) is an important regulatory cytokine that has a function central to the initiation and regulation of cellular immune responses. It has the capacity to regulate the differentiation of naive T cells into TH1 cells, which is crucial in determining resistance and the type of response that will be elicited in response to a particular pathogen or in this case to the tumour. It stimulates the growth and function of T cells and alters the normal cycle of apoptotic cell death.

1.4.1.2.2 Structure of Interleukin 12

IL-12 is one of a large group of cytokines that folds into a bundle of four alpha-helices. It is a 75 kDa heterodimeric protein consisting of two covalently linked glycosylated chains; p35 and a p40 subunit. The smaller p35 subunit is required for transducing intracellular signals through the *IL-12* receptor and the larger p40 facilitates protein binding interactions (Zhou *et al.*, 2005). The *IL-12* p40 and p35 chains are encoded by two separate genes that bear no apparent homology (Brandhuber *et al.*, 1987). The gene encoding the p40 chain is mapped to chromosome 5q31-q33, a region that encodes many cytokines and cytokine receptors, and the gene encoding the p35 chain is located on chromosome 3p12-3q13.2. The two genes that encode the murine p40 and p35 counterpart chains contain 70 and 60% sequence homology, respectively, to the human genes (Xiaojing *et al.*, 1996). Only a single receptor chain has been identified for *IL-12*, labeled the *IL-12Rbeta1* receptor. It has a structure of about 100 kDa in humans and mice is very homologous to the leukemia inhibitory factor (LIF) receptor (Chua *et al.*, 1995).

1.4.1.2.3 Function of Interleukin 12

The main function of *IL-12* is to direct the immune system towards a cell mediated (or Th1) $CD4^+$ type response; it promotes the differentiation of naïve T-cells into Th1 cell thereby activating them. It also activates cytotoxic $CD8^+$ T-cells and natural killer (NK) cells. *IL-12* enhances the cytotoxic effects of $CD8^+$ T-cells and NK cells by inducing their proliferation and IFN-θ production. *IL-12* also acts on $CD4^+$ T-cells and this induces IFN-θ production. IFN-θ has a powerful effect on phagocytes and dendritic

cells; it induces them to produce *IL-12* and this produces a powerful positive feedback loop. This positive feedback loop provides a strong arsenal of cytokines that protect against intracellular pathogens (Colombo *et al.*, 2002.). *IL-12* is produced mainly by activated monocytes and dendritic cells on encountering potentially infectious agents. *IL-12* is secreted by other cells, for example, neutrophils and B-cells (Adorini, L. 1999).

1.4.1.2.4 Interleukin 12 Receptors

IL-12 receptors (*IL-12*R) are type I transmembrane cytokine receptors. *IL-12* receptors are primarily expressed on activated T ($CD4^+$ and $CD8^+$) and natural killer (NK) $CD56^+$ cells but they have been found on other cell types (Zitvogel *et al.*, 1995). The receptor consists of two subunits designated as *IL-12*R_1 and *IL-12*R_2. Both these subunits belong to the gp 130 subgroup and have intracellular domains which interact with the Janus family kinases JAK2/TYK2 on receptor activation (*IL-12* binding) (Colombo *et al.*, 2002). This brings the receptor associated JAK2 into close proximity to the receptor intracellular domains and intramolecular phosphorylation leads to activation of JAK2. JAK2 phosphorylates and activates STAT4. The protein encoded for by STAT4 is a member of the STAT family of transcription factors. In response to cytokines and growth factors, STAT family members are phosphorylated by receptor associated kinases, and then form homo- or heterodimers that translocate to the cell nucleus where they act as transcription activators. This protein is essential for mediating responses to IL-12 in lymphocytes, and in regulating the differentiation of T helper cells. The binding of *IL-12* also activates the map kinase pathway which activates downstream MKK6 and p38. Full activation of STAT4 also requires its phosphorylation by p38.

Fully activated STAT4 translocates to the nucleus where it promotes the transcription of the ERM gene (BioCarta, 2006). The ERM belongs to the Ets family of transcription factors. ERM promotes the transcription of *IL-12* inducible genes such as IFN-θ, IL-18 receptor and the CCR5 chemokine. All these gene products are necessary for the full maturation of $CD4^+$ T-cells. It has also been established that Ets transcription factors are important in the development of NK cells (Ouyang *et al.* 1998). $CD8^+$ Cytotoxic T cells are possibly activated via the *IL-12*/STAT4/ERM pathway.

STAT1 is the key transcription factor for IFN-θ mediated gene expression and for full transcriptional activation it requires both serine 727 phosphorylation and tyrosine 701 phosphorylation (Jove, 2000). The activation of STAT1 is necessary for interaction with regulatory molecules such as MCM5, BRCA1, and p53 as well as to enhance IFN-θ mediated apoptotic gene expression, such as the up-regulation of FasL, tumour necrosis factor (TNF)–related apoptosis-inducing ligand, p21, and p27 (Ouchi *et al.*, 2000). STAT1 activation in the host is required for dendritic cell maturation and IL-12–mediated generation of CTL activity. Expression of STAT1 in tumour cells negatively regulates angiogenesis, tumourigenicity, and metastasis and knockout of STAT1 in the host abrogates IFN-θ mediated tumour regression and IL-12 tumour vaccine–mediated effect (Fallarino *et al.*, 1999). Torrero *et al.*, (2006) found that STAT1 deficiency in the host significantly enhances electroporative IL-12–mediated tumour regression, resulting in tumour eradication from 60% of SCCVII tumour–bearing mice. This effect was found to be independent of IFN-θ and FasL.

1.4.1.2.5 Interleukin 12 and the Fas ligand

The Fas ligand or FasL is a type II transmembrane protein that belongs to the tumour necrosis factor (TNF) family. The binding of Fas ligand with its receptor induces apoptosis. Fas ligand-receptor interactions play an important role in the regulation of the immune system and the progression of cancer. One of the most common ways that tumour cells evade detection by the immune system is deregulation of the Fas/Fas ligand pathway. *IL-12* is a potential modulator of FasL expression on T-cells. It has been shown that decreased Fas expression is associated with increased metastatic potential of tumour cells (Zhou *et al.*, 2005). It has already been stated that *IL-12* indirectly activates $CD4^+$ and $CD8^+$ T cells. The Fas ligand is expressed on mature $CD4^+$ and $CD8^+$ T cells and it binds to Death receptors (Fas/CD95, TNFR, TRAIL) on target tumour cells. Ligation of death receptors causes their trimerisation and death domains within the cytoplasm of the target cells bind to adaptor molecules like FADD/MORT1. The death effector domain of FADD recruits initiator Caspases (8, 10) and these activate downstream caspases (3,6 and 7) by proteolytic cleavage. This leads to target or tumour cell death by apoptosis (Roitt *et al.*, 2001). One study showed that Fas expression is directly up-regulated in human osteosarcoma LM7 and Ewing's sarcoma TC71 cell lines indicating that up-regulation of Fas by *IL-12* was not tumour specific. This Fas expression was dependant on NF-θB binding to a promoter region of the Fas gene and possibly implements a different *IL-12* activation pathway (Zhou *et al.*, 2005). Interestingly the NF-θB transcription factor is activated by tumour necrosis factor (TNF) binding to TNF receptor in an alternative pathway to apoptosis activation. Activated T and NK cells also secrete TNFθ in addition to IFN-θ. This NF-θB

activation is thought to offset apoptosis initiation (Roitt, *et al.*, 2001). The above study (Zhou, *et al.*, 2005) may link TNFR/NF-θB pathway in the up-regulation of Fas-like receptors and enhanced death signal transduction rather than in the offsetting of apoptosis. Tumour cells tend to down regulate the normal process of antigen presentation via the MHC class I pathway. This down regulation prevents tumour specific antigens from being presented to $CD8^+$ cytotoxic T cells and their subsequent destruction via death receptor ligation. A minority of cytotoxic T cells are $CD4^+$ and recognise tumour antigens bound to MHC class II molecules. Natural killer (NK) cells are also induced via the *IL-12*/IFN-θ pathway and this leads to their increased lytic activity on target cells. NK cells specifically recognise target or tumour cells that lack MHC class molecules. The molecules that detect the presence (or absence) of MHC class are called killer inhibitory receptors and are designated CD94. CD94 interact with MHC class I dependant HLA-E which are expressed along side MHC class I molecules. Failure of target cells to express MHC class I (along with the associated HLA-E) initiates killing mechanisms. NK cells deposit cytotoxic granules (perforin and granzymes) in a Ca^{2+} dependant phase which polymerise on the surface of target cells. Polymerisation of perforin creates pores in the target cells membrane which allows the entry of granzymes which kill target cells by direct interaction with initiator caspases or disruption of biochemical pathways essential for cell survival (Roitt *et al.*, 2001).

1.4.1.2.6 The Anti-angiogenic effects of Interleukin 12

IL-12 has a strong anti-angiogenic effect on tumours and it acts indirectly through the induction of IFN-θ. IFN-θ mediates its anti-angiogenic affects in two main ways. Firstly it induces the production of interferon inducible protein-10 (IP-10), which belongs to the CXC chemokine family. IP-10 is produced by a number of different cells including: activated monocytes, keratinocytes, fibroblasts, endothelial cells and T-cells. IP-10 has been found to inhibit basic fibroblast growth factor induced neovascularisation in athymic murine models. It has also been found to suppress endothelial cell differentiation into tubular capillaries. IFN-θ inhibits metalloproteinase which are required for the breakdown of the extra-cellular matrix (ECM). Breakdown of the ECM is necessary for angiogenesis and the production of new capillaries (Siddiqui *et al.*, 2006). *IL-12* induced (via IFN-θ) IP-10 has been shown to induce chemotaxis of activated T-cells. IP-10 binds to the seven-transmembrane spanning G-protein coupled receptor CXCR3 which is expressed on activated T-cells leading to chemotaxis. Therefore IP-10 is responsible for the recruitment of $CD8^+$ T-cells and NK cells to the site of tumour production (Pertl, *et al.*, 2001). CXCR3 receptors have also been found on endothelial cells and studies have suggested that ligation of CXCR3 may directly inhibit angiogenesis at endothelial cell level. Another cytokine called Mig seams to act in an analogous way to IP-10 and has a similar specificity for the CXCR3 receptor. Signalling molecule called I-TAC and possibly also *IL-12* may act directly to inhibit angiogenesis. Some studies have found the anti-tumour/anti-angiogenic effect of *IL-12* are dependant on the induction of IFN-θ and CXCR3 ligands but do not require the presence of T or NK cells. It has been demonstrated that recombinant *IL-12*

secreting fibroblasts are able to transduce their anti-angiogenic effects on both human and murine models lacking NK cells. It has also been found that *IL-12* inhibits the mitogen; vascular endothelial growth factor which is essential for angiogenesis. Overall it seems that IP-10/Mig, a full complement of $CD4^+$ and $CD8^+$, and NK cells are all needed to orchestrate the full anti-angiogenic effects of *IL-12* (Colombo *et al.*, 2002).

1.4.1.2.7 Interleukin 12 and antigen presenting cells

Activation of the *IL-12*/ IFN-θ pathway at the site of tumour formation can lead to the recruitment and infiltration of one of the most effective antigen presenting cells; dendritic cells. These phagocytotic APCs (antigen presenting cells) can present tumour antigens via the MHC class II restricted pathway to $CD4^+$ T cells. Intercellular adhesion molecules like ICAM-1/LAF1 and co-stimulatory CD28/B7-1 or LFA-3/CD2 may enhance the interaction between APCs and T helper cells. Activated $CD4^+$ T cells bearing co-stimulatory molecules may enhance the actions of dendritic cells and other phagocytes. All these processes can lead to enhance tumour recognition (Roitt *et al.*, 2001). *IL-12* promotes differentiation of naïve Th cells by enhancing the secretion and responsiveness of IL-2. IL-2 can augment proliferation and/or induce IFN-θ secretion which directly up-regulates APC costimulatory molecules such as B7 family, and ICAM-1. *IL-12* can also prevent the death of naïve Th cells and promote their expansion by an as yet unknown mechanism (Palmer, *et al.*, 2001). *IL-12* can also modulate the production of $CD4^+/CD25^+$ regulatory T cells (Treg). Treg cells are necessary to prevent over activation of the immune system which can lead to

autoimmune diseases. *IL-12* has been shown to significantly repress these cells in some mammary carcinomas (Spadaro, *et al.*, 2005).

1.4.1.2.8 Additional anti-tumour actions of Interleukin 12

Additional anti-tumour actions of *IL-12* are directly related to the induction of Th1 type responses. There is some evidence of complement activation, leading to the opsonisation of certain IgG antibodies and these complexes demonstrate anti-tumour activity *in vivo*. The hyperinduction of IFN-θ by *IL-12* can result in activation of inducible nitric oxide synthase (iNOS) and Nitric oxide (NO) production in macrophages. NO is responsible for transient but profound immunosuppression which results in decreased T cell proliferation and impaired immune responses. The exact mechanism(s) by which nitric oxide suppresses lymphocyte function is unknown but likely to be related to its ability to inhibit ribonucleotide reductase activity and/or mitochondrial respiration (Schwacha *et al.*, 1997). In addition to oxygen-derived free radicals, nitric oxide can act as a free radical and be converted to an even more reactive anion. Free radicals cause lipid peroxidation in cell membranes, oxidation of amino acids and proteins which results in fragmentation, and protein- protein cross linkages. Altered proteins are acted on by the proteosomes with further cell damage. Free radicals may be a common pathway for most types of cell damage, particularly oxygen derived free radicals (oxidative stress). This decrease in T cell proliferation and impaired immune response leads to an overall decrease in the anti-tumour action of *IL-12* (Colombo *et al.*, 2002.).

Systemic administration of *IL-12* results in inflammatory syndromes similar to the immune systems responses to severe infections. *IL-12* could be used as a powerful therapeutic weapon in the treatment of cancer but systemic toxicity to the patient limits its everyday use. Its use as an adjuvant in cancer vaccines and its targeted use in gene therapy applications has a potentially promising future.

1.4.1.2.9 IL-12 electroporative tumour treatment

IL-12 has been examined as a potential tumour gene therapy delivered by electroporation. None of the previous studies has involved the application of an SFV based plasmid and all involved the delivery of DNA rather than RNA. The pulse form that was applied generally for the delivery of the IL-12 DNA electroporative gene therapy was two pulses of 450 v/cm and 20 msec duration (Liu *et al.*, 2006, Torrero *et al.*, 2006, Li *et al.*, 2005). This pulseform was compared to the optimized SFV RNA intratumoural electroporation pulseform but was found to result in a lower transfection efficacy (see appendix 2A).

Previous studies examining the electroporative IL-12 treatment of SCCVII tumours following a single electroporative treatment resulted in a transient decrease in the rate of tumour growth with mice surviving up to 30 days longer than the control mice (Liu *et al.*, 2006). A similar result was observed by Torrero *et al.*, (2006) where a single electroporative treatment of 30 Δg of IL-12 DNA under the control of a CMV promoter resulted in a transient inhibition of SCCVII tumour growth. In this same study a single intratumoural electroporation with 0.5 units of Bleomycin was examined resulting in a similar but more significant transient inhibition of tumour growth. Only a combination

of IL-12 and Bleomycin resulted in complete tumour regression in 40% of the treated group (n=5). A 60% survival was obtained by Torrero *et al.*, 2006 when two treatments of the electroporativley delivered combination of IL-12 and Bleomycin were given eight days apart though two treatments did not significantly increase the tumour inhibition when IL-12 or Bleomycin were used as individual electroporative treatments.

Multiple electroporative IL-12 treatments resulted in the eradication of SCCVII tumours in 40% of treated mice (Li *et al.*, 2001, Li *et al.*, 2005). Treatments were initiated once tumours had reached 4 – 6 mm in diameter, with a second administration performed one week later and subsequent administrations performed every four days. Two pluses of 400 v/cm and 20 msec duration was used in each case but when the voltage was increased to 500 v/cm and the pulses given every ten days this resulted in the eradication of SCCVII tumours in 80% of treated mice but this data was not shown (Li *et al.*, 2001).

1.4.2 Pro-apoptotic cancer gene therapy

Evidence that antitumour therapies function by inducing apoptosis is revealing the crucial role apoptosis plays in tumourigenesis and antitumour therapy. Because cells have varying susceptibility to apoptosis induction, chemotherapy or radiation therapy may induce apoptosis in tumour cells and merely arrest the cell cycle of their normal counterparts, thereby opening a therapeutic window (Levine *et al.* 1994). Correspondingly, insensitivity to apoptosis induction may be a major mode of resistance to antitumour therapy. Apoptosis has also been shown to directly regulate tumourigenesis. The widely expressed *Bax* gene is one of the well-characterized

proapoptotic genes, and its overexpression leads to apoptosis in a wide variety of cells, with or without other additional stimuli (Xiang *et al.* 1996).

1.4.2.1 *Bax*

1.4.2.1.1 *Bax* and the Bcl-2 family of proteins

Bax belongs to the Bcl-2 family of proteins. Members of the family are involved in the regulation of apoptosis with both pro- and anti-apoptotic function. In mammals over 30 such relations have been described. The Bcl-2 family of proteins can be categorised into subfamilies based on the conserved sequence motifs known as Bcl-2 homology domains (BH1-BH4) that they contain. The *Bax* subfamily consists of *Bax*, *Bak* and *Bok* and they all posses the BH1, BH2 and BH3 domains (in contrast to other pro-apoptotic Bcl-2 member that only contain the BH3 domain). *Bax* is a cytosolic protein monomer in healthy cells. Initiation of *Bax* mediated apoptosis begins with a conformal change in the protein at both termini and it translocates to the other mitochondrial membrane (OMM). Oligomerization of *Bax* at the OMM is aided by the hydrophilic C-terminal domain which facilitates OMM attachment. *Bax* may form homodimers with *Bak*, *Bax* or aggregates which create pores in the OMM by insertion. Whatever the mechanism the outcome is the same; the loss of the electrical membrane potential and the release of pro-apoptotic factors, like cytochrome c, endonuclease G, Diablo, Dmi, etc., causing the activation of initiator caspases further downstream. Anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-XL etc.) sequester pro-apoptotic Bcl-2 members by binding to their BH3 domains. This prevents *Bax* or *Bak* oligomerization

and/or aggregation thereby protecting the OMM from permeablisation. Etopic over expression of anti-apoptotic members of the Bcl-2 family (Bcl-2, Bcl-XL etc.) would inhibit apoptosis. Similarly over expression of pro-apoptotic Bcl-2 members would induce cellular apoptosis. Therefore a mechanism has been proposed for the mitochondrial dependent apoptosis pathway; the death decision occurs at the mitochondria and it is dependant on the balance of pro-apoptotic and anti-apoptotic proteins at the OMM. Therefore etopic *Bax* expression induces cellular apoptosis in a dose dependent way (Gewies, 2003, Adams, 2003).

1.4.2.1.2 *Bax* and *p53*

Studies have shown that the *Bax* gene is a direct transcriptional target of p53 (Miyashita *et al.*, 1995) and tumours with a loss of p53 function are found to contain low levels of Bax protein (Basu *et al.*, 1998). p53 is a proapoptotic tumour suppressor gene. It is thought that the induction of irreversible cell suicide processes or the imposition of reversible growth arrest is how p53 yields it's tumour suppression effect through its function as a transcriptional modulator (activates or suppresses the transcription of other genes) (White, 1996). It has also been shown that the induction of p53-independent apoptosis by the SFV vector is an inherent function of the vector RNA and not dependent on expression of cloned heterologous genes (Glasgow *et al.*, 1998). Previous work has further developed the SFV vector induction of p53-independent apoptosis by incorporation of the Bax gene with the SFV vector resulting in p53-independent apoptosis in H358a human lung carcinoma xenografts in BALB/c nu/nu mice (Murphy *et al.*, 2000).

1.4.2.1.3 *Bax* activation

While the p53 protein drives the transcription of *Bax* protein, *Bax* is also activated by BH3 only proteins that trigger apoptosis in response to environmental signals (Adams, 2003). The c-Jun N-terminal kinase (JNK) is activated when cells are exposed to stress including ultraviolet radiation. JNK phosphorylates the two BH3 only proteins Bim and Bmf that are normally sequestered by dynein and myosin. Phosphorylation of Bim and Bmf allows their disassociation with dynein and myosin. The cytoplasmic release of Bim and Bmf is presumably due to cytoskeleton disruption in response to cellular damage. This leads to activation of Bak/Bak dimers at the mitochondrial membrane and influences the cell to undergo apoptosis (Lei, & Davis, 2002). Bid is another BH3 only protein that is responsible for the activation of *Bax*. Ligation of a death receptor (TNFR, CD95/Fas or DR5) at the cells surface leads to the cytoplasmic activation of pro-caspase 8. Caspase 8 cleaves bid into two fragments, a C-terminal fragment tc-Bid and an N-terminal fragment tn-Bid. tn-Bid translocates to the mitochondria where it triggers *Bax* and *Bak* oligomerization by inducing a conformal change in the N-terminal domain of *Bax* (Roucou *et al.*, 2002). Withdrawal of survival signals like cytokines (especially IL-3) and insulin-like growth factor prevents the phosphorylation of BH3 only protein Bad at three key sites (Ser-112, 136 and 155). Phosphorylated Bad is normally sequestered in the cytoplasm by 14-3-3 and free cytoplasmic Bad interacts with the BH3 domains of anti-apoptotic Bcl-2 members. This alters the balance of anti-apoptotic and pro-apoptotic factors at the OMM and apoptosis is initiated by the loss of apoptogenic mediators from the inner mitochondrial space (Kelekar *et al.*, 1997).

1.5 Drug delivery systems for cancer

A major limitation of molecular approaches to the treatment of cancer is delivery of the drugs to the cells in solid tumours. The drug must first pass into the blood vessels of the tumour, through the vessel wall, and then into the substance of the tumour. Innovative anticancer therapies in development face similar problems. Obstacles to these steps are inherent in tumours. Drug delivery to solid tumours consists of multiple processes, including transport via blood vessels, transvascular transport, and transport through interstitial spaces. These processes are dynamic and change with time and tumour properties and are affected by multiple physicochemical factors of a drug, multiple tumour biologic factors, and as a consequence of drug treatments. Factors that influence drug transport and delivery to solid tumours include the following (Jang *et al.*, 2003):

1. Differences in the vasculature in normal and tumour tissues
2. Transport of drugs and particles across tumour vasculature into surrounding tumour tissues.
3. Barriers of drug transport, accumulation, and retention in tumours.

1.5.1 Challenges of cancer drug delivery

Chemotherapy for cancer has adverse effects, which can be modified by appropriate methods of delivery. Similarly efficacy of anticancer drugs can be improved by optimal delivery. However, cancer drug delivery provides some challenges. Proteins and peptides being developed for treatment of cancer face the general problems of delivery of this category of pharmaceuticals and will be discussed in the following section. An

anatomical barrier to targeted drug delivery to the tumour is the tumour blood vessel pore barrier.

1.5.2 Approaches used to enhance drug delivery and transport in solid tumours

The effectiveness of cancer therapy depends in part on adequate delivery of the therapeutic agents to tumour cells. A better understanding of the processes and contribution of these factors governing drug delivery may lead to new cancer therapeutic strategies.

Changes in drug delivery as well as new drug development have had a major impact on cancer care in the past twenty years. Several modified release chemicals now have a role in cancer care, and new routes of drug delivery have been established as part of standard care (Tattersall and Clarke, 2003).

Several drug delivery systems have been investigated for possible administration of therapeutics for cancer. Protein and peptide therapeutics for cancer require special methods of delivery, including oral, nasal, and pulmonary routes. Intravenous drug delivery, antineoplastic drug implants, and use of monoclonal antibodies (MAbs) are promising methods of cancer drug delivery. One unique feature of cancer is that localized delivery of drugs directly into the tumour mass is a possibility. One innovative method of delivery of therapeutic substances in cancer patients is gene therapy.

1.5.3 Routes of drug delivery in cancer

Intravenous injection is a common method. Innovations in intravenous delivery of anticancer agents are described briefly. The main emphasis in this section will be on oral anticancer drug delivery. Local delivery to the tumour will be discussed in the following section on strategies.

1.5.3.1 Intravenous delivery

Currently, most chemotherapeutic drugs are administered intravenously in intermittent doses, typically via two-hour infusions of the drug every six hours. Previous studies have reported that frequent dose adjustments are needed to maintain the desired level of drug in patients and that metabolism of the drug varies from patient to patient (Algozzine, 2002). It has recently been shown that continuous-infusion achieved more predictable levels of the drug than the usual intravenous delivery method. In the small number of patients tested with continuous infusion, there did not appear to be a change in concentration or clearance of the drug during the infusion period with less than 10% variability. In the standard intermittent delivery method, the patients metabolized the drug more slowly as the intermittent doses continued and the variability between the test-dose prediction and the actual levels was more than 20% (Walko *et al.*, 2005). Continuous infusion may prevent side effects and increase effectiveness by maintaining a more consistent level of drug in the blood and avoiding extreme high and low blood levels. There are many difficulties with intravenous drug delivery including high cost (as administration can only be done by medical professionals and the high doses required) and short-term systemic effects (Nicolucci *et al.*, 2008,Wang, 2005).

1.5.3.2 Regional intra-arterial delivery

Regional intra-arterial chemotherapy has been used for a number of cancers often in combination with other methods of treating cancer. The main advantage is marked reduction of toxicity observed with systemic administration of the same drugs (Yamshita *et al.*, 2005).

1.5.3.3 Oral delivery

Most conventional anticancer agents are injectables. Oral formulations of anticancer agents are desirable for ease of administration and patient convenience. Interest in oral anticancer drugs is growing because they may reduce costs and improve patient satisfaction. Oral therapy can potentially reduce resource utilization and health care system costs, improve drug safety, and enhance patient satisfaction. There are some problems in oral delivery of anticancer agents. An appreciation of the role of transporters on the absorption and secretion of drugs is critical in understanding the poor and erratic bioavailability observed when the oral route is utilized for administration of anticancer drugs (Wang, 2005). Approximately 17% of the drugs in clinical development are intended for oral delivery. This figure will surely become more significant in the near future, providing a quick implementation of technologies for improving inter-subject variability, which is particularly erratic in cancer patients, and to appropriately formulate highly potent cytotoxic drugs (about a quarter of the drugs under development). Active research is underway on the inhibition of natural detoxification mechanisms of the body, such as gastrointestinal P-glycoprotein, as a way of improving the oral bioavailability of drugs (Ghebre-Selassie, 2004).

1.5.3.4 Nasal Delivery

The nasal cavity has been exploited as a route of systemic drug delivery due to ease of accessibility and because this route often suits the absorption profile of many drugs not suitable for oral delivery (Wang, 2005).

1.5.3.5 Transdermal drug delivery

Transdermal drug delivery systems have a limited application in cancer because only a few suitable agents are available for transdermal delivery of anticancer agents (Jain 2006). The technologies for administration of large molecule proteins are limited by this route except by electroporation and ultrasound. Transcutaneous electroporation has been used to deliver anticancer drugs and vaccines across the skin. Methods to extend the upper molecular weight limit of transcutaneous electroporation have been developed. The pro-photosensitizer drug, delta-amino levulinic acid, the anticancer drug methotrexate, and peptide vaccines designed for cancer prevention and immunotherapy have been delivered transcutaneously by electroporation (Hui 2002). These studies hold promise for the treatment of cancers in human.

1.6 Electroporation

Electroporation is the delivery of short and intense electric pulses into living cells (either in tissue or suspension), which results in a transient and reversible alteration of the cell transmembrane potential (Neumann *et al.*, 1999). This alteration of the cell transmembrane potential has been experimentally observed and may easily be calculated using Maxwell's equations (see page 76) (Bonnafous *et al.*, 1999). The alteration of the transmembrane potential induces transient membrane permeability hence a large number and variety of hydrophilic molecules, DNA etc., are able to diffuse through the plasma membrane (Mir *et al.*, 1995). This permeability is a result of a change in the distribution of the membrane proteins in response to the electrophoretic drag caused at the inter-electrode space when a voltage is applied.

1.6.1 Theory of electroporation

There have been many theoretical models proposed to explain the mechanisms of electroporation and gene transfer (Wells *et al.*, 2004), but no clear evidence has yet been obtained on how the DNA molecules interact with the electropermeabilized cell plasma membrane. The most widely accepted theoretical models proposed in the case of mammalian cells are the molecules cross the cell membrane due to:

(i) the formation of small hydrophobic openings or pores in the plasma membrane (Neumann *et al.*, 1997) (Figure 1.1),

(i) a binding step at the cell surface followed by a electroporation driven diffusion through the pores formed on the membrane surface (Xie *et al.*, 1993),

(i) electrophoretic forces associated with the external field (Sukharev *et al* 1992),

(i) adsorption by sphingosine-DNA interactions, insertion, and passage of DNA through a hydrophilic percolated porous zone (Neumann *et al* 1997).

The models outlined above suggest that molecules can enter the cell either during or after the delivery of the electric pulses. Current results have shown that the plasmid DNA for gene therapy has to be present prior to or during the electroporation pulse delivery for electroporative gene transfer. No gene transfer has been detected when a plasmid is added post electroporation. This has been shown for bacteria, yeast, and mammalian cell lines (Golzio *et al.*, 1998).

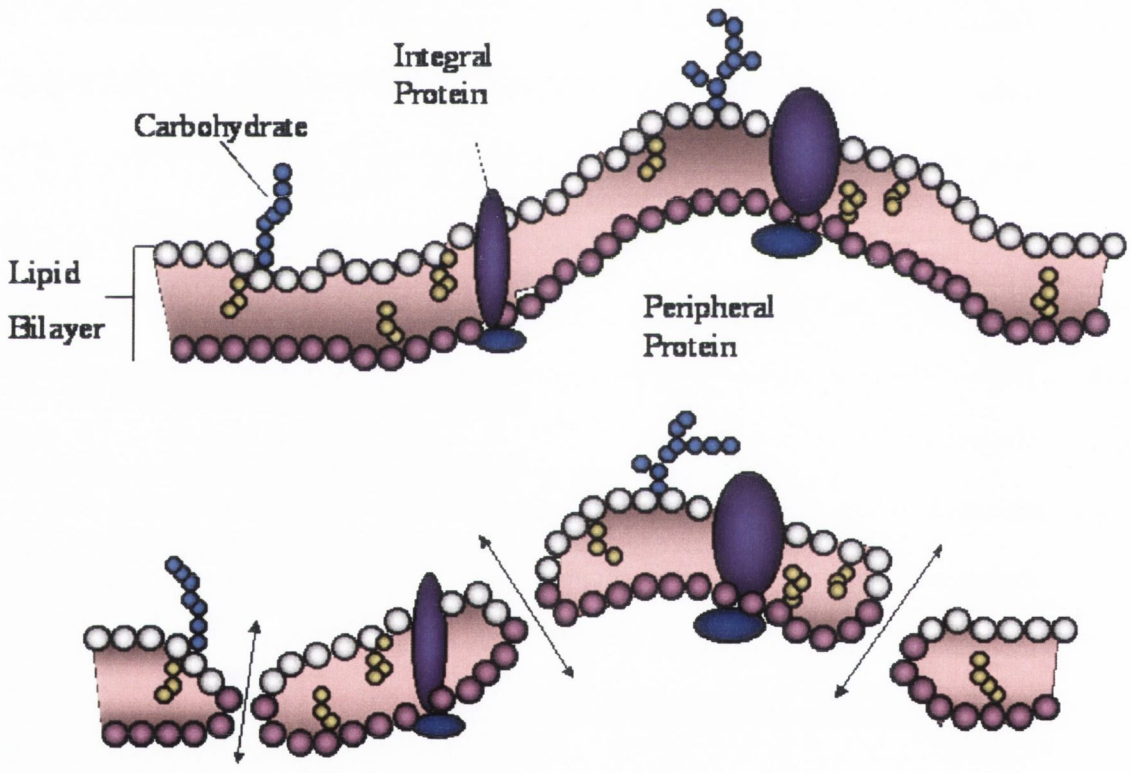


Figure 1.1: Pictorial representation of the theoretical cell prior to and post electroporation

Each of the different theories described above are based on confluent biological membranes that are subjected to electrical fields such as when capacitors are subjected to an applied voltage. Hence the mathematical models that are used to develop these theories are based on the bipolar lipid membrane mimicing capacitor behavior. The lipid membrane will build up a transmembrane potential (as with a capacitor) upon the application of an electrical field until the dielectric strength of the membrane is exceeded resulting in an increase in transmembrane electrical conductivity and transient permeation of the membrane. The biologic affect of electric fields on living cells has been under investigation from the early 1980s (Neumann *et al.,* 1989, Chang *et al.,* 1990, Zimmerman *et al.*, 1982). While it has been well established that extracellular molecules excluded by cell membranes can gain entry into the cell after treatment with an electric field very few structural investigations of the field effect on membranes are available. The electron microscope pictures published by Chang *et al.*, (1990) showing "craters" are thought to be misleading experimental artefacts as they were obtained specifically on red blood cells pulsed under strongly hypo-osmolar conditions and were detected only several milliseconds after the pulse. They have been reported to be reflecting the creation of hemolysis pores induced by the cell swelling. No such defects have been observed under iso-osmolar conditions or on other cell models (Escande-Geraud *et al.,* 1988). The toroidal pore as proposed by Chizmadzhev *et al.,* (1979) and others does not take into account the contributions of proteins and protein submembraneous networks. While the formation of pores is still a widely accepted theory, molecular descriptions of the membrane transitions remain highly speculative

due to a lack of information of the membrane transitions supporting the transmembrane traffic of polar compounds (Tessie *et al.*, 2005).

1.6.2 In vivo electroporation

In the case of in vivo electroporation, current electroporative theories and simulations may not be completely adequate since the extracellular milieu surrounding cells is not a homogeneous fluid. Cells within tissues are configured in a three- dimensional array that is usually modeled as a matrix of capacitors, conductors, and resistors. Furthermore, cells in tissues can also be considered to be a matrix in a fixed orientation within an electrochemical space. Therefore, from an electrical and chemical potential perspective, the complexity and variability of both tissue and cellular architecture inhibits accurate modeling. Even the initial step of in vivo delivery by electroporation that involves the association, binding, or proximity between cell membranes differs for drugs and nucleic acids. Virtually all studies that examine temporal relationships between electroporation and nucleic acid administration have demonstrated that optimal results are achieved by administering the nucleic acid before electrical treatment. It has been found that many low molecular weight compounds may be administered either before or immediately after field pulsing without influencing uptake (Gehl, 2003). This indicates that there may be a difference in the mechanisms by which genes and drugs cross the cell membrane following electroporation. This difference has not been adequately explained and hence in vivo application of these basic electroporative theoretical principles requires further analysis.

1.6.2.1 Electroporation and drug delivery

There is no advantage in combining electroporation with lipophilic drugs as permeabilizing the cell membrane does not increase the level of delivery of these drugs; however there is considerable gain in combining electroporation with hydrophilic drugs (Gehl *et al.*, 1998, Jaroszeski *et al.*, 2000). After the first report of the clinical use of electroporation (Belehradek *et al.*, 1993), several studies have reported the use of electroporation for delivery of chemotherapy (electrochemotherapy) to cancer patients (Mir *et al.*, 1995, Panje *et al.*, 1998, Sersa *et al.*, 1999, Sersa *et al.*, 2000, Sersa *et al.*, 2006).

1.6.2.1.1 Electrochemotherapy

The major disadvantage with current established chemotherapeutic treatments is that they lack sensitivity. It has been found that high dose of these drugs are needed to produce an antitumour effect, which often results in systemic toxicity and severe side effects. Some drugs while having a good potential antitumour effect are hampered by an inability to cross the plasma membrane. One such drug is bleomycin. Bleomycin is a hydrophilic and charged molecule, which under normal circumstances, is only internalized in limited amounts (Mir *et al.*, 1996). Under unassisted circumstances bleomycin is transported across the nonpermeabilized plasma membrane by carrier proteins which internalize it by an endocytotic pathway. This process is limited by the low number of these carrier proteins that are exposed on on the surface of cells as well

as withdrawal of these proteins from the membrane because of endocytosis (Mir *et al.*, 2006).

Upon entry into the cell the bleomycin molecule acts as an enzyme and creates several DNA double strand breaks (Tounekti *et al.*, 1993). The application of electroporation allows entry of the bleomycin directly into the cell cytosol and its cytotoxicity can be increased 300–5000 fold (Orlowski *et al.*, 1988, Gehl *et al.*, 1998, Jaroszeski *et al.*, 2000). Several different cancer types have been treated by electrochemotherapy using bleomycin: basal cell carcinoma (Glass *et al.*, 1997), malignant melanoma (Heller *et al.*, 1998, Gehl & Geertsen, 2000, Sersa *et al.*, 2000), adenocarcinoma (Mir *et al.*, 1998), squamous cell carcinoma (Belehradek *et al.*, 1993, Panje *et al.*, 1998), transitocellular carcinoma (Kubota *et al.*, 1998) and renal cell carcinoma (Sersa *et al.*, 2000). Electrochemotherapy has been shown to be highly effective in each of these histologically different cancer types. As electrochemotherapy is a localized treatment, little or no side effects have been reported. Brief contractions of the underlying musculature are the main side-effect with adequate local anaesthesia (Miklavcic *et al.*, 2000). Although until recently principally skin tumours have been treated, electrode systems for deeper situated tumours have been developed.

Besides membrane electroporation the application of electric pulses to the tumour tissue induces a transient, but reversible reduction of blood flow. The decrease in tumour blood flow by 80% immediately after the application of the electric pulses induces drug entrapment in the tissue for several hours and hence provides more time for the drug to act. The tumour blood flow has been observed to restore to normal levels within 24 hours after the pulse application (Sersa *et al.*, 1999). In normal tissue (not tumour

tissue) the restoration of normal blood flow is within seven hours. The reduction of blood flow prevents bleeding from the tissue which is important in some clinical settings such as when treating haemorrhagic tumours (Sersa *et al.*, 2006).

A difference in the antitumour effectiveness of electrochemotherapy was been observed by some groups between immunocompetent and immunodeficient mice. Sersa *et al.*, (1997) observed a tumour growth delay in immunocompetent mice of over twice that of immunodeficient mice with an 80% cure rate for immunocompetent mice and no cures with immunodeficient mice. The difference in responses between immunocompetent and immunodeficient mice indicates that there is an involvement of the immune system in the antitumour effectiveness of electrochemotherapy. A high degree of tumour antigen shedding has been observed following electrochemotherapy (Sersa *et al.*, 1997) hence systemic immunity can be induced which can be up regulated by an additional transient treatment with immune gene therapies such as IL-2, *IL-12*, GM-CSF or TNF-θ (Torrero *et al.*, 2006).

1.6.2.2 Electroporation and gene therapy

As has been stated earlier, the transfer of DNA to cells is a process whereby the cell is permeabilized by the electrical pulse and the DNA is thought to be moved by electrophoretic forces to enter the cell cytosol during the following pulses. It has been shown that no gene transfer occurs if the DNA is added immediately after the pulse but small molecules can diffuse into permeabilized cells in the minutess prior to membrane resealing (Mir *et al.*, 1999).

