

Exploring the relationship between cancer metabolism and oncolytic adenoviruses



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For Mark – you make it all worthwhile

“Once you know things, you’re a different person. You can’t help it.” – Sir Terry Pratchett.

A statement addressing the impact of the COVID-19 global pandemic upon this thesis

I was unable to access any laboratory from March 2020 until late August 2020, owing to the SARS-CoV-2 outbreak and the nationwide restrictions imposed as a result. All students have been advised to attach documentation of the effects that these restrictions had upon their research. In addition, I feel it important to mention the significant emotional toll of not being able to access the labs to conclude this thesis in the way I was planning to during a global pandemic.

During this lost time, my main research priorities were as follows:

- 1) EnAd-CMV-TIGAR-GFP
 - To assess the ability for EnAd-CMV-TIGAR-GFP to inhibit glycolysis.
 - To assess the ability for EnAd-CMV-TIGAR-GFP to replicate in and kill a panel of cell lines used in figure 3.9.
 - To assess the effect of expressing TIGAR upon all metrics used in this thesis, including speed of kill, genome replication, IC_{50} and protein production in comparison to EnAd.
- 2) EnAd + 2DG in xenograft mouse models
 - To analyse the effect of EnAd + 2DG treatment on tumour burden and survival time in the murine xenograft model used in figure 6.8.
- 3) WT SKOV3 and GI SKOV3 cells
 - To assess alterations in signalling pathways between wildtype SKOV3 cells and glycolysis-independent SKOV3 cells using western blotting techniques.

If these goals were met, then additional experiments were to be conducted as follows:

- 4) WT and GI PC9 cells
 - To repeat findings in SKOV3 cells in PC9 cells.
- 5) Ascites Cells
 - Results using primary human ascites cells were to be repeated in several new *ex vivo* specimens.
- 6) Ascites Fluid and glycolysis inhibition
 - To assess if viral replication could be rescued in cancer cells cultured in ascites fluid

Whilst I am unable to present this work (owing to these exceptional circumstances), I have made an effort to highlight the gaps that exist within this thesis and to bridge these with analysis of literature which provide possible insights into these experiments as well as to admit the limitations of certain results. I also hope to address research priority 1) in the time between handing in my thesis and my viva.

With the increased opportunity to explore the literature surrounding my thesis during the lockdown, I also wrote and published a review of oncolytic viruses and metabolism, with the help of another final year DPhil student, Sally Frost, in a peer-reviewed journal (Dyer *et al.* 2020).

Abstract

Tumour cells exhibiting the Warburg effect rely mostly upon aerobic glycolysis for ATP production and have a notable addiction to anaplerotic use of glutamine for macromolecular synthesis. This strategy maximises cellular biosynthetic potential and provides an attractive anabolic environment for viral infection. Here, we evaluate infection of highly permissive cells (A549 cells) and poorly permissive cells (SKOV3) with wildtype adenoviruses, and the oncolytic chimeric adenovirus enadenotucirev (EnAd) to probe the role of metabolism on the success of oncolytic virotherapy with EnAd.

All adenoviruses caused an increase in glucose and glutamine uptake along with increased lactic acid secretion in their host cells, mirroring the canonical Warburg effect phenotype. Through identifying the IC_{50} of a panel of cancer cells, we identify a correlation showing that cells which rely upon aerobic glycolysis tend to be more permissive to EnAd oncolysis.

The virus lifecycle of both wildtype adenoviruses and EnAd were critically dependent on exogenous glutamine. Virus activity in glutamine-free cells was rescued with exogenous membrane-permeable α -ketoglutarate, but not pyruvate or oxaloacetate, suggesting an essential role for reductive carboxylation in glutamine usage. We propose that this unusual glutamine metabolism is beneficial for the production of biosynthetic intermediates, particularly under conditions of hypoxia or decreased cellular respiration as is often seen during adenovirus infection.

Counterintuitively, restricting glycolysis using 2-deoxyglucose or by limiting glucose supply strongly improved virus activity in both cell types. Antagonism of glycolysis also boosted EnAd replication and transgene expression within human tumour biopsies, and in xenografted tumours *in vivo*. We further bolstered this finding through weaning SKOV3 cells off of glucose. This process created a cell line which is highly permissive to EnAd infection and lysis. Finally, we engineered an “armed” EnAd expressing the glycolysis inhibitor TIGAR which was able to lyse cells whilst expressing TIGAR without causing acidification of the media.

These findings emphasise the importance of metabolism on productive viral infections which is of particular significance for viruses restricted to replicate in tumours, that already display aberrant metabolic phenotypes.

Declaration of Authentication

The work presented in this thesis is wholly my own; any contributions made by others have been acknowledged accordingly. The content submitted here does not form part of another thesis in this or at any other university. This work was carried out under the supervision of Professor Leonard Seymour and Dr Emma Stone at the Department of Oncology, Medical Science Division, University of Oxford.

Arthur Dyer

Publication notes

The work presented in chapters 3,4 and 5 has been accepted for publication in Cancer Research (Dyer *et al.*, 2019):

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If I end up being half as generous with my time, or half as knowledgeable as Professor Len Seymour, I will consider myself a terribly lucky person. Professor Seymour's ability to create a group of people around him who all work together so well is testament to his remarkableness. I will be forever grateful to him for allowing us to ask silly questions and not to be afraid of the unknown.

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List of abbreviations

α KG – alpha-ketoglutarate

μ g – microgram

μ l – microliter

μ m – micrometre

μ M – micromole

2DG – 2-deoxy-D-glucose

2HG – 2-hydroxyglutarate

2-NBDG – 2-deoxy-2((7-nitro-2,1,3-benzoxadiazol-4-yl)amino

3' or 5': three or five prime end of a DNA or RNA molecule

$^{\circ}$ C – degrees Celsius

Ad – adenovirus

Ad11 – adenovirus serotype 11

Ad11p – adenovirus serotype 11 Slobitski strain

Ad5 – adenovirus serotype 4

ADP – adenosine diphosphate

ADP – adenovirus death protein

AdPol = adenovirus polymerase

AKT – protein kinase B

AMPK – AMP-activated protein kinase

ANOVA – analysis of variance

AST – aspartate transaminase

ATP – adenosine triphosphate

bp – base pair

CAR – coxsackie and adenovirus receptor

CBP – CREB-binding protein

CD – cluster of differentiation

CD46 – complement regulatory protein 46

CI – cell index

CMV - cytomegalovirus

CO₂ – carbon dioxide

CPE – cytopathic effect

CR = conserved region

DAMP – danger-associated molecular pattern

DMEM – Dulbecco's modified Eagle's medium

DNA – deoxyribonucleic acid

dsDNA – double-stranded DNA

DSR – differential stress response

E – adenovirus early gene

E1ACR2 – adenovirus early gene 1A conserved region 2

E1B-55k – adenovirus early protein 1B 55kDa protein

ECAR – extracellular acidification rate

EDTA - ethylenediaminetetraacetic acid

EnAd – enadenotucirev

EpCAM – epithelial cell adhesion molecules

ER – endoplasmic reticulum

FAD – Flavin adenine dinucleotide

FADH₂ – flavin adenine dinucleotide + hydrogen

FAP – fibroblast activation protein

FBS – foetal bovine serum

FDA – Food and Drugs Administration

FDG - [18F] fluoro-2-deoxyglucose

FFU – foci-forming units

FLuc – firefly luciferase

g – gravity

G6P – glucose-6-phosphate

GCN5 = general control of amino acid synthesis protein 5

GFP – green fluorescent protein

GI – glycolysis independent

Gln – glutamine

GLS – glutaminase

Gluc – glucose

GMCSF = granulocyte-macrophage colony-stimulating factor

GTP – guanosine triphosphate

h – hour

H – hydrogen

H₂O – water

HCMV – human cytomegalovirus

HEK – human embryonic kidney

Her2 – human epidermal growth factor receptor 2

HIF – hypoxia-inducible factor

HK – hexokinase

HPI – hours post-infection

HSP70 – heat shock protein 70

HSV – herpes simplex virus

hTERT – human telomerase reverse transcriptase

IC₅₀ – half-maximal inhibitory concentration

ICP34.5 – infected cell protein 34.5

ICP47 – infected cell protein 47

IDH – isocitrate dehydrogenase

IGF-1 – insulin-like growth factor 1

ITR – inverted terminal repeat

kb – kilobase

kDa – kilodalton

kg – kilogram

L – adenovirus late gene

L – litre

LDH – lactate dehydrogenase

LDHA – lactate dehydrogenase A

MHC = major histocompatibility complex

mins – minutes

mL – millilitre

MLP – adenovirus major late promoter

mM – millimole

MOI – multiplicity of infection

Mol – molar concentration

mpH – milli pH

mRNA – messenger ribonucleic acid

Ms – millisecond

mTOR – mechanistic target of rapamycin

mTORC1 – mechanistic target of rapamycin complex 1

NAD – nicotinamide adenine dinucleotide

NADH - nicotinamide adenine dinucleotide + hydrogen

NDV – Newcastle-disease virus

ng – nanogram

NHBE – normal human bronchial epithelial cells

nm - nanometre

NNT – nicotinamide nucleotide

OAA – oxaloacetic acid

OCR – oxygen consumption rate

ORF – open reading frame

OV – oncolytic virus

OX40 = Tumour necrosis factor receptor superfamily member 4

Oxphos – oxidative phosphorylation

PAMP – pathogen-associated molecular pattern

PBS – phosphate-buffered saline

PCAF = P300/CBP-associated factor

PCR – polymerase chain reaction

PDK – pyruvate dehydrogenase

PET – positive emission tomography

PFK – phosphofructokinase

PFU – plaque-forming unit

pH – potential of hydrogen

PI3K – phosphoinositide 3-kinase

PK – pyruvate kinase

pM – picomole

POD = promyelocytic leukaemia oncogenic domains

PP2A = protein phosphatase 2A

PPC – virus particles per cell

PPP – pentose phosphate pathway

pRb – phosphorylated retinoblastoma protein

pTP – pre-terminal protein

Pyr – pyruvate

qPCR – quantitative polymerase chain reaction

Rb = retinoblastoma protein

RGD-4C = integrin $\alpha V\beta 3$

RID = receptor internalisation and degradation

RIPA – radioimmunoprecipitation assay buffer

RNA – ribonucleic acid

RPMI – Roswell Park Memorial Institute medium

SA – splice acceptor

SD – standard deviation

SDS – sodium dodecyl sulphate

STR – short tandem repeat

STS – short-term starvation

TBST – tris-buffered saline with tween-20

TCA cycle – tricarboxylic acid cycle

TIGAR – TP53-induced glycolysis regulatory phosphatase

TK – thymidine kinase

TPL – tripartite leader

TRAIL = TNF-related apoptosis-inducing ligand

TRRAP = transactivation/transformation domain-associated protein

U – units

UV – ultraviolet

V – volt

VA = Viral associated

VACV – Vaccinia virus

VHL – Von Hippel-Lindau

WT – wildtype

1 Introduction

1.1 Cancer

Cancer is a name given to a collection of related diseases characterised by uncontrolled division and a propensity to invade into other tissues (Hanahan and Weinberg 2000, 2011). Ultimately, the disease is caused by inherited or acquired genetic mutations in a cell's DNA, leading to alterations in cellular processes that can include cell division and death. Cancer represents a significant health burden throughout the world, and in the UK it is estimated that, for individuals born after 1960, at least one in every two people will be diagnosed with some form of the disease during their lifetime (Ahmad, Ormiston-Smith and Sasieni 2015; CRUK 2020). From 2015 to 2017, there were 367,167 new cases of cancer in the UK with 164,901 deaths from cancer recorded in the same period. Because of improvements in cancer treatments and diagnosis, the mortality rate from cancer in the UK has fallen by 16% since the 1970s, with ten-year survival rates doubling from 24% to 50% in that period (CRUK 2020).

Whilst surgical resection and radiation therapy are powerful techniques, their strength is in the treatment of locoregional disease, and they often fail to eradicate metastases that can lead to cancer recurrence. Chemotherapeutic drugs can be more effective against metastatic tumour deposits owing to systemic delivery; however, these drugs usually lack specificity and are often associated with severe side effects which limit the therapeutic dose (Park and Choi 2016). Tumour recurrence is often observed in

patients treated with one or more of these treatment modalities, and it is estimated that some 90% of cancer deaths in the UK are associated with disseminated disease.

The advancement in our understandings of cancer at a cellular level has expanded dramatically in the past few decades leading to a more targeted treatment approach directed at some of the underlying cellular mechanisms and much earlier diagnosis increasing the probability of curing the disease effectively. One example of this is the highly promising field of cancer immunotherapy which has widely been described as a breakthrough in cancer treatment (Couzin-Frankel 2013). Successful cancer immunotherapy is most common in diseases such as melanoma and lymphoma, however, and the results in more complex heterogenic carcinomas are often poor, any responses transient and usually followed by a relapse of treatment-refractory disease.

1.2 The Hallmarks of Cancer

Cancer cells have altered cellular programming, resulting in increased survival, growth, and proliferation. These fundamental characteristics were documented in the seminal review “The Hallmarks of Cancer” by Hanahan and Weinberg in 2000 (Hanahan and Weinberg 2000). In 2011, the same authors released an updated list including two new hallmarks of cancer which were found to be of importance in the 11 years between publications. These two emerging hallmarks were immune evasion and deregulated metabolism, both of which are fundamental to the success of many modern cancer treatments in the clinics (Hanahan and Weinberg 2011).

1.3 Central Carbon Metabolism

1.3.1 Metabolism Overview

The basic principles underlying our current understanding of cellular metabolism were deciphered in the 1920s to the 1960s through the work of eminent scientists such as Otto Warburg, Albert Lehninger, Hans Krebs, and Sidney Weinhouse, many of whom are luminaries in the field of oncology as well as metabolism (Warburg, Wind and Negelein 1927; Eagle 1955; Warburg 1956a, 1956b; Lewis and Scott 1962).

Glucose catabolism has been thought of as the central core pathway for animal cells since the advent of our modern understanding of cellular metabolism. Whilst it is true that glucose catabolism is of vital importance in a wide array of purposes, it is clear now that metabolism is a highly complex web of interactions. Whilst we cannot encompass the entirety of these pathways within this thesis, it is always valuable to be mindful of this fact when interpreting metabolic data. In this study, we will be focussing on the metabolism of two of the most prevalent nutrients in the human body that are of importance to both the fields of oncology and virology, namely glucose and glutamine.

1.3.2 Glycolysis

Intracellular catabolism of glucose can be broadly divided into glycolysis, the tricarboxylic acid cycle, and the pentose phosphate pathway; however, many other pathways feed into, affect, or are affected by these central pathways. Glycolysis was

the first cellular metabolic pathway to be understood and is considered to have two separate end products, namely pyruvate or lactate (Warburg, Wind and Negelein 1927). Glycolysis itself is broadly divided into two main phases, the energy-requiring phase, and the energy-releasing phase. The first phase consists of the conversion of the six-carbon glucose into two phosphorylated three-carbon molecules (glyceraldehyde-3-phosphate), which consumes two adenosine triphosphate (ATP) molecules. The second phase involves the conversion of these three carbon products into pyruvate resulting in the production of four ATP molecules and two nicotinamide adenine dinucleotide + H (NADH) molecules for every glucose molecule. The whole of glycolysis consists of three irreversible and rate-limiting reactions: the conversion of glucose into glucose-6-phosphate (G6P) by hexokinase (HK), the conversion fructose-6-phosphate into fructose-1,2-biphosphate by phosphofructokinase (PFK), and the conversion of phosphoenolpyruvate into pyruvate by pyruvate kinase (PK). This reaction is summarised in **figure 1.1**.

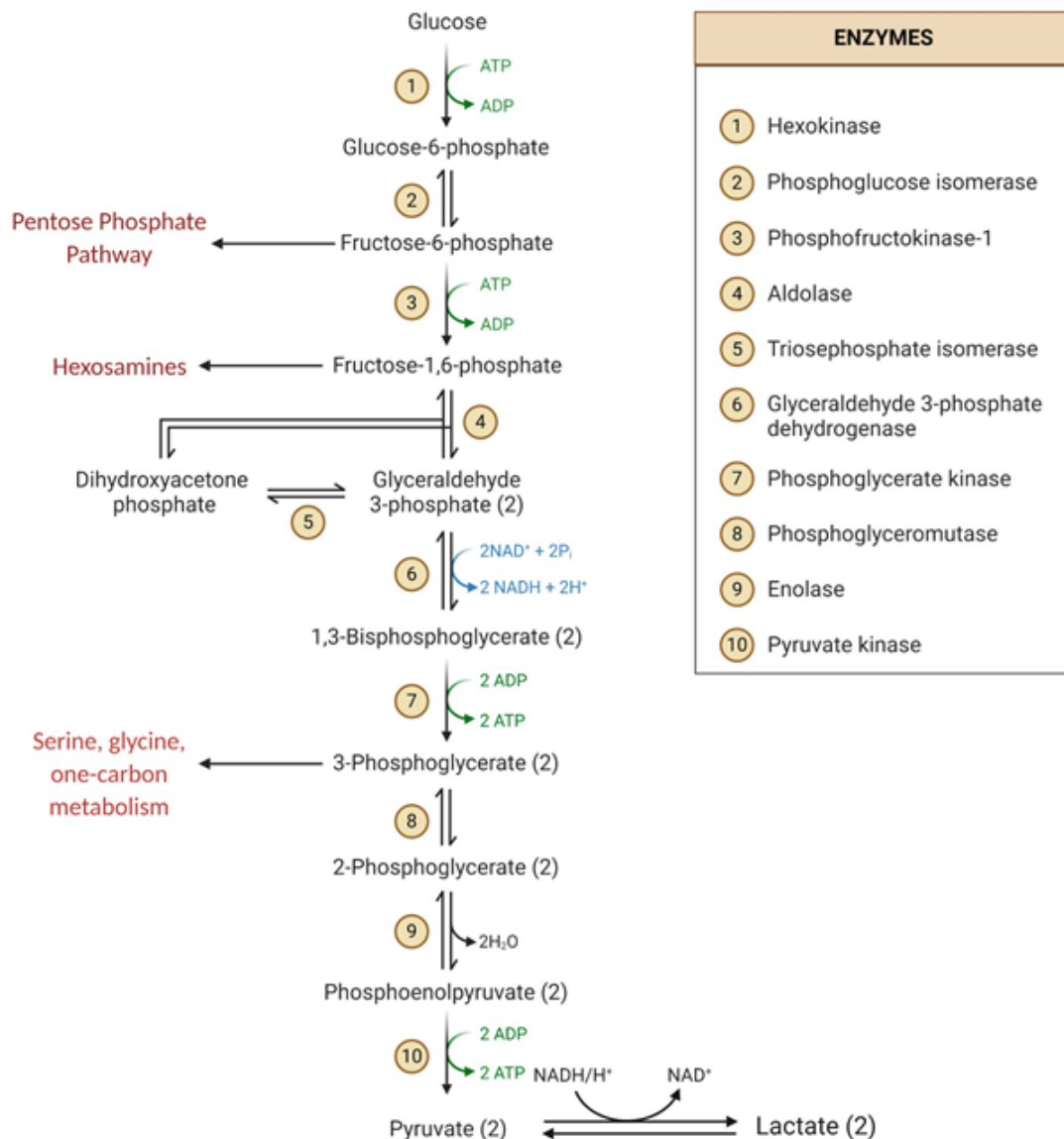


Figure 1.1 Schematic representation of glycolysis.

Glycolysis is a metabolic pathway which converts six-carbon glucose into three-carbon pyruvate or lactate through a series of chemical reactions via a series of metabolic intermediates. Broadly speaking, glycolysis can be divided into two phases: the energy-consuming phase (in which ATP is consumed through the conversion of glucose to fructose-1,1-phosphate), and the energy-producing phase (in which ATP and NADH is produced through the conversion of glyceraldehyde-3-phosphate into pyruvate). Created with Biorender.com.

1.3.3 The Tricarboxylic Acid Cycle

The tricarboxylic acid (TCA) cycle (also referred to as the Krebs's cycle or the citric acid cycle), is a series of chemical reactions used by all eukaryotic cells to produce metabolites needed for biomass along with the reducing agents NADH and FADH_2 , guanosine triphosphate (GTP), and CO_2 . Because of the ability to create many useful

precursors for cells, it is widely believed to be one of the earliest established components of cellular metabolism (Keller *et al.* 2017). In normal mammalian cells, the majority of Acetyl-CoA that is used to fuel the TCA cycle comes from the oxidation of pyruvate derived from glucose (Singh and Ward 1997). This Acetyl-CoA is combined with oxaloacetic acid (OAA) to produce citric acid, which is then converted into its isomer isocitrate, and then oxidised into α -ketoglutarate (α KG). Through a series of steps summarised in **figure 1.2**, α KG is converted into OAA allowing the cycle to continue. Even though the TCA cycle is referred to as a cycle, it is not necessary for metabolites to follow one particular route and, depending on the presence of enzymes and cellular stresses, many parts of the cycle can occur in bidirectional manners, this is particularly true of some cancer cells as will be discussed in later sections.

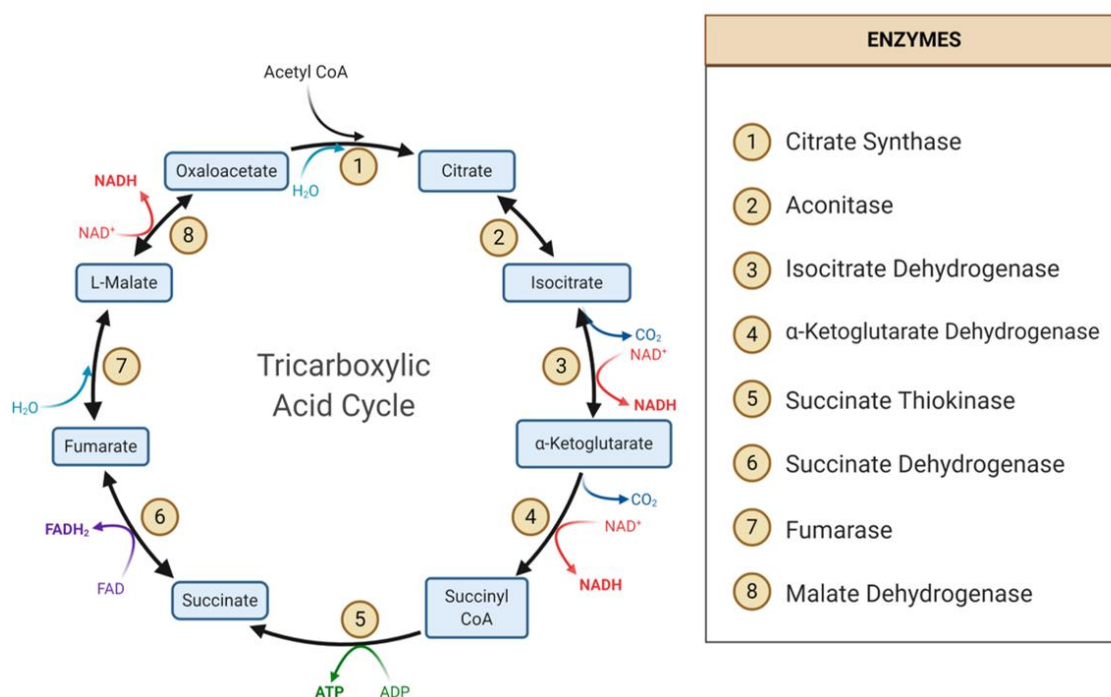


Figure 1.2 Schematic representation of the tricarboxylic acid cycle.

The tricarboxylic acid (TCA) cycle – also known as the citric acid cycle or the Krebs cycle – is a series of chemical reactions to generate energy and metabolites through the oxidation of acetyl CoA alongside the creation of the reducing agents NADH and FADH_2 which are used in many different biochemical reactions. Created with Biorender.com.

1.3.4 Pentose Phosphate Pathway

The pentose phosphate pathway (PPP) is a cytoplasmic pathway that runs in parallel to glycolysis and constitutes one of the best-studied pathways that is connected to glucose metabolism (Horecker 2002; Patra and Hay 2014). The PPP is a process that produces NADPH and 5-carbon sugars (pentoses). The first part of the PPP reaction is the oxidative conversion of glucose-6-phosphate into ribulose-5-phosphate in which two NADP⁺ molecules are reduced to NADPH. The second phase is the non-oxidative phase which ultimately results in the synthesis of 5-carbon sugars which are used in a variety of different processes such as the production of nucleotides and nucleic acid (from ribose-5-phosphate), the synthesis of aromatic amino acids (from erythrose-4-phosphate), or the production of other pentose-phosphates that can feed back into glycolysis (Pusapati *et al.* 2016).

