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Cork Cancer Research Centre



Ex vivo culture of patient tissue and examination of gene delivery

Thesis presented by

Simon Rajendran, MB BCh BAO., MSc.

Under the supervision of

Dr. Mark Tangney and Ms. Deirdre O'Hanlon

for the degree of

Doctor of Philosophy

June 2014

"Twenty years from now you will be more disappointed by the things that you didn't do than by the ones you did do. So throw off the bowlines. Sail away from the safe harbor. Catch the trade winds in your sails. Explore. Dream. Discover."

Mark Twain

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Thesis Abstract

Cancer remains a leading cause of morbidity and mortality despite better understanding of cancer causation and progression. Current anticancer regimens are associated with non-specific cytotoxicity leading to treatment failure due to serious toxic effects to normal tissue and the development of treatment resistant disease. Gene therapy offers the potential for selective targeting of tumour cells with little or no effects on normal tissue. However, gene therapy clinical trials have failed to replicate preclinical success. This may be due to the use of models that do not accurately resemble the complexity of *in vivo* conditions or mimic the heterogeneity of tumour tissues. As a consequence, we hypothesise that the use of patient tumour tissue would provide a promising preclinical model for the study of gene delivery methods prior to clinical application.

Chapter 1 begins with an introduction to cancer and the current challenges facing its management. The review discusses various gene delivery vectors and looks at ways of enhancing gene delivery by incorporating a strategy of organ selective gene delivery based on vector and organ characteristics. The review aims to identify the most appropriate gene delivery method and route of administration, taking into consideration characteristic anatomical features and physiological barriers of the affected organ together with clinical hallmarks of the disease.

In Chapter 2 we discuss the development of an *ex vivo* patient tissue model for the study of various gene delivery methods. We found that patient tumour samples could be maintained viable in culture conditions for *ex vivo* cultivation and appropriately respond to gene delivery treatments. We developed a novel tumour slice model culture system that is universally applicable to gene delivery methods, using a real-time luminescence detection method to assess gene delivery. This study demonstrated that Ad-mediated delivery and gene expression

was generally superior to other methods examined while US proved the optimal non-biological gene delivery method in patient tumour slices. The nature of the *ex vivo* culture system permitted examination of specific physiological variables and the influence of intratumoural factors. Parameters shown to diminish Ad gene delivery included blood, regions of low viability and secondary disease. The *ex vivo* model was also suitable for examination of tissue specific effects on vector expression. Ad under the control of the human CXCR4 promoter demonstrated a 'tumour on' and 'normal off' expression profile when compared with the ubiquitously active CMV promoter when tested in patient breast tumour tissue. For the investigation of vector efficacy, toxicity and target cell specificity we optimised a dissociation technique, which provides representative cells from the entire slice for the accurate assessment of viability and identification of targeted cell types. Tumour population analysis demonstrated that Ad mediated by CXCR targeted a higher percentage of tumour cells when compared with CMV.

Chapter 3 looks at the potential for colorectal tumour targeting using transcriptional targeting strategies. An *ex vivo* cultured patient colorectal tumour model was employed to examine Ad transduction of colorectal tumours. Ad under the control of the human CXCR4 promoter demonstrated low reporter gene expression in normal colon and liver tissue while providing high expression in colorectal tumours when compared with the CMV promoter. In addition, we investigate the effects of hypoxia on adenoviral gene delivery. Hypoxia is an important feature of solid tumours as a consequence of a structurally and functionally disturbed microcirculation and directly facilitates the development of treatment resistance. We developed an *ex vivo* system of changing oxygenation using the hypoxia inducer, cobalt, to mimic the transient hypoxic conditions found in solid tumours. We found that Ad-related transgene expression varied depending on the level of hypoxia, with significantly reduced levels observed with prolonged hypoxia. However, transgene expression was robust in the

cycling hypoxic conditions relevant to solid tumours and re-oxygenation of chronically hypoxic tissue enhanced transgene expression.

In Chapter 4, we developed an AAV-based tumour targeting vector using the tumour-selective promoter CXCR4. The AAVCXCR4 vector demonstrated efficient tumour-selective expression when locally administered to subcutaneous tumours and normal tissue. Population analysis showed that AAVCXCR4 preferentially targets epithelial tumour and CXCR4-expressing cells while the CMV promoter demonstrated majority expression in non-tumour and non-CXCR4-positive cell types. Comparison of the expression kinetics of CMV with CXCR4 following systemic delivery in hepatic tumour murine models revealed that AAV mediated by the CXCR4 promoter retained high expression in tumours-bearing livers while maintaining low expression in tumour free livers. Furthermore, AAVCXCR4 retained the tumour-selective nature in *ex vivo* patient breast tumour tissues, confirming the translational potential of this vector.

This is the first study incorporating patient tissue models in comparing gene delivery from various vectors, providing knowledge on cell-type specificity and examining the crucial biological factors determining successful gene delivery. We also demonstrate the potential of tumour specific promoters using viral vectors to enhance tumour selectivity over normal tissue. The results highlight the importance of in-depth preclinical assessment of novel therapeutics and may serve as a platform for further testing of current, novel gene delivery approaches.

Publications

1. **Adenovirus-mediated transcriptional targeting of colorectal cancer and effects on treatment-resistant hypoxic cells.** Simon Rajendran, Gerald C O'Sullivan, Deirdre O' Hanlon, and Mark Tangney. Clinical Colorectal Cancer, 2013 Sep;12(3):152-162. PMID: 23313233.
2. **Targeting of breast metastases using viral gene vector with tumour selective transcription.** Simon Rajendran, Sara Collins, Jan P van Pijkeren, Deirdre O' Hanlon, Gerald C O'Sullivan and Mark Tangney. Anticancer Research 2011 May;31(5):1627-35. PMID: 21617219
3. **Preclinical evaluation of gene delivery methods for the treatment of loco-regional disease in breast cancer.** Simon Rajendran, Deirdre O'Hanlon, David Morrissey, Tracey O'Donovan, Gerald C. O'Sullivan, Mark Tangney. Experimental Biology and Medicine 2011 Apr 1;236(4):423-34. PMID 21444371
4. **Control and augmentation of long-term plasmid transgene expression *in vivo* in murine muscle tissue and *ex vivo* in patient mesenchymal tissue.** David Morrissey, Jan P van Pijkeren JP, Simon Rajendran, Sara A Collins, Gareth Casey, Gerald C O'Sullivan, Mark Tangney. Journal of Biomedicine and Biotechnology. 2012;2012:37984. PMID: 22811595
5. **A Listeria monocytogenes-based DNA delivery system for cancer gene therapy.** . Jan Peter Van Pijkeren, David Morrissey, Michelle Cronin, Simon Rajendran, Gerald C. O'Sullivan, Cormac G.M. Gahan, Mark Tangney. Human Gene Therapy. 2010 Apr;21(4):405-16. PMID: 20105075

6. **Anti-metastatic effects of viral and non-viral mediated Nk4 delivery to tumours.** Alexandra Buhles, Sara A Collins, Jan P van Pijkeren, Simon Rajendran, Michelle Miles, Gerald C O'Sullivan, Deirdre M O'Hanlon and Mark Tangney. Genetic Vaccines and Therapy, 2009 Mar 9;7:5. PMID: 19272140
7. **Orally administered bifidobacteria as vehicles for delivery of agents to systemic tumours.** Michelle Cronin, David Morrissey, Simon Rajendran, SM El Mashad, D van Sinderen, Gerald C O'Sullivan, Mark Tangney. Molecular Therapy**,** 2010 Jul;18(7):1397-407. PMID: 20389288

Video Articles

1. ***Ex vivo* culture of patient tissue and examination of gene delivery.** Simon Rajendran, Slawomir Salwa, Xuefeng Gao, Sabin Tabirca, Deirdre O'Hanlon, Gerald C. O'Sullivan and Mark Tangney. Journal of Visualized Experiments, 2010 Dec 20;(46). PMID: 21326169
2. **Murine bioluminescent hepatic tumour model.** Simon Rajendran, Slawomir Salwa, Xuefeng Gao, Sabin Tabirca, Deirdre O'Hanlon, Gerald C. O'Sullivan and Mark Tangney. Journal of Visualized Experiments. 2010 Jul 17;(41). PMID: 20689502

Book Chapters

1. Book title: **Gene Therapy - developments and future perspectives** (ISBN 978-953-307-617-1) Chapter title: **Comparison of DNA Delivery and Expression Using Frequently Used Delivery Methods**. Sara Collins, David Morrissey, Simon Rajendran, Garrett Casey, Martina Scallan, Patrick Harrison, Gerald O'Sullivan and Mark Tangney

Oral Presentations

1. **Adenovirus-mediated transcriptional targeting of colorectal cancer and effects on treatment resistant hypoxic cells.** Simon Rajendran, Gerald C O'Sullivan, Deirdre O' Hanlon, and Mark Tangney. Society of Academic and Research Surgery Meeting, Nottingham 2012

2. **Targeting of breast metastases using a viral gene vector with tumour-selective transcription.** Simon Rajendran, Sara Collins, Jan P van Pijkeren, Deirdre O' Hanlon, Gerald C O'Sullivan and Mark Tangney. Plenary session, Sir Peter Freyer Memorial Lecture, Galway 2011

3. **A novel *ex vivo* model for the study of gene delivery to patient tissue.** Simon Rajendran, Deirdre O'Hanlon, David Morrissey, Tracey O'Donovan, Gerald C. O'Sullivan, Mark Tangney. Irish Association for Cancer Research, Athlone 2009

Declaration

I hereby declare that I am the sole author of this thesis.

I authorise University College Cork to lend and photocopy this thesis to other institutions or individuals for the purpose of scholarly research.

Simon Rajendran

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Abbreviations

AAV	Adeno-associated virus
Ad	Adenovirus
ATCC	American Type Culture Collection
BBB	Blood Brain Barrier
BLI	Bioluminescent Imaging
CAR	Coxsackievirus -adenovirus receptor
CCRC	Cork Cancer Research Centre
CMV	Cytomegalovirus
cm	Centimetre
CNS	Central Nervous System
CRC	Colorectal Cancer
CSF	Cerebrospinal Fluid
CXCR4	CXC - Chemokine Receptor 4
Cy5	Cyanine 5
DMEM	Dulbecco Modified Eagle Medium
DNA	Deoxyribonucleic Acid
EGF	Epidermal Growth Factor
EP	Electroporation
FBS	Foetal Bovine Serum
FUS	Focussed Ultrasound
h	Hour
HCC	Hepatocellular Carcinoma
HIV	Human Immunodeficiency Virus
HSPG	Heparin Sulphate Proteoglycans
HSV	Herpes Simplex Virus
IV	Intravenous
IVIS	*In vivo* Imaging System
kb	kilo Bases
Lipo	Lipofection
LPS	Lipopolysaccharide
MEM	Minimum Essential Medium
min	Minutes
ml	Millilitres
mm	Millimetres

mRNA	Messenger RNA
msec	Millisecond
NaCl	Sodium Chloride
O_2	Oxygen
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PEI	Polyethyleneimine
PI	Propidium Iodide
RNA	Ribonucleic Acid
ROI	Region of Interest
RT	Room Temperature
X-SCID	X-linked Severe Combined Immunodeficiency Disease
SRE	Skeletal-Related Events
ssDNA	Single-Stranded DNA
ssRNA	Single-Stranded RNA
TLR9	Toll-like Receptor 9
TSP	Tumour Specific Promoter
U	Units
US	Ultrasound
VATS	Video-assisted Thoracoscopy
VEGFR	Vascular Endothelium Growth Factor
µg	micrograms
µl	microlitres
µsec	microseconds
° C	Degrees Celsius

Chapter 1

Literature Review

The Road Ahead - Breast Cancer Gene Therapy

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Abstract

The major challenge of anti-cancer therapy is lack of specificity leading to treatment resistant disease. The ideal therapeutic would selectively eradicate tumour cells with minimum effects on normal tissue. Gene therapies have emerged as realistic prospects for the treatment of cancer due to their potential for selective tumour cell targeting. The most significant hurdle for clinical application however, depends on the ability to efficiently deliver sufficient therapeutic genes to the target site. This chapter reviews the suitability of various gene delivery methods as potential vehicles of therapeutic genes detailing the mechanisms of action and major obstacles along with recent advances in vector development to overcome or circumvent these difficulties. The merits of different gene delivery methods are discussed to assess the current feasibility of each in specific stages of cancer progression. The review discusses ways of enhancing gene delivery by incorporating a strategy of organ selective gene delivery based on vector and organ characteristics. The review aims to identify the most appropriate gene delivery method and route of administration, taking into consideration characteristic anatomical features and physiological barriers of the affected organ together with clinical hallmarks of the disease. We end with a brief perspective of the ethical concerns and commercial viability of cancer gene therapy.

1.1 Introduction

Cancer is the uncontrolled growth of cells in the presence of environmental stimuli leading to tissue invasion which may be localised or distant. Cancer can present at any age and afflicts all tissue types, including epithelial, mesenchymal, haematopoietic or embryonic tissue, making it the leading cause of mortality in all ages. Malignant cancer cells have the ability to invade, migrate and survive in distant organs and therefore the initial diagnosis is made at various stages in their progression, from being localised to widespread disease. In the last decade significant strides have been made in improving cancer treatment due to the better understanding of cellular, genetic and molecular mechanisms, which provide targets for treatments to prevent, detect, eradicate or control disease (1). The identification of cancers at earlier clinical stages has been shown to improve treatment outcome and long term prognosis. Successful treatment outcomes are often achieved after complete surgical resection of disease that is localised within a single organ. Remarkably, a surgical cure was already hypothesised in the 18th century by the "Father of Scientific Surgery" Scottish surgeon John Hunter (2) (**Figure 1.1**). However the lack of knowledge in the area of anaesthesia at the time prevented implementation. The success achieved from treating early localised disease encouraged widespread cancer awareness initiatives and the development of universal screening programs targeting numerous cancer types, including breast, prostate, cervical and colorectal. Changes in cancer risk behaviour, new detection and screening strategies and the development and implementation of new and more effective treatments have decreased cancer related mortality in certain cancer types (1). Despite these efforts, patients continue to present with disease at late stages leading to poor treatment outcomes and cancer related mortality remain high for certain cancers. The main reasons hypothesised for this are the indolent nature of certain cancers, the unpredictable nature of cancer progression and despite

widespread cancer awareness initiatives, late presentation of patients in spite of symptom development.

Major advances made in the understanding of the genetic basis of cancer is driving the development of alternative treatment strategies. Gene therapy is a relatively new member of the anti-cancer armamentarium which currently include traditional strategies of surgery, chemotherapy and radiation and more recent additions involving hormonal manipulation and biological agents. Gene therapy is the transfer of exogenous genes, called transgenes into cells which can be accomplished by a number of methods (3). The product of the transgene serves to either cure or restrict advancement of disease. The concept of gene therapy initially arose in the 1960s, and began to gain prominence in the early 1970s; where strategies initially focused on the replacement of loss of function as a treatment for monogenetic disorders, including cystic fibrosis and muscle dystrophy (4). While the first successful gene therapy case was conducted in 1990 in a 4-year old child with adenosine deaminase severe combined immunodeficiency (ADA-SCID) (5, 6), the first complete gene therapy cure however, was only achieved ten years later in children with X-linked severe combined immunodeficiency disease (X-SCID). The optimism generated by the first true success using gene therapy was subsequently challenged by significant failures. Two of the ten children cured from X-SCID developed T-cell leukaemia, which was the direct result of the gene delivery vector, Retrovirus (RV); integrating near the LMO2 proto-oncogene promoter, a phenomenon known as insertional mutagenesis, leading to aberrant transcription and expression of LMO2 which plays a crucial in haematopoietic development (7, 8). Furthermore, the first gene therapy death, Jesse Gelsinger in September 1999, occurred as a result of a severe immune response to the gene delivery vector, Adenovirus (Ad) raising concerns regarding the safety of gene therapy treatments. Despite these setbacks, gene therapy offers enormous therapeutic

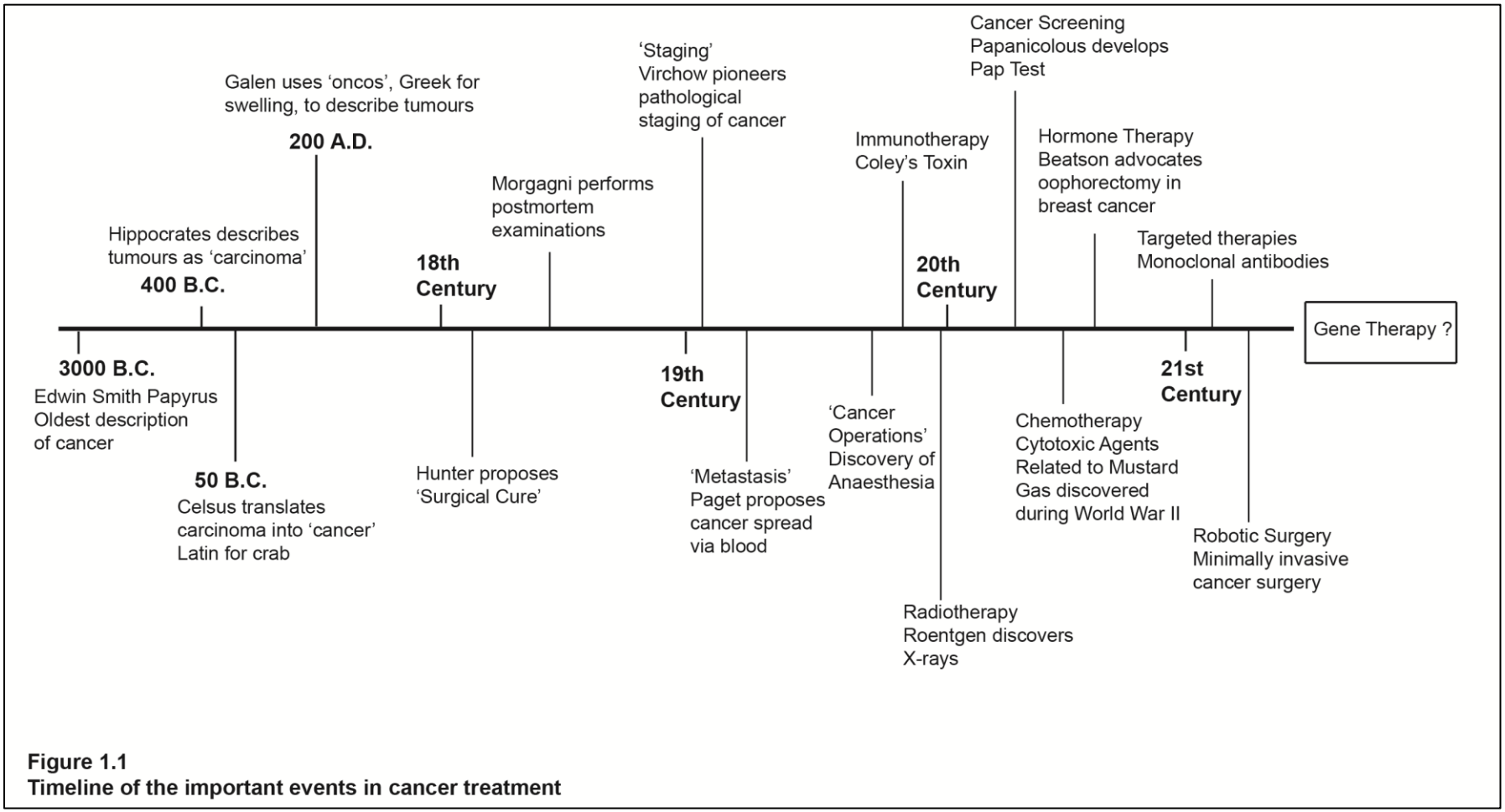


Figure 1.1
Timeline of the important events in cancer treatment

potential and as of June 2012 more than 1840 gene therapy clinical trials are being conducted in 31 countries worldwide using a number of gene therapy techniques (8). In addition to the treatment of genetic disease, gene therapy has been shown to have significant potential as a cancer therapeutic. Conventional non-invasive cancer treatment frequently causes serious side effects due to their non-specific effects, which eventually limits their potential therapeutic dose (9). Owing to this narrow therapeutic window, complete eradication of localised advanced or metastatic disease is rarely achieved, resulting in residual disease becoming refractory to treatment (9). Furthermore, approximately 30 % of all patients with early-stage breast cancer develop recurrent disease which is metastatic in most cases (10). In this respect, the application of gene therapy to cancer has attracted great attention because of its potential capability for selective targeting and killing of cancer cells with reduced normal tissue toxicities. Furthermore, gene therapy can theoretically be used in the adjuvant, neoadjuvant and metastatic settings and therefore may offer an alternative treatment option in patients with local and widespread metastatic disease. Currently, nearly 65 % of all gene therapy trials have been aimed at the treatment of cancer (8). Over the past decade, many gene therapy strategies have been devised. As has been the case with previous gene therapy trials, despite the effectiveness of any therapeutic strategy, the potential success of treatment is largely dependent on the application of a suitable gene delivery method. With this background in mind we have reviewed various gene delivery methods and their suitability for cancer gene therapy. Because of the huge amount of novel gene delivery methods we have primarily focused on important clinical aspects of gene delivery for breast cancer and the advantages it may offer as a new treatment option in this subset of patients.

1.2 Gene Delivery Methods

1.2.1 Identification of suitable strategy

The main objective in cancer gene therapy is efficient and effective delivery of therapeutic genes to cancer cells with little or no effects on normal cells. Therefore, a major hurdle for clinical application of gene therapy in cancer is the development of a gene delivery strategy that is both efficient and non-toxic and commercially viable. However, the specific requirements of the delivery system will also vary according to the type of cancer and clinical stage of disease. Various gene delivery methods have been developed and each has its own merits as a suitable vector in breast cancer gene therapy. The most commonly used gene delivery methods for cancer treatment and their physical attributes that make them the ideal cancer gene delivery agent in a particular setting are the focus of this review.

In order to achieve a therapeutic effect any gene delivery method should be able to deliver genes of interest to the designated target and to ensure their expression for an appropriate amount of time. Gene expression is regulated by a complex interplay of factors that function in a cell-type-specific manner to produce diverse effects. Numerous gene delivery methods have been developed during the past two decades and can be broadly divided into two distinct groups; non-biological and biological. **(Figure 1.2**). Biological methods involves the use of viruses which can be either RNA or DNA viruses and bacterial vectors. Non-biological methods involve the use of chemical or physical approaches to transfer genetic material carried on plasmid DNA. Although non-viral approaches are being used increasingly, viral vectors remain by far the most common approach, having been used in approximately two-thirds of clinical trials performed to date (8). This part of the review will include a brief discussion on commonly used gene delivery methods in gene therapy clinical trials (8), highlighting the properties that make them suitable for cancer gene therapy. We will then discuss the current challenges in the management of breast cancer and the potential role gene

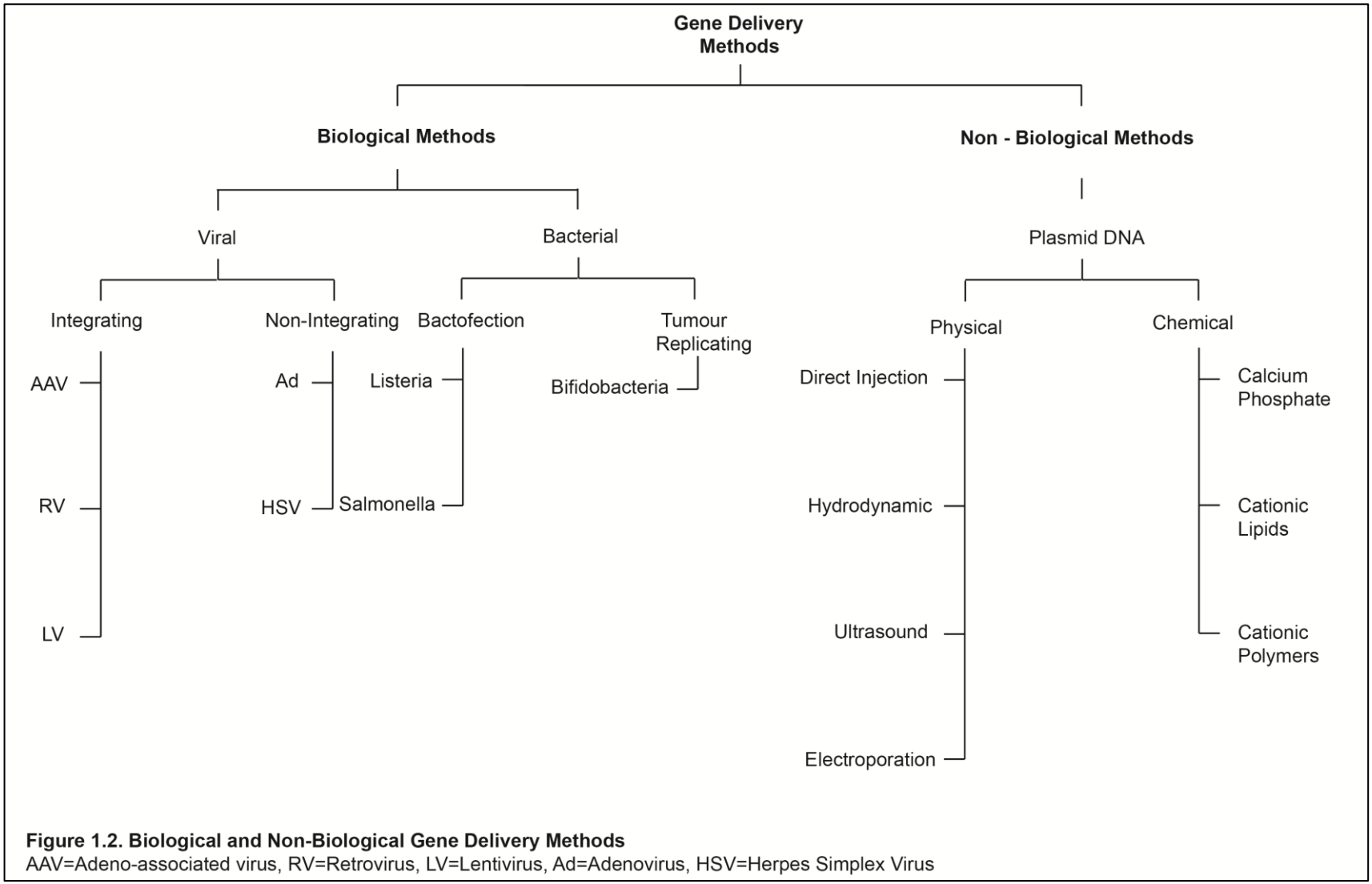


Figure 1.2. Biological and Non-Biological Gene Delivery Methods
AAV=Adeno-associated virus, RV=Retrovirus, LV=Lentivirus, Ad=Adenovirus, HSV=Herpes Simplex Virus

therapy may play in this setting. We focus on the suitability of various gene delivery methods, examining both potential advantages and limitations depending on the stage and site of disease

1.2.2 Biological methods

For more than two decades there was been widespread scientific interest in the utilisation of biological agents for the delivery of cytotoxic or therapeutic genes to cancer cells. This strategy aims to exploit the natural ability of disease causing microorganisms to evade the body's natural surveillance system and invade tumour or tumour associated cells. The first virus used for gene therapy was from the family of murine RV. Since then a wide variety of both DNA and RNA viruses have been studied for gene delivery. More recently bacterial vectors have shown promise as a tumour specific gene delivery vehicle.

1.2.2.1 Viral vectors

Viruses are natural DNA carriers that can very efficiently introduce foreign DNA into host cells. This innate ability was first harnessed in the 1970s and since then viruses have become the most widely used gene carrier in clinical trials. The most commonly used viral vectors in cancer gene therapy are derived from Ad, Adeno-associated virus (AAV), Lentivirus (LV), RV and Herpes Simplex Virus (HSV). The vast majority of cancer gene therapy has been carried out using replication-defective viral vectors with replication-defective Ad being the most commonly used vector (8). Viral vectors may be categorised into integrating and non-integrating, with AAV, RV and LV able to integrate their viral genome into the chromosomal DNA of the host, while both Ad and HSV deliver viral genomes to the nucleus of the host and remain episomal.

1.2.2.1.1 Retrovirus

RV belong to a family of enveloped RNA viruses, the *Retroviridae*, found in all vertebrates and can be classed into oncoretroviruses, LV and spumaviruses. RV consists of a double stranded RNA encapsulated by a protein core surrounded by a lipid bilayer (11). This lipid envelope contains polypeptide chains including receptor binding proteins. These receptor binding proteins link to membrane receptors of the host cell. Following attachment of the RV envelope to the target cell receptors, a fusion event between the viral lipid membrane and cellular membrane allows viral entry into target cells. Therefore, RV receptors and entry co-factors determine the tropism of this vector. RV also contain the enzymes reverse transcriptase (RTase) and integrase which mediate reverse transcription into viral DNA using the virus RNA as a template. During transduction, the virus injects viral RNA into the cytoplasm of the target cell along with the reverse transcriptase enzyme. The DNA produced from the RNA template contains the virally derived genetic instructions and allows transduction of the target cell to proceed. Viral DNA enters the nucleus and viral DNA is integrated into the target cell genome. The most important advantage that RV offer is high efficiency gene delivery to target cells that allow for long term and stable expression. This is due to their ability to transform their single stranded RNA into double stranded DNA that stably integrates into the target cell genome (12). A major limitation in their use is the inability to infect non-dividing cells. RV require nuclear envelope breakdown in mitosis to allow the viral genome to access the target cell genome and undergo integration (13). Furthermore, non-specific integration leading to deleterious effects such as insertional mutagenesis as was seen in the X-SCID clinical trial illustrated the potential dangers involved as a consequence of using RV. These factors have resulted in a decline in the use of RV vectors (8).