Electroporation for gene transfer *in vivo* has proven highly efficient, with most studies showing up to two log increase in gene expression when comparing direct injection with electrogenetransfer (Aihara and Miyazaki, 1998, Mir *et al.*, 1999). In many cases, a broader distribution of cells expressing the reporter gene is also observed (Mathiesen, 1999, Jaroszeski *et al.*, 1999, Somiari *et al.*, 2000). Electroporation as a method for gene transfer has been reported in a variety of different tissue types such as skin (Titomirov *et al.*, 1991, Heller *et al.*, 2001), liver (Heller *et al.*, 1996), brain (Nishi *et al.*, 1996), tumour (Rols *et al.*, 1998) and muscle (Aihara & Miyazaki 1998, Mir *et al.*, 1999). Two of the main areas of research for electroporative gene transfer are muscle and tumour tissue. Muscle tissue is an ideal candidate for electrogene transfer as the myofibers neither die nor divide leading to long term expression which may be transient in other cell types (Mir *et al.*, 1999). There have been many studies examining the muscle as a target for the development of electroporative DNA vaccines (Glasspool-Malone *et al.*, 2000, Somiari *et al.*, 2000, Drabick *et al.*, 2001, Liu and Huang 2002). Generally vaccination strategies are expensive due to the need to keep stores of vaccines fresh and to transport them cold, particularly in warm climates. As electroporation equipment can easily be transported, and as DNA is very stable, this may prove a more viable vaccination strategy. It has been found that local gene transfer can be performed using electroporation giving rise to antigen production either in skin or skeletal muscle (Gehl *et al.*, 2003, Wildera *et al.*, 2000). Electroporation is also increasingly being used for RNA gene transfer, particularly to dendritic cells. Both for murine and human dendritic cells, high RNA transfection efficiencies have been shown (Saeboe-Larssen *et al.*, 2002, Van Meirvenne *et al.*, 2002).

The level and duration of gene expression after in vivo electroporation is specific to the type of tissue being electroporated and the distribution of expression can be controlled by modification of electrodes to direct field pulses to specific areas of tissues/organs. Another method of controlling gene expression and distribution following electroporation is in the use of tissue-specific promoters (Somiari *et al.*, 2000). The presence of bacterial sequences within electroporated polynucleotides may also have adverse consequences on the duration of transgene expression. However, the effect of these variables is only now beginning to be examined. While recent studies demonstrated the feasibility of electrogene therapy using a range of therapeutic genes for the treatment of experimental tumours (Heller *et al.*, 1999), the transfection efficiency of electrogene therapy is still low compared to viral methods and there is a clear need to optimize this approach.

1.7 Viral vectors

Conventional viral based vector systems for transfer of genes encoding therapeutic proteins have had major problems concerning safety and expression . There is also the problem of the induction of highly antigenic virus related gene products which could lead to an inappropriate immune response. The most commonly used vectors are derived from retroviruses, adenoviruses, adeno-associated viruses and herpes viruses (Zeng *et al.*, 2004).

1.7.1 Retroviral vectors

Retroviral vector systems have a relatively simple RNA genome of approximately 9,000 nucleotides. By reverse transcription the RNA viral genome is used as a template for DNA synthesis and viral genes are randomly integrated into the host's genome. These randomly integrated viral genes are stably transmitted to progeny cells. The vectors can be produced by the use of packaging cell lines that constitutively express proteins to produce virus particles. The gene encoding the heterologous protein and viral packaging signal are encoded on one construct and the reverse transcriptase / integrase (pol gene), envelope (env gene) and the internal structural proteins (gag gene) on another trans acting construct. Co-transfection of both gene constructs or transfection of a purposely constructed cell line yields replication defective viral particles that have the foreign gene incorporated. There are advantages of retroviral vectors for cancer gene therapy. The virus is incapable of transducing non-dividing cells and only targets dividing cells. There are a large number of vector and producer cell lines available for research and clinical applications (e.g. the Lox6 cell line (Vavin *et al.*, 1997), the 293 cell line (Ghani *et al.*, 2006), the FNX cell line (Rattmann *et al.*, 2007)). The virus particles have a high transduction efficiency. Unfortunately there are disadvantages, especially for their use as vectors for therapeutic cancer genes. The most serious disadvantage is that retroviruses are associated with causing an array of malignancies, immunodeficiencies and neurological disorders (Cornetta *et al.*, 2005). Some retroviruses of the genus lentivirus are derived from HIV-like viruses and there is always the chance that recombination could lead to infective virion production. The random integration into the host genome could juxtapose the transgene upstream of a

proto-oncogene and up-regulate its expression (or similarly inactivate a tumour suppressor gene) (Kitchingman and Garcia, 1999). In one French clinical study using retroviral vectors to deliver genes to correct a severe X-linked immunodeficiency SCID-X1 syndrome two of the youngest patients developed a leukaemia-like clonal T-cell proliferation disease 3 years after treatment (Hacein-Bey-Abina, 2003). Genomic integration is also undesirable because this can lead to possible mutagenesis.

1.7.2 Adenoviral vectors

Adenoviral vectors have double stranded linear DNA genomes approximately 36,000 nucleotides long. The whole genome is divided into early (E) and late (L) transcripts with each region coding for a multiplicity of mRNAs and protein products (Kitchingman and Garcia, 1999). Up to 30 kb of the viral genome can be replaced with foreign gene sequences if required (Smith, 1995). All early and late genes are required for viral replication with the exception of early region 3 (E3), which is not required for growth in tissue culture. Any deleted genes must therefore be supplied by trans acting plasmid constructs or helper viruses. There are two methods of insertion of foreign gene into adenoviral vectors. Homologous recombination can be carried out in tissue culture, bacteria or yeast cells and it involves using a shuttle vector carrying the foreign gene flanked by 2-4 kb of viral genome at each end. This shuttle vector is co-transfected with a DNA construct representing the remainder of the viral genome. Recombinants can be detected by including marker genes and selecting individual plaques. The second method involves cloning the full length adenoviral genome in bacterial plasmids. This method has advantages over the former in that all plasmid

clones that form plaques must contain the inserted transgene (because the foreign gene product was carried and cloned within the plasmid construct) (Kitchingman and Garcia, 1999). The major advantage for cancer gene therapy is that adenoviral vectors do not integrate into the hosts' genome therefore there is no risk of insertional mutagenesis. The DNA vectors exist in host cells as stable episomes and only persist as long as the cell lives. Adenoviral vectors have a broad host range and can infect most cells with the exception of haematopoietic stem cells. Adenoviruses have a high transduction efficiency, infect both dividing and non-dividing cells and viral titres are high. There has been some research to show that adenoviral vectors are selectively toxic to certain tumours when used in conjunction with chemotherapeutic agents. The rationale behind this is that either the adenovirus E1A protein is a potent chemo-sensitizer or that it induces high levels of p53 leading to apoptosis (Khuri, *et al.*, 2000). The major disadvantages of adenoviral vector constructs are that high levels of viral proteins are expressed along with the transgene and these are immunogenic. Development of adenoviral vectors to deliver therapeutic or toxic compounds to cancer cells has been hampered by underestimation of host immunity to the virus. There have been a number of efforts to counteract this problem, including the use of immunosuppressive therapies, but none have been successful (Kitchingman and Garcia, 1999).

1.7.3 Adeno-associated (AAV) viral vectors

Adeno-associated viruses (AAV) belong to the family Parvoviridae and the genus *Dependovirus*. As suggested by the genus the virus is replication defective on its own and it is dependant on 'helper viruses' (usually adenovirus or herpes virus) for

replication (Kitchingman & Garcia, 1999). The AAV has a single-stranded DNA genome of only 4700 nucleotides. At each end of the genome there are 145-150 bp palindromic nucleotide sequences or inverted terminal repeats (ITRs). These ITRs are all that are required for integration into the host's genome. The AAV vectors can be constructed to carry up to a 5 kb inserted transgene and this insert replaces the two wild type genes rep and cap (Smith, 1995). The rep and cap genes are necessary for replication and capsid structural protein assembly respectively. Any transgene sequence must carry its own enhance and or promoter sequence. There is potential for wild-type AAV to target the human genome at chromosome 19 and at specific location (13-qter) (Kotin, *et al.*, 1990). This occurs at high frequency with the wild-type AAV in the absence of a helper virus and in the presence of rep. AAV vectors seem to have lost this ability and integrate randomly into the hosts' genome. A number a different systems have been developed for using the AAV vector for transfer of foreign genes. They all rely on the fact that replication requires three components: a construct that supplies the cap and rep genes, the transgene flanked by the ITRs and the helper virus gene products (from the adenovirus genome). One of the major advantages is that they are incapable of independent replication, nonpathogenic to humans and therefore very safe. They are able to transduce many cell types and there is some potential for site specific genomic integration. Generally they lack the ability to generate an immune response (however helper virus products that are co-transfected maybe highly immunogenic). The biggest disadvantages are their limited capacity to package large genes and they are difficult to produce in high titres. One of the reasons for a low titre is the inhibitory effect of the rep gene product on adenovirus replication which is required as a helper virus. The

need for helper viruses results in vector preparations with high levels of contamination. Contamination with helper viruses is undesirable especially in clinical applications (Kitchingman and Garcia, 1999).

1.7.4 Herpes simplex viral vectors

The herpes simplex virus type 1 (HSV-1) is a human neurotrophic virus. The wild type virus can exist in a non-immunogenic latent state as an episome or it can proceed to the lytic stage of its life-cycle. HSV-1 has a double stranded DNA genome 152,000 nucleotides long and encoding 81 gene products (Marconi *et al.*, 1996). There are three general classes of genes; immediate early, early and late genes. Immediate-early genes encode trans acting factors that control expression of early genes. Early genes control nucleotide metabolism and genomic DNA replication. Early genes also activate late genes which encode viral structural proteins (Kitchingman and Garcia, 1999). Many of these genes are not necessary for viral replication and can be deleted. The virus can accommodate a maximum insert in the region of 40-50 kb (Glorioso *et al.*, 1995). There are two methods of vector construction. Amplicons are hybrids of bacterial plasmids and contain HSV-1 and *E. coli* origins of replications, packaging sequences, transgene under the control of the immediate-early promoter and a selectable marker. Transfection of the amplicons into a cell line that provides all the deleted structural and regulatory gene components or co-transfection of a helper virus and the amplicon yields high-titer amplicon containing virus particles carrying the transgene. Recombinant HSV are constructed by deleting certain immediate-early genes. Deletion of these genes renders the virus replication deficient affecting its cytotoxicity and pathogenicity.

Immediate-early gene deletion allows the expression of promoters that could be utilised for constitutive expression which would otherwise be silent in the wild-type latent virus (Kitchingman and Garcia, 1999). Because the virus targets neuronal tissue it has been used to deliver genes to treat brain malignancies. Glioblastoma multiforme is an intracranial malignancy where long term survival after diagnosis is very rare. Delivery of gene encoding interlukin-12 using a mutant HSV-1 that lacked the neurovirulence late gene _134.5 and only replicated in proliferating cells gave promising results in animal models. Interleukin-12 up-regulates tumour antigen presentation via MHC Class I & II pathways and these cells are more immunogenic (Hellums *et al.*, 2005). A major disadvantage is that the requirements for the maintenance of gene expression during the latent state in neuronal cells are currently unknown. Another problem is that vectors that have certain gene deletions and have been rendered defective for growth only grow on complementing cell lines. These vectors tend to revert to the lytic life-cycle in model systems and cause cytotoxic cell death. The virus also has a large and complicated genome and in vitro manipulation is exceedingly difficult. The requirement for a helper virus is also a source of contamination in HSV preparations (Kitchingman and Garcia, 1999).

1.7.5 Semliki Forest viral vectors

The Semliki Forest virus (SFV) was first isolated in Uganda in 1942. SFV belongs to the family of sense (positive stranded) RNA viruses Togaviridae and the genus *Alphavirus*. This taxonomic classification is based on their serological cross-reactivity with one or more members of their genus. The genomes of more than ten different

alphaviruses have been examined by researchers and the amino acid sequence homology of the structural proteins is approximately 45% (Strauss and Strauss, 1994). The amino acid sequence homology of the non-structural proteins is closer to 60% indicating that this is a more highly conserved part of the genome. Alphaviruses have a wide geographical distribution and have been isolated on every continent except Antarctica (Strauss and Strauss, 1994).

1.7.5.1 Alphaviruses

Alphaviruses are natural parasites of arthropods and vertebrates and have a rather broad host range. They cause persistent life-long infection in arthropods while their infection in vertebrates is acute and transient. Replication of Alphaviruses in arthropods requires an intact nucleus. In contrast, replication in vertebrate cells proceeds almost normally in enucleated cells and replication is entirely cytoplasmic. The virions are spherical, 70-80 nm in diameter with an icosahedral symmetry and a triangulation number of 4. The nucleocapsid is surrounded by a tight fitting lipid membrane bilayer with embedded multiple copies of virus encoded heterodimeric glycoproteins. The lipid bilayer virus envelope is derived from the host's cells plasma membrane (Schlesinger, 1999).

The SFV genome is 11,442 nucleotides long, excluding the 5'methylated nucleotide cap and polyadenylated tail. Therefore the genome resembles mRNA and it encodes just 9 functional proteins. The genomic RNA contains two open reading frames (ORFs): the 5' region (two-thirds of the genome) encoding the non-structural proteins and the 3' region encoding the structural. The non-structural proteins are translated into a polyprotein that undergoes self cleavage to form a replicase structure nsP1-4. This

replicase structure is required for anti-sense strand synthesis and promotion of the S26 sub-genomic sense strand synthesis. The Alphavirus structural proteins are translated from the sub-genomic 26S mRNA. This second polyprotein undergoes both co-translational and post-translational modification to yield the 4 structural proteins that are necessary for virus assembly (Strauss and Strauss, 1994).

1.7.5.2 SFV infection

Infection begins with the virus particles binding to receptors on host cell surfaces. Attachment is sensitive to local ionic conditions and is usually mediated by highly conserved laminin receptors on mammalian cells. Insect cells may also possess similar laminin like receptors. The virus spike protein is the most important protein for receptor binding and membrane fusion. In most cells receptor mediated endocytosis is the main virus uptake method: clathrin-coated pits and acidified endosomes traffic virus particles through the host cell membrane into the cytosol. Cholesterol and sphingolipid are essential components in the host cell for virus fusion and initial binding. Upon cellular infection the viral genome is unpacked from the nucleocapsid (by an unknown mechanism) and protein translation begins at the 5' positive strand end of the genomic RNA. The capsid protein forms the nucleocapsid following synthesis of full length genomic RNA. During synthesis it folds to a serine like protease that autocatalytically cleaves the capsid from the polyprotein structure. There is an RNA hairpin structure near the N-terminal that acts as an enhancer for the translation of virus structural proteins in infected host cells. Protein p62 is a precursor to E2 with a transmembrane glycoprotein that is both palmitylated and glycosylated. It is cleaved in the trans-Golgi

by a furin-like protease and it is rarely incorporated into the viral structure. Protein E3 at its N-terminal contains the p62 signal sequence and it is glycosylated. E2 is the component of the heterodimeric virus spike and contains ligands for binding to host cell receptors. E2 is a transmembrane protein and binds to the capsid protein at its C-terminal end. Its glycosylated, palmitylated and some of its amino acid residues are possibly phosphorylated. Amino acid changes in E2 affect the viruses' assembly, stability, virulence and tropism. Protein 6K is a membrane associated palmitylated structure. The C-terminus contains the signal sequence of protein E1. Protein E1 is the other component of the heterodimeric virus spike: it's involved in host cell membrane fusion and contains amino acid sequences that function at low-pH. E1 is a transmembrane protein that is both glycosylated and palmitylated (Schlesinger, 1999 and Strauss and Strauss, 1994). The progeny viruses are assembled in two stages. There is a self association between the genomic RNA at specific regions and the newly synthesized nucleocapsid subunits. The other viral structural proteins undergo proteolytic processing, glycosylation, fatty acylation, oligosaccharide trimming and final processing as they move from the ER via the Golgi to the plasma membrane. The preformed nucleocapsid bind to arrays of E1-E2 trimers localised at the plasma membrane of the host cell and fusion of membrane with lipid bilayer allows for the release of the newly formed progeny virions (Schlesinger, 1999).

1.7.5.3 SFV non-structural proteins

Two-thirds of the genome is actively transcribed forming a non-structural polyprotein made up of 4 subunits nsP1-4. Protein nsP1 is a methyl and guanyl transferase and is

required for its 5' capping activity. It also plays a role in the initiation of anti-sense RNA strand transcription and modulation of nsP2 proteinase activity. Protein nsP2 is a cysteine protease at the C-terminal end. This protein functions in both cis and trans acting conformations modifying cleavages of the non-structural polyprotein complex (nsP1-4). At the N-terminal there are nucleoside triphosphatase and RNA helicase motifs. These N-terminal motifs are required for RNA binding activity and 26S transcription. Protein nsP3 is a threonine/serine phosphoprotein. Protein nsP4 is a RNA polymerase which is rather unstable and its concentration is strictly regulated and controlled by ubiquitination. The nsP1-4 polyprotein is cleaved by a cis acting protease encoded within the nsP2 C-terminus. This nsP1-4 is possibly complexed with host cell proteins and it interacts with a sequence of 19 nucleotides at the 3' end of the sense strand (genomic RNA). Initiation of anti-sense strand synthesis by the replicase complex occurs and complementary strand synthesis proceeds in the 5' to 3' direction. Transcription of the genomic strand (using the anti-sense strand as a template) requires modification of the nsP1-4 (replicase complex) by a trans acting protease located in the nsP2 protein region. This leads to an alteration in the individual subunits of the replicase structure. Once alteration by proteolytic cleavage is complete, the replicase complex can associate with the anti-sense strand sub-genomic 26S promoter, and synthesize the 5 structural proteins at very high levels. The 26S mRNA predominates in infected cells and yields of virus structural gene products can approach 25% of the host cells protein production. There are 6 proteins involved in structural viral assembly; capsid protein, p62, E3, E2, 6K and E1. Synthesis of full length genomic RNA by the altered replicase complex also occurs (Schlesinger, 1999).

1.8 Electroporation and SFV

The most common method for gene delivery is by the use of viral vectors. The viruses used as vectors include adenoviruses, adeno-associated viruses, retroviruses, lentiviruses, poxviruses, and herpes viruses. The different viral vectors are selected on the basis of how well they transfer genes to the cells they recognize and are able to infect, whether they alter the DNA of the cell, as well as the size and type of the gene being delivered. Yet there are many concerns with the application of viral vectors mainly due to the fact that viruses can usually infect more than one type of cell. Thus, when viral vectors are used to carry genes into the body, they might infect healthy cells as well as cancer cells. Another danger is that the new gene might be inserted in the wrong location in the DNA, possibly causing harmful mutations to the DNA or even cancer. Other concerns include the possibility that transferred genes could be "overexpressed," producing so much of the missing protein as to be harmful; that the viral vector could cause inflammation or an immune reaction; and that the virus could be transmitted from the patient to other individuals or into the environment. This is not just an issue with viral vectors when other methods of gene delivery are applied such as liposomes or electroporation there is a slight chance that this DNA could unintentionally be introduced into the patient's reproductive cells. If this happens, it could produce changes that may be passed on if a patient has children after treatment.

One method of overcoming these concerns is in the use of the Semliki Forest viral vector as a method of gene delivery. One of the main advantages of the SFV system from a safety point of view is that there is no DNA stage in the virus's life-cycle and repication is entirely cytoplasmic. Therefore there are no concerns surrounding

genomic integration of viral genes into the host's genome. Insertional mutagenesis is not a threat to the host. The disadvantage of using SFV is that there are still safety concerns due to the fact that it can infect multiple cell types and is not a completely localized treatment. Also there are issues of developing an immune response as with all viral vectors. Electroporation is a physical method that is used for localized gene delivery. The use of electroporation to been shown to be a highly effective method of gene delivery that overcomes many of the safety issue of other gene delivery systems. The combination of electroporation as a method of RNA delivery with the nonstructural proteins of SFV will allow for gene delivery with all the advantages of the SFV system (high gene expression and cytoplasmic replication) with the safety and localized delivery associated with electroporation (Figure 1.2).

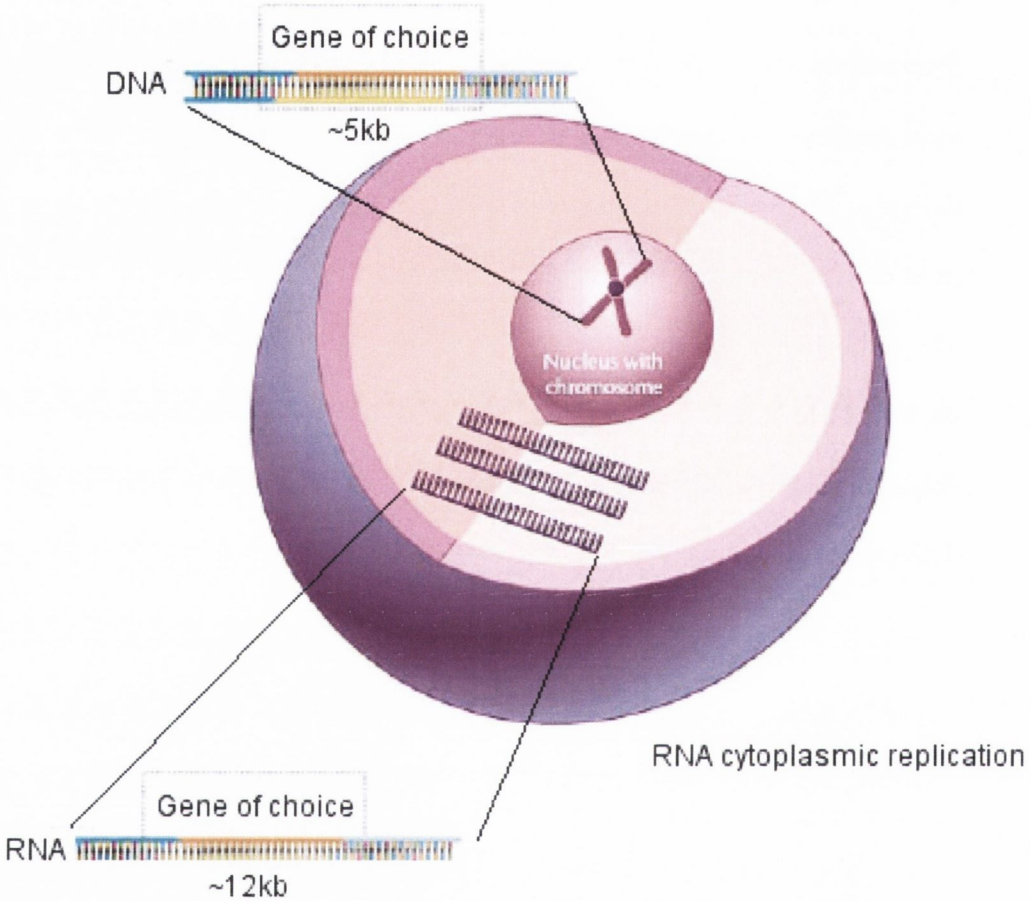


Figure 1.2: Schematic contrasting the delivery of the SFV based RNA delivery to that of DNA delivery into the cell following electroporation. Replication of the SFV RNA is entirely cytoplasmic and RNA replication is increased by the incorporation of the SFV replicase complex into the SFV based RNA plasmid resulting is high levels of protein production within the cell.

1.9 Research Hypothesis

1) RNA plasmids that contain the SFV replicase complex should be capable of transiently producing high levels of protein expression following entry into the target cell. Electoporation is a viable alternative to viral gene delivery that when combined with the SFV RNA replicase complex provides all of the advantages of viral gene delivery but using this safe physical method. While it is theoretically achievable to obtain transfection efficiencies above 90% with electroporation a variety of pulse parameters need to be optimized for each cell type and for the plasmid vector being applied. The *in vitro* optimisation of the electroporation parameters allows for an increased understanding of the effects of electroporating naked RNA or DNA directly into cells as well as providing information on the effectiveness of electroporating SFV based plasmids to a variety of different cell types.

1) The ability of SFV to induce apoptosis independently of viral structural proteins in a variety of cell lines has been well established (Glasgow *et al.,* 1997; Scallan *et al.,* 1997, Murphy *et al.*, 2000). Given the previous success in exploiting this cytopathic effect by treating tumours it was decided to investigate the possibility of electroporatively delivering the SFV vector with previously examined cancer gene therapies (Bax e.g. Murphy *et al.*, 2000 and IL-12 e.g. Gowda *et al.,* 2005) to enhance the antitumoural effect of the SFV vector.

1) DNA vaccines have been shown to induce both cell mediated and humoral immunity, which are long lasting (Donnelly *et al.*, 1997). This requires efficient membrane permeabilization to allow the DNA vaccines to enter the cells

(Nishikawa *et al.* 2001). This is not the case with SFV based RNA vaccines where it is simply necessary to deliver the gene through the cell membrane. The SFV RNA will replicate solely within the cytoplasm of the cell (Strauss & Strauss, 1994). Antibody responses were examined following electroporation of the SFV RNA plasmids showing the effectiveness of the electroproatively delivered SFV based plasmids in comparison of that of viral gene delivery or delivery of non SFV based plasmids.

Chapter 2

Materials & Methods

2.1 Materials

2.1.1 Cell Lines

The BHK-21 is a baby hamster kidney (BHK-21) fibroblast cell line, CT26 is an undifferentiated fibroblast colon carcinoma murine cell line, Caco2 is a human colorectal adenocarcinoma cell line that upon reaching confluence express characteristics of enterocytic differentiation and Cos7 is an African green monkey kidney fibroblast-like cell line. All cell lines were obtained from the American Type Culture Collection (ATCC) (Maryland, USA). The BHK cell line, sBHK (a BHK cell line that has been modified by the Karolinska Institute to be more amenable to SFV viral particle production), was a gift from Prof. P. Liljeström, (Microbiology and Tumourbiology centre, Karolinska Institute, Stockholm, Sweden).

2.1.2 Expression Vectors

The SFV RNA and DNA vectors, pSFV-*IL-12*, pSFV-LacZ, pSFV-EGFP and SFV helper vectors pSFV-SP6-helper-S2 and pSFV-SP6-CS219A were a gift from Prof. P. Liljeström, Karolinska Institute, Stockholm, Sweden. The SFV Luciferase vectors were a gift from Dr. C Smerdou, University of Navarra, Pamplona, Spain and the pSFV1-Bax RNA vector was produced by Mary Murphy, Trinity College Dublin, Ireland.

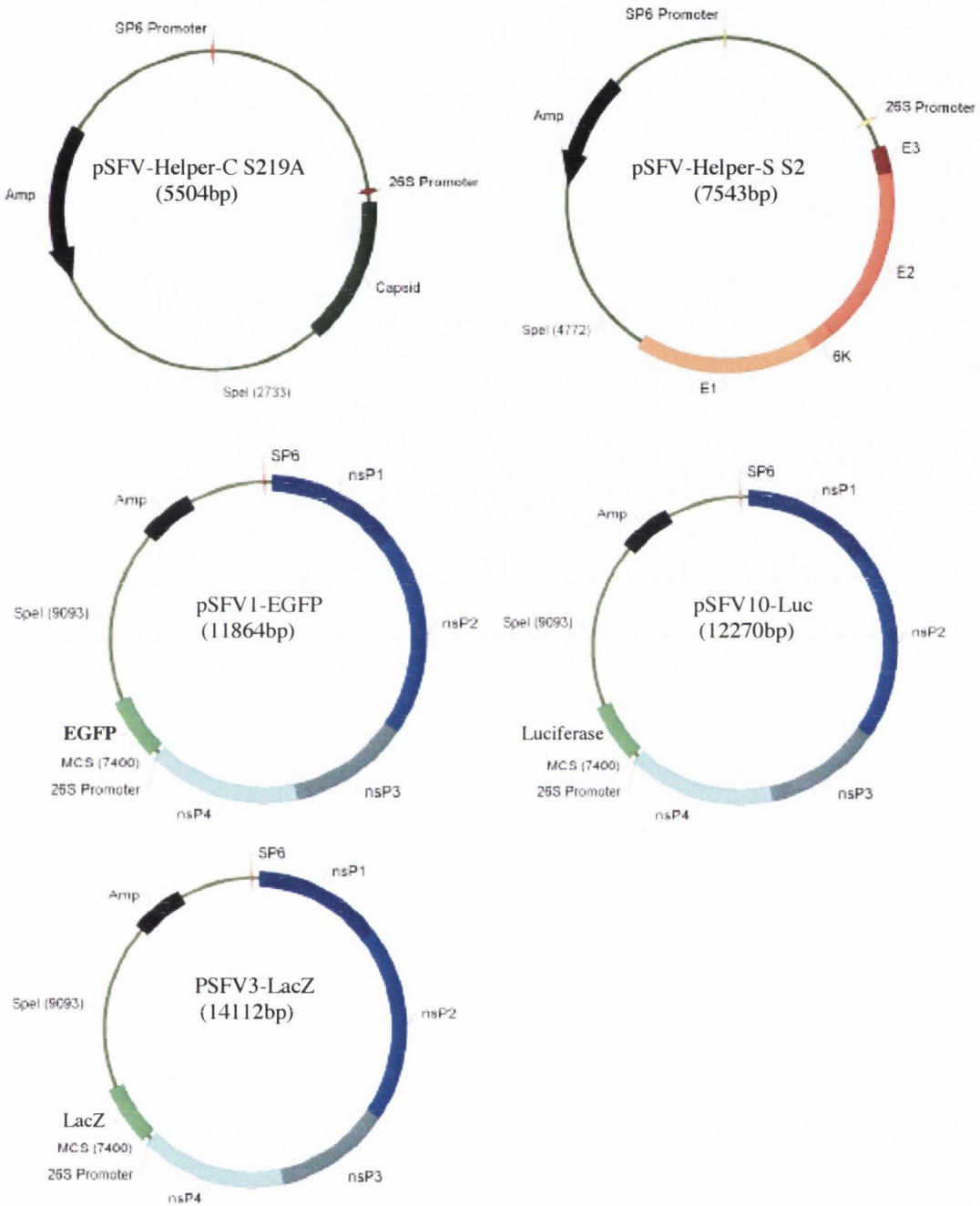
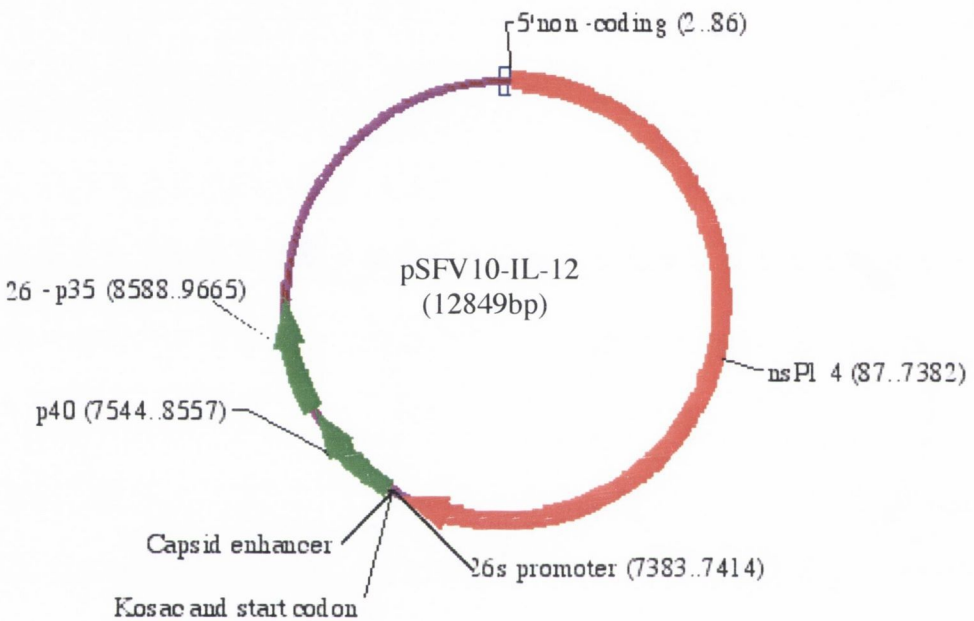


Figure 2.1: The recombinant vectors pSFV-EGFP and pSFV1-Luc as well as helper vectors, pSFV-Helper S2 and pSFV-Helper CS219A are shown. The recombinant vector incorporates the four genes encoding the SFV replicase complex (nsP1-4), but lacks the

structural genes (C, p62, 6K, E1), which are located on the helper vectors. All plasmids contain the unique *SpeI* site used for linearization, and SP6 promoter, which drives *in-vitro* transcription. Each of the SFV expression vectors has practically the same size and only differs in their polylinker regions (Liljestrom *et al.*, 1991).

a)



b)

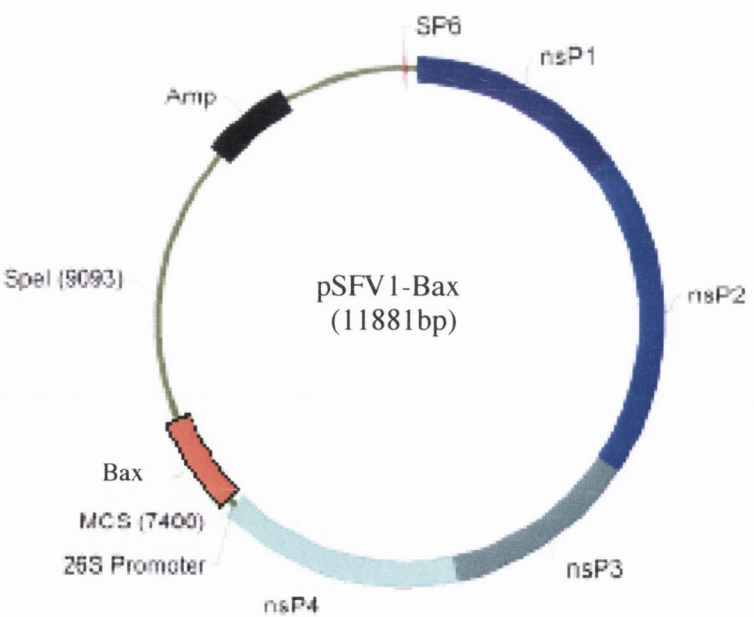


Figure 2.2: The expression vectors a) pSFV10 showing murine IL-12 subunits p40 and p35 in the multiple cloning site (MCS) of pSFV10 with their own 26S sub-genomic promoter and b) pSFV1-Bax showing the human 848bp subunit in the multiple cloning site of pSFV1 under the control of the 26S sub-genomic promoter.

2.1.3 Mice

Specific pathogen-free (spf) 4-6 weeks old female BALB/c mice were obtained from Harlan, UK and maintained in accordance with the principles outlined in S1 17/94 European Communities regulations 1994, for care and use of laboratory animals. Female mice were selected to facilitate mixing of groups and were all of similar size and weight at the beginning of experiments.

2.2 Methods

2.2.1 Cell Culture

The BHK-21, CT26, Cos7 and Caco2 cell lines were used for the *in vitro* studies. Swedish BHK (sBHK) cells were used for the production of high titre virus-like particles (VLPs). BHK-21, CT26, Cos7 and Caco2 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% (v/v) fetal bovine serum and 2 mM L-Glutamine and maintained at 37 °C in a humidified atmosphere of 5% CO_2. sBHK cells were grown in BHK medium supplemented with 5% fetal bovine serum, 10% tryptose phosphate broth, 20 mM HEPES buffer, 100 U/ml penicillin, 100 Δg/ml

streptomycin, and 2 mM L-Glutamine and maintained at 37 °C in a humidified atmosphere of 9% CO_2.

Splenocytes were grown in RPMI-1640 containing 10% foetal bovine serum (FBS), 10 mM HEPES, 2 mM glutamine, 100 U Penicillin 100 Δg/ml streptomycin, 10 mM sodium pyruvate, 1% non-essential amino acids, 50 ΔM Mercaptoethanol and maintained at 37 °C in a humidified atmosphere of 5% CO_2

Confluent monolayers of BHK-21, sBHK, Cos7, CT26 or Caco2 cells were detached using Trypsin-EDTA. The confluent monolayers were washed with Dulbecco's phosphate buffered saline without calcium, magnesium and sodium bicarbonate (PBS-) and incubated with 0.5% Trypsin 5.3mM EDTA at 37 °C until cell detachment was observed and their specific cell culture medium was added to terminate trypsinisation. Stocks of cells were prepared by slow freezing (at the rate of -1°C/min) to -80°C in medium containing 10% dimethylsulfoxide (Aldrich) and subsequently stored in liquid nitrogen.

2.2.2 Transformation

2.2.2.1 Preparation of competent *E. coli* DH5θ cells

Cells used for transformation of various plasmids were *Escherchia coli* strain DH5θ (New England Biolabs, USA). 500 ml of *E. coli* cells were grown in a 2 litre flask containing L broth incubated at 37 °C with shaking (200 rpm) until the cells reached the mid-log phase of growth (OD. 600= 0.4-0.5). The culture was then chilled on ice for 2 h

after which it was centrifuged at 800g for 20 minutes at 4°C. The supernatant was discarded and the cells were resuspended gently in 2 x 30 ml ice-cold 100 mM $MgCl_2$ (BDH, UK) and pelleted as above. Pellets were resuspended gently in 2 x 100 ml of ice-cold 100 mM $CaCl_2$ (Merc, Germany) and placed on ice for 1 h. Cells were pelleted as above and resuspended gently in 2 x 20 ml ice-cold 100 mM $MgCl_2$. 80 % glycerol (BDH, UK) was then added drop-wise with gentle swirling to a final concentration of 10% w/v. This cell-glycerol suspension was then aliquoted into 0.5 ml quantities on ice and snap-frozen in liquid nitrogen before storage at -80 °C.

2.2.2.2 Transformation of *E. coli* DH5θ cells (heat shock)

Competent DH5θ cells were transformed with each specific plasmid vector by adding the transforming DNA (1Δg to 200 Δl of competent DH5θ cells) to competent cells thawed on ice. This mixture was stored on ice for 30 minutes and transferred to a 42°C waterbath for 90 seconds. The DNA cell mixture was then incubated on ice for a further 1-2 minutes after-which 800 Δl of SOC media was added to each tube. Tubes were placed at 37 °C and shaken for 45 mins to allow cells to recover and to express antibiotic resistance. Transformed cells were plated onto L-agar plates (up to 200Δl per plate) containing 1mg/ml ampicillin/ kanamycin. The plates were incubated upside down overnight at 37°C and observed for colony growth.

2.2.2.3 Screening of the plasmid DNA from the transformed colonies

Isolated transformed colonies were inoculated in 15 ml of L-broth containing 100 μg/ml ampicillin/ kanamycin separately. The cell cultures were grown for ~18 h shaking at 37

°C. From each culture, a 5 ml aliquot was used to purify the plasmid, using the QIAGEN Miniprep Plasmid Purification Kit. Following confirmation of plasmid DNA by restriction digestion the remaining 10 ml of L-broth containing transformed colonies was transferred to 1500 ml of L-broth containing 100 μg/ml ampicillin/ kanamycin. The cell cultures were grown for ~18 h shaking at 37 °C and then entire culture used for plasmid purification using the QIAGEN Endotoxin free Megaprep Plasmid Purification Kit.