1.3.5 Glutamine Metabolism

Glutamine is the most abundant amino acid in the body and, as such, represents an essential resource for cells, particularly at times of rapid consumption of energy or resources such as in cancer or cell proliferation. Glutamine has a wide range of uses for cells including metabolism, nitrogen exchange, pH homeostasis, nucleotide synthesis (purines, pyrimidines, and amino sugars), NADPH creation, antioxidant regulation, and many other biosynthetic pathways (Lewis and Scott 1962; Labow, Souba and Abcouwer 2001; DeBerardinis and Cheng 2010; Lu, Pelicano and Huang 2010; Daye and Wellen 2012; Cruzat *et al.* 2018). It is reported that in plasma samples, glutamine concentrations range from 500-800 μM , representing approximately 20 % of the total

free amino acids pool (Roth 2008). In solid tissues, this concentration can be elevated with glutamine representing up to 60 % of the total amino acid pool (Labow, Souba and Abcouwer 2001; Cruzat *et al.* 2018).

1.4 Tumour Metabolism

1.4.1 Tumour Glucose Metabolism

As discussed in section 1.2, in the 11 years after the publication of the seminal review “The Hallmarks of Cancer” by Hanahan and Weinberg, it became apparent to the authors that there were two additional hallmarks of cancer, namely immune evasion and deregulated metabolism, both of which played crucial roles in the complex multi-cell type tumour microenvironment.

One well-studied feature of metabolic reprogramming in cancer is the “Warburg Effect” in which tumour cells increase glucose consumption and lactate secretion, even in the presence of oxygen (Warburg 1956b). This provides several key advantages to cancer cells. The rate of metabolism through aerobic glycolysis occurs 10-100 times faster than oxidative phosphorylation (oxphos) such that the amount of ATP synthesised over a given period is comparable (Shestov *et al.* 2014). The production of lactate can inhibit many immune cell functions effectively creating an “immunologically cold” cancer, and the metabolic plasticity in most cancer cells allows for alternative metabolites (such as glutamine) to be used for biosynthetic purposes. It

has also been shown that malignant cells require a high metabolic flux to synthesise building blocks such as nucleotides, proteins, and lipids.

This metabolic phenotype induces a unique extracellular microenvironment, including decreased concentrations of nutrients and oxygen, and an increase in metabolic waste products. This microenvironment is profoundly protective for tumours and provides resistance against conventional therapies such as chemotherapy and radiotherapy (Schlappack, Zimmermann and Hill 1991; Sauvant *et al.* 2008; Chiche, Brahimi-Horn and Pouysségur 2010; DeClerck and Elble 2010; Wojtkowiak *et al.* 2011; Thews *et al.* 2014). Additionally, the increased metabolic competition, accumulation of oncometabolites and metabolic waste products all aid in the creation of immunosuppression, facilitating cancer growth. For example, low extracellular pH strongly inhibits T cell activity (Wu, Ying and Hu 2016). Whilst this particular topic is beyond the scope of this thesis, we would be remiss not to remain cognisant of the effects of metabolism upon tumour immunity and the global immune system, and this subject is the topic of study within our laboratory.

1.4.2 Tumour Glutamine Metabolism

Despite recent advances in the field of tumour metabolomics, much work focusses on changes in glucose metabolism. The observation that mammalian cells also rely on glutamine (Reitzer, Wice and Kennell 1979; Moreadith and Lehninger 1984; Board, Humm and Newaholme 1990) helped move the field of cancer metabolism towards a more all-encompassing field, and in the past 40 years, increasing evidence has shown a

critical role for glutamine in cancer progression, proliferation and survival (Eagle 1955; Eagle and Habel 1956; Kovacevic and McGivan 1983; Board, Humm and Newaholme 1990; DeBerardinis *et al.* 2008a; Mullen *et al.* 2012).

Many cancer cells are currently considered to be “glutamine addicted”. Cancer cells, in particular, are highly cataplerotic (they remove a lot of metabolites from the TCA cycle for biomass) leading to a requirement for a high influx of intermediates to maintain the TCA cycle. Indeed, it is now believed that a high anaplerotic glutamine flux is a more specific marker of high cell growth than glycolytic flux as the latter can be triggered through many stressors such as hypoxia, independent of the demand for metabolites (Still and Yuneva 2017). As with glucose, glutamine can be oxidised to produce metabolites such as NADPH and lactate; however, unlike glucose, this also has the benefit of producing TCA intermediates for use as precursors for biosynthetic pathways.

In many cancer cells, there is a marked deficiency in mitochondrial function, whether due to defects in the mitochondria, hypoxia, or a mixture of genetic and environmental factors. Since the 1950s scientists have known that glycolysis is an important metabolic pathway, particularly in the absence of mitochondria or oxygen, but how the biosynthetic precursors arising from the TCA cycle could be created was not understood. In contrast to glucose, glutamine can be metabolised via both oxidative decarboxylation and reductive carboxylation, and the latter pathway can yield a range

of TCA cycle intermediates (DeBerardinis *et al.* 2007; Fendt *et al.* 2013; Fernandez-De-Cossio-Diaz and Vazquez 2017). Reductive carboxylation has since been shown to be the dominant pathway in cells with mitochondrial defects and in conditions of hypoxia (Mullen *et al.* 2012). Indeed, maintaining glutamine metabolism without oxygen can be argued to be of higher importance than glycolysis, given that the majority of TCA cycle intermediates are created through glutamine anapleurosis in many cancer cells. The pathways of reductive carboxylation or glutamine are documented in **figure 1.3** however as a brief explanation, α KG is converted to isocitrate consuming CO_2 in the process then this is converted to citrate which can either be used to form TCA intermediates or for the fatty acid synthesis pathway.

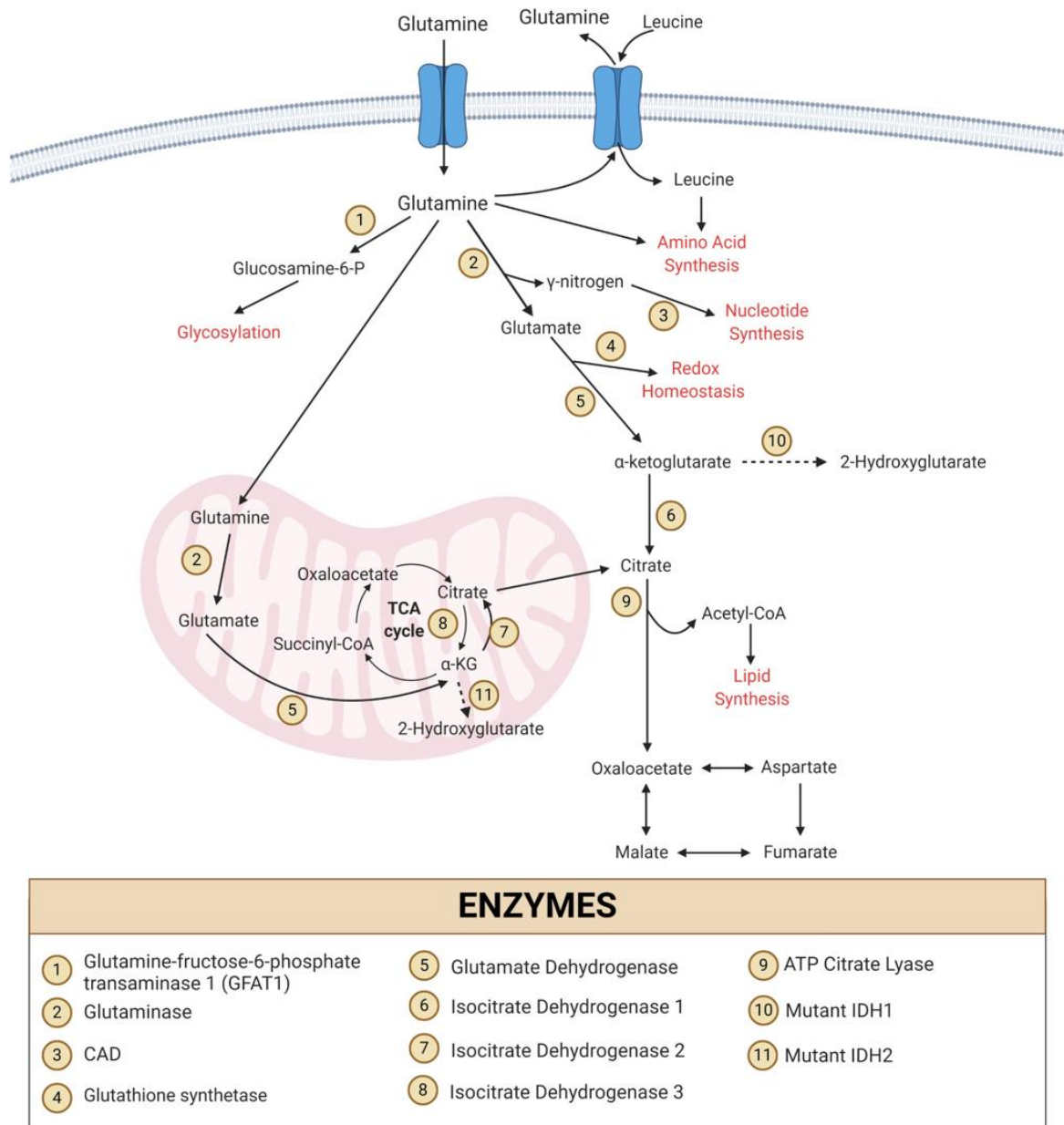


Figure 1.3 Schematic representation of the mammalian glutamine metabolism.

Glutamine is an important amino acid with many vital functions in the body. It can be used by cells for the production of glucosamine and hexosamine intermediates for processes such as glycosylation. In the cytosol, glutamine can donate its amide (γ) nitrogen for the synthesis of nucleotides producing glutamate in the process which is critical for maintaining redox homeostasis through production of glutathione, as well as protecting cells from oxidative stress. Glutamine can also be used for amino acid metabolism and synthesis through various pathways including aiding the transport of amino acids, such as leucine into cells and through the creation of cytosolic TCA metabolites. Glutamate can be converted to the TCA cycle intermediate α -ketoglutarate (α KG) either within the mitochondria or within the cytosol, driven by isocitrate dehydrogenases (IDHs) in a process known as anapleurosis. α KG can either be metabolised oxidatively, via the TCA cycle, or reductively as is pictured in the diagram. Some cancers harbour mutations in IDHs, resulting in the production of the oncometabolite 2-hydroxyglutarate (2HG). Created with Biorender.com.

1.4.3 Targeting Cancer Metabolism

Because cancer cells are fast-growing and display such unique metabolic profiles, several groups have tried to exploit this therapeutically with varying results. Many of the drugs targeting cancer metabolism are thought to be highly selective; however, some studies have found monotherapies targeting metabolic pathways to be ineffective (Pusapati *et al.* 2016). This is likely because cancer cells have a fairly high metabolic plasticity allowing them to circumvent inhibition of one pathway, eventually leading to resistance. Examples of this finding include reports that pancreatic cancer cells treated with glutaminase inhibitors resulted in an increase in glutamate from non-glutamine sources (Biancur *et al.* 2017) or findings that in various cells exposed to glycolysis inhibition, reprogramming via mTORC1 allows for treatment resistance (Pusapati *et al.* 2016). In both of these cases, however, the authors note that these cells only have limited metabolic plasticity. Treatment of these resistant cells with a combination of therapies targeting multiple arms of metabolism results in highly effective cancer treatments because these cells are unable to adapt to inhibition of multiple metabolic pathways. These results are promising steps forward in targeting cancer metabolism but highlight the complexity of metabolism beyond the understanding of simple glucose metabolism.

Intriguingly, although many papers discussing tumour metabolism highlight binary metabolic extremes when considering aerobic glycolysis and glucose oxidation, this is rarely observed in practice. Whilst some tumour cells in culture, and specific tumour cells within a heterogeneous tumour *in vivo* will display a true Warburg effect

phenotype, many cells will experience fluctuations in the availability of nutrients and will adapt to this temporally. Likewise, many cells exist on a spectrum of these two polar extremes with most cells displaying varying levels of glycolysis whilst glucose oxidation is maintained. It is still widely accepted that most normal cells obey the “Crabtree effect” – namely that cells which display a high rate of glycolysis do not maintain oxphos, but this does not appear to be the case in most cancer cells. The plasticity of cancer cells mentioned above, in conjunction with the acknowledgement of this spectrum of metabolic phenotypes, highlight the issues with traditional methods of targeting tumour metabolism or studying tumour metabolism using *in vitro* models (De Berardinis and Chandel 2016).

Short-term fasting can sensitise malignant cells to cancer treatments whilst at the same time protecting non-malignant cells in a phenomenon that is referred to as the differential stress response (DSR, Lee et al., 2010). The principal reasoning to explain the DSR is that during tumourigenesis, various mutations are gained, including the ability to proliferate in unfavourable conditions with or without the presence of external growth factor signalling such as insulin-like growth factor 1 (IGF-1). Because of these mutational gains of function, these cells become unresponsive to protective signalling generated by fasting (such as falling IGF-1). This means that, when combined with a cancer therapy such as chemotherapy, cancer cells will be unable to decrease their metabolic demands even when nutrients are in short supply, making them more vulnerable to chemotherapy (Hanahan and Weinberg 2011; Lee *et al.* 2012; De Groot *et al.* 2019). This response is in contrast to non-malignant cells which, during calorific

restrictions or fasting have been shown to protect themselves by decreasing, for example, AKT and RAS signalling pathways. In support of this, pre-clinical studies have shown that intermittent fasting in animals can extend lifespans, delay type II diabetes and cardiovascular and neurodegenerative diseases as well as reducing cancer rates (Berrigan et al., n.d.; M N Harvie et al., 2011; Michelle N. Harvie et al., 2016; Most, Tosti, Redman, & Fontana, 2017; O’Flanagan, Smith, McDonell, & Hursting, 2017).

Similarly, Raffaghello *et al.* published in 2008 that starvation in mice allowed researchers to increase the tolerable dose of chemotherapy up to three times higher than the maximal dose approved in humans (Raffaghello *et al.* 2008). Lee *et al.* published in 2012 that 15 out of 17 mammalian cancer cell lines researched are sensitised to various chemotherapeutics by fasting 24 hours before- and 24 hours post-chemotherapy. Remarkably, this paper also showed that two cycles of fasting in mice was as effective as two cycles of chemotherapy in reducing tumour burden and that the combination of starvation plus chemotherapy was the most effective regimen (Lee *et al.* 2012).

Because of this apparent differential ability of cancer cells and normal cells to protect themselves from nutrient restriction, various clinical trials and pre-clinical trials are investigating the effects of calorie restriction (reducing intake to 75% fewer calories) and short-term starvation (no food intake) on cancer treatments. Interestingly, preliminary reports have found that fasting for between two to five days can protect

patients from the side effects of chemotherapy without causing chronic weight loss (Most *et al.* 2017). However, any improvement in efficacy still has to be established.

1.5 Oncolytic Virotherapy

1.5.1 Introduction to oncolytic virotherapy

Oncolytic virotherapy is a form of cancer treatment whereby viruses, which are restricted to infect and kill cancer cells, are administered to patients. Through multiple rounds of infection, cancer cells are lysed and progeny virions released, creating a self-amplifying treatment. Ultimately, the virus is usually intended to spread systemically and infect any micrometastases present. The theory of oncolytic virotherapy is that, alongside the direct damage from lysis of cells, the lytic death pathway will also expose more cancer antigens alongside danger-associated molecular patterns (DAMPs) and pathogen-associated molecular patterns (PAMPs) creating an immunologically “hot” tumour where the immune system itself will begin to recognise and potentially control the cancer. This theory has been proven, in part through murine studies in which an abscopal effect is observed with non-infected cancers also regressing or in tumour re-challenge models (Liu *et al.* 2003; Fend *et al.* 2017; Havunen *et al.* 2018; Kuryk, Møller and Jaderberg 2019; Martin *et al.* 2019; Romano and Marino 2019). Oncolytic virotherapy has recently received renewed interest since promising clinical results for the first agent (the herpes simplex virus known as Imlygic) which received its product licence in 2015 from the federal drugs administration in America (Russell, Peng, and Bell 2012; Chiocca and Rabkin 2014; Seymour and Fisher 2016; Arthur Dyer *et al.* 2018; “FDA Approves First Oncolytic Virus Therapy” 2015).

Many wildtype viruses (including some adenoviruses) show an intrinsic selectivity for replication within cancer cells. The reason behind the apparent intrinsic cancer selectivity of many wildtype viruses is a topic of wide debate but likely reflects the hyperactivated phenotype of tumour cells, providing an environment that promotes increased virus replication. Indeed, many publications highlight the apparent overlap between the hallmarks of cancer and the hallmarks of virally infected cells. For example, the oncolytic effect of wildtype Newcastle disease virus (NDV) is thought to be based on its strong preference towards apoptosis-resistant cells (Mansour, Palese and Zamarin 2011). An additional explanation for the intrinsic oncotropism of many wildtype viruses is thought to be partly because of an upregulation of receptors used for viral entry. Some receptors such as the coxsackie and adenovirus receptor (CAR) and the complement regulatory protein 46 (CD46), which are the receptors for adenovirus serotypes Ad5 and Ad11 respectively are upregulated in many cancers (Korn *et al.* 2006; Reeh *et al.* 2013; Do *et al.* 2018; Su *et al.* 2018).

1.5.2 Rational design of oncolytic viruses

Since the discovery of the natural oncotropism of many viruses, our understanding of viruses has grown along with our ability to design and conduct novel scientific techniques. We are now able to rationally design oncolytic viruses to ensure that the virus is selective for cancer cells.

One technique used in the rational design of oncolytic viruses is spatial retargeting through altering the receptor-binding proteins or through coating the virus in a synthetic coating containing new receptor binding proteins. Examples of this include

retargeting herpes simplex virus (HSV) to the Her2 receptor, which is associated with breast and ovarian cancer or the retargeting of measles to the urokinase receptor, upregulated in multiple malignancies (Shiple *et al.* 1999; Jing *et al.* 2009; Shobana, Samal and Elankumaran 2013; Leoni *et al.* 2015). A second strategy to ensure tumour-specificity is to place essential viral genes under the control of tumour-associated promoters such as the human telomerase reverse transcriptase (hTERT) promoter or hypoxic response elements (Post and Van Meir 2003; Ahn *et al.* 2009; Xie *et al.* 2009; Hu *et al.* 2010; Kwon *et al.* 2010; Hashimoto *et al.* 2012).

A third, and commonly used strategy, is to delete viral genes which are trans-complemented by the tumour phenotype. This process of trans-complementation can best be described in the example of ONYX-015. Early in adenovirus replication, the virus expresses the E1B-55k protein which inhibits apoptosis of the infected cell to allow for increased viral progeny production. E1B-55K prevents apoptosis through interaction with p53, and this led to the rationale that deletion of this gene would limit the replication of the virus to cells in which p53 is already dysregulated, as is common in many cancers (Heise' *et al.* 1997; Rothmann *et al.* 1998). After expanding to clinical trials, it was revealed that the cancer selectivity of ONYX-015 was independent of p53 and rather was due to an increased export of late viral mRNA in cancer cells (O'Shea *et al.* 2004). Additionally, ONYX-015 contained partial deletion of the E3 region, which regulates immune responses, owing to safety concerns, and this partially led to macrophage clearance of virally-infected cells (Wang *et al.* 2003).

Despite this, ONYX-015 led the way for several oncolytic viruses with trans-complimenting deletions such as attenuation of adenoviruses through removal of the E1A gene, vaccinia virus through deletion of TK or HSV through deletion of ICP34.5 and ICP47.

1.5.3 Directed evolution of oncolytic viruses

The rational design of oncolytic viruses (OVs), as discussed above can be a challenging undertaking as it requires a deep and thorough understanding of the biology of cancer cells, the surrounding supporting cancer milieu, and the virus. The complexity and heterogeneity of solid cancers makes this a particularly difficult feat. For this reason, a promising alternative is that of directed evolution, or bioselection. This approach involves using a pool of viruses to infect a target cell of choice in conditions that encourage viral recombination. After several rounds of recombining, the most effective virus is isolated and tested for maximal proliferation in tumour cells whilst negatively selecting those viruses which can replicate in non-cancerous cell lines. The archetypal example of this is enadenotucirev (EnAd), a chimeric oncolytic adenovirus currently undergoing clinical trials. Because of the process of directed evolution, as opposed to rational virus design, the exact reasons for the selectivity and toxicity EnAd displays are unknown, as will be discussed below.

1.6 Oncolytic Adenoviruses

1.6.1 Introduction to adenoviruses

Adenovirus is a medium-sized (90-100 nm) non-enveloped virus which contains a double-stranded DNA genome. The genome is approximately 36 kb in size encoding over 40 proteins (Saha, Martuza and Rabkin 2017) which are divided broadly into early genes and late genes depending on whether they are expressed before, or after, DNA replication (Paul Thomas and Mathews, 1980).

Since the first isolation of human adenoviruses from adenoid tissue in 1953 there have been approximately 68 types identified in humans which are classified into seven groups (groups A – G) as denoted in **table 1.1** (Berk 2007; Ghebremedhin 2014). The traditional grouping of adenoviruses is based on the resistance to neutralisation by the antisera from other adenovirus types. More recently, attempts have been made to focus typing on protein and nucleotide sequence alignment in loop 1 and loop 2 of the hexon protein - the type-specific epitopes involved in the virus neutralisation tests (Madisch *et al.* 2005; Guimet and Hearing 2016).

The function of many of the adenovirus early proteins are relatively well understood, both in the context of viral replication and when expressed independently in cells. We have listed the main functions of each of the early proteins in **table 1.2**, but we will give a brief overview of some of the pivotal functions below.

Table 1.1 Human adenovirus species and their main tropisms and associated virus types. Table was adapted from Berk *et al.* 2007, and Ghebremedhin *et al.* 2014

Species	Types	Major site(s) of infection	Common Disease associations
A	12, 18, 31, 61	Gut, urinary tract, and respiratory tract	Gastroenteritis, meningoencephalitis
B	3, 7, 11, 14, 16, 21, 34, 35, 50, 55, 66, 68, 72, 79	Respiratory tract, renal and eye	Acute respiratory disease
C	1, 2, 5, 6, 57	Eye and respiratory tract	Acute febrile pharyngitis
D	8–10, 13, 15, 17, 19, 20, 22–30, 32, 33, 36–39, 42–49, 51, 53, 54, 56, 58–60, 62–65, 67, 69, 70, 71, 73–75, 81, 83–85, 90	Gut and eye	Epidemic keratoconjunctivitis, gastroenteritis
E	4	Respiratory tract and eye	Pneumonia
F	40, 41	Gut	Gastroenteritis
G	52	Gut	Gastroenteritis
Unclassified	76, 77, 78, 80, 82, 86–89		

Table 1.2 An overview of the early transcriptional units of human adenoviruses listing the protein products and their primary functions, adapted from Ghebremedhin *et al.* 2014. ORF = open reading frame, TRAIL = TNF-related apoptosis-inducing ligand, MHC = major histocompatibility complex, PODs = promyelocytic leukaemia oncogenic domains, PP2A= protein phosphatase 2A

Transcriptional Unit	Protein	Primary Functions
E1A	5 isoforms	Transcription activation (binding E2F)
E1B	55K	Inhibition of apoptosis and mRNA transport
	19K	Apoptosis inhibition and promotion of autophagy
E2A	DNA Binding Protein (DBP)	Stimulating the initiation and elongation of viral DNA
E2B	Polymerase	Viral polymerase
	Pre-terminal protein (pTP)	Attachment to nuclear matrix and priming site for DNA replication
E3	12.5K	Unknown
	6.7K	Inhibitor of TRAIL
	gp19K	Inhibition of MCH class peptide presentation
	Adenovirus death protein (ADP)	Host cell lethality to allow for viral spread
	Receptor internalisation and degradation complexes (RIDs)	Protection from apoptosis
	14.7K	Protection for apoptosis
E4	ORF1	Binds PDZ proteins
	ORF2	Unknown
	ORF3	Inhibits PODs, inhibits p53, and blocks the DNA damage response
	ORF4	Binds to PP2A and induces tumour-specific cell killing
	ORF6	Induces ubiquitination and degradation of cellular proteins, blocks the DNA damage response, and inhibits p53.
	ORF6/7	Regulates E2Fs

1.6.2.1 Adenovirus Early Gene expression – E1

The early genes E1A and E1B are the first genes to be expressed and optimise the host cell for viral replication followed by E2 which controls DNA replication whilst E3 and E4 genes control host immune responses and cell signalling. The major late promoter (MLP) promotes the expression of late genes post DNA replication which comprises of L1-L5 genes that code primarily for capsid proteins. At each end of the genome is an inverted terminal repeat (ITR) from which DNA replication begins. The genome structure of adenoviruses is represented in **figure 1.4**.

The E1A gene is composed of two exons leading to the formation of several E1A polypeptides following alternative splicing of the primary RNA transcript, with E1A-13s and E1A-12s being the most abundant (**figure 1.4B**). 13s and 12s (which differ only in the exclusion of the conserved region CR3 from E1A-12s) act in several ways to encourage viral replication including as a regulator of viral transcription through their interaction with many host-cell proteins, many of which are involved in the regulation and control of transcription, some of which are indicated in **figure 1.4B**. When expressed in cells independent of the other adenovirus proteins, E1A can induce immortalisation in primary cell lines and can induce S phase progression in quiescent cells (Ruley 1983; Frisch and Mymryk 2002).