1.2.2.1.2 Lentivirus

LV, a subset of the *Retroviridae* are unique among RV because of their ability to infect target cells independently of their proliferation status (14). Much of the development of these vectors has focussed on human immunodeficiency virus (HIV). The advantage of using HIV based vectors is that these vectors can accommodate relatively large gene inserts and possess a pre-integration complex than allows viral genome to move through the nuclear membrane without requiring mitosis. Therefore, unlike retroviral vectors, this mechanistic feature allows LV to target both dividing and non-dividing cells whilst still demonstrating sustained stable gene expression through chromosomal integration. Commercial limitations include difficulty in producing high titre viral stocks. Regarding safety, questions still remain as to the potential risk of insertional mutagenesis and furthermore, scrutiny regarding the use of vectors derived from HIV remain due to the serious clinical consequences of the wild-type virus. This has resulted in the study of other LV, including HIV-2, primate LV such as Simian Immunodeficiency Virus and non-primate LV such as Feline Immunodeficiency Virus and Equine Infectious Anaemia Virus and these vectors are currently under preclinical development for gene therapy (15).

1.2.2.1.3 Herpes Simplex Virus

Herpesviridae encompass a large family of DNA viruses that include HSV-1 and 2, Varicella Zoster Virus, Epstein-Barr Virus and Cytomegalovirus (CMV). *Herpesviridae* are ubiquitous in the human population with more than 90 % of adults been infected with at least one of these viruses. Among the herpesviruses, neurotrophic herpesviruses such HSV-1 and HSV-2 are unique among viruses currently under development as gene delivery vectors in that they have the ability to infect axonal nerve terminals before retrograde transport to neuronal cell bodies. In the neuronal cell bodies, latency, a state where viral genomes persist for the entire

life of the host as intranuclear episomal elements is established (16, 17). During latency HSV is not naturally cleared by the immune system and host cells remain undamaged. HSV has many favourable properties for clinical application. The main advantages of using HSV vectors include easy production of non-pathogenic vector, efficient gene delivery to a broad range of tissues due to the wide expression of cellular receptors recognised by the virus, the ability to accommodate large or multiple gene inserts due to the large viral genome and finally it has the capability of persisting in a lifelong non-integrated latent state without causing disease in the immune-competent host (18, 19), therefore demonstrating the potential for a long-term therapeutic benefit. The limitations associated with HSV vectors include an inability to retain expression of inserted genes during latency, as these genes like the majority of the HSV genome during latency, become rapidly transcriptionally inactivated. Long-term expression can be achieved by using elements from the latently active region of the virus to confer a long-term activity onto a number of promoters which otherwise function only in the short term and also allows multiple inserted genes to be expressed from HSV vectors during latency (18-20).

1.2.2.1.4 Adeno-Associated virus

AAV is a small non-enveloped, non-pathogenic human virus commonly found in the human respiratory or gastrointestinal tract. AAV has rapidly gained popularity in the field of gene since the establishment of the first infectious clone of AAV serotype 2 in 1982 (21). AAV are members of the Parvovirus family, specifically the Dependovirus subfamily and require a helper virus, usually Ad or HSV in order to complete its life cycle (22). The AAV genome is a small (~4.7 kb), linear single-stranded DNA molecule. AAV are capable of both latent and lytic infections. They can remain dormant in an integrated state, or replicate in the cell, induce lysis with release of particles. Viral entry by AAV is mediated by binding to its

primary receptor heparin sulphate proteoglycans (HSPG) (23). Reported co-receptors of AAV include $\alpha_v\beta_5$ integrin, fibroblast growth factor receptor 1 (FGFR-1) and hepatocyte growth factor (c-Met), although this has been disputed by some groups (24, 25). Following entry into the cell and transfer into the nuclear compartment, the single stranded AAV genomes are slowly converted to double-stranded molecules. The second strand synthesis is dependent on cellular DNA polymerases, and appears to be the rate-limiting step in AAV expression (26). In the presence of helper functions such as those provided by Ad, transcription generated from three distinct viral promoters, p5, p19 and p40 is upregulated, producing rep and cap proteins which play a role in viral replication. While in the absence of a helper virus, AAV can either integrate into the host genome at a specific site on chromosome 19, known as AAVS1 or persist as an episomal form (27-29). There are currently twelve different human serotypes of AAV described in the literature (30, 31) and more than a hundred serotypes from non-human primates have been discovered to date (32). AAV2 is the most widely used human serotype for gene delivery studies (32). The variation in capsid proteins between serotypes impart different binding characteristics, altering the biodistribution and transduction efficiency. This provides the ability to cross-package or pseudotype AAV vectors into capsids from other serotypes. Cross-packaging has shown to combine characteristics from two serotypes generating a vector with the desired target cell profile whilst maintaining a serotypes characteristic safety profile. AAV are considered favourable gene delivery vectors as they offer stable and persistent gene expression (33) to both dividing and non-dividing target cells. The widespread distribution of its primary receptor HSPG on many cell types explains the broad tropism of AAV vectors. AAV in general has an excellent safety profile which when combined with its many serotypes and reduced potential for activation of inflammatory or cellular immune responses has made this vector an attractive option for cancer treatment and clinical application (32).

1.2.2.1.5 Adenovirus

Ad are non-enveloped icosahedral medium sized (90 - 100 nm) viruses with a 36 kb double stranded DNA genome. Since their isolation from human adenoid tissue by Rowe and colleagues in 1953 (34), Ad have become the most extensively studied gene delivery vectors (35, 36) being used in more than 24 % of gene therapy trials worldwide (8). Fifty-seven different serotypes of Ad have been identified so far, and these are divided into six subgroups A-F (37). The most commonly used serotypes are serotype 2 (Ad2) and 5 (Ad5) of subgroup C. Host cell entry of Ad requires two distinct, sequential steps, binding and internalisation. All subgroups excluding subgroup B, attaches to the cell by binding to the primary cellular receptor Coxsackie-Adenovirus Receptor (CAR) (38, 39). The globular knob domain of the Ad trimeric fibre capsid protein is responsible for binding to CAR and initiating internalisation. Besides CAR, Ad may use other molecules as receptors for binding, including the major histocompatibility complex I (MHCI) and heparin sulfate glycosaminoglycans. Internalisation is mediated via integrin ($\alpha_v\beta_{3/5}$) mediated endocytosis following the interaction of the integrins with the viral penton base protein. The virion then escapes from the endosome and localises to the nuclear pore, whereupon its genome is translocated to the nucleus where the primary transcription events are initiated. The genome remains extra-chromosomal, which minimises the risk of insertional mutagenesis. Expression of the adenoviral genes is temporally regulated. E1A is the first transcription unit to be expressed. The E1A proteins activate transcription from other adenoviral early regions. The expression of the early adenoviral genes sets the stage for replication of the viral DNA (37, 40, 41). The expression of the late Ad genes commences with the onset of DNA replication. A non-replicating Ad has complete deletion of the E1A region, preventing expression of the E2 genes and thus blocking viral DNA replication and synthesis of late structural proteins

except in the context of E1A-expressiong packaging cells. E1-deleted or the so-called "first-generation" replication-deficient Ad vectors are the most widely used in gene delivery.

The main advantages of Ad vectors are in their ability to deliver large therapeutic genes at high transduction efficiency in both dividing and non-dividing cells. The virus is relatively easy to produce in high titres for clinical use, possesses a broad tropism allowing targeting of a wide range of cells and comparable safety as the viral genome is not integrated into the host genome (41). Safety concerns about the use of Ad were raised following the death of Jesse Gelsinger from multi organ failure as a result of a severe immune response to the Ad vector used in the trial. The viral capsid proteins trigger an acute inflammatory response leading to the rapid release of inflammatory cytokines and recruitment of immune effector cells. The acute-phase toxicity depends on the dose of vector and does not require viral gene expression. Ad gene expression is only transient as the viral genome is not integrated into the host cell genome and furthermore, in many cases, the expressed transgene product has also been shown to be immunogenic. As a consequence of elimination of transduced cells by the acquired cellular immune response, transgene expression can be short lived. In addition, a humoural immune response is generated towards the Ad vector, leading to reduced effectiveness in repeat vector administration. Moreover, exposure to wild-type Ad is common in humans, therefore the presence of neutralising antibodies present in patients previously exposed to viruses will result in an immune response against the vector.

1.2.2.2 Bacterial vectors

The association of bacterial infections and tumour colonisation with regression is by no means a recent discovery. At the beginning of the 19th century, Vaultier observed that cancers regressed in patients with bacterial infections (42, 43). In 1867, the German physician, W Busch reported the regression of an inoperable sarcoma in a patient with

Erysipelas infection, which was later identified to be *Streptococcus pyogenes*. William Coley, American bone surgeon and pioneer of cancer immunotherapy also characterised concomitant bacterial infections of tumours and attributed the regression of tumours to the induction of an immune response to bacterial products. This concept led to the creation of Coley's Toxins, a mixture of killed bacterial infusions, that were administered by injection directly into the tumour mass (Figure 1.1) (44). Despite achieving considerable treatment success, the unpredictable nature and serious effects of Coley's Toxins, coupled with the development of radiation and chemotherapy led to the gradual disappearance of bacterial therapy in anti-cancer strategies until more recent times. The discovery of bacteria that specifically infiltrate and replicate preferentially within tumours and the advent of genomic sequencing and genetic engineering, began a renewed interest in the use of bacteria as gene delivery vectors.

Bacteria, like viruses are natural DNA carriers that permit the efficient delivery of genes to target cells. Bacterial gene delivery is achieved by two broad approaches. The first approach known as bactofection is achieved by the direct entry of the entire bacterium into the cell (45-47). Bactofection applies to both active invasion of non-phagocytic cells and to the passive uptake by phagocytic immune cells. For this review, we focus primarily on the use of active invasion of cells for gene delivery. The ability to actively invade a cell is typically found in pathogens. Different bacterial strains use different methods of cell invasion and following invasion they can be localised either primarily in the cytoplasm, vacuoles or in extracellular space. Once inside the cell, spontaneous or induced bacterial lysis leads to the release of plasmid DNA for subsequent gene expression. This trait was discovered less than 20 years ago and since then, various bacterial strains have demonstrated this ability. However, prior to clinical application, their pathological potential to invade normal cells must be eliminated (48-52) whilst retaining bactofection efficacy.

The second approach entails bacterial replication outside the target cells (47). Certain bacteria have shown the propensity to accumulate in tumours. Several theories have been postulated as to why bacteria replicate and survive, in some cases, specifically within tumours. Traditionally, the main mechanism believed to be responsible for tumour colonisation by bacteria was the hypoxic nature of solid tumours providing an ideal growth environment for anaerobic and facultative anaerobic bacteria. Recent theories propose that the leaky tumour vasculature allows bacteria to enter and lodge into tumours, necrotic areas within tumours serve as a source of nutrients in the form of purines for certain bacteria and that tumours provide immunological sanctuaries for bacteria where clearance mechanisms are inhibited allowing bacterial accumulation (53). Furthermore, the presence of chemoattrant compounds within necrotic zones may promote bacterial chemotaxis. This unique tumour homing characteristic allows the use of non-invasive strains to achieve tumour localised expression which occurs locally outside the tumour cells.

Both these gene delivery approaches have their advantages however, to achieve success using either strategy, bacterial strains must ideally be non-pathogenic, non-immunogenic, have the ability to preferentially accumulate or replicate around or within the tumour cells and be susceptible to antibiotic for plasmid release and bacterial clearance. In addition significant effort must be made to prevent lateral gene transfer to other bacteria and to limit environmental spread of the vector (54). A safety property unique to bacterial vectors is the potential sensitivity to antibiotics, enabling vector control post-administration. We review two of the most commonly investigated bactofection vectors, Salmonella and Listeria along with Bifidobacteria which has been shown to have a strong predisposition to tumour colonisation following intravenous and oral administration (55).

1.2.2.2.1 Salmonella

Salmonella are motile Gram-negative facultative anaerobes known to cause gastrointestinal infections in humans. Salmonella strains are known to colonise various types of cancers. Being a facultative anaerobe, they can colonise all areas of tumours independent of oxygen levels. Salmonella is currently the most widely used vector for bactofection. For bactofection, entry into the cytosol is the crucial step in plasmid transfer. Following cell entry, Salmonella remain trapped in the target cell phagosome. The main advantages of bactofection are the simplicity of application, economical, and ability to transfer large DNA constructs. The major drawbacks to using Salmonella in cancer gene therapy is that it is highly immunogenic which might result in rapid clearance of bacteria and /or induce tumour necrosis factor alpha (TNF-α) mediated septic shock.

1.2.2.2.2 Listeria monocytogenes

Listeria monocytogenes (*L. monocytogenes*) is a facultative, intracellular Gram-positive bacterium named after the imminent British surgeon Joseph Lister. *L. monocytogenes* is efficient in mediating internalization into host cells. Once inside the host cell, the bacterium produces specific virulence factors which lyse the vacuolar membrane and allow escape into the cytoplasm (56). Once in the cytosol, *L. monocytogenes* is capable of actin-based motility through the production of bacterial actin assembly-inducing protein which results in actin polymerisation. The continuous actin polymerization promotes motility in the cytoplasm and subsequent cell-to-cell spread (57). Cell-to-cell spread is achieved through the formation of protrusions into neighbouring cells, thus down-stream spread is achieved without an extracellular phase. When compared with other bactofection vectors, *L. monocytogenes* offers a number of advantages. *L. monocytogenes* is a gram positive organism, and therefore does not possess lipopolysaccharide (LPS) in the outer membrane like gram negative organisms

which act as endotoxins and elicit strong immune responses resulting in more rapid elimination of vector. The cytoplasmic location of *L. monocytogenes* unlike with other bacterial vectors such as *E.coli* and Salmonella which remain trapped in the host cell phagosome (58) is beneficial for the delivery of DNA. *L. monocytogenes* permits along with DNA the delivery of RNA or protein. It demonstrates amplification, local spread throughout the tumour and long-tem expression, which can be induced or silenced by antibiotic administration. Furthermore, unlike viral vectors, where vector synthesis is generally extremely cumbersome, time consuming and expensive, bacterial vectors are an economical and technically easier vector to manufacture (58). For successful bactofection, as with Salmonella, strains must have to be non-pathogenic, non-immunogenic, have the ability to preferentially accumulate and replicate within the tumours and be susceptible to antibiotic for plasmid release and bacterial clearance.

1.2.2.2.3 Bifidobacteria

Bifidobacteria is a non-motile, Gram positive, facultative anaerobic bacterium, first isolated in 1900 by Henry Tissier from a breast-fed infants. They are native, commensals of the human gastrointestinal system, and certain strains have been used as probiotics (53). Bifidobacteria have recently been shown to be effective delivery vehicles in the specific targeting of solid tumours when delivered by both intravenous and oral route. After application, its growth is limited to hypoxic areas such as the tissue of solid tumours. This feature potentially offers targeting of both primary and systemic disease (53, 55). The advantages of Bifidobacteria are its safety, easy to manufacture and its inert ability to home to and selectively replicate in tumours (55).

1.2.3 Non-biological methods

Non-biological gene delivery generally refers to the transfer of plasmid DNA into cells by using chemical or physical methods. Chemical methods promote gene delivery by means of nano-carriers while physical methods either depend on the local delivery of a large volume of plasmid DNA, high pressure or the utilisation of various energy forms to generate transient pores on the cell membrane to facilitate gene delivery.

1.2.3.1 Plasmid based gene delivery

The term "plasmid" was introduced by Joshua Lederberg in 1952 to describe any extrachromosomal genetic particle (59, 60). Since about 1970, plasmids have become important reagents in molecular genetic research and biotechnology. For successful plasmid based gene delivery and subsequent expression in mammalian cells, plasmid DNA needs to overcome three major hurdles. The plasmid needs to travel from the site of administration to the surface of target cells. DNA are sensitive to the nucleases in biological matrices. Once at the surface the DNA needs to cross the plasma membrane into the cytoplasm. Cell membranes are like sheets of amphipathic molecules that separate cells from their environment and form the boundaries of organelles within cells. DNA are large hydrophilic anionic polymers that are unable to cross the cell membrane without assistance. Once inside the cell, plasmids have to travel through the cytoplasm and across the nuclear membrane into the nucleus to initiate gene expression. Plasmids move through the cytoplasm utilising the microtubule network and its associated end-directed motor dynein which in general is an ineffective process (60). Plasmid based methods do not generally replicate in mammalian cells and have a better safety profile when compared with other vectors. However plasmids possess considerably less gene delivery capability and, thereby potentially limiting future

clinical application. In order to improve transfection efficiency using plasmid DNA, chemical or physical strategies can be used.

1.2.3.1.1 Chemical methods

To function as a synthetic vector, chemical methods must protect DNA against nuclease degradation, effectively shepherd DNA across the target cell plasma membrane, through the hostile intracellular environment, and into the nucleus. The major chemical methods used to improve plasmid based gene delivery are calcium phosphate, cationic lipids and polymers. The major advantage of chemical methods are their simplicity, ease of production and relatively low toxicity. We discuss the mechanisms underlying these three approaches and compare their relative advantages and potential for clinical application.

1.2.3.1.1.1 Calcium phosphate

The use of calcium phosphate for gene delivery was developed by Graham and van der Eb in 1973 and is currently one of the most commonly used *in vitro* gene delivery systems (61) because it is easy to use, cost-effective and a very safe technique. This technique utilises the formation of small, insoluble calcium-phosphate-DNA precipitates that can be adsorbed onto the cell surface and be taken up by cells through endocytosis. However, highly effective calcium-phosphate-DNA precipitates can only be generated under strict biochemical and physical conditions. Therefore, the transfection efficiency and expression levels are difficult to replicate in animal models. To counter these disadvantages, new methods based on the calcium-phosphate-DNA precipitation are being developed. Calcium phosphate nanocomposite particles encapsulating plasmid DNA are reported to demonstrate significantly higher transfection efficiency when compared to that of standard calcium phosphate transfection (62, 63).

1.2.3.1.1.2 Cationic lipids

The use of cationic lipids for DNA delivery was pioneered by Felgner and colleagues (64) and the first cationic lipid synthesised for this purpose was N-(2,3-dioleoxypropyl) N, N, N-trimethylamonium chloride (DOTMA). Many novel cationic lipids have been developed since then, including N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl-sulfate (DOTAP), 3β[N-(N′, N′-dimethylaminoethane)-carbamoyl] cholesterol (DC-Chol) and dioctadecylamidoglycylspermine (DOGS) (65). The principle aim when using cationic lipids is the formation of DNA / liposome complexes (lipoplexes) (64, 66, 67). When hydrated, cationic lipids form liposomes, which when mixed with DNA, the positive charges at the liposome surface electrostatically interact with the negative charges on the phosphate backbone of the DNA to form DNA / liposome complexes (lipoplexes). The lipoplexes bind to the cell surface and enters by endocytosis. It then escapes from the endosome and dissociates to release the plasmid. The plasmid enters the nucleus and initiates gene expression. These lipoplexes significantly increase plasmid DNA transfection efficiency from 5 % to more than 90 % in certain cell types. Numerous efforts have been made to optimise the transfection activity of cationic lipids. The most important physicochemical properties dictating transfection activity is the cationic lipid structure. All cationic lipids have three common structures, a positively charged head group, a hydrophobic anchor and a linker connecting the head group and the hydrophobic anchor. Many complex structures between DNA and cationic liposomes have been identified (68, 69), however the most important parameters affecting transfection activity of cationic polymers appear to be the particle size of the lipoplex and the charge ratio (amines to DNA phosphate ratio, + / -) (64, 67, 70). Transfection efficiency is attributed to efficient condensation of DNA by electrostatic interaction between the positively charged liposome and the negatively charged DNA, efficient interaction between a net positive charge of the cationic liposome / DNA complex

with the negatively charged cell membrane and effective intracellular DNA release owing to the fusogenic properties of cationic liposomes that form and destabilise the plasma membrane. As a gene delivery method, cationic liposomes have many advantageous features, including their ease of preparation and production, providing several routes of administration, and at therapeutic doses they show non-toxicity and non-immunogenicity. However, significant success has been limited to *in-vitro* studies. Cationic lipids face significant challenges *in-vivo.* Deterrents include inactivation of lipoplex after systemic delivery, cell barriers preventing translocation into the target cell and intracellular inactivation of the plasmid mediated by factors that include low pH exposure and deoxyribonuclease (DNAse) activity. To help improve transfection, the addition of a 'helper lipid' like dioleoylphosphatidyl ethanolamine (DOPE) or cholesterol may improve stability and transfection efficiency in certain cell types (71).

1.2.3.1.1.2 Cationic polymers

Cationic polymers are a group of hydrophilic molecules that readily self-assemble with DNA and generate small tortoidal or spherical structures called polyplexes. Polyplexes are approximately 40-100 nm in size, depending on the polymer size and structure, DNA to polymer ratio and the type and concentration of ions in the buffer. The general type of polymers used are linear (polylysine, spermine and histone), branched and spherical. The most extensively studied and used cationic polymers are polyethyleneimine (PEI) and dendrimers. PEI may be a linear or highly branched organic polymer produced by polymerising aziridine. They have very high cationic charge density enabling the binding of anionic DNA within the physiological pH range to generate small condensates called polyplexes. Polyplexes can be manipulated to interact with a negatively charged cell surface to maximize DNA uptake. PEI, with every third atom in the polymer being amino nitrogen,

allows effective buffering of the sudden decrease in pH from the extracellular environment to the endosomal compartment. This feature is important for the protection of DNA as it travels to the nucleus. Dendrimers are a class of branched, spherical and starburst molecules. They differ in their initiator structure and in the number of layers or generations of building blocks in each molecule. The common initiators include ammonia as trivalent initiator and ethylenediamine as a tetravalent initiator. Polymerization takes place in a geometrically outward fashion, resulting in a branched polymer with spherical geometry and containing interior tertiary and exterior primary amines. Their precisely controlled size and shape allows the formation of more homogeneous and reproducible DNA complexes. Cationic polymers offer the advantage of simplicity, ease of production and relatively low toxicity as they will not elicit major immune responses or integrate into the host genome.

1.2.3.1.2 Physical methods

Physical methods use some form of physical force to overcome the physical barriers presented by tissues. Physical techniques include the application of energy waves to cells to create transient pores in the cell membrane, thereby permitting entry of plasmid without killing the cell. The major physical methods developed for cancer therapy are direct needle injection, particle-bombardment or gene gun, hydrodynamics, electroporation and US.

1.2.3.1.2.1 Direct needle injection

Gene expression by the direct injection of plasmid DNA was first reported by Wolff et al in skeletal muscle 1990 (72). As previously discussed, the important variables dictating gene expression using this method are the factors affecting the transport of plasmid DNA from the outside of the cell, through the cytoplasm into the nuclear envelope. Gene expression using

this method is significantly lower than with other methods, however this method is likely to pose the lowest risk of toxicity or other unwanted effects. In addition to skeletal muscle, gene expression after intra-organ injections of various organs including liver, heart and brain and intra-tumour injection has also been demonstrated. The actual mechanism of plasmid DNA uptake by cells *in vivo* is unknown. To improve gene delivery efficacy, the pre-injection of various agents including bupivacaine and lignocaine prolong local concentration of plasmid DNA increasing the efficiency of direct injection. The main disadvantages of direct injection are the low transfection efficiency, lack of cellular specificity and the brief expression in most tissues.

1.2.3.1.2.2 Hydrodynamics

The systemic delivery of plasmid DNA by needle injection results in poor gene expression in major organs because of the rapid degradation of the DNA by nucleases and the clearance by the mononuclear phagocytic system. Hydrodynamic gene delivery was developed to enable efficient gene delivery to internal organs. It involves a rapid injection of a large volume of plasmid DNA systemically. This method has been shown to induce high levels of gene expression in internal organs, with the highest level of expression observed in the liver. One of the major concerns about hydrodynamics-based gene delivery is its safety and invasiveness since it involves a rapid intravenous injection of an extremely large volume of plasmid DNA. Miao et al reported transient focal acute liver damage in animal studies after hydrodynamic treatment involving less than 5 % of the hepatocytes, which rapidly recovered with complete recovery, while Lie et al correspondingly showed a transient rise in the liver enzyme, alanine aminotransferase (ALT) after delivery (73). This rise was shown to occur at the earlier phase of treatment, suggesting that the increased permeability may account for considerable enzyme leakage from the hepatocytes. Taking account of these findings and the known intrinsic

ability of the liver for regeneration, the minor toxic effects and invasiveness would be acceptable for clinical application. The advantages of hydrodynamic gene delivery are its efficiency, reproducibility and the potential to apply organ restricted gene delivery. Wolff et al (74) was the first to report on the concept of organ-restricted hydrodynamics-based gene delivery, where hypertonic solutions of DNA were injected intraportally in murine models with transiently occluded hepatic veins. Zhang et al (75) demonstrated delivery via the portal and hepatic vein and bile duct while Eastman et al (76) demonstrated a catheter mediated hydrodynamics based delivery to individual lobes in rabbit models.

1.2.3.1.2.3 Particle bombardment

Particle bombardment first developed by Stanford and colleagues for plant cells involves the use of high pressure to drive DNA mixtures into the target tissue (77). The DNA mixture may be composed of gold or tungsten particles pre-coated with plasmid DNA. When fired, the gene-carrying particles enter the cytoplasm of the cells and the DNA is gradually released and then expressed. Factors affecting the efficiency of this method include the coating of DNA onto particles, particle size and the timing of the delivery. The major disadvantage of this method is the accessibility of target tissue. Currently, this technique has largely been used for nucleic acid vaccination (78).

1.2.3.1.2.4 Electroporation

Electroporation (EP) is based on the principle that a short-pulsed electric field to a living cell causes a transient permeability in the outer membrane of the cell resulting in the cellular uptake of DNA (79). This permeability is manifested by the generation of pores across the membrane which closes in approximately 1 - 30 min after the field is discontinued, without

causing significant damage to the exposed cells. It has been shown to work in a wide variety of cell types and by varying the electric field strength and the length of time the cells are exposed to the electric field, it is possible to improve transfection to any cell type. The negatively charged DNA molecule can enter through a concentration gradient that is facilitated by electrophoretic and electro-osmotic transport. Electroporation has shown to increase gene expression in a range of tissues including liver, muscle, skin and solid tumours. In tumour delivery, plasmid DNA is injected into the interstitial spaces of the tissue and the required electric pulses are applied with needle or calliper-type electrodes. The advantages of electroporation include targeting expression as only the area of tissue exposed to the electric field will express the gene, the use of plasmid DNA is economical and simplifies gene preparation, the ability to apply large DNA constructs (80) and the relative safety to tissues. EP when compared to cationic polymers has been shown to result in reduced toll-like receptor 9 (TLR9) signalling and therefore a relatively low acute inflammatory response to plasmid DNA (81). An additional degree of specificity can also be achieved by using a tumour specific expression plasmid. EP has been tested extensively in preclinical melanoma models. Several of the preclinical therapies tested (Interleukin-12 and interleukin-2) have demonstrated therapeutic effect with minimal toxicity against melanomas and have now advanced to clinical trials.

1.2.3.1.2.5 Ultrasound

US mediated gene delivery was first reported by Fechheimer et al in 1987 (82). US (US) causes the transient formation of small pores of up to 100 nm in effective diameter with a half-life of a few seconds in cell membranes to enable the uptake of DNA. The biophysical effect of US most clearly implicated in the mechanism of reversible pore formation in the cytoplasm is acoustic cavitation. Applying US to liquid leads to the formation of vapour-

filled bubbles or cavities. The formation and collapse of the US-induced bubbles is called cavitation. The collapse of these bubbles can be violent enough to transiently form pores on the cell membrane allowing DNA entry (83, 84). US exposure in the presence of microbubble echo-contrast agents have been shown to enhance acoustic cavitation and result in higher levels of gene expression (85). The advantages of US are it is non-invasive, well tolerated, with an exceptional safety record over a wide range of frequency and intensity and has high levels of public acceptability and understanding. Furthermore, there are available highly sophisticated, flexible, cost-effective and readily available diagnostic and therapeutic systems that can achieve site-specific transfer of US almost anywhere in the body.

1.3 Clinical relevance

1.3.1 Which method when?

The suitability of various gene delivery methods for cancer gene therapy depends on a number of clinical variables, including the site of disease (breast, liver, bone, brain or lung), type of disease (primary, recurrence), extent of disease (clinical stage) and patient factors. In addition to clinical factors, the choice of method needs to be safe for general hospital use and commercially viable. In most cases, the site and type of disease will dictate the route of delivery (intra-tumoural, intravenous, intra-arterial, oral) while the duration and level of expression will depend on the type and extent of disease. In addition toxicity will affect patient compliance and immunogenicity will dictate the effectiveness of repeated dosing. This review looks at the potential application of gene therapy from a gene delivery perspective. It is based on our current knowledge of gene delivery and explores its relevance in the management of breast cancer.

1.3.2 Breast Cancer

1.3.2.1 Localised Disease

Breast cancer is the second most common cause of cancer related death in Irish women and the main cause of death in women ages 40 to 59. Despite advances in early detection and the understanding of the molecular bases of breast cancer biology, about 30 % of patients with early-stage breast cancer develop recurrent disease (10). Surgical excision followed by adjuvant therapies in the form of chest wall irradiation, chemotherapy and hormonal therapy remains the mainstay of breast cancer treatment. While current strategies have proven very effective in the treatment of localised disease they offer challenges of their own. The breast, while being a superficial structure, lies in close proximity to the heart, lungs and major arterial and lymphatic vessels. Surgical excision of localised disease is in general a straight forward procedure. Following surgery, radiation of the chest wall and regional lymph nodes is performed, while hormonal treatments and systemic adjuvant chemotherapy is indicated in selected cases based upon hormone receptor status and prognostic risk factors. Despite the use of adjuvant therapies a substantial number of patients develop local recurrence due to residual disease. In addition, radiation and chemotherapeutics pose serious complications. Complications from radiotherapy include overlying skin damage, lung injury leading to diffuse scarring and fibrosis, cardiac toxicity and lymphoedema in patients who have undergone lymph node dissection (86, 87). Chemotherapy can cause life threatening conditions including acute myeloid leukaemia and myelodysplastic syndrome while hormonal therapies are associated with cardiac toxicity, osteoporosis, joint disorders, kidney and liver dysfunction (88, 89). Furthermore, a subset of patients present with locally advanced disease require preoperative cytoreduction in order to obtain maximum local control of disease to facilitate excision of the primary tumour (90, 91). While significant

improvements in local disease control have been seen with the use of systemic chemotherapeutics, these treatments are associated with serious side-effects. They may also fail to control local disease in a large number of cases due to a phenomenon known as primary resistance to therapy. Furthermore, breast conservation surgery is only possible in a selected group of cases and these patients are at significantly higher risk of local recurrence due to residual disease (10). Therefore, despite advances in adjuvant and neoadjuvant therapeutics, patients with localised disease still pose a significant clinical problem.