2.2.3 SFV RNA production

2.2.3.1 Linearization of plasmid DNA for *in vitro* transcription

For the production of SFV RNA, each plasmid was linearized using a specific restriction enzyme. In each of the SFV plasmids a unique restriction site preceding the non-structural protein genes and SP6 promoter was selected. The Spe 1 site was used to linearise the pSFV-EGFP, pSFV-LacZ, pSFV-Bax and helper SFV plasmids whereas the NruI site was used for the pSFV-IL12 plasmid. A total of 30 Δg plasmid DNA was linearised in a 100 Δl volume containing 9 Δl NEBuffer 2 and 9 Δl BSA for Spe1) or 18 Δl NEBuffer 3 (Nru1), and 3 Δl of Spe I or NruI enzyme.

Following 1.5 hour digestion in a waterbath at 37 °C, cut and uncut plasmids were visualized by gel electrophoresis along with a 1 kb molecular weight marker (NEB). Linearized plasmids were then cleaned using the Qiagen nucleotide extraction kit and resuspended in a final volume of 50 Δl sterile nuclease-free water. The concentrations of the linearized plasmid DNA were estimated on a 0.8% agarose gel by comparing band

intensities with those of the DNA molecular weight markers and confirmed by spectrophotometry using the Eppendorf Biophotometer.

2.2.3.2 *In vitro* RNA transcription

The cleaned linearized SFV plasmids were used for *in vitro* RNA transcription (a minimum of 1.5 Δg of DNA was used). 60 Δl of the cleaned linearised DNA was added to 30 Δl SP6 buffer (40 mM HEPES-KOH pH 7.4, 6 mM MgOAc, 2 mM Spermidne-Hcl), 30 Δl 1 mM $m^7G(5')ppp(5')G$, sodium (CAP), 30 Δl 5 mM dithiothreitol (DTT), 30 Δl rNTP mixture (1 mM each rATP, rCTP, rUTP, 0.5 mM rGTP), 9 Δl RNasin and 9 Δl SP6 RNA polymerase made up to a 300 Δl volume with nuclease free water. This reaction was incubated at 37°C for 1.5 – 2 hours and the results analyzed by running the 1 Δl of RNA on 0.6% (w/v) agarose gel comparing band intensities with those of the DNA molecular weight markers and quantitated by spectrophotometry using the Eppendorf Biophotometer. RNA was aliquoted into 100 Δl volumes and stored at -80°C.

2.2.4 *In vitro* electroporation

Cells were grown to ~ 85 % confluency, washed and trypsinized. The cells were resuspended in 10 ml of fresh medium and centrifuged at 1500 rpm for 10 minutes at room temperature. Pellets were gently resuspended in 1 ml of medium, counted, resuspended in 10 ml PBS and centrifuged again. The cells were then resuspended in PBS (+ MgCl) to a concentration of $1x10^6$ in 750 Δl. 750 Δl was transferred to a 4 mm electroporation cuvette (MSC) and 50 Δl of plasmid DNA/RNA added (typically 1 Δg/

Δl concentration). The cuvette was then pulsed with the desired pulse parameters using either the BioRad Gene Pulser Excel or the BTX ECM 2001 Electro cell manipulator. Immediately following electroporation the cells were removed from the cuvette and resuspended in 12 mls of warm media (~ 37 ° C). Dependent of the experiment the cells resuspended in the 12 ml of media were added to a T75 flask (Sarstedt) or the cells were added to a six well plates in 3 ml aliquots. Cells are then incubated for 12 – 14 hours at 37 °C in a humidified atmosphere of 5% CO_2 prior to analyses.

2.2.5 Recombinant Semliki Forest virus like particle (VLP) production

2.2.5.1 Preparation of recombinant and helper SFV plasmids

Competent DH5θ cells were transformed with pSFV-IL12, pSFV-LacZ, pSFV-EGFP, pSFV-Helper S2 and pSFV-Helper CS219A separately as outlined in the section 2.2.2. RNA was transcribed and quantitated as described in section 2.2.3.

2.2.5.2 High titre rSFV-IL12 VLP production

In vitro transcription of each of the RNA reporter plasmids and the helper vectors pSFV-S2 spike and pSFV-C219A capsid were made in 300 Δl volume as described in section 2.2.3.2. For each of the virus like particle (VLP) stocks three T175 flasks (Sarstedt) of confluent sBHK (Swedish BHK) cells were prepared for electroporation per SFV construct. Cells grown to ~ 85 % confluency in the T175 flasks were trypsinized as normal, suspended in 20 ml volumes and centrifuged at 1500 rpm for 10 min. The pellets were combined, resuspended in 50 ml of PBS and centrifuged at 1500

rpm for 10 min. The pellet was resuspended in 3.9 ml media and 300 Δl of the desired RNA non structural vector (see section 2.2.3.2 e.g. pSFV-EGFP, pSFV-LacZ or pSFV-*IL-12*) was added in combination with 300 Δl of each of the helper vectors pSFV-SP6-helper-S2 (Spike) and pSFV-SP6-CS219A (Capsid). This 4.8 ml total was then aliqouted into six 800 Δl volumes into 4 mm electroporation cuvettes and electroporated with two pulses of 850 v/cm, 5ms duration. The electroporated cells were resuspended in media and divided into two T175 flasks with a final volume of 50 ml in each.

2.2.5.3 Harvesting and concentration of VLPs by ultracentrifugation

The supernatants of the two T175 flasks were pooled (100 ml) 36 – 40 hours post transfection and clarified by centrifugation at 6,000 x g for 30 minutes at 4 °C in a Sorval Rc 5C Plus centrifuge. The supernatants were then decanted into fresh centrifuge tubes and re-centrifuged under the same conditions for 15 min. Clarified supernatants were then pooled and aliquoted into three Ultra-Clear SW28 centrifuge tubes (Beckman, USA) in 20 ml amounts. A 20% sucrose (w/v) solution was prepared in TNE buffer and 5 ml of this was carefully added to the base of each tube using a Pasteur pipette to form a sucrose cushion. Tubes were then filled to within 2-3 mm from the top with the remaining clarified supernatant and balanced before being spun at 25000 rpm for 2 hours at 4 °C in a Beckman L8-60M ultracentrifuge. The supernatants and sucrose cushions were removed using a pipette and a sterile cotton swab was utilized to remove residual sucrose from the sides of the tubes.

In order to resuspend the viral pellets, 300 Δl TNE buffer was added to the base of each tube and aspirated gently. Tubes were then covered with parafilm and incubated on ice

for 2 hours after which the TNE was aspirated, removed and pooled on ice. A further 300 Δl TNE buffer was added to each of the tubes which were then vortexed for 1 minutes and incubated on ice for 1 hour. The TNE was again removed and pooled on ice and 300 Δl TNE was used to wash each tube in succession by aspiration before being pooled along with the other viral suspensions to a final volume of 1800 Δl. The virus suspension was then aliquoted into 50 and 100 Δl volumes on ice and snap-frozen in liquid nitrogen. The production of high-titre stocks of rSFV-*IL-12*, rSFV-LacZ, rSFV-Luc and rSFV-EGFP, was performed as described above and stored frozen at -80°C.

2.2.5.4 High titre rSFV particle titration

A T75 flask of ~80% confluent sBHK cells was grown, trypsinized and seeded onto 22 mm^2 coverslips in a six-well cell culture dish and incubated until confluent. Serial dilutions (5 Δl, 0.5 Δl, 0.05 Δl, and 0.005 Δl) of VLPs were prepared on ice and used to infect, in duplicate, successive wells of the confluent sBHK monolayers in a total volume of 500 Δl infectious medium for 1 hour, with rocking every 10 min. The inocula were then aspirated and the monolayers washed twice with 1 ml PBS before the addition of 2.5 ml fresh BHK-21 medium at 37 °C to each well. Following 16 hours incubation at 37 °C, medium was removed, cells washed in PBS, fixed with 4 % paraformaldehyde (20 – 30 min) and washed in PBS. Expression was detected as described in section 2.2.6 based on the gene being expressed. Positive cells were enumerated and VLP titres (Infectious unit/ml) were calculated at a magnification of x 400.

2.2.6 Determination of electroporation transfection \ VLP infection efficiency

2.2.6.1 Fluorescence (microscopy)

To determine the transfection efficiency of the electroporation by fluorescence, BHK-21, Cos7, CT26 or Caco2 cells were seeded in 24-well cell culture dishes onto 22x22 mm glass cover slips at a concentration of 1.67 x 10^5 cells/well in 2 ml medium post electroporation (see section 2.2.4) and allowed to adhere for ~12 – 14 hours. In the case of the VLPs the infection efficiency was analyzed by seeding the cells in 24-well cell culture dishes onto 22x22 mm glass cover slips at a concentration of 5 x 10^3 cells/well in 1 ml maintenance medium and allowed to adhere for ~12 - 14 hours. Cells were then washed with 1 ml PBS+ and infected with the VLPs in triplicate at a multiplicity of infection (MOI) of 10 in 200 Δl of infectious medium for 1 h at 37 °C with rocking every 10 min. After two 1 ml PBS+ washes, 1 ml maintenance medium at 37 °C was added to each well and cells were maintained for 14 hours at 37°C in a humidified atmosphere of 5% CO_2.

Supernatants were removed from either the VLP infected or the electroporation transfected cells after ~14 hours. The cells were fixed to the slides with 4% paraformaldehyde and stained with VECTASHIELD hardset mounting medium with DAPI. Expression was examined by fluorescent microscopy using a Nikon eclipse E400 microscope and appropriate filters (Enhanced Green Fluorescent Protein (EGFP) expression - FITC and fluorescein filter; DAPI nuclear stain - DAPI filter). Transfection/ infection efficiencies were calculated as the number of fluorescent cells as a percentage of the total number of DAPI-positive cells in each microscopic field.

2.2.6.1 Fluorescence (flow cytometry (FACS))

To determine the transfection efficiency of the electroporation by flow cytometry, BHK-21, Cos7, CT26 or Caco2 cells were seeded in T75 tissue culture flasks at a concentration of ~1 x 10^6 cells/flask in 12 ml medium post electroporation (see section 2.2.4) and allowed to adhere for ~12 – 14 hours. Supernatants were removed from cells after ~14 hours. The cells were trypsinised as detailed 2.2.1, resuspended in 10 ml media / flask and centrifuged at 1500 rpm for 10 min. The cells were resuspended in 10 mls PBS and centrifuged as before. Cells were resuspened in 1 ml of PBS, 5 Δl of propidium iodide was added and left to stand for 5 minutes in the dark. Expression levels (EGFP– transfection efficiency (cell exhibit green fluorescence upon expression of EGFP protein), PI – cell death) were analyzed using a Becton Dickinson FACS caliber FACS machine and the actual percentage readings were derived using the CellQuest software program for analysis of FACS data

2.2.6.3 Luminescence

To determine the transfection efficiency of the electroporation by luminescence, BHK-21, Cos7, CT26 or Caco2 cells were seeded in 24-well cell culture dishes at a concentration of 1.67 x 10^5 cells/well in 2 ml medium post electroporation (see section 2.2.4) and allowed to adhere for ~12 – 14 hours at 37°C in a humidified atmosphere of 5% CO_2. Supernatants were removed from the transfected cells after ~14 hours, cells were washed twice in 1 ml PBS and 400 Δl of 1 x Reporter Lysis buffer (Promega) added to each well. The plated was stored at – 80 °C for 15 minutes, left to thaw for 30

minutes at room temperature and cells removed by scraping and centrifuged at 12,000 rpm for 15 minutes. 20 Δl of sample was mixed with 100 Δl of luciferase reagent (Promega) and analyzed using the Berthold Luminometer at 250 nm. The remaining sample was kept for protein quantification.

2.2.7 Protein quantification

The level of protein from the luciferase experiments was quantified using the BSA Protein Assay (MSC) as per manufacturer's instructions. Results were combined with the luciferase results and graphed as RLU/Δg protein.

2.2.8 Comparative analysis of rSFV-*IL-12* VLPs and electroporated pSFV-*IL-12* for biologically active expression of IL12

2.2.8.1 Analysis for the *IL-12* expression

Analyses of expression and secretion levels of IL12 were carried out as described by Gowda *et al.*, (2005) using BHK-21 and CT26 cells. Cells were seeded in 24-well cell culture dishes at a concentration of 1.67 x 10^5 cells/well in 2 ml medium post electroporation (see section 2.2.4) and allowed to adhere for ~12 – 14 hours. In the case of the VLPs the infection efficiency was analyzed by seeding the cells in 24-well cell culture dishes at a concentration of 5 x 10^3 cells/well in 1 ml maintenance medium and allowed to adhere for ~12 - 14 hours. Cells were then washed with 1 ml PBS+ and infected with the VLPs in triplicate at a MOI of 10 and incubated at 37°C in 5% CO_2. The supernatants of the transfected /infected cells were collected at different time points

(12, 24, 48 and 72 hours post infection / electroporation) and centrifuged 10,000 rpm for 10 minutes to remove the cell debris. The level of secreted *IL-12* protein (1:20, 1:200 and 1:250) in triplicate for each time point sample was estimated by capture ELISA using OptEIA™ Set Mouse *IL-12*p (70) Kit in flat-bottomed 96-well ELISA plates. Capture ELISAs were performed according to the kit manufacturer's instructions and results plotted to see the difference in the level of IL12 secreted by the rSFV-*IL-12* VLPs and electroporated pSFV-*IL-12* in both BHK-21 and CT26 cell lines.

2.2.8.2 Examination of biological activity of the secreted *IL-12* (as described by Gowda *et al.*, (2005))

Biologically active *IL-12* induces splenocytes (T-cells) to secrete interferon-θ (IFN-θ). A spleen from BALB/c mouse was collected in Hank's Balanced Salt Solution (HBSS) on ice. The tissue was homogenized with a syringe plunger and passed through a 70 Δm cell strainer. The cells were centrifuged at 1500 rpm for 10 minutes at 4°C. The supernatant was discarded and 5 ml of 1x red blood cell (RBC) lysis buffer was added. Cells were incubated for 2 minutes at room temperature. 10 mls HBSS was added to each tube followed by centrifugation at 1500 rpm for 10 minutes at 4°C. The supernatant was decanted off and the cells were resuspended in 20 ml RPMI-1640 (containing 10% heat inactivated FBS, 20 mM HEPES buffer, 100 Δg/ml streptomycin, 2 mM L-Glutamine, 1% Sodium pyruvate, 1% non-essential amino acids and 50 ΔM Mercaptoethanol). The cells were counted (using a haemocytometer) and resuspended in HBSS at $1x10^6$ /250 Δl. 0.5 ml (per well) of the cells were dispensed into two 24 well plates. 100 Δl (neat) orl Δl (1:100) of the supernatant from the rSFV-*IL-12* VLPs

infection or electroporated pSFV-*IL-12* or pSFV-EGFP medium from BHK-21 or CT26 cells were added to the splenocytes in the wells. Each well was made up to a final volume of 1 ml HBSS /well and incubated at 37°C. The supernatants were harvested at different time points (24, 48, and 72 hours). The cells were separated by centrifuging the harvested supernatant from the splenocytes at 10,000 rpm and stored at -20°C for further analysis. The OptEIA™ Set Mouse IFN-θ kit was used to measure the production of IFN-θ from murine splenocytes induced by supernatant from the rSFV-*IL-12* VLPs infected cells, electroporated pSFV-*IL-12* cells, electroporated pSFV-EGFP infected cells and medium from untransfected /infected BHK-21 or CT26 cells. The capture ELISAs were performed according to the manufacturer's instructions. The absorbances were read at 450 nm and mean concentrations at each time point plotted.

2.2.8.3 Annexin V Staining

Cell were electroporated with 50 Δl of pSFV1-Bax as described in section 2.2.4, stained for evidence of early stage apoptosis (Annexin V positive) using the Annexin V-FITC Apoptosis Detection Kit I (BD Pharmingen Cat. No.556547) according to manufacturers instructions and analyzed by FACS as described in section 2.2.6.2.

2.2.9 In vivo tumour treatment studies

2.2.9.1 Induction of tumours (CT26 or OE19) in vivo

The right flank of each female BALB/c mouse (six / treatment group) of 6-8 week old mice was shaved in order to facilitate tumour induction and measuring, this was not

necessary for BALB/c *nu/nu* mice. CT26 or OE19 cells were propagated in T175 culture flask as described in section 2.2.1 and harvested by trypsinization when in the exponential phase of growth. Cells were centrifuged at 1500 rpm at 4 °C and resuspended by gentle aspiration in non-supplemented medium. Cells were centrifuged and resuspended as before, adjusted to a concentration of 1 x 10^6 cells /100 Δl (CT26) or 1 x 10^8 cells /100 Δl (OE19) and injected subcutaneously (s.c.) into the right flank of each mouse using a 1 ml syringe (Beckton Dickenson, UK) and 21 G needle in a 100 Δl volume. Mice were examined daily for signs of s.c. tumour formation and when detected, tumour diameters were measured using linear calipers in 2 perpendicular diameters with the average tumour diameter calculated as the square-root of the product of cross-sectional diameters, assuming spherical shape. Tumour diameters were measured daily and treatment was initiated once tumours reached an average diameter of 4 mm.

2.2.9.2 Treatment of tumours (CT26 or OE19) by electroporation

The plasmid RNA (pSFV-Bax, pSFV-*IL-12*, pSFV-Luc or pSFV-EGFP) was prepared as described in section 2.2.3.2 and adjusted to a concentration of 1 Δg/Δl with nuclease free water. Mice were anesthetized using a xylazine/ketamine mixture (0.1 ml / 10 g). The plasmid RNA was injected 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. The electroporation needles were inserted to either side of the site of the plasmid injection and after 1 – 2 minutes the tumours were electroporated with eight pulses of 600 v/cm and 5 ms duration (see Appendix 2A) for

pulseform selection). A total of six mice were used per treatment group. Tumours were measured daily as described in section 2.2.9.1 and mice were euthanised by CO_2 asphyxiation and cervical dislocation when tumours approached 10 % of the mouse body weight.

2.2.9.3 Treatment of tumours (CT26) with VLPs

High titre rSFV-*IL-12*, rSFV-EGFP, rSFV-Luc VLPs were prepared as described in section 2.2.5.3, titrated (section 2.2.5.4), and adjusted to a concentration of 1 x 10^6 IU/ml in TNE buffer. Mice received one intratumoural injection of 50 Δl TNE buffer alone, untreated, rSFV-EGFP (control groups) or the TNE containing high-titer rSFV-*IL-12* VLPs using a 0.5 ml insulin microsyringe (Beckton Dickenson, UK). 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. Only one injection was administered to each tumour upon reaching 4 mm in diameter (day 0), or a second treatment was given day 8 (see section 4.2.1.4). Groups of six mice were used per treatment group. Tumours were measured daily as described in section 2.2.9.1 and mice were euthanised by CO_2 asphyxiation and cervical dislocation when tumours approached 10 % of the mouse body weight.

2.2.10 Immunization of BALB/c mice

2.2.10.1 Immunization with electroporated pSFV-LacZ

The plasmid DNA (pCMV-LacZ, pBK-SFV-LacZ or pBK-SFV-EGFP) was prepared as described in section 2.2.2.3, RNA (pSFV-LacZ, pSFV-Luc or pSFV-EGFP) was prepared as described in section 2.2.3.2 and each was adjusted to a concentration of 1 Δg/Δl with nuclease free water. Mice were anesthetized using a xylazine/ketamine mixture (0.1 ml / 10g). Groups of mice to be immunized received a 50 Δl intramuscular (i.m.) injection of the RNA or DNA at a concentration 1 Δg/Δl in the left tibialis anterior. The electroporation needles were inserted to either side of the site of the plasmid injection and after 1 – 2 minutes the tumours were electroporated with six pulses of 400 v/cm and 10 ms duration. Mice were boosted 13 days later with the same injection and electroporation in the right tibialis anterior. As naïve controls, groups of mice were simultaneously injected with plasmid alone, electroporated alone, or treated with pSFV-Luc/EGFP in the same manner. Groups of six mice were used per treatment group.

2.2.10.2 Immunization with rSFV-LacZ VLPs

For immunization, rSFV-LacZ VLPs were prepared as described in section 2.2.5.3, titrated (section 2.2.5.4), and adjusted to a concentration of 1 x 10^6 IU/ml in TNE buffer. Groups of mice to be immunized received a 50 Δl intramuscular (i.m.) injection of the rSFV-LacZ VLPs in TNE at a concentration of 1 x 10^6 IU/ml in the left tibialis anterior and were boosted 14 days later with the same injection in the right tibialis

anterior. As naïve controls, groups of mice were simultaneously mock-immunized with TNE alone, or rSFV-Luc/EGFP in the same manner. Groups of six mice were used per treatment group.

2.2.10.3 Harvesting of serum from BALB/c mice

Blood was harvested from mice via the facial vein using a 28 gauge needle on day 0, 7, 13, 20 and 26. The blood was subsequently incubated at 4 °C for 12 hours and clotted blood was centrifuged at 10,000 rpm for 10 minutes. The serum was decanted and aliquoted before storage at -80 °C.

2.2.10.4 Analysis θ-galactosidase antibody expression

ß-galactosidase was selected as an ideal reporter gene for analysis of electroporative antibody responses and has been well characterized as a vaccine reporter (Wildera *et al.*, 2000). ß-galactosidase can also be detected within the tissue by histological analysis. Serum from blood samples collected on day 0, 7, 13, 20 and 26 from inoculated (and boosted) BALB/c mice was analyzed by ELISA. Flat-bottomed 96-well ELISA plates were coated with coating buffer and 2.5 Δg/ 100 Δl of ß-Galactosidase (Roche) (100 Δl/well) and stored for 12 hours at 4 ^{o} C. The plate was then washed and coated with blocking buffer (100 Δl/well), stored at room temperature for 3 hours, rewashed and the serum added with serial dilutions in blocking buffer (diluted in sep plate 1/50 – 1/102400). The plate was stored at room temperature for 1 hour, washed, coated with secondary antibody, anti-mouse –hrp (BD Biosciences), (1/500 dilution 50Δl/well) and incubated in the dark at room temperature from 1 h. 3,3',5'5-Tetramethylbenzidine

(TMB) (Sigma) (100Δl/well) was added to the plate once washed and the stopping buffer to stop reaction (50Δl/well) added once the reaction had reached completion (~ 4 minutes). The absorbances were read at 450 nm and mean antibody titer concentrations at each time point plotted.

2.2.11 Histopathology

2.2.11.1 Preparation of tumour and muscle cryosections

Excised tumours and muscle were embedded in OCT compound (TissueTec, USA) on cork disks (Lamb, UK), snap-frozen in liquid nitrogen-cooled isopentane and stored at -80 °C. A Leica CM 1900 cryostat was used to prepare ~4 - 6 Δm thick sections at –15 °C which were placed on poly-L-lysine coated glass slides, fixed in acetone at -20 °C for 5 min, air-dried overnight and stored in airtight containers at -80 °C.

2.2.11.2 Haematoxylin and eosin staining for routine histology

Tumour and muscle cryosections were examined routinely using the haematoxylin and eosin (H&E) staining method. Sections were then placed in Harris' haematoxylin (BDH, UK) 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 (BDH, UK) for 2 minutes and washed under running tap water for a further 5 minutes or until cleared. H&E stained slides were prepared from a minimum

of two separate sections of each tumour to provide a more representative depiction of the microscopic changes.

2.2.11.2 Routine histology

Coded H&E stained tumour sections were examined blind 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 haemorrhages, presence of TILs and their location in the tumour microenvironment. Coded H&E stained muscle sections were examined blind for the presence of necrosis, thromboses and haemorrhages.

2.2.11 *In vivo* imaging of BALB/c / BALB/c *nu/nu* mice

To determine the transfection efficiency of the electroporation by luminescence mice were anesthetized using a xylazine/ketamine mixture (0.1 ml / 10 g) 48 hours post luciferase transfection (electroporation 50 Δl of RNA (1 Δg/Δl))/ infection (Luciferase VLPs (1 x 10^6 IU/ml)). 100 Δl of luciferase reagent (Promega) was delivered ip and the mouse analyzed after 3 minutes using the Xenogen *in vivo* imager. It should be noted that less varation may have been observed in the results had the mice been analyzed 15 minutes post delivery of the luciferase reagent. In the case of tumour gene expression 100 Δl of luciferase reagent (Promega) was injected 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 in the cases of tumour expression. The

expression was quantified according to manufacturers instructions and mean RLU/ treatment group plotted.

2.3 Statistical Analysis

The t test was used to analyse normally distributed data where the standard deviations were similar. This test can only be used to compare the means of two groups as there is an increase in error when more than two groups are analyzed. When analyzing more than two groups the ANOVA test was used. This detects if there is a difference between independent samples of the same variance, while the Tukey test was used to examine what the difference was (following the initial AVOVA test). A p value of less than 0.05 was considered significant in each case. The co-efficent of variation was used to analyze the standard devaiation as a fraction of the mean.

Chapter 3

In vitro SFV plasmid electroporation

3.1 Introduction

Although *in vitro* electroporation has been shown to be effective in a wide variety of cell types, each different type of cell requires slightly different electroporation conditions. There are a variety of different parameters that effect the electroporation efficiency, these include the shape of the target cells, the cell density, temperature, conductivity of the poration medium, molecular size of the transferred molecules, electric field strength, number of pulses, duration of pulse and the frequency applied (Cemazar *et al.*, 1998, Rols *et al.*, 1990, Wolf *et al.*, 1994). Thus, while it is theoretically achievable to have over 90% of cells permeabilized with less than 5% cell death the optimal electroporation parameters must be determined empirically for each cell line individually (Gehl *et al.*, 1998).

The main parameters that should be examined when optimizing in vitro electroporation will now be discussed.

3.1.1 Cell size and shape

It has been well established that cell size is inversely correlated to the size of the external field needed to create permeabilization based on the equation $\Delta V = f\ r\ E \cos\theta$, where ΔV is the transmembrane voltage (V), θ is the angle made with the direction of the external field E, f is the geometric factor (in the case of a sphere, the factor takes a constant value of 1.5), r is the radius of the cell and E is the field strength (V/r) (Figure 3.1) (Sale *et al.*, 1968, Neumann *et al.*, 1989, Chang *et al.*, 1992). Therefore, electroporation parameters must be optimized for each cell type. Based on this equation

the homogeneity in cell size is an essential parameter when electroporating any population of cells as variations within this will decrease the transfection efficiency. The growth phase of the cell is an essential element also to be considered as cells have been shown to be more trasfectable when in logarithmic growth rather than in stationary growth phase (Tatebe *et al.*, 1995).. This is based not only on the fact that in dividing cells the nucleus is more accessible to the transfected DNA but also due to the fact that the cell size varies between these growth phases, with larger cells being observed in the stationary phase (Tatebe *et al.*, 1995). The most common difficulty when electroporating cells is that in many cases cells are not truly spherical and hence there will be a variation in the transfection efficiency due to differences in cell orientation.

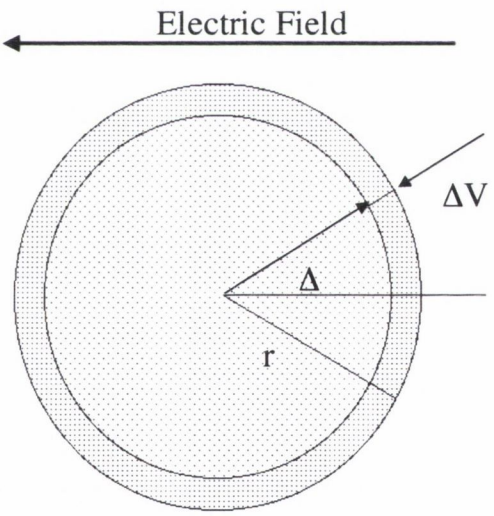


Figure 3.1 Schematic of transmembrane voltage of a cell induced by an applied electrical field.

3.1.2 Temperature

It has been well established that temperature has a direct effect on the permeation and resealing of the cell membrane following electroporation. When cells are electroporated at 4°C the necessary field strength of the pulse is nearly twice as high as those values for room temperature though in most cases mammalian cells are electroporated at room temperature (Zimmermann *et al.*, 1996). Where the effect of temperature on electroporation is most commonly observed is when examining membrane resealing. It has been shown that membrane resealing post electroporation is strongly temperature dependent, with slow closure at low temperatures (Kinosita & Tsong 1977). Electroporation at room temperature causes the permeated membrane areas to seal up more rapidly than at low temperatures. This has been observed to accelerate membrane regeneration and thus increases the cell survival rate. However, the transfection rate may be lower than that obtained when electroporation is carried out at low temperatures. It has been shown that cooling at the time of permeabilization and subsequent heating in an incubator increases transfer efficacy of DNA but this will decrease cell viability in some cell types (Rols *et al.*, 1994). The temperature can also effect the conductivity of the electroporation buffer. Increasing the temperature causes the conductivity of a solution also to increase, which may lead to lower transfection rates. For this reason, it is advisable not to work at temperatures in excess of 33°C (Zimmermann *et al.*, 1996).

3.1.3 Pulsing medium.

Some authors advocate the use of low-conductivity media for DNA transfer in order to increase viability and increase transfection efficacy (Rols *et al.*, 1994). Calcium

concentration in the medium during electroporation should be considered in order to avoid sudden high intracellular levels of this electrolyte. However, some authors advocate the use of calcium or magnesium in the buffer when performing DNA transfer as this will act as a positive charge between the negatively charged exterior of the cell and the polyanionic DNA, approximating the DNA molecules to the membrane prior to the electroporation (Gehl, 2003).

3.1.4 Pulse parameters

The most important parameters that impact on the electroporation effectiveness are electric field strength, pulse duration and the number of pulses (Gehl *et al.*, 1999). As can be seen in figure 3.2 the relationship between the electric field strength (voltage) and the pulse duration is an inversely proportional one. The higher the voltage applied the lower the pulse duration recommended for electroporation. If the applied voltage sufficiently exceeds the normal transmembrane potential (~ 0.7 – 1 V), the membrane is permeabilized with aqueous pores formed theoretically through which molecules can penetrate the membrane. The field strength or pulse length can be increased to enhance the permeability of the cell membrane but increasing this beyond a certain threshold can lead to an unstable situation where the cell can not repair the damage and hence will die (lyse). If the field strength is too low or the pulse length too short, the alteration of the transmembrane potential will not be achieved as the membrane will not be charged enough to reach the electroporation membrane potential. If the goal is intracellular delivery of a chemotherapeutic agent to kill tumour cells, exceeding the upper limit of

electroporation which leads to lysis of the cells may be acceptable, whereas for the delivery of genes for vaccines or gene therapy, it is essential to select the experimental parameters within the region of effective electroporation and high cell survival.

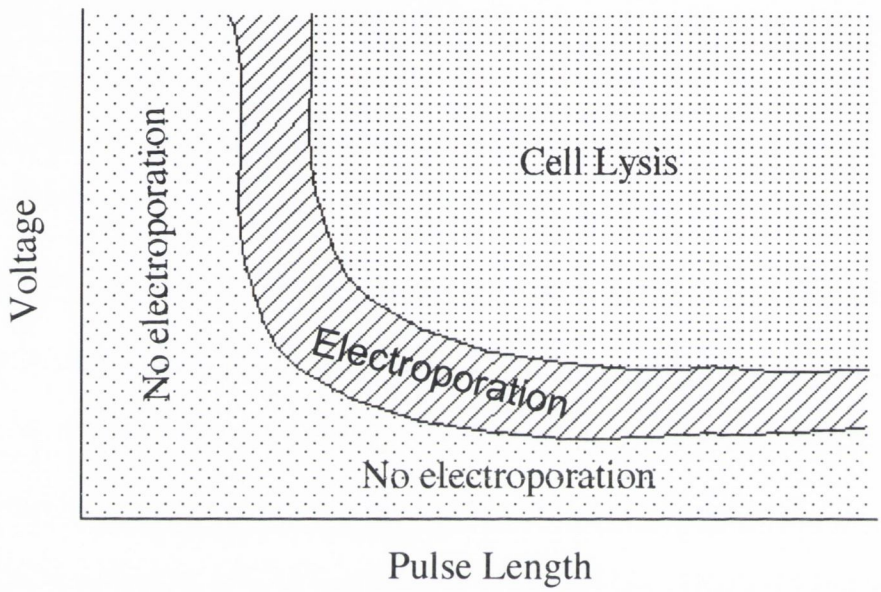


Figure 3.2 : Image depicting the relationship between field strength and pulse length for the electroporation of cells

3.1.5 In vitro electroporation

It was necessary to examine the effectiveness of the electroporation *in vitro* prior to the *in vivo* experiments. This is done to approximate the *in vivo* analysis of electroporation efficacy and as such reduce the number of animals used in experimentation (in accordance with the concepts of replacement, refinement and reduction, Russell *et al.*, 1954). The *in vitro* optimization of the electroporation parameters allows for an increased understanding of the effects of electroporating naked RNA or DNA directly

into cells as well as providing information on the effectiveness of electroporation on a variety of different cell types. The parameters examined include cell shape and size, pulse field strength (v/cm), pulse duration, pulse number, pulse type, electroporation temperature and pulsation medium. The examination of these parameters will provide more information on the *in vitro* electroporation of SFV based plasmids but will not impact of the paramter selection for the *in vivo* pulseforms. These parameters have been compared for efficiency of electroporation and cell viability. Flow cytometry, luminometry and microscopy were used to assess the electroporation efficiency as well as the cell viability.

3.2 Results

3.2.1 Cell size and shape

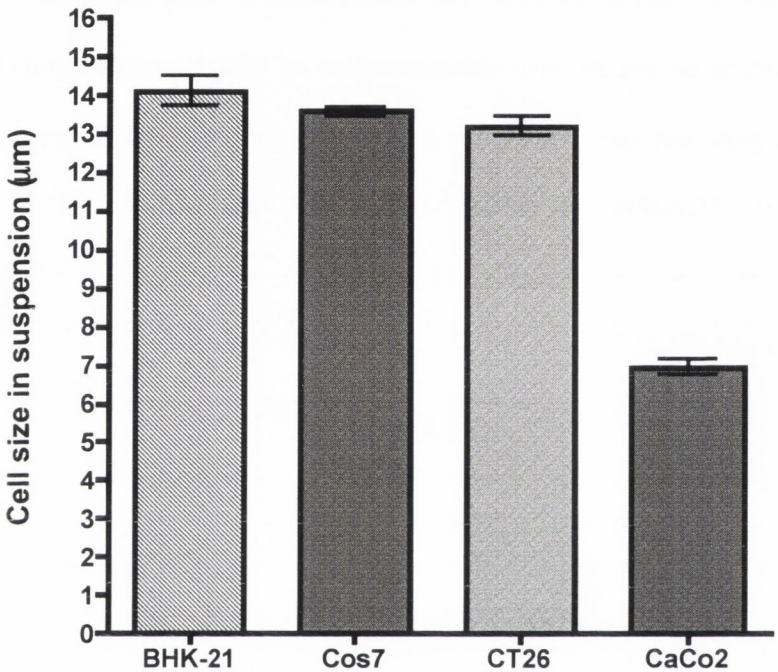


Figure 3.3: Size and shape of a variety of different cell types in suspension examined using the Cell A Life Sciences Imaging Software (Olympus). A minimum of 68 cells was measured for each cell type with the arbitory horizontal distance across the cell being used in each case.

Cells were suspended in PBS (+MgCl) and the cell size and shape examined by microscopy. The BHK-21 cell line is commonly used for transfection with the SFV vector system and is considered an easily transfectable cell line by electroporation. This cell line was observed to have a large (14.68 μm±1.67) and uniform spherical shape when analyzed in suspension. Cos7 is a commonly used cell line for transfection by a

wide variety of different methods. This cell line while observed to have a smaller cell size (13.57 μm±1.05) than the BHK-21 cell line, are as uniform in cell shape (spheroid). The CT26 cell line has a similar size (13.16 μm±1.8) to the Cos7 cell line but the cells while having a spherical shape were found to be less uniform than either the BHK-21 or Cos7 cell lines (the coefficient of variation for the CT26 cell was 13.98% in comparison to 7.73% for the Cos7). The Caco2 cell line has been found to be difficult to transfect by any method. These cells were found to have the smallest (6.99 μm±2.37) and least uniform cell size and shape (the coefficient of variation for the Caco2 is 33.92%).

3.2.2 Pulse parameters

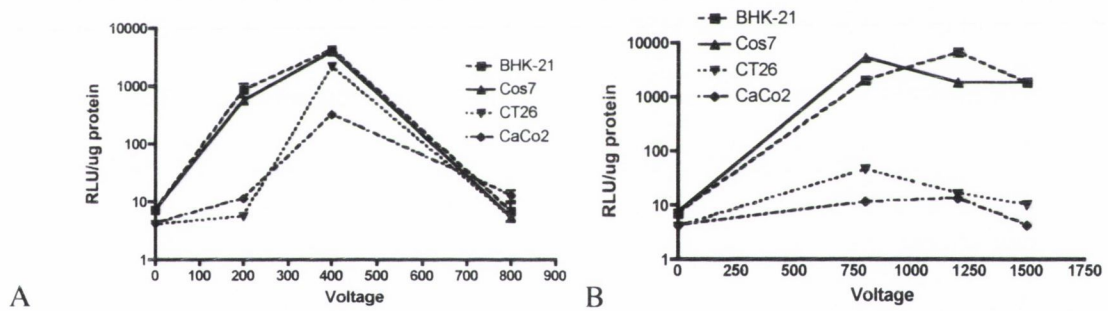


Figure 3.4: Effect of voltage (v/cm) on transfection efficiency at 100 Δs (A) and 20 ms (B) pulse duration were examined for each cell line using eight pulses. 50 μg of luciferase was delivered in each case and detected 14 hours post electroporation as described in section 2.2.6.3. A minimum of three replicates were used in each case and the luminescence (RLU) per μg of protein graphed.

A range of different cell types were examined for transfection efficiency with two pulse durations. These two pulse durations were selected to contrast the effectiveness of a long and short duration pulse over a range of voltages for each of the cell types examined. 100 μs pulse duration was selected based on the electroporation in vitro optimization work of Cegovnik *et al.*, 2004 where lower duration pulses were examined on a range of different cell lines. 20 ms was selected as a longer pulse to contrast with the short pulse over the voltage range as it has be hypothosized by a number of researchers (Lucas *et al.*, 2001, Satkauskas *et al.*, 2005) that DNA will be driven into the cell by an electrophoretic effect using lower duration pulses. This pulse duration has been previously used for *in vitro* electroporation by Lewis *et al.*, (1999). As the pulse duration and the voltage are related it is essential that each of these parameters were examined in conjunction. 1 x 10^6 cells were electroporated with 50 Δg of DNA/RNA in each case. The CT26 cell line and the Caco2 cell line were observed to give low transfection efficiencies at 20ms pulse duration while the BHK-21 cell line and the Cos7 cell line both showed in high levels of luciferase expression when pulsed under these conditions (B). The BHK-21 and Cos7 cell lines also showed a high level of transfection efficiency when voltage was examined at 100 Δs pulse duration (Figure 3.5 (A)). Both the BHK-21 and the Cos7 cell lines had a higher transfection efficiency for 800 v/cm 100 Δs duration while the CT26 were found to have optimal expression at 400 v/cm for 20 ms duration. The Caco2 cell line was found to have extremely low luciferase expression levels throughout the range of voltages examined.