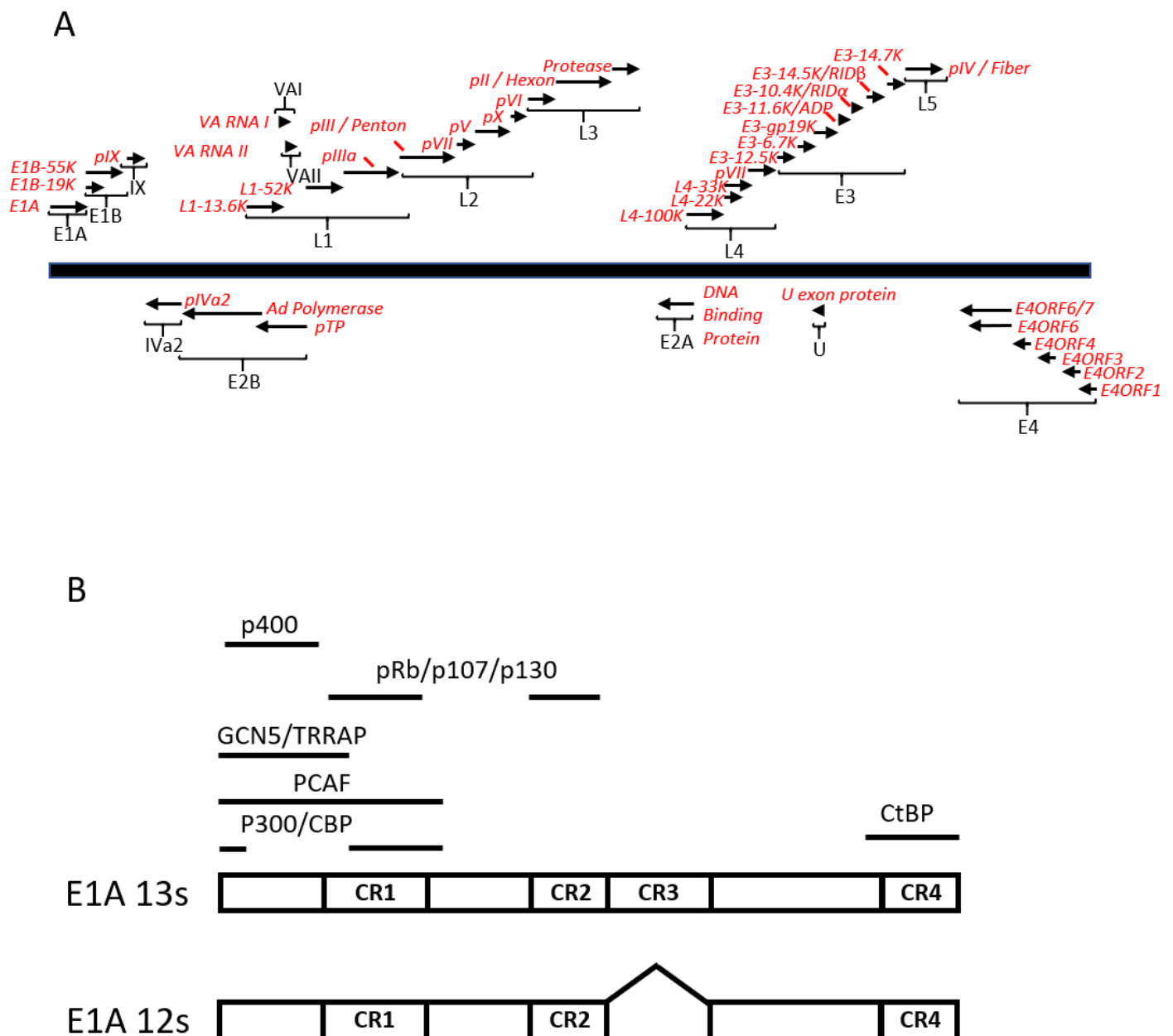


Figure 1.4 Schematic representation of the adenovirus genome.

- (A) A diagram depicting the genome structure of adenoviruses with transcriptional units denoted in black and the respective proteins coded for denoted in red. VA = viral associated, E = early, L = late, ADP = adenovirus death protein, RID = receptor internalisation and degradation, p = protein, pTP = pre-terminal protein.
- (B) A depiction of the early adenovirus region E1A showing the conserved regions (CR1-4) and the two most common spliced isoforms: E1A-12s and E1A-13s. Binding sites for cellular proteins are indicated by bars above the E1A proteins. CR = conserved region, CBP = CREB-binding protein, PCAF = P300/CBP-associated factor, TRRAP = transactivation/transformation domain-associated protein, GCN5 = General Control Of Amino Acid Synthesis Protein 5, pRb = phosphorylated retinoblastoma protein. Adapted from Guimet and Hearing 2016

Predominant amongst the interactions of E1A with host cell processes is the activation of gene expression through the E2F family of transcription factors. This family of transcription factors play an important role in the expression of genes controlling cell cycle progression, in part through interactions with the retinoblastoma gene family (Rb). Rb interactions with E2Fs are controlled through phosphorylation of Rb by cyclin-dependent kinases resulting in their dissociation from E2Fs, allowing the repression of E2Fs to be lifted (Frisch and Mymryk 2002; DeCaprio 2009). Adenovirus E1A proteins allow the virus to subvert this tight control of cell cycle progression by E2Fs by binding directly to Rb proteins resulting in their sequestration, allowing E2Fs to activate both viral and host cell gene expressions. The CR4 domain of E1A at the C-terminal exon contains a nuclear localisation signal and a binding site for the transcriptional corepressor c-terminal binding protein (CtBP) (Chinnadurai 2011; Yousef *et al.* 2012). One other protein interacting with E1A is p300 which is usually in a complex with the CREB-binding protein (CBP) acting as a transcriptional regulator with binding sites identified in CR1 and CR3 of E1A. Overall E1A can both inhibit p300/CBP activity in some contexts and promote it in others through redirecting p300/CBP from cellular promoters allow for the histone acetyltransferase activity of P300/CBP to occur.

A promising advance in the creation of tumour-specific oncolytic adenoviruses replies upon modifying the E1A region to prevent the virus from altering host cell defences (such as pRb mediated apoptosis) allowing the virus to only replicate within cells in which such pathways are already defective (such as tumour cells). Notable amongst these examples is the 24-base pair deletion in the CR2 region of E1A. This region is

responsible for binding pRb, ensuring that viruses deleted in this region will only replicate within cell sin which abnormal Rb control is already active. Defective Rb/p16 pathways have been identified in a vast majority of cancers making this deletion particularly pertinent (Sellers and Kaelin 1997; Fueyo *et al.* 2000; Öberg *et al.* 2010; Cherubini *et al.* 2011; Konecny *et al.* 2011; Aguirre-Hernández *et al.* 2018; Hulin-Curtis *et al.* 2018).

Early region 1B (E1B) encodes the E1B-19k and E1B-55K proteins which predominantly function to inhibit apoptosis as well as creating an environment which is hospitable to viral protein production and DNA replication. The importance of E1B proteins can best be seen in the significantly reduced viral yield in E1B mutated viruses owing to premature cell death by apoptosis. The E1B-55k protein binds to p53, resulting in an inhibition of p53-induced transcription. Interestingly, the 55K protein also promotes cell transformation through an unknown mechanism through to be partly through the inhibition of the proapoptotic protein Daxx. Additionally, E1B-55K acts in conjunction with the Ad protein E4-ORF6 to promote the degradation of p53 and other cellular protein targets (Blackford and Grand 2009; Tarakanova and Wold 2010; Schreiner, Wimmer and Dobner 2012).

The E1B-19K protein is also involved in the inhibition of apoptosis, although this is through blocking apoptotic pathways which do not rely upon p53 such as the tumour necrosis factor- α pathway. Essentially, E1B-19K is a functional homologue of the cellular suppressor of apoptosis Bcl-2 by binding and inhibiting the proapoptotic

proteins Bax and Bak (Han *et al.* 1996; Degenhardt, Perez and White 2000; Lomonosova, Subramanian and Chinnadurai 2005; Tarakanova and Wold 2010). As with Bcl-2, E1B-19K acts with beclin-1 to also promote autophagy. Building upon the promising success of E1ACR2 deleted oncolytic adenoviruses, viruses deleted in both E1ACR2 and E1B-19K show greatly enhanced cell killing and highly promising results. The double deleted adenoviruses are particularly powerful when combined with DNA-damaging agents or to apoptosis-inducing agents such as mitoxantrone, in part through the loss of the viral manipulation of autophagy (Leitner *et al.* 2009; Pantelidou *et al.* 2016; Aguirre-Hernández *et al.* 2018).

1.6.2.2 Adenovirus Early Gene expression – E2 and E3

The E2 transcription unit encodes proteins required for the replication of viral DNA. The DNA-binding protein (DBP) binds to single-stranded DNA to simulate the initiation and elongation of viral DNA. The precursor terminal protein (pTP) precursor terminal protein (pTP) binds DNA as well as forming a heterodimer with adenovirus-polymerase (ad-pol) resulting in the formation of the preinitiation complex for viral DNA replication. The pTP also is involved in the attachment of viral DNA to the nuclear matrix allowing efficient transcription and DNA replication. Additionally, through binding to viral DNA, pTP blocks the accessibility of 5' exonucleases preventing the host-cell recognition of the ends of the viral genome (Schaack *et al.* 1990; de Jong, Meijer and van der Vliet 2003; De Jong, Van Der Vliet and Brenkman 2003).

The E3 region contains seven open reading frames which encode proteins involved with modulating the host immune system to favour infection. The E3-6.7K is one of several adenovirus genes that interferes with apoptosis. In the case of E3-6.7K, inhibition of apoptosis is achieved through manipulation of the tumour necrosis factor-related apoptosis-inducing ligand (TRAIL). The E3 proteins encoding for receptor internalisation and degradation complexes α and β (RID α and RID β), and E3-14.7K proteins also inhibit TRAIL apoptosis as well as interfering upstream with tumour necrosis factor α (TNF α) and the FAS ligand (Benedict *et al.* 2001; Lichtenstein *et al.* 2004a, 2004b; Moise *et al.* 2004). Many viruses contain proteins which inhibit apoptosis (a cellular response to stresses including viral infection). Avoiding premature host-cell death pathways is vital for all viruses as it ensures that the virus can lyse cells only when the viral progeny is packaged.

Cytotoxic t cells respond to antigens presented by major histocompatibility complexes (MHCs). Upon recognition of an antigen presented in such a way, t cells will release perforins and granzymes that promote killing of the infected cell. Many viruses have developed ways to inhibit this immune signalling pathway and antigen presentation to avoid detection, and ultimately clearance, by a host's immune system. The E3-19k protein functions effectively to inhibit antigen processing and counters both the innate and adaptive immune responses. Through forming a complex with MHC class I molecules, E3-19K prevents peptide presentation allowing an avoidance of detection of infected host cells by cytotoxic t cells (Fessler, Delgado-Lopez and Horwitz 2004; Horwitz 2004; Lichtenstein *et al.* 2004b). Additionally, to avoid detection by natural

killer cells, E3-19K also acts to suppress expression of the natural killer group 2D (NKG2D) ligands MHC class I chain-related proteins A and B (MICA and MICB) thus avoiding the innate immune system (McSharry *et al.* 2008).

One protein from the E3 region, which does not have immunomodulatory functions is the adenovirus death protein (ADP). The ADP unlike all the other E3 proteins is not expressed at early stages of infection. ADP is synthesised from mRNA, which contains the adenovirus tripartite leader, (TPL). The TPL allows for the translation of late proteins even during host cell protein translation shut down, which occurs late in adenovirus infection. As discussed earlier, viruses need to be able to control the moment at which a host cell is lysed to ensure that viral genomes are packaged ready for a new round of infection, and ADP is understood to kill cells through interfering with the nuclear membrane integrity. Indeed, both caspase-dependent and caspase-independent mechanisms of cell death have been observed when ADP is overexpressed in cells, and the exact killing mechanism is not fully understood (Abou El Hassan *et al.* 2004; Yun *et al.* 2005; Guimet and Hearing 2016). We have recently shown that the oncolytic adenovirus EnAd which is deficient in ADP kills via a mechanism known as oncosis, perhaps in part due to the absence of the ADP (Dyer *et al.* 2017).

Often, the E3 region of adenoviruses is deleted or removed from oncolytic adenoviruses, in part to allow for more than 2kb of space in the genome which allows researchers to insert transgenes. In addition to allowing for an increased transgene

capacity, the partial or complete deletion of E3 allows for presentation of viral antigens on the surface of infected tumour cells which can trigger an anti-cancer immune response and aid in turning an immunologically “cold” tumour into an immunologically “warm” tumour.

1.6.2.3 Adenovirus Early Gene expression – E4

Compared to the other early regions of adenoviruses, E4 gene products display quite a wide range of activities including transcription regulation, DNA replication, mRNA processing, host cell protein synthesis shutoff, oncogenic transformation, and apoptosis inhibition (listed in **table 1.2**). The E4 proteins are identified by the presence of multiple open reading frames (ORFs)

E4ORF1 has a wide range of activities, in part thanks to the C-terminal sequence motif referred to as a PDZ domain. The PDZ binding motif allows binding to a select group of cellular PDZ domain-containing proteins which causes activation of PI3K and AKT (Javier 1994; Frese *et al.* 2003; Chung *et al.* 2007). IT has also recently been shown that E4ORF1 activates C-Myc and promotes cellular anabolic glucose and glutamine metabolism, which work to promote viral replication (Thai *et al.* 2014, 2015).

The E4ORF3 protein is highly conserved between adenoviruses with multiple functions. The primary reported functions of E4ORF3 appear to be the inactivation of cellular stress response pathways, including interferon signalling, p53 signalling, and the DNA

damage response machinery. Adenoviruses with deletions in E4ORF3 are deficient in DNA replication in cells displaying an antiviral response through expression of interferons (Ullman, Reich and Hearing 2007). This control of interferon signalling is understood to be through interaction with promyelocytic leukaemia oncogenic domains (PODs), which E4ORF3 has been shown to localise with (Doucas *et al.* 1996). Normally, Mre11-Rad50-Nbs1 complexes (MRN complexes) will be recruited to the ends of damaged DNA, if left unprotected, the ends of the viral genome will be recognised by the host cell as a double-stranded DNA break and will elicit a DNA damage response. E4ORF3 effectively inhibits this DNA damage response by sequestering MRN proteins in the nucleus (Weitzman and Ornelles 2005). Additionally, E4ORF3 blocks p53 DNA binding and transactivation by inducing heterochromatin formation at the site of p53 promoters (Soria *et al.* 2010).

As with E4ORF3, the E4ORF4 protein is also multifunctional; however, the majority of its actions are owing to the E4ORF4 binding partner, PP2A. Once E4ORF4 is bound to PP2A, it activates the phosphatase activities of PP2A resulting in the dephosphorylation of target proteins including MAP kinases as well as the decreased phosphorylation (and therefore inactivation) of various transcription factors (Branton and Roopchand 2001; Kleinberger 2015). Additionally, E4ORF4 acts to decrease the activity of the adenovirus E1A, thereby effectively aiding the switch from early to late gene expressions. E4ORF4 has interestingly been shown to induce selective apoptosis of malignant cells with papers showing that oncogenic transformation of non-malignant cells renders them susceptible to killing by E4ORF4. However, this has only

been shown when E4ORF4 is expressed alone. This cancer-specific cell death is again dependent on the E4ORF4 protein binding to PP2A and requires modulation of Src-family kinases (Shtrichman *et al.* 1999; Branton and Roopchand 2001).

Finally, the E4ORF6 protein binds to p53 thereby inhibiting it as well as forming a protein complex with the E1B-55K protein to target p53, and other cellular proteins for proteasome-dependent degradation through polyubiquitination (Ben-Israel and Kleinberger 2002; Schreiner, Wimmer and Dobner 2012).

1.6.2 Adenovirus lifecycle

The precise features, and divisions of the adenoviral life cycle remains a topic of scientific debate and is broadly based upon research conducted with the serotype ad5 in non-cancerous cells. Nevertheless, it is essential to understand the broad divisions of the virus life cycle. The adenoviral life cycle can be divided into cell attachment and uptake, endosomal escape, nuclear import, early gene expression, DNA replication, late gene expression, assembly, and release (as depicted in **figure 1.5**).

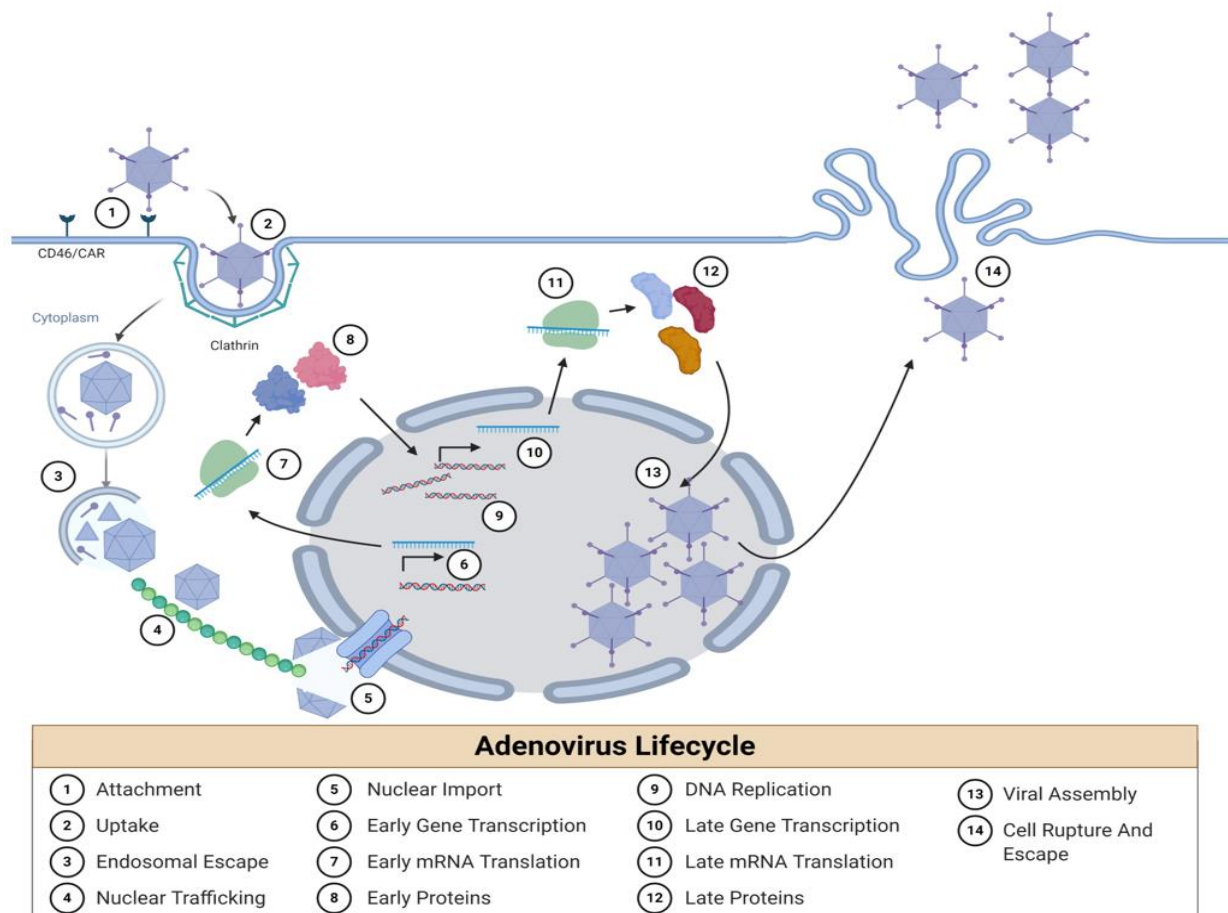


Figure 1.5 Schematic representation of the adenovirus lifecycle.

Adenoviruses infect cells through binding to their receptor (examples include the coxsackievirus and adenovirus receptor (CAR) or CD46 and viruses are internalised in via clathrin-coated endocytic vesicles. Once the virus has escaped the endosome, it is then trafficked to the nucleus via microtubules. The genome is transported into the nucleus, and the early genes are transcribed and translated to produce early proteins. After DNA replication, the late genes (which are mainly structural) are transcribed and translated and genomes are loaded into the virus capsids whereupon the cell is lysed, and the viruses escape. Created with Biorender.com.

1.6.2.1 Cell attachment and uptake

The primary receptor for adenovirus groups A, C, E and F, is the CAR receptor which binds the knob domain of the adenovirus fibre protein (Bewley *et al.* 1999). Group B adenoviruses do not bind to CAR but rather bind to CD46 (Gaggar, Shayakhmetov and Lieber 2003). CAR is upregulated in many human tissues, including erythrocytes, and the liver leading to hepatotropism, which is undesirable for systemically delivered OV.

CD46 is present on all nucleated cells, however it is upregulated in cancer (Do *et al.* 2018; Su *et al.* 2018). Attachment to receptors ultimately leads to host cell cytoskeletal rearrangement leading to internalisation in clathrin-coated vesicles.

1.6.2.2 Endosomal escape and nuclear import

As with all viruses that enter via endocytosis, adenoviruses must escape the endosomal compartment before degradation occurs in the lysosomes. The current theory on how this escape occurs is that acidification of the endosome results in capsid disassembly, which exposes the viral protease and a minor viral protein VI which ultimately ruptures the endosome (Wiethoff *et al.* 2005; McGuire *et al.* 2011). It has also been shown that, owing to their binding of CD46 and not CAR, group B adenoviruses require a lower pH for endosomal escape (Dales 1962; Chardonnet and Dales 1970; Miyazawa, Crystal and Leopold 2001). The virus is then trafficked by microtubules towards the nucleus where the hexon protein binds to nuclear pore complexes. Viral DNA is then imported through the nuclear pore in a mechanism that is still poorly understood.

1.6.2.3 Early gene expression and DNA replication

The E1A gene is immediately transcribed upon reaching the nucleus producing a single pre-mRNA which is alternatively spliced to produce splice products, with 12S and 13S proteins being the most abundant. The E1A protein interacts with a wide range of host cell pathways to reprogram cellular gene transcription, including metabolic genes as will be discussed later. E1A also allows for the activation of the transcription of other early viral genes such as E2 which encodes for the viral DNA polymerase, a pre-

terminal protein (pTP) and a DNA binding protein. The viral DNA polymerase and the pTP then bind to the ITRs at the 5' end of the virus genome and initiate viral DNA replication.

1.6.2.4 Late genes, capsid assembly, and cell lysis

After DNA replication, late proteins under the control of the major late promoter (MLP) are transcribed as a single mRNA construct that is alternatively spliced. Every spliced mRNA contains a tripartite leader (TPL) sequence at the 5' end which functions as an mRNA cap, thereby avoiding the requirement for a host cell-derived cap. The use of a cap-independent translation initiation allows these proteins to be expressed despite global downregulation of protein translation induced by adenovirus infection (Ramke *et al.* 2017). The late protein products are largely structural and assemble into empty capsids which are then loaded with DNA before cell lysis. In group C adenoviruses (such as Ad5), death occurs upon expression of the adenovirus death protein (ADP), causing a death pathway similar to apoptosis. In contrast, group B adenoviruses lack this protein and therefore have a unique death mechanism (Dyer *et al.* 2017).

1.6.3 Oncolytic Adenoviruses

As discussed earlier in this chapter, there are multiple methods to ensure that a chosen oncolytic virus is cancer-selective. Few virus platforms have received as much attention as adenoviruses, likely due to several including a deep understanding of the adenovirus genome, a low level of pathogenicity in the wildtype virus and the relative

ease of genetic manipulation. Because of these factors, there are many examples of oncolytic adenoviruses with varying degrees of success. At the time of writing, there are currently 231 clinical trials utilising adenoviruses for cancer treatment listed on clinicaltrials.gov (clinicaltrials.gov 2020).

The most commonly used tactic to restrict adenovirus replication to tumour cells is to alter the early genes, although the precise mutations and early gene deletions vary quite widely. ONYX-015 was the first oncolytic adenovirus to reach clinical trials and is deleted in the early genes E1B55K and E2B. We have discussed this virus earlier in section 1.5.2. Contrary to intentions, cancer-selectivity of the virus is not actually defined by p53 status and clinical trials in the west showed relatively limited efficacy, nevertheless it is interesting to note that the Chinese FDA approved a nearly identical virus (named H101) for combination therapy with chemotherapeutics in head and neck cancers in 2005. Many patients have now been treated with no reported side-effects (Garber 2006; Yu and Fang 2007).