The superficial location of breast tissue lends itself to the application of both biological and non-biological methods. Breast tumours may be approached by either using simple injection techniques under imaging guidance or via invasive surgery. Intratumoural delivery maximises initial delivery of vector into the tumour. Prolonged and sustained gene expression may not be an essential requirement for the eradication or cytoreduction of localised disease while long-term expression would most likely be necessary to prevent or contain disease recurrence in clinically indicated cases. RV are effective gene delivery vectors to a variety of cells and stable integration should provide long-term expression. However retroviral vectors integrate only into actively dividing cells. Breast tumours have been shown to have a heterogeneous population of both dividing and non-dividing cells. LV, a subset of RV, on the other hand are equally effective in both dividing and non-dividing cells (92). Breast tumours can range in size from a few mm to a few cm. For complete irradiation or effective cytoreduction large quantities of vector would likely be needed. Therefore, a major obstacle in using this vector is the cost of producing high viral titres for this purpose. Furthermore, the potential controversy and opposition to the use of vectors derived from HIV in the hospital setting cannot be overlooked. An alternative option is Ad, which demonstrates effective gene delivery in a large number of tumour types including breast cancer (92). While Ad does not offer prolonged transgene expression like RV, threshold expression is among the highest in

all gene delivery methods, and is reached rapidly in a variety of tumour cells including stromal and hypoxic tumour cells. Furthermore, Ad is relatively easy to produce in high titres and therefore economically viable, however, vectors must be administrated under level 2 biosafety containment measures. The foremost consideration prior to clinical use is toxicity as has been tragically demonstrated in the past. The development of acute-phase toxicity is dependent on the dose of vector used, which is expected to be high in order to achieve complete tumour eradication. In addition, humoural immune response generated towards the vector will preclude multiple vector administrations. Therefore, regarding clinical application, the expression and safety profile would make Ad suitable for cytoreduction of locally advanced tumours prior to surgical resection as its cytotoxic effect is likely to be rapid but short-lived allowing for early surgical intervention following Ad application. AAV has the ability to effectively penetrate the stroma of solid tumours due to its small size, and offers a safer delivery option. While threshold expression is lower than Ad, transgene expression is stable with long term gene expression capability in both dividing and non-dividing cells. The major disadvantage with using AAV in this setting is the slow onset of gene expression. While the kinetic profile of this vector would preclude managing tumours in the acute setting, long term transgene expression would be suitable for the prevention of local recurrence. One of the drawbacks of intraoperative adjuvant therapy in the form of radiation or chemotherapy is tissue damage and poor healing leading to reduced cosmesis and the application of suboptimal treatment doses. In this regard, AAV expression kinetics would be suitable for intraoperative administration as the slow progression to achieve threshold expression would allow for tissue healing following surgical resection. The other main drawback of using AAV is that the small size of its genome significantly limits the amount of genetic material that it can carry and therefore limits therapeutic options applicable using this vector.

Non-viral gene delivery methods offer a safer but less efficient alternative strategy. Current plasmid based methods, due to overall gene delivery inefficiency, are unlikely to achieve complete eradication or offer cytoreduction properties. For solid tumours, plasmid based methods would likely require large quantities of DNA and energy to achieve gene expression levels comparable to those of viral vectors. The ability of plasmids to accommodate large DNA inserts and the non-toxicity and non-immunogenicity properties of plasmid based delivery strategies may prove effective in patients with early breast cancer. While overall survival in these patients is high, a small subset develop disease recurrence, therefore, some form of local recurrence deterrent is warranted. Current deterrent strategies such as chest wall irradiation or chemotherapy have significant toxic side-effects which are very probable to negate their therapeutic benefits in this subset of patients. Plasmid based methods may be applied intraoperatively to sterilise the operative field following surgical resection. The devices used for electroporation and US can be easily modified for the hospital setting and the non-toxic and non-immunogenic nature of this method has little or no negative effect on wound healing, whilst potentially reducing risk of local recurrence from isolated tumour cells.

The lymphatic system is an important pathway for breast cancer spread. The breast has two major lymphatic basins, the axillary and internal mammary lymph nodes (93). Enlarged and/or suspicious lymph nodes are detected using regional ultrasonography, however, micro-metastatic disease in lymph nodes require histological evaluation (94, 95). Sentinel lymph node biopsy is routinely used as an assessment tool for guiding loco-regional management of micrometastases in axillary lymph basins (96-98). The current recommendation for sentinel lymph node biopsy containing metastatic foci is axillary node dissection which is associated with significant complications including seroma, shoulder dysfunction, axillary web syndrome and lymphoedema (93, 99), while radiation of internal mammary basin is difficult

and increases cardiovascular mortality (100). For the potential management of lymph node disease by gene therapy, a promising strategy would be the use of a lymphatic targeting approach. The lymphatic system, while serving its function in cancer spread, is also a critical route for bacterial drainage making bacteria an ideal vector for treating lymphatic disease (101). Bacterial vectors can target tumours either by bactofection or through tumour-specific replication. Bacterial chemotaxis occurs towards chemo-attractant compounds which are present in conditions such as necrotic tissue, aberrant neovasculature or in immune sanctuaries (47, 102). However, these conditions are not expected to be present in lymph node basins containing micrometastases. Therefore, it is possible to hypothesis that bacterial vectors demonstrating bactofection such as *Salmonella* or *L. monocytogenes* may potentially be more effective for targeting nodal micrometastases. However, recent reports have demonstrated tumour-specific bacterial growth in small prehypoxic tumours, suggesting that hypoxia in necrotic centres may not be a vital component for tumour-specific growth (55). Intra-tumoural administration of bacteria will locate to lymphatic basins however, the required bacteria loads through this route is likely to be high due to tumour and systemic absorption. An alternative approach would be to deliver bacteria directly into the lymph channels (103). Intra-lymphatic administration will allow bacteria to reach and target micrometastases within lymph nodes. Furthermore, intra-lymphatic administration will result in the need for lower doses of bacteria and should reduce the adverse effects of systemic absorption.

1.3.2.2 Metastatic Disease

Metastatic disease is diagnosed at presentation in 1 - 5 % of women with breast cancer, while nearly 50 % of breast cancer patients will develop distant metastases (104-107). Patients with metastatic disease are unlikely to be cured of their disease by presently available treatment

strategies. Complete remission in patients with metastatic disease following current systemic treatments is uncommon and only a small percentage of those remain disease free for a prolonged period (108, 109). Survivors tend to be young and otherwise healthy women with oligometastatic disease. The five year survival rate in patients with metastatic disease remains poor. The poor long term survival rates have made the development of new treatment options particularly important for this subset of patients. More aggressive approaches, such as high dose chemotherapy or autologous stem-cell transplantation do not appear to improve overall survival (91, 110). It is evident that prolonged chemotherapy is associated with significant therapy-related toxicity with little or no benefit to overall survival. Where curative treatment by complete eradication of tumour burden is not possible, the focus of treatment shifts to symptom control and the improvement of quality of life. Factors which influence the choice of gene delivery method used to treat metastatic disease include the site and extent of metastasis, the number of metastatic sites and impending catastrophe. The most common sites of metastatic breast disease are the liver, bone, lungs and brain. The review looks at identifying the most appropriate gene delivery method and route of administration, taking into consideration characteristic anatomical and physiological features of the affected organ together with clinical hallmarks of the disease.

1.3.2.2.1 Liver

Liver metastasis is by far the most common liver malignancy in the Western World. Nearly 15 % of newly diagnosed patients with metastatic breast cancer have liver metastasis which may be the only site of distant disease (91, 105). Therapies to treat patients with hepatic malignancies have been investigated for many decades yet effective options for treating metastatic liver tumours remain few (111, 112). Resection of liver metastases has become the mainstay of curative management but only a minority of patients are candidates for surgery.

Since fewer than 20 % of liver tumours can be surgically resected, metastatic liver cancer is often fatal, with up to 90% of patients dying from liver failure (113, 114). Systemic chemotherapy with 5-fluorouracil (5-FU) modulated by leucovorin is the treatment of choice in patients unsuitable for surgical resection. It is associated with a major response rate in only a quarter of patients and median survival of 9 – 12 months (105, 107). To improve therapeutic efficacy, intravascular regional therapies by portal venous and hepatic arterial delivery of chemotherapy have been extensively studied (115) but have had little or no success in improving survival outcomes, while radiation therapy for liver metastasis has proven especially challenging as doses delivered must be limited to minimise damage to healthy liver tissue (111, 112). In view of the low response rate to 5-FU based chemotherapy and limited options for a second-line treatment, alternatives to systemic chemotherapy have been evaluated in patients with liver metastases.

The liver is a complex organ in both anatomy and physiology. These characteristics present challenges, as well as offering opportunities when evaluating alternative treatments options. The liver is the largest organ in the body and regulates a wide variety of normal vital functions. Therefore, despite the ability to regenerate, extensive liver injury from non-specific therapeutics are poorly tolerated leading to multi-organ failure and eventual death. Gene therapy to isolated liver lesions can be delivered by injection under US guidance or by minimally invasive laparoscopic surgery. However, many patients with liver metastasis have multi-focal disease at time of presentation and consequently local treatment strategies are generally unsuitable. The most applicable treatment strategy in this case is systemic delivery. For systemic administration, methods that specifically target hepatocytes will avoid broad biodistribution and extrahepatic effects. Plasmid based methods have shown reduced efficiency following systemic delivery due to rapid degradation of plasmids by various nucleases present in blood extracellular matrix. While bacterial vectors have thus far not

demonstrated evidence of hepatic targeting, solid tumour targeting characteristics evident in several preclinical trials would suggest a similar response in metastatic liver disease (47, 53, 55). In contrast, several viral vectors have shown hepatic transduction following systemic delivery. It is well established that intravenous administration of Ad results mostly in hepatocyte transduction because hepatocytes express high levels of the primary Ad receptor CAR (116, 117). However, high immunogenicity associated with Ad triggers a complex immune response, that leads to hepatotoxicity (117). This high immunogenicity also induces a long lasting cellular and humoural immunity that impairs subsequent re-administration. LV illicit little or no immune or inflammatory responses and have demonstrated long term expression through integration in hepatocyte preclinical models (118, 119). AAV serotype 8 is highly liver tropic and AAV have shown preferential hepatic transduction in therapeutically relevant levels *in vivo* following systemic delivery (120).

The precision of gene delivery to the liver can be further regulated by the route of vector delivery. The liver has a dual blood supply, receiving blood from the hepatic portal vein and hepatic artery. This unique anatomic feature provides systemic ports of entry either by the portal vein or hepatic artery for localised delivery of therapeutics. Intra-arterial administration demonstrates higher gene delivery efficiency as compared with the intraportal route, reflecting the fact that tumours within the liver are found to be supplied by the hepatic artery rather than the portal vein. Hydrodynamic gene delivery involves the rapid administration of a high volume of plasmid DNA through systemic ports and has shown high levels of gene expression in the liver (121). Under image guidance, sequential lobe-specific hydrodynamic gene delivery can be performed, whereby individual or multiple hepatic segments can be sequentially targeted with no little or no evidence of gene delivery in other organs (121). The advantages of using a plasmid based method is that plasmid is easy to make, is less immunogenic and can safely be modified for hospital use. One of the major concerns about

hydrodynamic-based gene delivery is hepatic toxicity as it involves a rapid portal delivery of an extremely large volume of plasmid DNA over a short period of time. However, liver injury using this technique is usually temporary and completely reversible. Other vectors including viral and bacterial can also be administered using systemic ports avoiding unnecessary systemic biodistribution. However, large liver lesions have demonstrated increasing resistance to viral transduction following intravascular delivery in preclinical models. The opposition to intravascular transduction has been attributed to the maturation of the blood-tumour barrier. The blood-tumour barrier is a physical barrier between blood and tumour cells. The thickness and impermeability of the vascular barrier increases as tumours increase in size and may serve to isolate tumour cells from systemic homeostatic mechanisms including the host immune system. Vasoactive compounds such as histamine and nitric oxide can augment the permeability of vascular endothelium. Histamine achieves this by opening intercellular junctions and creating endothelial pores, while nitric oxide, which normally regulates vascular integrity at physiological levels, induces vasodilatation and microvascular leakage at therapeutic levels (122-124). Thus, the intra-arterial infusion of vasoactive compounds such as histamine or nitroglycerin, a nitric oxide donor, before vector administration has demonstrated the capacity to enhance transgene expression.

1.3.2.2.2 Brain

Despite surgical and medical advances, the prognosis for patients with brain metastasis remain grim (106, 125). In spite of major technical advances in neurosurgery such as microsurgery and stereotaxy, brain metastasis remain very difficult to resect due to their infiltrative nature and multifocal or multilobular presentation. (126). Radiation has shown to have little or no effect on long term survival while the major reason for chemotherapy failure is the difficulty of delivering therapeutic to the brain (106). Several effective therapeutics

cannot be used in brain metastasis, because the blood-brain barrier (BBB) prevents the transport of many systemically-delivered molecules into the brain. Gene delivery may be particularly useful in this situation for the delivery of therapeutics, which are otherwise difficult to deliver into the brain. While current therapeutics are a long way from achieving a cure, strategies providing symptom relief should be considered such as cytoreduction which would result in relieving raised intracranial pressure and reversing progressive neurological deficits. Using our knowledge of gene delivery methods and by exploiting specific central nervous system (CNS) delivery strategies we examine approaches for gene therapy to the brain.

The intravenous administration of gene delivery vectors appears to be ineffective for CNS delivery due to the BBB blockage (127). The BBB is formed by tight junctions between the endothelial cells of the cerebral capillaries and blocks diffusion and entrance from the blood stream of even small vectors such as AAV to the brain parenchyma. Any systemically administered vector must penetrate the BBB to enter the brain. Lipid-soluble agents that can freely diffuse through the capillary endothelial membrane may passively cross the BBB. While plasmid DNA are not transported through this barrier, cationic lipids and polymers bind to plasmid DNA due to electrostatic interactions leading to hydrophobic collapse with compaction in the nanometer range (typically between 100-200 nm in diameter) so that complexes are small enough to be used to deliver plasmid DNA to the brain (128-131). While compaction protects plasmid DNA from nuclease degradation in systemic circulation, intravenous delivery can result in large molecular weight aggregates depositing in the pulmonary vascular bed, decreasing bioavailability to the brain. To reduce systemic loss, delivery can be administered intra-arterially into the internal carotid artery (127, 132). The main disadvantage to using cationic lipids and polymers in the brain is that highly charged complexes can inhibit important cellular processes such as cell survival signalling, leading to

substantial CNS toxicity (133). Current viral delivery strategies rely on direct invasive stereotactic injection that can circumvent the BBB; however, this technique is invasive and delivery is only limited to the CNS tissues surrounding the delivery tract and cannot be widely spread due to the potential risk of serious neurological damage. Convection-enhanced delivery administers viral particles actively though an infusion tube and has been shown to increase the distance of penetration after viral vector direct injection (134). However the insertion of the infusion tube is an invasive procedure and risk of infection is high, while gene delivery distribution remains relatively restricted (134). Certain agents including mannitol and microbubble-enhanced focused US (FUS) have shown to locally and temporally disrupt the BBB allowing increased penetration by viral vectors (135). FUS is capable of transcranial delivery and at present is the only known non-invasive method for localised and transient disruption of the BBB (135-137). An alternative route bypassing the BBB to the CNS that has been studied extensively is intrathecal or intraventricular, where therapeutics are administered directly into the cerebrospinal fluid (CSF) (138, 139). When compared to intravascular delivery using AAV in preclinical trials, intra-CSF administration results in an immediate high global CNS gene delivery with reduced peripheral organ distribution. The difficulty associated with intra-CSF administration is that only a small volume can be infused due to the resulting increase in intracranial pressure and the associated risk of intracerebral haemorrhage, CSF leak, CNS infection and neurotoxicity (139). Intranasal delivery to the CNS is another novel technique under investigation. Intranasal delivery of HSV in preclinical models demonstrated widespread CNS distribution with less cytotoxicity when compared with intravenous and stereotactic delivery methods (140).

1.3.2.2.3 Bone

Bone is the most frequent site for distant spread of breast cancer and the axial skeleton is predominantly affected (104). Patients with bone metastasis suffer from significant morbidity because of pain and skeletal related events (SRE) that occur due to tumour-induced osteolysis resulting in loss of the structural integrity of bone (104, 141). Radiation therapy is the most common treatment for bone metastases. However, if fractures are already present, radiation will not relieve pain and the high doses needed for treating disease will further impair the healing process. Bisphosphonates are potent inhibitors of osteoclast-mediated bone resorption and have been shown to prevent and delay SRE and to reduce bone pain (104). Nevertheless, in spite of aggressive treatment with bisphosphonates more than one third of these patients will develop further SRE within 2 years of initiating therapy (104). Clinical application of gene therapy to metastatic bone disease is not yet on the near horizon, although certain strategies have shown potential. There is substantial preclinical literature documenting successful delivery and intraosseous expression of genes using Ad, retroviral or plasmid based vectors (142, 143). These delivery methods can be used for both systemic and local transgene delivery. Systemic delivery aims to disseminate and express the transgene widely in the skeleton and is particularly useful in widespread bony disease. Bacterial vectors have the potential to home to bony metastasis after systemic administration (47, 55). Local delivery on the other hand introduces and expresses the transgene in a limited and defined area such as an isolated osseous lesion or pathological fracture (144). Local gene delivery has the additional advantage of minimizing side-effects in non-target organs, with little or no transgene expression outside the point of delivery unless initial transgene expression is exceptionally high (145). In addition to the delivery of cytotoxic genes, gene therapy may also be used to readdress the imbalance that occurs in metastatic bone disease between the action of osteoblasts and that of osteoclasts, resulting in net bone loss (141). Local delivery of

osteogenic transgenes, has shown great promise in a number of applications where it is necessary to regenerate bone (144-146). The stable and controlled delivery of growth factors and stimulation of osteoblastic new bone formation can potentially enhance and accelerate functional bone formation in diseased bone allowing application of higher doses of conventional therapeutics.

1.3.2.2.4 Lung

Breast cancer metastasis frequently occurs in the lungs, and is associated with a poor prognosis (91, 147). Isolated lung metastases have been reported to occur in 10 – 20 % of all women with breast carcinoma. Pulmonary metastasectomy can be carried out for multiple and bilateral disease (148). Regardless of complete resection, 5-year survival rate remains low (≤ 45 %) (147-149). In addition, conventional therapies such as chemo- and radiotherapy are ineffective, with nearly three quarters of patients who die of breast cancer having pulmonary metastases (147, 150).

The lung is a complex anatomical and physiological organ. For discussion, the lung can be anatomically divided into the conducting airways (trachea, bronchi and bronchioles) and the parenchyma (gas-exchanging alveolar cells). The conducting airways branch from the hilum of the trachea towards the terminal airways and are responsible for the delivery of oxygenated air to the parenchyma. The parenchyma provides a very large surface area for blood-air interface and is highly vascularised from blood supplied by the pulmonary arteries (151). In addition the lung is also supplied with systemic blood for nutrition via the bronchial arteries which arise from the descending aorta.

The lung is a particularly attractive organ for gene therapy due to potential accessibility through the airways and vasculature. Successful gene delivery to the lungs has been achieved in preclinical trials using direct injection, intranasal and systemic delivery. In the clinical

setting, gene therapy by direct injection can in theory be achieved by invasive surgery or by minimal access techniques such as video-assisted thoracoscopy (VATS) or transthoracic injection under imaging guidance. Bronchoscopy may be used for delivery to large conducting airways. Viral, bacterial and in particular plasmid based methods such as electroporation and US can be administered using these techniques. Electroporation has shown enhanced gene delivery, with reduced toxicity and inflammation when compared with cationic lipids in lung tissue (81, 152). The intranasal route by droplet or aerosol delivery provides a comparatively less invasive option. Viral and cationic polymers delivered by aerosol have demonstrated effective and uniform distribution in preclinical trials (153). LV demonstrated long-term gene expression via aerosol delivery while the intranasal route was found to be superior to intravenous administration in Ad lung transduction (150, 154). While aerosol techniques have their advantages, certain clinical situations may preclude effective aerosol delivery such as bronchial inflammation due to pneumonia, lung collapse from tumour mass effect on main bronchus or large pleural effusions. In such situations, intravenous delivery may prove superior. Following intravenous administration, cationic lipids and polymers accumulate in the pulmonary vasculature because of the first passage effect. Successful gene expression was found in lung parenchyma after intravenous administration of cationic polymer and lipid based delivery methods (153). The duration of gene expression was biphasic in nature, with a initial dramatic but transient expression followed by a prolonged gene expression at a lower level. AAV vectors have shown potential for a delayed but persistent pulmonary expression while the systemic administration of Bifidobacteria have demonstrated gene expression in pulmonary metastatic nodules in preclinical models (55).

1.4 Discussion

The leading cause of conventional treatment failure in clinically advanced disease is dose-limiting toxicities leading to the development of treatment resistant tumour cells. For patients with disease recurrence following treatment, further management options are limited. The foremost challenge with current anti-cancer therapies is the lack of tumour cell specificity. The lack of specificity results in undesirable systemic side effects with little or no improvement in long term survival. Gene therapy has the potential to selectively target tumour cells and can be used either to deliver a therapeutic or to stimulate an immune response against tumour cells (155). Additional advantages of gene therapy include the ability to express therapeutics for extended periods of time and to regulate therapeutic level both quantitatively and temporally. The primary goal of any gene delivery system is to achieve adequate gene delivery to the target cells with minimal damage to the host. Therapeutic efficacy is thought to be related to the tumour-associated vector load. The tumour associated vector load is dependent on the route of delivery. The review outlines gene delivery methods and routes of delivery available for the treatment of primary and metastatic breast cancer. We discuss gene delivery strategies for localised disease, cytoreduction techniques and micro or macro-metastatic disease in various organs. In this review we propose ways to enhance gene delivery by incorporating a strategy of selective disease based gene delivery. This approach aims to offer more effective and less toxic treatment by selecting gene delivery strategies based on clinical and disease characteristics.

To achieve eradication of all tumour cells using a therapeutic approach, a very high level of transgene expression is likely be required. The duration of expression may not be clinically significant in this case. If complete eradication is unattainable, a containment strategy is the next best treatment option. For containment long-term expression of therapeutic is likely to be required to reduce the incidence of relapse and thus improving long-term survival.

However, a potential downside to using containment strategies is that treatment resistant cells will ultimately develop and overcome treatment. The best candidates for achieving long term transgene expression are integrating viruses such as RV and LV or AAV which offer persistent expression by remaining episomal. Certain non-viral constructs based upon transposons are also known to integrate and offer long term gene expression (117, 156). For life-threatening disease or when cytoreduction of locally advanced disease is required, short-lived high bursts of transgene expression are best applied and can be achieved using vectors such as Ad. In patients with low risk of recurrence less toxic alternatives, such as plasmid based methods should be considered. For the treatment of metastatic disease, an organ specific approach needs to be taken, where the ideal delivery method is identified based on tissue tropism, route of administration and potential toxicity. Experimental evidence indicates that in addition to the vector used, the route of administration has a major influence on the transduction / transfection efficiency. By localising vectors at their desired site of action toxicity can also be reduced. The various factors that determine which route of administration favours a high transduction rate in a particular setting must be carefully considered. Optimizing the administration techniques with the vector used to maximise gene distribution and gene expression is an important step in selective disease based gene delivery. For targeting multiple metastatic sites, vectors that are suitable for systemic delivery, with minimal side effects on healthy tissue are needed. While viral vectors have demonstrated tissue-selective expression, transduction with these vectors following systemic delivery are generally low and toxic effects remain. An attractive alternative may be the use of bacterial vectors demonstrating tumour specific replication. These vectors are generally safe and can potentially be given via the oral route. An additional option is to use multiple vectors in an organ specific manner, where different gene delivery strategies are used for different organs.

When clinical translation is intended, additional factors, such as cost effectiveness, safety, intellectual property and public perception become very important. In addition to production cost, other factors need to be considered, such as cost development for hospital setting, safety precautions, ethical and public health approval for hospital use and general public acceptance. Depending on the vector and clinical features, there may also be advantages in terms of overall cost. Clinical grade viral vectors are demanding to manufacture and need to be used at high multiplicities of infection; both of these factors increase costs. Bacterial vectors, while considerably easier to manufacture and regarded as safer, still require biosafety measures for clinical use. Clinical application of gene therapy, especially for non-lethal diseases, such as in early breast cancer is hindered by its pervasive reputation for being unsafe and the belief that gene therapy is inherently more risky than conventional therapy. The application of vectors such as LV derived from HIV is unlikely to see clinical application in any but life-threatening diseases. Perceptions concerning the safety of adenoviral vectors have had to recover from the death of Jesse Gelsinger. Although gene therapy clinical successes so far have been limited to small numbers of patients it is hoped that the improved perception of gene therapy as a whole will enable greater and more sustained investment to supply the needed momentum for further development. Research into vector design needs to proceed in parallel with the clinical arena, where identification of obstacles to clinical gene delivery can inform the next phase of basic research. Symbiosis between the scientific and medical establishments will encourage rapid and significant improvements and innovations in gene / cell therapy technologies for anticancer treatments. In addition, a tailored personalised gene therapy protocol based on predictive factors and clinical features may become a reality.

1.4.1 Limitations

This review is based on a combination of preclinical and clinical trials and the authors recognise its inherent limitations due to this. In addition, transductional and transcriptional

viral targeting strategies, replicating viral vectors and the use of mesenchymal cells as delivery platforms were not discussed.

1.5 Conclusions

Gene therapy is an important advancement with the potential to revolutionise clinical care of cancer patients. However, current technologies to deliver genes are not sufficient to make gene therapy a one stop treatment strategy even under optimum conditions, let alone under stresses associated with malignancy. However, combining our current knowledge on vector characteristics with existing understanding of the clinical features of cancer may offer the best hope for moving forwards with gene therapy.

Chapter 2

Preclinical Evaluation Of Gene Delivery Methods For The Treatment Of Loco-Regional Disease In Breast Cancer

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1. **Preclinical evaluation of gene delivery methods for the treatment of loco-regional disease in breast cancer.** Simon Rajendran, Deirdre O'Hanlon, David Morrissey, Tracey O'Donovan, Gerald C. O'Sullivan, Mark Tangney. Experimental Biology and Medicine 2011 Apr 1;236(4):423-34. PMID 21444371
2. ***Ex vivo* culture of patient tissue and examination of gene delivery.** Simon Rajendran, Slawomir Salwa, Xuefeng Gao, Sabin Tabirca, Deirdre O'Hanlon, Gerald C. O'Sullivan, Mark Tangney Journal of Visualized Experiments, 2010 Dec 20;(46). PMID: 2132616
3. **Control and augmentation of long-term plasmid transgene expression *in vivo* in murine muscle tissue and *ex vivo* in patient mesenchymal tissue.** David Morrissey, Jan P van Pijkeren JP, Simon Rajendran, Sara A Collins, Gareth Casey, Gerald C O'Sullivan, Mark Tangney. Journal of Biomedicine and Biotechnology. 2012;2012:37984. PMID: 22811595

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Abstract

Preclinical results with various gene therapy strategies indicate significant potential for new cancer treatments. However, many therapeutics fail at clinical trial, often due to differences in tissue physiology between animal models and man, and tumour phenotype variation. Clinical data relevant to treatment strategies may be generated prior to clinical trial through experimentation using intact patient tissue *ex vivo*.

We developed a novel tumour slice model culture system that is universally applicable to gene delivery methods, using a real-time luminescence detection method to assess gene delivery. Methods investigated include viruses (Ad and AAV), lipofection, US, electroporation and plasmid DNA. Viability and tumour populations within the slices were well maintained for 7 days, and gene delivery was qualitatively and quantitatively examinable for all vectors. Ad was the most efficient gene delivery vector with transduction efficiency > 50%. US proved the optimal non-viral gene delivery method in human tumour slices. The nature of the *ex vivo* culture system permitted examination of specific elements. Parameters shown to diminish Ad gene delivery included blood, regions of low viability and secondary disease. US gene delivery was significantly reduced by blood and skin, while tissue hyperthermia improved gene delivery. US achieved improved efficacy for secondary disease. The *ex vivo* model was also suitable for examination of tissue specific effects on vector expression, with Ad expression mediated by the CXCR4 promoter shown to provide a tumour selective advantage over the ubiquitously active CMV promoter.

This is the first study incorporating patient tissue models in comparing gene delivery from various vectors, providing knowledge on cell-type specificity and examining the crucial biological factors determining successful gene delivery. The results highlight the importance of in-depth preclinical assessment of novel therapeutics and may serve as a platform for further testing of current, novel gene delivery approaches.

2.1 Introduction

Breast cancer is estimated to account for nearly 27% of predicted new cancer cases in the United States and Europe this year (157, 158). Despite early detection methods and advanced conventional treatments, loco-regional recurrence rates can be as high as 13% (159). Nearly half of these patients will develop treatment resistant loco-regional disease, which is an independent prognostic factor associated with poor outcome (160). These figures clearly indicate the urgent need for novel therapies in the treatment of this disease and gene therapy is believed to offer exciting therapeutic approaches (161) and thus may prove suitable in altering the course of recurrent loco-regional disease.