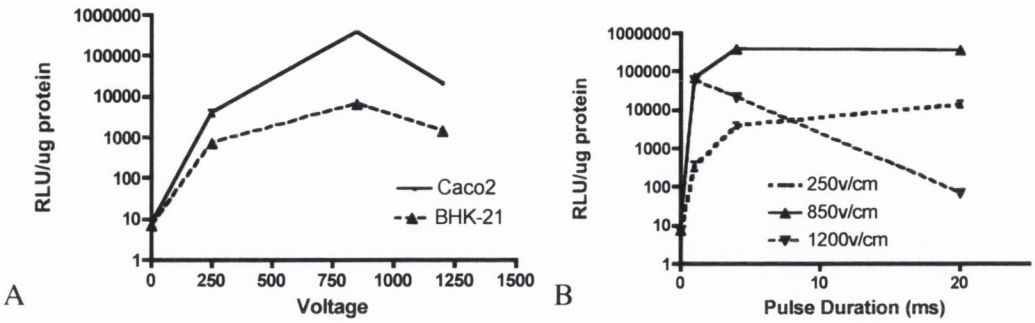


Figure 3.5: Effect of voltage on transfection efficiency at 5 ms with BHK-21 cells (two pulses) and Caco2 cells (four pulses) (A) and pulse duration on Caco2s (B). 25 μg of luciferase was delivered in each case and detected 14 hours post electroporation as described in section 2.2.6.3. A minimum of three replicates were used in each case and the luminescence (RLU) per μg of protein graphed.

The 100 Δs and 20 ms pulse forms resulted in low levels of luciferase expression for the Caco2 cell line and so it was further examined at a range of pulse numbers, durations and voltages. Two pulses of 850 v/cm and 4 ms duration have been shown to result in effective SFV RNA delivery to BHK-21 cells (Lundstrom, 1999) and so this pulseform was selected as a basis for further analysis with the Caco2 cell line. No statistically significant difference (95% confidence interval) in luciferase expression was obtained when the previously optimised pulseform (eight pulses of 800 v/cm and 100 Δs duration) (Figure 3.4) is contrasted with the Lundstrom (1999) optimised pulseform (two pulses of 850 v/cm and 4 ms duration) for BHK-21 cells (Figure 3.5 A). The Caco2 cell line was initially examined for luciferase expression following two pulses of 850 v/cm and 5ms duration but low levels of expression were obtained and so the number of the 850 v/cm pulses was increased resulting in an increase in luciferase

expression with the Caco2 cell line. Four pulses were found to be the optimal number of pulses for use with the Caco 2 cell line (Figure 3.5 A). The Caco2 cell line was further examined at a range of different voltages at 5ms pulse duration at the optimal pulse number. 850 v/cm was found to be the optimal voltage (Figure 3.5 A) and a lower voltage (250 v/cm) and a higher voltage (1200 v/cm) were selected to be examined over the range of pulse durations. 250 v/cm was selected as Lucas *et al.*, (2001) and Satkauskas *et al.*, (2005) among others suggest that lower voltages for a longer duration have the effect of driving the gene/ drug into the cell by an electrophoretic effect and further suggest that a high voltage pulse for a short duration is all that is needed for the initial portation of the cell. Satkauskas *et al.*, (2005) demonstrated that once a high voltage pulse is applied to initially porate the muscle cells the DNA transfection efficiency is governed by the electrophoretic effect of the low voltage pulse. While this type of combined pulseform was not achievable using the electroporation equipment available 1200 v/cm was selected to examine the effect of a high voltage short duration pulse on the transfection of the Caco2 cell line (Figure 3.5 B). It is clear from Figure 3.5 A that a high voltage short duration pulse is as effective as a low voltage long duration pulse as discussed in section 3.1.4, but four pulses 850 v/cm for 5 ms remained the optimal pulse for electroporation of Caco2 cells.

The optimal pulse conditions were found to be:

Cos7: 800 v/cm 100 Δs duration x 8 pulses

BHK-21: 800 v/cm 100 Δs duration x 8 pulses / 850 v/cm 5 ms duration x 2 pulses

[850 v/cm 5 ms duration x 2 pulses was used for further examination of the BHK-21 cell line as this is a well established pulseform used to electroporate this cell line]

CT26: 400 v/cm 20 ms duration x 8 pulses

Caco2: 850 v/cm 5 ms duration x 4 pulses

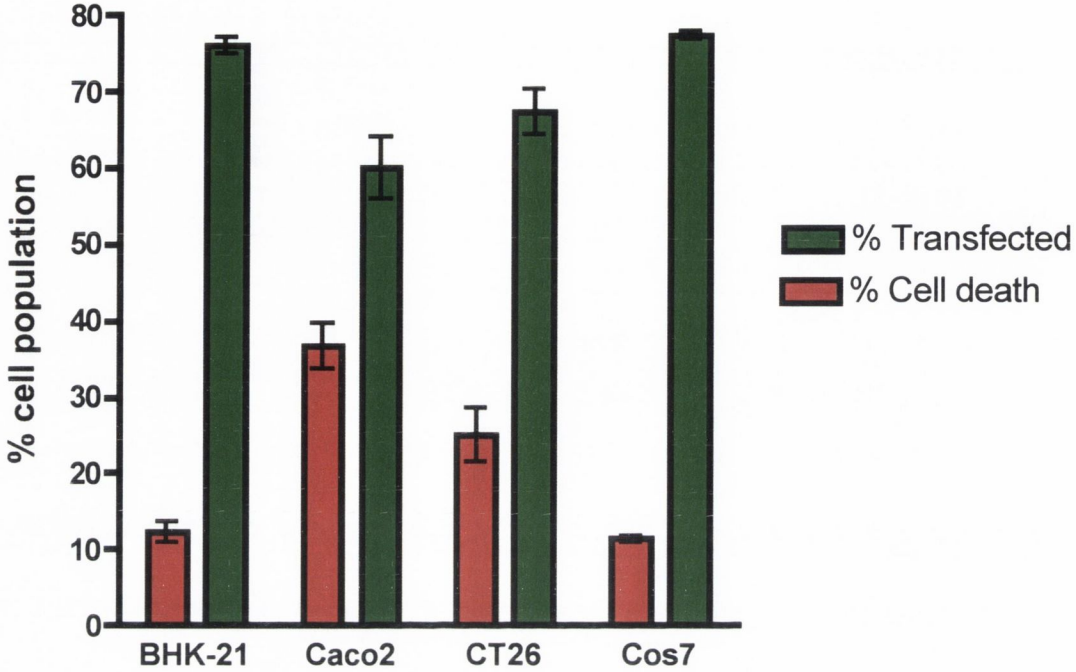


Figure 3.6: Examination of various cell lines for transfection efficiency (pSFV1-EGFP expression) and cell death (PI expression) 14 hours post electroporation using the optimal pulse conditions (as listed above) with 50 Δg pSFV-EGFP RNA.

The relationship between cell death and electroporation efficiency was examined for each of the optimised cell lines. The BHK-21 cell line was examined at both optimal conditions with no significant difference (95% confidence interval) observed between these pulse parameters. The results were analysed using flow cytometry to detect the fluorescence due to the expression of the Enhance Green Fluorescent Protein (EGFP) as an indication of transfection efficiency and propidium iodide (PI) uptake and fluorescence as an indication of cell death 14 hours following electroporation with 50 Δg pSFV-EGFP. When the percentage cell death following electroporation of the BHK-21 cell line is contrasted to that of the Cos7 cell line no significant difference in the

percentage of cell death is observed (99% confidence interval), this is also the case when the percentage transfection for these two cell lines are contrasted. A similar result is observed when the Caco2 cell line or the CT26 cell lines are examined for a significant difference in the percentage of cell death or the percentage transfection, but this is most likely due to the high standard deviation observed in the results with these cell lines. A significant difference may have been observed if larger groups had been examined. $1x10^6$ cells were examined in each case with the cells in logarithmic growth phase. It is essential that the cells are pulsed just prior to confluency (~80%) as any variance around this will lead to an increased level of cell death and a decrease in the level of gene expression.

3.2.3 Temperature

Each of the cell lines was examined for the effect of temperature change on the transfection efficiency and cell death. Cells were typsinized as normal, stored at 4°C for five minutes and resuspended in cuvettes prior to electroporation. Cells were immediately placed within warm media (stored at 37°C) following electroporation.

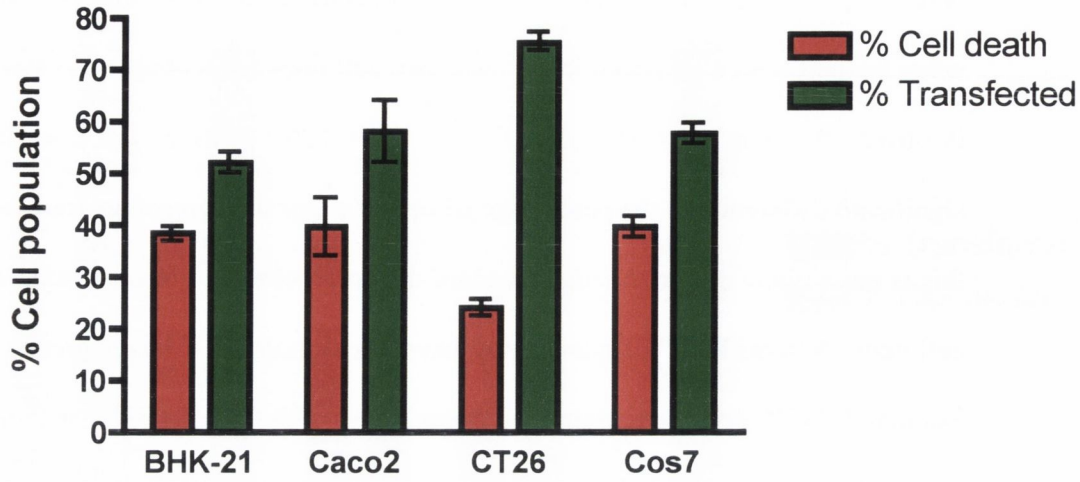


Figure 3.7: Examination of the effect of lowering cell temperature at the time of electroporation on various cell lines for transfection efficiency (pSFV1-EGFP expression) and cell death (PI expression) 14 hours post electroporation using the optimal pulse conditions (section 3.2.2) with 50 Δg pSFV-EGFP RNA.

A higher percentage cell death was observed for all cell types when the temperature was lowered for electroporation. While in some cases (Caco2 and CT26) an increase in gene expression was observed there was no statistically significant difference (95% confidence interval) in the level of gene expression than when cells were electroporated at room temperature. For the BHK-21 and Cos7 cell lines a statistically significant increase (95% confidence interval) in cell death was observed when the electroporation temperature was lowered.

3.2.4 Pulsing medium

Four different pulsing media were selected for comparison using the BHK-21 cells with the 850 v/cm 5 ms duration and two pulses. Two different PBS solutions were examined contrasting the effect MgCl in PBS solution has on the percentage transfection. The PBSucrose solution is an electroporation solution that is being used by a number of research groups (e.g. CNRS, France) to optimise in vitro electroporation and serum free media is a commonly used electroporation solution.

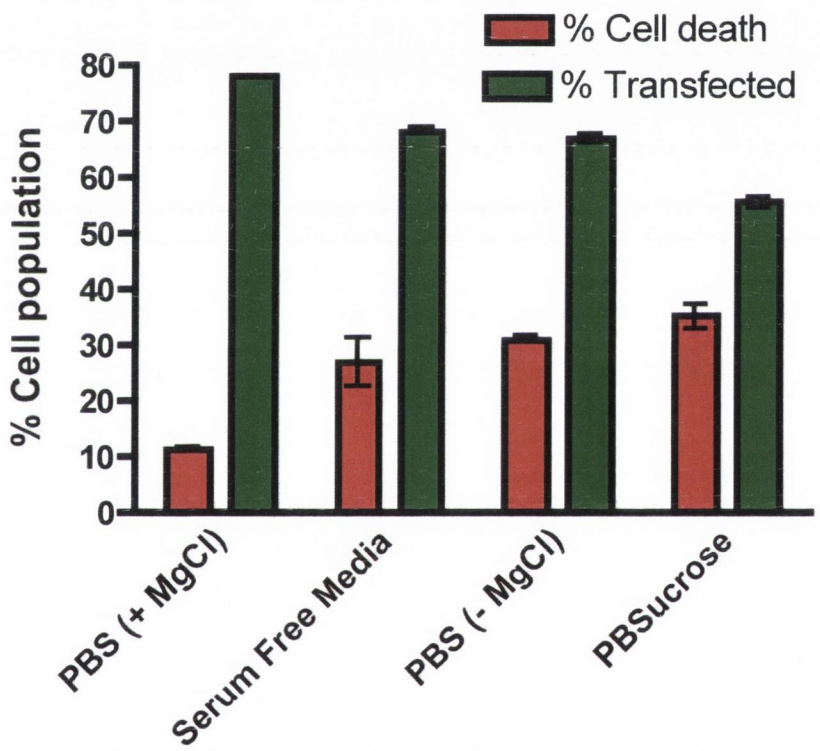


Figure 3.8: Examination of the effect of the pulsing medium on the electroporation of the BHK-21 cell line for transfection efficiency (pSFV1-EGFP expression) and cell death (PI expression) 14 hours post electroporation using the optimal pulse conditions (section 3.2.2) with 50 Δg pSFV-EGFP RNA.

There was found to be no significant difference (95% confidence interval) in the level of tranfection efficiency found when various pulsing media were examined. A higher percentage of gene expression was observed with the PBS (+ MgCl) when compared to the other media and a lower percentage of cell death was found with this pulsing medium. A statistically significant difference was observed between the level of cell viability and EGFP expression following use of the PBS (+ MgCl) and the PBSucrose (95% confidence interval). A higher percentage of cell death and lower gene expression was observed when cells were electroporated in the PBSucrose.

3.2.5 Contrasting SFV RNA and DNA plasmids

Both the SFV RNA and DNA plasmid contain the sequence for the non-structural proteins of the SFV virus. This enables enhanced RNA replication within the cytoplasm of the transfected cell. While the RNA plasmid only has to be delivered to the cytoplasm of the cell to initiate this RNA replication the DNA plasmid must enter the nucleus of the cell where the CMV element of this plasmid initiates replication. Once the RNA has been shunted into the cytoplasm enhanced RNA replication based on the SFV replication complex is initiated.

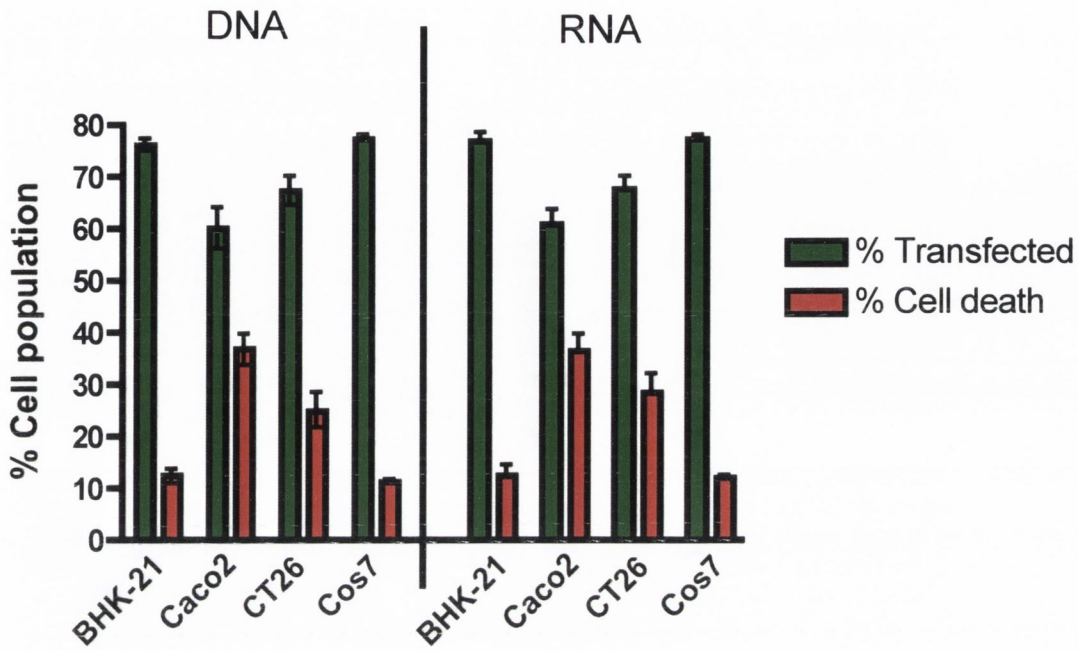
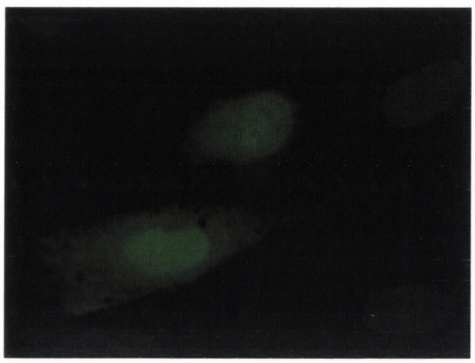
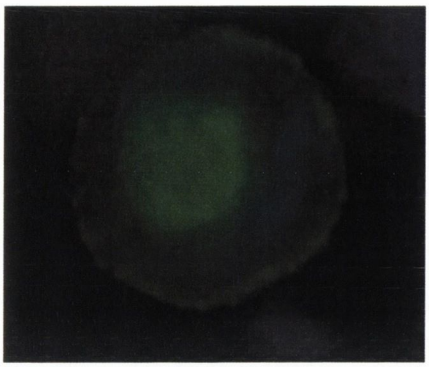


Figure 3.9: Examination of the various cell lines for transfection efficiency (SFV-EGFP expression) and cell death (PI expression) 14 hours post electroporation using the optimal pulse conditions (section 3.2.2) with 50 Δg of either SFV-EGFP RNA or DNA.

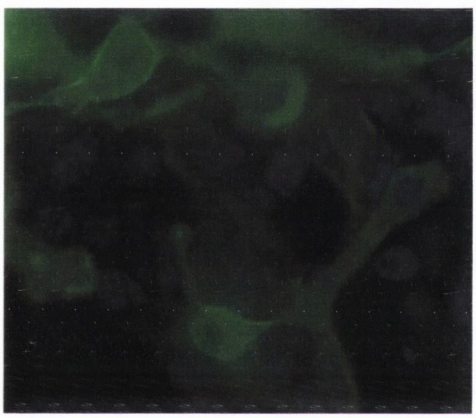
There was found to be no difference in the level of gene expression or cell death observed when either the SFV RNA or DNA was applied to each of the cell lines. It is thought that while the DNA plasmid must enter the nucleus in order to have gene expression the electroporation of the cell drives this and so as both plasmids have the SFV replication complex both plasmids have an equal degree of EGFP expression and hence fluorescence.



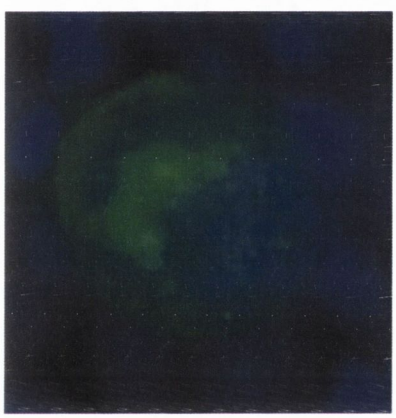
BHK-21 cells x 400



Cos7 cells x 1000



CT26 cells x 400



Caco2 cells x 1000

Figure 3.10: Fluorescent microscopy analysis of various cell lines 14 hours post electroporation with pSFV1-EGFP RNA (50 Δg). The optimal pulse conditions were used for each cell line (section 3.2.2). Fluorescence was used to determine transfection efficacy based on EGFP expression.

There was found to be a high degree of gene expression for the BHK-21 cell line and the Cos7 cell line. The CT26 cell line was found to have a high level of fluorescing cells interspaced with non-fluorescing cells. The cells that were fluorescing were found to be larger and more rounded in shape. Only the larger cells were found to be fluorescing when the Caco2 cell line was transfected while there was a large degree of cell death and a high number of non-fluorescing smaller cells observed.

3.3 Discussion

3.3.1 Cell size and shape

The results clearly show *in vitro* electroporation to be effective in a variety of different cell types. Each different type of cell requires slightly different electroporation conditions for optimal gene expression and minimal cell death. The impact of the pulsing conditions on the different cell types can be related back to the size and shape of the various cells when examined suspended in the pulsing medium (PBS (+ MgCl)). The impact of cell size on electroporation has been well documented. Gehl *et al.*, (2003) outlines how the smaller the cell radius, the larger the external field needed to achieve permeabilization. It has been observed that cells that are larger and more uniform in size (e.g. BHK-21 and Cos7) can be electroporated using milder pulse conditions. There was found to be less cell death and higher transfection efficiencies with these cell types. According to simulations by Neumann *et al.*, (1999) this is due to the electrical field acting on each cell type in a similar fashion when the cells are all symmetrical spheroid shaped i.e. each cell has an equal amount of power acting on it's membrane and hence

will permeate to a similar degree. Based on these theories (Neumann *et al.*, 1999, Krassowska *et al.*, 2007) asymmetric cells such as the CT26 and Caco2 cell lines have a greater field strength at the ends of the cells that are parallel to the electrical field. The ends of such cells may also be more readily porated because the radius of curvature is smaller than that of the membrane surrounding the cell body. Somiari *et al.*, 2000 describes in the case of radially symmetric cells such as myofibers, that the electroporation threshold at the surfaces perpendicular to the field is quite different if the field is administered parallel to the myofiber rather than perpendicular to the long axis.

A similar effect would be observed with the CT26 and Caco2 cell line where there would be a higher electrical field acting on the surface of the cell if the more curved side of the cell is parallel to the electrodes than if it is perpendicular to the electrodes. Hence the variability in the shape of the CT26 cells can increase the variability of the results. If there are more cells in suspension with the curved side parallel to the electrodes there is a higher electrical field acting on these cells, hence there is a higher degree of permeability and so increased gene delivery and cell death (Golzio *et al.*, 2001). If there are a higher number of cells with the less curved side parallel to the electrodes then the electrical field acting on these cells is lower and so there is decreased permeability and decreased gene delivery and cell death. As there is no method to align the cells for electroporation in suspension the lack of uniformity of cell shape in the CT26 cells when in suspension will result in a higher degree of variability in the transfection efficiencies and cell death (for CT26 cells the coefficient of variation in cell size is 13.93%).

This effect of variability in cell shape is even more apparent with the Caco2 cell lines as not only are these cells observed to be erratic in shape when in suspension but they are also found to be of smaller size (the coefficient of variation is 33.92%). As a result of the small size of the Caco2 cell line a higher electrical field is necessary to electroporate them (Neumann *et al.*, 1999, Heller *et al.*, 1999) and so when there is variability in the size and shape a higher degree of cell death occurs (Somiari *et al.*, 2000, Golzio *et al.*, 2001).

3.3.2 Pulse parameters

The most important parameters that impact on the electroporation effectiveness are electric field strength, pulse duration and the number of pulses. As described earlier the relationship between the electric field strength (voltage) and the pulse duration is an inversely proportional one (Weaver *et al.*, 1994). Each of the cell lines were examined at a range of different voltages, pulse durations and pulse numbers. As can be observed from the results each of the cell lines had an increase in expression as the voltage or pulse duration was increased until the maximum field strength and duration for transfection was achieved. At voltages above the maximum field strength and pulse duration there is a decrease in the gene expression. This is due to the increase in cell death at higher voltages and pulse durations.

The increase in transfection efficiency as the voltage increase has been observed by many authors over a range of cell lines (Chu *et al.*, 1987, Prausnitz *et al.*, 1993, Rols *et al.*, 1998, Delteil *et al.*, 2000, Canatella *et al.*, 2001, Cegovnik *et al.*, 2004). The effect of the pulse parameters is determined by the cell size and shape as has been described

earlier. For a larger uniform cell type such as the BHK-21 cell line there are a range of voltages that may be effective for efficient transfection. This type of cell line will have a large electroporation threshold (Figure 3.2). The pattern for the BHK-21, Cos7 and CT26 cell lines largely followed that observed by Cegovnik *et al.*, (2004) when examining SA1, LPB, L929 and CHO cells; Delteil *et al.*, (2000) when examining CHO cells and Prausnitz *et al.*, (1993) when examining red blood cells. Namely, the increase of the electric field strength or duration was followed by an increase in the proportion of cells expressing the reporter gene and a reduction in the number of viable cells.

The results were slightly different with the Caco2 cell line due to the nature of the shape of this cell line leading to an extremely narrow range of electric field strengths or durations in which an optimum transfection efficacy and cell survival is achieved. The results for this cell line were similar to those found by Cegovnik *et al.*, (2004) when examining B16F1 cells and Rols *et al.*, (1998) when examining B16 cells. These cells like the Caco2 cells are found to be small (~10 Δm) and non-uniform in shape when in suspension. It is thought that with these cell lines a more complex pulseform combining a high electric field for a short duration and a low electric field for a long duration may be more effective (Lucas *et al.*, 2001).

3.3.3 Effect of temperature on electroporation

The effect of changing the temperature at the time of electroporation has been described by some authors as a method for increasing electroporation efficiency (Gehl *et al.*, 2003). When cells are held at 4^0C at the time of electroporation the cell will remain porous for longer durations (Weaver *et al.*, 2000). It was found that by

decreasing the temperature at the time of electroporation there was no significant increase in transfection efficiency for any of the cell lines examined but there was an increase in cell death. This is most likely due to a decrease in the transient nature of the electroporation. A similar result was found by Rols *et al.*, (1994) when examining CHO cells. There was found to be no significant increase in transfection efficiency due to the decrease in cell viability. It is thought that if electroporation conditions (i.e. the pulse parameters applied) been optimized at the lower pulse conditions there may have been more effective transient permeabilisation of the cells. Since lower temperatures allow the pore to remain open for longer durations, a pulse of smaller duration may have been more effective. It should also be considered while there is data to suggest that the pores in the cell membrane may remain open for longer durations at low temperatures (Weaver *et al.*, 2000, Rols *et al.*, 1994) there is no real evidence to show that gene transfer occurs through these pores (Tessie *et al.*, 2005).

3.3.4 Effect of pulsing medium on electroporation

As with the change in temperature there was found to be no significant increase in gene expression found in each of the cells examined by changing the pulsing medium. These results correspond to the results of Rols *et al.*, (1990) where it was found that the threshold value for permeabilization of CHO cells was independent of the ionic strength of the pulsing medium. While no significant increase in gene expression was observed by electroporating in a range of different pulsing media, there was a difference found in cell viability post electroporation. With the PBSurose there was a significant decrease in cell viability for all cell lines. This result corresponds with research by Djuzenova *et al.*,

(1996) where it was found that decreasing the media conductivity results in lower viability of the murine myeloma cells. Though it should be noted that an increase in gene expression was also observed by Djuzenova *et al.*, (1996). As with the change in temperature it is likely that the pulse parameters need to be optimized in each of the different pulsing media. It is thought that a lower pulse field strength may have yielded lower cell death as simulations by Lojewska *et al.*, (1989) implied that lower pulse voltages are needed with increased medium conductivity.

3.3.5 Comparison of SFV RNA and SFV DNA electroporation

Each of the cell lines were electroporated using the optimized pulseforms with EGFP RNA or DNA. There was found to be no difference in the level of gene expression or cell death observed when RNA or DNA was applied. While some authors have found there to be a difference in the level of gene expression when either RNA or DNA has been applied this is highly dependent on the cell type being electroporated. RNA is finding increased application in the area of dendritric cell electroporation where both on murine and human dendritic cells, high transfection efficiencies have been shown (Tendeloo *et al.*, 2001, Saeboe-Larssen *et al.*, 2002, Van Meirvenne *et al.*, 2002). The lack of difference between the RNA and DNA in this case is most likely due to the fact that both the RNA and DNA were of similar size as both were SFV based plasmids.

3.3.6 Conclusion

There were a variety of different parameters analyzed for the effect on electroporation efficiency, these included the shape of the target cells, temperature, conductivity of the poration medium, electric field strength, number of pulses, duration of pulse and the frequency applied. It is clear from the results that each of these parameters are related and hence each has to be analyzed in conjunction with the other parameters. While theoretically it is achievable to have over 90% of cells permeabilized with less than 5% cell death it is unlikely due to slight variations in cell size, density and growth phase. The results have shown *in vitro* electroporation of SFV RNA and DNA plasmids is effective in a wide variety of cell types but needs to be optimized based on the characteristics of each cell type.

Chapter 4

In vivo SFV plasmid electroporation for use in cancer gene therapy

4.1 Introduction

The ability of SFV to induce apoptosis in a variety of cell lines has been well established (Glasgow *et al.*, 1997; Scallan *et al.*, 1997). This capacity to induce apoptosis is also shared by the SFV vector and rSFV VLPs and occurs independently of viral structural proteins and the status of cellular p53 (Glasgow *et al.*, 1998; Murphy *et al.*, 2000). Given the previous success in exploiting this cytopathic effect by treating tumour xenografts in BALB/c *nu/nu* mice with rSFV VLPs (Murphy *et al.*, 2000; Murphy *et al.*, 2001), it was decided to investigate the possibility of electroporatively delivering the SFV vector with previously examined cancer gene therapies in order to enhance the antitumoural effect of the SFV vector.

Preliminary work developing this gene therapy has resulted in the cloning of murine *Bax* into a Semliki Forest Virus (SFV) vector and exploited the inherent ability of rSFV VLPs to induce p53-independent apoptosis in the treatment of H358a human lung carcinoma xenografts in BALB/c *nu/nu* mice (Murphy *et al.*, 2000). An antitumoural effect also observed in xenografts of the rat prostate cancer cell line AT3-*bcl*-2, was enhanced with the use of rSFV VLPs expressing the pro-apoptotic gene *Bax*, but was not as effective as that observed in the slower growing H358a xenografts (Murphy *et al.*, 2001). The therapeutic effect of the SFV vector encoding the *Bax* gene *in vivo* was compromised by a low yield of recombinant SFV particles due to induction of apoptosis in the packaging cells as a result of the *Bax* expression. This highly effective induction of apoptosis post electroporation *in vitro* makes *Bax* RNA an ideal candidate for development as a gene therapy in combination with *in vivo* electroporation. The objective of this section is to examine the proapoptoic efficiency of electroporatively

delivering this pSFV1-Bax RNA, a plasmid that encodes not only for the proapoptoic Bax gene but also the non-structural proteins of the SFV virus (the replicase complex).

4.2 *Bax*

Advances in our understanding of the mechanisms by which tumour cells detect drug-induced DNA damage leading to apoptotic death has aided in the design of novel, potentially more selective strategies for cancer treatment. Several of these strategies use proapoptotic factors and have shown promise in sensitizing tumour cells to the cytotoxic actions of traditional cancer chemotherapeutic drugs. One such strategy is the application of the proapoptoic gene Bcl-2 associated X protein (*Bax*) to the tumour site. *Bax* is a downstream effector of the tumour suppressor gene p53 and is upregulated by transcriptional activation. The *Bax* gene is one of the well-characterized proapoptotic genes, and its overexpression leads to apoptosis in a wide variety of cells, with or without additional stimuli (Xiang *et al.* 1996). Sturm *et al.*, (1999) found that low *Bax* expression was a negative prognostic factor in patients with resected liver metastases from colorectal cancer. Also, there is evidence that genetic damage to *Bax* genes causes resistance towards apoptosis as in tumourigenesis, but also resistance to anti-tumour therapy. In patients with breast cancer, a reduced expression of *Bax* was correlated with a poor response to chemotherapy and shorter overall survival (Krajewski *et al.*, 1995). Restoration of *Bax* expression in breast cancer cell lines increased sensitivity to cytotoxic drug therapy and also suppressed tumourigenesis (Yin *et al.*, 1997).

4.2.1 In vitro *Bax* results

The effectiveness of the electroporative delivery of SFV-*Bax* RNA was examined *in vitro* prior to the *in vivo* experiments. This was done to help direct the in vivo analysis of electroporation efficacy and as such reduce the number of animals used in experimentation (in accordance with the concepts of replacement, refinement and reduction, Russell *et al.*, (1954)). This *in vitro* analysis also provided information on the effectiveness of the electroporatively delivered *Bax* RNA and on the proapoptotic nature of this plasmid. Though the ability of the *Bax* gene to induce apoptosis has been well established , the combination of the SFV non-structural proteins (shown by Murphy *et al.*, (2000) to be proapoptotic) and the Bax gene are examined. The in vitro optimization of the electroporation parameters allows for an increased understanding of the effects of electroporating naked RNA into cells as well as providing information on the effectiveness of the *Bax* RNA.

While the proapoptotic effect of electroporated pSFV-*Bax* RNA has previously been shown *in vitro* (Murphy *et al.*, 2000) it was necessary to contrast the proapoptotic nature of the SFV vector with that of the pSFV-*Bax* RNA using the previously optimized protocol from the *in vitro* electroporation optimization (Figure 2.5).

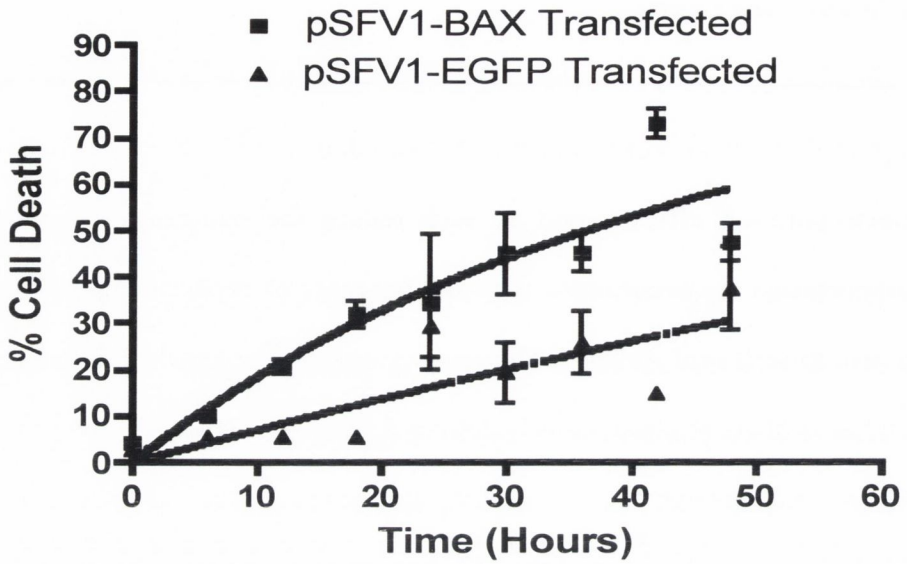


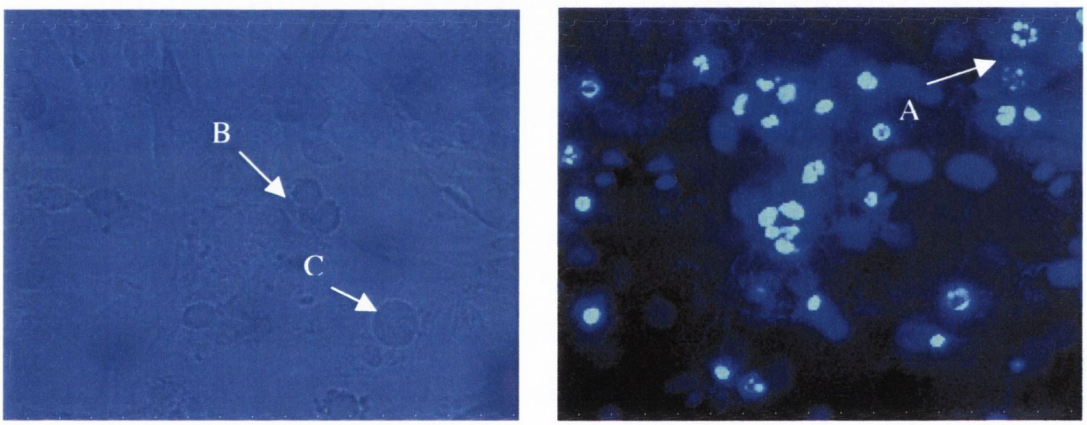
Figure 4.1: Effect of electroporatively delivered pSFV-*Bax* RNA on cell death. BHK-21 cells were electroporated with pSFV-*Bax* RNA using two pulses of 5 ms duration and of 850 v/cm (based on optimal pulse conditions section 3.2). Cells were harvested every six hours post electroporation and propidium iodide was added. Cell death (PI fluorescence) was analysed by flow cytometry.

The optimized *in vitro* electroporation pulse conditions (section 3.2) were applied to transfect BHK-21 cells with pSFV-*Bax* to examine the effectiveness of the electroporated pSFV-*Bax* at induction of apoptosis. Figure 4.1 shows the effect of electroporated pSFV-*BAX* RNA and SFV1-EGFP RNA over a 48-hour period. Flow cytometry was used to measure the fluorescence of propidium iodide as an indication of cell death at time points of every six hours after electroporation. pSFV-*Bax* is observed to be over twice as effective as the apoptotic SFV vector encoding EGFP (pSFV-*Bax* inducing apoptosis at a rate of 1.5%/hour in comparison to 0.7%/hour with the EGFP expressing vector). There was no difference in the percentage of cell death for

unelectroporated cells and those electroporated without any vector. It was observed that post 76 hours the percentage of cell death for the pSFV-*Bax* RNA and the rate of cell death for the SFV control vector were not significantly different (both being approximately 38%).

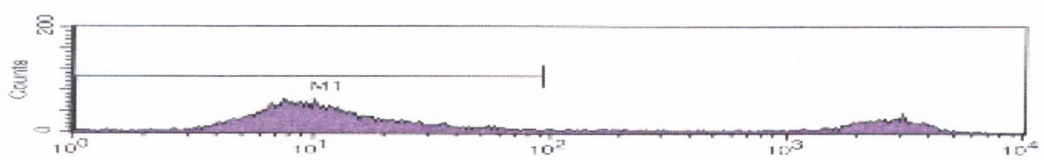
While there is an increase in the percentage of cell death when cells are transfected with pSFV-*Bax* RNA, it is necessary to look at morphological changes in the cell population to examine what type of cell death is occurring. pSFV-*Bax* has been well characterized to induce cell death through apoptosis both when applied in combination with the SFV vector (virally and non-virally delivered) (Murphy *et al.*, 2000) and when applied with other vector systems (Xiang *et al.*, 1996).

a)



b)

(Bax)(I)



(Control (II)

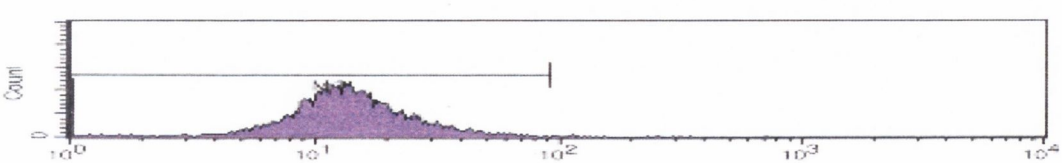


Figure 4.2: *a)* Effect of electroporatively delivered SFV-*Bax* RNA on cell death analysed by microscopy. BHK-21 cells were electroporated with SFV-*Bax* RNA using two pulses of 5 ms duration and of 850 v/cm. Cells were fixed with 4% paraformaldehyde 16 hours post electroporation and stained with VECTASTAIN DAPI Hardset mounting medium. Arrows show defragmented nuclei (A), blebbing (B) and membrane disintegration (C). Cells were also analyzed by flow cytometry for FITC expression using the Annexin V kit (BD Biosciences) 16 hours post electroporation with SFV-Bax RNA. Morphological changes in the cells were analysed using a fluorescent microscope and confirmed by flow cytometry.