Because of the reduced replication observed with E1B55K deleted mutants and with our increased understanding of adenovirus genome, we can now be more inventive with deletions of early adenovirus genes to ensure cancer selectivity and retain replicative potency. One pertinent example of this is a deletion of one of the four conserved regions of the E1A gene (E1A conserved region 2 or E1ACR2) which binds to the retinoblastoma protein (pRb). When E1ACR2 is deleted, the virus is restricted to grow in cells with a dysregulated cell cycle and is therefore rendered partially cancer-

selective (Fueyo *et al.* 2000; Heise *et al.* 2000). There are many examples of E1ACR2 deleted adenoviruses, and this class of oncolytic adenoviruses presents the basis most promising results in clinical trials, especially when combined with an arming of the virus with a therapeutic transgene (Rojas *et al.* 2009; Nokisalmi *et al.* 2010; Öberg *et al.* 2010; Cherubini *et al.* 2011; Kuryk *et al.* 2017; Aguirre-Hernández *et al.* 2018; Nattress and Halldén 2018).

Another useful deletion of oncolytic adenoviruses is the deletion of the anti-apoptosis gene E1B19K protein which, when present in adenoviruses, inhibits apoptosis to allow greater viral replication (Han *et al.* 1996; Degenhardt, Perez and White 2000; Tarakanova and Wold 2010). When combined with a pro-apoptotic treatment such as chemotherapeutics, E1B19K deleted oncolytic adenoviruses cause a marked improvement in potency and viral replication (Öberg *et al.* 2010; Cherubini *et al.* 2011; Aguirre-Hernández *et al.* 2018).

Several other approaches to restrict OV to tumour cells have been used to good effect in adenoviruses. Through altering the outer proteins of adenoviruses to bind to cancer-specific markers, we can effectively retarget the virus to only enter cells which express those proteins. The tactic of retargeting oncolytic adenoviruses is particularly useful for those using Ad5 as an OV platform because the usual receptor of entry is CAR. CAR is expressed on a wide variety of cells which can lead to sequestration of systemically delivered Ad5 in the blood through binding to erythrocytes (Carlisle *et al.* 2009; Coughlan *et al.* 2012). Finally, cancer selectivity can be garnered through placing the

essential E1A gene expression under the control of a tumour-specific promoter. One example of this is the human telomerase reverse transcriptase promoter which is upregulated in a variety of cancer cells (Nemunaitis *et al.* 2010).

Some of the most promising oncolytic adenoviruses have been discussed above and fall into one of three categories: retargeted viruses, viruses under the control of tumour promoters, and viruses with trans-complimentary deletions. Often these viral backbones are then armed with a therapeutic transgene (typically either an immunostimulatory gene, a pro-drug, or an anti-cancer drug) allowing highly specific expression of a payload directly within tumours avoiding some of the toxicities that would be associated with systemic delivery of such drugs (Seymour and Fisher 2016; Dyer *et al.* 2019). We have listed some of the oncolytic adenoviruses currently undergoing clinical trials in **table 1.3** adapted from a recent review by Cunliffe *et al.* (Cunliffe, Bates and Parker 2020). The most promising of these candidates are the viruses which are truly restricted to only replicate within tumour cells (either through altering the receptor binding or through trans-complementation) without losing the immunostimulatory nature of the virus or without attenuating the viruses so much that it loses the ability to lyse cells and spread through a tumour.

Table 1.3 A summary table of oncolytic adenoviruses currently in clinical trials, including their genome, modifications and clinical target. Table adapted from Cunliffe *et al.* 2020. GMCSF = Granulocyte-macrophage colony-stimulating factor, Rb = retinoblastoma protein, Ad = adenovirus, OX40 = Tumour necrosis factor receptor superfamily member 4, E1ACR2 = adenovirus early gene 1A conserved region 2, RGD-4C = integrin $\alpha\beta3$, E319K – adenovirus early gene 3 - 19kda protein, E3 = adenovirus early gene 3.

Adenovirus type	Modifications	Clinical Target	NCI identifier
Ad5/3 (capsid-modified)	Replaced Ad5 knob with Ad3. Expressing GMCSF	Rb/p16 defective cells containing Ad3 receptors	C98287
Ad5	Expresses OX40 ligand. Missing E1ACR2	Rb/p16 defective cells	C160192
Ad5/3	Missing E1ACR2. RGD-4C motif. Mutation in E319K gene (increasing Ad5 release). Replaced Ad5 knob with Ad3.	Rb/p16 defective cells containing Ad3 receptors	C168607
Ad5	E2F-1 promotor. Missing E3 region. Expressing GMCSF	Rb/p16 defective cells	C48412
Ad5/Ad35	Missing E1ACR2. Contains L5 segment of fiber from Ad35. Expresses CD40 ligand	CD46-expressing cells, Rb/p16 defective cells	C148462
Ad5 (delivered inside bone marrow-derived mesenchymal stem cells)	Contains RGD-4C motif (allowing integrin binding), missing E1ACR2	Rb/p16 defective cells, not reliant upon CAR expression	159798
Ad5-DNX-2401	Contains RGD-4C motif (allowing integrin binding), missing E1ACR2	Rb/p16 defective cells, not reliant upon CAR expression	C74067
Ad5	Encodes murine interleukin 12 in the E3 region and a suicide fusion gene in the E1 region (E1B55K)	E1B55K deletions target cells with defective p53 targeting and cell cycle disruptions. Affects late viral mRNA export	C123930
Chimeric Ad3/Ad11p (EnAd)	Chimeric adenovirus EnAd with chimeric E2 region, deletion in E3 regions and E4ORF4	CD46-expressing cells, not reliant upon CAR expression. Mechanism of selectivity not fully understood	C113786

1.6.4 Enadenotucirev

EnAd has a 98.6% sequence homology to that of the group B adenovirus Ad11p. The differences between Ad11p and EnAd are depicted in **figure 1.6**. EnAd has a near-complete deletion in the E3 region (responsible for host immune modulation), a partial deletion in E4orf4 (responsible for activating mTOR through a PI3K-independent mechanism in a manner similar to that of limiting glucose, as well as dampening E1A), and a chimeric ad3/ad11p E2b region (believed to allow the viral polymerase to act faster).

The E3 deletion restricts EnAd to regions with immune-suppressed environments (such as in tumours). The E4ORF4 deletion is of particular interest. Many groups have shown that transfection of cancer cells with the E4ORF4 region results in cancer-specific cell death, therefore, it is surprising that this is missing in EnAd (Shtrichman *et al.* 1999; Dziengelewski *et al.* 2020). We have speculated a number of theories as to why EnAd has lost this region. One possibility is that in Ad5 (a group C adenovirus), E4ORF4 activates mTOR through a PI3K-independent mechanism in a manner similar to that of limiting glucose. Although it is not known if group B E4ORF4 acts similarly, it may be the case that EnAd is restricted to cells which already have dysregulated mTOR signalling or aberrant metabolism.

Despite these questions, the process of directed evolution leads to an oncolytic virus which can lyse tumour cells whilst being unable to replicate within normal cells (Kuhn *et al.*, 2008). In clinical trials, EnAd has been shown to reach the tumour following

systemic intravenous delivery and to have good safety and tolerability whilst also leading to a relatively strong antitumour immune response, in part thanks to a lack of antibodies to this strain in most of the population (Garcia-Carbonero *et al.* 2017; Machiels *et al.* 2019).



Figure 1.6 Comparison of the genome of the oncolytic adenovirus enadenotucirev (EnAd) and the parental wildtype adenovirus Ad11p.

Schematic representation of the wildtype adenovirus and oncolytic adenovirus enadenotucirev EnAd. EnAd was created through the process of directed evolution and contains a chimeric E2B region containing portions of Ad5 E2B and Ad11p. EnAd also contains a 2444 base pair (bp) deletion in the E3 region alongside a 25 bp deletion in the E4 region equating to E4ORF4.

We have also shown that EnAd lyses cells in a unique death pathway known as oncosis whereby ATP rapidly declines in the host cell leading to a distinctive single membrane blister alongside expression of immune-stimulatory markers such as HSP70 and calreticulin (Dyer *et al.* 2017). The reason for this unique death pathway is speculated to be because EnAd has lost E4ORF4, which interacts with the mTOR-AMPK pathway. In addition, E4ORF4 acts to dampen the viral protein E1A, which partly is responsible for creating a Warburg-like metabolic phenotype (high aerobic glycolysis, lactate production and glutamine metabolism, Prusinkiewicz *et al.* 2020). It is possible that

because EnAd has lost E4ORF4, it cannot inhibit this metabolically active signalling and eventually the cells rupture due to ATP loss.

With regards to the relationship between the novel chimeric virus EnAd and the parental virus Ad11p, the EnAd genome contains three large modifications leaving a 98.6% sequence identity (Kuhn *et al.* 2008). The genetic differences between the parental wildtype adenovirus Ad11p and the widely studied adenovirus Ad5 have been reviewed previously but are summarised in **table 1.4** (Mei *et al.*; Stone *et al.* 2003).

Table 1.4 Genetic differences of the three affected regions between Ad11p to EnAd, and commonly used wildtype Ad5. Details of the aa identity between Ad5 and Ad11p as adapted from Mei *et al.* 2003 and Kuhn *et al.* 2008. E = adenovirus early gene, pTP = pre-terminal protein, Ad = adenovirus, AdPol = adenovirus polymerase, bp = base pair.

Gene	Ad11p	Ad5 (% amino acid identity with Ad11p)	EnAd
E2 pTP	+	+ (78.7%)	Ad3 substitution
E2 <u>AdPol</u>	+	+ (57.1%)	Ad3 substitution
DNA Binding Protein	+	+ (85.0%)	+
E3 12.5K	+	+ (53.3%)	+
E3 16.1 K	+	-	+
E3 18.5K	+	+ (35.8%)	Fused to E3-15.3K
E3 15.3K	+	+ (53.1%)	Fused to E3-15.3K
E4ORF4	+	+ (44.3%)	- (24bp deletion)

As previously discussed, EnAd was created through a process known as directed evolution.

The specific process that created EnAd was carried out by super-infecting the colon

carcinoma cell line, HT29 with a library of adenoviruses from different serotypes. The

super-infection conditions encouraged recombination between the serotypes producing a

highly diverse library where Darwinian evolution dictates that any redundant genomic

information for survival in the very particular cancer cell line was likely lost. After two such rounds of infection, supernatants from the infected cells were then diluted and infected flasks of the following cell lines: the colon carcinoma cell line HT29, the pancreatic cancer cell line Panc-1. The breast cancer cell line MDA-231 and the prostate cancer cell line PC3. After the first signs of cytopathic effect (CPE), infected cells were collected and passaged onto new cells a total of 20 times.

After 20 passages, viruses were isolated through two rounds of plaque purification on A549 cells allowing for the purification of single viral colonies. From the second round of plaque purification, these purified viral populations were then analysed, and EnAd was selected as the most suitable candidate for investigation owing to the oncolytic potency on a range of cancer cells coupled with its low toxicity on non-malignant primary endothelial and epithelial cells HUVEC and HMEC. Ultimately, it was shown that EnAd is 2-3 logs more potent and selective than the parental serotypes and more potent than the Ad5-based oncolytic virus ONYX-015 (Kuhn *et al.* 2008). Additionally, because EnAd shares a high level of similarity with Ad11p, it uses CD46 as a receptor and not CAR making it suitable for intravenous delivery without the fear of either sequestration in erythrocytes or reactivity with pre-existing Ad5 antibodies which are present in approximately 50% of the population. The near-complete deletion of E3 also allows for quite a large transgene capacity in EnAd when compared to other oncolytic viruses; this provides extra appeal when contemplating the next generation of oncolytic viruses.

Because EnAd was designed using evolution and not intelligent design, it is not currently known what the reasons are for the deletions generated in EnAd. However, given that we

know the function of the missing genes, we can speculate. Indeed, much speculation on the area has been widely published both by our groups and others (Dyer *et al.* 2017, 2018, 2019; Cunliffe, Bates and Parker 2020). The first alteration is the chimeric E2B region which has been hypothesised to enhance the adenovirus pre-terminal protein (pTP) and the viral DNA polymerase activity allowing for faster replication of viral DNA.

The second genomic mutation present in EnAd is a large deletion of E3. We have previously discussed that E3 proteins, which are responsible for immune-modulation, are thought to be non-essential for growing adenoviruses in cultured conditions *in vitro* without the pressure of the host's immune system. Five E3 proteins from Ad5 are present in Ad11p, however the amino acid identity of these proteins is quite low. Additionally, two E3 proteins (20.3K and 20.6K) are only found in group B viruses and have unknown functions. The EnAd E3 deletions likely render the virus unable to manipulate the host cell immune responses; this may mean that viral antigens will be presented more on MHC class-I molecules leading to the creation of a more immunologically warm tumour. It would be intriguing to carry out directed evolution in the presence of a functioning immune system; however, this is limited with adenoviruses as mice are not permissive to adenovirus replication (Lei *et al.* 2018). Additionally, correct and consistent culture systems would be difficult to achieve.

The third genomic alteration in EnAd is a deletion in E4ORF4. The functions of E4ORF4 in Ad5 have been described (section 1.6.2.3); however, the sequence identity between Ad5 and Ad11p E4ORF4 is only 55.7% therefore it is likely that the two proteins have different functions. Ad5 E4ORF4 has been shown to inhibit E1A transcription, thus aiding the switch from the early portion of the viral lifecycle to the late portion. Additionally, E4ORF4

activated the mTOR pathway through interaction with protein phosphatase 2A (PP2A).

Overall, we have hypothesised that the E4ORF4 mutation is likely to render EnAd unable to hijack certain cellular processes, leading to a rapid drop in ATP and an unorganised, immunogenic cell swelling and death known as ischemic cell death or oncosis (Dyer *et al.* 2017).

The molecular target of rapamycin (mTOR) and the pathway downstream of it, integrate signals from multiple sources such as growth factors and nutrients to regulate the translation of proteins important to cell growth and regulation. Growth factors stimulate mTOR through inactivation of the TSC1/2 complex, which in turn lifts TSC1/2 inhibition of mTOR. This, in turn, induces the phosphorylation of many key downstream proteins such as the translation initiation factor 4E binding protein 1 (4EBP1). Low glucose has also been shown to activate mTOR through inhibition of AMPK, resulting in decreased Rheb-GTP. Adenoviruses have been shown to encode two proteins, E4ORF1 and E4ORF4, that activate mTOR even under conditions of nutrient/growth factor deprivation. E4ORF1 activates mTOR through a similar pathway to growth factors, whereas E4ORF4 mediated activation of mTOR occurs in the absence of increases in Rheb-GTP loading; thus, it is believed to substitute for glucose signalling to mTOR. It is thought that this is mediated through the binding of PP2A to E4ORF4 (O'Shea *et al.* 2005; Brunton *et al.* 2013). Interestingly, many tumours have constitutive mTOR activation leading some to hypothesise that EnAd may be restricted to such cells which are unable to respond to low glucose levels of already have aberrant growth factor sensing and mTOR signalling.

1.7 Oncolytic viruses and metabolism

As viruses are obligate intracellular pathogens, they need to hijack cellular processes to create a favourable environment for efficient production of infectious progeny, and this includes metabolism. In many ways, their demands for macromolecular synthesis appear similar to those of a proliferating or cancer cell (Dyer *et al.* 2018, 2020).

Metabolomic analysis of virally infected cells was first conducted with cells infected with human cytomegalovirus (HCMV; Munger, Bajad, Coller, Shenk, & Rabinowitz, 2006). During the lytic portion of its lifecycle, HCMV was found to induce a range of metabolic changes including an upregulation of the tricarboxylic acid (TCA) cycle, nucleotide biosynthesis, and glycolysis. Such alterations are now known to be critical for replication of many viruses, including Kaposi's sarcoma-associated herpesvirus, herpes simplex virus 1 (HSV-1), adenovirus, hepatitis C virus, human immunodeficiency virus and dengue virus (Munger *et al.* 2006; Birungi *et al.* 2010; Diamond *et al.* 2010; Hollenbaugh, Munger and Kim 2011; Vastag *et al.* 2011; Delgado *et al.* 2012).

Despite there being some common metabolic alterations to host cells, the methods through which these alterations are achieved vary quite significantly between viruses, and there is no common target used by all viruses. Indeed, even closely related viruses can have vastly different metabolic requirements, such as HSV-1, and HCMV, which are both members of the family Herpesviridae. HSV-1 requires glucose for replication and supplementation of glutamine during primary infections, or viral latency suppresses the virus, whereas HCMV preferentially requires glutamine over glucose to meet its metabolic demands (Courtney, Steiner and Benyesh-Melnick 1973; Chambers, Maguire

and Alwine 2010; Abrantes *et al.* 2012). Although metabolic requirements differ based on which virus is used, each OV requires a specific (yet definable) intracellular metabolic environment and they provide an obvious and, as yet, underexplored platform with which to produce these environments through the expression of metabolic reprogramming agents.

We have reviewed the metabolic demands of various OV platforms in a recent review (Dyer *et al.* 2020) however there are several recent publications harnessing OVs and tumour metabolism which are of note for this thesis. Several OVs have been shown to benefit from short-term starvation and triggering the DSR. Esaki *et al.* reported in 2016 that transient fasting improved the replication of oncolytic herpes virus in glioblastoma models with striking results shown both *in vitro* and *in vivo* (Esaki *et al.* 2016). In 2019, Scheubeck *et al.* reported that low-glucose, low serum starvation can synergistically enhance measles virus-mediated oncolysis efficacy in HT-29 cells (Scheubeck *et al.* 2019). In 2019, Rivadeneira *et al.* showed that the adipokine leptin could metabolically reprogramme exhausted tumour-resident T Cells, when expressed from oncolytic vaccinia virus (Rivadeneira *et al.* 2019). The authors show that engineering oncolytic viruses to express leptin in tumour cells induced complete responses in murine models alongside a long-lasting T cell memory response. Meng *et al.* also reported that infection with oncolytic Newcastle disease virus caused an increase in immunosuppressive factors which dampened the immune response (Meng *et al.* 2020). Through targeting aerobic glycolysis, Meng *et al.* showed a decrease in lactate

accumulation in the tumour leading to improved antitumour efficacy and prolonged survival in mice models (Meng *et al.* 2020).

In two seminal papers, Thai *et al.* reported that wildtype adenoviruses increase aerobic glycolysis and glutamine consumption and that this alteration in metabolism is dependent upon the expression of the adenovirus E4 open reading frame 1 (E4ORF1) (Thai *et al.* 2014, 2015). Upon infection of non-cancerous primary lung epithelial cells, Thai *et al.* report that E4ORF1 localises to the host cell nucleus and binds to MYC. Once bound to MYC, E4ORF1 enhances MYC binding to and promoting the expression of glycolytic and glutaminolysis target genes specifically. These findings were further bolstered in 2020 by results showing that transfection of cells Ad5 E1A resulted in a similar metabolic phenotype of increased aerobic glycolysis, decreased cellular respiration and increased glutamine anapleurosis (Prusinkiewicz *et al.* 2020). These papers aimed to investigate adenovirus metabolism to identify antiviral targets; however, for our research, they help to provide vital aspects of central carbon metabolism which we can interrogate.

EnAd has been shown to have a broad potential to target solid tumour carcinomas with intravenous administration resulting in measurable delivery of viruses to the tumour. There is a possibility that this selection for solid epithelial cancers is through a dependency upon metabolic alterations as the metabolism of solid tumours differs quite widely from liquid tumours which have fewer metabolic demands placed upon them, although this has not been researched.

The metabolic response of cancer cells to oncolytic viruses remains unexplored, and we believe that interrogation of this field could identify strategies to improve viral replication and lysis of host cancer cells.

1.8 Thesis Overview and Hypothesis

1.8.1 Thesis overview

There is a current paucity of work surrounding the interactions between the aberrant metabolism of tumour cells and the activity of OV. Additionally, in the majority of *in vitro* OV work, the levels of glucose used ranges from 5-25 mM glucose whereas, in solid cancers, glucose concentrations are as low as 0.1-0.4 mM, or around six mM for blood cancers (Hirayama *et al.* 2009; Lagziel, Gottlieb and Shlomi 2020).

In nearly all of the studies listed so far, cells were grown in unrealistic conditions with nutrients such as glucose and glutamine far in excess of what is to be found even in normal blood plasma. The impact of high levels of nutrients on these experiments is a crucial consideration for future studies as it is essential to test new drugs targeting cancer cells under physiologically relevant conditions. One example is that of metformin (a drug currently being tested for a wide range of cancer treatments) which is much more effective in enhancing the sensitivity of cancer cells to chemotherapy only when cells are grown in reduced glucose conditions (Yu *et al.* 2016). In 2016, Pusapati *et al.* showed that weaning cancer cell lines off of glucose makes them

significantly more susceptible to co-treatment with mTORc inhibitors and anti-glycolysis drugs (Pusapati *et al.* 2016).

We believe that, through studying the metabolic alterations following infection with OV's such as EnAd in cancer cells, we can identify any crucial metabolites required for efficient viral replication and cell lysis. Likewise, through reducing metabolites to levels seen *in vivo*, we believe that we may reveal previously unknown insights into the viral lifecycle and allow us to narrow the current gap between bench and bedside. Finally, analysis of crucial metabolic conditions required for efficient oncolysis will enable us to artificially create this metabolic profile either through the use of drugs co-administered with EnAd or through arming EnAd with a metabolic transgene.

1.8.2 Hypotheses to be addressed in this thesis

1. Adenovirus infection induces an increase in aerobic glycolysis and glutamine metabolism in host cells.
2. Removal of glutamine from the media will inhibit EnAd oncolytic activity.
3. Removal of glucose from the media will inhibit EnAd oncolytic activity.
4. Chemical induction of a preferential metabolic profile will potentiate EnAd oncolytic activity.

In the final chapter, based on the interpretation of the evidence presented in the previous chapters, a new hypothesis is proposed:

5. EnAd can be armed with glycolytic inhibitors which will alter host cell metabolism and improve its oncolytic potential.

2 Materials and Methods

2.1 Cell culture

2.1.1 Cells passaging and growth

Immortalised cell lines (summarised in **table 2.1**) were maintained in high glucose Dulbecco's modified Eagle's medium (DMEM, Sigma-Aldrich UK) supplemented with 10% heat-inactivated foetal bovine serum (FBS, Gibco, UK, #10500064) unless otherwise stated. Cell lines were split every 2-4 days, to avoid over-confluency. Cell medium was removed, and the monolayer was washed with phosphate-buffered saline (Thermo Fisher, UK) and detached using enzymatic dissociation with trypsin/ethylenediaminetetraacetic acid (EDTA) (0.25% trypsin, 0.02% EDTA; Sigma-Aldrich UK) for a maximum of 5 minutes at 37°C and 5% CO₂ at which point, cells were split at a ratio of 1:4-1:10 depending on their growth rates and maintained in humidified atmosphere in 5% CO₂ at 37°C.

2.1.2 Cell Verification and maintenance

All cell lines were authenticated by STR profiling (CRUK Cambridge Institute). All cell lines were routinely tested for mycoplasma using MycoAlert™ Mycoplasma Detection Kit according to the manufacturer's protocols and were free from contamination during these studies. All cells were maintained for a maximum of 20 passages before being discarded and replaced with a cryopreserved stock.

Table 2.2 – Immortalised cell lines used in this study

Cell Line	Description	Source
HEK293A	- Originally derived from human embryonic kidney cells - Adherent - Contains a stably integrated copy of the adenovirus E1 gene - Cultured in DMEM	ATCC
DLD-1	- Human colorectal adenocarcinoma cell line - Adherent - Cultured in DMEM	ATCC
PC9	- Human lung adenocarcinoma cell line - Formally known as PC14 - Adherent - Cultured in DMEM	ECACC
SKOV3	- Human ovarian adenocarcinoma cell line - Adherent - Cultured in DMEM	ATCC
A549	- Human lung adenocarcinoma cell line - Adherent - Cultured in DMEM	ATCC

ATCC = American Type Culture Collection

ECACC = European Center of Authenticated Cell Cultures

2.1.3 Cryopreservation and recovery

Cells to be cryopreserved were resuspended in a solution of FBS containing 10% dimethyl sulfoxide (DMSO, Sigma Aldrich UK D2650) then aliquoted into cryovials (Sigma Aldrich UK) and cooled slowly in freezing containers (Cryo 1°C Mr Frosty, Nalgene) at -80°C. For long-term storage, cells were transferred to liquid nitrogen tanks (-196°C). Cryopreserved cells were reanimated by thawing at 37°C in a fixed temperature water bath then resuspended dropwise into pre-warmed complete medium followed by centrifugation (400 x g, 5 mins). The supernatant

was discarded carefully, and the cell pellet was then resuspended in complete medium.

2.2 Metabolic assays

2.2.1 Glucose Uptake Analysis

Cells were seeded in 24 well plates (Corning UK) at 100,000 cells per well and left to adhere at 37°C and 5% CO₂ overnight in 1mL high glucose DMEM + 10% FBS.