Various gene therapy strategies may be applicable for the targeted treatment of loco-regional disease. Clinical application however, depends on the ability to delivery sufficient genes to the target site. Six methods of gene delivery were examined in this study. While the use of other potentially more efficient and less immunogenic vectors, such as nanoparticles (162), is increasing, there are currently numerous ongoing human clinical trials utilising the methods described in this paper. The Ad vector, due to its exceptional gene delivery capability, has been the most widely used in preclinical and clinical trials (163). The concurrent success of non-viral gene delivery systems, including electroporation (EP) and Ultrasound (US) has also encouraged significant work in this field (164-166). Recent failure of promising gene therapy strategies in the clinical setting has highlighted the importance of testing strategies on the most stringent model available prior to entering clinical trials (167-169). The factors that are thought to play a major role in treatment failure are the unpredictable variation in tissue physiology between rodent and man, tumour heterogeneity and complex tumour phenotypes. This research quagmire has led to the demand for alternative models and has prompted the emergence of *ex vivo* model systems utilising intact human tissue. The tissue slice model system was first introduced by Krumdieck (170) several decades ago, however, its relevance

and potential in cancer research has only been realised of late. It involves the use of fresh human tumour tissue, cut into thin slices and maintained *ex vivo*, and represents a more clinically relevant primary model as it allows strategies to be tested on intact tissue. Several reports have utilised the tissue slice model of human tumours to study Ad mediated gene delivery (171-174); however, little is known about the effects of other gene delivery systems on human tumours and none of the current models offer the opportunity to investigate other gene delivery methods. Furthermore, previous reports on *ex vivo* models used analysis techniques requiring sacrifice of the tissue for quantification of gene expression. We introduce a novel *ex vivo* system with the capability of examining all delivery methods and offering real-time analysis of gene delivery. The universal application is achieved by utilising a slice thickness that does not limit nutrient and oxygen diffusion throughout the slice and allows for the physical delivery of genes by direct injection or the use of devices for electroporation and sonoporation. Furthermore, a thicker slice, which maintains all cellular components and diffusion gradients, would more closely represent the true intra-tumoural conditions of solid tumours.

For measurements of *ex vivo* gene expression, we introduce a novel bioluminescence imaging technique based on the expression of luciferase, the light emitting enzyme of the firefly *Photinus pyralis* (175). This non-toxic detection system enables the sequential quantification of gene expression without sacrifice of tissue, allowing for measurement of duration of expression and thereby enabling assessment of vectors requiring longer incubation periods for maximum expression such as Adeno-associated virus (AAV) (176). For the investigation of vector efficacy, toxicity and target cell specificity we have optimised a dissociation technique, which provides representative cells from the entire slice for the accurate assessment of viability using automated cell counting and for identification of cell types by flow cytometric analysis. Using this model system we evaluate the efficacy of various gene

delivery methods and the factors that limit or enhance gene expression in patient tumour samples. We also investigate the potential of tumour specific promoters to enhance tumour selectivity over normal tissue. This model would broaden our understanding of the biological factors that promote and hinder successful gene delivery to tumours and provide valuable insight for identifying novel gene delivery systems for the treatment of locoregional breast cancer. This would permit further assessment of the clinical relevance of new treatment strategies prior to entering clinical trial.

2.2 Materials and Methods

2.2.1 Human Tissue Samples

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals and informed consent was obtained from the patients the day before surgery. Breast tumour samples were obtained from 80 patients undergoing wide local excision or mastectomy because of breast cancer, their age ranging between 35 and 75 years. The tissue was obtained from tumours greater than 2 cm in diameter as surgical waste from patients at the South Infirmary Victoria Hospital and Mercy University Hospital immediately after surgical resection. Liver tissue was obtained from three patients undergoing partial hepatectomy for metastatic liver disease. Colon tissue was obtained from three patients undergoing bowel resection for malignancy.

2.2.2 Tissue Slice Culture

Fresh tumour material upon collection was placed in collection media (Dulbecco's Modified Eagle's Medium (DMEM) supplemented with penicillin (200 IU/L), streptomycin (200 µg/L) and fungizone (250 µg/ml) at 4°C. Prior to slicing, tissue was washed with wash buffer (Phosphate buffered saline (PBS) supplemented with penicillin, streptomycin and fungizone. Slicing was performed aseptically using a Vibrotome (Leica, Germany). Tissue was held using Dermabond (Johnson & Johnson, UK) and the base discarded as waste. Slice thickness was set at 2000 microns and cut using a reciprocating blade at 22-26 rpm depending on the consistency of the tissue. Slices were incubated in 6-well plates (1 slice/well) containing culture media at 37°C with 5% CO2 in a humidified environment. **http://www.jove.com/video/2378/ex-vivo-culture-of-patient-tissue-examination-of-gene-delivery**

2.2.3 DNA Constructs

pCMV-luc plasmid, which expresses firefly luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter, was purchased from Plasmid factory (Bielefeld, Germany). Replication incompetent recombinant Ad 5 particles under the transcriptional control of the CMV promoter and CXC chemokine receptor 4 (CXCR4) promoter were kind gifts from Prof. Andrew Baker, University of Glasgow, and Prof. David Curiel, University of Alabama respectively, and generated as described previously (171, 177). Infectious particle concentration was determined by plaque assay using the ViraPower Adenoviral Expression Kit (Invitrogen, Dublin) according to manufacturer's instructions. An AAV plasmid expressing firefly luciferase under the transcriptional control of the CMV promoter was constructed and purified as described previously (176).

2.2.4 Plasmid / Viral Delivery

Cell number for slice thickness was estimated at 8×10^6 cells per slice based on an 80-cell thick slice (2000 μm) and 8-mm slice diameter. 50 μg of plasmid DNA in a volume of 50 μl (Buffer TE) was used for all non-viral methods. This was to ensure that plasmid DNA was not a limiting factor for gene expression, based on standard *in vitro* recommendations (1 μg per 1.9×10^5 cells) DNA was injected directly into the breast tumour slices. After treatment, slices were incubated at 37 °C with 5 % CO2 in a humidified environment. Media was supplemented with serum after two hours.

Viral Vectors: The titre employed for different particles was based on optimum particle concentration. For Ad 2.1×10^7 infectious particles per slice (average of 2.6 infectious particles per cell) were administered. However, in AdCMV, this viral load saturated the Xenogen IVIS-bioluminescent detection system, and therefore 1×10^7 infectious particles (1.25 viral particles per cell) per slice was used for all subsequent trials with this vector. AAV

titre employed was 1.1×10^8 infectious particles per slice (13.75 infectious viral particles per cell) was based on experience with AAV used in murine therapeutic trials in our laboratory (176). 50 µl of viral particles AdCMV (1×10^7), AdCXCR (2.1×10^7), AAVCMV (1.1×10^8) were injected directly into the centre of the tumour slice.

Electroporation: Plasmid DNA was injected into the centre of the slice. After 90 sec, pulses were delivered using a needle pair electrode. The tip of the electrode was carefully placed near the centre of the slice, by controlling depth of insertion relative to slice thickness. Pulses were generated using the Cliniporator (IGEA, Carpi, Italy) at 1 high-voltage pulse of 360 V followed by, 8 low-voltage pulses at 36 V (178).

Sonoporation: Plasmid DNA was injected into the centre of the tumour slice. After 90 sec, low-intensity US was delivered for 5 min using a 1-MHz US transducer (CRM-1, UK) with a surface area of 5 cm^2. The probe was held against the tumour throughout the duration of treatment. The parameters 1.0 W/cm^2, 20 % duty cycle were used (179).

Lipofection: 50 µg of plasmid DNA was combined with 25 µl Lipofectamine2000 (Invitrogen, UK) (2:1) and incubated at RT for 5min. The mixture was injected directly into the centre of the tumour slice.

2.2.5 Tissue Viability Assessment

Viabilities of individual tissue samples were determined using propidium iodide (PI) by Nucleocounter (ChemoMetec, Ireland) or FACS. Tissue samples are dissociated into a single cell suspension using a combination of physical dissociation and enzymatic digestion. Briefly, the tumour slice is cut with a scalpel and incubated in culture media supplemented with Collagenase IV (1500 U per slice), Dispase (4.8mg per slice) and DNAse (0.01MU per slice) for 45 min at 37 °C. Dissociated cells were removed at 15 min intervals and passed

through a 70 μm nylon cell strainer (BD Falcon, UK). Both systems are based on the uptake of Propidium Iodide (PI) by non-viable cells.

2.2.6 Histology and Immunohistochemical detection of Ki-67 Positive Cells

For histological examination, tumour slices were fixed in 10% formaldehyde solution and embedded in paraffin. Sections (4 μm) were prepared and for conventional histology were stained with Haematoxylin and Eosin (H & E). In parallel sections, Ki-67 positive cells were immunohistochemically highlighted using the monoclonal antibody MIB1(MIB1, Dianova, DEA 505) as described by Gerdes et al (180).

2.2.7 *Ex vivo* and *in vivo* comparison of gene delivery

All animal experiments were approved by the Ethics Committee of University College Cork. Female MF1 nu/nu mice were obtained from Harlan Laboratories (Oxfordshire, England). For MCF-7 tumour induction, $1x10^6$ tumour cells, suspended in 200 μl of serum free DMEM were injected subcutaneously into the flank. When tumours reached 1 cm diameter; mice were anaesthetised using 100 μl intraperitoneal injection of PBS containing 1.5 mg ketamine hydrochloride (Veroquinol, Ireland) and 300 μg xylazine (Chanelle, Ireland) and tumours removed. Tumours were sliced, treated and analysed for gene expression at 24 h as described above. For *in vivo* experiments, mice were anaesthetised before viral, lipofectamine and plasmid vectors were slowly injected into the centre of the tumour. After 90 sec, this was followed by either US or electroporation treatment. In US treatment, high viscosity coupling gel (Aquasonic 100) was used to improve skin contact.

2.2.8 Xenogen IVIS Imaging

Tissue slices were maintained in 6-well plates for analysis. 100 µl of 30 mg/ml firefly luciferin (Biosynth, Switzerland) was injected into the slices and 100 µl was added into media. After 10 min of incubation, slices were imaged for 5 min using an intensified CCD camera (IVIS Imaging System, Xenogen). For Whole Body Imaging of mice, 100 µl of luciferin was injected intratumourally and 100 µl intra-peritoneally. 10 min-post luciferin injection, mice were anaesthetised and imaged for 3 min. All measurements were obtained using Living Image 2.6 application (181).

2.2.9 Epithelial cell analysis of tumour slices

Tumour slices were dissociated into a single cell suspension fixed using 70% ethanol and permeabilised using IFA-tx buffer (2 % FCS, 10mM Hepes, 0.1% Triton X-100, 0.1 % Sodium Azide and 150mM NaCl) buffer. Cells were incubated for one hour with pan-cytokeratin (3 µg) antibody tagged with PE (clone C-11) (Abcam, UK).

2.2.10 Cell type specific expression

Slices were dissociated, fixed and permeabilised as described above. Cells were dual stained sequentially for one hour on ice with pan-cytokeratin antibody tagged with Phycoerythrin (PE) (3 µg), and primary luciferase antibody (10 µg) (clone Luci17) (Abcam, UK) and a secondary Cyanine 5 (Cy5) antibody (1:500) (Abcam, UK).

2.2.11 Statistics

Calculation of means, standard deviation (SD), and standard error of the mean (SEM) was performed using GraphPad Prism (V 3.0, San Diego, CA, USA). Statistical significances of

the differences between the individual groups were determined by using the two-tailed Student's *t*-test for paired values. Differences with a *p*-value <0.05 were considered significant.

2.3 Results

2.3.1 Optimisation of tissue slice model system

The optimum temperature for collection and storage of tissue was determined to be 4 °C (data not shown). At this temperature, viability was unaffected over a 12 h period and it prevented the occurrence of microbial infections before processing. The minimum thickness for accurate intra-tumoural gene delivery by direct injection and for the application of electroporation and sonoporation devices was 2 mm. The greatest transfection efficiency was found to occur in the absence of serum (data not shown) and consequently this was employed in all treatments. Serum was subsequently added 2 h after treatment for all methods.

2.3.2 Cell viability and characterisation of cultured tumour slices

To mimic *in vivo* conditions, efforts were aimed at maintaining slices in their constituent cellular components. In order to avoid stimulation of particular cellular populations, slices were maintained in standard culture medium. PI viability assays were employed to assess total cell viability at various time points after surgical resection prior to cultivation. The optimum harvesting time after surgical resection for breast tumours was within 30 min, with viability dropping by 80.2 % after 90 min **(Figure 2.1a)**. All samples used for subsequent analyses were harvested within 30 min of resection from patients.

The efficiency of the culture method in retaining tissue viability over time was examined **(Figure 2.1b)**. An initial decrease in viability observed at 24 h may be attributed to an adaptability period to culture conditions and therefore prior to all treatments, slices were incubated in culture medium for 2 h at 37 °C to ensure slice adaptation prior to treatment. Viability assays showed that slices were able to maintain 80 % of original viability for up to 96 h **(Figure 2.1b)**. The decrease in slice viability after 96 h is most likely due to a negative cell turnover (cell death > cell proliferation). Previously reported studies have only maintained *ex vivo* tissue viability for a shorter period of 4 days (172). In order to examine the uniformity of viability throughout the tissue section, intra-slice viability was assessed. No marked difference was observed between regions within the slice and there was a proportionate decrease in viability in all regions over time **(Figure 2.1c)**. Flow cytometric analysis of epithelial cells indicated that a significant proportion of tumour cells (82.3 +/- 12.3 %) were still present after 24 h of culture when compared with pre-cultured tissue **(Figure 2.1d)**. Histological analysis at 0 and 24 h revealed no significant change in morphology or apoptosis as detected by H&E (ii). Proliferative cells were present at 0 and 24 h detected by anti-Ki 67 staining (iii). Thus, this culture system was capable of maintaining the desired viability and tumour component required for the application and assessment of gene delivery methods.

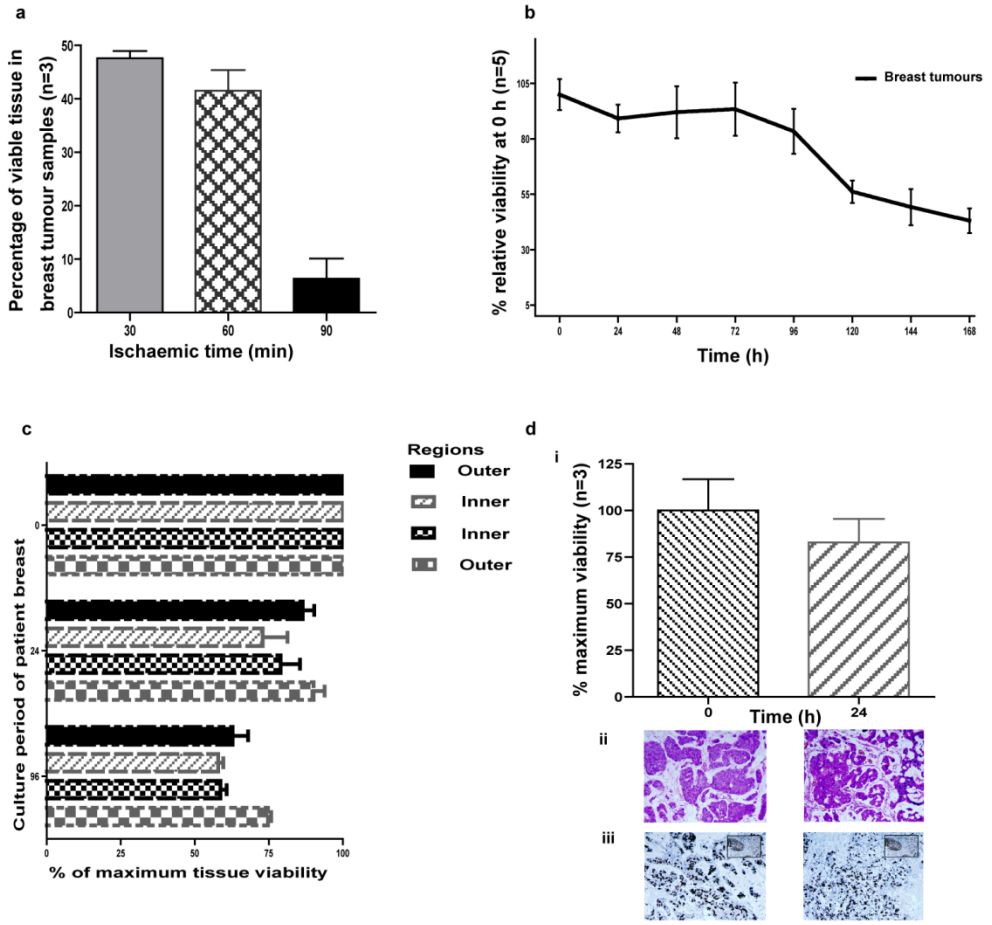


Figure 2.1 Cell Viability and Characterisation In Cultured Tumour Tissue
(a) Influence of time on sample recovery A propidium iodide assay was used to assess cell viability at various time points (30, 60, 90 min) after surgical resection prior to cultivation. **(b) Viability of cells in culture over time** Slices were cultured and viability at various time points assessed over a period of 7 days. **(c) Intra-tissue variability in viability** Intra-slice viability was assessed by further dividing cultured slices into 500 µm regions at either 24 or 96 h and viability assessed. **(d) Cell type characterisation and histological analysis** Epithelial cell component of slices was examined by flow cytometry before cultivation and 24 h after using pancytokeratin marker (i). Histological assessment by H&E (ii) and anti-Ki67 (iii) staining was carried out at 0 and 24 h. Inset demonstrating positive control.

2.3.3 Real-time luminescence imaging of *ex vivo* tissue permits assessment of gene delivery and expression over time

The optimised culture model was employed to assess and compare gene delivery systems. Breast tumour slices were administered CMV - firefly luciferase DNA cassette according to materials and methods using various gene delivery methods and expression analysed at

various time points post treatment. Parameters for various gene delivery methods were optimised in various cell lines and MCF-7 tumours *in vivo* . A method for real-time IVIS imaging of *ex vivo* tissue slices transfected with luciferase was developed. Luminescence per gene copy administered was determined and results are displayed in **Figure 2.2a** which represents maximum gene expression for various methods at their optimum time point. Ad has highest gene expression at 48 h, while AAV peaked at 120 h. The highest expression for lipofectamine was found at 72 h, while no further increase in gene expression was found after 24 h for both US and EP unlike all other delivery methods. The results obtained with AAV, which requires the longest time for optimum expression (5 days) correlates with various published *in vivo* time course studies. Ad proved the most efficient vector overall with US providing highest reporter gene expression among the non-viral methods in breast tumour tissue.

2.3.4 Comparison of *ex vivo* and *in vivo* analyses

To assess consistency with the *in vivo* setting, we performed *in vivo* and *ex vivo* MCF-7 xenograft gene transfections / transductions using all methods. A xenograft model was used instead of spontaneous or induced models because of the relative ease in obtaining tumours of similar volumes. Spontaneous or induced models would more closely represent the tumour state in patients in relation to organ specific physiology and immunogenicity. They however have slow and unpredictable growth rates and therefore require large numbers to obtain sufficient homogenous tumour experimental groups. The time point for maximum expression *in vivo* for each method was used to measure luminescence. The pattern of relative efficiencies of methods *in vivo* (Ad > AAV > Lipo > EP > US > plasmid) was reflected *ex vivo* except in the case of US which provided higher relative efficiency *ex vivo*. Both EP and Lipo performed better in the *in vivo* setting. However, there was a marked reduction in the

efficiency of Ad and US as delivery methods *in vivo* . This suggests the activity of *in vivo* factors adversely affecting gene delivery and/or expression, which are absent in the *ex vivo* model for these methods **(Figure 2.2b).**

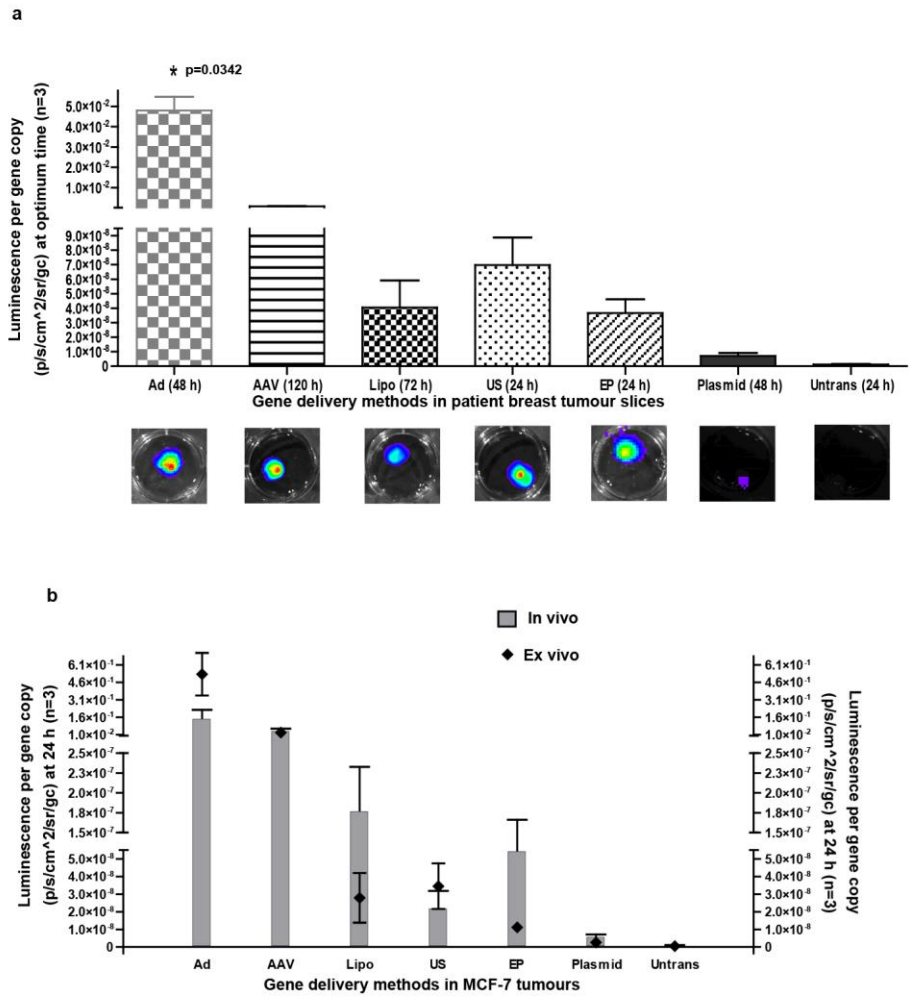


Figure 2.2 Figure 2 Assessment of gene delivery in breast tumour tissue
(a) Gene delivery in patient breast tumour samples Breast tumour slices were treated *ex vivo* with various gene delivery methods. Luciferase activity at optimum time points for each delivery method (EP and US – 24 h, Ad – 48 h, Lipo - 72 h, Plasmid – 24 h, AAV – 120 h) was detected by IVIS-Luminescence system following addition of substrate to culture medium. Luminescence units are displayed per gene copy administered. Representative images of luminescence readings are displayed below. **(b) Comparison of gene delivery methods in ex vivo and in vivo models** MCF-7 tumour xenografts were treated *ex vivo* (squares, z axis) or *in vivo (bars, y axis)* with the various methods as above. The pattern of relative efficiencies of methods *in vivo* (Ad>AAV>Lipo>EP>US>Plasmid) was generally reflected *ex vivo* except in the case of Ad and US which provided higher relative efficiency *ex vivo*.

2.3.5 Examination of biological barriers to US gene delivery

Among the non-viral methods examined here, US provided the greatest gene expression *ex vivo* in patient tumour tissue. In murine trials, the high level of US transfected gene expression was only seen *ex vivo* with significant reduction *in vivo*. To investigate the reasons for the dramatic differences between US results *ex vivo* and *in vivo*, **(Figure 2.2b)**, we examined the involvement of physical factors that are not present *ex vivo*, as potential biological barriers to US gene delivery *in vivo*. We investigated the following physical factors; presence of blood, skin and temperature and tumour variables of tumour type, tumour stage (primary versus metastatic tumour) and tumour US wave penetration.

The presence of whole patient blood acted as a barrier to all gene delivery methods, with the most significant effect on US as shown in **Figure 2.3a**. Separation of blood into cellular and serum components showed that US was only adversely affected by the cellular component of blood (data not shown). US may enhance gene delivery by either thermal or non-thermal effects. The thermal effect caused by US is due to the absorption of US waves. US application results in a 0.5 - 1.0 °C rise in tissue temperature, yet increasing tissue temperature to 39 °C, only moderately increased gene expression by plasmid delivery, which was still significantly lower than US (data not shown). However, combining hyperthermia (39 °C) with US improved transfection by 200 %, without affecting viability **(Figure 2.3b)**. Increasing temperature beyond 39 °C resulted in dramatic reduction in viability and gene expression per viable cell (data not shown).

As evidenced by *in vivo* US-mediated delivery to mouse liver, overlying skin reduced transfection by 40 % when compared with direct contact **(Figure 2.3c)**. The optimal distance from the US probe for highest transgene expression **(Figure 2.3d)** was also investigated. Maximal luminescence was seen in the tissue area 500 - 1000 μm from the US probe. The layer closest to the probe did not show significantly reduced viability following US

application (data not shown) indicating that the observed luminescence was due to maximum efficiency of gene delivery sonic waves 500 - 1000 μm from the US probe, rather than a high rate of cell killing outweighing DNA delivery in the tissue closest to the probe. US gene delivery was as effective in secondary nodal tumours compared with primary ductal tumours from the same patients **(Figure 2.3e)**. However, gene expression was reduced in tissue slices originating from lobular tumours when compared with ductal tumours from three different patients **(Figure 2.3f)**.

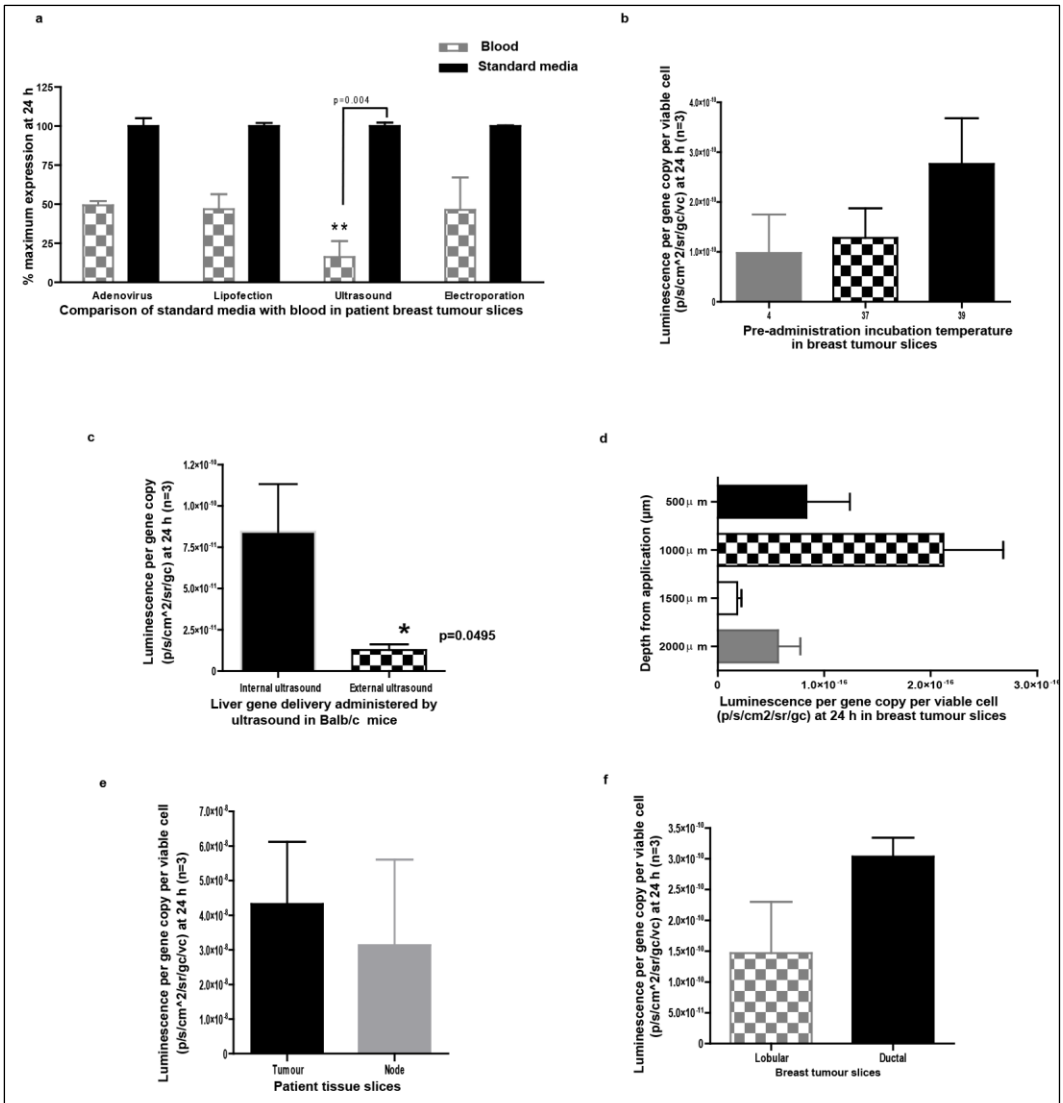


Figure 2.3 Biological barriers to ultrasound related reporter gene expression **(a)** Breast tumour slices were treated with various gene delivery methods in the presence of the patient's own blood. After treatment, slices were removed from blood, washed, cultured and assessed for gene expression and viability. **(b)** Tumour slices were incubated at various temperatures for 2 h prior to gene delivery and culture as normal. Resulting expression and viability was analysed at 24 h. **(c)** The effects of the presence of skin between the US probe and target tissue were assessed in a Balb/C murine model, where treatment was performed on the liver either under the skin or over it and in vivo luminescence assessed using IVIS imaging. **(d)** Tumour slices were examined for maximum reporter gene expression and viability with respect to distance from US probe. **(e, f)** US treatment was examined in ductal, metastatic nodal and lobular tissue.

2.3.6 Examination of biological barriers to Ad gene delivery

Ad vectors are extremely efficient gene delivery agents and target a wide host tissue range. However, various physical factors can limit gene expression. As previously mentioned, in

Figure 2.3a, blood is a barrier to all methods, reducing Ad gene expression by 50%. Solid tumours are associated with regions of reduced viability due to hypoxic conditions. Regional tissue viability can affect treatment in two ways, by reducing gene delivery and/or by its effect on gene expression. In our studies, viability was observed to have a linear relationship with plasmid DNA mediated transgene expression (data not shown). With Ad, reporter gene expression was unchanged at viability rates above 40 % of maximum viability, with significant reduction at viabilities below this threshold, suggesting saturation in luminescence readings or gene delivery above this limit **(Figure 2.4a)**. Ad demonstrated good penetration of solid tumours, with comparable expression in all regions of the slice **(Figure 2.4c)**. However, gene expression was significantly reduced ($p=0.045$) in nodal metastatic disease **(Figure 2.4b)** suggesting reduced transduction in metastatic tissue unlike observations with US **(Figure 2.3e)**. Measurement of gene expression over time showed maximum expression at 48 h, correlating with *in vivo* data, with 70 % reduction in gene expression at day 5 **(Figure 2.4d)**.