Figure 4.2 above shows the effect of electroporated pSFV-*Bax* RNA and pSFV-EGFP (or pSFV-Luc) on BHK-21 cells at a 16 hour timepoint. There have been clear morphological changes observed in the cells transfected with pSFV-*Bax* RNA showing defragmented nuclei (A), blebbing (B) and membrane disintegration (C), all clear indications of the induction of apoptosis. These changes while present in the electroporated, unelectroporated cells or those cells transfected with the pSFV-EGFP or pSFV-Luc vectors are not observed at a high frequency. The morphological indications of apoptosis induced by the *Bax* expression are confirmed by FITC expression in the Annexin V assay as only cells undergoing apoptosis will fluoresce (I) in comparison to the control unelectroporated cells (II).

4.2.2 *In vivo Bax* results

Once the ability of electroporatively delivered pSFV-*Bax* RNA to induce apoptosis *in vitro* was established it was then appropriate to examine the ability of the electroporated pSFV-*Bax* RNA to inhibit the growth of subcutaneous CT26 tumours in BALB/c mice and OE19 tumours in nude mice.

CT26 cells were centrifuged and adjusted to a concentration of 1 x 10^6 cells/50 Δl and injected subcutaneously into the right flank of each mouse using a 1 ml syringe (Beckton Dickenson, UK) and 21 G needle in a 50 Δl volume. Mice were examined daily for signs of s.c. tumour formation and when detected, tumour diameters were measured using linear calipers in 2 perpendicular diameters with the average tumour volume calculated as the square-root of the product of cross-sectional diameters, assuming spherical shape. Mice were treated once a 4 mm tumour volume was obtained and were euthanised when tumours reached an average over 15 mm in diameter (above 1.7 cm^3).

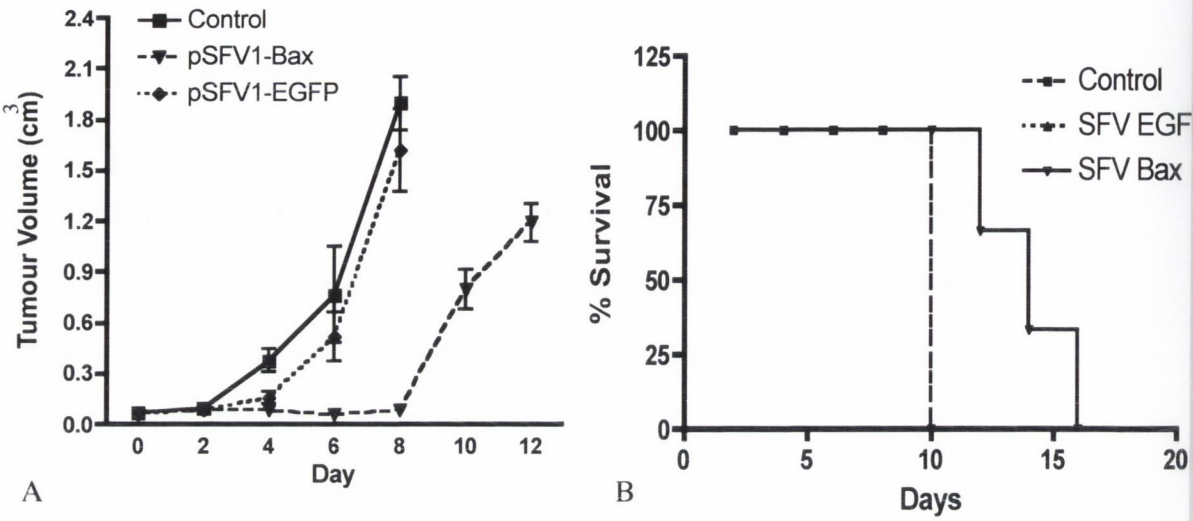


Figure 4.3: Treatment of subcutaneous CT26 tumours in immunocompetent BALB/c mice. Mice were treated with 50 Δl pSFV-*Bax* RNA electroporatively delivered using optimized intratumoural pulseform (Appendix 2A). Tumour volume was calculated every two days post treatment and mice euthanised when the average tumour volume reached above 1.7 cm^3 (day 8 for control groups). A) The average tumour volume per group of six mice was calculated and plotted against time for each group until tumour volume reached ~1.7 cm^3. B) The percentage survival per group post electroporative treatment (n=6) was calculated and plotted against time for the duration of the experiment.

There was no significant difference in the growth curve for the nuclease free water control group, the electroporation alone group and no treatment control group and so only the no treatment group is shown above.

Each of the control tumour groups grew in the same manner observed in untreated BALB/c mice. All six control tumours (in each control group) had reached an average

diameter above 15 mm by day 8 and mice were euthanised accordingly. Tumours treated with SFV-EGFP displayed some inhibition of tumour growth initially but the tumours still reached an average diameter above 15 mm by day 8 and the mice were euthanised. The SFV-*Bax* electroporately treated group showed significant inhibition of tumour growth until day 8. Post day 8 there was a significant increase in the tumour growth rate with 4 out of 6 tumours in this group reaching an average tumour volume above 15 mm by day 12 and the remaining 2 tumours by day 14.

Upon statistical analysis using a one-way repeated measures ANOVA with Tukey's multiple comparison post test, this inhibition of tumour growth was found to be insignificant in comparison to control tumours (comparison of all groups over entire experiment). It can therefore be concluded that electroporatively delivered pSFV-*Bax* is incapable of significantly inhibiting CT26 tumour growth in the BALB/c mouse model following one treatment. The data was analysed solely at day 8 (prior to the regrowth of the tumours) and showed a highly significant ($P = <0.001$) inhibition of tumour growth when the electroporatively pSFV-*Bax* treated group is compared to that of the untreated tumours using the student t test.

The tumour sections from six days after the electroporative treatment were stained using haematoxylin and eosin (H&E) and examined histologically to evaluate the effect of the electroporative treatment on the CT26 tumours. Tumours that were untreated, treated with nuclease free water alone or electroporation alone and no treatment control groups all showed lobular proliferations of polyhedral tumour cells separated by thin-walled blood vessels (Figure 4.4 (D)). While tumours that were electroporatively treated with

the pSFV1-Bax showed areas of necrosis and oedema adjoining islands of tumour cells. These tumour cells are closely packed around thin-walled blood vessels (Figure 4.4 (A)). The areas of necrosis and oedema are surrounded by intact tumour cells in each of the pSFV1-Bax treated tumours (Figure 4.4 (B)). Pockets of tumour cells with round to spindle-shaped, coarsely granular nuclei are loosely arranged in oedematous stroma. Apoptotic forms and necrosis are prominent in these areas (Figure 4.4 (C)).

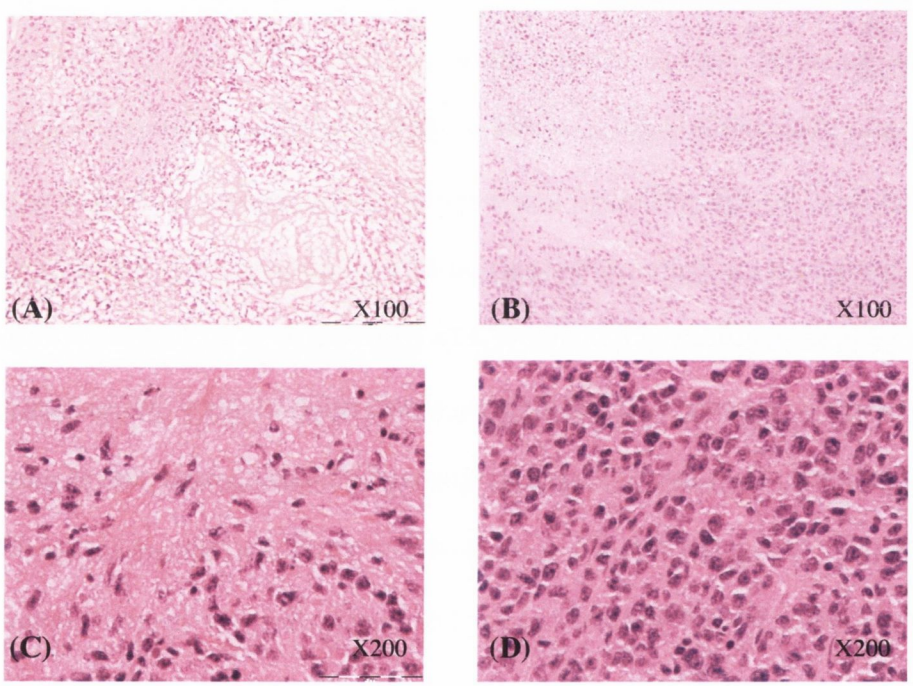


Figure 4.4: Histopatholgical examination of H&E stained (as described in section 2.2.11) CT26 tumour sections six days following electroporation with pSFV1-Bax RNA (50 μl) (A-C). Histopatholgical examination of H&E stained electroporated only CT26 tumour sections six days following electroporation (D). All control tumours showed a similar pattern of histopathology to that of the electroporation only group on day 6 (D).

It was necessary to examine more than one cell line and so a slow growing OE19, a human esophageal cell line was selected to contrast with the fast growing CT26 murine colon adenocarinoma cell line.

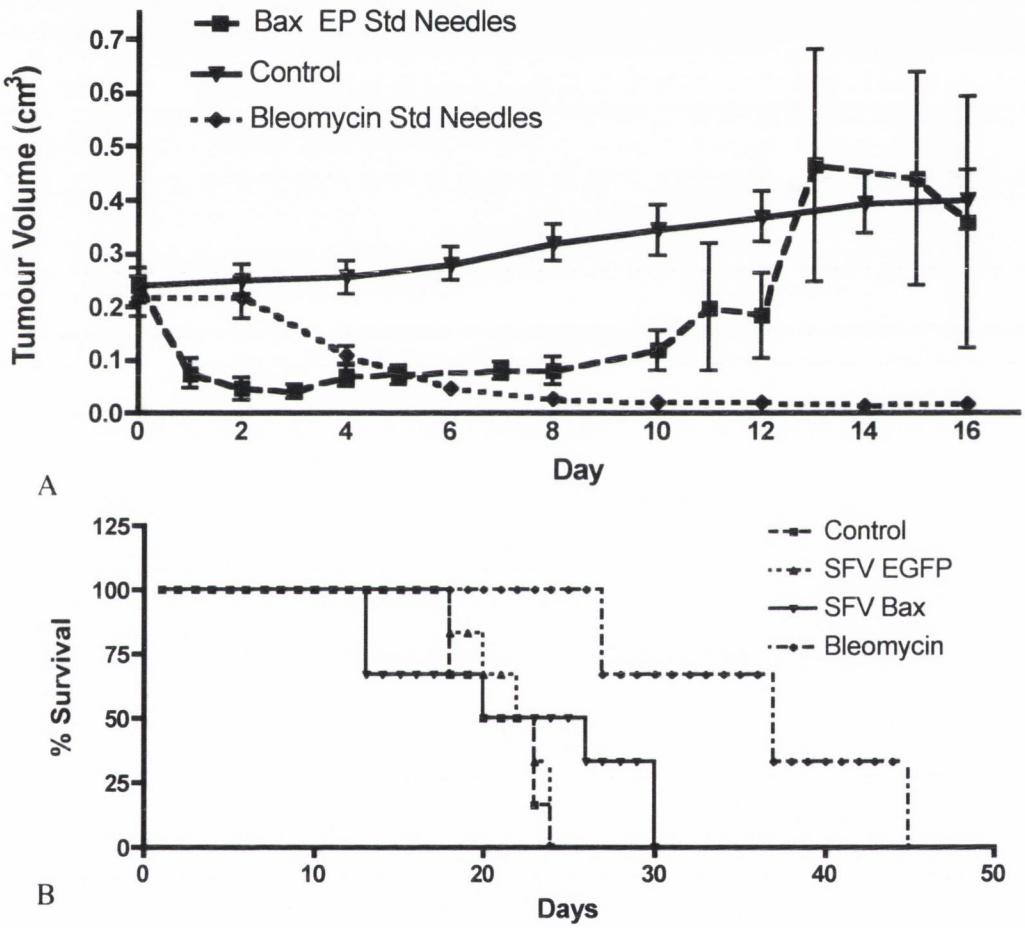


Figure 4.5: Treatment of subcutaneous OE19 tumours in nude mice. Mice were treated with 50 Δl pSFV-*Bax* RNA (or 1500 units of Bleomycin) electroporatively delivered using optimized intratumoural pulseform (Appendix 2A). Tumour volume was calculated every two days post treatment and mice euthanised when the average tumour volume reached above 1.7 cm^3 (day 18 for control groups). A) The average

tumour volume per group of six mice was calculated and plotted against time for each group until the tumour volume reached ~1.7 cm^3. B) The percentage survival per group post electroporative treatment (n=6) was calculated and plotted against time for the duration of the experiment.

OE19 tumours were induced in female nude mice by s.c. injection of 50 Δl of OE19 cell suspension in non-supplemented RPMI at a concentration of 1 x 10^8 cells/50 Δl (a high cell concentration was used as this is a slow growing human cell line). Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of six mice were used per treatment group. Tumours received one 50 Δl intratumoural injection of nuclease free water alone, bleomycin alone, electroporation alone, no treatment or electroporatively delivered SFV-*Bax*, SFV-EGFP RNA (50 Δg) or Bleomycin (3000 units/ml). Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when the average tumour diameter reached above 15 mm. There was no significant difference in the growth curve for the nuclease free water alone, bleomycin alone, electroporation alone and no treatment control groups and so only the no treatment group is shown above.

Each of the control tumour groups grew in the same manner observed in untreated nude mice. While there was more variability in the tumour growth rate with this cell line all six control tumours (in each control groups) had reached an average diameter above 15 mm by day 23 (e.g. untreated control: 2 day 18, 3 day 20, 1 day 23) and mice were

euthanised accordingly upon reaching the 15 mm tumour volume. Tumours treated with SFV-EGFP displayed no inhibition of tumour growth with this cell line but reflected the growth pattern of the untreated control group. The SFV-*Bax* electroporately treated group showed significant inhibition of tumour growth until day 10 (student t test p=<0.05). Two of the six SFV-*Bax* electroporatively treated tumours had reached a tumour volume comparable with that of the untreated control group by day 13. By day 23, when all of the control group tumours had exceeded a volume of 15 mm, only 2 of the SFV-*Bax* electroporately treated tumours remained. The final 2 mice were euthanised from this group on day 29 once the tumours had exceeded 15 mm in diameter. There appeared to be an almost complete regression in the bleomycin treated group but these tumours had all regrown by day 30 and had all exceeded 15 mm in diameter by day 44.

Upon statistical analysis using a one-way repeated measures ANOVA with Tukey's multiple comparison post test, this inhibition of tumour growth was found to be significant ($P<0.05$) in comparison to control tumours (comparison of all groups over entire experiment). It can therefore be concluded that electroporatively delivered SFV-*Bax* is capable of significantly inhibiting OE19 tumour growth in the nude mouse model following one treatment. The overall experimental statistical significance noted with the OE19 tumour treatment and not with the CT26 tumour treatment using the one way ANOVA may be due to the fact that OE19 is a slower growing tumour cell line and hence a difference in growth pattern may be more prevalent over time with this cell line. The SFV-*Bax* treated groups was observed to be significantly different when compared to each of the control groups (having a P value < 0.001, when contrasted to the

untreated control group using the student t test) at day 8, though no statistical significance is observed post day 11 using the student t test. A more significant difference in the tumour growth rate is observed between the bleomycin electroporatively treated group (P value < 0.001, when contrasted to the untreated control group using the student t test up as far as day 16). While there was a significant inhibition of tumour growth with the electroporatively delivered SFV-*Bax* RNA, the electroporatively delivered bleomycin (electrochemotherapy) had a significantly higher inhibition of tumour growth (with a P value <0.05 using the student t test).

4.3 *IL-12*

The SFV RNA vector has been shown to be successful when used in combination with the proapoptotic gene *Bax* both *in vitro* and *in vivo*. To further examine the combination of the SFV RNA and electroporation *IL-12* was selected as a potential immunogenetherapy. Intratumoural injections of SFV expressing the cytokine *IL-12* showed significant tumour regression and inhibition of tumour angiogenesis in a mouse B16 melanoma model (Zhang *et al.*, 1997, Yamanaka *et al.*, 2000). *IL-12* has previously been shown to be highly effective when used in combination with the SFV viral vector system (Gowda *et al.*, 2006) and SFV-IL12 was shown to cause significant cytotoxicity in solid tumours in pre-clinical animal models of human breast, lung, prostate and pancreatic cancer in mice (Murphy *et al.*, 2000; Hardy *et al.*, 2000).

Several studies in animal models have indicated that the expression of various cytokine genes in tumour cells generally results in a dramatic alteration of tumour cell growth and the induction of tumour specific immunity (Rakhmilevich *et al.*, 1996).

Furthermore, the use of various viral vectors in the delivery of *IL-12* has demonstrated a promotion of tumour regression and inhibition of metastases (Lasek *et al.*, 1997, Ren *et al.*, 2003), but the *IL-12* concentration at the tumour site is critical for tumour regression (Colombo *et al.*, 1996). In order to sustain high *IL-12* concentration at the tumour site approaches such as frequent intratumoural injections with *IL-12* encoding virus particles that have enhanced expression may be required.

Cytokines are hormones produced mainly by lymphocytes and monocytes (Macatonia *et al.*, 1995) that act primarily within the immune system and can modulate the intensity of the immune response. Cytokines and related immunomodulators have been shown to produce antitumour effects in various types of cancers. The reason for this ability is that they directly inhibit tumour growth (e.g., interferon-alpha) and deliver immune factors to the tumour site. Cytokines also augment the effector function of T cells in recognizing MHC-presented peptides on tumour cells (Manetti *et al.*, 1994). Immunotherapy with cytokine-secreting tumour vaccines is considered to be most beneficial in patients whose tumours have been resected and in whom immune responsiveness has been restored. This treatment has been examined as an adjuvant therapy to prevent the growth of micrometastases and examined for its ability to cause regression of established tumours (Colombo *et al.*, 1996, Liu *et al.*, 2002).

IL-12 also known as natural killer cell stimulator factor is produced primarily by stimulated macrophages. Emerging experimental evidence suggests that the cytokine *IL-12* can enhance the development of an effective immune response against tumours. *IL-12* exerts a potent antitumour effect following local or systemic administration. Gene

gun-mediated delivery of plasmid encoding *IL-12* to intradermal tumours in mice has shown that gene therapy provides a safer alternative to *IL-12* protein therapy for clinical treatment of cancers (Rakhmilevich *et al.*, 1996).

Microencapsulated murine fibroblast cells have been genetically engineered to produce *IL-12* and act as a source of continuous release of *IL-12* (Zheng *et al.*, 2003). These cells have a significant therapeutic effect on the experimental colon tumour by activating cells secreting cytokines may thus be an extremely versatile tool for tumour gene therapy.

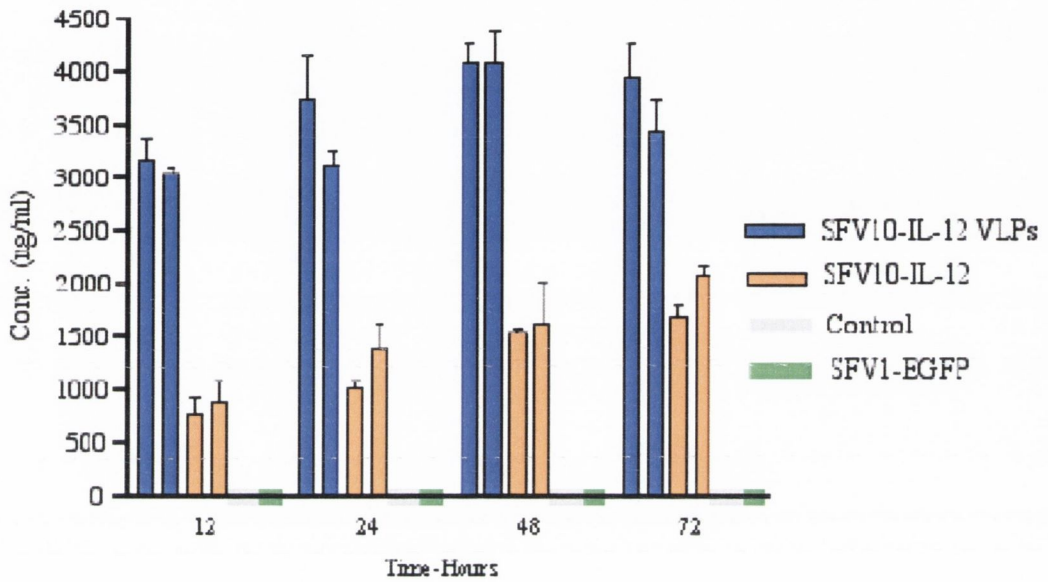
Adeno-associated virus vectors have been shown to be effective vehicles for delivering the *IL-12* gene to leukemic cells, and these cells can be used to generate cancer vaccines. Encouraging pilot clinical results have been recently obtained from the first phase I trial studying adenovirus mediated *in vivo* gene transfer of *IL-12* into lesions of advanced cancer patients. Further improvements will follow from: i) increases in the efficacy of gene transduction; ii) development of tumour specific promoters; iii) development of regulatable and long-term expression vectors and iv) combination with other immunological and non- immunological anticancer therapies (Mazzolini *et al.*, 2003).

4.3.1 *In vitro IL-12* results

Initially the effectiveness of electroporatively delivered *IL-12 in vitro* was examined. Previous studies by Gowda *et al.*, (2005) showed that SFV viral particles have been very effective at inducing the release of active *IL-12* both *in vitro* and *in vivo* and so it

was necessary to contrast the effectiveness of SFV viral vectors encoding the *IL-12* to electroporatively delivered SFV-*IL-12* RNA.

A



B

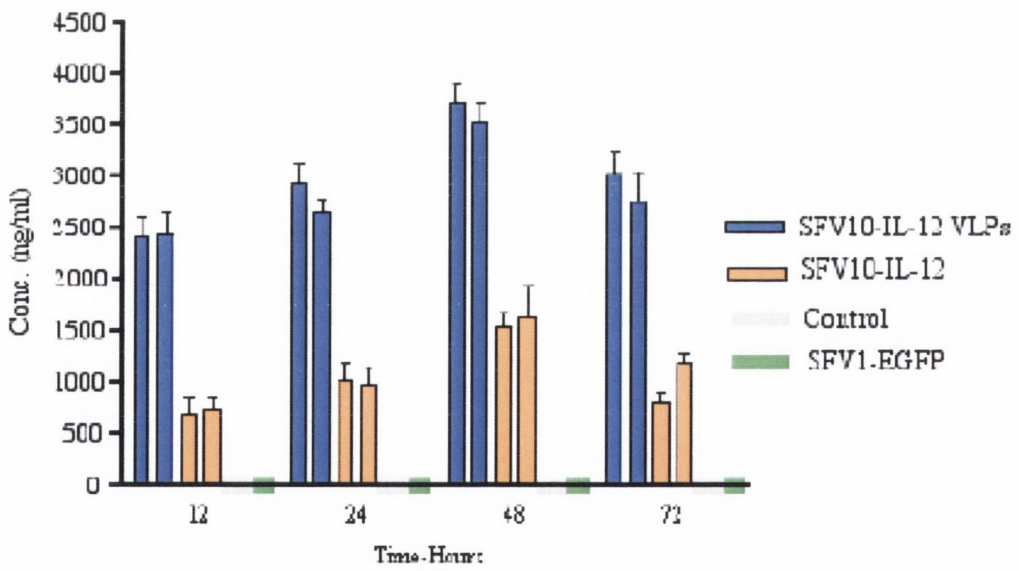


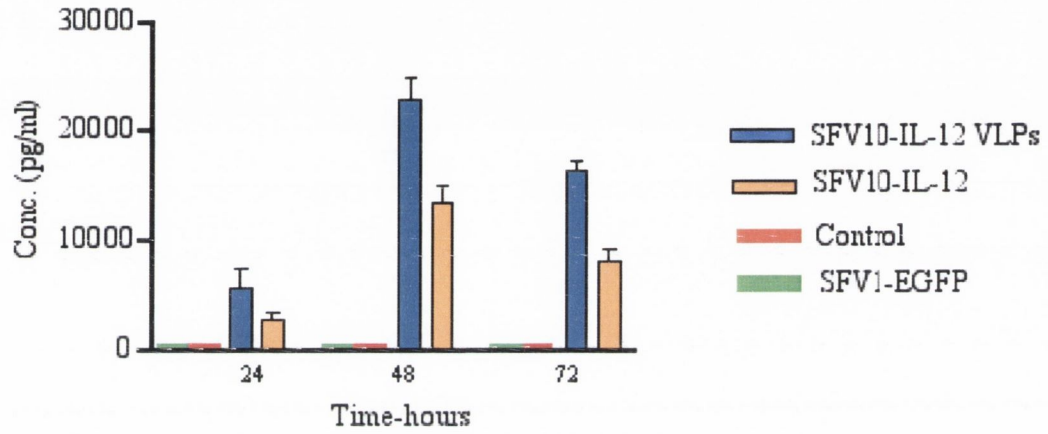
Figure 4.6: BHK-21 (A) and CT26 (B) cells were infected with VLPs of rSFV10-IL12 at MOI of 10 or electroporated with the SFV10-IL12 RNA. The levels of secreted *IL-12*

from the supernatants of transfected BHK-21 (A) and CT26 (B) cells were analyzed by OptiEIA ELISA as described by Gowda.*et al.*, (2005).

A high level of *IL-12* was detected when both the BHK-21 and CT26 cells were either electroporated or infected using SFV vectors. Approximately $1x10^6$ cells were either transfected or infected in each case for the BHK-21 and CT26 cells. The levels of *IL-12* detected from the supernatant of the virally infected cells was almost double that found when the cells were electroporated with the SFV-*IL-12* RNA. The level of *IL-12* in each case follows the same pattern with an increase over time up to 48 hours for both the electroporated and virally delivered *IL-12* for the BHK-21 cells. When the supernatant from the CT26 cells was analysed there was a lower level of *IL-12* detected for both the electroporated and virally delivered *IL-12* compared to the BHK-21 transfected cells. The level of *IL-12* for both the electroporated and virally delivered *IL-12* follows the same pattern with an increase over time up to 48 hours and a decrease in the level of *IL-12* detected at 72 hours.

Biologically active *IL-12* induces T-cells to secrete interferon-gamma (IFN-θ) and tumour necrosis factor-alpha (TNF-θ) from both NK cells and helper T cells. It has been found that since *IL-12* is responsible for the production of IFN-θ, immunological action must be directed primarily to those cells that are capable of producing IFN-θ. It was necessary to examine if the *IL-12* found within the supernatant (the *IL-12* secreted post electroporation/viral infection) was biologically active. Murine splenocytes were exposed to the supernatants containing the biologically active *IL-12* inducing the splenocytes to secrete IFN-θ.

A



B

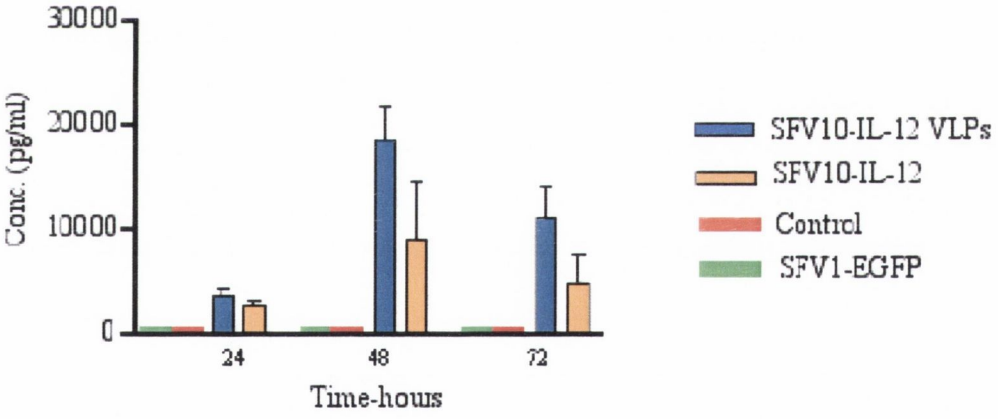


Figure 4.7: The production of IFN-θ from murine splenocytes ($1x10^6$) treated with 100 µl of 24 hours naive *IL-12* supernatants of *IL-12* transfected BHK-21 (A) and CT26 (B) cells was measured using the OptiEIA ELISA. The supernatants of SFV-IL12 VLP infected, electroporated SFV-*IL-12* RNA, electroporated SFV-EGFP RNA and

untreated control were added to BHK-21 (A) and CT26 (B), where active secreted *IL-12* activated splenocytes to produce IFN-θ (Gowda.*et al.*, 2005).

These results confirmed the expression and secretion of biologically active *IL-12* from both the virally delivered pSFV10-IL12 and electroporated pSFV10-IL12 vectors. Supernatants from control groups (electroporated SFV-EGFP RNA, uninfected control) were unable to induce splenocytes to produce IFN-θ. 100 Δl of supernatant was taken from approximately $1x10^6$ cells that were either transfected or infected in each case for the BHK-21 and CT26 cells. There is a decrease in the level of IFN-θ for both the electroporated and virally infected cells after 48 hours indicating that a lower level of biologically active *IL-12* is detected post 48 hours. There is less production of IFN-θ in the CT26 cells for both the virally infected and electroporated treatments than is observed with the BHK-21 cells. There is almost half the level of IFN-θ observed when the supernatant of electroporatively SFV-*IL-12* transfected BHK-21 cells or CT26 cells are added to the splenocytes in comparison to the virally infected cells, but a lower concentration of *IL-12* was detected within the supernatant of the electroporated cells initially when compared to the virally delivered *IL-12*.

4.3.2 *In vivo IL-12* results

Once the ability of electroporatively delivered pSFV-*IL-12* RNA to induce active *IL-12 in vitro* was established it was then appropriate to examine the ability of the electroporated pSFV-*IL-12* RNA to inhibit the growth of subcutaneous CT26 tumours in BALB/c mice.

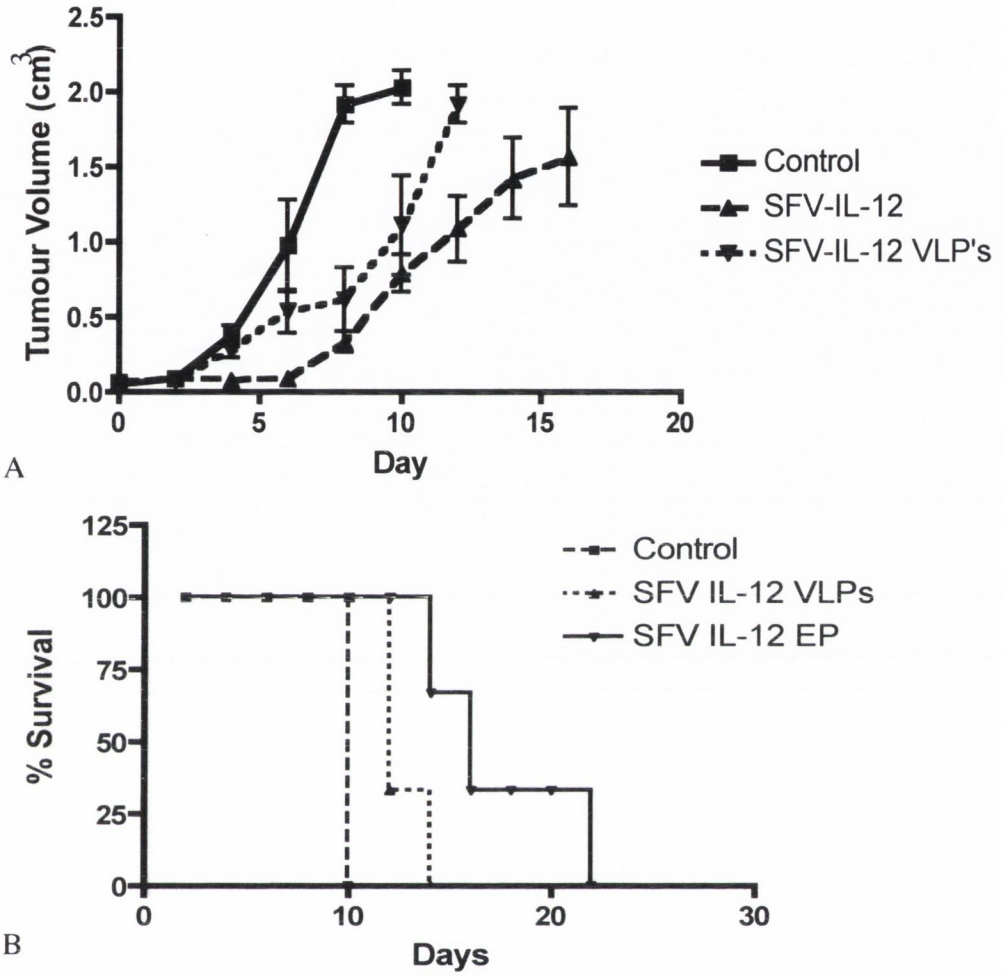


Figure 4.8: Treatment of subcutaneous CT26 tumours in nude mice. Mice were treated with 50 Δl pSFV-*IL-12* RNA electroporatively delivered using optimized intratumoural pulseform (Appendix 2A) or intratumourally injected with 1 x 10^6 SFV VLPs encoding for *IL-12*. Tumour volume was calculated every two days post treatment and mice euthanised when the average tumour volume reached above 1.7 cm^3 (day 10 for control groups). A) The average tumour volume per group of six mice was calculated and plotted against time for each group until the tumour volume reached ~1.7 cm^3. B) The

percentage survival per group post electroporative treatment (n=6) was calculated and plotted against time for the duration of the experiment.

CT26 tumours were induced in female immunocompetent BALB/c mice by s.c. injection of 50 Δl of CT26 cell suspension in non-supplemented DMEM at a concentration of 1 x 10^6 cells/50 Δl. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of six mice were used per treatment group. Tumours received one 50 Δl intratumoural injection of nuclease free water alone, electroporation alone, no treatment or electroporatively delivered SFV-*IL-12* (1Δg/Δl in 50 Δl volume) or virally delivered SFV-*IL-12* (5x10^9 IU in 50 Δl volume). Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when the average tumour diameter reached above 15 mm. There was no significant difference in the growth curve for the nuclease free water alone, electroporation alone and no treatment groups and so only the no treatment group is shown above.

Each of the control tumour groups grew in the same manner observed in untreated BALB/c mice. All six control tumours (in each control group) had reached an average diameter above 15 mm by day 10 and mice were euthanised accordingly. Tumours treated with SFV virally delivered *IL-12* displayed some inhibition of tumour growth initially but the tumours had still reached an average diameter above 15 mm by day 12 and the mice were hence euthanised. The SFV-*IL-12* electroporatively treated group showed significant inhibition of tumour growth until day 6. Post day 6 there was significant increase ($P<0.05$, student t test) in the tumour growth rate with 4 out of 6

tumours in this group reaching an average tumour volume above 15 mm by day 16 and the remaining 2 two tumours were found to be above 15 mm in diameter by day 20. Upon statistical analysis using a one-way repeated measures ANOVA with Tukey's multiple comparison post test, this inhibition of tumour growth was found to be insignificant in comparison to control tumours (comparison of all groups over entire experiment). It can therefore be concluded that electroporatively or virally delivered SFV-*IL-12* is incapable of significantly inhibiting CT26 tumour growth in the BALB/c mouse model following one treatment. While if the data is analyzed solely at day 6 (prior to the regrowth of the tumours) there is a highly significant ($P = <0.05$) inhibition of tumour growth when the electroporatively SFV-*IL-12* treated group is compared to that of the untreated tumours using the student t test. When the virally delivered SFV-*IL-12* is contrasted at the same day there is no significant inhibition of tumour growth observed. It is necessary to examine if more than one treatment will have an effect of increasing the inhibition of the growth of the CT26 murine colon adenocarinoma cell line.

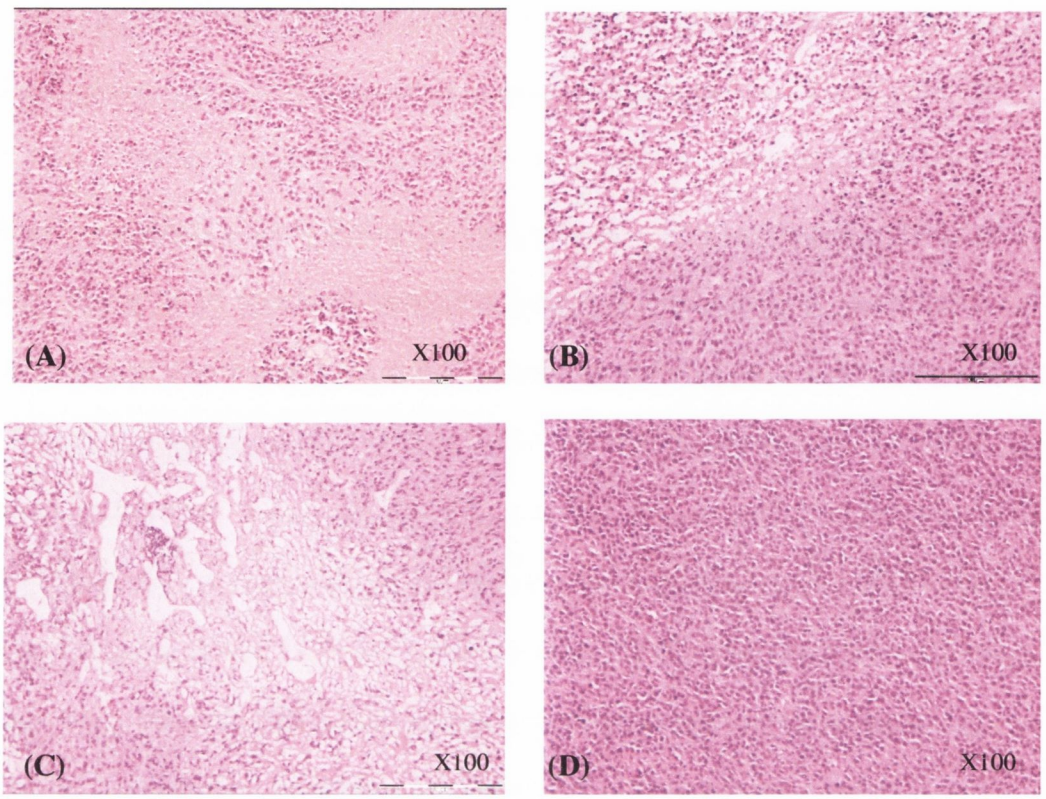


Figure 4.9: Histopatholgical examination of H&E stained (as described in section 2.2.11) CT26 tumour sections six days following electroporation with pSFV10-IL-12 RNA (50 µl) (A and C). Histopatholgical examination of H&E stained CT26 tumour sections six days following treatment with SFV-IL-12 ($1x10^6$ VLPs) (B) Histopatholgical examination of H&E stained untreated CT26 tumour sections on day six of experiment (D). All control tumours showed a similar pattern of histopathology to that of the untreated group on day 6 (D).