Cells were then infected with viruses at the stated concentrations in DMEM + 2% FBS, after 90 mins the medium was removed, the monolayer was washed with PBS and replaced with 1 mL DMEM high glucose + 2% FBS containing 300 µMol 2-deoxy-2((7-nitro-2,1,3-benzoxadiazol-4-yl)amino (2-NBDG, Abcam UK) then incubated at 37°C and 5% CO₂. At fixed time points, cells were detached using non-enzymatic dissociation buffer (Gibco, UK), fixed in 4% formalin for 5 minutes washed in PBS then resuspended in MACS buffer. Fluorescence was analysed by flow cytometry using a BD FACS Calibur (BD Biosciences) and data processed with FlowJo v10.0.7r2 software (Treestar Inc., USA).

2.2.2 Glutamine Uptake Assay

Glutamine uptake from the medium was measure using the glutamine-glutamate-glo-assayTM assay kit (Promega, UK) according to the manufacturer's protocol. Briefly, cells were seeded at 10,000 cells per well in a 96 well plate and

left overnight to adhere then infected with viruses at stated concentrations in DMEM (high glucose) + 2% FBS or treated as stated in the text. Ninety minutes post-infection medium was removed from the cells, and they were washed with PBS then 200 μ L DMEM + 2% FBS was added to the wells. At set time points, the medium was removed from wells followed by a wash with PBS. 50 μ L of inactivation solution was added to the samples followed by 50 μ L Tris solution and samples were stored at -20°C. 25 μ L aliquots of samples were pipetted into a white 96-well plate. 25 μ L of Glutaminase Buffer was added to the wells followed by a 40 minutes room-temperature incubation. 50 μ L of Glutamine Detection Reagent was added to all wells, and luminescence was measured using the victor plate reader (1.0 seconds per well).

2.2.3 Lactate Secretion Assay

Lactate secretion into medium was measure using the Lactate-Glo™ assay kit (Promega, UK) according to the manufacturer's protocol. Briefly, cells were plated in a 96 well plate at 10,000 cells per well and left to adhere at 37°C and 5% CO₂ overnight. Cells were then infected with viruses in DMEM + 2% FBS, and after 90 mins the medium was removed and replaced with 200 μ L DMEM high glucose + 2% FBS or were treated according to the text. At fixed time points post-infection, 10 μ L of medium was removed and diluted in 1000 μ L of PBS then stored at -20°C until all the samples were collected. 50 μ L of each sample was transferred to a sterile white 96-well assay plate in triplicate. 50 μ L of Lactate

Detection Reagent was added to each well, and the plate was placed on a shaker for 60 seconds to mix then incubated for 60 minutes at room temperature. Luminescence was recorded using the victor plate reader (1.0 second per well). The data is presented as fold increase in luminescence of uninfected cells.

2.2.4 Seahorse XF Analyser Assay

Extracellular acidification rate (ECAR) and oxygen consumption rate were measured on a Seahorse XF96 analyser (Agilent Technology UK) according to previously established manufacturer protocols. Briefly, cells were plated at 10,000 cells per well in a Seahorse XF96 well microplate and left to adhere overnight at 37°C and 5% CO₂. 200 µl of pH 7.4 calibrant was added to each well of a Seahorse XF96 calibration microplate and left to hydrate overnight at 37°C. The day of the experiment, the supernatant was aspirated from the cell monolayers and replaced with 175 µl of Seahorse Assay medium. The machine was calibrated using the calibration plate, and then ECAR and OCR were recorded for each well. The media was then removed from the plate and replaced with 100 µl of -20 °C methanol and incubated at -20 °C for 5 minutes. The methanol was aspirated carefully and replaced with 200 µl of 4 µg/mL Hoechst (Sigma Aldrich) in 1 x PBS. Cell numbers were assessed using a Celigo imaging cytometer (Nexcelcom) and used to normalise the results. This procedure was performed in collaboration with Sally Frost (DPhil student, Seymour Group, Department of Oncology, University of Oxford).

2.2.5 IC₅₀ assay for 2-deoxy-D-glucose or Enadenotucirev.

Cells were plated at 10,000 cells per well in 200 µl DMEM high glucose + 10% FBS and left to adhere overnight at 37°C and 5% CO₂. Cells were treated in triplicate with 2DG (Sigma Aldrich) from 35 mM to 0 mM or with EnAd from 5000 virus particles per cell to 0 virus particles per cell mM in 200 µl DMEM + 10% FBS. At 72 hours post-treatment for 2DG or 120 hours post-infection for EnAd, media was removed, and cell viability was assessed using CytoTox 96 Non-Radioactive Cytotoxicity assay (Promega, UK) including controls of cells at 100% viability, PBS alone and 100% lysed cells. IC₅₀ concentrations were established using GraphPad software.

2.3 Cell-Based Experiments

2.3.1 Analysis of cell viability using the xCELLigence Real-Time Cell

Analysis system

Cells were seeded at 10,000 cells per well in E-Plate 16 plates (ACEA Biosciences, UK) and incubated overnight to adhere in a humidified atmosphere in 5% CO₂ at 37°C. Impedance measurements were taken every 10 minutes for the duration of the experiment and displayed as Cell Index (CI), which is used to represent cell viability in real-time.

2.3.2 Analysis Light Microscopy analysis of cell morphology

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in humidified atmosphere in 5% CO₂ at 37°C. Cells were then treated as described in the text and incubated in humidified atmosphere in 5% CO₂ at 37°C for 96 hours. Images were taken using a Zeiss Axiovert 25 inverting light/fluorescence microscope, and images were recorded by a Nikon DS-U2 camera and processed with NIS-element AR 3.00 software.

2.3.3 Polyacrylamide gel electrophoresis (PAGE) and transfer to a nitrocellulose membrane

Protein expression was assessed and quantified by western blotting, as previously described (Towbin, Staehelin and Gordon 1979). Briefly, after treatment as described in the text, cell monolayers were scraped using cell lifters and transferred to microtubes for centrifugation (400 g for 5 minutes at room temperature). The supernatant was carefully aspirated, and cell pellets were resuspended in RIPA buffer (Thermo Fisher, UK) supplemented with 1 x Halt™ protease/phosphatase inhibitor cocktail (Thermo Fisher, UK) and incubated on ice for 5 minutes. To remove debris the samples were centrifuged (10 000 g for 10 mins) and the supernatant was removed and placed in a fresh microtube. Cells were incubated with 0.25 U benzonase (EMD, Millipore, USA) for 10 minutes on ice and were stored at -20°C until required. Lysate concentrations were measured using the QuantiPro BCA Assay Kit (Sigma-Aldrich, UK, QPBCA-1KT). Samples

containing 30 µg of each protein lysate in 1 x reducing Laemmli sample buffer were heated at 95°C for 5 min. Proteins were separated on a 4-20% Mini- PROTEAN TGX Precast Protein Gel (Bio-Rad, UK) in Tris-glycine running buffer with sodium dodecyl sulphate (SDS) and transferred onto a 0.2 µm nitrocellulose membrane using the semi-dry blot method through using the Trans-Blot Turbo Transfer System (Bio-Rad, Germany) according to the manufacturers' instructions.

2.3.4 Immunoblot and detection

After transfer of protein to membranes, membranes were washed in TBST (Tris-buffered saline with 0.05% Tween-20, Sigma Aldrich, UK). Transfer efficiency was analysed through exposure of the membrane to ponceau staining solution (Bio-Rad, Germany) for 5 minutes on a benchtop rocker followed by 3 x 5 minutes wash in TBST. Non-specific binding was blocked by incubation of the membrane with 10 mL of EveryBlot Blocking Buffer (Bio-Rad, Germany) for 5 minutes at room temperature. Membranes were then incubated with the appropriate primary antibody at the appropriate dilution in EveryBlot Blocking Buffer at 4°C. The next day, membranes were washed three times in TBST for five minutes. Next, membranes were incubated with the appropriate secondary antibody at the appropriate concentration in EveryBlot Blocking Buffer with constant agitation for two hours at room temperature. Membranes were washed three times for five minutes in TBST. Membranes were incubated with SuperSignal West Dura Extended Duration Substrate (Thermo Fisher, UK, #34075) and bands detected using the Gel Doc system (Bio-rad).

2.4 Virus-Based Experiments

2.4.1 Fluorescence Microscopy analysis of viral infection

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in humidified atmosphere in 5% CO₂ at 37°C. Cells were then infected in triplicate with virus particles at the stated concentration of either EnAd-CMV-GFP, EnAd-SA-GFP, or EnAd-CMV-TIGAR-GFP in DMEM + 2% FBS. Ninety minutes after infection, the medium was removed from the cells, and they were washed with PBS then 1 mL DMEM + 2% FBS was added to the wells unless otherwise stated and cells were incubated in humidified atmosphere in 5% CO₂ at 37°C for 96 hours. Images were taken using a Zeiss Axiovert 25 inverting light/fluorescence microscope using a 400 ms exposure, and images were recorded by a Nikon DS-U2 camera and processed with NIS-element AR 3.00 software.

2.4.2 Flow cytometry analysis of viral protein production

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in a humidified atmosphere in 5% CO₂ at 37°C. Cells were then infected in triplicate with EnAd-CMV-GFP or EnAd-SA-GFP in DMEM + 2% FBS. Ninety minutes after infection, the medium was removed from the cells, and they were washed with PBS then 1 mL DMEM + 2% FBS was added to the wells unless otherwise stated. At set time points cells were washed with 1 x PBS detached using non-enzymatic cell dissociation buffer (Thermo Fisher UK), fixed

in 4% formalin (Sigma Aldrich UK) for 10 minutes then washed and resuspended in MACS buffer and analysed for fluorescence in FL-1. The percentage of fluorescent cells was gated against uninfected cell control, and the mean fluorescence represents the geometric mean of GFP expression.

2.4.3 Determination of Adenovirus Genomes by qPCR

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in humidified atmosphere in 5% CO₂ at 37°C. Cells were then infected in triplicate with virus in DMEM + 2% FBS at the concentrations stated in the text. Ninety minutes after infection, the medium was removed from the cells, and they were washed with PBS then 1 mL DMEM + 2% FBS was added to the wells. At set time points wells were scraped, and the lysate was stored at -20°C until analysis. Samples were extracted with the Purelink genomic DNA mini kit (Thermo Fisher, UK) according to the manufacturer's protocol. Primers and FAM-TAMRA probes (Sigma, UK) recognised the fibre gene. Primers and probe were added to QPCRBIO probe mix Hi-Rox (PCR Biosystems, UK) master mix. The qPCR was run on an ABI PRISM 7000 (Applied Biosystems, UK). qPCR primers and probes used in this thesis are listed in appendix 3.

2.4.4 Determination of Infectious Adenovirus Particles by 50% cell culture infectious dose TCID₅₀

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in humidified atmosphere in 5% CO₂ at 37°C. Cells were then infected in triplicate in DMEM + 2% FBS at the concentrations stated in the text. Ninety minutes after infection, the medium was removed from the cells, and they were washed with PBS then 1 mL DMEM + 2% FBS was added to the wells. At set time points wells were scraped, and the lysate was stored at -20°C until analysis. A549 cells were seeded in 96 well plates at 20,000 cells per well in 200 µL DMEM High Glucose + 10% FBS and incubated in humidified atmosphere in 5% CO₂ at 37°C. 1:10 serial virus dilutions were created in DMEM high glucose + 2% FBS and used to infect the A549 cells. Cells were incubated in humidified atmosphere in 5% CO₂ at 37°C for five days then scored for the presence of viral plaques. The highest dilutions showing cytopathic effect (CPE) were counted, and the infectious particle unit (plaque-forming units [PFUs] per millilitre) calculated using the Karber formula.

2.4.5 Analysis of viral infection using the Celigo Micro-Well Imaging Cytometer

Cells were seeded at 100,000 cells per well in a 24 well plate and incubated overnight to adhere in a humidified atmosphere in 5% CO₂ at 37°C. Cells were then infected in triplicate with EnAd-CMV-GFP or EnAd-SA-GFP in DMEM

supplemented with 2% FBS at the concentrations stated in the text. Ninety minutes after infection, the medium was removed from the cells, and they were washed with PBS then 1 mL RPMI + 2% FBS was added to the wells. At set points post-infection, cells were imaged using the celigo image cytometer (Nexcelom Biosciences, UK) and the celigo software analysed the number of GFP positive cells.

2.4.6 Large-scale virus preparation and purification

EnAd-CMV-GFP, EnAd-SA-GFP and EnAd-SA-FLuc were kind gifts from PsiOxus Therapeutics (Abingdon, UK). EnAd-CMV-GFP is a replication-competent derivative of EnAd, a chimaera of Ad3 and Ad11p with a 2444 bp deletion in E3ORF and a 25 bp deletion in E4ORF4 containing a CMV immediate-early promoter-driven GFP cassette inserted after the fibre gene. EnAd-SA-GFP and EnAd-SA-FLuc are derivatives of EnAd containing GFP or luciferase under the control of the viral major late promoter/splice acceptor. Concentrated and purified virus stocks were prepared through a caesium chloride gradient. Crude or purified virus stocks were used to infect HEK 293 cells grown to confluency in a Corning HYPERFlask. Purified stocks were characterised using the Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher Scientific) and TCID₅₀ using A549 cells. Infectious particles were measured by TCID₅₀ assay on A549 cells using 1:10 serial virus dilutions. Plates were observed day five post-infection. The highest dilutions showing cytopathic effect (CPE) were counted, and the

infectious particle unit (plaque-forming units [PFUs] per millilitre) calculated using the Karber formula.

2.5 Malignant ascites Models

2.5.1 General Statement

Primary human malignant peritoneal ascites were received from the Churchill Hospital, Oxford University Hospitals (Oxford, UK) following written informed consent from patients with indications of advanced carcinoma, predominantly ovarian cancer. The research ethics committee of the Oxford Centre for Histopathology Research approved this work (Reference 09/H0606/5+5) and studies were conducted in accordance with the UK Human Tissue Act of 2004 and the Declaration of Helsinki.

2.5.2 Malignant Ascites processing and characterisation

Upon receipt, cellular and fluid fractions were separated, and fluid used immediately, or aliquots stored at -80°C for future analysis. To collect the cellular fraction, samples were centrifuged (400 g for 10 mins at room temperature). The collected cell pellet was treated with red blood cell lysis buffer (Qiagen, UK, #158904) and cryopreserved at 20 million cells per mL in freezing medium until further use.

For characterisation, cells were stained with Live/Dead Fixable Near IR stain (Invitrogen, UK, #L10119) and antibodies targeting CD4, CD8, EpCAM, FAP, PD-L1, CD11b, CD206, and FR β (Biolegend, UK) and analysed by flow cytometry using an Attune™ NxT (Thermo Fisher, UK) and data were analysed using FlowJo v10.07r2 software (TreeStar Inc., USA). Samples that were chosen for further analysis were placed in a suitable cell culture flask in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% FBS and left to adhere overnight in a humidified atmosphere in 5% CO₂ at 37°C. The supernatant was then aspirated, and adherent cells were then used as stated in the text. This procedure was performed in collaboration with Sally Frost (DPhil student, Seymour Group, Department of Oncology, University of Oxford), Dr Eleanor Scott (DPhil student, Seymour Group, Department of Oncology, University of Oxford), and Dr Sarah Larkin (Post-doctoral research assistant, Human Tissue Facility, Department of Oncology, University of Oxford).

2.6 Murine Subcutaneous xenograft experiments

2.6.1 General Statement

All animal experiments were performed in accordance with the terms of UK Home Office guidelines and the UKCCCR Guidelines for the Welfare of Animals and were approved by the University of Oxford Animal Welfare and Ethical Review Body (AWERB). All animals were held in individually ventilated cages in specific pathogen-free barrier units and allowed to acclimatise for one week

before any procedures were conducted. *In vivo* animal work was performed under licence number P096ECC10.

2.6.2 DLD1 Subcutaneous Model

For *in vivo* co-administration studies, 6-week old female athymic nude mice Crl:NU(NCr)-*Foxn1*^{nu} (Charles River, Margate, UK) were injected subcutaneously with 5×10^6 DLD1 cells. Once xenografts developed to $\sim 100 \text{ mm}^3$, mice were randomised and dosed intratumorally with 10 μL containing 5×10^9 particles of enadenotucirev or vehicle control in two separate locations. Ninety-six hours post-infection mice received 500 mg kg^{-1} 2DG every other day for two weeks, at which point they were sacrificed, and the xenografts were removed. Tumours were homogenised in 1 x reporter lysis buffer (Promega) using a motorised homogeniser (Ultra Turrax IKA T18 Basic; Fisher Scientific, Loughborough, UK) to obtain a 150 mg/mL homogenate. Luciferase assays were conducted according to manufacturer's protocols (Promega, UK) and normalised to protein content using the bicinchoninic acid assay (QuantiPro, Sigma, UK).

2.7 Molecular cloning techniques

2.7.1 Restriction digests

Enzymatic restriction digests were performed for diagnostic purposes or to linearise the EnAd viral backbone before transfection. Digests were performed in

accordance with the manufacturer's instructions (NEB, UK), incubating for a total of 2 hours at 37°C. For the preparation of vector backbones, alkaline phosphatase (1 µL, #M0290S, NEB, UK) was added to the reaction mixture for the final 1 hour of incubation to minimise re-ligation.

2.7.2 Polymerase chain reaction (PCR)

PCR amplifications were performed with Phusion polymerase in 20 µL reactions comprising 1 ng template DNA, 10 µL Phusion Mastermix (2X; NEB, UK, #M0531L) and 0.5 µM forward and reverse primers (for primer list see Appendix 3). The following thermocycling conditions were used:

1. **Initial denaturation:** 98°C for 30 s
2. **Denaturation:** 98°C for 10 s
3. **Annealing:** Primer-specific annealing temperature for 30 s
4. **Extension:** 72°C for 1 min/kb
5. **Repeated cycles:** Return to 2, x 34
6. **Final extension:** 72°C for 10 min
7. 4°C forever

PCR reactions were performed using a PTC-225 Peltier Thermal Cycler DNA Engine Tetrad (MJ Research, US).

2.7.3 Agarose gel electrophoresis

Restriction digest and PCR products were resolved and visualised by agarose gel electrophoreses. Briefly, 1% (w/v) agarose gels were prepared using 1x TAE (50x TAE Buffer: 242 g trisaminomethane (Tris), 100 mL 0.5 M EDTA and 57.1 mL Glacial Acetic Acid in 1 L H₂O) and contained GelRed nucleic acid gel stain (Biotium, UK, #BT41003) for DNA visualisation. Samples were mixed with 6x loading dye (NEB, UK, #B7025S) and ran alongside a DNA ladder (Quick-load 1 kb DNA ladder, NEB, UK, #N0468S, unless otherwise specified) in TAE buffer at 120 V for 30-60 minutes. Gels were subsequently visualised under UV light using an Alpha Imager (Alpha Innotech, USA).

Where necessary (i.e. for all DNA fragments intended for Gibson assembly), bands were excised by visualising under blue light using a Dark Reader trans-illuminator (GRI, UK). DNA was then extracted and purified with a Monarch DNA gel extraction kit (NEB, UK, #T1020L), eluting in 10 µL H₂O.

2.7.4 Gibson assembly

Gibson assembly is a cloning method which enables the joining of multiple DNA fragments in a single reaction. This technique relies upon the presence of overlapping regions of DNA (20-40 bp) between adjacent fragments, which anneal following the generation of complementary overhangs by a 5' exonuclease (Pearce *et al.* 2009). Overlapping fragments were generated through PCR amplification of

insert DNA (primers are listed in appendix 3) and restriction digest of plasmid backbones.

Gibson assembly was performed using HiFi DNA Assembly Master Mix with HF buffer (NEB, UK, #E2621L). According to the manufacturer's recommendations for 2-3 fragment assemblies, for most reactions 50 ng vector and a vector:insert molar ratio of 1:2 were utilised. As an exception, all reactions involving the EnAd viral backbone were performed at a vector:insert ratio of 1:15 owing to the large size of this vector. Following incubation for 1 hour at 50°C, the reaction products were used immediately for bacterial transformation or stored at 4°C until further use.

2.7.5 Bacterial transformation

After being thawed on ice, competent XL10 *Escherichia coli* (*E. coli*) cells were mixed with 2 µL HiFi DNA assembly reaction product and incubated on ice for 30 mins. Cells were then heat-shocked at 42°C for 45 seconds, before returning to the ice for a further 2 minutes. 500 µL SOC outgrowth medium (#15544-034, Thermo Fisher, UK) was added and the culture incubated for 1 hour at 37°C on a shaker. Finally, cells were spread onto agar plates containing 50 ng/mL of the appropriate antibiotic and incubated at 37°C overnight.

2.7.6 Plasmid preparation and purification

Bacterial colonies were selected and grown in Lysogeny broth (LB) medium (3 mL or 150 mL for mini and maxi-preparations respectively: #L3022, Sigma-Aldrich, UK) under ampicillin or kanamycin selection, where appropriate. Cultures were incubated overnight at 37°C in an orbital shaker at 200 rpm. Plasmid purification was then performed using the QIAprep Spin Miniprep Kit (#27106, Qiagen, UK) or Qiagen Plasmid-Plus Maxiprep Kit (#12945, Qiagen, UK) as appropriate, following manufacturer's instructions.

For purification of linearised recombinant EnAd constructs (before transfection), an isopropanol precipitation method was used. In brief, DNA was mixed with 0.6 volumes of isopropanol and centrifuged at 12,000 g for 30 mins at 4°C. After removing the isopropanol, the pellet (containing precipitated DNA) was washed with 70% ethanol, then centrifuged again at 12,000 g for 2 mins at room temperature. Residual ethanol was removed by pipetting and subsequent air-drying. Finally, the DNA pellet was resuspended in a small volume (<10 µL) of dH₂O.

2.7.7 Sanger Sequencing

Plasmid clones with the correct restriction digest patterns were sequenced by Sanger sequencing at Eurofins Genomics (Germany), and aligned with the predicted sequences using SnapGene software (USA).

2.8 Generation of recombinant adenoviruses

2.8.1 Cloning to generate EnAd-CMV-Tigar-GFP

A transgene cassette encoding TIGAR-GFP promoted with an exogenous CMV promoter, and a 3'polyadenylation sequence was inserted into SbfI-linearised parental EnAd plasmid (EnAd2.4) with Gibson assembly technology. Successful transgene insertion was confirmed by restriction digest and Sanger sequencing (GATC, UK).

2.8.2 Virus transfection

Before transfection, EnAd-CMV-Tigar-GFP plasmid was linearised and purified by isopropanol precipitation. HEK293A cells were seeded in T25 flasks (1 million cells/flask) and transfected 24 h later with linearised EnAd-CMV-TIGAR-GFP. In brief, 5 µg of DNA was diluted in 50 µL Opti-MEM, whilst 20 µL Lipofectamine 2000 was diluted in 30 µL Opti-MEM. The two solutions were incubated separately for 5 min at RT, then mixed and incubated for a further 20 min. After topping up to 4 mL with Opti-MEM, the transfection mixture was added to cells. Four hours later, the transfection medium was replaced with DMEM containing 2% FBS. Cells and supernatant were harvested upon observation of extensive cytopathic effect (CPE) alongside GFP expression.

2.8.3 Plaque purification and plaque assays to determine viral titres.

A549 cells were seeded in complete medium in 6-well plates at a density of 800,000 cells per well to generate a monolayer. Plaque purification was performed by applying 10-fold serial dilutions of virus, which were removed after 4 h and replaced with an overlay of 1% agarose-DMEM (supplemented with 2% FBS). Single virus plaques were picked by careful scraping with a pipette tip, then amplified through re-infection of HEK293A cells (seeded in 6-well plates in DMEM 2% FBS). Amplified viruses were harvested upon observation of extensive CPE and GFP expression, then assessed for TIGAR-GFP expression, before proceeding to large-scale virus preparation. For assessment of viral titres, we used the following calculation according to previously established methods (Baer and Kehn-Hall 2014):

$$\frac{PFU}{Ml} = \frac{\text{number of plaques counted}}{(\text{volume of lysate infected with}) \times (\text{dilution})}$$

2.8.4 Large-scale virus preparation and purification

To generate large-scale viral stocks, A549 cells were seeded in 10-layer Hyperflasks (Corning, UK, #CLS10034) and, at 80-90% confluency, infected with the selected virus clones (diluted in DMEM containing 2% FBS). Upon the first signs of CPE (48-120 h later), cells were harvested, and intracellular virus particles released by

performing three freeze/thaw cycles with liquid nitrogen and a 37°C water bath. After 1 h incubation at 4°C with water-saturated n-butanol (diluted 1:100), cellular debris was pelleted through centrifugation (500 g, 10 min, 4°C). The supernatant containing free virus particles was subsequently collected and applied to a discontinuous caesium chloride gradient, prepared in Beckman Coulter Ultra-Clear centrifuge tubes (Fischer Scientific, UK, #12706558).