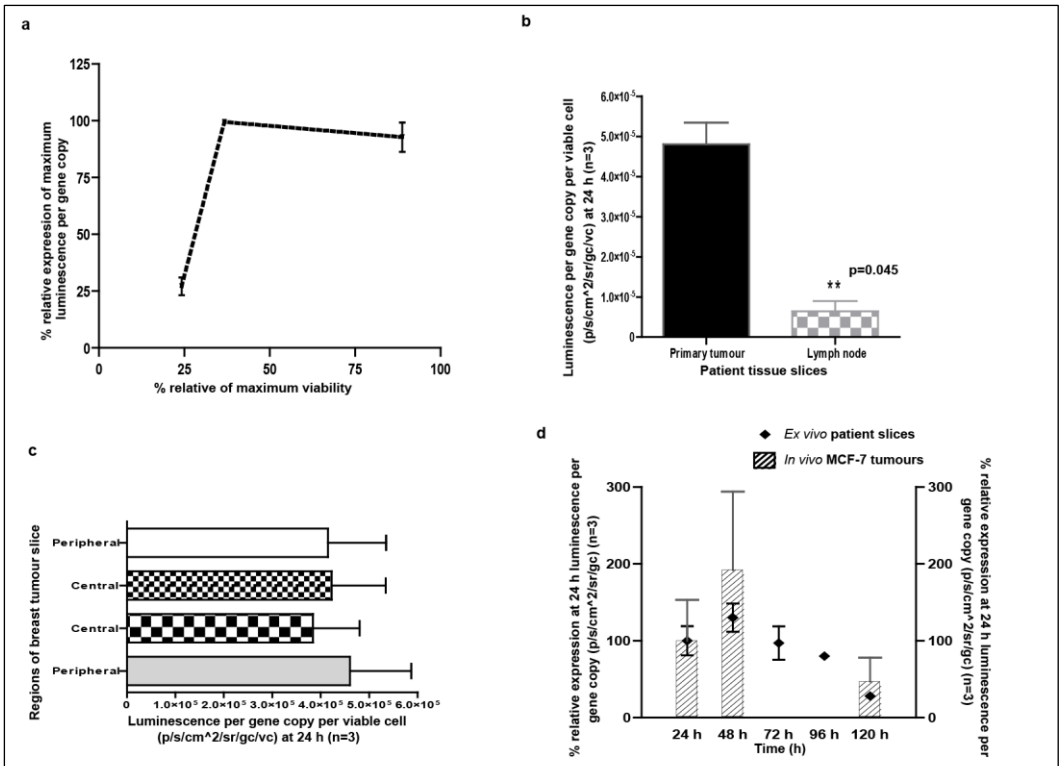


Figure 2.4 Biological barriers to adenovirus related reporter gene expression **(a)** Breast tumour slices were administered AdCMV and incubated at 37 °C for 24 h. Slices were further sliced into 500 µm regions and luciferase expression and viability assessed at 24 h. Viability for each region was analysed by creating a single cell suspension and performing propidium iodide assay (n=3). **(b)** Breast tumour slices from the primary tumour and metastatic nodes were assessed for AdCMV gene expression and viability at 24 h (n=3). **(c)** Breast tumour slices at 24 h after central AdCMV administration were sliced into 4 equal regions and assessed for gene expression and viability. **(d)** Gene expression for AdCMV administered slices was assessed every 24 h for 5 days compared with corresponding in vivo MCF-7 tumours (n=3).

2.3.7 Validation of system using malignant and non-malignant tissues

In order to further examine the culture and imaging system in a setting aimed at qualifying as well as quantifying reporter gene expression, we compared the tissue / cell selectivity of *luciferase* expression under the control of gene promoters with disparate expression profiles in malignant and normal tissues. While CMV is ubiquitously expressed, CXCR4 is considered to be differentially expressed in tumour cells (182-184). Ad vectors featuring the firefly luciferase gene under the control of either the CMV (AdCMV) or CXCR4 (AdCXCR4) promoter were examined in breast tumour, normal colon and normal liver

tissues. Normal liver and colon was used rather than normal breast due to the fatty consistency of normal breast tissue making slicing impractical. When examined by bioluminescent imaging, AdCXCR demonstrated tumour tissue selectivity, with significantly improved normal to tumour ratio when compared with AdCMV **(Figure 2.5a)**. To quantify transduction efficiency within tumour and normal tissue with both Ad vectors we optimised a method of gentle tissue dissociation using both chemical and physical means to isolate a representative population of cells. Ad mediated luciferase expression was examined by flow cytometry in cell suspensions derived from slices 48 h after treatment. We demonstrated transduction efficiency (% luciferase positive cells) of AdCMV (30.9 +/- 7.7 %) and AdCXCR4 (11.3 +/- 0.9 %) in breast tumour slices **(Figure 2.5c and d respectively)**. This specificity was confirmed in analysis of liver samples, with 10.6 % (+/- 3.4) liver epithelial cells luc+ in AdCMV administered samples, compared with 0.6 % (+/- 0.4) in the case of AdCXCR4 **(Figure 2.5e and f respectively)** and in colon samples, with 20.3 % (+/- 8.4) colon cells luc+ in AdCMV compared with 0.77% (+/- 0.15) in AdCXCR4 **(Figure 2.5g and h respectively)**. Graphical representation of transduction efficiency ratios in tumour, colon and liver is shown in **Figure 2.5b**. However, human tumours are composed of a variety of cells, of which only ~30% represent the tumour population. Hence, to assess cell type selectivity within the slices we co-localised transfected populations with an epithelial tumour cell marker, pancytokeratin. Analysis of tumour cell populations revealed that AdCXCR4 had a higher selectivity for expression in epithelial tumour cells [93.7 % (+/- 6.2) epithelial cells luc+; 6.25 % (+/- 3.46) non-epithelial cells luc+] when compared with AdCMVLuc [75 % (+/- 8) epithelial cells luc+; 25% (+/- 8) non-epithelial cells luc+] as shown in **Figure 2.5i and j** respectively. **Figure 2.5k** demonstrates graphical representation of epithelial cell transduction in tumours with both Ad vectors confirming selectivity of AdCXCR4 expression in tumour epithelial cells.

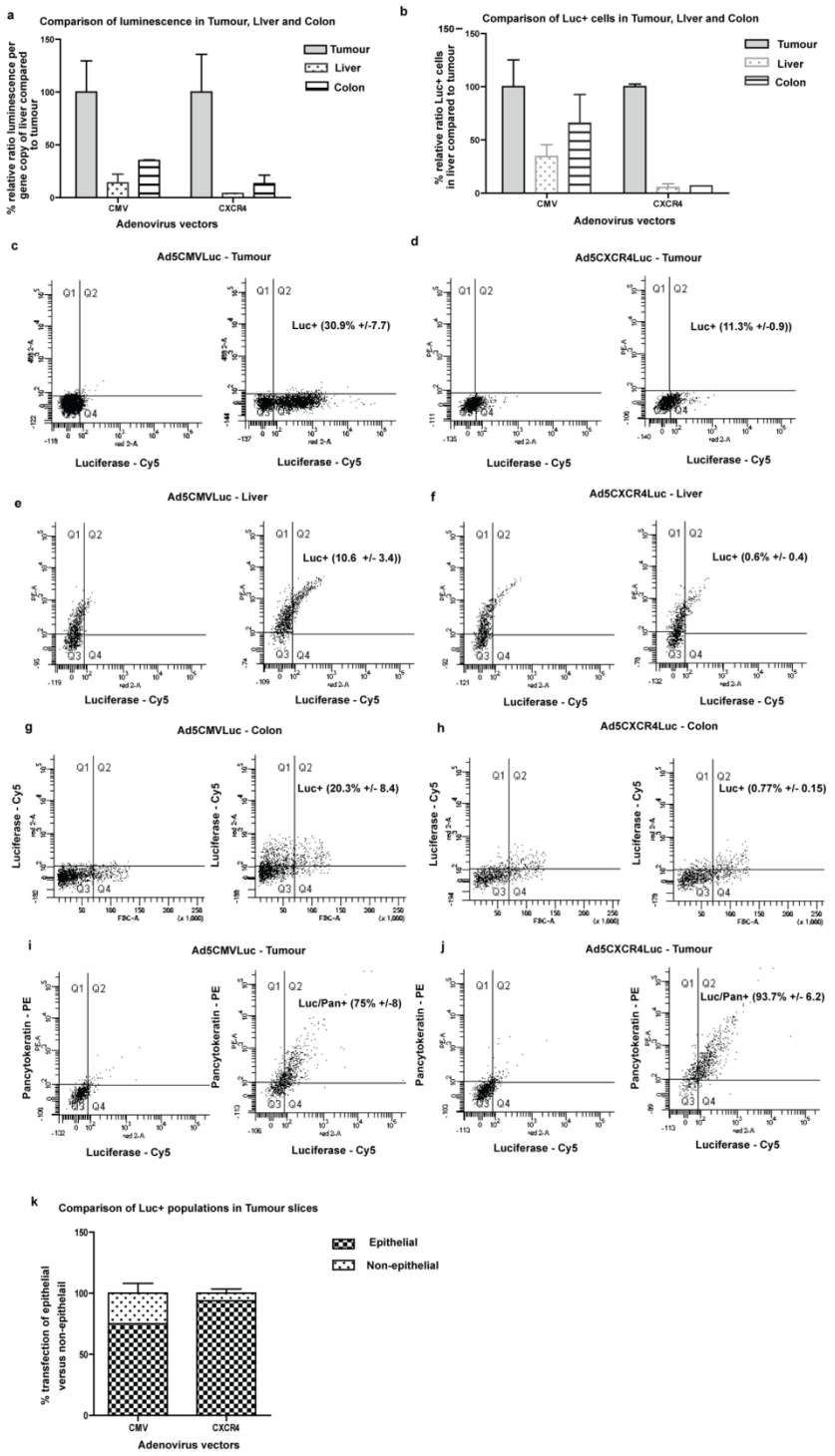


Figure 2.5 Comparative analyses of CMV and CXCR4 promoter activity in tumour, liver and colon patient samples
Breast and liver patient samples were administered Ad5CMVLuc or Ad5CXCR4Luc and incubated at 37°C for 24 h. **(a)** Bioluminescent imaging at 48 h demonstrates a greater tumour specificity of AdCXCR4Luc when compared to Ad5CMVLuc. Percentage luminescence in liver and colon is expressed relative to tumour. Slices were dissociated into single cell suspensions and fixed with 70% ethanol. To establish transduction efficiency (% luc+ cells) cells were stained with anti-luciferase antibody (detected as Cyanine 5 labelled cells [red 2A]) and to identify epithelial tumour cells, populations were stained with a pan-cytokeratin antibody (detected as Phycoerythrin labelled cells [PE-A]).

Figure 2.5 (continued)
(b) Comparison of transduction efficiency of AdCMV and AdCXCR4 in tumour, colon and liver samples. Percentage luciferase expression in liver and colon is expressed relative to tumour. **(c, d)** Representative flow cytometric analysis of tumour cells with AdCMV (c) and AdCXCR4 (d) stained with luciferase antibodies (right panels) and corresponding non-specific IgG (left panels). Percentage of cells in Q4 represent those cells expressing luciferase in Ad5CMVLuc (30.9 % +/- 7.7) and Ad5CXCR4Luc (11.3 % +/- 0.9). **(e, f)** Representative flow cytometric analysis of liver patient samples with AdCMV (e) and AdCXCR4 (f) stained with luciferase antibody (right panels) and corresponding IgG (left panels). Percentage of cells in Q2 represents those cells expressing luciferase in AdCMV-Liver (10.6 % +/- 3.4) and AdCXCR4-Liver (0.6 % +/- 0.4). **(g, h)** Representative flow cytometric analysis of colon patient samples with AdCMV (g) and AdCXCR4 (h) stained with luciferase antibody (right panels) and corresponding IgG (left panels). Percentage of cells in Q1-Q2 represent those cells expressing luciferase in AdCMV (20.3% +/- 8.4) and AdCXCR4 (0.77 % +/- 0.15). Tumour samples transduced with AdCMV and AdCXCR4 were counter-stained with pancytokeratin. Percentage luc+ epithelial tumour cells in AdCMV-Tumours (75 % +/- 8) and AdCXCR4-Tumours (93.7 % +/- 6.2) are presented in Q2 (right panels) and corresponding IgG (left panels). **(i, j)** Quantification of epithelial and non-epithelial fractions of luc+ tumour cells from AdCMV [75 % (+/- 8) epithelial cells luc+; 25% (+/- 8) non-epithelial cells luc+] and AdCXCR4 [93.7 % (+/- 6.2) epithelial cells luc+; 6.25 % (+/- 3.46) non-epithelial cells luc+]. Percentages shown are averages of three independent experiments.**(k)** Graphical representation of epithelial cell transduction in tumours with both Ad vectors.

2.4 Discussion

The major focus of this study was to determine whether *ex vivo* tumour slices could be used to compare various gene delivery methods using a real time bioluminescence detection system for quantitative assessment of gene expression in patient samples. This *ex vivo* model enables the assessment of gene delivery methods on the complex 3D architecture, tumour heterogeneity and phenotypic variation that is present *in vivo* . The maintenance of significant viability is crucial for *ex vivo* cultivation (185). In order to evaluate the suitability of this novel system, we studied the effects of culture conditions on tumour cell viability and variations in tumour cell populations over time. The tissue slices were maintained for up to 7 days with sustained viability, ensuring appropriate response to various gene delivery experiments and allowing the use of methods requiring longer incubation periods for maximum expression such as AAV. There have been a number of publications over the last ten years on the use of *ex vivo* tumour slices with specific individual viral vectors (171, 172). However, only single methods were applicable using these models, valuable insights on tumour biology *ex vivo* and their effects on gene delivery were not fully explored. Our model uses a comparatively thicker slice of tumour tissue when compared with other model systems. Through optimisation, we achieved an important balance between ensuring adequate

diffusion of nutrients and oxygen to the inner regions of the slice and facilitating the physical delivery of genes by direct injection, the application of electroporation and sonoporation devices and enabling in-depth intra-slice analysis. Our approach of tumour dissociation for the analysis of PI uptake, cell markers and transduction efficiency allows for the examination of a representative, viable cell population in the relative proportions found in the intact tumours. This, we believe, gives a more detailed and accurate representation of the cells in the slices that may be unavailable in the intact tissue (186). Access to high quality screening in developed countries, has resulted in the detection of breast cancer at an earlier stage, leading to a decrease in the incidence of large tumours and thus less patient tissue available for research purposes. Even though our model uses a large slice of tumour, the bioluminescence detection system we have developed allows the accurate analysis of gene expression over time without sequential sacrifice of tissue, as with previous models, minimising wastage of valuable patient material. To allow for differences in tissue composition and variation in transfectability between patient samples, it was important to compare all methods on tissue from the same patient. Despite marked heterogeneity between patient samples, we found Ad to be the most effective vector overall, correlating with existing evidence in the field (187). The finding that US provided the optimal transgene expression for *ex vivo* human breast tumour slices among non-viral methods was surprising. When we repeated these experiments in MCF-7 xenografts, we found a similar trend in the *ex vivo* setting; however both methods had a significantly lower transfection efficiency in the *in vivo* setting. This suggested to us that physical conditions were inhibiting both methods from demonstrating their maximum transfection ability *in vivo*.

The nature of the *ex vivo* system permitted the examination of specific physical factors individually, that can account for this reduction in gene delivery. Furthermore, comparing delivery to distinct histological types and stages of disease in breast cancer can be studied.

The physical factors investigated were blood, regional viability, tumour penetration, skin, tumour type and temperature. Large bulky tumours, fluctuations in body temperature and the skin barrier are frequently encountered in the clinical setting. Solid tumours are known to possess a tortuous and leaky vasculature and can have significant variations in regional viability (188) while regional lymph nodes are the commonest site for metastatic spread in breast cancer. Our experiments show that blood, reduced viability, and nodal tissue are factors that adversely affect Ad gene delivery. The reduction of gene expression in nodal tissue is likely to be due to physical properties of nodal tissue rather than phenotypic variation in nodal tumour cells. Lymph nodes are composed of a fibrous capsule and a supporting meshwork of connective tissue and extracellular matrix (ECM) components which have previously been shown to reduce adenoviral gene delivery (189). The use of degradation enzymes of ECM components, such as hyaluronidases may improve Ad gene delivery to involved lymph nodes. In primary tumours, Ad demonstrated a transduction efficiency of up to 50%, and was potent at penetrating all regions of the slice. However, this efficiency was not accompanied by long term gene expression with >70% reduction in expression within 5 days. Reduction in gene expression may be attributed to either cell turnover or cell death in the *ex vivo* tissue. In MCF-7 tumours, when comparing *ex vivo* with *in vivo* gene expression there was significant reduction (50 and 74 % respectively) in gene expression *in vivo* which may be linked to the presence of a systemic immune response *in vivo* which is absent in an *ex vivo* model . US gene delivery offers good penetration of nodal tissue and a temperature rise of 2 °C greatly enhanced gene delivery. Expression is significantly reduced by the presence of overlying skin and blood, and it offers a reduced penetration of tumour slices. These findings indicate that Ad may be a better candidate vector for the targeting of large solid tumours. US offer a better safety profile albeit a less efficient alternative to Ad. Its greatest benefit may be in the treatment of superficial, small, and relatively avascular

involved nodes of the chest wall or axilla. The targeting of viable regions and a temporary blood-free field would improve transfection ability for all methods. The use of imaging modalities such as Colour Flow Doppler to identify neo-vascularity (190) and hence target viable regions and the application of vasoconstrictor agents to reduce blood exposure prior to treatment will improve gene delivery. In addition, heat delivery systems may improve US mediated treatment delivery.

The model is not confined to tumour studies and allows for inter-tissue variations to be assessed. The chief safety concern with Ad use is activation of the inflammatory response syndrome (191). Slow intratumoural delivery of Ad and the use of polymeric delivery systems (192) reduces systemic dissemination. Yet, there can be significant viral loads systemically disseminated due to a delivery-induced convective transport into leaky vessels which leads primarily to accumulation in the liver (193). The predilection for liver accumulation *in vivo* may be due to the detoxifying activity of the liver making it inherently prone to "capture" foreign particles. This unwanted effect from dissemination may be limited by the integration of unique promoters in viral vectors, which are highly expressed in tumour cells, with little or no expression in normal cells. Ad expressing *luciferase* under CXCR4 control was found to improve tumour cell targeting with relative sparing of non-tumour cells in patient tumour slices, and moreover reduced gene expression in non-target tissue (liver slices). Comparison of tumour selectivity of the CXCR4 promoter evaluated by bioluminescence and FACS revealed good correlation with previous publications with regard to both tissue type and cell population. The tumour-cell selective transgene expression would suggest that Ad utilising the CXCR4 promoter would also support the systemic targeting of disseminated disease with minimal non-target effects and moreover allow early post surgical treatment.

Conclusions

Our study demonstrates, for the first time that bioluminescence imaging of luciferase-transfected patient tumour slices can be used for the assessment of gene delivery methods and to study physical barriers to gene delivery. Bioluminescent imaging provides a rapid, economical and simple method for monitoring gene expression. Furthermore, it offers an attractive alternative to animal trials and may provide a more accurate representation of patient response. A range of commonly employed gene delivery methods with potential for breast tumour therapy were examined with this strategy. While we did not examine other vector types in this study such as nanoparticles (194-196) or bacterial vectors (197), we have previously demonstrated bactofection using *L. monocytogenes* in *ex vivo* patient tumour tissue (197). It is therefore likely that the model system developed here is applicable to other vector types. In addition, a large number of patient tissue types may be examined using this model system including ovarian (197), colorectal (198) and mesenchymal tissue (199). Furthermore, the model is not confined to gene delivery assessment. The extended duration of culture period and 3D architecture of this model makes it applicable to studies of novel gene therapeutic strategies where the interplay between tumour cells and the microenvironment are important. The findings from this study and potential relevance in the clinical setting highlight the importance of meticulous evaluation prior to entering clinical trial.

Chapter 3

Adenovirus mediated transcriptional targeting of colorectal cancer and effects on treatment resistant hypoxic cells

Sections from this chapter have been published as

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Abstract

Colorectal cancer is the second leading cause of cancer-related mortality and frequently presents with locally advanced or metastatic disease. Ad vectors are important gene delivery agents as they offer efficient and broad tissue transducability. However, their ability to penetrate through multi-cell layers in colorectal cancers and maintain expression in colon tumour-related hypoxic conditions has yet to be analysed. Furthermore, their broad tissue tropism presents safety concerns. An *ex vivo* cultured patient tumour sample model was employed to examine Ad transduction of colorectal tumours. Results obtained from Ad delivery of the firefly luciferase (FLuc) reporter gene indicated that colon tumour tissue was more amenable to Ad transduction than other tumour histological types examined (breast and ovary). Ad transduction levels were significantly higher than a range of viral and non-viral methods examined in patient colon tissue. Control of transgene expression using the CXCR4 promoter was examined as a strategy to confine expression to tumour cells. An Ad construct carrying FLuc under the control of the human CXCR4 promoter demonstrated low reporter gene expression compared with the ubiquitously expressing CMV promoter in normal colon and liver tissue while providing high expression in tumours, demonstrating a 'tumour-on' and 'normal-off' phenotype in patient tissue. The effects of changing hypoxia on Ad-related transgene expression were examined in an *in vitro* model of hypoxic conditions relevant to clinical colorectal tumours. Reporter gene expression varied depending on the level of hypoxia, with significantly reduced levels observed with prolonged hypoxia. However, transgene expression was robust in the cycling hypoxic conditions relevant to colorectal tumours. This study provides novel, clinically relevant data demonstrating the potential for efficient gene delivery to colorectal tumours using Ad.

3.1 Introduction

Colorectal cancer (CRC) is the third most common cancer in both men and women and the second leading cause of cancer-related mortality in both sexes in the United States and Europe (158, 200). Approximately 5–15% of newly diagnosed CRC cases present with locally advanced disease, while the liver is the most common site for metastatic spread (201). Extensive locally advanced or metastatic disease may require down-staging by chemotherapy to improve resectability. While current neoadjuvant chemotherapy regimens are efficient at down-staging disease, they are associated with significant side effects because of their relatively non-specific toxicity which can either delay the timing of surgical resection resulting in repopulation of tumour cells or increase postoperative complication rates (202).

In this regard, gene therapy may serve as a novel neoadjuvant approach for the down-staging of locally advanced or metastatic disease prior to surgical resection. Various gene delivery methods have been developed to enhance tumour transduction efficiency (TE). Ad vectors are effective gene delivery vectors due to their efficient ability to target a wide range of cells. Preclinical trials have demonstrated the gene delivery capability of Ad in a number of different cancers (171, 174); however, its potential in clinical colorectal cancer has yet to be explored. In order to reduce toxicity to normal healthy cells from gene therapy, transcriptional targeting strategies can be employed. Transcriptional targeting involves regulating the expression of the gene of interest by utilising promoters, that maintain a 'tumour-on' and a normal tissue-off' status. A number of tumour selective promoters have been explored in the context of cancer gene therapy. The CXCR4 promoter has been shown to have an optimal profile of activity and specificity in various other tumour types (183, 203-205). CXCR4 is also known to be over-expressed in colorectal carcinoma tissues when compared with normal tissues (206-208). Higher CXCR4 expression has also been found in

colorectal liver metastasis when compared to the primary tumour (209). In this study, we evaluate gene delivery using Ad and the transcriptional selectivity of Ad under the control of CXCR4 in an *ex vivo* human colorectal cancer model. We performed our studies on human tumour tissue as we felt this model system, though lacking a competent immune response, provides a reliable model for direct extrapolation to the clinical setting.

We also explored the feasibility of gene delivery under conditions which mimic tumour hypoxia. Hypoxia is an important feature of solid tumours as a consequence of a structurally and functionally disturbed microcirculation and, in some cases, a reduced O_2-carrying capacity of the blood due to tumour-associated anaemia (210). Hypoxic cells can also arise from perfusion driven changes in oxygen supply, resulting in rapid and reversible changes in oxygenation (211). Hypoxia has been related to the up-regulation of gene products that may promote tumour progression, by enabling tumour cells to adapt to nutritional deprivation or to escape a hostile environment. It is a well-established factor influencing treatment resistance to both chemo- and radiotherapy and is a prognosticator for poor survival (212, 213). While, stress response genes are up-regulated under hypoxia, the majority of cell metabolism is decreased, with a reduction in overall transcription and translation (214). These effects may hinder gene delivery to hypoxic cells within solid tumours, and therefore the effects of hypoxia on Ad gene delivery warrant further investigation as the effective targeting of these cells may be an important predictor of treatment response in viral gene therapy.

3.2 Materials and Methods

3.2.1 Human Tissue Samples and Tissue Slice Culture

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals and informed consent was obtained from the patients the day before surgery. Tissue samples were collected and cultured as described previously (46, 92, 215, 216). Briefly, colon tumour samples were obtained from 30 consecutive patients undergoing bowel resection for colorectal cancer, their age ranging between 50 and 70 years. Patients who received neoadjuvant chemo-radiotherapy and those with small tumours with little surgical waste were excluded from the study. Type of surgical procedure was not considered here. The tissue was obtained as surgical waste from patients at the South Infirmary Victoria Hospital and Mercy University Hospital immediately after surgical resection. Liver tissue was obtained from three patients undergoing partial hepatectomy for colorectal metastatic liver disease. Fresh tumour material upon collection was placed in collection media, Dulbecco's modified eagle's medium (DMEM) (GIBCO, Invitrogen Corp., Scotland) supplemented with penicillin (200 IU / L), streptomycin (200 μg / l) fungizone (250 μg / ml) and gentamicin (125 μg / ml) at 4°C. Prior to slicing, tissue was washed with wash buffer, phosphate buffered saline (PBS) supplemented with penicillin (200 IU / L), streptomycin (200 μg / L), gentamicin (125 μg / ml) and fungizone (250 μg / ml). Slicing was performed aseptically using a Leica Vibrotome (Laboratory Instruments and Supplies, Meath, Ireland) as previously described (215, 217). Slice thickness was set at 2000 μm and cut using a reciprocating blade at 22-26 rpm depending on the consistency of the tissue. Slices were incubated in 6-well plates (1 slice / well) containing culture media at 37°C with 5 % CO_2 in a humidified environment. Slices were injected with Ad constructs and maintained at culture conditions.

3.2.2 Vector constructs

pCMV-luc plasmid, which expresses firefly luciferase under the transcriptional control of the cytomegalovirus (CMV) promoter, was purchased from PlasmidFactory (Bielefeld, Germany). Replication incompetent recombinant Ad particles under the transcriptional control of the CMV promoter (AdCMV) and CXC chemokine receptor 4 (CXCR4) promoter (AdCXCR4) were a kind gift from Prof. Andrew Baker, University of Glasgow, and Prof. David Curiel, University of Alabama respectively and generated as described previously (171, 177). Briefly, the Ad vectors encode the reporter gene, Firefly luciferase, regulated by CXCR4 or CMV promoter, which drives the reporter gene expression, inserted in the E1-deleted region of the adenoviral vector backbone. For details of construction, see Zhu et at (182). The levels of luciferase expression related to these vectors reflects the activity of either CMV or CXCR4 promoters. Infectious particle concentration was determined in our lab by plaque assay using the ViraPower AdExpression Kit (Invitrogen, Dublin) according to manufacturer's instructions.

3.2.3 Comparison of gene delivery methods

Cell number for slice thickness was estimated at 8×10^6 cells per slice based on an 80-cell thick slice (2000 μm) and 8-mm slice diameter. 50 μg of plasmid DNA in a volume of 50 μl (Buffer TE) was used for all non-viral methods. This was to ensure that plasmid DNA was not a limiting factor for gene expression, based on standard in vitro recommendations (1 μg per 1.9×10^5 cells). DNA was injected directly into the colon tumour slices. After treatment, slices were incubated at 37 °C with 5 % CO2 in a humidified environment. Media was supplemented with serum after two hours. We did not use a range of multiplicity of infection concentrations (MOI). The titre of different particles was based on optimum particle

concentration. For Ad we used 2.1×10^7 per slice (2.6 infectious particles per cell) per slice. However, in AdCMV, this viral load saturated the IVIS detection system, and therefore we chose 1×10^7 infectious particles (1.25 viral particles per cell) per slice for all subsequent trials with this vector.

Electroporation: Plasmid DNA was injected into the centre of the slice. After 90 sec, pulses were delivered using a needle pair electrode. The tip of the electrode was carefully placed near the centre of the slice, by controlling depth of insertion relative to slice thickness. Pulses were generated using the Cliniporator (IGEA, Carpi, Italy) at 1 high-voltage pulse of 360 V followed by, 8 low-voltage pulses at 36 V (178).

Ultrasound: Plasmid DNA was injected into the centre of the tumour slice. After 90 sec, low-intensity US was delivered for 5 min using a 1-MHz US transducer (CRM-1, Rich-Mar Corporation, Oklahoma, USA) with a surface area of $5cm^2$. The probe was held against the tumour throughout the duration of treatment. The parameters 1.0 W/cm^2, 20 % duty cycle were used (179).

*Lipofection (Lipo):*50 µg plasmid DNA was combined with 25 µl Lipofectamine2000 (Invitrogen, UK) (2:1) and incubated at RT for 5min. The mixture was injected directly into the centre of the tumour slice (176).

Viral vector transduction: 50 µl volumes of viral particles AdCMV (1×10^7) and AdCXCR4 (2.1×10^7) were injected directly into the centre of the tumour slice (92).

3.2.4 Tissue Viability Assessment

Viability of the individual tissue samples were determined using propidium iodide (PI) by Nucleocounter (ChemoMetec, Bioimages Ltd, Cavan, Ireland) or FACS as previously described (92). Briefly, tumour slices were dissociated into a single cell suspension using a combination of physical dissociation and enzymatic digestion. Optimisation experiments

were carried out using physical, chemical and a combination of both methods. The combination method had a slightly higher dissociation rate than chemical means, while physical methods alone had the lowest yield (results not shown) (217). The tumour slice is then cut with a scalpel and incubated in culture media supplemented with Collagenase IV (1500 U per slice), Dispase (4.8 mg per slice) and DNAse (0.01 MU per slice) for 45 min at 37 °C. The dissociated cells are removed at 15 min intervals and passed through a 70 μm nylon cell strainer (BD Falcon, UK). Both systems are based on the uptake of PI by non-viable cells.