The tumour sections from six days after the electroporative treatment were stained using haematoxylin and eosin (H&E) and examined histologically to evaluate the effect of the electroporative pSFV10-IL-12 treatment on the CT26 tumours. Tumours that were treated with nuclease free water alone, electroporation alone or not treated all showed

dence populations of polyhedral tumour cells (Figure 4.9 (D)). While tumours that were electroporatively treated with the pSFV10-IL-12 showed areas of necrosis and oedema adjoining islands of tumour cells with the tumour cells closely packed around thin-walled blood vessels. (Figure 4.9 (A)). The areas of tumour cell necrosis are in apposition of intact and necrotic cells in each of the pSFV10-IL-12 electroporatively treated tumours (Figure 4.9 (B)). The SFV-IL-12 VLP treated tumours all display areas of vascular dilatation within the areas of tumour cell necrosis and oedema. Also small aggregates of leucocytes are found to be present (as can be observed within the perivascular space of the tumour section in Figure 4.9 (C)).

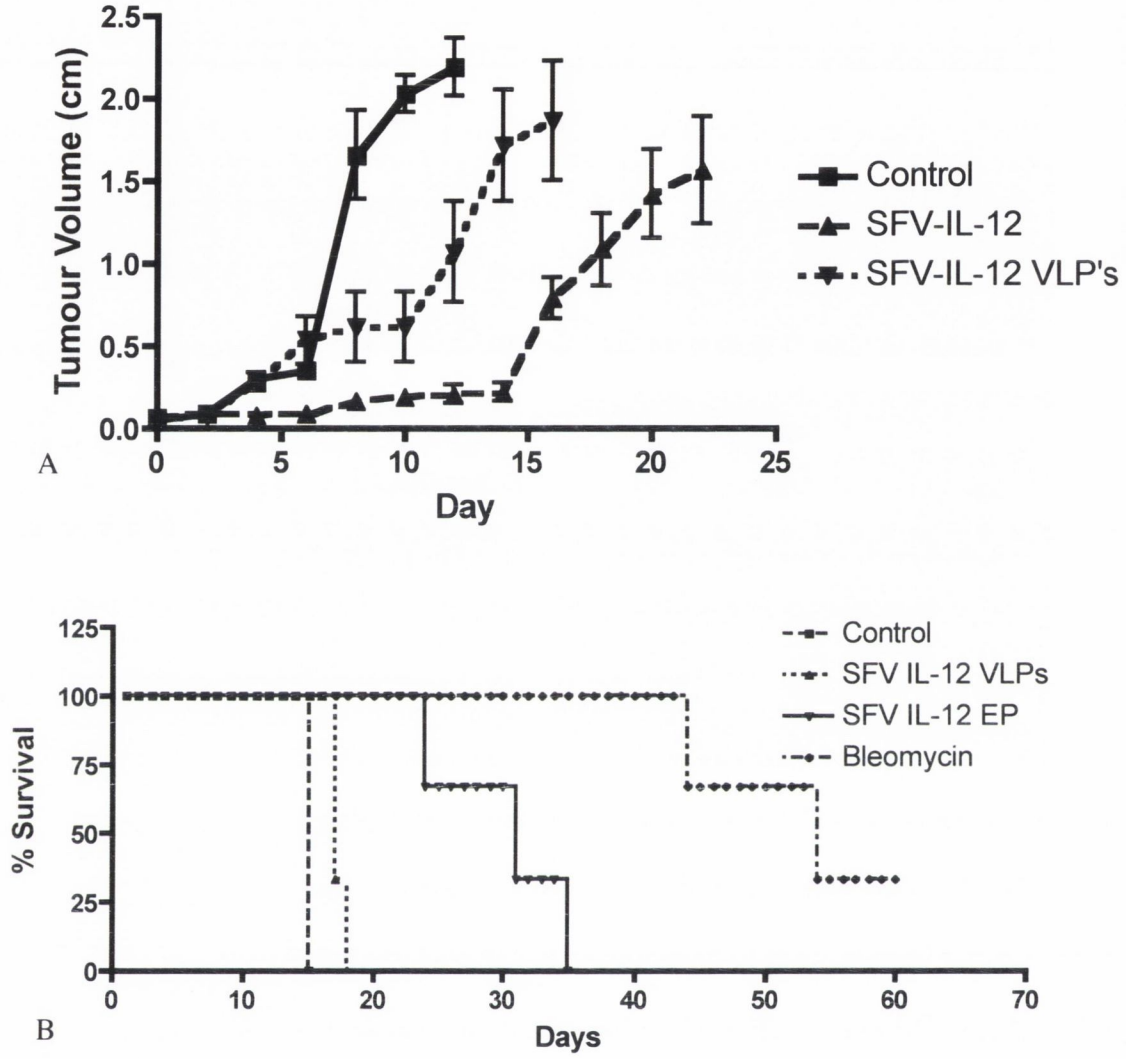


Figure 4.10: Treatment of subcutaneous CT26 tumours in nude mice. Mice were treated twice (treatment 1 day 0, treatment 2 day 8) with 50 Δl pSFV-*IL-12* RNA electroporatively delivered using optimized intratumoural pulseform (Appendix 2A) or intratumourally injected with 1 x 10^6 SFV VLPs encoding for *IL-12*. Tumour volume was calculated every two days post treatment and mice euthanised when the average tumour volume reached above 1.7 cm^3 (day 14 for control groups). A) The average tumour volume per group of six mice was calculated and plotted against time for each

group until the tumour volume reached ~1.7 cm^3. B) The percentage survival per group post electroporative treatment (n=6) was calculated and plotted against time for the duration of the experiment.

CT26 tumours were induced in female immunocompetent BALB/c mice by s.c. injection of 50 Δl of CT26 cell suspension in non-supplemented DMEM at a concentration of 1 x 10^6 cells/50 Δl. Treatment was initiated in individual tumours upon their reaching an average diameter of 4 mm and groups of six mice were used per treatment group. Tumours received two 50 Δl intratumoural injection of nuclease free water alone, electroporation alone, no treatment or electroporatively delivered SFV-*IL-12* (1 Δg/Δl in 50 Δl volume) or virally delivered SFV-*IL-12* (5x10^9 IU in 50 Δl volume), one on day 0 and a second on day 8 following the initial treatment. Average tumour diameters were calculated as the square root of the product of two perpendicular measurements (assuming spherical shape) and mice were euthanised when the average tumour diameter reached above 15 mm. There was no significant difference in the growth curve for the nuclease free water alone, electroporation alone, no treatment and so only the no treatment group is shown above.

Each of the control tumour groups grew in the same manner observed in untreated BALB/c mice. All six control tumours (in each control group) had reached an average diameter above 15 mm by day 14 and mice were euthanised accordingly. The delay in the growth of the tumours in comparison to Figure 4.8 could be an impact of the second treatment on day eight. Tumours treated with SFV virally delivered *IL-12* displayed some inhibition of tumour growth initially but the tumours had still reached an average

diameter above 15 mm by day 17 and the mice were hence euthanised. The SFV-*IL-12* electroporatively treated group showed significant inhibition of tumour growth until day 14. Post day 14 there was a significant ($P<0.05$, student t test) increase in the tumour growth rate with 2 out of 6 tumours in this group reaching an average tumour volume above 15 mm by day 24. Of the remaining four, two tumours were found to be above 15 mm in diameter by day 30 and two by day 34.

Upon statistical analysis using a one-way repeated measures ANOVA with Tukey's multiple comparison post test, this inhibition of tumour growth was found to be insignificant in comparison to control tumours (comparison of all groups over entire experiment). While when the data is analyzed at day 12 (following two treatments) the inhibition of tumour growth for both the virally delivered and electroporated *IL-12* is significantly different when compared to the controls (student t test P value < 0.001 in each case). When the inhibition of electroporatively delivered and virally delivered SFV-*IL-12* are contrasted at day 12 or day 16 it is found that there is a significantly higher inhibition of tumour growth with electroporative delivery (student t test P value < 0.001 at both time points). It can therefore be concluded that electroporatively or virally delivered SFV-*IL-12* is capable of significantly inhibiting CT26 tumour growth in the BALB/c mouse model following two treatments but only for a short duration (up to 14 days) after which tumours grow at a usual rate.

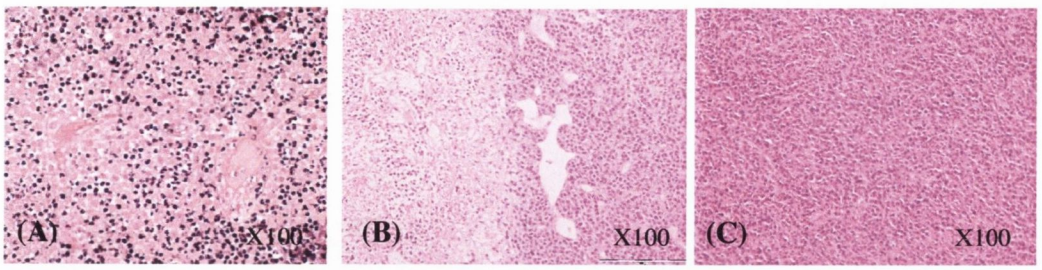


Figure 4.11: Histopatholgical examination of H&E stained (as described in section 2.2.11) CT26 tumour sections 12 days following electroporation with pSFV10-IL-12 RNA (50 μl) (A). Histopatholgical examination of H&E stained CT26 tumour sections 12 days following treatment with SFV-IL-12 ($1x10^6$ VLPs) (B) Histopatholgical examination of H&E stained untreated CT26 tumour sections on day six of experiment (C). All control tumours showed a similar pattern of histopathology to that of the untreated group on day 6 (C).

The tumour sections from 12 days after the electroporative treatment were stained using haematoxylin and eosin (H&E) and examined histologically to evaluate the effect of the electroporative pSFV10-IL-12 treatment on the CT26 tumours. Tumours that were treated with nuclease free water alone, electroporation alone and no treatment control groups all showed dence populations of polyhedral tumour cells on day 6 (Figure 4.11 (C)). While tumours that were electroporatively treated with the pSFV10-IL-12 showed areas of necrosis, throbosis and neutrophil infiltration. (Figure 4.8 (A)). The SFV-IL-12 VLP treated tumours all displayed areas of vascular dilatation within the areas of tumour cell necrosis and oedema. Also small aggregates of leucocytes were found to be present (Figure 4.11).

4.4 Discussion

There have been several strategies investigated that exploit both viral and non-viral vectors for cancer therapy ranging from specific approaches such as oncogene inactivation, to less direct methods such as proapoptotic or antiangiogenic strategies. Induction of apoptosis in tumour cells is a goal common to many cancer treatment regimens and gene therapy is no exception, with much promising research in the introduction of pro-apoptotic factors to the tumour microenvironment (Hughes *et al.*, 2004; Waxman & Schwartz, 2003). The SFV vector system encodes its own apoptosis induction mechanism that can itself be exploited for tumour therapy, effectively enhancing the effect of the expression of proapoptotic genes by this vector (Murphy *et al.*, 2000). This proapoptotic effect provides an additional opportunity for the expression of other potentially therapeutic genes by this vector system. The expression of the anti-angiogenic cytokine *IL-12* and of the pro-apoptotic gene *bax* by rSFV VLPs have both been employed successfully in the treatment of murine tumour models using the SFV viral vector system (Asselin-Paturel *et al.*, 1999; Murphy *et al.*, 2001).

4.4.1 pSFV1-Bax RNA tumour treatment

The antitumour effects of the *Bax* gene have been assessed by many different approaches Coll *et al.*, (1998) showed that transfecting the *Bax* gene to two cultured bronchioloalveolar carcinoma cell lines killed 70 –90% of the transfected cells using naked DNA transfer, whereas p53 killed only 40% of them and both *Bax* and p53 share

a similar antitumour activity *in vivo* (Coll *et al.*, 1998). These results suggest that perhaps *Bax* may be more effective *in vitro* than *in vivo*, Coll *et al.*, (1998) further implies that inefficient delivery of this transgene to the target cells was a limiting factor in assessing the therapeutic value of the *Bax* gene.

Many attempts have been made to increase the transfection efficiency of this gene; one of these strategies involved the use of adenoviral vectors, which are widely used for *in vivo* gene delivery in gene therapy (Kovesdi *et al.*, 1998), but constructing such a vector was not viable because of the toxic effect of the transgene product on the packaging cell line 293. The SFV viral like particle packaging system was examined for use with the *Bax* gene but constructing such a vector was also not viable because of the toxic effect of electroporating the SFV-*Bax* RNA on the packaging BHK-21 cell line (Murphy *et al.*, 2000).

Here electroporation was applied as a physical gene delivery system to assess the antitumour effects of the *Bax* gene *in vitro* and *in vivo* in a range of cell lines. Because this is an RNA system it may also be useful for testing SFV RNA activities in a variety of tumour models post electroporation. The results obtained demonstrate that the *Bax* gene can effectively induce apoptosis and suppress tumour growth both *in vitro* and *in vivo* but only transiently. This transient apoptosis induction may be as a result of the short duration of SFV expression, which has been shown to only persist up to seven days *in vivo* (Morris *et al.*, 2001). When the inhibition of tumour growth is contrasted with both the CT26 and the 0E19 cell lines both of which display very different growth characteristics the tumour inhibition induced by electroporative delivery of SFV-*Bax* RNA is comparable, with a only a slight difference observed in the inhibition of the

OE19 tumours as they display a slower growth rate. Although we have only tested one intratumoural electroporation in this study, other administration schedules or subsequent challenges with the *Bax* expressing vectors may theoretically improve the therapeutic effects further by minimizing untransduced tumour cells, unless clonal resistance to *Bax*-mediated apoptosis occurs. Experiments have shown that overexpression of *Bax* enhances intracellular accumulation of chemotherapeutics (Strobel *et al.*, 1998) and may improve the clinical outcome of chemotherapy (Tai *et al.*, 1998). The combination of the *Bax* gene therapy with other therapeutic agents may also improve the therapeutic effects. As can be seen from the results, electrochemotherapy is highly effective when compared to the *Bax* electrogenetherapy and it is thought that the combination of these two therapies may offer a more long-term therapeutic strategy. Though as can be seen from the results in both cases, electrochemotherapy and *Bax* electrogenetherapy, complete tumour regression was not obtainable with just one treatment. It is thought that only with multiple treatments covering the entire tumour volume and margins can total tumour regression be obtained (Mir, 2001; Mekid *et al.*, 2003, Li *et al.*, 2005) using the SFV-*Bax* electrogenetherapy. Unfortunately evidence has shown overexpression of the *Bax* gene may also induce apoptosis in normal cells, raising a safety issue in the application of the *Bax* gene to healthy tissue (Kagawa *et al.*, 2000).

4.4.2 pSFV1-IL-12 RNA tumour treatment

IL-12 activates innate immune cells and promotes their production of cytokines and chemokines, and thus mediates local recruitment of cells of innate and adaptive immunity (Cavallo *et al.*, 1999). Furthermore, an *IL-12*-conditioned microenvironment

is suitable for APC activation, antigen presentation, and prevention (or reversal) of the induction of tolerance toward tumour antigens (Koido *et al.*, 2000). It has previously been shown that an antibody response (Boggio *et al.*, 2000) and production of IFN-θ (Nanni *et al.*, 2001) are essential for successful preventive vaccination, and that combination of *IL-12* with electroporation does not obviate the need for an active IFN-θ and antibody response, but markedly enhances the number of T cells that specifically release IFN-θ (Spadaro *et al.*, 2005).

Studies have shown that the amount of *IL-12* available at the tumour site is critical for tumour regression and evidence has been provided showing that maximum antitumour effects can be achieved by transient *IL-12* expression systems instead of prolonged high-dose treatment (Colombo *et al.*, 1996). The Semliki Forest virus vector is a transient expression vector and has been previously used in vaccine studies e.g. against louping ill virus (Fleeton *et al.*, 2000) as well as in the successful delivery of *IL-12* for cancer treatment (Colmenero *et al.*, 1999, Asselin-Paturel *et al.*, 1999) but only as part of the SFV viral vector system. It is thought that electroporative delivery of the SFV based gene will not only have the advantage of transient high level protein expression but also will eliminate the safety issues associated with viral delivery systems. Electroporation also has the advantage in complete localized gene delivery (unlike viral vectors which can be detected elsewhere within the system e.g. the lymph nodes (Morris *et al.*, 2001)) and electroporation may enhance *IL-12* activation by priming the innate immune system (Wildera *et al.*, 2000).

Expression and secretion levels of *IL-12* by virally delivered pSFV10-*IL-12* and electroporation were tested *in vitro* in BHK-21 and CT26 cells using the infectious virus

like particles at a multiplicity of infection (MOI) of 10 and electroporation of 50 Δl of plasmid at a concentration of 1 Δg/Δl. Expression levels of *IL-12* from rSFV10 infected BHK-21 and CT26 cells was almost two-fold higher than that of the electroporated SFV10-IL12 cells. These results confirm expression of the electroporated SFV10-*IL-12*, although at a lower level when compared to SFV10-*IL-12* viral vector. Although SFV replicons have the capacity to infect a variety of cell lines *in vitro*, very little is known about their infectivity (Lundstrom *et al.*, 1999, Wahlfors *et al.*, 2000). A difference in the IL-12 levels was observed between the CT26 and BHK-21 cells, perhaps reflecting a infection variability of the replicons with different cell lines (similar observation were made by Smyth *et al.*, (2005) and Gowda *et al.*, (2005)). With electroporation a higher number of BHK-21 cells can be transfected without decreasing the viability than CT26 cells and so this may be the factor influencing the differences in IL-12 levels found when these cell lines are electroporated with pSFV10-IL-12.

Biologically active *IL-12* induces T-cells to produce IFN-θ. To confirm secretion of the expressed protein and its biological activity, the ability of supernatants from SFV-*IL-12* virus like particle (VLP) infected cells and SFV-*IL-12* electroporated cells to induce IFN-θ production by mouse splenocytes was assessed. While there is an array of different cells to be found within the spleen the production of IFN-θ to levels above that of the control is clearly indicative of the biological activity of the IL-12 found within the supernatants from the SFV-*IL-12* virus like particle (VLP) infected cells and the SFV-*IL-12* electroporated cells (Gowda *et al.*, 2005). The expression of *IL-12* in the supernatant by the infectious VLP and electroporated BHK-21 and CT26 cells and the ability to induce IFN-θ production in splenocytes indicated expression and secretion of

functionally active *IL-12*. IFN-θ levels induced by the VLPs and electroporated BHK-21 and CT26 infected supernatants were found to be not significantly different though the CT26 results for both electroporated cells and VLPs were more variable than that of the BHK-21. This may be due to more variability in the transfection efficiency of this cell line and hence increased variability in the levels of active *IL-12* secreted. This ELISA was used to test the bioactivity of the secreted *IL-12* by the SFV-IL-12 VLPs and electroporated *IL-12* RNA, not to quantitate the amount of secreted *IL-12*. This is because the level of secreted IFN-θ relates not to the overall amount of IL-12 found within the media of each cell sample (at each time point) but the level of biological activity of the IL-12 found within this media. There is a significantly higher ($p=0.05$, student t test) level of IFN-θ detected for the VLPs when the IFN-θ levels are contrasted for the BHK-21 cells. There is no significant difference for the CT26 transfected/infected cell line but this is most likely due to the increased variability within the levels of *IL-12* secreted. It is thought that with an increased number of samples a significant difference may have been apparent.

The *in vivo* antitumour effectiveness of electroporatively delivered SFV10-*IL-12* was contrasted to that of virally delivered SFV10-*IL-12* with CT26 tumours. *IL-12* has been shown to be quite effective in inducing antitumoural immune responses and increasing both CTL and NK activities. It has been demonstrated that production of IFN-θ is essential for the antitumour effect of *IL-12* (Oshikawa *et al.*, 1999). It has been shown that both methods of *IL-12* delivery induced IFN-θ production *in vitro*.

Only one treatment was administered once the tumours had grown to a 4mm diameter as with prior electroporative research. A single electroporative treatment resulted in a

transient suppression of growth with both methods of *IL-12* delivery, though a significant difference (p= 0.05, student t test) was observed between the electroporatively delivered and the control tumours at day 5 while no significant difference was observed between the virally delivered SFV10-*IL-12* and the control at this time point. There was increased tumour growth post day 6 for the electroporatively delivered *IL-12* mimicking the results observed when the SFV-*Bax* is electroporatively delivered. Also Torrero *et al.*, (2006) and Liu *et al.*, (2006) found a transient decrease in tumour growth following a single electroporative treatment with IL-12 DNA.

Previous studies using the SFV viral vector as a method of *IL-12* delivery showed clear tumour regression post six treatments for both CT26 and K-BALB tumours (Gowda *et al.*, 2005). Six consecutive treatments was not possible with electroporation as this would result in a high level of tissue damage and would have been a highly invasive procedure for the mice. Two treatments were selected for comparative purposes to examine if there is a synergistic effect on tumour suppression by having multiple treatments. Day 8 was selected for the second electroporative treatment as there was found to be a significant increase in growth rate at this time point following one electroporative treatment. The results show electroporative SFV-*IL-12* leads to a significant suppression of tumour growth (p=0.001) in comparison to both the controls and virally infected tumours until day 14 after which the tumour begin to grow at the same rate as the control and SFV virally infected tumours. This result is similar to that found by Kishida *et al.*, 2001 where two intratumoural electroporative treatments resulted in transient suppression of tumour growth for 8 to 10 days in CT26 tumours. Increased inhibition of SCCVII tumours was observed following two electroporative

treatments (8 days apart) with IL-12 DNA (30 Δg) under the control of a CMV promoter (Torrero *et al.*, 2006). No significant difference was observed between the application of either one or two treatments in this case. A significant difference in tumour growth was observed with the SFV10-IL-12 RNA CT26 tumour treatment most likely due to the high level of expression obtained by the SFV-based plasmid vector, ascribed to multiple functions of SFV, such as the cytoplasmic replication.

It was found that systemic administration of recombinant *IL-12* causes dose-dependent adverse effects in mice (Orange *et al.*, 1995) and human (Motzer *et al.*, 1998). IFN-θ production brings about an acute systemic inflammatory response with marked injury to some particular organs, i.e. the liver, lungs and intestine (Carson *et al.*, 2000). While some signs of inflammation were observed within the tumour from the electroporative SFV10-*IL-12* delivery, no sign of inflammation or cell damage was observed in any other organ analyzed with either the SFV viral particle or electroporative SFV10-*IL-12* delivery. Li *et al.*, (2005) found that intramuscular electroporation with IL-12 DNA was less effective than intratumoural treatment but did not cause any adverse effects. While gene expression following multiple treatments has been detected post administration in other organs e.g. the lymph nodes (Morris *et al.*, 2001) with SFV VLP delivered genes there were no side effects observed when the SFV viral particles were intratumourally delivered (Gowda *et al.*, 2005). Electroporative gene transfer was found to provide a relatively low level of *IL-12* at the target tissue (Kishida *et al.*, 2001) in comparison to the levels detected with the viral vectors but the high expression levels resulting from the SFV vectors may have resulted in a more efficient gene therapy with expression levels comparable to both the AAV and SFV viral vectors. When tumour growth is

compared to that of previous studies two treatments using the SFV viral vector is not observed to be effective (Gowda *et al.*, 2005). Multiple treatments are always applied over a number of days to induce a tumour suppression response using IL-12 delivered either by viral vectors (Gowda *et al.*, 2005, Puisieux *et al.*, 1998) or by electroporation (Li *et al.*, 2002, Li *et al.*, 2005). While SFV10-*IL-12* was observed to be somewhat more effective there was no significant difference between the proapoptotic gene therapy or the immunotherapy following one treatment. It is thought that only through multiple treatments covering the entire tumour volume and margins can total tumour regression be obtained (Mir, 2001; Mekid *et al.*, 2003, Li *et al.*, 2002).

Chapter 5

In vivo SFV plasmid electroporation for use in vaccine delivery

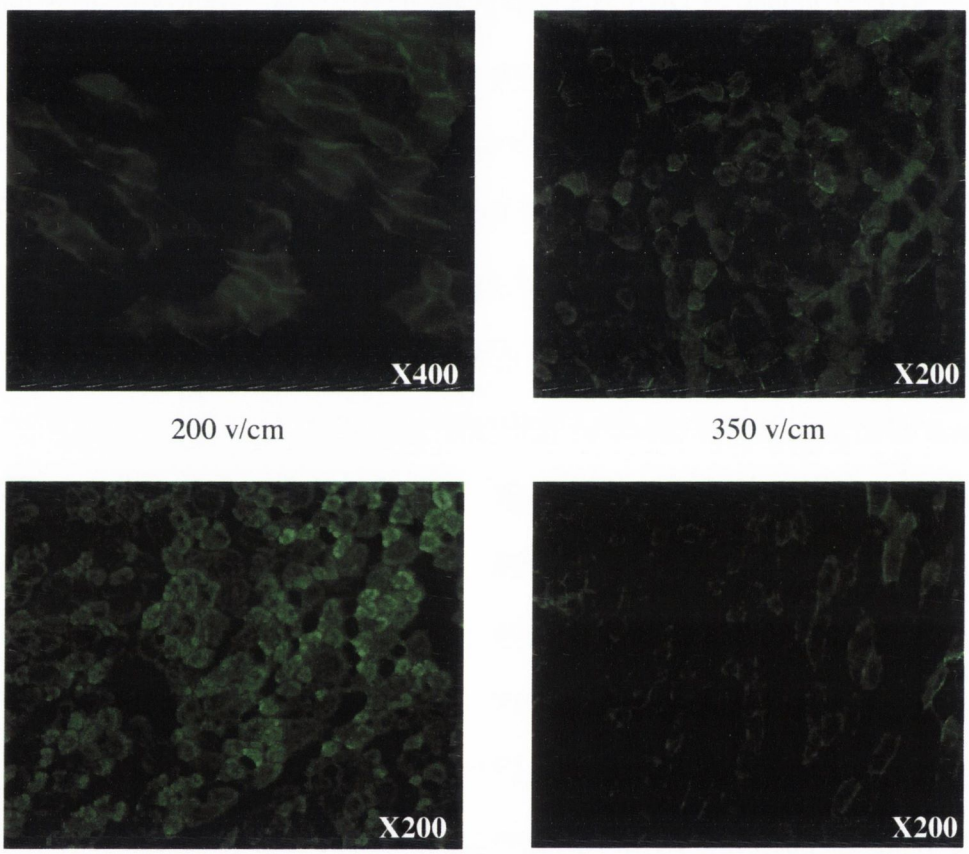
5.1 Introduction

Nucleic acid vaccines offer a novel way to immunize individuals against diseases. The development of these vaccines is one of the most promising applications of recent advances in gene based therapy (Srivastava *et al.*, 2003). Nucleic acid vaccines are easy to produce, do not replicate and encode only the antigen of interest, as opposed to live attenuated or viral carrier systems. These vaccines express antigens *in vivo*, thus conserving the native conformation of the antigenic epitopes, which is critical for the induction of specific humoral antibody and cellular immune responses. The endogenously synthesized antigens can access both major histocompatibility complex (MHC) classes I and II pathways for antigen presentation (Leitner *et al.*, 2000). DNA vaccines have been shown to induce both cell mediated and humoral immunity, which are long lasting (Donnelly *et al.*, 1997). They may be constructed to have more than one antigen-coding gene, thus potentially decreasing the number of vaccinations required. To be therapeutically useful, the DNA vaccine must be delivered inside the nucleus before it can express antigen molecules. This requires efficient membrane permeabilization to allow the DNA vaccines to enter the cells (Nishikawa *et al.* 2001). This is not the case with SFV based RNA vaccines where it is simply necessary to deliver the gene through the cell membrane. The SFV RNA will replicate solely within the cytoplasm of the cell (Strauss & Strauss, 1994). There have been several reports of gene expression and the possibility of vaccination using plasmid DNA coding antigens (DNA vaccines) *in vivo* after direct plasmid DNA injection (Wolff *et al.*, 1990, Ulmer *et al.*, 1993, Raz *et al.*, 1994, Lagging *et al.*, 1995). Evidence has shown that although direct injections of DNA vaccines do induce immune response in smaller animals, the

delivery of the DNA to target cells is not optimal, especially in large animals (Whalen, 1996, Waalen, 1997, Mumperet *et al.*, 2001) but this may not be the case with RNA vaccination. The amount of DNA used to induce immunity with direct injections is very high (Wang *et al.*, 1998). One reason for the lack of efficacy in larger animals may be inefficient uptake of DNA by cells *in situ*. Hence, it is necessary to test whether cellular uptake of DNA is a significant limitation to efficient transfection *in vivo* and subsequent induction of immune responses. Several non-viral methods have been reported to enhance the immunogenicity of DNA vaccines, primarily by increasing the transfection efficiency. Most of these methods are not suitable for routine use either because of the safety concerns or due to the complex procedures and expenses involved (Herweijer and Wolff, 2003). Electroporation has already been demonstrated to substantially increase the effectiveness of nonviral gene therapy *in vivo* and could circumvent the barrier to the delivery of nucleic acids to the cytoplasm. *In vivo* electroporation substantially increases DNA delivery and DNA vaccine potency and appears to be well tolerated by the animals (Wildera *et al.*, 2000). This has been shown for reporter genes and for genes of interest for therapeutic applications, such as erythropoietin (Rizzuto *et al.*, 1999) and HSV-TK (Gato *et al.*, 2000). Among the tissues targeted for *in vivo* electroporation are skin (Titomirov *et al.*, 1991), liver (Heller *et al.*, 1996), melanoma (Sersa *et al.*, 2000) and muscle (Selby *et al.*, 2000, Widera *et al.*, 2000, Zucchelli *et al.*, 2000 and Babiuk *et al.*, 2002). Facilitation of gene expression *in vivo* by electroporation of plasmid DNA has implications for both vaccine and gene therapy applications. In this study, increased antigen expression following electroporative delivery of SFV based DNA or RNA plasmids into muscle, significantly

increases the potency of DNA vaccines in mice. This is shown to be not only a result of the optimized electroporation conditions but also due to the indicative nature of the SFV plasmids.

5.2 In vivo intramuscular gene delivery



200 v/cm 350 v/cm

400 v/cm 450 v/cm

Figure 5.1: Fluorescent images of 8 Δm sections of mouse quadriceps muscle 48 hours post electroporation with 50 Δg pSFV-EGFP RNA. The pulses in each case were of 10 ms duration (5000 Hz) and 6 pulses were delivered 1 – 2 minutes following

intramuscular injection of the RNA. A minimum of 12 tumours were analyzed for each voltage.

While the most important of the pulse conditions are the voltage and pulse duration, the effect of all pulse conditions that encompass the electroporatic pulseform on intramuscular electroporation were examined (Appendix 3A). The voltage and pulse duration influences the number of cells that are electroporated, the duration of the electroporation and the level of tissue damage that is caused as a result of the electroporation. Figure 5.1 shows fluorescent images of tissue sections taken 48 hours post intramuscular electroporation with pulses of 10 ms duration but with a varying voltage applied. It is clear from the images that 400 v/cm is the optimal pulse voltage for intramuscular electroporation. At voltages below 200 v/cm or above 600 v/cm no expression of the pSFV-EGFP plasmid was observed. No difference in expression was observed when these experiments were repeated using pulses of 20 ms duration, though a decrease in expression was observed in sections electroporated with pulses of 100 Δs duration (Appendix 3A). The pulses were all examined at 48 hours post electroporation as this was observed to be the optimal time of EGFP expression post electroporation. Figure 5.2 displays fluorescent images of sections taken at both 24 and 48 hours post intramuscular electroporation. There is clearly a higher expression observed 48 hours post electroporation. This can be contrasted with the optimal time of EGFP expression when delivered by SFV viral like particles, which has been shown to be 24 hours post intramuscular injection (Morris *et al.*, 2001). A decrease in the level of expression of EGFP is observed when SFV viral like particles are analyzed at 48 hours post

electroporation (i.e. a lower number of fluorescent cells are observed). Cells expressing EGFP are in a more localized area when electroporated, in comparison to cells that express the virally delivered EGFP.

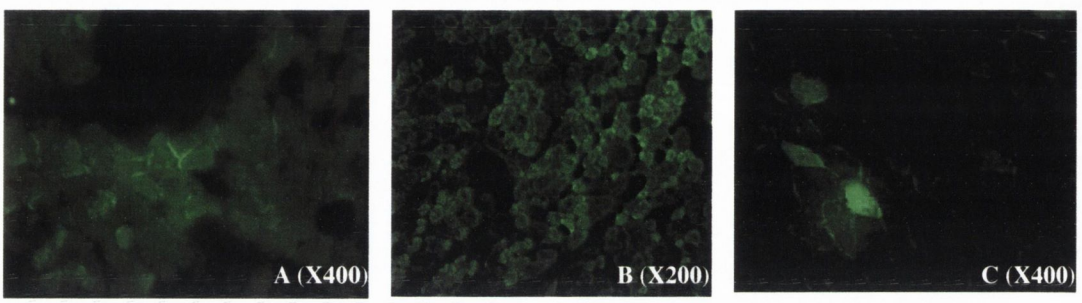


Figure 5.2: Fluorescent images of 6-8 μm sections of mouse quadriceps muscle taken 24 hours (A) and 48 hours (B) post electroporation with 50 μg of pSFV-EGFP RNA. The pulses in each case was of 400 v/cm and 10 ms duration with 6 pulses delivered 1 – 2 minutes following intramuscular injection of the RNA. Image C displays a fluorescent image of an 8 μm section of mouse quadriceps muscle taken 48 hours post injection with SFV viral like particles ($1x10^6$) encoding EGFP.

There are a number of reasons why EGFP cannot solely be used as a method of comparing the different pulseforms, mainly that some quantitative comparison of the different conditions is required and examination of cryosections by fluorescent microscopy can only be used for qualitative purposes. Luciferase was used to examine quantitatively the differences between the various pulses in order to select the optimal pulseform. The results obtained from the EGFP reduced the number of parameters for examination and allow for microscopic analysis of electroporation, while luciferase is quantitative and can be only examined on a macro scale.

Three pulse voltages were compared to examine the effectiveness of square wave pulseforms at intramuscular electroporative transfection. These pulses were selected based on both the available literature (Wolf *et al.*, 1994, Wildera *et al.*, 2000, Mathiesen *et al.*, 1999), from preliminary experiments (Appendix 3A) and data already obtained for the voltage experiments using pSFV-EGFP RNA. It is clear from the graph (Figure 5.3) that 400 v/cm is the optimal pulse voltage, this corresponds with the results obtained from the EGFP experiments. These pulses were delivered for 10 ms based on the results of the EGFP transfection and six pulses were delivered in each case. Eight pulses were also examined using 400 v/cm but no significant difference was observed (Appendix 3A).

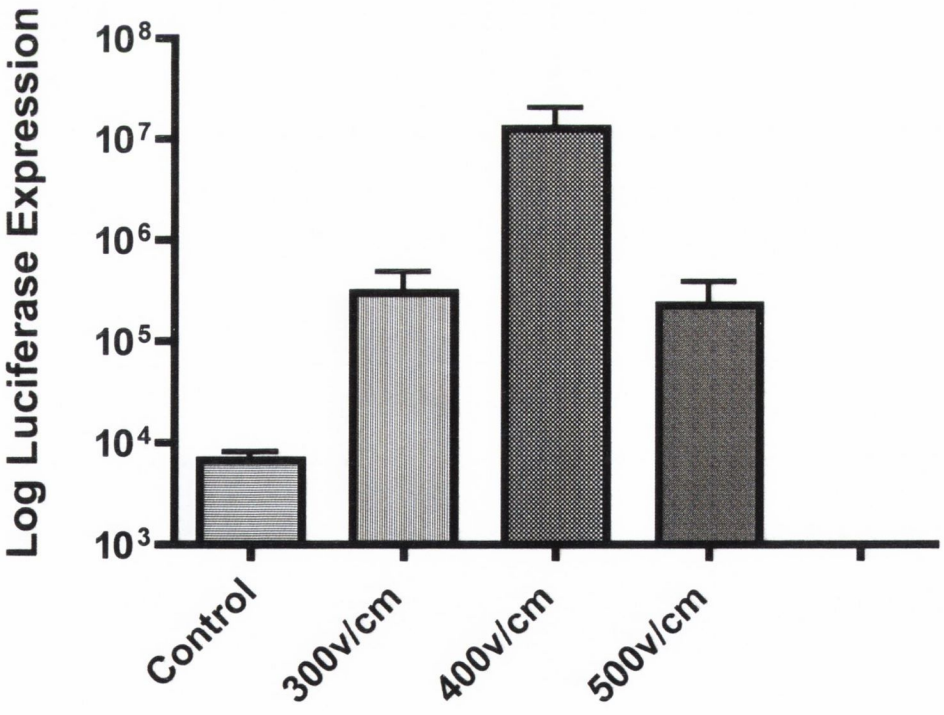


Figure 5.3: Examination of luciferase expression following intramuscular electroporation with the pSFV-Luciferase plasmid (50 μg) using different pulse voltages

48 hours following electroporation Luciferase levels were examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) was intramuscularly injected but without any pulse delivery was also analyzed. Six pulses of 10 ms duration were applied in each case to groups of six mice.

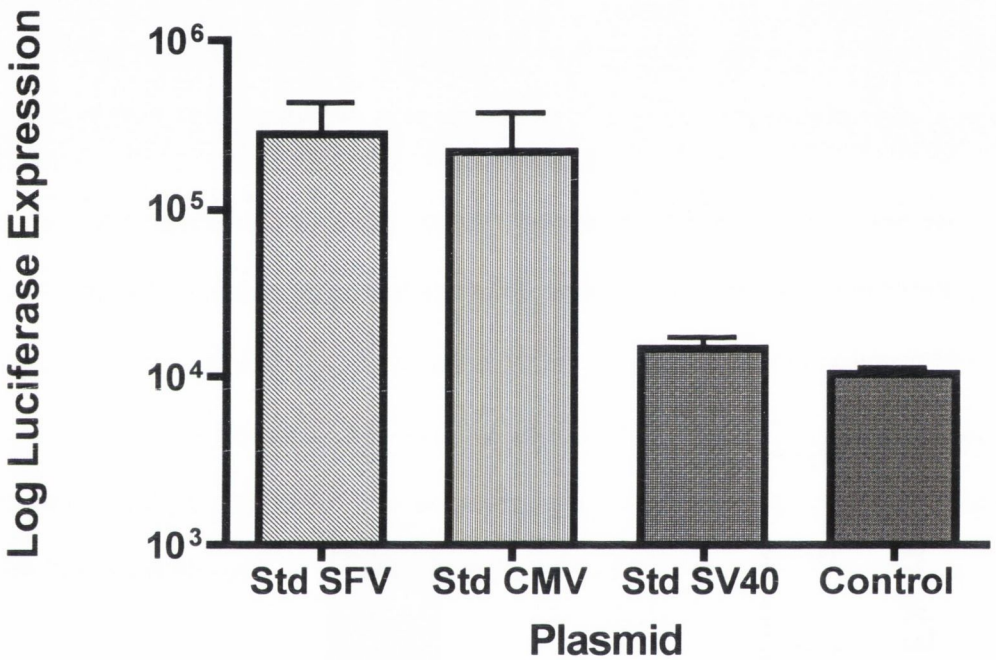


Figure 5.4: Examination of luciferase expression following electroporation with the three different plasmid promoters (50 μg), the SFV based RNA 26 subgenomic promoter, the CMV based DNA equivalent and a SV40 based commercial pGL3 DNA plasmid. These were applied using 400 v/cm and analyzed 48 hours following electroporation. A control of non-electroporated CMV DNA intramuscularly injected was also analyzed. Luciferase levels were examined using the Xenogen live body imager. Six pulses of 10 ms duration were applied in each case to groups of three mice.