In brief, the caesium chloride gradient consisted of 3 mL of 1:32 ρ caesium chloride carefully underlaid with 2 mL of 1:45 ρ caesium chloride. Two mL of 40% Glycerol was then carefully added on top then finally overlaid with virion-containing supernatant to the top of the tube. Tubes were centrifuged at 25,000 rpm at 10°C for 1 hr 30 min in a Beckman L8-70M Ultracentrifuge with SW40TI rotor. After centrifugation, two discrete bands were visible: a lower band containing intact virus particles, and an upper band containing incomplete virus particles. The lower band was collected by carefully puncturing the tube with a 21 g needle and aspirating into a 10 mL syringe. Caesium chloride was removed through three rounds of dialysis in PBS using a 3-12 mL Slide-A-Lyzer dialysis cassette (ThermoFisher, UK, #66810). The resultant virus-PBS solution was extracted with a 21 g needle and a syringe, then treated for 1 h at room temperature with magnesium chloride (2 mM) and benzonase (1 μ L/mL) to degrade unpackaged DNA. After the final dialysis, the virus solution was diluted in glycerol at a final concentration of 20%, and viruses were stored at -80°C until further use.

2.8.5 Picogreen assay for adenovirus quantification

Estimates of viral genomes/mL were obtained using the Picogreen assay (ThermoFisher, UK, #P7589). Purified viral stocks were diluted 1:10 in TE buffer before addition of SDS (0.05%) and heat treatment (56°C, 30 mins) to remove non-encapsidated DNA. Further dilutions of virus stocks (up to 1:50) were performed with TE to ensure their concentrations fell within the range of the standard curve. Quantities of dsDNA were determined according to the manufacturer's instructions, using a standard curve of bacteriophage lambda DNA. Estimates of viral genomes/mL were calculated by assuming a ratio of 1 µg DNA per 2.7×10^{10} adenovirus particles.

2.9 Statistics

Various statistical analyses were employed to evaluate whether differences between datasets were significant. When comparing two datasets, a Student's two-tailed t-test was used. Where more than two groups were compared, the one-way Analysis of Variance (ANOVA) test was used with Tukey's posthoc analysis. For grouped datasets, two-way ANOVA test was used with Bonferroni posthoc analysis. All data is presented alongside bars indicating $\pm$ standard deviation (SD). The significant levels used were $p = 0.01 - 0.05$ (*), $0.001 - 0.01$ (**), ≤ 0.001 (***). All experiments were performed in triplicate, unless otherwise stated. For analysis of correlation, GraphPad (Prism, UK) was used to calculate a two-tailed Pearson's correlation calculation with a 95% confidence

interval. Correlation coefficient values are presented on the graphs and r values of 0-0.19 is regarded as very weak, 0.2-0.39 as weak, 0.40-0.59 as moderate, 0.6-0.79 as strong and 0.8-1 as very strong correlation.

3 The metabolic response of cancer cell lines to infection with adenoviruses

3.1 Introduction

Central carbon metabolism, and in particular, the metabolism of glucose and glutamine, the central macronutrients, allow for energy to be harnessed in the form of adenosine triphosphate (ATP) and for the creation of metabolites used for all cellular processes and homeostasis. It has long been documented that cancer cells have altered cellular programming, which results in increased survival, growth, and proliferation leading to tumour formation and progression. These fundamental changes were documented in the seminal reviews “The Hallmarks of Cancer” by Hanahan and Weinberg in 2000 and 2011 (Hanahan and Weinberg 2000, 2011).

One well-studied hallmark of cancer is metabolic reprogramming. In particular, the “Warburg Effect” in which tumour cells increase glucose consumption and lactate secretion, even in the presence of oxygen has been heavily studied (Warburg 1956b). This “aerobic glycolysis” phenotype is relatively inefficient for ATP production, compared to normal mitochondrial respiration and could,

theoretically limit tumour growth in glucose-depleted conditions. It has, however, been shown in many different studies that aerobic glycolysis is beneficial to malignant cells as it is faster than oxidative phosphorylation and they require a high metabolic flux to synthesise building blocks such as nucleotides, proteins, and lipids. As well as increasing the metabolic flux, the aberrant tumour metabolism helps to create a unique metabolic microenvironment including decreased concentrations of nutrients such as glucose, glutamine, arginine, tryptophan and oxygen concentrations, and an increase in immunosuppressive metabolic waste products such as lactate, reactive oxygen species, and kynurenine (De Berardinis and Chandel 2016).

Many wildtype viruses display an inherent ability for replication within cancer cells with some viruses showing a preference for replication within tumours (Kelly and Russell 2007; Pasin *et al.* 2020). The mechanism underpinning this inherent cancer selectivity, however, is a topic of intense discussion. It has been postulated that the hallmarks of tumour cells provide a cellular pathology that is conducive to efficient virus replication and many publications highlight that there appears to be considerable overlap between the hallmarks of cancer and the hallmarks of virus infection (reviewed in Seymour and Fisher 2016). As viruses are obligate intracellular pathogens, they need to hijack cellular processes to create a favourable environment for efficient production of infectious progeny. In many ways, viral demands for macromolecular synthesis appear similar to those of a proliferating or cancer cell.

The chimeric adenovirus enadenotucirev (EnAd) was created through a process referred to as “directed evolution” whereby several different wildtype strains of adenovirus were used to infect cancer cells in conditions that favoured recombination of viruses. After many rounds of this process, the candidate virus that emerged was a chimaera of Ad3 and Ad11p with a 2444 base pair (bp) deletion in E3ORF and a 25 bp deletion in E4ORF4 (Kuhn *et al.* 2008). Because of the process of directed evolution, EnAd is an oncolytic virus that has naturally, rather than synthetically, adapted to replicate only within cancer cells and to lyse these cells quickly. Additionally, EnAd may be uniquely reliant upon the phenotypes present in the cells used to create the virus such as the Warburg effect.

Given this context, there is a need to understand how metabolism impacts oncolytic viruses which are, by design, restricted to proliferation in cancer cells where metabolism is highly aberrant, as this has the potential to improve the outcome of oncolytic virotherapy. Additionally, considering that viruses are inherently reliant upon the conditions in which the host cells reside, it is imperative to understand how oncolytic viruses respond to infections in conditions more closely mirroring those found in tumours *in vivo*, where nutrients are often present at much lower conditions. To do so, we first need to understand the metabolic response of cancer cells to infection with such viruses, and this will underly the results presented in the following chapter.

3.2 Chapter Aims

1. Analyse the basal metabolic phenotypes of cancer cell lines used in this thesis.
2. Investigate the metabolic response of cancer cells to infection with wildtype adenoviruses.
3. Investigate the metabolic response of cancer cells to infection with the oncolytic adenovirus enadenotucirev (EnAd).

3.3 Results

3.3.1 Measuring the basal metabolic profiles of cancer cell lines

Central carbon metabolism uses a relatively complex series of cellular enzymes to convert sugars such as glucose and glutamine into metabolic precursors. These precursors can then be used to generate cellular biomass needed for homeostasis along with ATP generated through the transfer of electrons via nicotinamide adenine dinucleotide (NAD) and flavin adenine dinucleotide (FAD). Whilst the entire metabolome of cells is of importance and scientific interest, studies in this chapter focus on the metabolism of the two most abundant metabolites in the body, namely glutamine and glucose.

We first sought to determine the basal metabolic phenotypes of the cancer cell lines that will form the basis of much of the work presented in this thesis. Analysis

of oxygen consumption rate (OCR, a measurement of mitochondrial respiration) and extracellular acidification rates (ECAR, a measurement of glycolysis) were performed using the Seahorse XF analyser and plotted against each other according to previous protocols (Potter, Newport and Morten 2016). A549 cells showed a typical phenotype commonly referred to as the Warburg effect, namely low or little OCR with high ECAR, whereas SKOV3 cells and PC9 cells showed relatively normal metabolic phenotype (moderate ECAR and moderate OCR) as is shown in **figure 3.1**.

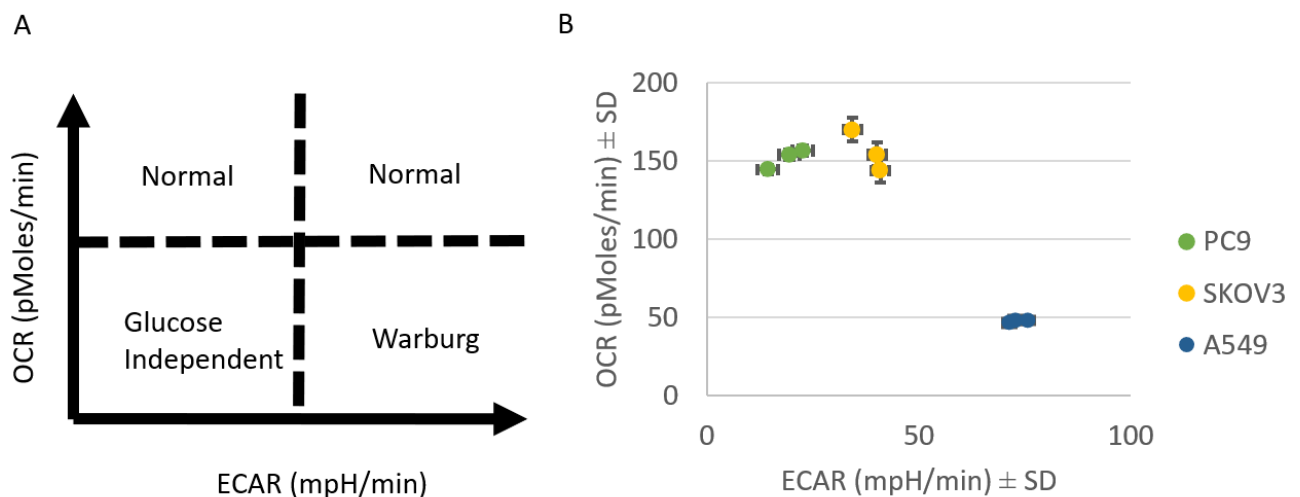


Figure 3.1 Bioenergetic profiles of PC9, SKOV3, and A549 cancer cell lines.

Oxygen consumption rate (OCR – a measurement of mitochondrial respiration) and extracellular acidification rate (ECAR – a measurement of glycolysis) were measured using a Seahorse XF analyser and normalised to cell number. **(A)** A simplified diagram representing the different bioenergetic profiles of cells and what they represent and **(B)** the metabolic profiles of PC9, A549 and SKOV3 cells. Data shown represent the mean ± SD of biological triplicates.

To further understand the basal metabolism of these three cell lines, we measured glucose uptake, glutamine uptake and lactic acid production (**figure 3.2**). A549 cells exhibited a significantly higher level of glucose uptake, glutamine uptake and

lactic acid secretion than both SKOV3 and PC9 cells, again confirming that A549 cells display a “Warburg” metabolic phenotype whereas PC9 and SKOV3 cells display a more regular metabolic profile where glucose is used to fill the TCA cycle.

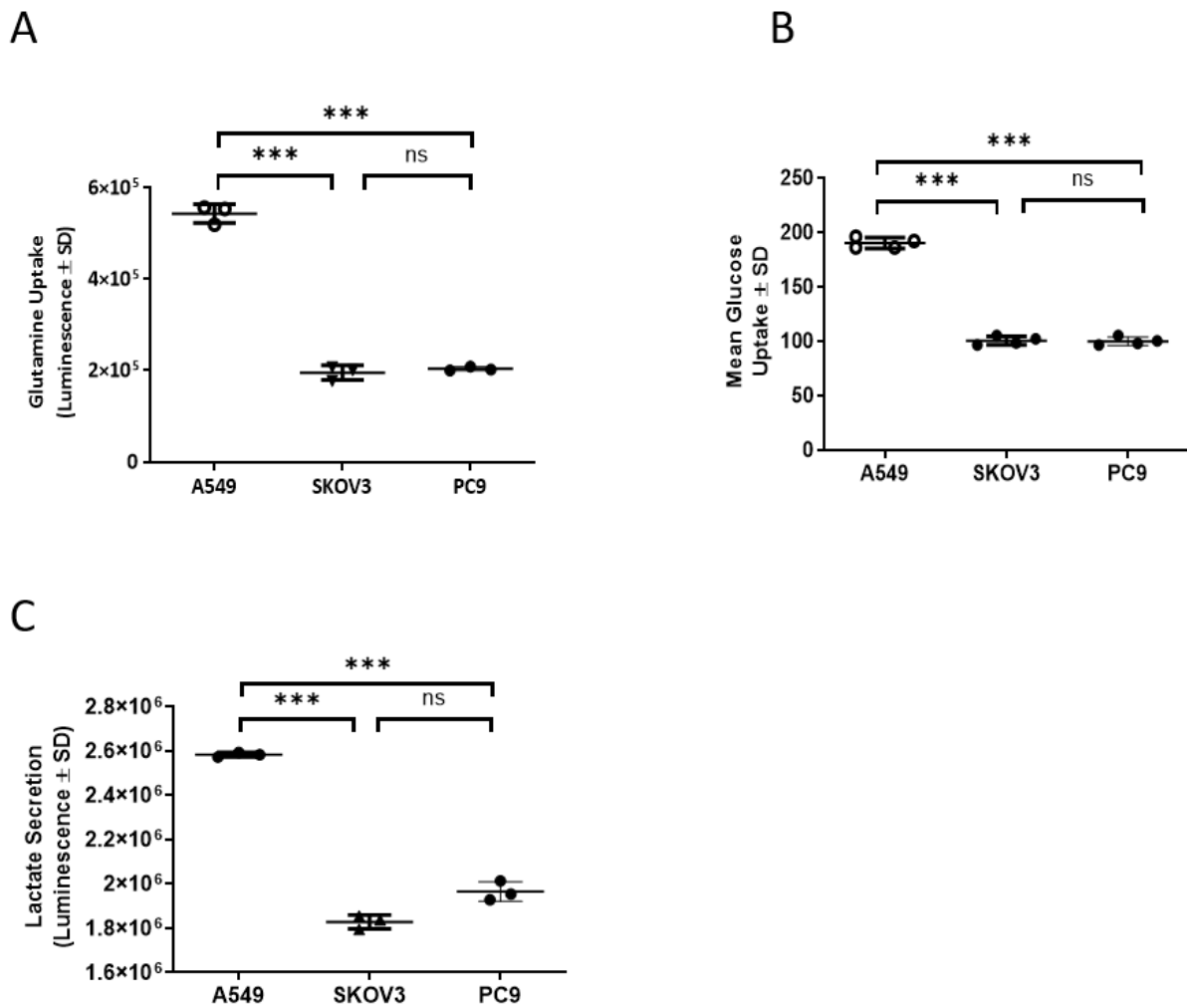


Figure 3.2 Basal metabolic properties of PC9, SKOV3, and A549 cancer cell lines.

Analysis of glutamine uptake (A), glucose uptake (B), and lactate secretion (C) in A549, SKOV3, and PC9 cells. Data shown represents the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Cancer cells have previously been shown to have a wide range of dependency on glucose for survival and homeostasis (Potter, Newport and Morten 2016; Pusapati *et al.* 2016). Indeed, very few tumour cells investigated show a true Warburg phenotype with no glucose entering the TCA cycle. Instead, most cancer cells rely upon aerobic glycolysis despite using glucose oxidation. To establish the glucose dependency of A549, SKOV3 and PC9 cells, we measured cell viability after treatment with the glucose analogue 2-deoxy-d-glucose (2DG), a widely used inhibitor of glycolysis. Seventy-two hours post-treatment, viability was analysed, and an IC_{50} was established for each cell line.

Previous publications have defined cells which have an IC_{50} of ≥ 3 mM as being glycolysis independent. In contrast, cells which have a mean IC_{50} below 3 mM can be defined as being glycolysis addicted (Pusapati *et al.* 2016). SKOV3 cells had a mean IC_{50} of 1.4 mM, and PC9 cells had a mean IC_{50} of 1.8 mM, meaning that both cell lines can be described as having a glycolysis addicted phenotype (**figure 3.3**). A549 cells had an IC_{50} of 9.2 mM, meaning that, unlike the other two cell lines, these cells can be described as glycolysis independent (**figure 3.3**).

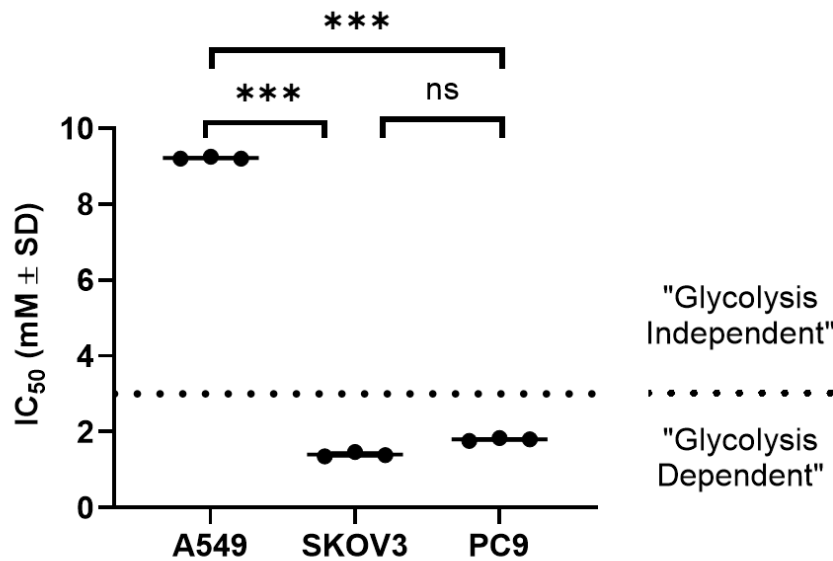


Figure 3.3 Classification of cells as glycolysis-independent and glycolysis-dependent.

Analysis of the IC₅₀ of A549, SKOV3, and PC9 cells when treated with the glycolysis inhibitor 2-deoxy-d-glucose (2DG). Cell viability was measured 72 hours post-treatment, and the IC₅₀ was determined. The broken line represents a “dependency threshold” of 3 mM 2DG. Data shown represents the mean ± SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

3.3.2 Measuring the metabolic alterations of cancer cell lines infected with adenoviruses

We next aimed to understand how metabolism is altered in cancer cell lines in response to infection with the chimeric oncolytic adenovirus EnAd or with two wildtype viruses, the archetypal adenovirus type 5 (Ad5, a group C adenovirus) or the parental adenovirus type 11p (Ad11p, a group B adenovirus). To allow for a valid comparison of the effects of the viruses, we first analysed the viral particle to infectivity ratio of the three viruses (**table 3.1**). All three viruses had a similar viral particle to infectivity ratio allowing further experiments to compare between the viruses fairly without skewing the data owing to an unequal number of viral proteins infecting each cell using equivalent multiplicities of infection (MOIs). We

also analysed the viral particle to infectivity ratio of two viruses expressing GFP (EnAd-CMV-GFP and EnAd-SA-GFP), which are commonly used as reported viruses within our laboratory as we will use these later within our thesis.

Table 3.1 Particle numbers and infectious titres of virus stocks used in this thesis.

Analysis of the particle number and infectious titres of virus stocks used in this thesis

	Particle number per ml (VP/ml) *	Infectious particle number per ml (PFU/ml)*	VP//PFU ratio
Ad5	2.11E12	2.45E10	86.12
Ad11p	2.76E12	6.15E10	44.88
EnAd	2.65E12	4.77E10	55.56
EnAd-CMV-GFP	4.16E12	4.86E10	85.60
EnAd-SA-GFP	3.53E12	5.39E10	65.49

*VP/ml was measured by Picogreen assay and PFU/ml was measured by 7 days TCID₅₀ assay on A549 cells.

We first analysed the glucose uptake of A549 cells when infected with adenoviruses. Glucose uptake was analysed using the fluorescent glucose analogue 2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) which was added to the medium at the point of infection and, at set time points, cells were detached and analysed for fluorescence by flow cytometry. The cells were infected with 100 particles per cell (ppc) for each virus which equates to an approximate MOI of 1 (**table 3.1**).

A549 cells showed an increase in uptake of the fluorescent glucose analogue 2-NBDG following infection with each of the viruses, despite already having a very high basal glucose uptake. This response occurred relatively early in infection (48 hours post-infection at an MOI of 1 (100 PPC), and 72 hours post-infection at an

MOI of 0.1 (**figure 3.4**). Ad5 causes a higher glucose uptake than Ad11p and EnAd increases glucose uptake at an even higher level. Cells incubated with a lower viral dose (10 PPC, which equates to an MOI of roughly 0.1) showed a similar pattern (**figure 3.4**).

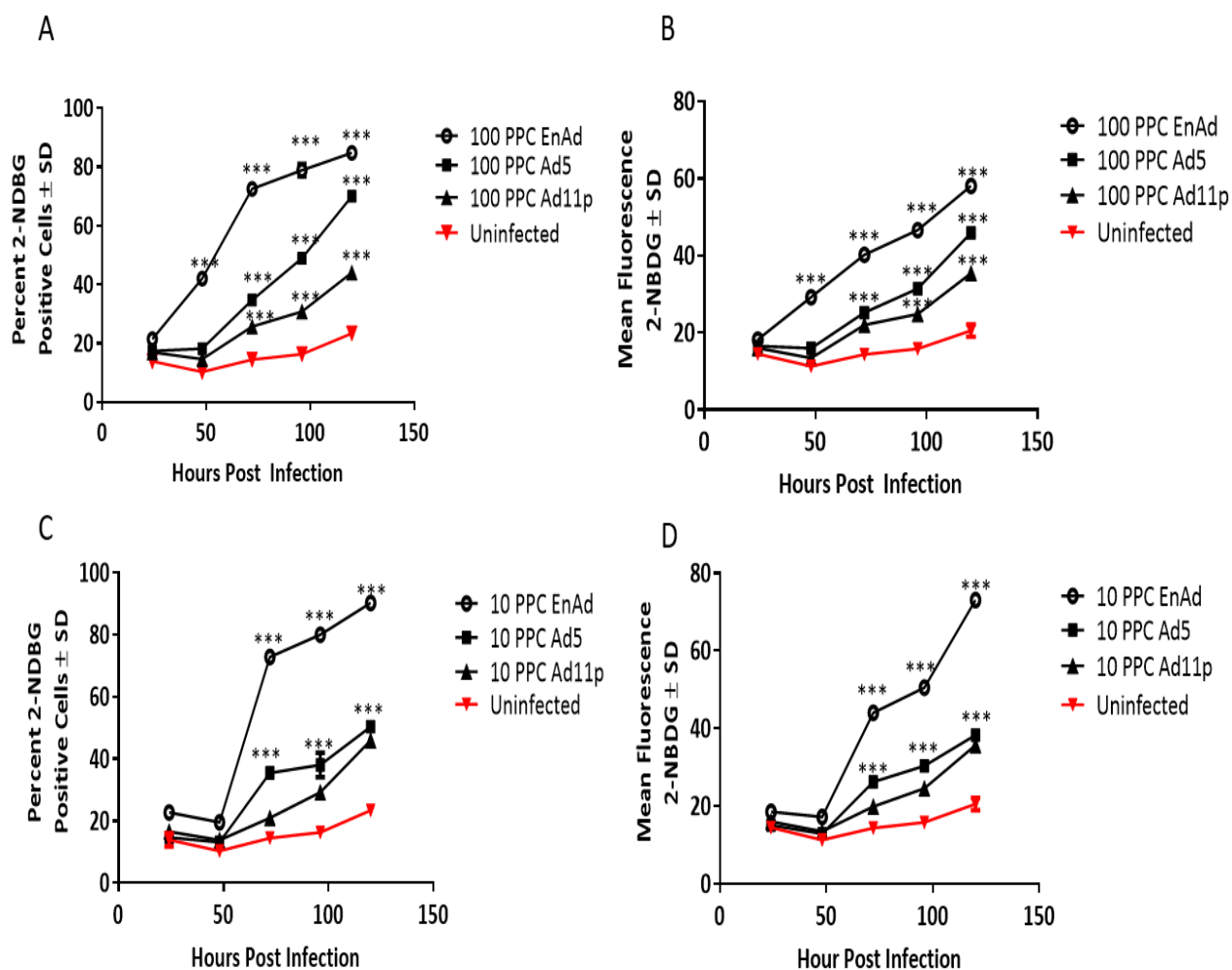


Figure 3.4 Adenovirus infection of A549 cells causes an increase in glucose uptake. Flow cytometry analysis of the fluorescent glucose analogue 2-NBDG uptake in A549 cells infected with EnAd, Ad5, Ad11p or uninfected in the presence of 300 μ M 2-NBDG. (A) The percentage of 2-NBDG positive A549 cells after infection with 100 viral particles per cell (ppc) of each virus. (B) The mean fluorescence of A549 cells after infection with 100 ppc of each virus. (C) The percentage of 2-NBDG positive A549 cells after infection with 10 ppc of each virus. (D) The mean fluorescence of A549 cells after infection with 10 ppc of each virus. Data shown represents the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Adenovirus-infected cells are previously reported to show increased acidification of their culture medium (Thai *et al.* 2014). Thus far, this has only been noted with wildtype adenoviruses infecting normal (non-cancerous) cell lines. We first analysed changes in the acidification of the media of infected cancer cells using phenol red, a pH indicator commonly used in cell culture. In agreement with previous reports, we observed an acidification made apparent by the yellowing of the media of A549 cells infected with EnAd, Ad11p compared to mock-infected cells indicating a pH below 7 (**figure 3.5**). To assess whether the observed increase in glucose uptake and acidification of the media did reflect an increase in aerobic glycolysis, we measured secretion of lactic acid by A549 cells infected with Ad5, Ad11p, and EnAd (**figure 3.5**). All viruses caused increased lactic acid secretion, with Ad5 and EnAd showing significant effects (up to 1.4-fold rises) by 72 hours post-infection and Ad11p showing an increase after 96 hours post-infection. Cells infected with EnAd showed a fall in lactic acid production, perhaps reflecting earlier cell death. This was reflected by the earlier release of lactate dehydrogenase (LDH) from cells infected with EnAd (**figure 3.5**).