3.2.5 Bioluminescent Imaging

Tissue slices were maintained in 6-well plates for analysis. 100 μl of 3 mg / ml firefly luciferin (Biosynth, Switzerland) was injected into the slices and another 100 μl was added into media. After 10 min of incubation, slices were imaged using the IVIS 100 Imaging system (Caliper Life Sciences, Cheshire, UK) and photons were counted for 5 min. Bioluminescence per slice was quantified using Living Image software (Caliper). Luminescence is represented as expression per gene copy administered.

3.2.6 Flow cytometric analysis

Tumour slices were dissociated into a single cell suspension using Collagenase IV (1500 U per slice) (Sigma-Aldrich, St. Louis, MO, USA), Dispase (4.8 mg per slice) (Sigma-Aldrich) and DNAse (0.01 MU per slice) (Sigma-Aldrich) for 45 min and then passed through a 70 μm cell strainer (BD Falcon, UK). The cells were fixed using 70 % ethanol and permeabilised using IFA-tx buffer (2 % FCS, 10 mM Hepes, 0.1% Triton X-100, 0.1 % Sodium Azide and 150 mM NaCl) buffer. Transduction efficiency (luciferase positive cells) was assessed by

staining cells sequentially for one hour on ice with primary luciferase antibody (10 µ) (clone Luci 17) (Abcam, Cambridge, UK) and a secondary Cyanine 5 (Cy5) antibody (1:500) (Abcam). For analysis of cell type specific expression, Cells were dual stained sequentially for one hour on ice with pan-cytokeratin antibody tagged with FITC (2 µg) (clone C-11) (Sigma-Aldrich), and primary luciferase antibody (10 µg) (clone Luci17) (Abcam,) and a secondary Cyanine 5 (Cy5) antibody (1:500) (Abcam). For Hif-1α analysis, cells were stained with anti-human Hif-1α (5 µg) antibody tagged with PE (clone 241812) (R&D systems, Abington, UK). For CXCR4 cell analysis, cells were stained with anti-mouse CXCR4 (5 µg) antibody tagged with phycoerythrin (clone 2B11) (eBioscience, San Diego, CA, USA).

3.2.7 Modelling of hypoxic conditions in *ex vivo* tissue

A system of changing oxygenation was developed to mimic the transient hypoxic conditions found in solid tumours. Hypoxic conditions were achieved by incubating slices in 20 µmol / ml cobalt chloride (Sigma-Aldrich). Three hypoxic states as previously described were studied. This analysed the effect of hypoxic or normoxic pre-conditioning, transduction and after transduction had on gene expression. The three hypoxic states included cycling (Hp-O_2-O_2, O_2-Hp-O_2), prolonged (Hp-Hp-O_2, O_2-Hp-Hp) and chronic (Hp-Hp-Hp) hypoxia (214). Each state consisted of 3 cycles. Each hypoxic (Hp, in the presence of cobalt) or normoxic (O_2, in the absence of cobalt) cycle was 8 h long. Briefly, tumour slices were incubated in the presence or absence of cobalt chloride for 8 h. Following the first cycle, tumour slices were washed in PBS and transferred to the second cycle where treatment with Ad in the presence of hypoxia or normoxia. Following the second cycle, tumour slices were washed in PBS and incubated in the 3rd cycle. After completion of the 3rd cycle at 24 h, gene expression was assessed using Bioluminescent Imaging.

3.2.8 Statistical analysis

Calculation of means, standard deviation (SD), and standard error of the mean (SEM) was done in GraphPad Prism (V 3.0; GraphPad Prism Software Incorp., San Diego, CA, USA). The significance of the differences between the individual groups was carried out using the two-tailed Student's *t*-test for paired values. Differences with a *p*-value < 0.05 were considered significant.

3.3 Results

3.3.1 Gene delivery to colorectal and other tumour tissue

While there have been no previous gene delivery studies using patient colorectal tumour tissue, prior studies by us and others have demonstrated Ad vectors to be effective gene delivery vectors in a number of other types of patient tissue including breast and ovarian tumours (92, 174, 218). To assess the potential of Ad gene delivery in colorectal cancer, Ad gene expression in colorectal tumour slices was compared with both breast and ovarian tumour slices. There were significantly higher levels of gene expression per viral particle per viable cell in colorectal tumour slices when compared with both breast ($p = 0.001$) and ovarian ($p < 0.001$) tumour slices **(Figure 3.1a)**. We also compared Ad with several gene delivery methods in tumour slices. The highest level of luciferase expression was found at 48 h for all methods (data not shown). Ad was found to have significantly greater gene expression levels per gene copy per viable cell ($p < 0.001$) in patient tumour samples when compared with other standard gene delivery methods **(Figure 3.1b)**. These results validate the clinical potential for Ad as an effective gene delivery vector for colorectal cancers.

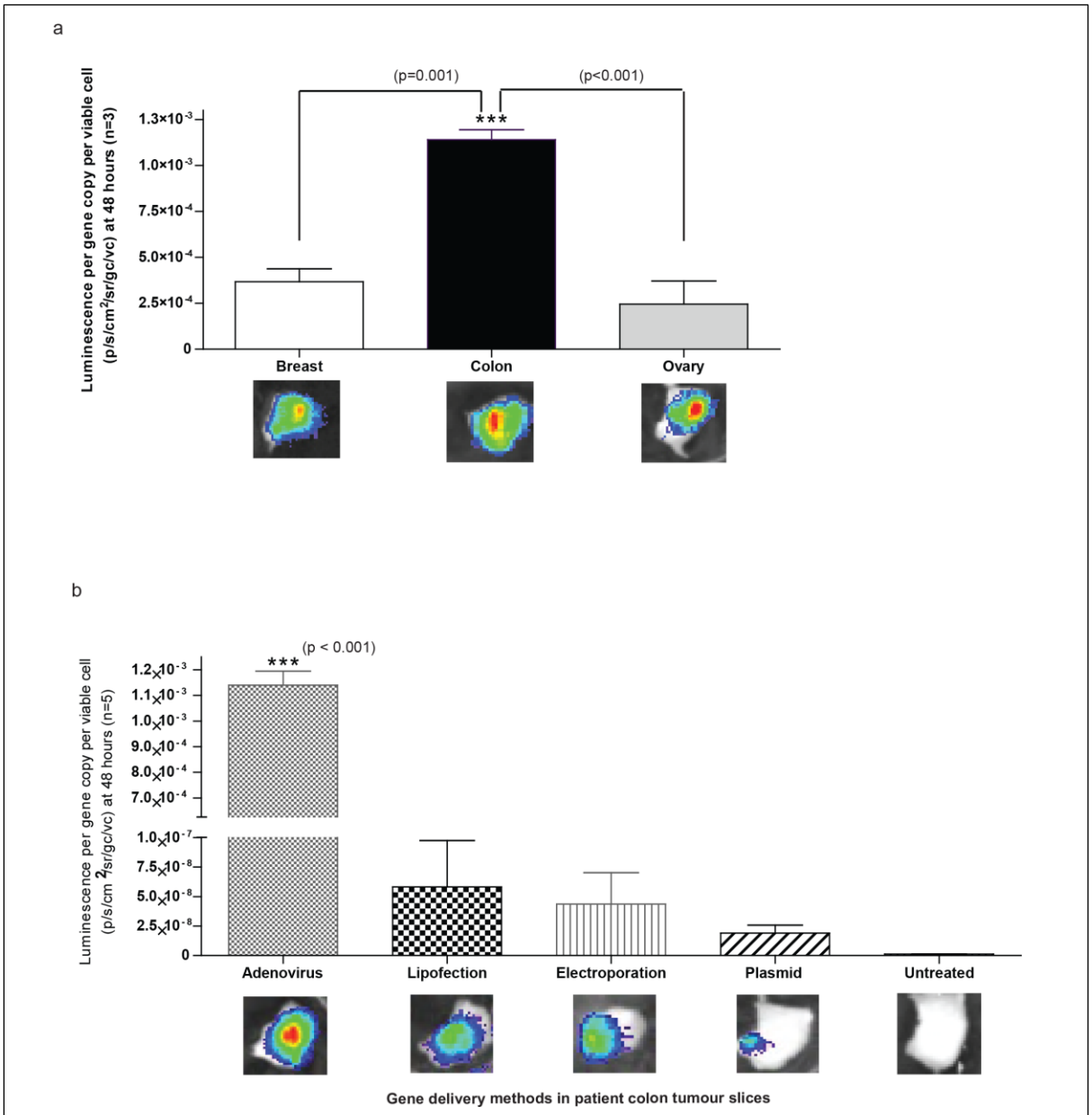


Figure 3.1 Assessment of Ad gene delivery in patient tumour tissue
(a) Ad gene delivery in patient tumour samples Patient breast, colorectal and ovarian tumour slices were administered ex vivo with AdCMV and reporter gene expression assessed at 48 h by bioluminescence imaging. A significantly higher level of bioluminescence per gene copy administered per viable cell was observed in colon tumour slices when compared with both breast ($p=0.001$) and ovarian ($p<0.001$) tumour slices. Representative IVIS images are displayed. **(b) Comparison of gene delivery methods in patient tumour samples** Colorectal tumour slices were administered FLuc using various gene delivery strategies. Bioluminescence per gene copy per viable cell was determined for each vector and results displayed represent values for various methods at 48 h post transfection / transduction. Ad was found to have the highest gene expression per gene copy administered per viable cell in patient colorectal tumour samples when compared with the other gene delivery methods ($p < 0.001$). Representative IVIS images are displayed.

3.3.2 Tumour-selective promoter-mediated restriction of transgene expression to colorectal tumour cells

The tissue selectivity of Ad under the control of either the CMV or CXCR4 promoter was examined. While CMV is ubiquitously expressed, CXCR4 is considered to be differentially expressed in tumour cells (182-184). AdCMV or AdCXCR4 were examined in tumour and normal colon and normal liver tissues. The efficiency of Ad vector is likely to be different in cancer tissue from different patients. Therefore, tissue from each patient was used to conduct each entire experiment, and for every experiment conducted, colon cancer tissue samples from 3 different patients were used. To determine the tumour expression profile between AdCMV and AdCXCR4, colorectal tumour tissue were treated with both vectors, and expression detected by bioluminescent imaging at 48 h revealed no significant difference between both vectors in tumour slices ($p = 0.285$) **(Figure 3.2a)**. Examination with normal tissue demonstrated that AdCXCR4 shows tumour tissue selectivity, with improved normal colon and normal liver to tumour ratio when compared with AdCMV **(Figure 3.2b)**. The TE in tumour slices was determined by flow cytometric analysis. Tumour slices were dissociated using both chemical and physical means to isolate a representative population of single cells which were fixed and stained with anti-luciferase antibody. We demonstrated a comparable TE (% luciferase positive cells) of AdCMV (28.03 +/- 0.73 %) and AdCXCR4 (23.9 +/- 3.25 %) ($p=0.201$) in colon tumour slices **(Figure 3.2c)**. Graphical representation of TE ratios in tumour and normal colon and liver is shown in **Figure 3.2d**. Analysis of colon and liver samples revealed minimal transduction with AdCXCR4, while a greater number of normal cells expressing luciferase with AdCMV. For effective anticancer gene therapies, it is required that a high percentage of the tumour population are targeted by the vector. To determine the percentage of tumour cells transduced, luciferase positive cells were co-localised with the tumour marker, pancytokeratin, and found that AdCXCR4 targeted a

higher percentage of tumour cells (67.4 ± 7.27 %) when compared with AdCMV (38.6 ± 5.83 %) (p=0.037) **(Figure 3.3a)**. To further validate tumour selective expression with CXCR4, gene expression in tumour-bearing slices was compared with tumour-free liver slices from patients with liver colorectal metastasis. Expression was found predominantly in slices containing tumour (p = 0.085) confirming restricted expression in tumour **(Figure 3.3b)**.

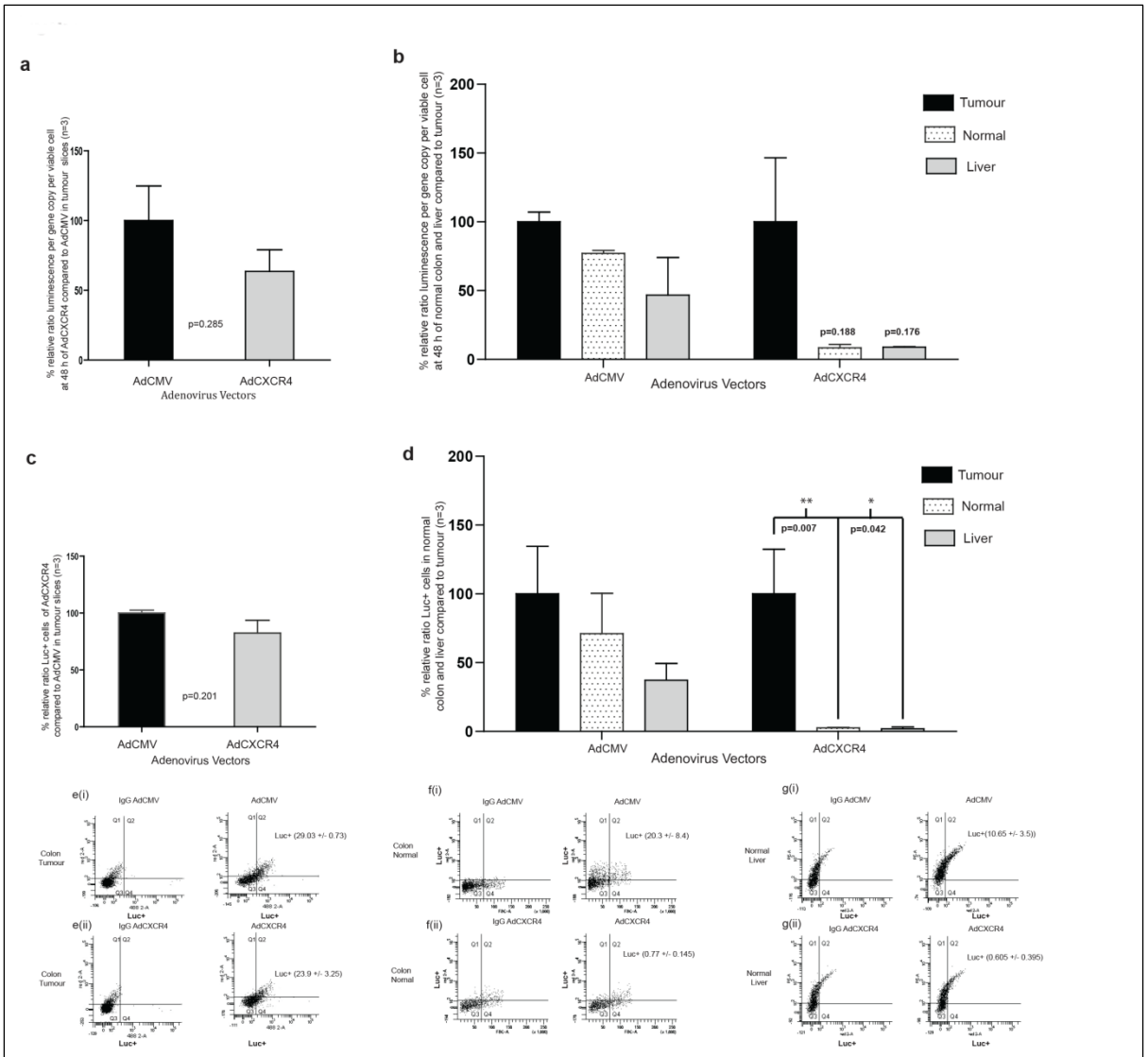


Figure 3.2 Comparison of AdCMV and AdCXCR4 reporter gene expression in tumour, colon and liver patient samples
(a) Patient tumour slices were administered AdCMV or AdCXCR4. Bioluminescence imaging at 48 h revealed a higher reporter gene expression per gene copy per viable cell with AdCMV which was not significant (p=0.285).
(b) When evaluating reporter gene expression in normal tissue, AdCXCR4 was found to have reduced reporter gene expression per gene copy per viable cell in both normal liver and normal colon tissue, while AdCMV reporter gene expression remained high in both normal colon and normal liver tissue when compared with tumour tissue. Comparison of transduction efficiency of AdCMV and AdCXCR4 in tumour, colon and liver samples The TE (% of luciferase positive cells) of both vectors in tissue slices was examined by FACS analysis. **(c)** A comparable transduction efficiency was found across both vectors (p=0.201) when examined in tumour slices. Representative flow cytometric analysis of tumour cells treated with AdCMV **[Figure e(i)]** and AdCXCR4 **[Figure e(ii)]** stained with luciferase antibody (right panel) and corresponding non-specific IgG (left panel). Percentage of cells in Q1+2 represent those cells expressing luciferase in AdCMV (29.03 ± 0.73 %) and AdCXCR4 (23.9 ± 3.25 %) (d) With AdCXCR4, TE was significantly lower in both normal colon (p=0.007) and liver (p=0.042) tissue slices, while TE from AdCMV remained high in both tumour and normal tissue. Representative flow cytometric analysis of normal colon and normal liver patient samples with AdCMV **[Figure f(i) and Figure g(i)]** and AdCXCR4 **[Figure f(ii) and Figure g(ii)]**. Percentage of cells in Q1+2 represents those cells expressing luciferase in normal colon with **[Figure f(i)]** AdCMV (20.3 ± 8.4 %) and **[Figure f(ii)]** AdCXCR4 (0.77 ± 0.145 %), while percentage of cells in Q2+4 represents those cells expressing luciferase in normal liver with **[Figure g(i)]** AdCMV (10.65 ± 3.5 %) and **[Figure g(ii)]** AdCXCR4 (0.605 ± 0.395 %).

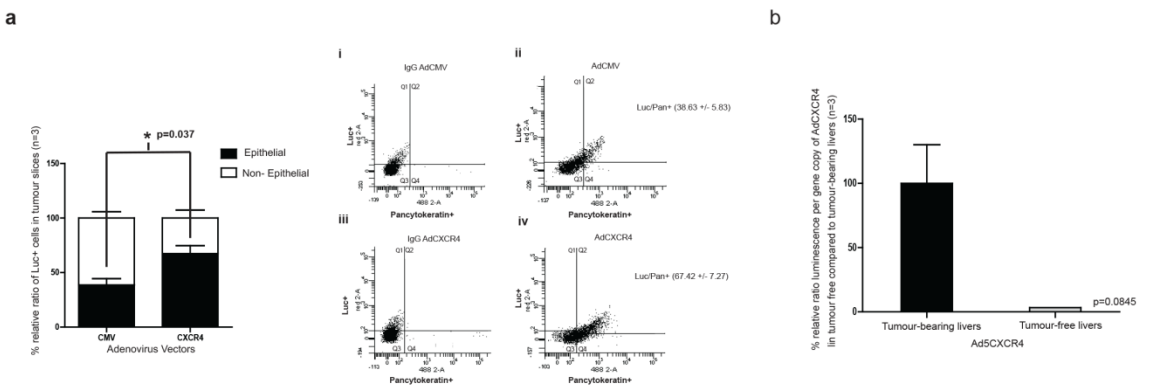


Figure 3.3 Characterisation of transgene expressing cell populations and analysis of transgene expression in tumour-bearing patient liver samples
Characterisation of luciferase expressing cell populations (a) Tumour slices were treated with both vectors. Graphical representation of luciferase positive cell populations expressing pancytokeratin within tumour slices. Co-localisation of luciferase positive cells with the tumour marker, pancytokeratin are presented in Q2 (right panels) and corresponding IgG (left panels) and showed that (iv) AdCXCR4 targeted 67.4 ± 7.27 % and (ii) AdCMV 38.6 ± 5.83 % of epithelial tumour cells (p=0.037).
Comparison of transduction efficiency of AdCXCR4 in tumour-bearing and tumour-free patient liver samples
Tumour-bearing and tumour-free liver slices were obtained from patients with colorectal liver metastasis. **(b)** Liver slices were treated with AdCXCR4 and analysis at 48 h found reporter gene expression confined to tumour-bearing liver slices (p=0.085). Percentages shown are averages of three independent experiments.

3.3.3 Modelling of hypoxic conditions in *ex vivo* tissue

A therapy that maintains therapeutic profile in hypoxic zones within solid tumours should improve the therapeutic to toxicity index and treatment outcomes when dealing with large or advanced tumours. Transgene expression levels may be modulated in response to hypoxia, and hence we investigated the effects on gene expression from the Ad vector in patient colorectal tissue slices, using the hypoxia inducer, cobalt. The key regulator of gene expression under hypoxia is hypoxia inducible factor-1 (Hif-1) (219, 220). Hif-1 is an oxygen sensitive transcription factor that accumulates under hypoxic conditions. Hif-1 is composed of two subunits, Hif-1α and Hif-1β. Hif-1α levels change in response to oxygen concentrations (220, 221). Cobalt mimics hypoxia by causing the stabilisation of Hif-1α (219, 222, 223).

Hif-1α expression was measured by flow cytometry after culture of healthy liver tissue in culture medium supplemented with cobalt chloride. Normal liver tissue was used in this study because of the difficulty in obtaining large amounts of colorectal tumour tissue from the same

patient to conduct the optimisation experiment. Large quantity of liver tissue was available from each patient and liver tissue demonstrated tissue slicing consistency. The highest concentration of cobalt chloride (250 μmol / ml) which did not significantly affect tissue viability at 48 h was used (data not shown). Liver slices cultured in cobalt chloride were assessed for Hif-1α by flow cytometry. Incubation in cobalt chloride was found to lead to a progressive rise in Hif-1α levels over time, validating the use of cobalt chloride to mimic hypoxia in this setting. Peak Hif-1α levels were found at 6 h after exposure, suggesting that 6 h is the minimum culture period required to achieve the hypoxic state **(Figure 3.4a)**. A system of changing oxygenation aimed at mimicking transient hypoxic and chronic hypoxic conditions found in solid tumours (224, 225) was developed to further test the effect of solid tumour conditions on gene expression. 8 h was taken as time point for each hypoxic (Hp, in the presence of cobalt) or normoxic (O_2, in the absence of cobalt) cycle. Once maximum hypoxia is reached, the tissue will remain in this hypoxic state. 8 h was chosen to allow comparison of data with other experiments, where gene expression was analysed after 24 h. Three hypoxic states were studied as previously described, cycling (Hp-O_2-O_2, O_2-Hp-O_2), prolonged (Hp-Hp-O_2, O_2-Hp-Hp) and chronic (Hp-Hp-Hp) hypoxia (214).

3.3.4 Effects of hypoxia on Ad gene delivery and expression

Ad gene expression was found to have similar levels in cycling hypoxia (Hp-O_2-O_2 [p=0.642], O_2-Hp-O_2 [p=0.603]) and prolonged hypoxia (Hp-Hp-O_2 [p=0.383], O_2-Hp-Hp [p=0.439]) when compared with normoxic conditions, while chronic hypoxia (Hp-Hp-Hp [p=0.239]) was found to have the most marked effect on gene expression **(Figure 3.4b)** with expression levels reduced by nearly 80%. The analysis of TE using Ad under the three physical conditions demonstrated a reduction in TE with increasing hypoxia. Significance

was found when comparing normoxia with prolonged (p=0.007) and chronic (p=0.003) hypoxia **(Figure 3.4c)**.

It is also known that some gene promoters including, CXCR4 are upregulated in hypoxic conditions (220). CXCR4 expression analysed by flow cytometery was found to increase in hypoxic conditions in the murine colon cancer cell line, CT26 **(Figure 3.5a)**. When comparing AdCMV and AdCXCR4 in chronic hypoxic conditions, AdCXCR4 was found to have an improved hypoxic to normoxic ratio when compared with AdCMV **(Figure 3.5b)** suggesting that the CXCR4 promoter mediates higher gene expression over CMV under anoxia. To study the recovery of chronically hypoxic tissue and the effect on transgene expression, we analysed the effects of gene expression after reversing chronic hypoxia to normoxic conditions. Reversing chronic hypoxia to normoxic conditions demonstrated increased gene expression, though less than achieved in complete normoxia, expression was nonetheless nearly 30 times higher for both vectors than in chronic hypoxia **(Figure 3.5c)**. This would suggest that re-oxygenation of chronically hypoxic regions in solid tumours may make them more susceptible to Ad gene therapies.

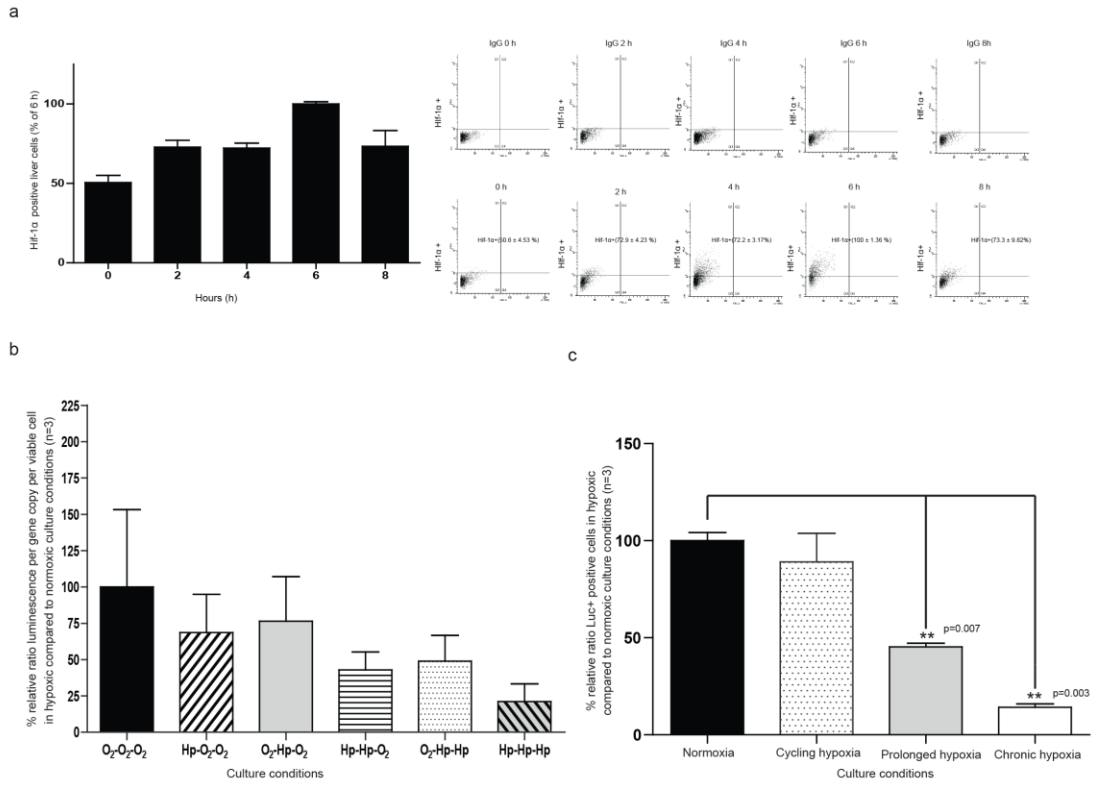


Figure 3.4 Ex vivo model of tissue hypoxia and effects on Ad vector gene expression
Optimisation of hypoxic conditions ex vivo using cobalt chloride Hif-1α surface expression was determined by flow cytometry at 0, 2, 4, 6 and 8 h. (a) Hif-1α levels were found to increase with time in the presence of cobalt with peak levels of 46.7 % of liver cells expressing HIF-1α in the presence of cobalt at 6 h of culture. We present the rest of the time points as a ratio of HIF-1α at 6 h. A graphical representation of Hif-1α expression at indicated time points as relative percentage of Hif-1α expression at 6 h is shown. Representative flow cytometric analysis of liver patient samples in the presence of cobalt chloride at indicated time points stained with Hif-1α antibody (lower panels) and corresponding IgG (upper panels) are shown on right. Percentage of cells in Q1 represents those cells expressing Hif-1α at 0 (50.56 ± 4.53 %), 2 (72.9 ± 4.23 %), 4 (72.2 ± 3.17 %), 6 (100 ± 1.36 %) and 8 (73.4 ± 9.82 %) h expressed as a relative percentage of cells expressing Hif-1α at 6 h. **(b, c) Effects of hypoxic conditions on Ad gene expression and transduction efficiency** Ad reporter gene expression was assessed in different oxygenation states, normoxia (O2-O2-O2), cycling hypoxia (Hp-O2-O2, O2-Hp-O2), prolonged hypoxia (Hp-Hp-O2, O2-Hp-Hp) and chronic hypoxia (Hp-Hp-Hp). **(b)** There was no significant difference across normoxic (O2-O2-O2), cycling (Hp-O2-O2, O2-Hp-O2), prolonged (Hp-Hp-O2, O2-Hp-Hp) and chronic (Hp-Hp-Hp) hypoxic conditions ($p > 0.239$). However, chronic hypoxia (Hp-Hp-Hp) was found to have the most detrimental effect with reporter gene expression levels reduced by 80% when compared with normoxia. **(c)** Transduction efficiency under both prolonged and chronic hypoxia was reduced significantly ($p = 0.007$ and $p = 0.003$ respectively) when compared with normoxia, while cycling hypoxia demonstrated a similar TE with normoxia.