The effectiveness of the different plasmid vectors was investigated, these included a SFV RNA plasmid and CMV DNA plasmid which are contrasted with the commercially available pGL3-Luc plasmid (Figure 5.4). The pGL3 plasmid differs in size (~5 kb) and in the SV40 promoter. No difference in expression levels were observed when the SFV based (26s subgenomic) RNA and CMV DNA plasmid were compared while a clear advantage in expression levels were observed utilising the pGL3-Luc plasmid. The difference in the overall expression between the SFV-Luciferase RNA may have been due to inefficient transfection, differences in the orientation of the electric field or *in vivo* degradation of the SFV RNA. Despite a lower level of luciferase expression being observed with the SFV RNA in this experiment, there is still a higher level of expression observed between this plasmid and the pGL3 luciferase plasmid.

All experiments were performed using standard 26 gauge needles, which were then compared to silicon microneedles (Figure 5.5). The type of electrode used for electroporation was also examined for transfection efficiency. Use of novel electrode designs may lead to a reduction in the level of tissue damage that occurs due to electroporation and may also provide a less invasive electroporative process.

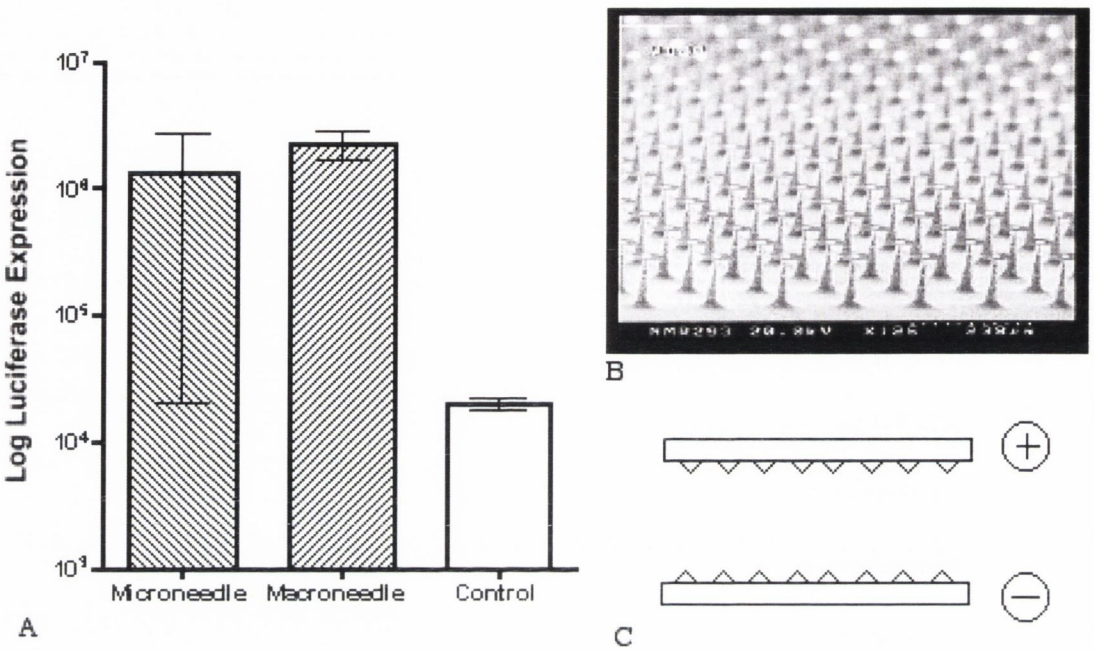


Figure 5.5: A novel silicon planar device with an array of microneedles (270 μm) etched onto the surface (B) was compared to standard 26 gauge needles. In both cases six pulses of 400 v/cm and 10 ms duration were applied across the clamped muscle (C) following intramuscular injection with pSFV-Luciferase (50 μg). There was no difference in the overall level of expression after 48 hours but there was an extremely large degree of variance in the expression in the mice electroporated with the microneedle electrode (A).

One of the main advantages of electroporation is that not only do you obtain high transfection, the expression is in a localized region and thus reduces the possibility of side effects that are often observed with systemic treatments. When examining the intramuscular luciferase expression it is possible to visualise the distribution of the gene expression within the muscle. This ensures that the transfected gene is being expressed

only locally and has not migrated to other areas of the body following electroporation. When the gene is virally delivered it may be detected in other areas of the body depending on the route of injection.

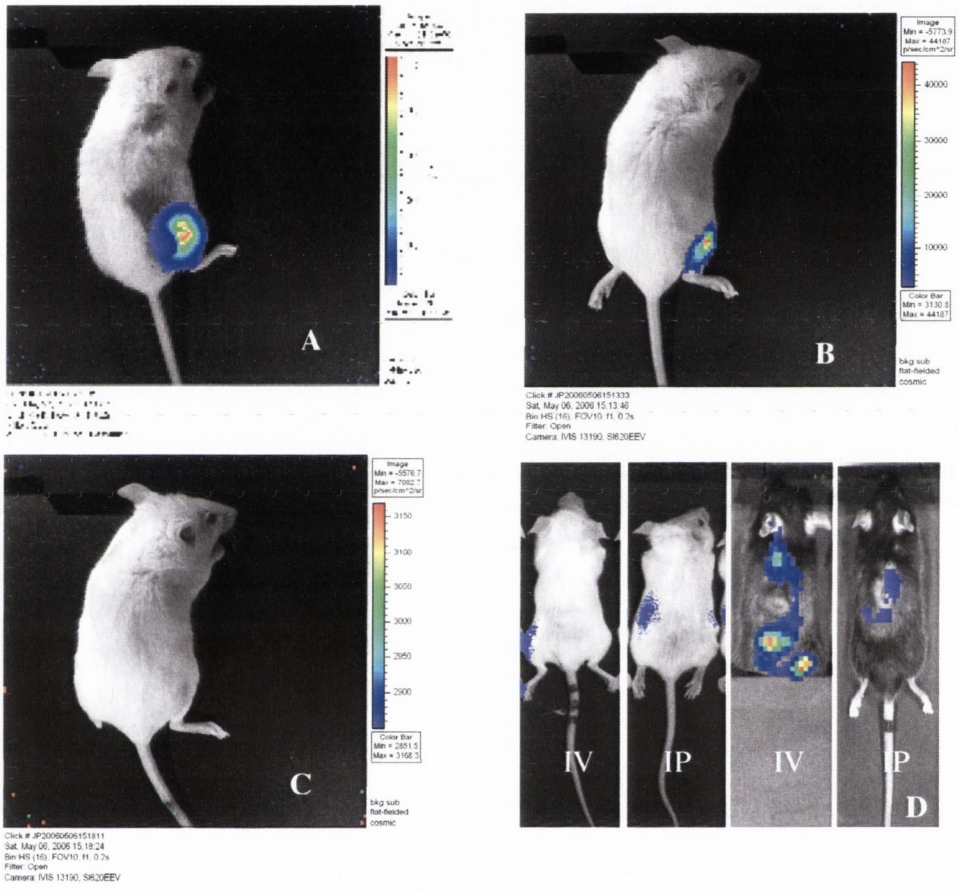


Figure 5.6: Images showing the distribution of gene expression following transfection with the pSFV-Luciferase plasmid (50 μg) or following infection with SFV viral like particles encoding luciferase. The highest level of expression is observed in the areas depicted by red and the lowest by purple with the actual unit values detailed in the corresponding colour chart. Six mice were used per group with group A being electroporated using standard needle electrodes inserted into the muscle, group B electroporated with the muscle clamped between two microneedle electrodes, C a

control with only SFV RNA injected into the muscle and group D virally transfected (IV or IP) Balb/c or C57BL mice.

It is important to examine the localization of the gene expression around the site of the plasmid injection when electroporation is applied (either using standard electrodes or microneedles) or when the plasmid is virally delivered. The highest degree of distribution of gene expression from the injection site is observed with electroporation using standard needle electrodes (A). The most localized expression is found directly at the site of plasmid injection with a decreasing gradient in the level of luciferase expression from this site. There was no difference in the distribution of the gene within the muscle to electroporative gene transfer when SFV viral like particles ($1x10^6$) are injected intramuscularly, but there is a decrease in the area of gene expression when the microneedles are applied (B). There is a wide degree of variation in the distribution of gene expression with the microneedles but it is always above the control (C). A clear difference in expression levels and distribution are observed when the SFV viral like particles ($1x10^6$) are delivered intravenously or intraperitionially. There is a lower degree of expression, more widely distributed over the entire system particularly when the C57 black mice (Harlan) are used rather than Balb/c (Harlan). This difference in expression levels was not observed for any of the electroporated mice (or those intramuscularly injected with SFV particles) where expression levels and distribution remained the same regardless of the mouse breed used.

5.3 Electroporative tissue damage

While high levels of gene expression are required the level of tissue damage occurring should also be considered, as it is desirable to have as efficient a trasfection as possible with minimal tissue damage. Tissue damage as a result of electroporation can affect gene transfer by reducing the number of viable cells that express the gene. When a high degree of tissue damage occurs an immune response can be raised, which may effect the overall vaccine treatment though there may be situations where a physical activation of the immune response may be desirable (Babiuk *et al.*, 2002). The level of tissue damage was examined on both a macro scale through direct visual observation of the damage to the muscle following the electroporation and through histological analysis of 4 μm sections taken from the muscle 48 hours post electroporation.

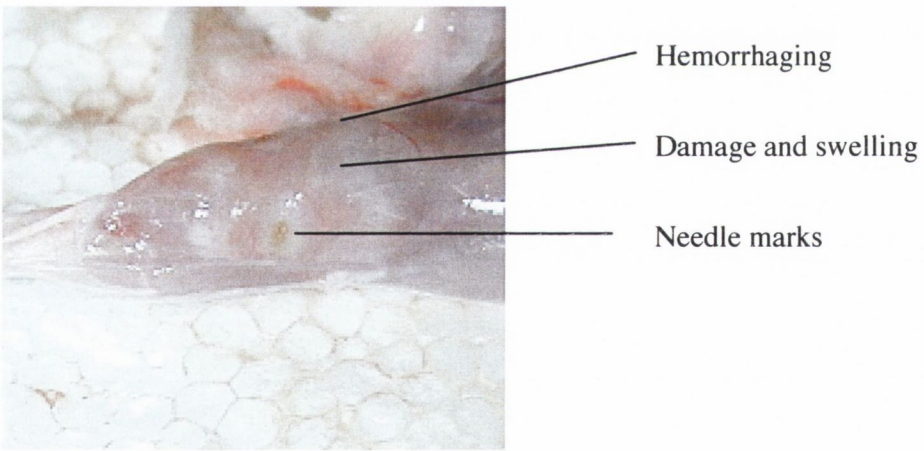


Figure 5.7: Image showing the level of damage occurring in the muscle following electroporation with six pulses of 400 v/cm of 10 ms duration. The image was taken 48 hours post electroporation where the highest degree of tissue damage is observed.

The damage was not obvious immediately following electroporation but was observed up to 72 hours post electroporation. There was no evidence of tissue damage 7 days following electroporation. While necrosis, swelling and needle marks were observed in almost all cases hemorrhaging was not as common and was observed in less than 30 % of cases. There was no evidence of discomfort for the mice either during or following the experiments. These experiments were also performed using the previously described microneedle electrodes. No visual difference was observed between the muscle of the control mice and that or those electroporated using the microneedles. A slight bruising was observed following electroporation of nude mice, but this may have been as a result of the electrode clamping procedure.

A lot of information can be derived from the visual observations of the tissue damage that occurred as a result of electroporation, examination at a cellular level was carried out for further clarification. Histology can provide more information about both the short and long term effects of intramuscular electroporation.

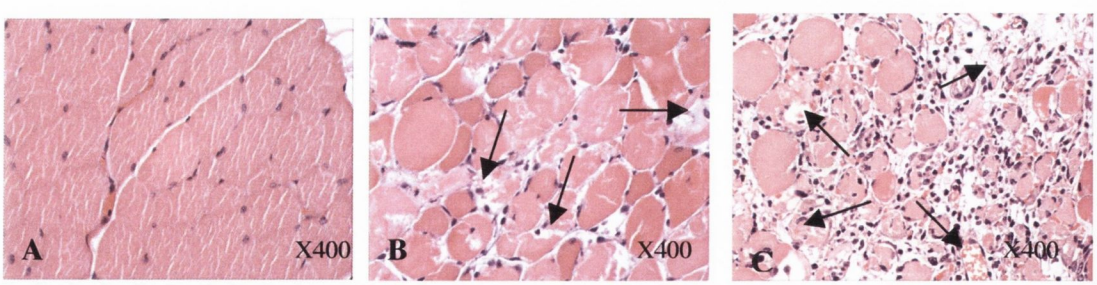


Figure 5.8: Images of 4 Δm paraffin embedded sections of muscle 48 hours post electroporation with six pulses of 400 v/cm and 10 ms duration. There was no damage observed in the control section (A). A mild degree of necrotic myofibers (arrows) was

observed when the microneedle electrodes were applied (B). When the standard needles were applied massive necrosis, oedema, haemorrhage and low-grade leucocytic infiltration were observed (C) (See Appendix 4A).

The tissue damage was examined using a range of different voltages and electrodes. The observed necrotic cells exhibited pyknosis of the nucleus (condensation of the chromatin into a small densely staining mass) and amorphous cytoplasmic eosinophilia. As is particularly noticed in Figure 5.8 (C) more advanced necrosis was characterised by nuclear karyorrhexis (fragmentation of the nuclear material), karyolysis (loss of nucleus) and cytolysis. There was no histological evidence of a difference in the mild level of tissue damage over the range of microneedle heights observed (Appendix 3A). The various voltages analyzed (Table 3.1) ranged from extremely an extremely high to low voltage potential. A decrease in tissue damage was observed when lower voltages were applied but this resulted in a corresponding decrease in gene expression. At all voltage potentials above 100 v/cm some degree of tissue damage was histogically evident. As the voltage was increased a proportional increase in leucocytic infiltration was observed (recognised by their small densely-stained nuclei and thin halo of poorly stained cytoplasm, see Figure 5.8) indicating an increase in inflammation within the muscle. It should be noted that some leucoytic infiltration was still observed at 400 v/cm when the microneedle electrodes were applied.

Voltage (v/cm)	Necrosis	Odema	Leucocytic infiltration	Calcification	Haemorrhage
0	-	-	-	-	-
100	+	-	+	-	-
250	+	-	+	+	-
350	++	-	+	+	-
400	+++	+	++	+	+
800	+++	++	+++	++	+
400 Microneedle	+	-	+	-	-

Table 5.1: Outline of tissue damage occurring 48 hours post electroporation was analysed histologically following H&E tissue staining. The severity of damage is noted by the use of "+" . "+" denoting mild damage and "+++" severe.

Six pulses were of 10 ms duration delivered in each case. The standard needle electrodes were inserted to a depth of 1cm into the mouse quadriceps muscle, which was clamped between the two microneedles electrodes. Minimal damage was observed with the microneedle electrodes. There was no histogical evidence of tissue damage 7 days post electroporation unless voltages above 600 v/cm were applied. When voltages greater than 600v/cm were applied evidence of massive necrosis and in some case odema were still evident up to 21 days post electroporation. It should be noted that the mice in these cases favoured the non-electroporated leg.

5.4 *In vivo* antibody response

The effectiveness of the optimized intramuscular electroporation pulse conditions in combination with the SFV vector system was assessed. Various SFV LacZ RNA and DNA vectors were examined for the ability to raise an antibody response to ß-galactosidase. ß-galactosidase was selected as an ideal reporter gene for analysis of electroporative antibody responses not only has it been well characterized as a vaccine reporter but can also be detected within the muscle through histogical analysis.

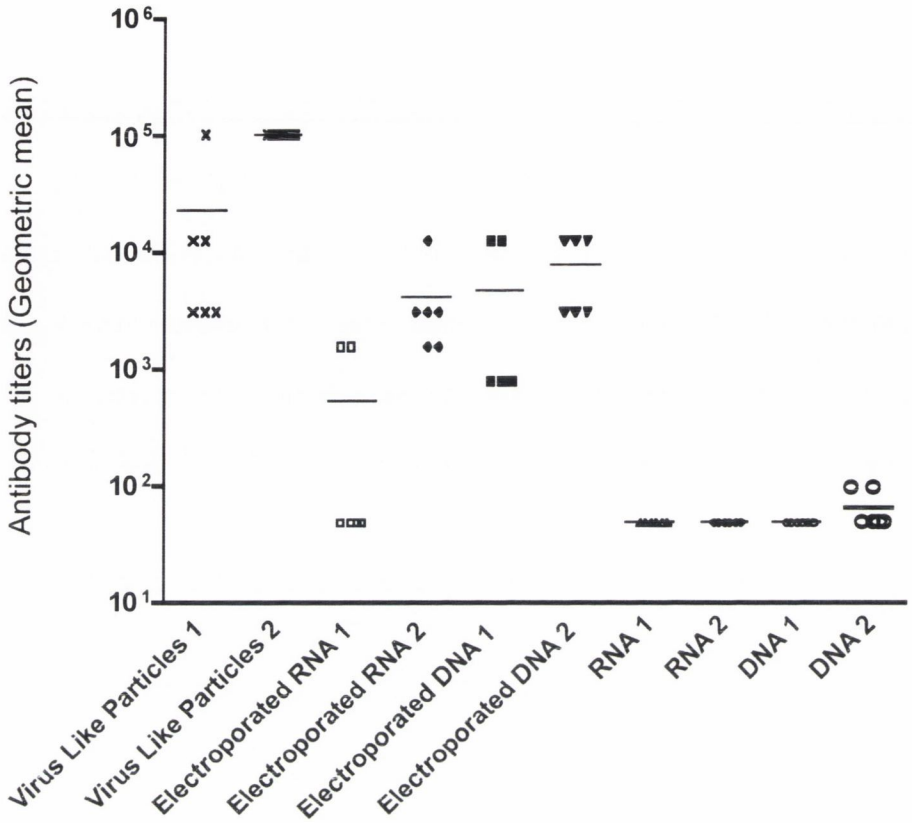


Figure 5.9: Graph representing the geometric mean blood serum antibody response in Balb/c mice following delivery of a ß-galactosidase reporter gene. In each case where electroporation was applied there were six pulses of 400 v/cm and 10 ms duration delivered to the mouse quadriceps muscle 1-2 minutes following injection with the RNA/DNA gene. The SFV viral like particles ($1x10^8$) encoding for LacZ were intramuscularly injected into the mouse quadriceps muscle (n=6).

Intramuscularly injected SFV RNA and DNA were contrasted with intramuscularly injected SFV RNA and DNA followed by electroporation. These results were also compared with SFV viral like particles ($1x10^8$) intramuscularly injected into the mouse quadriceps muscle. Blood was taken through the cheek vein 12 days following the first

immunization and the antibody serum levels analysed by ELISA (Figure 5.9 "1"). The mice were subjected to a second immunization on day 13 and bled again through the cheek vein on day 26 (2). There was a significant ($p=<0.001$) increase in the serum antibody levels when electroporation was applied following immunization with the ß-galactosidase gene. There was no difference in the antibody serum levels when mice were immunized with either pSFV1-LacZ RNA or pBK-SFV-LacZ DNA. There was a significant increase in the serum antibody levels observed when mice were immunized with the SFV viral like particles encoding LacZ. In all cases an aminastic response was observed following a second immunization.

Evidence has suggested that the method of gene delivery will strongly affect the type immune response (Li *et al.*, 2004). In order to examine if this was applicable in this study, i.e. did electroporation result in a TH1 type immune response or did the addition of the SFV replicase complex (and hence expression of viral proteins) result in a predominant TH2 type immune response. Also being examined here was the type of immune response raised as a result of the delivery of the SFV RNA or DNA and the impact this may have on the type of immune response obtained. In this case the blood serum levels were analysed for IgG1 and IgG2a. The functional properties of Ig are closely related to their isotype. For instance, IgG2a antibodies activate the complement system more readily than IgG1 antibodies, by binding to specific Fc receptors that are expressed on murine macrophages and are involved in phagocytosis. IgG2a antibodies are quite efficient mediators of antibody-dependent cell-mediated cytotoxicity (Kipps *et al.*, 1985). In contrast to anti-soluble protein or anti-carbohydrate antibodies, which are

generally restricted to the IgG1 (and IgG3) isotypes, most antiviral antibodies elicited by infection with RNA and DNA viruses belong to the IgG2a subclass (Markine-Goriaynoff et el 2000).

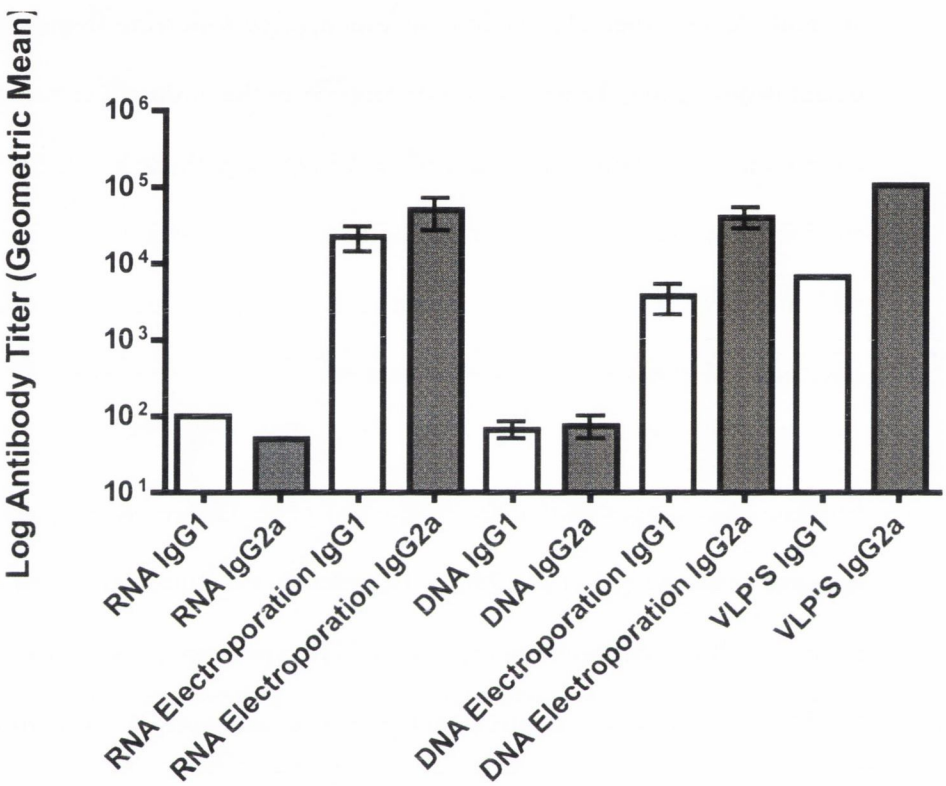


Figure 5.10: The blood serum following two immunizations with the SFV-LacZ RNA, DNA or SFV VLP's encoding for LacZ from Figure 5.9 was analysed for the type of IgG immune response by ELISA.

There was a significantly higher IgG2a antibody response observed with both the electroporatively delivered DNA and the viral delivery. This is expected due to the SFV replicase complex. While there was no real difference in the type of immune response of the intramuscularly injected RNA or DNA, this may be a result of very low over all antibody titers being detected within the serum. There was a significantly

(p=<0.05) higher IgG1 response observed in the electroporated pSFV3-LacZ RNA group (DNA vrs RNA IgG1 p = 0.031, DNA vrs RNA IgG2 p = 0.9048).

Due to the tissue damage observed following electroporation using standard needle electrodes (Fig. 5.8) a range of minimally invasive electrodes were examined. These included silicon microneedle electrodes provided by Dr. A Morrissey ,Tyndall National Institute, Cork, Ireland.

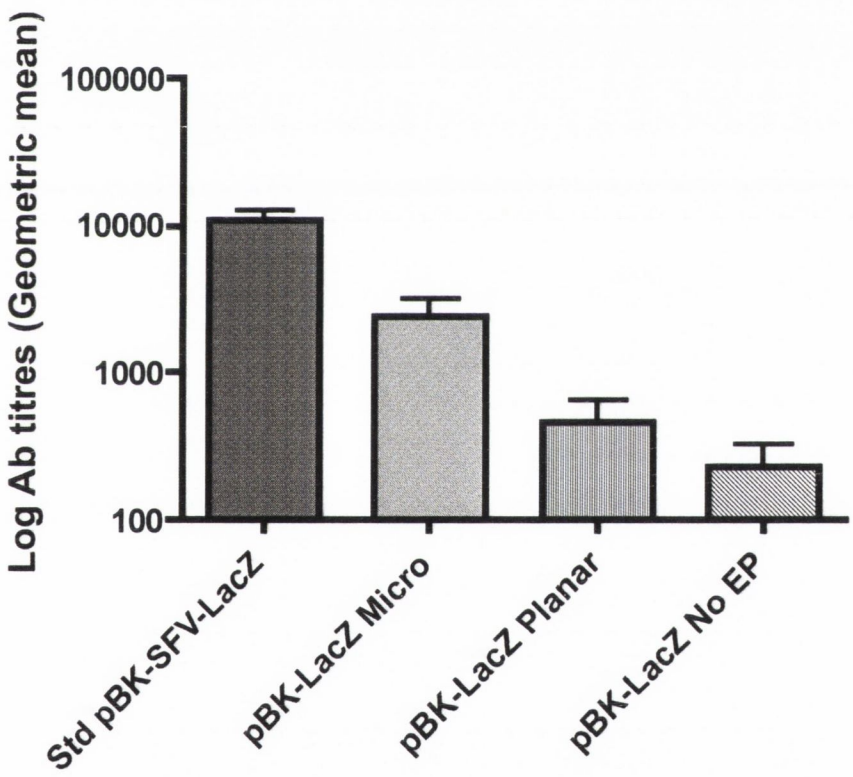


Figure 5.11: The blood serum antibody titre level analyzed by ELISA following one immunization with the pBK-SFV-LacZ (50 μg) using various electrode designs. In each case where electroporation was applied there were six pulses of 400 v/cm and 10 ms duration delivered to the mouse quadriceps muscle 1-2 minutes following injection with

pBK-SFV-LacZ (50 μg). The SFV viral like particles ($1x10^8$) encoding for LacZ were intramuscularly injected into the mouse quadriceps muscle (n=6).

The level of antibody response reflects that of geen expression. Compared to the standard needle electrodes, there was a reduced level of both gene expression and antibody response when the microneedle electrodes were applied.

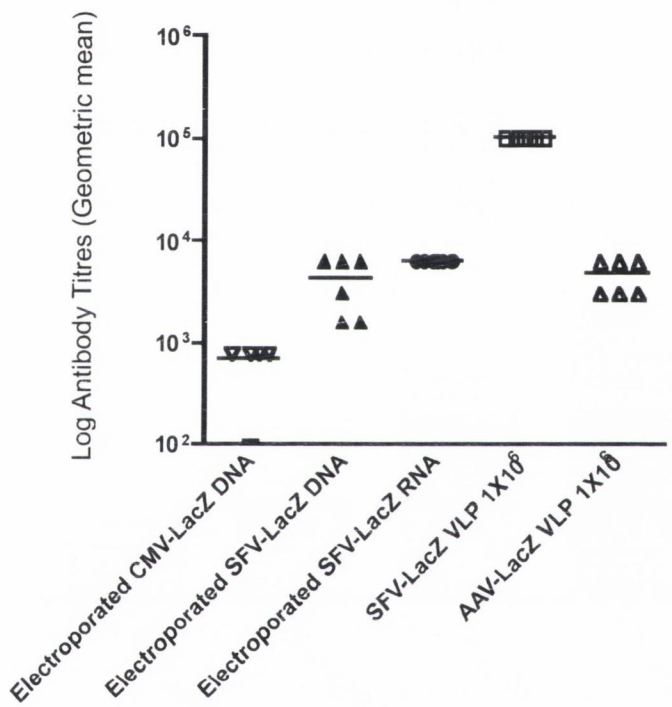


Figure 5.12: Antibody responses following intramuscular delivery of SFV viral like particles (1×10^6) encoding LacZ were contrasted with intramuscular delivery of AAV viral like particles (1×10^6) encoding LacZ. These results were compared with antibody response levels following intramuscular electroporation with SFV DNA or RNA (50 μg) as described in Figure 5.9.

There was no significant difference in the antibody responses observed when two immunizations were applied for each of the different gene delivery methods. This clearly shows that electroporative gene delivery is as effective as viral delivery for raising antibody titres.

5.5 Discussion

As has been shown in chapter 3 electoporatively delivered genes have the ability raise antibody responses comparable to that of commonly applied viral vectors. While these studies have been done solely in mice, the mouse represents a widely used experimental model for characterization of immune responses induced by DNA vaccines. Three promoters (SFV 26s, SV40 and CMV) were selected as a result of *in vitro* studies (both the SV40 and CMV are common plasmid promoters) where expression levels from these promoters were observed to vary over four-orders of magnitude. Interestingly, unlike *in vitro* no significant difference in gene expression was measured *in vivo* 48 hours following intramuscular injection in combination with electroporation between the SFV and CMV promoters, though a clear advantage was observed over the SV40 (Figure 5.4). One explanation for the similarity of gene expression *in vivo* is that the transcription factors recruited by the viral promoters *in vivo* may be different from those recruited in *vitro*. Also size of the plasmid may have impacted on the transfection efficacy *in vivo* as the SFV based plasmids are typically ~12kb in size while the CMV and SV40 based plasmids are typically ~5.5 kb in size. Molnar *et al.*, (2004) details how larger plasmids may be electroporated inefficiently into muscle fibers. It is shown that a

9 kb plasmid yields significantly higher intramuscular transfection than a 19 kb plasmid under the same electroporation conditions. Alternatively, the inflammation associated with electroporation may activate transcription factors not activated during the *in vitro* transfection (Miller *et al.*, 2004).

It has been demonstrated that the expression of a plasmid is solely observed in the muscle post electoporation (Figure 5.6) using both SFV based luciferase and ß-galactosidase plasmids. The distribution of gene expression after intramuscular (i.m.) plasmid electroporation was determined by luminometry. The injection of 50 Δl caused immediate swelling of the anterior epimysial sheath, spreading of DNA throughout the muscle, and DNA uptake by muscle cells. Such uptake is consistent with previous reports by other groups, which have shown that factors such as needle type, orientation and speed of injection, volume and type of injection fluid, and preinjection of hypertonic solutions can influence gene expression after intramuscular injection (Manthorpe et. al. 1993). This swelling may result in further dispersion of the gene throughout the muscle and increased uptake and expression of the gene at the site of injection. These results may provide insight into one of the variables leading to lower immunogenicity of DNA vaccines in larger animals, where the relative volume injected to the muscle is much smaller than that used in the mouse and hence the corresponding hydro static pressure is lower. The level of expression is dependent not only on the concentration of the DNA/RNA that has been delivered to the muscle but also on the volume that this has been delivered in. In each of the intramuscular experiments described a 50 Δl injection volume was applied. Dupuis *et al.*, (2000) found where the same concentration of DNA was delivered in a 5 Δl and a 50 Δl volume, a weaker

immune response was observed when the lower volume was applied. This may indicate that had a lower injection volume been applied to the muscle in these experiments that a lower immune response may have been observed.

The degree of muscle damage due to the electroporation was shown to be dependent on the pulse type applied. Previous data suggests that the level of tissue damage will reduce the transgene expression, but that the damage is reversible and dispels with time (Wang *et al.*, 2005). While there is less damage observed when the microneedle electrodes are applied (Figure 5.6) these electrodes are thought to less efficient for the delivery of the SFV based plasmids. It is thought that these electrodes have an application where lower levels of gene expression are desired while assuring a minimal level of tissue damage. While it was not examined within this thesis, the application of multiple treatments using these electrodes may result in higher gene transfer than standard needle electrodes due to the decrease in damage observed. Wang *et al.*, (2005) found that tissue damage with respect to electroporation negatively effected gene transfer with short interval repeat treatments.

There was no difference in luciferase gene expression levels (RLU) observed when the DNA/RNA was injected in either PBS, saline or water, as delivery is dependent upon the level of gene expression within the muscle (Appendix 3A). The more gene expression the higher the antibody titre or the level of luciferase expression. The luciferase expression levels were analyzed 48 hours post electroporation as it was shown to be the optimal time of expression for the electroporatively delivered RNA plasmids (Figure 5.2, See appendix 3A). While some expression was observed as early as 7 hours post transfection no expression has been observed prior to this time point

contrasting with the results of Manthorpe *et al.*, 1993 where transgene expression was detected as early as 2 minutes after injection of a luciferase reporter plasmid into mouse muscle. Extracellular DNA is rapidly degraded and cleared from muscle within hours (Lew *et al.*, 1995, Bureau *et al.*, 2004). Interestingly, Bureau *et al.*, (2004) found that while the amount of intact plasmid may be greatly reduced, the transfection efficacy remained the same when pulses were delivered either 20 seconds or 3 hours later suggesting that there are two pools of plasmid DNA upon intramuscular electroporation, one rapidly cleared and degraded and the other more stable. It has been shown that administration of inhibitors of DNA degradation do not increase expression of a luciferase reporter gene (Levy *et al.*, 1996), which suggests that in the mouse model, electroporatively driven uptake and expression of foreign DNA or RNA by muscle cells occurs very rapidly. This process is still unclear but one theory is that DNA/RNA enters into mature muscle cells by T tubules, which are found only in skeletal and cardiac muscle (Wolff *et al.*, 2000). In addition, the multiple nuclei in muscle cells as observed in the histology (Figure 5.8), may increase the probability of DNA reaching the nucleus (Dowty *et al.*, 1995) though no difference in expression was observed when either DNA or RNA were applied. It appears from the examination of the muscles following electroporation with the ß-galactosidase plasmid that there may be uptake and expression of DNA reaching the nucleus; this has also been suggested by other groups (Dowty *et al.*, 1995). There was no evidence of DNA/RNA uptake and expression in other tissues such as liver and lung after electroporation of the plasmid (Figure 5.6) while this has been observed by other groups following electroporation and also following direct injection (Zhang *et al.*, 1999). This may be due to a higher

concentration of DNA being applied in these cases. The method of injection has a high impact on the distribution of the gene expression within the host system (Figure 5.6). When the plasmid is directly injected into the muscle the expression distal to the injection site has been reported to occur only around the area where the needle injected DNA was disseminated. This has been observed in muscle (Aihara *et al.*, 1998), liver (Heller *et al.*, 1996) and melanoma (Rols *et al.*, 1998). If the plasmid is delivered through any other method e.g. i.p.,i.v. expression is observed in other organs though when plasmid DNA is delivered i.v. the highest level of gene expression is still observed in the electroporated area (Heller *et al.*, 2002, Tsujie *et al.*, 2001).

For each case of electroporative gene delivery 50 Δg of plasmid was used, this was found to be the optimal concentration of plasmid (see appendix 3A). As in other studies the transfection efficiency does increase in a dose dependent manner though it has be suggested that muscle damage will also increase in a parallel manner with respect to the concentration of plasmid delivered (Durieux *et al.*, 2004).

Electroporation parameters have been shown to greatly effect the gene expression (and hence effect the antibody titres), *in vitro* high voltage, short pulse duration conditions are typically used. Though this 400 v/cm pulse type was shown to be the optimal pulse parameters with this electroporation system (Figure 5.3) the voltages used differ to those used by other groups (Wang *et al.*, 2005, Bureau *et al.*, 2004). *In vitro*, electroporation pulses modify biological membranes and facilitate penetration of cells by DNA molecules, with only minor damage to these membranes (Chang *et al.*, 1990). It is also conceivable that these conditions can affect the integrity of the nuclear membrane and allow free passage of plasmid DNA into the nucleus. This may be

particularly relevant for delivery of DNA into the nuclei of non-dividing cells, such as muscle cells. The means by which electroporation increases DNA vaccine potency is not yet known, but is likely to be related to increased expression of encoded antigen. This could simply provide more antigen available for priming of immune responses. However, other factors may also be involved. For instance, whereas transfection of muscle cells is increased by electroporation, transfection of other cells, such as APCs, may also be facilitated although this has not yet been shown. Another possibility is that the application of an electric field directly in the tissue could result in an inflammatory response that aids in the priming of immune responses against the DNA-encoded antigens. It was observed that when novel electrode designs were applied to the muscle resulting a decrease in tissue damage (and inflammation) this yields a decrease in antigen expression, though a decrease in gene expression was also observed. While there is clear evidence of an inflammatory response from the histology (Figure 5.8), there is little to suggest the role of this inflammation in electroporative vaccine enhancement. It is more likely that the enhanced gene expression and hence increase in Ag expression in muscle cells with respect to the combination of the SFV vector and electroporation plays a predominant role in the enhancement of DNA or RNA vaccine potency by electroporation.

Chapter 6

Conclusions & Future work

6.1 Conclusions

Electroporation is a minimally invasive localized gene delivery method that can be applied to cancer therapy for both early and late stage cancers, reducing side effects often experienced when cancer therapies are applied systemically. Electroporative gene delivery has also been extensively applied for vaccination (Glasspool-Malone *et al.*, 2000, Somiari *et al.*, 2000, Drabick *et al.*, 2001, Liu & Huang, 2002), due to the expensive of many current vaccination strategies. It has been found that local gene transfer can be performed using electroporation giving rise to high gene expression in a variety of different tissue types such as skin (Titomirov *et al.*, 1991, Heller *et al.*, 2001), liver (Heller *et al.*, 1996), brain (Nishi *et al.*, 1996), tumour (Rols *et al.*, 1998) and muscle (Aihara & Miyazaki, 1998, Mir *et al.*, 1999). Electroporative gene delivery has also been shown to induce antigen production either in skin and skeletal muscle (Gehl *et al.*, 2003, Waldera *et al.*, 2004). Due to the effectiveness of electroporation as a method of gene delivery there are many advantages in combining this with the SFVbased plasmids (either DNA or RNA). The aim of this research was to investigate the combination of SFV based plasmids and electroporative gene delivery.