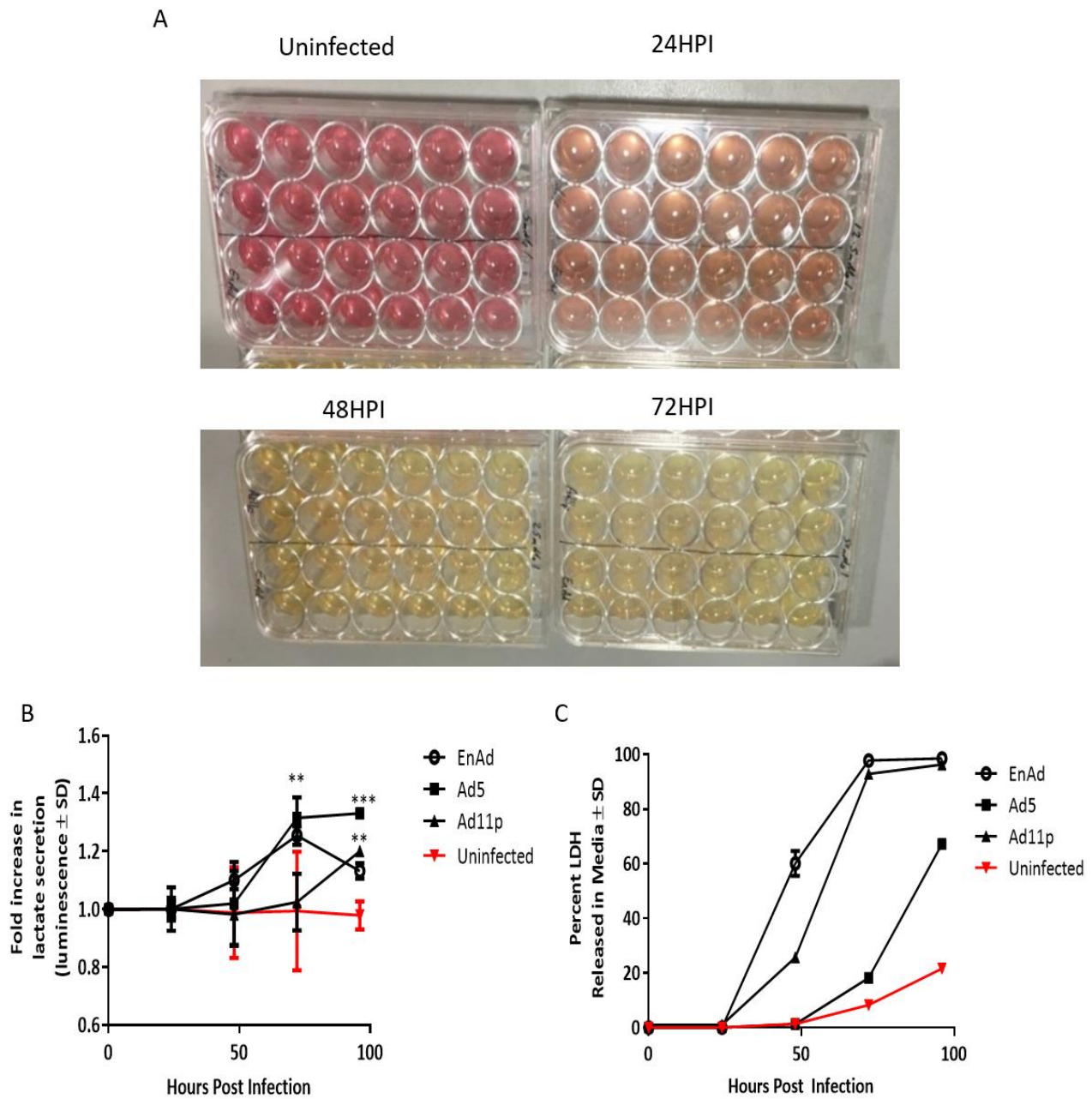


Figure 3.5 Adenovirus infection of A549 cells causes an increase in lactate secretion.

(A) A549 cells infected with EnAd (100 ppc, bottom 2 rows) and Ad11p (100 ppc, top 2 rows) are shown on day 1-4 post-infection. (B) Lactate secretion in A549 cell medium following infection with EnAd, Ad11p or Ad5 (all 100 ppc) using luminescence presented as fold increase compared to uninfected cells. (C) LDH secretion in medium of A549 cells following infection with 100 ppc EnAd, Ad11p or Ad5 with 100% denoting cells lysed with 0.8% Triton X-100. Data shown represents the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

As previously mentioned, A549 cells display a basal metabolic phenotype which is commonly referred to as the Warburg effect (aerobic glycolysis with less reliance upon glucose oxidation). We next sought to understand how viral infection affects the metabolism of cells which typically metabolise glucose via glucose oxidation and oxidative phosphorylation (oxphos). We have chosen SKOV3 cells for this analysis (Hatzivassiliou *et al.* 2005). Each of the viruses promoted increased glucose uptake in SKOV3 cells, with 40% to 50% of cells showing more uptake of 2-NBDG than uninfected control cells at 48 hours post-infection (**figure 3.6**). Interestingly, the mean levels of 2-NBDG uptake were approximately 10-fold higher following EnAd infection than for the wildtype Ad5 and Ad11p (**figure 3.6**). Whereas wildtype Ad5 and Ad11p showed only slight trends of virus dose on increased glucose uptake, EnAd showed a clear dose-effect with higher doses giving increased glucose uptake at much earlier times (**figure 3.6**).

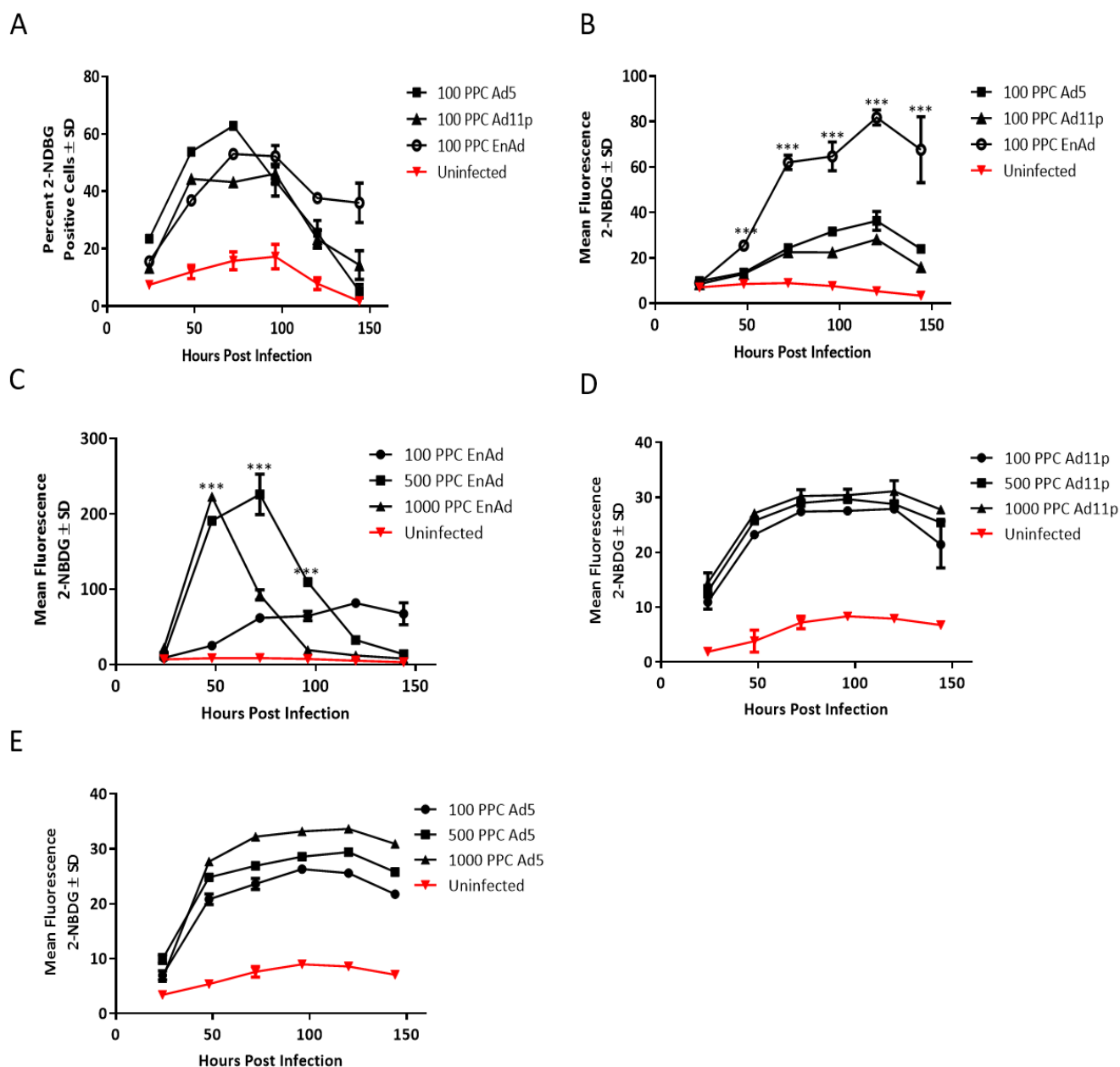


Figure 3.6 Adenovirus infection of SKOV3 cells causes an increase in glucose uptake.

Flow cytometry analysis of the fluorescent glucose analogue 2-NBDG uptake in SKOV3 cells infected with EnAd, Ad5, Ad11p or uninfected in the presence of 300 μ M 2-NBDG. **(A)** The percentage of 2-NBDG positive SKOV3 cells after infection with 100 viral particles per cell (ppc) of each virus. **(B)** The mean fluorescence of SKOV3 cells after infection with 100 ppc of each virus. **(C-E)** The Mean fluorescence of 2-NBDG positive SKOV3 cells after infection with 100, 500, and 1000 ppc of each EnAd **(C)**, Ad11p **(D)**, or Ad5 **(E)**. Data shown represents the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Because we observed that infection with adenoviruses increases aerobic glycolysis, we hypothesised that nutrients other than glucose were being used either for energy metabolism or to provide biosynthetic precursors. Glutamine offers an alternative energy source and supports many metabolic functions needed for cell survival, growth, and proliferation. Glutamine is particularly vital in tumours where glucose is often present at low levels with some tumour cells regarded as being “glutamine-addicted”.

To probe the hypothesis that glutamine is used to provide critical metabolites owing to the decrease in glucose entering the TCA cycle during adenovirus infection, we analysed glutamine uptake in cells infected with EnAd, Ad11p and Ad5. We showed an increase in glutamine uptake by A549 cells following infection with Ad5, Ad11p, or EnAd. However, the stimulation of uptake by EnAd was faster (48 hours post-infection) whereas Ad11p and Ad5 infected A549 cells showed a significant increase in glutamine uptake only at 96 hours post-infection (**figure 3.7**).

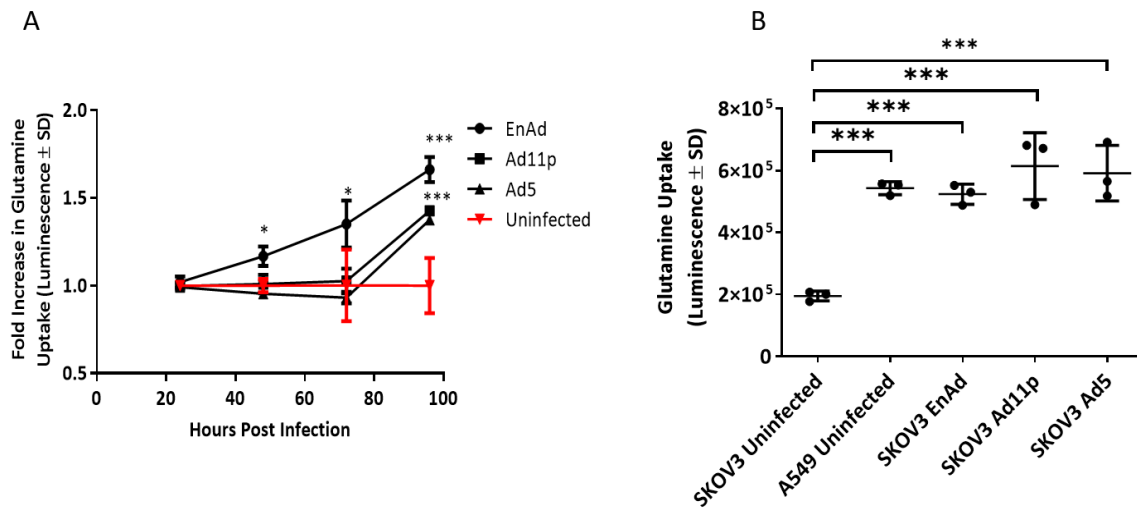


Figure 3.7 Adenovirus infection of A549 and SKOV3 cells causes an increase in glutamine uptake. (A) Analysis of glutamine uptake in A549 cells infected with EnAd, Ad11p, Ad5 (100 ppc of each) using luminescence presented as fold increase compared to uninfected cells. (B) Analysis of glutamine uptake in SKOV3 cells 96 hours post-infection with EnAd, Ad11p, Ad5 (100 ppc of each) using luminescence. Data shown represents the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

As previously discussed, A549 cells metabolise glucose via aerobic glycolysis, leaving a dependency upon glutamine for many of their metabolites. SKOV3 cells use glucose to fill the TCA cycle (Hatzivassiliou *et al.* 2005). We next aimed to understand how cells with a lower dependency upon glutamine (SKOV3 cells) altered their glutamine metabolism following infection with adenoviruses. At 96 hours post-infection, SKOV3 cells infected with EnAd, Ad11p and Ad5 all showed a significantly higher glutamine uptake compared to mock-infected cells. This increase in glutamine uptake was significantly similar to uninfected A549 cells (**figure 3.7 B**) These results indicate that post-infection with adenoviruses, SKOV3 cells exhibit a metabolic phenotype similar to A549 cells and similar the Warburg effect (**figure 3.8**).

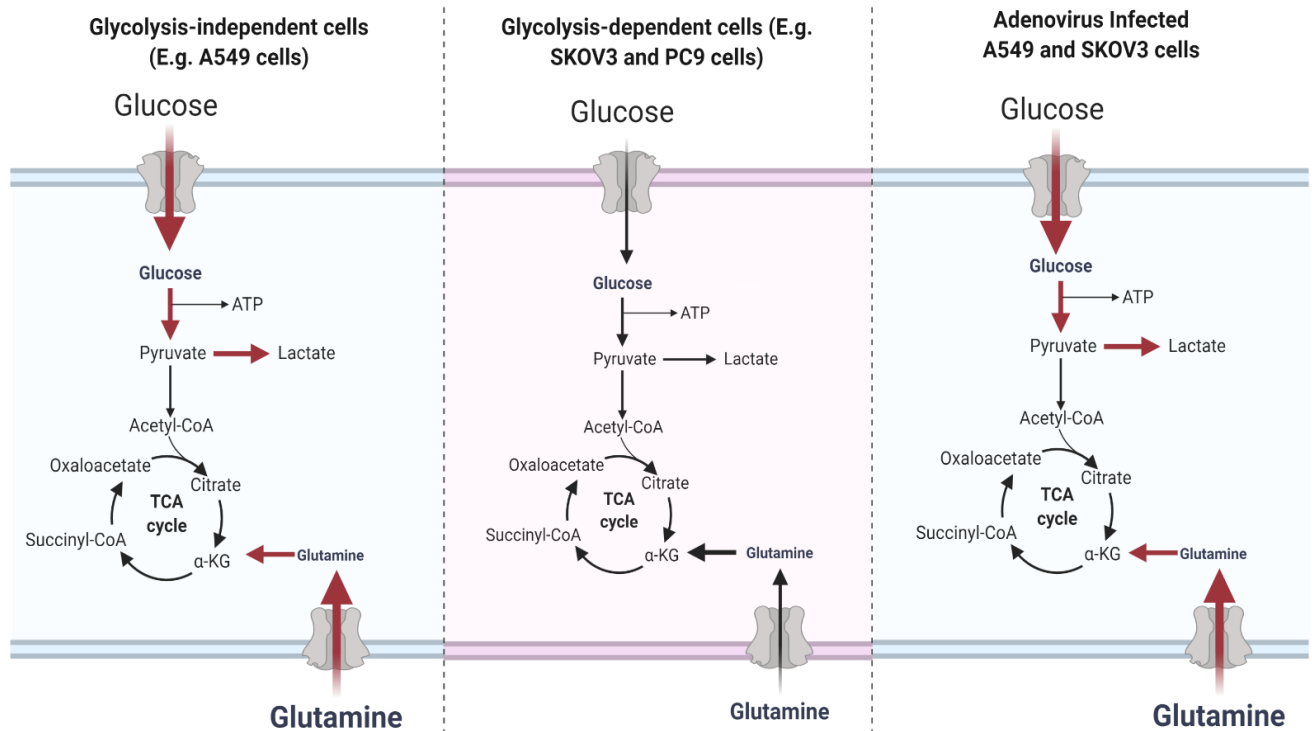


Figure 3.8 Central carbon metabolism of cancer cell lines used in this chapter pre- and post-infection with adenoviruses.

Simplified schematic representation of basal metabolic phenotypes of glycolysis-independent cells (e.g. A549, left panel), glycolysis-dependent cells (e.g. SKOV3, and PC9 cells middle panel); and the metabolic phenotype of A549 and SKOV3 cells post-infection with adenoviruses (right panel). The red arrows represent pathways that are increased under each circumstance. ATP, adenosine triphosphate; TCA, tricarboxylic acid cycle (Krebs cycle); α -KG, alpha-ketoglutarate.

3.3.3 Measuring the permissivity of A549, PC9, and SKOV3 cells to infection with EnAd

We next analysed the permissivity of several different cell lines to infection with the oncolytic virus EnAd to allow for further comparisons of cellular metabolism at specific points of the viral lifecycle such as when cytotoxicity occurs. We first analysed the mean half-maximal inhibitory concentration (IC_{50}) of various cell lines

when infected with EnAd at six days post-infection to determine how susceptible each of the cell lines are to being lysed by the virus. **Figure 3.9** shows that, in general, cell lines have a relatively wide range of IC_{50} doses. SKOV3, OVCAR8, PC9, OE33 and 7860 cells represent the highest IC_{50} results of between 10 viral particles per cell and 1000 viral particles per cell. Cell lines such as HEK293A, HT-29, DLD-1 and A549 all have very low viral IC_{50} results of below one viral particle per cell.

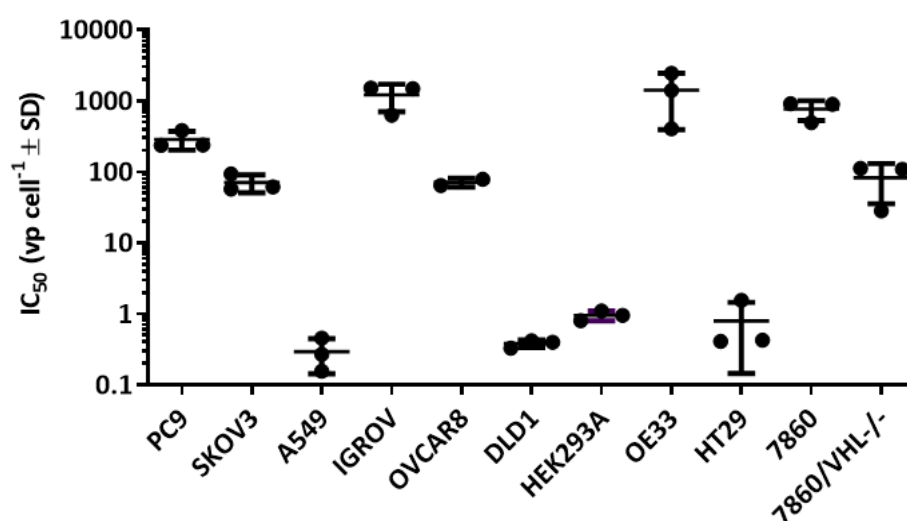


Figure 3.9 Analysis of the susceptibility of various cancer cell lines to oncolysis with the oncolytic virus enadenotucirev.

Analysis of the IC_{50} of various cancer cell lines to the oncolytic adenovirus EnAd. Values represent cells plated in a 96 well plate and exposed to 5-fold virus dilutions. After 5 days, cell viability was assessed with MTT, and IC_{50} values were calculated in Prism. Data shown represents the mean $\pm$ SD of biological triplicates.

After determining that SKOV3, PC9 and A549 cells show very different IC_{50} results (indicating that to kill 50% of the cells at day six post-infection requires almost a 10,000-fold difference in viral particles per cell), we next wanted to analyse how long the same viral dose took to kill each of these cell lines to mitigate for possible

viral toxicity levels that are likely to be observed at close to 1000 viral particles per cell. To analyse cell viability in real-time rather than at a single end-point assay, we used xCELLigence technology. The xCELLigence machine allows for a real-time label-free recording of cell impedance, giving a measurement known as a cell index (CI) which can be used to measure cellular adhesion (a proxy for cell viability). As can be seen from **figure 3.10**, at 100 ppc, A549 cells began to show cytotoxicity starting at around 60 hours post-plating, which is approximately 36 hours post-infection whereas SKOV3 cells infected with the same amount of virus began to show cytotoxicity at 100 hours post-infection and PC9 cells at 160 hours post-infection. For A549 cells, 50% cytotoxicity was reached at approximately 48 hours post-infection, and 100% cytotoxicity was observed at 60 hours post-infection. For SKOV3 cells 50% cytotoxicity was observed at 110 hours post-infection and 100% cytotoxicity at 150 hours post-infection and for PC9 cells, 50% cytotoxicity was observed at 270 hours post-infection and 100% cytotoxicity was reached at 370 hours post-infection (**figure 3.10**).

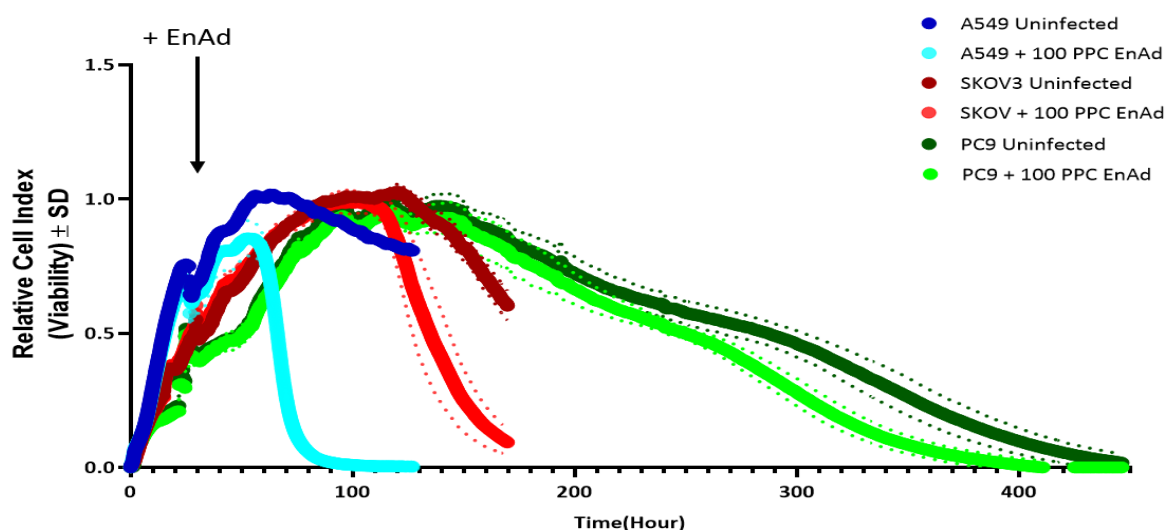


Figure 3.10 Real-time analysis of the cytotoxicity of various cancer cell lines following infection with the oncolytic virus enadenotucirev.

xCELLigence analysis of SKOV3 and A549 cells infected with EnAd (100 ppc). Cell index (a measure of cell viability) was measured every 10 minutes for 160 hours. Cells were plated at 0 hours and were infected at approximately 24 hours post-seeding as indicated on the graph. Data shown represents the mean $\pm$ SD of biological triplicates.