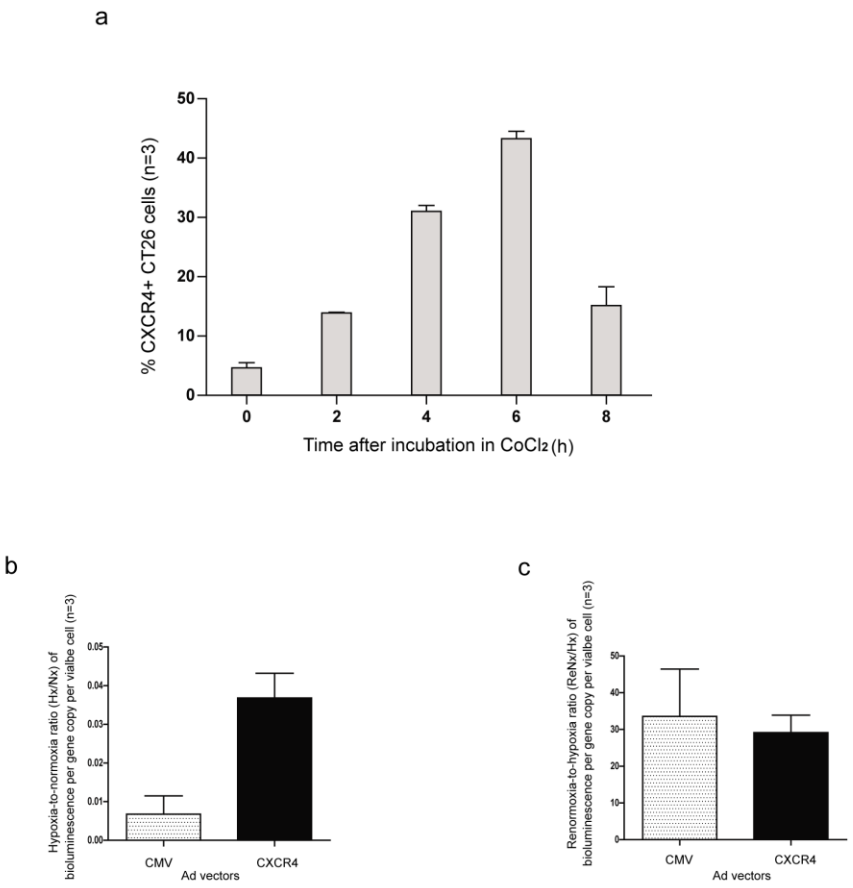


Figure 3.5 AdCXCR4 gene delivery and expression in hypoxic tissue
(a) Effect of hypoxia on CXCR4 expression in CoCl2 treated CT26 cells. CT26 murine colorectal cancer cells were cultured in the presence of cobalt chloride and CXCR4 expression determined by flow cytometry using anti-mouse CXCR4 antibody at 0, 2, 4, 6, and 8 h. CXCR4 levels were found to increase with prolonged culture in cobalt with peak levels found at 6 h. A graphical representation of CXCR4 expression at indicated time points expressed as relative percentage of Hif-1α expression at 6 h is shown. **(b) Comparison of CMV and CXCR4 activity in hypoxic conditions in patient tumour tissue.** The effect of CXCR4 promoter on gene expression in chronic hypoxic conditions was studied. Tissue slices were treated using both promoters at chronic hypoxic (Hp-Hp-Hp) and normoxic conditions and gene expression per viral particle per viable cell was analysed at 24 h. Gene expression for each promoter was expressed as a ratio of levels found at hypoxic to normoxic conditions. The CXCR4 promoter was found to have a higher hypoxia to normoxia ratio of gene expression per gene copy when compared with CMV. **(c) Effect of reversing chronic hypoxia to normoxia on gene delivery in patient tumour tissue.** Slices pretreated in chronic hypoxic conditions (Hp-Hp-Hp) were reintroduced to normoxic conditions. Slices were then administered both promoters and gene expression per viral particle per viable cell for each promoter was expressed as a ratio of gene expression found at re-normoxia to chronic hypoxia at 24 h. There was greater reporter gene expression per viral particle per viable cell for both promoters with CMV 33.6 times (p=0.079) and CXCR4 29.1 times (p=0.606) higher than in chronic hypoxic conditions. Means of ≥ 3 independent experiments, ± SEM are shown.

3.4 Discussion

The treatment of advanced colorectal cancer remains an elusive predicament. The failure of conventional therapeutics to maintain remission has called for newer treatment options to be explored. We propose the use of gene therapy in colorectal cancer as an adjunct to surgical resection. Gene therapy offers the potential of a targeted therapy resulting in less non-specific effects. Successful gene therapy depends on the ability to transfer therapeutic genes to tumour cells. Adequate gene expression in tumour cells remains the main stumbling block in the gene therapy of cancer. Numerous gene delivery methods are under investigation with each method offering its own merits (226). Ad is an efficient gene delivery vector (227) for numerous cancers (217, 228) including breast and ovarian cancer and has in this study been shown to be more effective in colorectal cancers when compared with breast and ovarian cancer. Ad also demonstrated higher levels of gene expression when compared with other standard gene delivery methods including electroporation and lipofection with FACS analysis demonstrating TE > 30 %.

To reduce the risk of nonspecific gene expression, transcriptional targeting offers promise. CXCR4, identified as a co-receptor for HIV-1, is a chemokine receptor functionally expressed on a multitude of tissues and cell types. CXCR4 and its ligand, CXCL12 (stromal cell-derived factor-1), play an important role in lymphocyte trafficking and recruitment, haematopoiesis and development processes such as organogenesis, vascularisation and embryogenesis. The binding of CXCL12 to CXCR4 (CXCR4 / CXCL12) initiates intracellular signalling through several divergent pathways including the Ras-MAPK signalling pathway which can result in a variety of responses such as chemotaxis, cell survival and/or proliferation, increase in intracellular calcium, and gene transcription (229, 230). The up-regulation of CXCR4 in cancer cells leads to the activation of the Ras-MAPK signalling pathway resulting in increased proliferation, defects to apoptosis, invasiveness and

neovascularisation. The high expression levels of CXCR4 in cancer cells and low or absent expression levels in normal cells enabled the use of CXCR4 as a tumour- selective promoter to regulate Ad gene expression.

When comparing AdCXCR4 with AdCMV, while gene expression was lower in tumour slices with AdCXCR4, we found relatively low expression in normal tissue (colon and liver), while levels in normal tissue with AdCMV remained high (Figure 2b). The TE in tumour slices was similar between both vectors (Figure 2c), while there was a significantly lower TE in normal tissue (colon [p=0.007] and liver [p=0.042]) when using AdCXCR4. Solid tumours are known to be composed of a variety of cells, of which only 30 - 40 % comprise tumour cells. Analysis of luciferase-positive cells demonstrated that AdCXCR4 targeted 67 % of epithelial tumour cells, a higher percentage of tumour cells within the tumour slice when compared with AdCMV which targeted only 39 % (Figure 3a). Previous reports have demonstrated therapeutic response using similar transduction efficiency (231)**.** Therefore, selective targeting of tumour cells by AdCXCR4 will lead to more effective tumour destruction. The improved tumour to normal ratio will also reduce the non-specific effects to normal tissue, allowing normal tissue to recover to an adequate functional level. The selective tumour targeting will enable a higher therapeutic index when considering treatment using this vector. The tumour microenvironment is characterised by heterogeneous blood flow, resulting in varying regions of hypoxia. Hypoxia provides an environment directly facilitating chemo- and radio-resistance and also encouraging the evolution of phenotypic changes inducing permanent resistance to treatment (232).

The one fundamental question remaining from Ad tumour delivery was the feasibility of gene delivery to hypoxic regions of solid tumours. Work on the effects of hypoxia on Ad gene delivery has only been demonstrated *in vitro*. Unlike previously published studies, we have analysed the effects of hypoxia on Ad gene delivery in patient tissue slice. We utilised a

cobalt-induced hypoxia model. Hif-1α levels are known to accumulate when exposed to proteasomal inhibitors, transition metals (e.g. cobalt), iron chelators or reducing agents (219, 233). Cobalt mimics hypoxia by two mechanisms (31, 222, 223). It inhibits the hydroxylation of Hif-1α by binding to the iron-binding domain of Hif hydroxylase, inactivating the hydroxylase activity, while in the event that Hif-1α becomes hydroxylated, cobalt can also bind directly to the hydroxylated proteins to prevent the interaction between Hif-1α and pVHL, thereby preventing Hif-1α degradation (219). Cobalt was used instead of hypoxic chambers, as we aimed to study the isolated effects of hypoxia on gene delivery. Changing oxygen tensions will have local effects such as changes to pH, which in a dynamic *in vivo* system will be equilibrated, however in a static *ex vivo* model, these factors may account for effects on gene delivery. The luciferase bioluminescent detection system used for analysing gene expression is a very powerful, non-invasive method allowing rapid and sensitive longitudinal follow-up of gene expression in this model. The disadvantages of this model include the requirement of a substrate to enable light emission, utilisation of an oxidation step that requires molecular oxygen and the dependence of light signal on tissue depth. Therefore, this detection system will be less sensitive at measuring gene expression in thick tumour slices and in hypoxic states (234). Furthermore, oxygen tension may vary within solid tumours. Our model utilises a relatively thin tumour slice, non toxic substrate and cobalt chloride was used to mimic hypoxia having no impact on oxygen levels or tissue viability.

The effects of Ad gene delivery to hypoxic cell *in vitro* have been previously studied in cell lines (214). However, effects on multi-cell layers or solid tissue and changing hypoxic conditions are to date unexplored. In this study, we highlight the importance of hypoxia as a potential barrier to gene expression. The fact that neither cycling hypoxia nor prolonged hypoxia, the major hypoxic states in solid tumours, had significant detrimental effects on gene expression is promising for future Ad gene therapy strategies for colorectal cancer.

However, chronic hypoxia was found to reduce gene expression by more than 80 %. This detrimental effect on gene expression was found to be reversible when slices were reintroduced to normoxia, with gene expression levels similar to those achieved in normoxic conditions. Chronically hypoxic cells are usually located in regions with inadequate and underdeveloped vascular bridges. Vascular normalisation using anti-angiogenic agents such as vascular endothelial growth receptor-2 antibodies and subsequent reduction in tumour hypoxia may be an alternative strategy to improve gene expression. The Coxsackie virus and Ad receptor (CAR) expression, which plays a crucial function in adenoviral cell entry, has been shown to decrease as tumours progress reducing efficacy of Ad vectors. This may be countered by using fibre mosaic Ad vectors, which utilise different receptors for cell entry (235). Ad can be delivered directly into the tumour mass limiting toxic effects on normal cells. Direct delivery can be achieved using endoscopy which is non-invasive, economical, well tolerated and relatively easy to perform (236).

Gene therapy is hampered by poor gene transfer to the tumour mass via both viral and non-viral vectors including adenoviral vectors. Many studies have explored the utilization of replicating Ad (oncolytic Ades) to enhance the viral distribution in the tumour mass, enhanced gene expression and oncolysis (237). There have been a few studies on the effects of hypoxia on gene expression and replication of Ad (238, 239). Hypoxia has been shown to reduce adenoviral replication in cancer cells by down-regulation of viral protein expression (239). A number of hypoxia-selective oncolytic Ad, generated by incorporating hypoxia-responsive elements into synthetic promoters have been shown to be effective and safe (240).

In conclusion, the Ad vector is a promising candidate for gene therapy in colorectal cancers. The AdCXCR4 vector demonstrates tumour selective expression with low normal and liver levels indicating that the CXCR4 promoter may allow tumour treatment with a low potential toxicity in normal tissue. The effective targeting in hypoxic conditions suggests that Ad may

offer a better strategy against the hypoxic component of human tumours than current therapeutics. A greater therapeutic index and enhanced hypoxic targeting may potentially result in better down-staging outcomes leading to improved surgical and hence treatment outcomes.

Chapter 4

Targeting of Breast Metastases Using a Viral Gene Vector with Tumour-selective Transcription

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1. **Targeting of breast metastases using viral gene vector with tumour selective transcription.** Simon Rajendran, Sara Collins, Jan P van Pijkeren, Deirdre O' Hanlon, Gerald C O'Sullivan and Mark Tangney. Anticancer Research 2011 May;31(5):1627-35. PMID: 21617219
2. **Murine bioluminescent hepatic tumour model.** Simon Rajendran, Slawomir Salwa, Xuefeng Gao, Sabin Tabirca, Deirdre O'Hanlon, Gerald C. O'Sullivan, Mark Tangney Video Article. Journal of Visualized Experiments. 2010 Jul 17;(41). PMID:20689502

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Abstract

AAV vectors have significant potential as gene delivery vectors for cancer gene therapy. However, broad AAV tissue tropism results in nonspecific gene expression. We investigated the use of the CXCR4 promoter to restrict AAV expression to tumour cells, in subcutaneous mouse models of breast cancer and in patient samples, using bioluminescent imaging and flow cytometric analysis. We demonstrated higher transgene expression in subcutaneous MCF-7 tumours relative to normal tissue (muscle) using the CXCR4 promoter, unlike a ubiquitously expressing CMV promoter construct, with preferential AAVCXCR4 expression in epithelial tumour and CXCR4-positive cells. Transgene expression following intravenously administered AAVCXCR4 in a model of liver metastasis was detected specifically in livers of tumour bearing mice. *Ex vivo* analysis using patient samples also demonstrated higher AAVCXCR4 expression in tumour compared with normal liver tissue. This study demonstrates for the first time, the potential for systemic administration of AAV vector for tumour-selective gene therapy.

4.1 Introduction

Breast cancer is the leading cause of cancer-related mortality in middle-aged women despite significant advances in conventional therapies (157). The prevalence of locoregional recurrence and distant metastasis can be as high as 13 % and 10 % respectively despite improvements in adjuvant treatment e.g. chemoradiotherapy and hormone therapy. The main stumbling block with both chemotherapy and radiotherapy is the induction of dose- limiting normal tissue toxicity, which reduce their clinical effectiveness. Liver metastases are present in 15 % of metastatic breast cancer patients and are the only site of distant disease in one-third of these patients. Therefore, targeted treatment of localised liver disease would have the potential to improve outcome in a significant proportion of these patients. Currently, surgical resection is the treatment of choice for isolated liver disease. The liver's unique anatomy presents numerous challenges when evaluating for surgical resection, resulting in fewer than 20 % of liver tumours being suitable for surgical resection, and up to 90 % of unresected cases undergoing chemotherapy fail to maintain long-term remission and die from liver failure. These failings warrant new treatment options for this subset of patients.

Gene therapy could potentially offer a safe and effective treatment modality in such patients. An ideal delivery system in patients with liver disease should offer effective transduction in the liver with prolonged gene expression restricted to infiltrative tumour cells only. Furthermore, it requires the capacity to be delivered by both local and systemic routes for isolated deposits and disseminated disease respectively, minimal expression in non-target organs and an acceptable safety profile. The use of an adeno-associated virus (AAV) approach has a number of features that make AAV an ideal vector for gene therapy strategies: it offers long-term gene expression, there is little or no cell-mediated immune response to the virus, and it is not associated with any disease (241). While AAV-derived vectors have shown promise in many clinical trials to treat a number of non-malignant conditions (33, 163,

168, 242), their use has not been examined widely in cancer settings (176, 228, 243). The suitability of AAV for tumour treatment lies in its ability to efficiently and stably transfect a wide range of cells, including dividing and non-dividing cells. It also has the ability to penetrate the stroma of solid tumours due to its small size and can offer an excellent safety profile combined with reduced potential for activation of inflammatory or cellular immune responses.

AAV2 serotype was the first parvovirus to be isolated from humans and the first to be used as a vector for gene therapy application (244). AAV2 has a broad tissue tropism due to the wide expression of AAV receptors in all tissues (245). This would enable the targeting of systemically disseminated disease and, furthermore, AAV vectors have been shown to direct stable gene transfer and expression in hepatocytes. This feature makes them an attractive tool for treatment of liver metastatic disease.

Currently, tissue targeting using AAV is mainly achieved by the localised delivery of treatments, and/or use of different, more recently identified serotypes with restricted tissue tropisms (155, 246). However, local delivery can only be achieved in limited situations such as for superficial deposits, skin and muscle, or may require invasive means for access. The most applicable treatment strategy for liver and distant metastasis is via systemic delivery. Yet the broad host spectrum of AAV may limit its systemic use due to transgene expression in normal tissue.

This paper studies the use of transcription targeting for AAV to restrict transgene expression to tumour cells, thereby sparing normal tissue, an outcome that conventional therapeutic approaches have failed to achieve. Transcription targeting using specific promoters is one of the oldest and most widely used strategies for targeting gene therapy (3, 9). Tumour-specific promoters (TSP) restrict the expression of genes of interest to tumour cells (182). Numerous TSPs have been applied in preclinical studies, including alpha-

fetoprotein, carcinoembryonic antigen, survivin, CXCR4 and osteocalcin (3). However, their use has not been investigated in AAV as widely as in Ad. CXCR4 is a chemokine receptor that has been shown to be expressed at high levels in many types of cancer, including breast cancer, but is repressed in normal tissue (183, 247). CXCR4 regulates the growth of primary and metastatic tumours and tumour cells are believed to adopt the expression of chemokine receptors to facilitate metastatic spread through chemokine gradients (184, 248).

The aim of our study was to develop a strategy to increase efficiency and specificity of tumour gene delivery. This paper incorporates the use of the CXCR4 as a TSP in an AAV vector and investigates its tumour selective potential in models of both primary and metastatic breast cancer.

4.2 Materials and Methods

4.2.1 Cell culture

The human adenocarcinoma breast cancer cell line, MCF-7 (ATCC, Manassas, VA, USA) was maintained in Dulbecco's minimal essential Medium (DMEM) (GIBCO, Invitrogen Corp., Scotland) supplemented with 10% foetal calf serum (FCS), 100 U / ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine and sodium pyruvate and grown at 37 °C in a humidified atmosphere of 5 % CO_2.

4.2.2 Animals and tumour induction

All murine experiments were approved by the Ethics Committee of University College Cork. Mice were obtained from Harlan Laboratories (Oxfordshire, UK). They were kept at a constant room temperature (22 ^{o}C) with a natural day/night light cycle in a conventional animal colony. Standard laboratory food and water were provided *ad libitum*. Before experiments, the mice were afforded an adaptation period of at least 7 days. Female mice in good condition, without fungal or other infections, weighing 16 – 22 g and of 6 – 8 weeks of age, were included in experiments. For routine tumour induction, 2×10^6 tumour cells, suspended in 200 µl of serum-free DMEM were injected subcutaneously into the flank of female MF1*nu/nu* mice. The viability of the cells used for inoculation was greater than 95 % as determined by Nucleocounter (ChemoMetec, Bioimages Ltd, Cavan, Ireland). Following tumour establishment, tumours were allowed to grow and develop and were monitored twice weekly. Tumour volume was calculated according to the formula $V=(ab^2)\ \Pi/6$, where *a* is the longest diameter of the tumour and *b* is the longest diameter perpendicular to diameter *a*.

When tumours reached approximately 100 mm^3 in volume, the mice were randomly divided into experimental groups.

4.2.3 Induction of hepatic metastases

Isolated MCF-7 liver tumours were induced in MF1 *nu/nu* mice as described elsewhere (165, 249). Briefly, mice were anaesthetised using 100 µl intraperitoneal injection of Phosphate buffered saline (PBS) containing 1.5 mg ketamine hydrochloride (Vetoquinol, Dublin, Ireland) and 300 µg xylazine (Chanelle, Loughrea, Ireland) and placed on a heating pad. The abdominal skin was disinfected with 10 % w/w iodinated povidone antiseptic skin cleaner (Videne, Ecolab, Leeds, UK). A 1 - 1.5 cm incision was made in the left subcostal margin. The abdominal muscles were divided, the peritoneum opened and the abdomen entered. The spleen was gently mobilised and the mid-body of the spleen divided between two 4 / 0 Vicryl ligatures (Ethicon, Johnson & Johnson, Berkshire, UK) placed between splenic vascular pedicles, such that each had its own vascular pedicle and was still in contact with the portal circulation. MCF-7 cells (5×10^5) were inoculated into one hemi-spleen, which was removed 10 minutes later. The other hemi-spleen was returned intact, leaving the mouse with half an immunologically competent spleen. Mass closure of peritoneum and muscle was performed using 4 / 0 PDS (Ethicon, Johnson & Johnson, Berkshire, UK) and the skin closed using 4 / 0 Prolene (Ethicon, Johnson & Johnson, UK). Animals were fed a moistened diet for 24 hours. Carpofen (5 mg / kg, Norbrook laboratories, Newry, UK) was used for postoperative analgesia. Animals were observed for the development of disease and treated at day 7 post inoculation.

4.2.4 Creation of splenic port

The use of a splenic port for hepatic delivery has been described previously (250). Liver metastases were induced as described above. For this model, the intact hemi-spleen was transposed to a subcutaneous position while carefully preserving its vascular pedicle. The defect in the abdominal wall was reapproximated using 4 / 0 PDS to hold the spleen in position, over which the skin was closed. Postoperative recovery was as above. The subcutaneously placed hemi-spleen was easily visible and palpable for direct delivery into the portal circulation.

4.2.5 Production of AAV vectors

A schematic representation of the relevant elements of the vector constructs utilised is shown in **Figure 4.1**. AAVCMV expressing firefly luciferase was generated (176). Firefly luciferase-expressing AAVCXCR4 vector was generated as follows: pDriveCXCR4LacZ plasmid was purchased from Invivogen (Cayla SAS, Toulouse, France). The *CXCR4* promoter DNA sequence was PCR amplified using primers designed with *M1u*I and *Eco*RI restriction overhangs, (forward- M1uI5' CATACGACGCGTAGTTGACAATTAATCAT CGGC3, reverse:*Eco*RI 5'CGAATTCGTAACCGCTGGTTCTCCAAGAT3') and cloned into pAS (AAV-luc). This *CXCR4* promoter DNA sequence was inserted upstream of the firefly luciferase gene. Thus the levels of luciferase expression reflect the activity of the *CXCR4* promoter. Clone sequence was validated by sequencing (MWG Biotech, Ebersberg, Germany) and restriction enzyme analysis. AAV particles were generated using the AAV Helper-Free System (Stratagene, Agilent, Dublin). AAV particles were purified using Virakit AAV Purification Kit (Virapur, San Diego, USA) as per manufacturer's instructions.

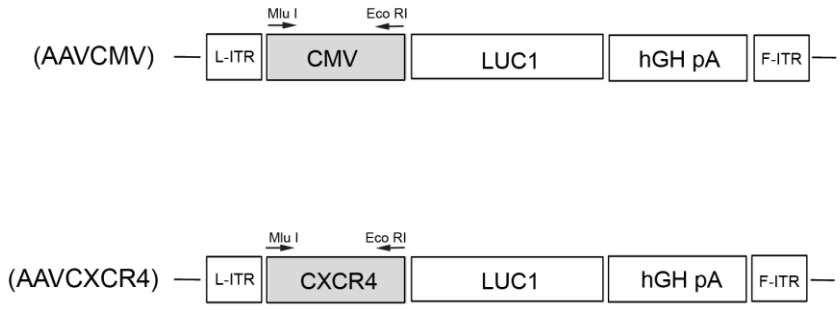


Figure 4.1 Vector Constructs
Schematic representation of the relevant elements of the AAV vector constructs utilised in this study

4.2.6 Murine AAV treatments

All treatments were carried out under general anaesthesia in Class 2 containment hoods. Viral vector particles were administered in a volume of 50 µl for all delivery routes. In subcutaneous tumour and muscle delivery, 1×10^8 infectious particles were injected slowly into the centre of the subcutaneous tumour or into the quadriceps femoris muscle of the lower limb. For direct intrahepatic delivery, a right subcostal incision was made and 1×10^8 infectious particles were injected under direct vision into the liver capsule. Due to difficulty generating large viral titres, we developed the splenic port model as direct intravenous access to the liver via the portal circulation. Following tumour cell delivery and excision of the inoculated hemispleen, the untreated hemispleen is used to create a port that can be used for hepatic portal delivery. The hemispleen is transposed to a superficial position in a subcutaneous pocket carefully preserving its vascular position. For splenic port injections, 1×10^8 infectious particles AAV vectors were injected into the subcutaneous hemi-spleen, allowing

direct delivery of vectors into the portal circulation and therefore into the liver. http://www.jove.com/video/1977/murine-bioluminescent-hepatic-tumour-model

4.2.7 Human tissue samples

This study was approved by the Clinical Research Ethics Committee of the Cork Teaching Hospitals and informed consent was obtained from patients. Tissues were collected and cultured as described previously (215, 251). Briefly, breast tumour samples were obtained from patients undergoing wide local excision or mastectomy because of breast cancer. Tumour free liver tissue was obtained from three patients undergoing partial hepatectomy for metastatic liver disease. Fresh tumour material upon collection was placed in collection medium DMEM supplemented with penicillin (200 IU / l), streptomycin (200 µg / l) and fungizone (250 µg / ml) at 4 °C. Slicing at 2000 µm was performed aseptically using a Leica Vibrotome (Laboratory Instruments and Supplies, Meath, Ireland). Slices were incubated in 6-well plates (1 slice / well) containing culture media at 37 °C with 5 % CO^2 in a humidified environment Slices were injected with AAV after 6 hours of incubation and maintained at culture conditions.

4.2.8 Bioluminescent imaging (BLI)

For *in vivo* imaging, mice were anaesthetised and were injected intraperitoneally with 100 µl of an aqueous solution of luciferin (3 mg / ml) and placed under the chamber of the CCD camera system of the Xenogen IVIS 100 Imaging system (Caliper Life Sciences, Runcorn, Cheshire, UK) and photons were counted for 3 min. Regions of interest were identified and quantified using LIVING Image software (Caliper Life Sciences, Runcorn, Cheshire, UK). For *ex vivo* imaging, tissues of interest were excised, placed in 6-well tissue plates with 150

μg/ml D-luciferin in PBS and imaged for 3 min. Luminescence is represented as expression per gene copy administered.

4.2.9 Flow cytometric analysis

Subcutaneous tumours were dissociated into a single cell suspension using Collagenase IV (1500 U per tumour) (Sigma-Aldrich, St.Louis, MO, USA), Dispase (4.8 mg per tumour) (Sigma-Aldrich, St.Louis, MO, USA) and DNAse (0.01MU per tumour) (Sigma-Aldrich, St.Louis, MO, USA) for 45 min and then passed through a 70 μm cell strainer (BD Falcon, Dublin, Ireland). The cells were fixed using 70% ethanol and permeabilised using IFA-tx buffer (2% FCS, 10 mM Hepes, 0.1% Triton X-100, 0.1 % Sodium Azide and 150mM NaCl). To assess the transfection efficiency (luciferase positive cells), cells were stained sequentially for one hour on ice with primary luciferase antibody (10 μg) (clone Luci17) (Abcam, Cambridge, UK) and a secondary Cyanine 5 (Cy5) antibody (1:500) (Abcam, Cambridge UK). For CXCR4 cell analysis, cells were stained with anti-mouse CXCR4 (5 μg) antibody tagged with phycoerythrin (clone 2B11) (eBioscience, San Diego, CA, USA). For epithelial cell analysis, cells were stained with pan-cytokeratin (3 μg) antibody tagged with PE (clone C-11) (Abcam, Cambridge, UK).

4.2.10 Statistical analysis

Calculation of means, standard deviation (SD), and standard error of the mean (SEM) was carried out in GraphPad Prism (V 3.0; GraphPad Prism Software Incorp., San Diego, CA, USA). The significance of the differences between the individual groups was carried out using the two-tailed Student's t-test for paired values. Differences with a p-value <0.05 were considered significant.

4.3 Results

4. 3.1 Preferential expression of AAVCXCR4 in subcutaneous and hepatic MCF-7 tumours compared with non-malignant tissue

A human breast cancer (MCF-7) murine model was used to study tissue specificity of AAV vectors under the control of CMV (AAVCMV) and CXCR4 (AAVCXCR4) promoters. To establish that it was possible to detect both AAVCMV and AAVCXCR4 in muscle and tumour, and also to determine the expression ratio between tissues over time, subcutaneous MCF-7 tumours and muscle for each mouse were treated with direct administration of AAVCMV or AAVCXCR. AAV vector transduction is often slow due to the necessity of conversion of the single-stranded genome into double-stranded DNA. Previous work by us and others has demonstrated a delay in AAV-related expression following administration; therefore, experimental animals were not imaged prior to day 7 post vector delivery (155). At day 7, AAVCMV-related luminescence was observed in both muscle and tumour with a relative expression ratio of 50 ± 5% in muscle when compared with tumour (**Figure 4.2a**). At day 10, AAVCMV expression remained relatively unchanged in tumour, while there was a further increase in expression in muscle (58 ± 25 %), surpassing that of the tumour. In the case of AAVCXCR4-related luminescence, tumour levels were markedly higher than in muscle, with expression ratios of 10 % in muscle relative to tumour at both days 7 and 10, although differences did not reach statistical significance.

To assess expression levels in a model for metastatic disease of the liver, confined hepatic tumours were established by intrasplenic administration of MCF-7 tumour cells. At 7 days post tumour induction, particles were injected directly into the liver. AAVCXCR4 demonstrated tumour to normal expression ratios in liver tumours similar to those observed in

subcutaneous tumour animals (Figure 4b). AAVCXCR4 muscle expression was 54 % that of tumour at day 7, although not statistically significant. Conversely, AAVCMV muscle expression was over 20-fold higher than tumour levels.