The *in vitro* experiments have shown the effectiveness of electroporative gene delivery over a range of different cell types. There is a large degree of variance in the optimal pulse parameters for each cell type. This can be seen to largely depend on the cell size and shape. Cells that are smaller in size and less uniform in shape are typically more difficult to electroporate (Neumann *et al.*, 1999, Gehl *et al.*, 2003) e.g. Caco2 cells, while cells that have a larger diameter and a uniform spherical shape give consistently high

levels of gene expression when electroporated. Cell lines such as OE19, a human oesophageal cell line, was found to be very difficult to transfect in vitro with transfection efficiencies below 30 % when the SFV based RNA or DNA or CMV DNA plasmids were used (less than 15 % when the pGL3 based plasmids were electroporated) but this cell line gave transfection efficiencies comparable to CT26 when electroporated *in vivo*. Increasing the voltage, the pulse duration or the pulse number of the electroporative pulse form may increase the level of gene expression within the various cells line but once the power needed to permeabilize the particular cell type is exceeded the transfection efficiencies will decrease, while cell death is increased due to irreversible cell poration. (Golzio *et al.*, 2001). No advantage was observed in changing the temperature of the pulse medium for transfection but this is most likely due to the optimization having been done at room temperate and using PBS. Examination of these conditions, which may result in cells be coming more susceptible to permeabilization (Zimmermann *et al.*, 1996, Rols *et al.*, 1994) should be done using milder pulse conditions. There was found to be no difference in the level of gene expression for either the SFV based RNA or DNA or CMV DNA plasmids when electroporated *in vitro* or *in vivo* though all plasmids gave higher results than the pGL3 based plasmids. While there was no difference in gene expression there was a significant difference in the antibody response with each of the different vectors. The SFV based plasmids induced the highest electroporative antibody titre when electroporated. This may be a result of the viral SFV element as the SFV viral like particles was found to induce the highest antibody titres. It could be that coupling the inflammatory response of the electroporative tissue damage with the immune response to the viral proteins produced as a result of the SFV non-structural proteins has a synergistic

effect inducing a higher antibody response. It could be argued that as the optimization both intratumoural and intramuscular were done using SFV based RNA that the electroporation conditions used *in vivo* favoured the application of SFV based plasmids. This is doubtful as the degree of difference in the level of gene expression for the CMV and SFV plasmids (LacZ or Luciferase) is minimal, while the differences in antibody titres are significant. It may be that the inflammation associated with electroporation may activate transcription factors that may lead to this difference in antibody response with these vectors (Miller *et al.*, 2004).

The optimal gene expression was found to be later (12 - 24 hours) following electroporative gene delivery when compared to viral delivery. It is thought that the cell undergoes a stress response due to the electroporation that shuts down protein synthesis, hence the delay in gene expression. This phenomenon has been observed in both tumour and muscle cells, but no difference in the optimal time of expression was observed *in vitro*; no difference in the time of expression was observed between the RNA and DNA plasmids either *in vitro* or *in vivo*.

The combination of electroporation and the SFV plasmids leads to transient intramuscular and intratumoural gene expression, due to the transient nature of the SFV plasmids. This is beneficial in terms of biosafety, and as RNA replication occurs exclusively within the cytosol, insertional mutagenesis (which can occur with some DNA vectors such as those derived from retroviruses) is not a concern. The electroporated RNA was found to persist at the site of injection for up to 7 days but was not found elsewhere within the system. The SFV viral vectors have also been shown to persist at the site of injection for up to 7 days but also have been detected in other organs post injection

e.g. the lymphoid organs (Morris-Downes *et al.*, 2001). Another advantage for combining electroporation and SFV as a vaccine is that most mammalian species have no preexisting immunity to alphaviruses (Atkins *et al.*, 1999) and therefore no immunity to the SFV proteins. In the case of multiple applications of a SFV viral based vaccine there is the possibility that there would be an acquired immunity to the SFV virus. This is less probable with the SFV-based electroporative vaccine as the structural proteins that are likely to induce this immunity are not present. There may be a marginal response to the non-structural proteins of the SFV although it is thought that this will be insufficient to illicit an immune response.

Electroporation has an advantage over many current viral based vaccines in that it has been shown to be effective for gene delivery over a range of tissue types such as skin (Titomirov *et al.*, 1991, Heller *et al.*, 2001), liver (Heller *et al*, 1996), brain (Nishi *et al.*, 1996), tumour (Rols *et al.*, 1998) and muscle (Aihara & Miyazaki 1998, Mir *et al.*, 1999). A limiting factor in the effectiveness of viral vectors in gene therapy is the efficiency by which they can successfully infect target cells and therefore achieve their desired therapeutic effect. Cell type has a major influence on viral infection efficiency, with viruses typically displaying tropism for particular tissues where they replicate most efficiently.

Electroporation has been shown to be highly effective in cancer drug therapies with many studies showing increased drug delivery (Orlowski *et al.*, 1988, Gehl *et al.*, 1998, Jaroszeski *et al.*, 2000) and tumour regression irrespective of histological cancer type (basal cell carcinoma (Glass *et al.*, 1997), malignant melanoma (Heller *et al.*, 1998, Gehl & Geertsen, 2000, Sersa *et al.*, 2000), adenocarcinoma (Mir *et al.*, 1998), squamous

cell carcinoma (Belehradek *et al.*, 1993, Panje *et al.*, 1998), transitocellular carcinoma (Kubota *et al.*, 1998) and renal cell carcinoma (Sersa *et al.*, 2000)) While all this data has been very promising for electrochemotherapy there still remains a number of different cancer types that do not respond, drugs are expensive (though lower doses can be delivered effectively with electroporation) and this type of treatment is applicable only to accessible tumours.

The combination of SFV plasmids with electroporation has been shown to lead to transient gene expression within the tumour. While persistent expression of certain proteins can be favorable, the transient high level of expression achieved using the electroporated SFV plasmids is particularly suited to cytotoxic and immunostimulatory cancer gene therapy, where prolonged expression is not necessarily desirable. Given the inherent ability of the SFV based plasmids to induce apoptosis in a variety of cell lines and to elicit strong immune responses when employed as prototype vaccines, the potential for the implementation of SFV in immunotherapy of cancer is clear (Fleeton *et al.*, 1999; Murphy *et al.*, 2000, Gowda *et al.*, 2006). In this study, SFV plasmids encoding either the *Bax* or *IL-12* genes were employed in the treatment of CT26 tumours in BALB/c mice. It was hoped that the expression of the viral antigens by the SFV plasmids would augment their antitumoural effect through the induction of host immune responses directed at infected tumour cells in combination with the proapototic *Bax* gene or the immune stimulatory *IL-12* gene.

Electroporatively delivered pSFV-*Bax* was shown to effectively induced apoptosis and transiently suppressed tumour growth both *in vitro* and *in vivo*. The inhibition of tumour growth appeared to only be effective while the plasmid was being

expressed within the tumour (~ 7 days (Morris-Downes *et al.*, 2001)) after this time the tumour appeared to grow at the same rate as untreated tumours. While a significant inhibition in tumour growth was observed with the OE19 in comparison to the CT26 tumours, it is more likely due to the slower growth rate of this tumour type rather than the electroporatively delivered pSFV-*Bax*. The results are comparable to those found by Murphy *et al.*, (2000) where a low titre rSFV-Bax was shown to transiently inhibit AT3-Neo and AT3-Bcl-2 tumour growth. Interestingly, electroporated pSFV-EGFP that was used as virus control showed the potential for CT26 tumour inhibition when compared to the untreated or electroporated only controls. This reflects the findings of Gowda *et al.*, (2006) and Murphy *et al.*, (2000) where some tumour inhibition was observed with the control rSFV-EGFP virus like particles. This is most likely due to the expression of the viral antigens by the SFV plasmids resulting in a mild antitumoural effect.

Electroporatively delivered pSFV-*IL-12* was shown to effectively induce production of active IL-12 and to transiently suppress tumour growth. The inhibition of tumour growth as with the electroporated pSFV-Bax appeared to only be effective while the plasmid was being expressed within the tumour after which the tumour growth rate returned to that of the untreated tumour. As a previous study by Colombo *et al.*, (1996) showed the amount of IL-12 available at the tumour site is critical for tumour regression, a second treatment was given 8 days following the initial tumour treatment. The tumour sizes were observed to remain static for a further 8 days. Evidence suggests that maximum anti-tumour effects can be achieved by transient IL-12 expression systems instead of prolonged high-dose treatment (Colombo *et al.*, 1996). While SFV plasmids have been shown to result in transient gene expression it thought that insufficient

electroporative delivery of the SFV10-IL-12 may have resulted in the incomplete tumour regression.

Complete tumour regressions have been observed in a number of tumour studies using the SFV viral like particles (Gowda *et al.*, 2006, Smyth *et al.*, 2005, Murphy *et al.*, 2000). In each of these studies the tumours were subjected to at least seven viral treatments, one day apart while only one or two treatments were applied to tumours in this case. To treat seven times a day apart was deemed too invasive for electroporative gene delivery using a mouse model. Bleomycin is typically delivered to the entire tumour volume with multiple applications leading to no change in normal healthy tissue, while causing apoptosis within the tumour, the reason however remains unclear. This would not be an option in this case as up-regulation of Bax has been shown to cause apoptosis in healthy cell populations (Jurgensmeier *et al.*, 1998) and hence may result in damage to healthy tissue. It is thought that if the electroporation of pSFV-*Bax* or pSFV-*IL-12* was to be repeated a number of times at the initial treatment, delivering the gene to a larger area of the tumour or over the entire tumour volume, that a significant difference in tumour growth would be observed as was found by Li *et al.*, (2002) who reported that multiple electroporative treatments with IL-12 resulted in tumour regression in 40 % of treated tumours. It should also be noted that while no difference in the level of gene expression was observed between the SFV and CMV based plasmids (Figure 5.4) a higher immune response was raised to the SFV based plasmids (Figure 5.12) therefore the pSFV-*IL-12* may offer more effective inhibition of primary tumour growth and secondary metastases than that found by Li *et al.*, (2002) upon multiple applications using a CMV based plasmid.

Successful gene therapy depends on the properties of gene delivery systems including antigenicity, bio-safety and the ability to express a functionally active product. The combination of electroporation and SFV based plasmids has been shown to offer high levels of transient gene expression while improving on possible bio-safety issues associated with SFV viral vectors (Atkins *et al.*, 1999). To conclude, electroporatively delivered SFV plasmids are capable of raising high antibody responses indicating their potential as candidate vaccination strategies (see chapter 5). Electroporatively delivered SFV proapoptotic or immuno- therapy inhibits cancer growth, showing the considerable potential for of this combination for cancer gene therapy (see chapter 4).

6.2 Future work

The work described in this thesis concerns the development of electroporation for use with the SFV based plasmids. Electroporative SFV plasmid delivery has great potential in the areas of vaccination and cancer gene therapy. The future work outlined details the ideal development of *in vivo* electroporation for use in vaccination or cancer gene therapy.

6.2.1 Electroporative vaccine delivery

After showing the effectiveness of electroporated SFV plasmids at inducing antibody responses, the next step would be to examine the ability of this combination to induce protection against a viral challenge. SFV viral like particles express louping ill virus antigens, inducing a better protective response than plasmid-based DNA vaccines or inactivated whole particle vaccines (Fleeton *et al.*, 2000). The SFV plasmid RNA vector

is already available and so it is recommended that this be electroporated intramuscularly using both the standard needle and microneedle electrodes. This should be assessed for expression of louping ill virus antigens and the ability to induce a protective response in comparison to the SFV viral like particles, a plasmid-based DNA vaccine or an inactivated whole particle vaccine.

6.2.2 Electroporative cancer gene therapy

6.2.2.1 pSFV-Bax cancer treatment

Electroporated pSFV-*Bax* has been shown to inhibit growth of CT26 and OE19 tumours, albeit transiently. It is thought that if the electroporation of pSFV-*Bax* was repeated a number of times at the initial treatment, therefore delivering the gene over the entire tumour volume, that a significant difference in tumour growth may be observed. It is recommended that the *in vivo* tumour treatments described in this thesis be repeated with multiple electroporations of the pSFV-*Bax*. This should be contrasted with multiple electroporations of a control reporter vector e.g. pSFV-Luc (also allowing quantification of gene expression), bleomycin, electroporation alone and untreated tumour groups.

6.2.2.2 pSFV-*IL-12* cancer treatment

As has been described in section 6.2.2.1 multiple electroporations at the initial tumour treatment, delivering the *IL-12* gene to the entire tumour volume should result in a significant difference in tumour growth. It is recommended that the *in vivo* tumour treatments described in this thesis be repeated with multiple electroporations of the pSFV-*IL-12* (Li *et al.*, 2005). This should be compared with multiple electroporations of

a control reporter vector, bleomycin, electroporation alone, SFV viral particles encoding *IL-12* and untreated tumour groups.

A second element of this work would be to examine the effectiveness of the electroporated pSFV-*IL-12* for the inhibition of 4T1, a mammary tumour cell line that can metastasize to the lung, liver, lymph nodes and brain, while the primary tumour is growing *in situ*. This should be examined with both intratumoural and intramuscular electroporation of the IL-12 with multiple electroporations and controls as outlined previously. Successful inhibition of metastases by intramuscular electroporation would lead to examination of the combination of electrochemotherpy for the treatment of the primary tumour and electroporated pSFV-*IL-12* for the inhibition of secondary metastases.

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Appendices

Appendix 1A

Miscellanious Materials List

TRIzol	Invitrogen
Enzymes	New England Biolabs
DNA Ladders (1kb and 100bp)	New England Biolabs
Plasmid Purification Kits	Qiagen
SP6 RNA polymerase	Promega
$m^7G(5')ppp(5')G$	Promega
10X Loading Dye	Promega
Antibodies	BD Biosciences
OptEIA mouse *IL-12* (p70) kit	BD Biosciences
OptEIA mouse IFN-θ kit	BD Biosciences
3,3',5'5-Tetramethylbenzidine (TMB)	Sigma
Phosphate buffered-saline (PBS)	Sigma
Cell lysis Buffer	MSC
NEB buffer set	New England Biolabs
Loading dye	Invitrogen
DNase/Rnase free water	Sigma
Ampicillin	Sigma
DMSO	Sigma
Agarose	Sigma
NaCl	Sigma
Sucrose	Sigma

Magnessium Chloride	Sigma
Kanamycin	Sigma
L-Glutamine	Sigma
T/EDTA	Sigma
FBS	Sigma
Tissue Culture Consumables	Sarstedt
Bradford Reagent	Sigma
27 gauge needles	Sigma
Propidium Iodide	Sigma
4mm Electroporation Cuvettes	MSC
Isopropanol	Sigma
BSA Protein Assay regent A	MSC
BSA Protein Assay regent B	MSC
Xylazine	Vetoquinol
Ketamine	Vetoquinol
Luciferase Assay System	MSC
Lysis Buffer	MSC
Nuclease free water	MSC
Polylysene Slides	Reagecon
Cover slips	Reagecon
Bleomycin	Mayne Phrama
VECTASHIELD hardset mounting medium with DAPI	Vector Labs

Appendix 2A

Selection of optimal intratumoural pulseform based on expression of luciferase in CT26 tumours, 48 hours post electroporation with SFV-Luciferase RNA.

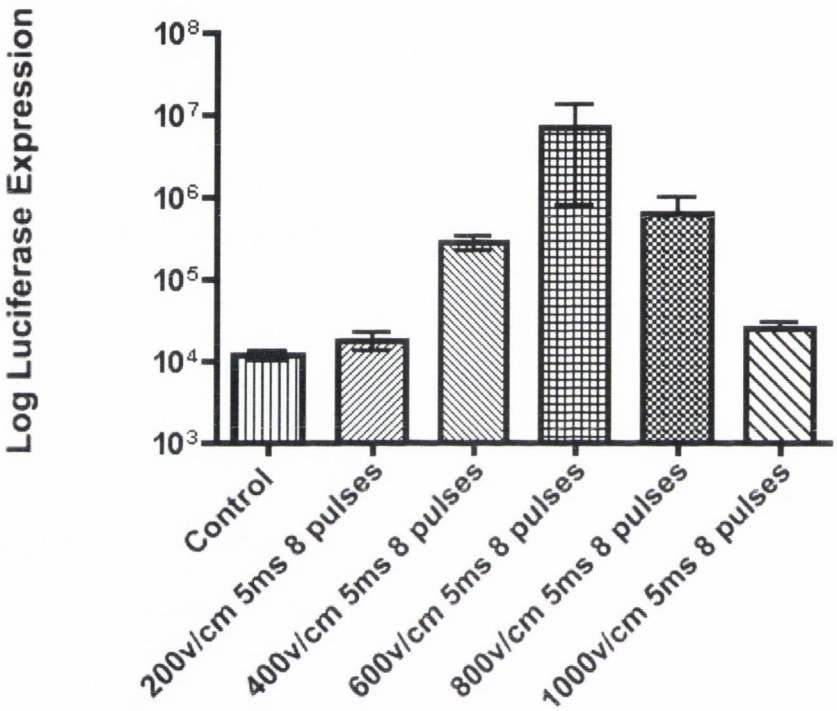

Figure 2A.1: Examination of luciferase expression following intratumoural electroporation with the pSFV-Luciferase plasmid (50 μg) using different pulse voltages 48 hours following electroporation Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intratumourly injected but without any pulse delivery was also analyzed. Eight pulses of 5ms duration were applied in each case to groups of three mice.

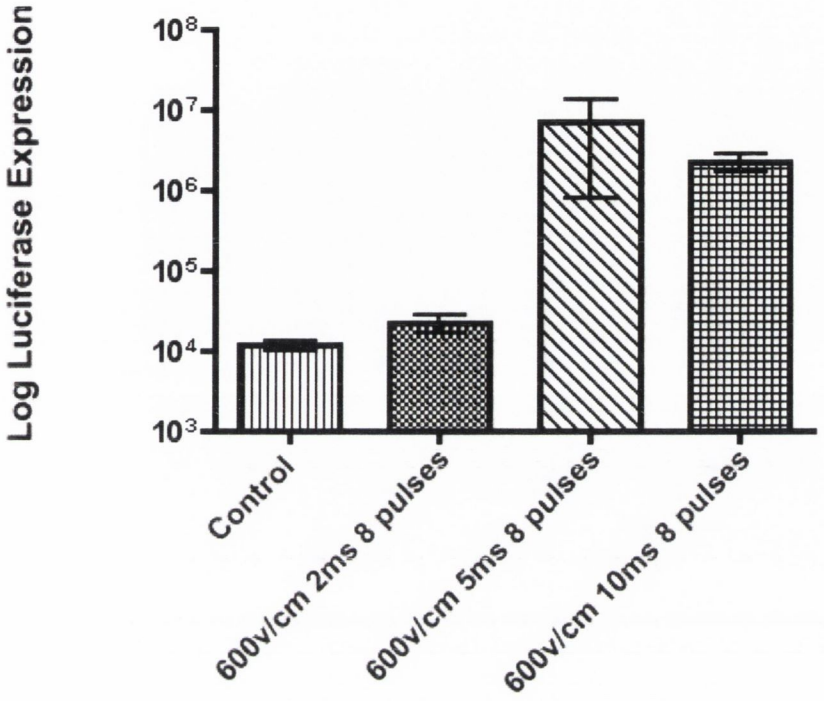

Figure 2A.2: Examination of luciferase expression following intratumoural electroporation with the pSFV-Luciferase plasmid (50 μg) using different pulse durations 48 hours following electroporation Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intratumourly injected but without any pulse delivery was also analyzed. Eight pulses of 600 v/cm were applied in each case to groups of four mice.

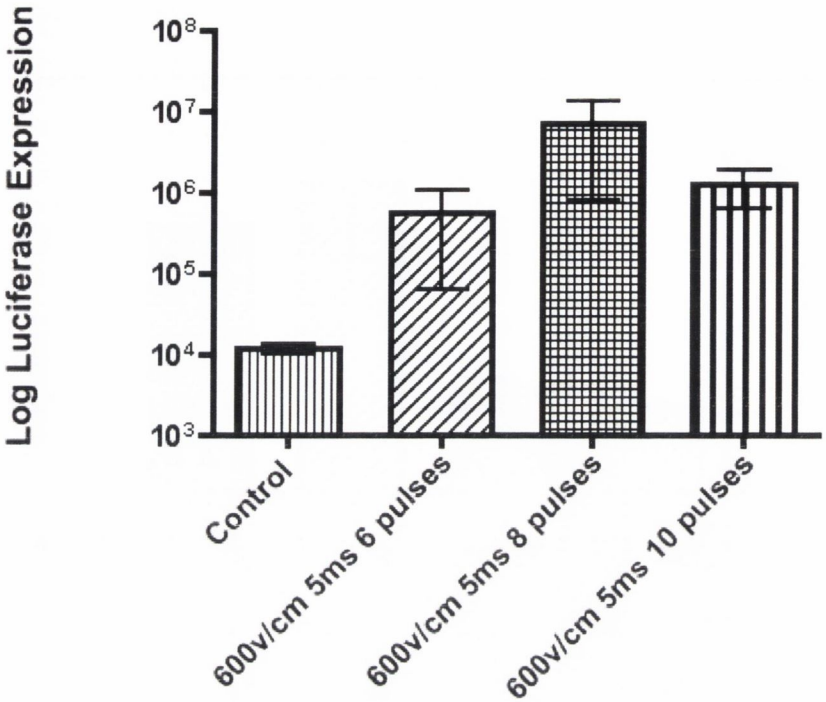

Figure 2A.3: Examination of luciferase expression following intratumoural electroporation with the pSFV-Luciferase plasmid (50 μg) using different pulse numbers 48 hours following electroporation Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intratumourly injected but without any pulse delivery was also analyzed. Pulses of 600 v/cm and 5 ms duration were applied in each case to groups of three mice.

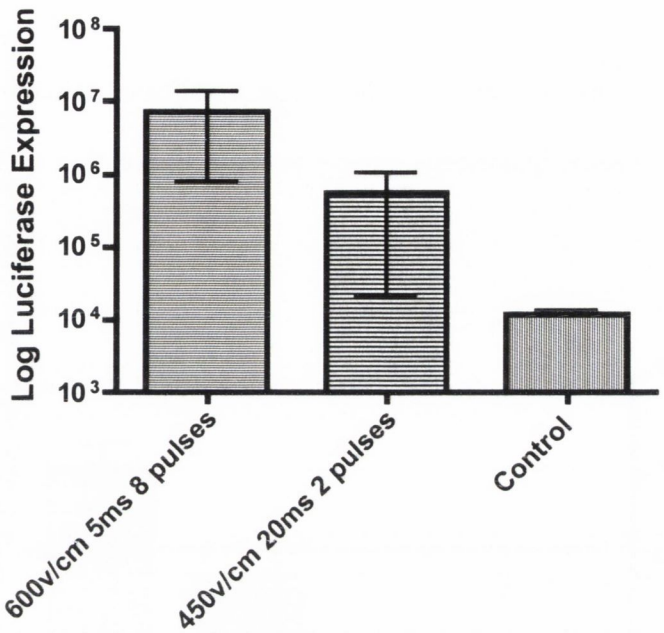

Figure 2A.4: Examination of luciferase expression following intratumoural electroporation with the pSFV-Luciferase plasmid (50 μg) using the selected optimal electroporation pulse conditions (similar to that found by Mesojednik *et al.*, (2007) and Wells *et al.*, (2000)) in comparision to that used by other groups (Li *et al.*, 2005) 48 hours following electroporation Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intratumourly injected but without any pulse delivery was also analyzed (n=3).

Appendix 3A

Selection of optimal intramuscular pulse conditions based on expression of luciferase in mouse quadriceps muscle post electroporation with SFV-Luciferase RNA.

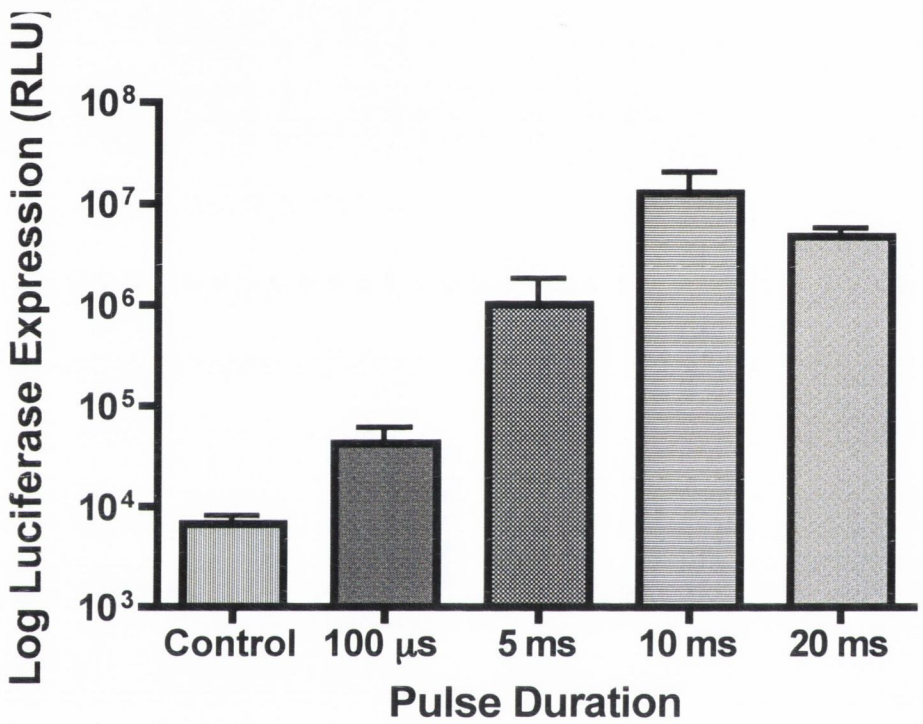

Figure 3A.1: Examination of luciferase expression following intramuscular electroporation with the pSFV-Luciferase plasmid (50 μg) 48 hours following electroporation with eight pulses of 400 v/cm. Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intramuscularly injected but without any pulse delivery was also analyzed (n=4).

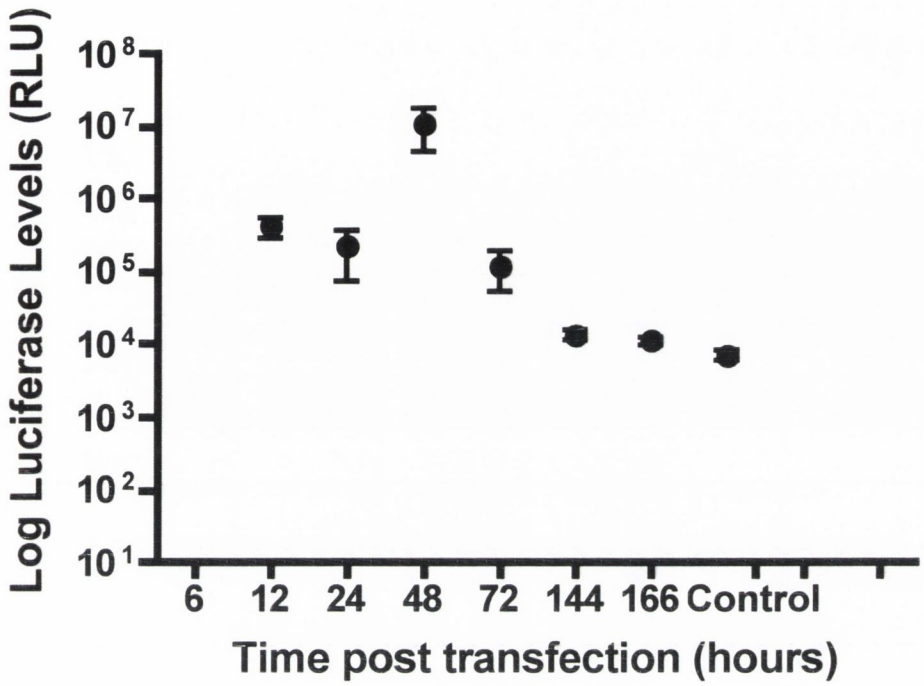

Figure 3A.2: Examination of luciferase expression following intramuscular electroporation with the pSFV-Luciferase plasmid (50 μg) at various time points following electroporation with eight pulses of 400 v/cm and 10 ms duration. Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intramuscularly injected but without any pulse delivery was also analyzed (n=3) (no difference was observed at each time point and so only 48 hours is shown).

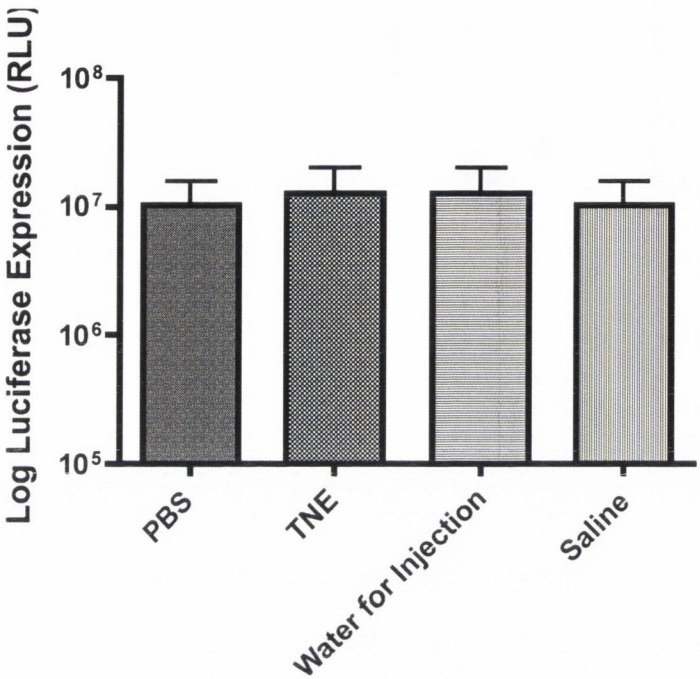

Figure 3A.3: Examination of luciferase expression following intramuscular electroporation with the pSFV-Luciferase plasmid (50 μg) 48 hours following electroporation with eight pulses of 400 v/cm and 10 ms duration. Luciferase levels are examined using the Xenogen live body imager. (n=3).

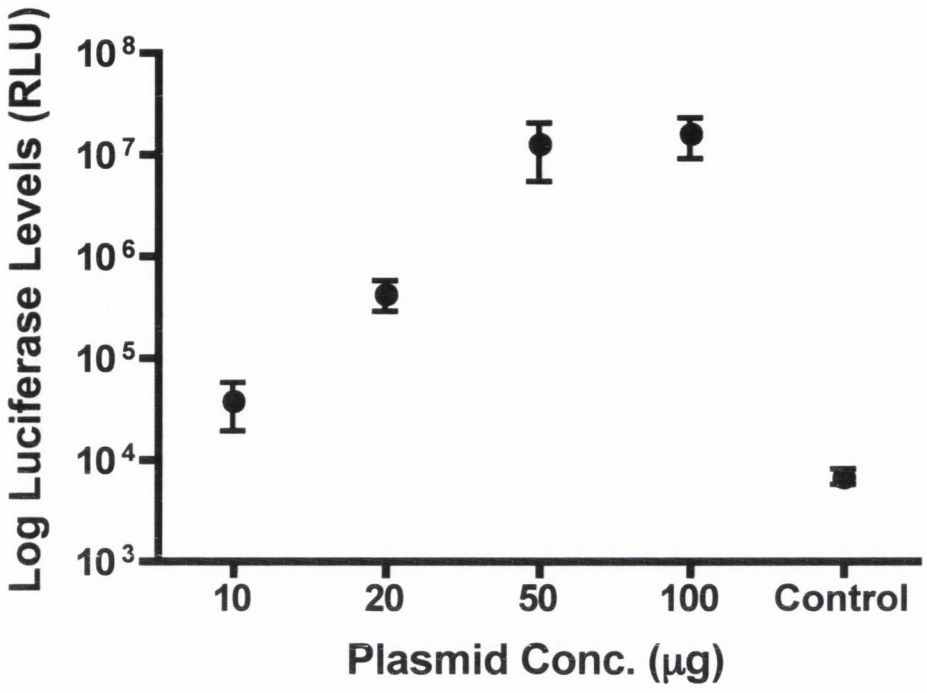

Figure 3A.4: Examination of luciferase expression following intramuscular electroporation with the various concentrations of the pSFV-Luciferase plasmid 48 hours following electroporation with eight pulses of 400 v/cm and 10 ms duration. Luciferase levels are examined using the Xenogen live body imager. A control group where pSFV-Luciferase plasmid (50 μg) has been intramuscularly injected but without any pulse delivery was also analyzed (n=4).

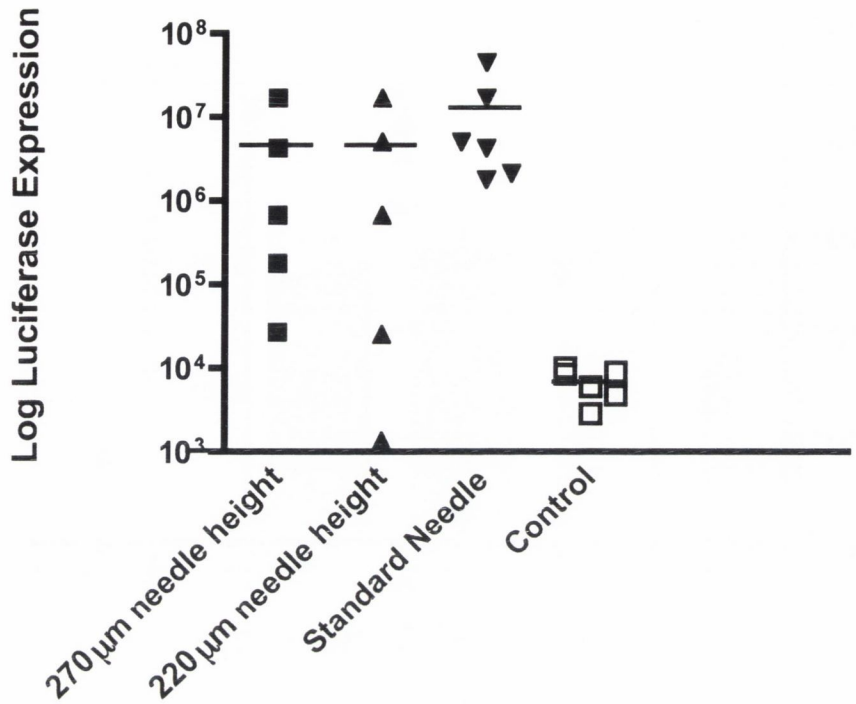

Figure 3A.5: Examination of luciferase expression following intramuscular electroporation with the pSFV-Luciferase plasmid (50 μg) 48 hours following electroporation with the microneedle electrodes. Eight pulses of 400 v/cm and 10 ms duration are used in each case. Luciferase levels are examined using the Xenogen live body imager (n=5). A control group where pSFV-Luciferase plasmid (50 μg) has been intramuscularly injected but without any pulse delivery and a control group where pSFV-Luciferase plasmid (50 μg) has been intramuscularly injected and electroporated using standard needle electrodes were also analyzed.

Appendix 4A

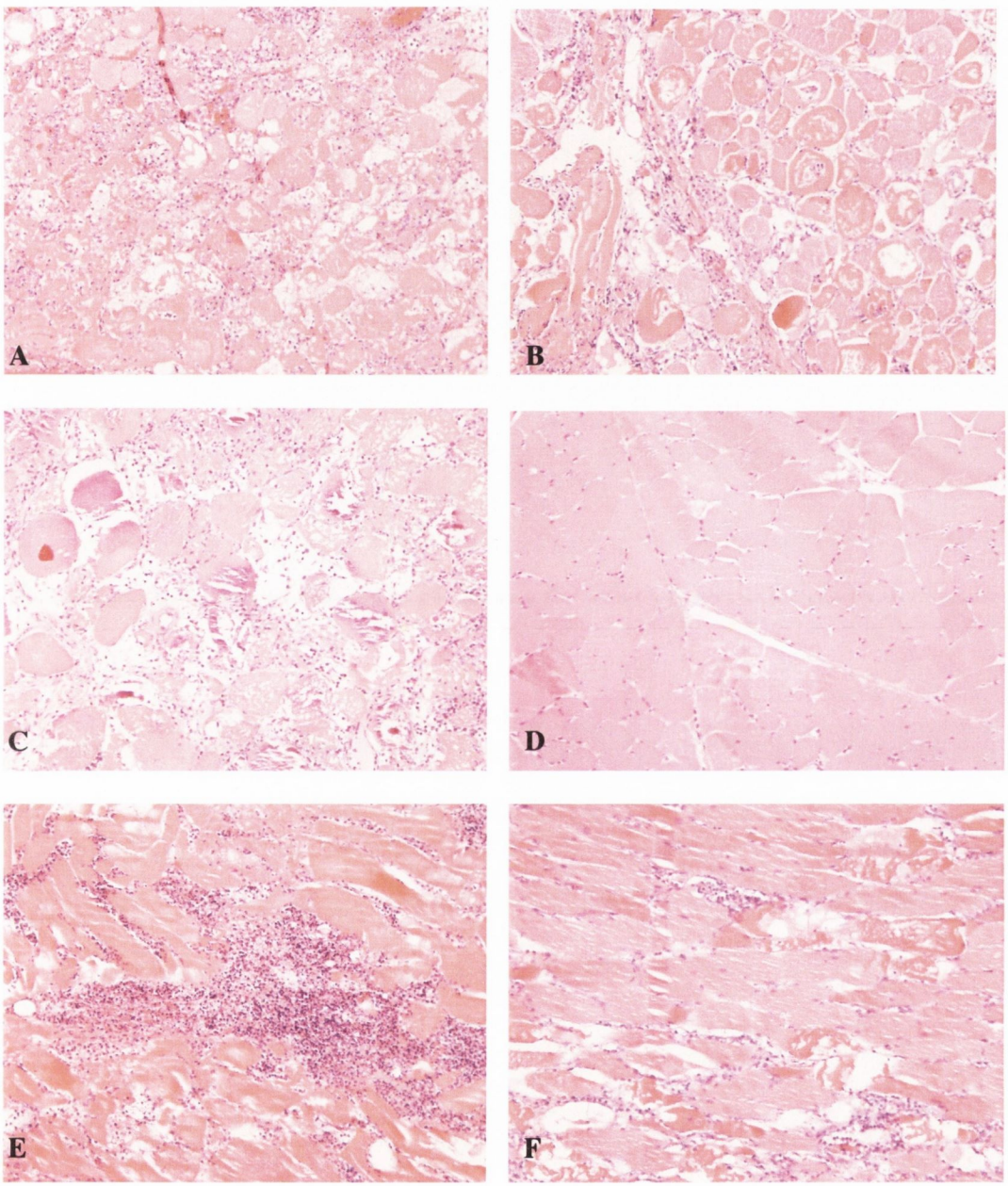

Figure A4.1: Images (x 400) of 4 μm paraffin embedded sections of muscle 48 hours post electroporation with six pulses of 400 v/cm and 10 ms duration. There was no damage observed in the control section (D). Some necrotic myofibers and leucocytic infiltration was observed when the microneedle electrodes were applied

(E and F). When the standard needles were applied massive necrosis, oedema, haemorrhage and leucocytic infiltration were observed (A, B and C).