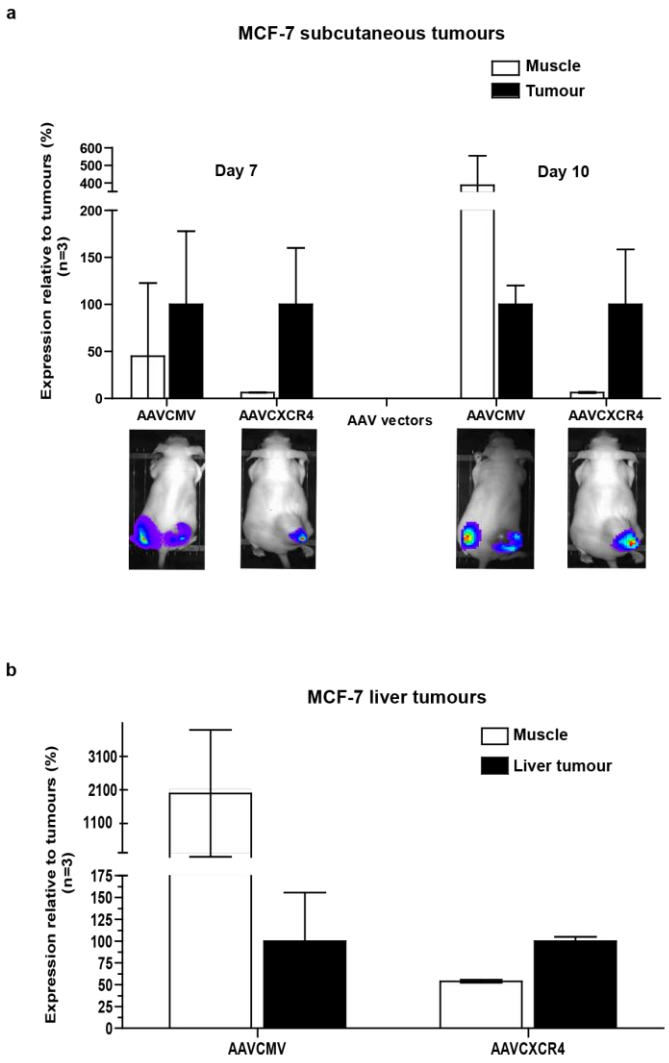


Figure 4.2 Relative reporter gene expression in muscle, subcutaneous tumours and liver tumours
Subcutaneous MCF-7 tumours greater than 100 mm3 and quadriceps muscle of MF1 nu/nu mice were treated with direct injection of 1 × 108 AAV particles. (a) Luminescence was detected in live mice 7 and 10 days after virus injection by whole body IVIS imaging. Region of interest (ROI) analysis was performed and the ratio of luminescence from tumour and muscle determined for each mouse. At each time point, for each vector, tumour luminescence readings were taken as 100%, and the percentage relative luminescence for corresponding muscle was expressed. Representative IVIS images of mice are displayed below the corresponding vector. AAVCXCR4 expression in muscle was less than 10% relative to tumour at both days 7 and 10 ($p<0.135$). (b) MF1 nu/nu mice bearing MCF-7 liver tumours were treated with direct injection of 1 x 108 AAV particles. Luminescence was detected in livers of mice 7 days post virus injection by whole body IVIS imaging and the percentage relative luminescence expressed as before. AAVCXCR4 expression in muscle was less than hepatic tumour expression at day 7, while AAVCMV expression in muscle was 20 times higher than that in liver tumours.

4.3.2 Characterisation of intratumoural cell populations expressing transgene

To determine transduction efficiencies and to identify cell populations targeted by both viral vectors, subcutaneous MCF-7 tumours were treated with either vector at day 10 post tumour induction. Treated tumours were harvested and dissociated into single cells as described previously (46). The transduction efficiency (luciferase-positive cells) of AAVCMV was greater than that of AAVCXCR4 (**Figure 4.3a**). To examine the correlation between reporter gene expression and a CXCR4 protein-positive phenotype, luciferase-positive populations were co-stained with anti-CXCR4 antibodies. Results shown in **Figure 4.3b** demonstrate that AAVCXCR4 shows transcriptional selectivity for CXCR4-positive cells (43 ± 5 %) when compared with AAVCMV (25 ± 5 %) within solid tumours.

Solid tumours are known to be composed of distinct subpopulations including tumour epithelial cells, stromal cells and immune-associated cells. When luciferase-positive cells were co-stained with an epithelial-specific antibody (pancytokeratin), there was a higher percentage of pancytokeratin-positive (tumour) cells expressing luciferase in tumours treated with AAVCXR4 (67 ± 5 %) when compared with tumours treated with AAVCMV (32 ± 7 %) (**Figure 4.3c**), demonstrating that AAVCXCR4 has transcriptional selectivity for CXCR4 positive and epithelial tumour cells within solid tumours, while AAVCMV, although achieving higher expression levels, mediates transcription in all cell types.

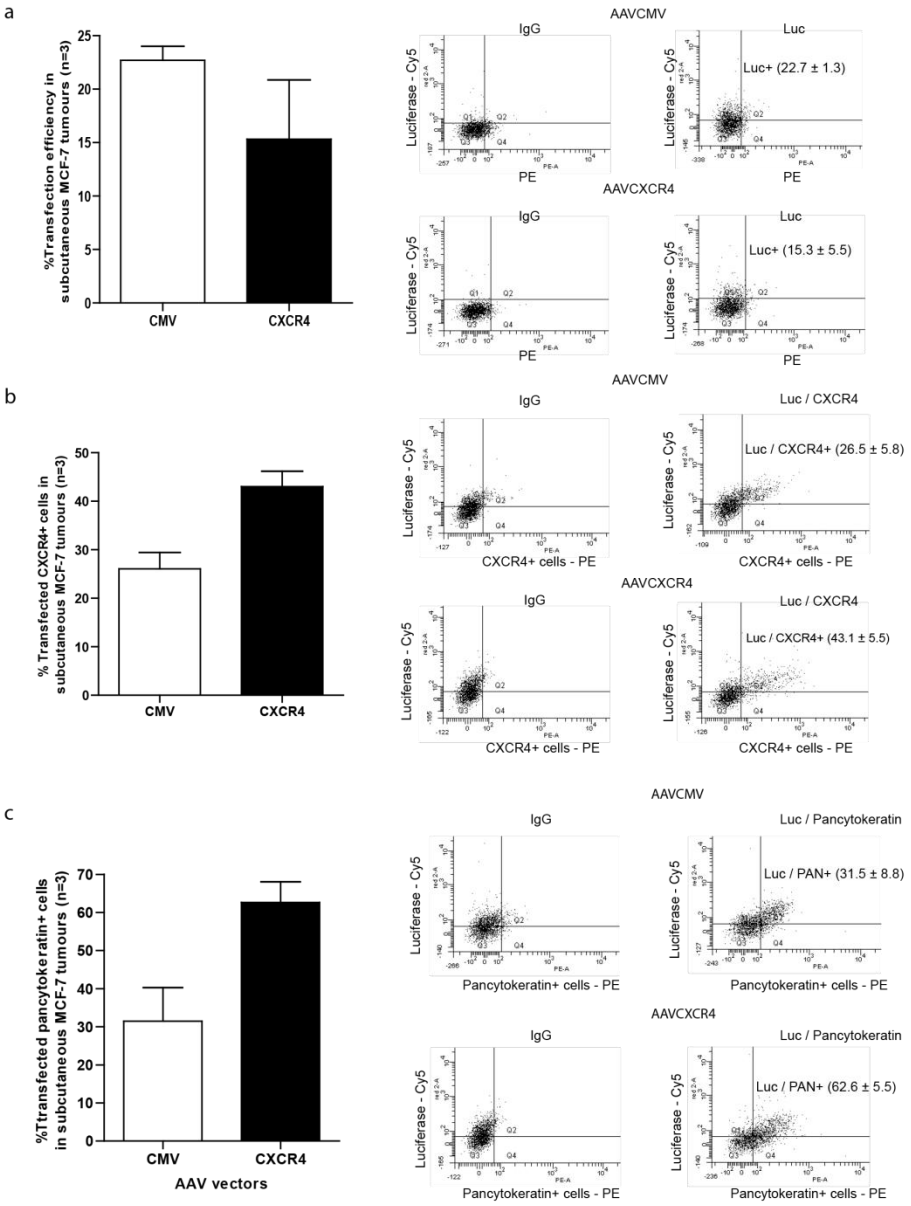


Figure 4.3 Characterization of cell populations expressing transgene in subcutaneous tumours
Subcutaneous MCF-7 tumours were directly administered AAVCMV or AAVCXCR4. Seven days after treatment, tumours were dissociated into single-cell suspensions and subjected to flow cytometric analysis. To establish transduction efficiency (% luc+ cells) cells were stained with anti-luciferase antibody (detected as cyanine 5 labelled cells [red]). Epithelial tumour cells were identified with a pan-cytokeratin antibody (detected as phycoerythrin-labelled cells [PE-A]) and CXCR4-positive populations were stained with a CXCR4 antibody (detected as phycoerythrin-labelled cells [PE-A]). **(a)** Comparison of transduction efficiency of AAVCMV and AAVCXCR4 in MCF-7 tumours and percentage luciferase expression with AAVCXCR4 relative to AAVCMV. Representative flow cytometric analysis of tumour cells with AAVCMV and AAVCXCR4 stained with luciferase antibodies (right panels) and corresponding non-specific IgG (left panels). Percentage of cells in Q1-2 represent those cells expressing luciferase in AAVCMV and AAVCXCR4. **(b)** Comparison of luciferase positive CXCR4 expressing cells in MCF-7 tumours percentage of CXCR4-positive cells in AAVCXCR4 relative to AAVCMV. Representative flow cytometric analysis of MCF-7 tumours with AAVCMV and AAVCXCR4 counterstained with anti-CXCR4 antibody tagged with PE (right panels) and corresponding IgG (left panels). Percentage of cells in Q2 represents CXCR4-positive cells expressing luciferase. **(c)** Comparison of luciferase positive epithelial tumour cells in MCF-7 tumours. Tumour samples transduced with AAVCMV and AAVCXCR4 were counter-stained with pancytokeratin. Percentage luc+ epithelial tumour cells are presented in Q2 (right panels) and corresponding IgG (left panels).

4.3.3 Systemic delivery of AAVCXCR4 mediates tumour-specific transgene expression in murine livers

The ideal cancer therapeutic strategy would selectively target malignant cells throughout the body, while having minimal effect on healthy cells. To examine the possibility for AAV to be used in a systemic cancer therapeutic strategy (previously unreported), mice bearing localised hepatic tumours were administered either vector through a splenic port. While luminescence from AAVCMV was significantly higher than that from AAVCXCR4, AAVCMV expression was concentrated at the site of administration (splenic port), unlike AAVCXCR4, where expression was detected only in the liver (**Figure 4.4a**). This pattern was confirmed by *ex vivo* BLI of the excised tissues (**Figure 4.4b**). The tumour-specific nature of AAVCXCR4 was further confirmed by examining its expression in both hepatic tumour-bearing and tumour-free animals. AAVCXCR4 gene transcription was up-regulated in livers bearing tumour, with only baseline levels evident in the livers of tumour-free mice (**Figure 4.4c**). *Ex vivo* analysis confirmed that gene expression of AAVCXCR4 was confined to tumour bearing organs (**Figure 4.4d**).

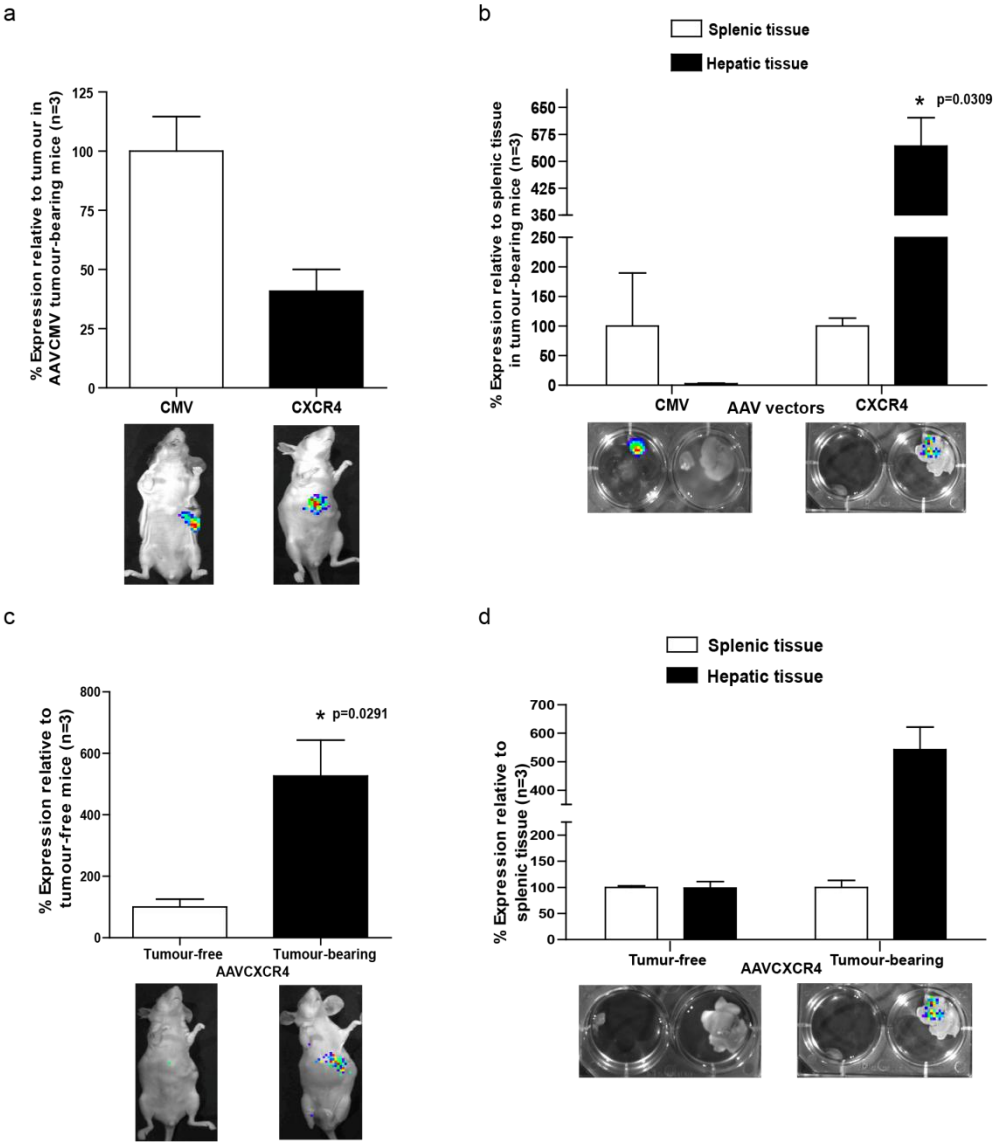


Figure 4.4 Systemic portal delivery and organ distribution of vector expression
MCF-7 liver tumours were induced in MF1 nu/nu mice. At 7 days post tumour induction, 5 × 108 infectious AAV particles were delivered to the liver by splenic portal inoculation. Gene expression was quantified by whole-body bioluminescent (BLI) imaging and subsequent BLI imaging of organs ex vivo. **(a)** While whole body IVIS imaging demonstrated higher total AAVCMV expression in tumour bearing mice when compared with AAVCXCR4, images indicate that AAVCMV expression was primarily localised to the splenic port while AAVCXCR4 expression was localised to the liver. **(b)** Ex vivo analysis of residual splenic and hepatic tissue from tumour bearing mice by IVIS imaging revealed that 98 % of AAVCMV expression was in the splenic tissue, while 85 % of AAVCXCR4 expression was localised to the liver (>5-fold increase compared with spleen; p=0.039). Data are presented as a relative percentage of splenic luminescence for each vector, where luminescence of the splenic tissue is taken as 100%. Representative images of corresponding tissue are shown below each. **(c)** Whole-body IVIS imaging 10 days post systemic portal delivery of AAVCXCR4 to tumour-bearing and tumour-free mice, revealed 5 fold higher gene expression in tumour-bearing mice when compared with tumour-free mice (p=0.029). Representative images are shown. **(d)** The ex vivo analysis of splenic and hepatic tissue from both tumour bearing and tumour free mice revealed that gene expression was localised to hepatic tissue (tumour-bearing mice) when compared with splenic tissue while there was no difference in gene expression in either tissue in tumour-free mice. Representative ex vivo images are shown.

4.3.4 Tumour-selective expression from AAVCXCR4 is maintained in patient tissue

To assess the translational aspect of using CXCR4 as a tumour specific promoter in an AAV vector, the transcriptional patterns of AAVCMV and AAVCXCR4 were assessed in *ex vivo* cultured normal (liver) and tumour (breast) patient tissue samples (n = 3) and BLI carried out five days post transduction. Analysis revealed AAVCXCR4 expression was 4 times greater in tumour tissue than in normal liver tissue, although not statistically significant, while expression of AAVCMV was comparable in both normal and tumour tissue (**Figure 4.5**). This confirms the tumour selectivity of AAVCXCR4 in patient tissue, a fact which, when combined with the systemic murine data from this study, supports the concept of utilising AAVCXCR4 as a reliable tumour-targeting systemic vector for treatment of metastatic cancer.

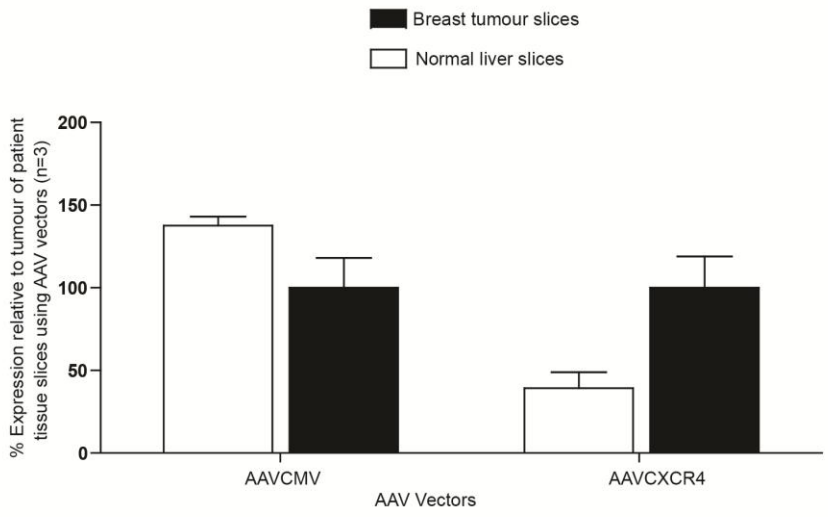


Figure 4.5 Tumour-selective transgene expression in patient samples
Patient tissue samples were obtained at the time of surgery, sliced into 2000 µm slices and maintained in culture media. AAV vectors were delivered into slices by direct injection and gene expression was measured using bioluminescent imaging at day 5 post delivery. AAVCMV expression levels were comparable in malignant and normal tissues. AAVCXCR4 expression was 4 times higher in tumour tissue when compared with normal liver tissue.

4.4 Discussion

The concept of developing gene delivery vectors that localise gene expression to tumour sites *via* systemic delivery is important for the advancement of cancer gene therapies. A systemically delivered targeted therapy allows biodistribution of therapeutic to metastatic disease in distant organs, with the advantage of reduced toxicity and improved therapeutic index and with a convenient and generally well-tolerated administration. This work provides proof of concept for the possibility of selective tumour targeting *via* AAV vectors following systemic delivery. We demonstrated reduced expression of AAVCXCR4 in normal tissue in which standard AAV vector (AAVCMV) is strongly expressed, which translated to a reduction in non-specific transgene expression. The AAVCXCR4 vector demonstrated efficient tumour-selective expression when locally administered to subcutaneous tumours and normal tissue (muscle). Population analysis by FACS showed that AAVCXCR4 preferentially targets epithelial tumour and CXCR4-expressing cells in subcutaneous MCF7 tumours, while the CMV promoter demonstrated majority expression in non-tumour and non-CXCR4-positive cell types.

AAV is known to mediate long-term expression in both normal and tumour tissue, unlike other widely used gene delivery vectors such as Ad, which only maintains minimal gene expression within days after delivery. Comparison of the expression kinetics of CMV with CXCR4 revealed that the CXCR4 promoter retained high expression in tumours while maintaining low expression in normal tissue for a period of up to 10 days, unlike AAVCMV. AAVCMV expression in tumours decreased by 65 % from day 7 to day 10. CMV promoter activity has previously been demonstrated to be silenced in tumour cells *via* methylation of ‘foreign’ (microbial) DNA CpG sequences (252-254). Therefore, even though the CMV promoter may provide higher expression levels initially, use of a native mammalian promoter sequence like that of CXCR4 is likely to provide long-term expression.

Our intravenous delivery experiments in the hepatic model demonstrated enhanced gene expression for AAVCXCR4 in tumour-bearing livers with minimal expression in tumour-free livers. Several serotypes of AAV exist and corresponding tissue tropism varies considerably (246). The AAV2 serotype examined here is known to transduce a wide range of tissue types, including liver and muscle, albeit at a lower efficiency than other serotypes more specific for individual cell types. Therefore, use of an AAV serotype with a high tropism for the target tissue would be expected to produce efficiencies higher than reported here for AAV, in addition to providing a further level of selectivity in terms of vector safety. In particular, it has been demonstrated that AAV8 has higher efficiency for delivery to liver, while AAV1 and AAV6 to lung (244, 255). Therefore, employment of other serotypes, or pseudotyping (cross-packaging AAV2 DNA with other serotype capsids) may further improve expression in liver and other distant metastatic sites (256).

CXCR4 expression represents an ideal tumour-selective system, as it has been shown to be up-regulated in breast tumour cells and suppressed in normal epithelial cells. Furthermore, this up-regulation of CXCR4 may contribute to metastatic progression through chemokine networks (184, 248). Therefore, the use of CXCR4 as a TSP would potentially enable selective targeting of CXCR4-positive tumour cells which are developing or already possess the potential for metastasis. Furthermore, *ex vivo* studies with patient tissue have demonstrated the tumour-selective nature of AAVCXCR4 over AAVCMV, confirming the translational potential of this vector.

Due to their distinctive properties, AAV vectors have the potential to play a significant role in the advancement of cancer care. Here we have demonstrated an AAV-based tumour targeting strategy using a tumour-selective promoter strategy. The transcriptional targeting approach displays significantly improved tumour to normal transgene expression ratio, allowing for the efficient targeting of AAV vectors to cancer cells

and the sparing of normal tissue in both subcutaneous and metastatic liver tumours using both local and systemic delivery routes. Intra-tumoural analysis emphasised the specific tropism for tumour cells. This study demonstrates the tumour-targeting flexibility which AAV-based vector systems offer and the ability to administer AAV systemically and achieve a high level of systemic tumour targeting. This vector strategy stands to play an important role in targeting distant, inaccessible, or undetected metastatic disease.

Chapter 5

Conclusions and future direction

5.1 Conclusions

Cancer remains a leading cause of morbidity and mortality despite better understanding of cancer causation and progression. Current anticancer regimens are associated with non-specific cytotoxicity leading to treatment failure due to serious toxic effects to normal tissue and the development of treatment resistant disease. Gene therapy has emerged as a realistic prospect for the treatment of cancer due to its potential for selective tumour cell targeting. The greatest challenge that cancer gene delivery vectors still face is the ability to safely and efficiently deliver genes in order to enable sufficient gene expression into target cells. A wide variety of biological and non-biological gene delivery vectors have been developed and explored over the past decade. However, gene delivery strategies used to date have significant delivery limitations which has slowed the progress of gene therapy to the clinical stage.

The overall objectives of this thesis are to evaluate the efficacy of various gene delivery methods in a clinically relevant tumour model and to also investigate potential strategies for tumour selective delivery. We began with the development of a tumour slice model system using patient waste tissue. This model involves the use of fresh human tumour tissue, cut into thin slices and maintained *ex vivo.* The viability of patient tissue samples were only moderately reduced under the chosen culture conditions and did not affect the proliferation capacity of the cells within the tissues as characterised by the expression of the proliferation marker Ki-67. This model potentially represents a more clinically relevant model as it allows strategies to be tested on intact patient tissue. Furthermore, a thicker slice, which maintains all cellular components and diffusion gradients, would more closely represent the true intra-tumoural conditions of solid tumours. Through optimisation of tumour slice thickness and culture conditions we developed an *ex vivo* system with the capability of examining both biological and non-biological gene delivery systems with real-time analysis of gene delivery.

The universal application is achieved by utilising a slice thickness that does not limit nutrient and oxygen diffusion throughout the slice while allowing the application of both biological and non-biological methods. For real-time measurements of gene expression, we applied a bioluminescence imaging technique based on the expression of luciferase. Bioluminescent imaging provides a rapid, economical and simple method for monitoring gene expression. This non-toxic detection system also enables the sequential quantification of gene expression without sacrifice of tissue, allowing for measurement of duration of expression and thereby enabling assessment of vectors requiring longer incubation periods. Using this model system we evaluated the efficacy of various gene delivery methods in a variety of tissue and tumour types. Ad was found to be the most efficient gene delivery vector in breast, colorectal and metastatic tumours (Chapter 2 and 3). Among the non-biological techniques, US yielded the highest transfection in all tissues. The nature of the *ex vivo* system also permitted the examination of specific physical factors individually, that can account for *in vivo* reduction in gene delivery. Parameters shown to diminish Ad gene delivery included patient blood, hypoxia and metastatic disease.

The *ex vivo* model was also suitable for examination of tissue specific effects on vector expression. Transcriptional targeting involves regulating the expression of the gene of interest by utilising tumour promoters, that maintain a ‘tumour-on’ and a 'normal tissue-off’ status. Ad expression mediated by the tumour specific promoter CXCR4 was shown to provide a tumour selective advantage over the ubiquitously active CMV promoter in breast, colorectal and metastatic liver tumours (Chapter 2 and 3). For the investigation of vector efficacy, toxicity and target cell specificity we optimised a dissociation technique, which provided representative cells from the entire slice for the accurate assessment of viability and identification of targeted cell types. Patient tumour cell population analysis demonstrated that

Ad mediated by CXCR targeted a higher percentage of tumour cells when compared with CMV.

Hypoxia is an important feature of solid tumours as a consequence of a structurally and functionally disturbed microcirculation. Hypoxia has been related to the up-regulation of gene products that has been shown to promote tumour progression, by enabling tumour cells to adapt to nutritional deprivation or to escape a hostile environment. Hypoxia also provides an environment directly facilitating chemo- and radio-resistance and also encouraging the evolution of phenotypic changes inducing permanent resistance to treatment. A therapy that maintains therapeutic profile in hypoxic zones within tumours would improve the therapeutic to toxicity index and treatment outcomes respectively. A system of changing oxygenation was developed using the hypoxia inducer, cobalt, to mimic the transient hypoxic conditions found in solid tumours. Ad-related transgene expression varied depending on the level of hypoxia, with significantly reduced levels observed with prolonged hypoxia. However, transgene expression was robust in the cycling hypoxic conditions relevant to solid tumours. This suggests that Ad may offer a better strategy against the hypoxic component of tumours. It is also known that some gene promoters including, CXCR4 are upregulated in hypoxic conditions. The CXCR4 promoter was shown to mediate higher gene expression over CMV under anoxia while the reversing of chronic hypoxia to normoxic conditions demonstrated increased gene expression of both promoters. Therefore, a strategy to re-oxygenate chronically hypoxic regions in solid tumours may make them more susceptible to Ad gene therapies.

A systemically delivered therapy would allow biodistribution of therapeutic to metastatic disease in distant organs. The concept of developing a systemically delivered vector that localises gene expression to tumour sites is important for the advancement of cancer gene therapies. We developed an AAV-based tumour targeting vector using the

tumour-selective promoter CXCR4. Comparison of the expression kinetics of CMV with CXCR4 following local delivery in murine tumour models revealed that AAV mediated by the CXCR4 promoter retained high expression in tumours while maintaining low expression in normal tissue (Chapter 4). The systemic delivery of AAVCXCR4 in a hepatic tumour model demonstrated enhanced gene expression in tumour-bearing livers with minimal expression in tumour-free livers. Furthermore, AAVCXCR4 retained the tumour-selective nature in *ex vivo* patient tumour tissues, confirming the translational potential of this vector.

5.2 Future direction

We performed gene delivery studies using various vectors in an *ex vivo* human tumour model as we felt this would provide a reliable model for direct extrapolation to the clinical setting. The extended duration of culture period and 3D architecture of the *ex vivo* patient tissue model make it universally applicable to studies of novel gene delivery and therapeutic strategies where the interplay between tumour cells and the microenvironment are important. In addition, the *ex vivo* tissue culture may also offer a new opportunity to improve the manner in which pharmaceutical companies identify potential candidates and bring new therapeutics to the market. Currently, immortalized cell lines and stem cells are being used for this purpose. While these systems have their advantages, these model fail to accurately represent a fully differentiated and functional tissue state. This work has demonstrated that the *ex vivo* model is both reproducible and predictable in a variety of tissue types, which as a result, would allow its use from high-throughput screening to safety and toxicology testing.

However, we also recognise that there are important limitations regarding the *ex vivo* model utilised here. Certain tissue types including breast and omental tissue were difficult to culture intact due to their lipomatous composition. This resulted in the use of alternative tissues as controls. In addition, the immune system and systemic barriers are underrepresented in the *ex vivo* tumour models. An immune competent model will provide a better understanding of additional barriers that vectors must overcome for successful clinical application. We have demonstrated that transgene expression can be regulated by transcriptional restriction using targeted tumour specific promoters. We have categorically established tumour specific targeting using the tumour specific promoter CXCR4. Future work would involve incorporating into these vectors therapeutic genes that target either the tumour cells, tumour neovasculature or tumour associated cells. An increasing number of studies have reported successful tumour targeting using other tumour specific promoters including survivin and cyclooxygenase-2 (257, 258). Future comparison and analysis between various tumour specific promoters in preclinical models will be invaluable in designing future cancer specific vectors. In addition, transcriptional targeting may potentially be extended to other settings including non-malignant conditions, such as autoimmune, traumatic or inflammatory conditions (199). Furthermore, AdCXCR4 vector demonstrates effective targeting in cobalt induced hypoxic conditions. Cobalt was used to mimic hypoxia due to the luciferase bioluminescent detection system requiring molecular oxygen for the production of light. Future work would involve the incorporation of a second reporter on each construct, such as a fluorescent protein, which would eliminate oxygen dependence, and permit gene delivery evaluation using an alternative detection system. Gene delivery to distant tumours for therapeutic approaches is a demanding task that urges the development of delivery vectors capable of overcoming many obstacles in the systemic circulation. We have demonstrated systemic hepatic tumour targeting using an AAV transcriptional targeting vector. Despite the potential

shown using transcriptional targeting systems, it is clear that further work is required to optimise delivery and to limit potential adverse side-effects. Enhanced gene delivery may be achieved by incorporating a strategy of organ selective gene delivery based on both vector and disease characteristics. In addition combining gene delivery strategies to achieve synergistic effects as previously reported may also be investigated. Further research into vector design and targeting will need to proceed in parallel with clinical studies, where proof of concept and identification of barriers to clinical gene delivery can inform the next phase of clinical research.

At the present time, gene therapy may offer the best path for a lifetime unencumbered by invasive surgery or toxic conventional therapeutics for the treatment of cancer. With continuing efforts alongside the significant progress already made, it is foreseeable in the near future that the challenge for the development of a safe, effective and clinically applicable gene delivery method for cancer therapy may be finally realised.

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"There are only two ways to live your life.

One is as though nothing is a miracle.

The other is as though everything is a miracle."

Albert Einstein