Both PC9 and SKOV3 cells are not very susceptible to killing by EnAd as can be observed by the high IC_{50} (**figure 3.9**) and the xCELLigence kill curves (**figure 3.10**).

When compared to A549 cells, which have a mean IC_{50} of 0.27 virus particles per cell and, at an MOI of 1, are completely lysed by 60 hours post-infection (**figures 3.9 and 3.10**), SKOV3 cells have a mean IC_{50} of 76 virus particles per cell and take 150 hours to fully lyse (**figures 3.9 and 3.10**).

It is interesting to note an apparent correlation between cells that display a glycolysis independent phenotype (defined as having an $IC_{50} \geq 3$ mM 2DG, **figure 3.3** (Pusapati *et al.* 2016)) and cells which are highly permissive to viral lysis. We next defined the 2DG IC_{50} of several cells for which the viral inhibitory

concentration is shown in **figure 3.9**. Cells were plated at 80% confluency and exposed to a 9-fold dilution of 2DG for 72 hours after which viability was measured, and an IC_{50} for 2DG was calculated for each cell line (**figure 3.11A**).

We next wanted to probe further the correlation between permissivity to oncolysis with EnAd and the dependency of a cell on glucose oxidation. To do this, we plotted the results from **figure 3.9** (IC_{50} against EnAd) against the results from **figure 3.11** (IC_{50} against 2DG). Cells which displayed a dependency upon glucose to maintain homeostasis (cells with an IC_{50} for 2DG of less than 3 mM) tend to be less permissive to viral lysis than those cells which display a glycolysis independent phenotype (**figure 3.8**). Indeed, statistical analysis of the correlation between these data sets resulted in a Pearson's correlation coefficient r value of -0.59, suggesting a moderate but statistically significant correlation between these values.

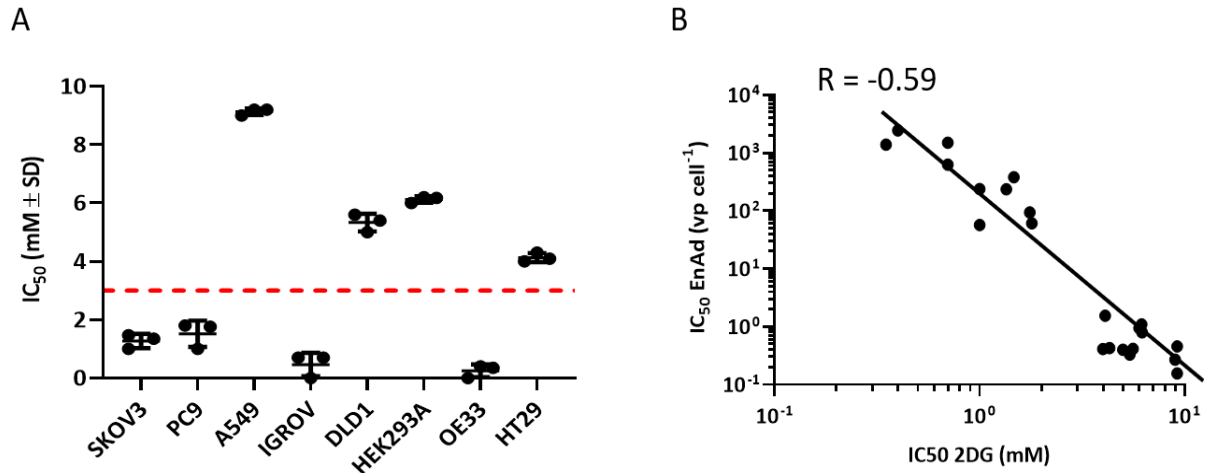


Figure 3.11 Analysis of the susceptibility of various cancer cell lines to treatment with the glycolysis inhibitor 2-deoxy-d-glucose (2DG) and the oncolytic virus enadenotucirev (EnAd). (A) Analysis of the IC₅₀ of various cancer cell lines when treated with the glycolysis inhibitor 2-deoxy-d-glucose (2DG). Cell viability was measured 72 hours post-treatment, and the IC₅₀ was determined in Prism. The broken line represents a “dependency threshold” of 3 mM 2DG. Data shown represents the mean ± SD of biological triplicates. (B) Analysis of the IC₅₀ of various cell lines against EnAd (y-axis, data taken from figure 3.9) plotted against the analysis of the IC₅₀ of the same cell lines against 2DG. Data shown represents the mean IC₅₀ of a biological singlet. Correlation analysis was calculated using a two-tailed Pearson’s correlation calculation with a 95% confidence interval.

3.4 Chapter Discussion

In this chapter, we aimed to test the first of our five hypotheses (listed in section 1.8.2), namely “Adenovirus infection induces an increase in aerobic glycolysis and glutamine metabolism in host cells.”

Increasing laboratory-based and clinical evidence has noted that the hallmarks of cancer and the hallmarks of viral infections overlap quite significantly (as reviewed in Seymour and Fisher 2016). Because viruses are obligate intracellular pathogens,

they are highly reliant on host cell processes. Given that oncolytic viruses are restricted to only grow within cancer cells, they are particularly reliant on the unique metabolic niche that cancer has created. Understanding how metabolism is altered upon infection of cancer cells with oncolytic viruses could provide key insights into vital pathways and metabolites that could be used to improve the outcome of oncolytic virotherapy (Dyer *et al.* 2020).

We aimed to understand how metabolism is altered during the lifecycle of the oncolytic adenovirus EnAd with an ultimate aim to harness this knowledge to create a more favourable environment for oncolytic virotherapy. We used three main cell lines, two of which have a normal metabolic phenotype whereby glucose is used to fuel the TCA cycle and metabolised via glucose oxidation (SKOV3 and PC9) and one of which has a more aberrant metabolism similar to the classically described Warburg effect (A549), whereby glucose is metabolised via aerobic glycolysis.

To prove that these cell lines had the expected metabolic phenotypes, we analysed aspects of their metabolism such as the extracellular acidification rate (ECAR) and oxygen consumption rate (OCR) using a Seahorse analyser (**figure 3.1**), alongside glucose and glutamine consumption and lactate secretion (**figure 3.2**). These findings are in agreement with other research which states that found A549 cells take up high levels of glucose that is mainly secreted as lactate without entering the TCA cycle and that SKOV3 cells route virtually all of their glucose into

the TCA cycle, secreting little lactate (Hatzivassiliou *et al.* 2005). PC9 cells have also previously been shown to exhibit regular oxidative glucose consumption in agreement with our results (Pusapati *et al.* 2016).

Previous publications have defined cells which rely upon glucose to fuel the TCA cycle (also referred to as “glycolysis-dependent” cells) as being cells which have an IC_{50} of 3 mM 2DG and below. In contrast, cells which have an IC_{50} of above 3 mM 2DG do not rely upon glucose for the majority of their TCA metabolites, which can be described as being “glycolysis independent” (Pusapati *et al.* 2016). This delineation, although somewhat subjective, does provide a useful boundary by which we can divide cancer cells. Our results shown in **figure 3.3** indicate that A549 cells are “glycolysis independent” whereas SKOV3 and PC9 cells have a “glycolysis dependent” phenotype, whereby they rely upon glucose being metabolised via oxidative phosphorylation (**figure 3.8**). It is important to reiterate that despite the seemingly polar extremes of these two metabolic phenotypes, most cells which display the Warburg effect still have glucose entering the TCA cycle (as is depicted in **figure 3.8**) but, unlike non-malignant cells, can maintain aerobic glycolysis in normoxic conditions despite the use of glucose oxidation and oxphos (De Berardinis and Chandel 2016).

To understand the importance of various metabolites upon oncolysis, we first sought to understand how cancer cells respond metabolically to infection with both the chimeric adenovirus EnAd and the wildtype viruses Ad11p and Ad5. We

show that A549 cells increase their glucose uptake and lactic acid secretion in response to infection with both of the wildtype adenoviruses and EnAd despite already having a high basal glucose uptake (**figure 3.4 and 3.5**). These results were even more pronounced in SKOV3 cells which, perhaps because they have a lower basal glucose uptake, showed a stark increase in the Warburg effect during EnAd infection (**figure 3.6**). This led us to conclude that, in agreement with previous findings of wildtype viruses in non-cancerous cells, infection of adenoviruses in cancer cells causes an increase in glucose uptake accompanied by an increase in aerobic glycolysis.

As can be observed in both A549 (**figures 3.4 & 3.5**) and SKOV3 cells (**figure 3.6**), the observed increases in glucose uptake and lactate secretion occur at around 50 hours post-infection. Previous papers have shown that, with wildtype adenoviruses in non-malignant cell lines, this increased glycolysis and decreased cellular respiration occurs as a result of expression of either early adenovirus gene products E1A (Prusinkiewicz and Mymryk 2019) or E4ORF1 (Thai *et al.* 2014) and that the biggest alterations in host cell metabolism occur at around 48-72 hours post-infection. If it were the case that the increase in glucose metabolism were a result of cellular stress alone, we would expect the metabolism to rise sooner than we have observed, as is the case with several RNA viruses (Miyake-Stoner and O'Shea 2014; Thaker, Ch'ng and Christofk 2019; Dyer *et al.* 2020). We have not specifically identified the viral genes responsible for this increased metabolic

profile, but we believe it likely to be similar to the results reported with wildtype Ad5 in non-malignant cells (Thai *et al.* 2014; Prusinkiewicz *et al.* 2020).

Because glucose is being metabolised in a relatively inefficient manner with fewer glucose molecules entering the TCA cycle, other metabolites must be required for producing viral progeny during adenovirus infection. The most abundant and likely alternative is glutamine, and we confirmed that glutamine uptake is also increased significantly at early time points during infection for EnAd, and for later timepoints for Ad5 and Ad11p (**figure 3.7**). Again, these findings are in agreement with results published by Thai *et al.* who reported that infection of non-cancerous cells with the wildtype Ad5 increased glutamine uptake (Thai *et al.* 2015). We do observe noticeable differences in glutamine uptake from non-infected cells relatively late in infection when compared with Thai *et al.* who report observed changes at 24-48 hours post-infection. This may be because the basal level of glutamine uptake is already very high in A549 cells (**figure 3.2 A**) so any changes are harder to observe. This difference may also reflect the increased sensitivity of using radiolabelled glutamine compared to a luciferase-based assay.

Having confirmed that the metabolic phenotype of EnAd infected cancer cells is similar to the Warburg effect, we next postulated that those cells which already display this phenotype might be more susceptible to viral lysis. We aimed to find two cell lines of similar origin with different susceptibility to EnAd for future comparisons. We hoped to use PC9, and A549 cells as both of these cell lines are lung adenocarcinoma cell lines; however, these cell lines have such vastly different

susceptibilities (**figure 3.10**). It is challenging to compare these cell lines without accounting for the stress of PC9 cells being left in culture for nearly ten days longer than A549 or, increasing the dose of virus approximately 1000 fold to have viral lysis occur at the same point without accounting for the stress that places on the cells. For future analysis within this thesis, we will use SKOV3 cells which, although of a different origin to A549 and PC9 cells, have a moderate susceptibility to EnAd whilst having an oxidative glucose metabolic phenotype to allow for a fairer comparison. We aim in the future to expand our results to a wider panel of cells as we are cognisant of the limitations of using one glycolytic cell line and one oxidative cell line.

We analysed IC_{50} of a panel of cancer cells to EnAd and found a broad range of susceptibilities to viral lysis (**figure 3.11**). When plotted against the IC_{50} of the same cells to 2DG, we observed a correlation between these two variables and, whilst being conscious that correlation does not equate to causation, this does suggest a link between metabolism and susceptibility towards viral oncolysis with EnAd. One reason for this apparent correlation may be that EnAd was created and evolved within HT-29 cells and PANC-1 cells, both of which show a classical Warburg phenotype (Kuhn *et al.* 2008; Lin *et al.* 2014; Wang *et al.* 2018) and this may have resulted in a preference for such cells or an evolution towards a dependency upon this host cell phenotype. One other possible reason for this correlation is that previous papers have reported that early adenovirus genes (either E1A or E4ORF1) are responsible for increasing glucose uptake, aerobic glycolysis, glutamine uptake

and decreasing cellular respiration (Thai *et al.* 2014; Prusinkiewicz and Mymryk 2019). It is conceivable that cells which already display this metabolic phenotype can move more quickly into later stages of viral replication as some of the early gene functions are already achieved in such cells. This led us to hypothesise that altering the metabolism of oxidative cells towards a phenotype similar to the Warburg effect in poorly susceptible cells may improve oncolysis, and we will explore this hypothesis in the remaining chapters.

3.5 Chapter Conclusions

1. A549 cells display a Warburg effect phenotype with high glucose uptake, high glutamine uptake, high lactate secretion, and a high tolerance to the glycolysis inhibitor 2DG.
2. SKOV3 and PC9 cells display a normal metabolic phenotype with lower glucose uptake, lower glutamine uptake, lower lactate secretion and a low tolerance to the glycolysis inhibitor 2DG.
3. Infection with wildtype adenoviruses and the chimeric oncolytic adenovirus result in an increase in glucose uptake, glutamine uptake and lactate secretion in A549 and SKOV3 cells.
4. Cells with a dependency on glucose tend to be less permissive to viral oncolysis by EnAd.

4 Altering glutamine metabolism during adenovirus infection

4.1 Introduction

To maintain homeostasis and to proliferate at an accelerated pace, cancer cells must increase the import of nutrients from their surrounding microenvironment. In mammalian cells, the two principal nutrients used for these demands are glucose and glutamine (depicted in a simplified version in **figure 4.1**).

Glutamine can contribute to central carbon metabolism. Additionally, owing to the presence of nitrogen within its structure, it can be utilised for the *de novo* biosynthesis of many nitrogen-containing compounds such as purines and pyrimidines as well as non-essential amino acids (Márquez *et al.* 1989; DeBerardinis *et al.* 2008b; Mullen *et al.* 2012; Jiang *et al.* 2016). Harry Eagle first described the high demand for glutamine in proliferating cancer cells in the 1950s for cultured HeLa cells, who observed that these cells require up to 100-fold molar excess of glutamine relative to other amino acids (Eagle 1955). Since this observation, many other researchers have noted the depletion of glutamine from the tumour microenvironment when compared to normal tissue (Rivera *et al.* 1988; Márquez *et al.* 1989; Yuneva *et al.* 2012; Pavlova *et al.* 2018). Cancer's high demand for glutamine is also useful for diagnosis in environments of heavy glucose

utilisation where the use of ^{18}F -labelled glucose is not feasible (for instance, glioblastomas) by using ^{18}F -labelled glutamine.

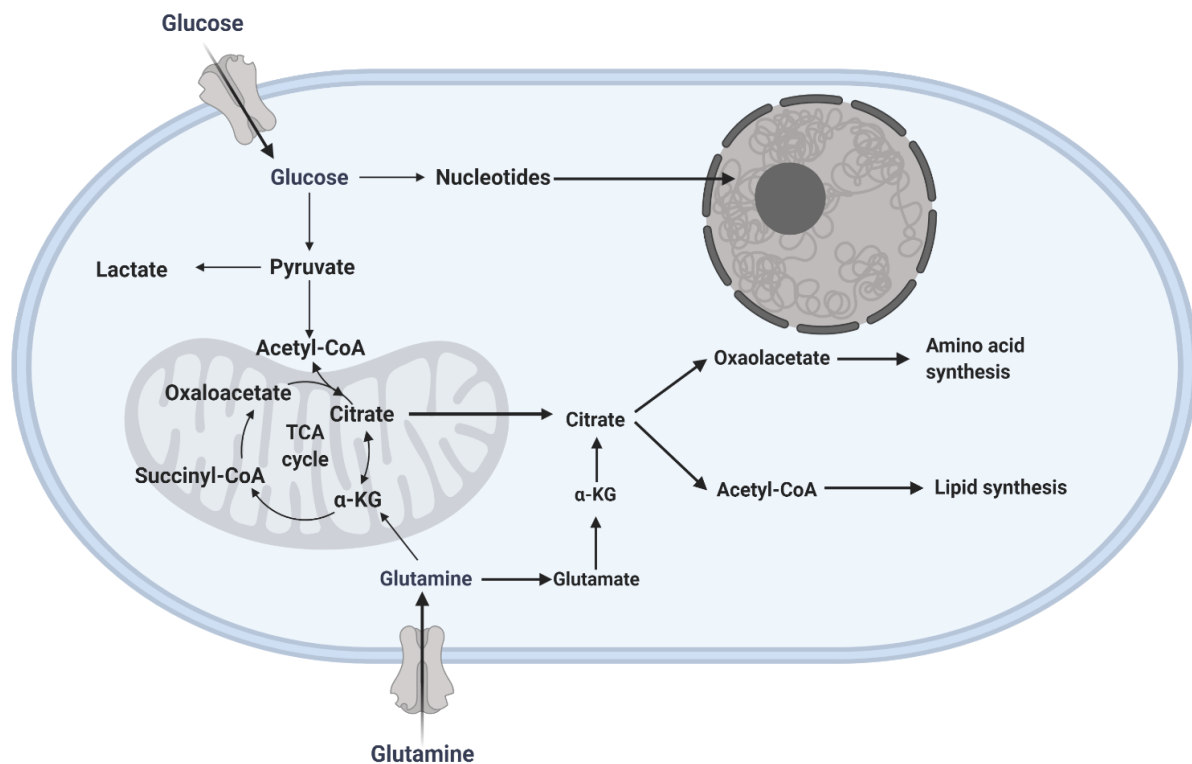


Figure 4.1 Central carbon metabolism of glucose and glutamine.

A simplified schematic representation of the metabolic fates of glucose and glutamine in mammalian cells. Extracellular glucose can be used to create nucleotides via the pentose phosphate pathway, or it can be catabolised via glycolysis to pyruvate which is either converted to lactate (in normoxic conditions this process is referred to as the Warburg Effect) or is used to fuel the TCA cycle. Glutamine, once intracellular, can be used for a myriad of purposes. Still, in this diagram, we have documented the oxidative metabolic pathway whereby glutamine is used to fuel the TCA cycle in the mitochondria or is reductively carboxylated to citrate either outside or inside the mitochondria. TCA, tricarboxylic acid cycle (Kreb's cycle); α -KG, alpha-ketoglutarate. Created with Biorender.com.

As with cancer cells, virally infected cells generally exhibit increased anabolic metabolism to support proliferation, and therefore some viruses depend on increased glutamine metabolism for the creation of infectious progeny (Ralser *et*

al. 2008; Chambers, Maguire and Alwine 2010; Fontaine, Camarda and Lagunoff 2014; Sanchez *et al.* 2015, 2017). The recent advances in the field of metabolomics have led to a much deeper understanding of metabolism and its effects upon viral lifecycles. It appears that, in general terms, viral infections cause an increase in global metabolism either through triggering cellular responses to the presence of viruses or by hijacking the cells own pathways (Miyake-Stoner and O'Shea 2014; Thaker, Ch'ng and Christofk 2019; Dyer *et al.* 2020).

It is evident from the differing tactics used by viruses that there is no single essential metabolic pathway or metabolite required by all viruses. Even closely related viruses can have very different metabolic demands such as human cytomegalovirus (HCMV) and herpes simplex virus 1 (HSV-1) which are both members of the Herpesviridae family (Chambers, Maguire and Alwine 2010; Abrantes *et al.* 2012). HSV-1 requires glucose for replication and supplementation of glutamine during primary infections, or viral latency suppresses the virus; whereas HCMV preferentially requires glutamine over glucose to meet its metabolic demands.

In 2015, Thai *et al.* reported that glutamine uptake is increased upon infection of primary human bronchial epithelial cells with wildtype Ad5 and that E4orf1 binding to MYC promotes a rewiring of host cell glutamine metabolism which in turn fosters virus replication. Through metabolomics with U-¹³C₅-glutamine, they revealed the pathways through which wildtype adenoviruses force host cells to

metabolise glutamine, namely an increased conversion of glutamine to aspartate, asparagine, and proline. They reported glutamine being metabolised via a process called reductive carboxylation, in which glutamine can be converted to citrate either in the cytoplasm or within the mitochondria without consuming oxygen (Thai *et al.* 2015).

In cancer cells, reductive glutamine metabolism is suggested to aid growth by providing a source of citrate without utilising oxygen which can then be used for macromolecule synthesis and acetyl-CoA production for lipid synthesis even in hypoxic environments or in malignant cells where the mitochondria are defective (Wise *et al.* 2011; Metallo *et al.* 2012; Mullen *et al.* 2012; Gameiro *et al.* 2013; Jiang *et al.* 2016).

Because viruses are by definition obligate intracellular pathogens, they are uniquely dependent upon the environment in which the host cell resides. For this reason, it is surprising to consider that a vast amount of pre-clinical evaluation of cancer treatments such as that of OV_s is done in media containing nutrients in concentrations far above those found even in healthy tissues. Whether glutamine uptake is essential for replication of oncolytic viruses in the context of aberrant cancer metabolism remains a largely under-investigated subject and whether pharmacological alterations of glutaminolysis can affect replication of oncolytic viruses remains undetermined.

We hypothesise that inhibiting or diminishing glutamine metabolism will be detrimental to viral replication. This hypothesis will underpin the results presented in this chapter.

4.2 Chapter Aims

1. Analyse the effect of reducing glutamine concentrations on the adenovirus lifecycle in cancer cells.
2. Analyse the effect of inhibiting glutaminolysis during adenovirus infection in cancer cells.
3. Improve enadenotucirev replication by altering glutamine metabolism

4.3 Results

4.3.1 Removal of glutamine in highly permissive A549 cells during adenovirus infection

A previous publication by Thai *et al.* in 2015 reported that glutamine and glutaminolysis was critical for the replication of wildtype adenovirus type 5 (Ad5) in normal human bronchial epithelial cells (NHBE). We hypothesised that the chimeric oncolytic adenovirus enadenotucirev (EnAd) also requires glutamine to replicate in cancer cell lines. We previously showed an increase in glutamine

uptake by A549 cells following infection with Ad5, Ad11p, or EnAd, although the stimulation of uptake by EnAd was faster and greater (**figure 3.7**).

To begin to assess whether glutamine was essential for a successful EnAd life cycle, we first evaluated the impact of removing glutamine altogether from the media of infected host cells. A549 cells were infected with two EnAd-based GFP reporter viruses, with GFP controlled either by a CMV promoter (EnAd-CMV-GFP) or using a splice acceptor under the virus Major Late Promoter (MLP; EnAd-SA-GFP). These constructs allow us to visualise proteins expressed early in infection or in the late stage of the infection lifecycle, respectively. Cells infected with either virus in the presence of glutamine (4 mM) showed a high expression of GFP at 72 hours post-infection. In the absence of glutamine, minimal GFP expression was observed with either virus (**figure 4.2**).

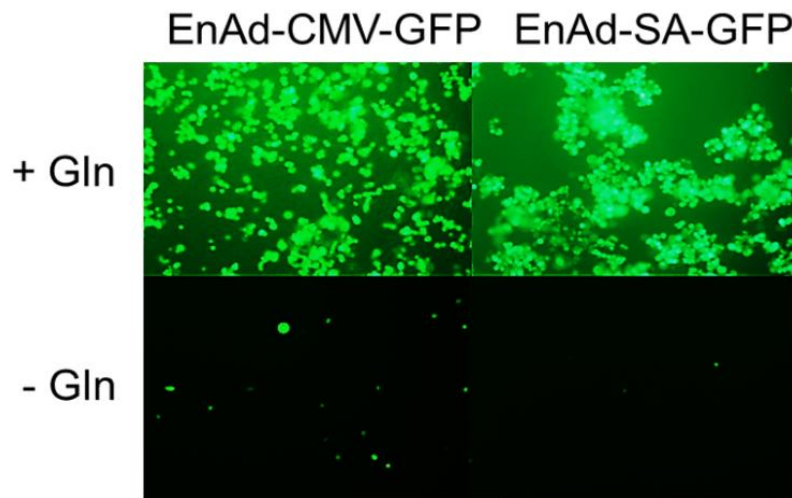


Figure 4.2 Removal of glutamine from A549 cells infected with EnAd decreases viral protein synthesis.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine (+ Gln) or without glutamine (- Gln). 5 days post-infection cells were imaged using a Nikon DS-U2 imaging microscope. The images are representative of biological triplicates.

We next aimed to quantify the observed reduction in fluorescence upon glutamine withdrawal using flow cytometry. The results show that around 90% of cells infected with EnAd-CMV-GFP or EnAd-SA-GFP at an MOI of 1 are GFP positive by 96 hours post-infection in the presence of glutamine. In contrast, fewer than 10% of cells were positive if A549 cells were infected in DMEM media without glutamine (**figure 4.3 A & C**). In GFP-positive cells, the levels of GFP expression per cell was 10- to 50-fold lower in the absence of glutamine (**figure 4.3 B & D**).

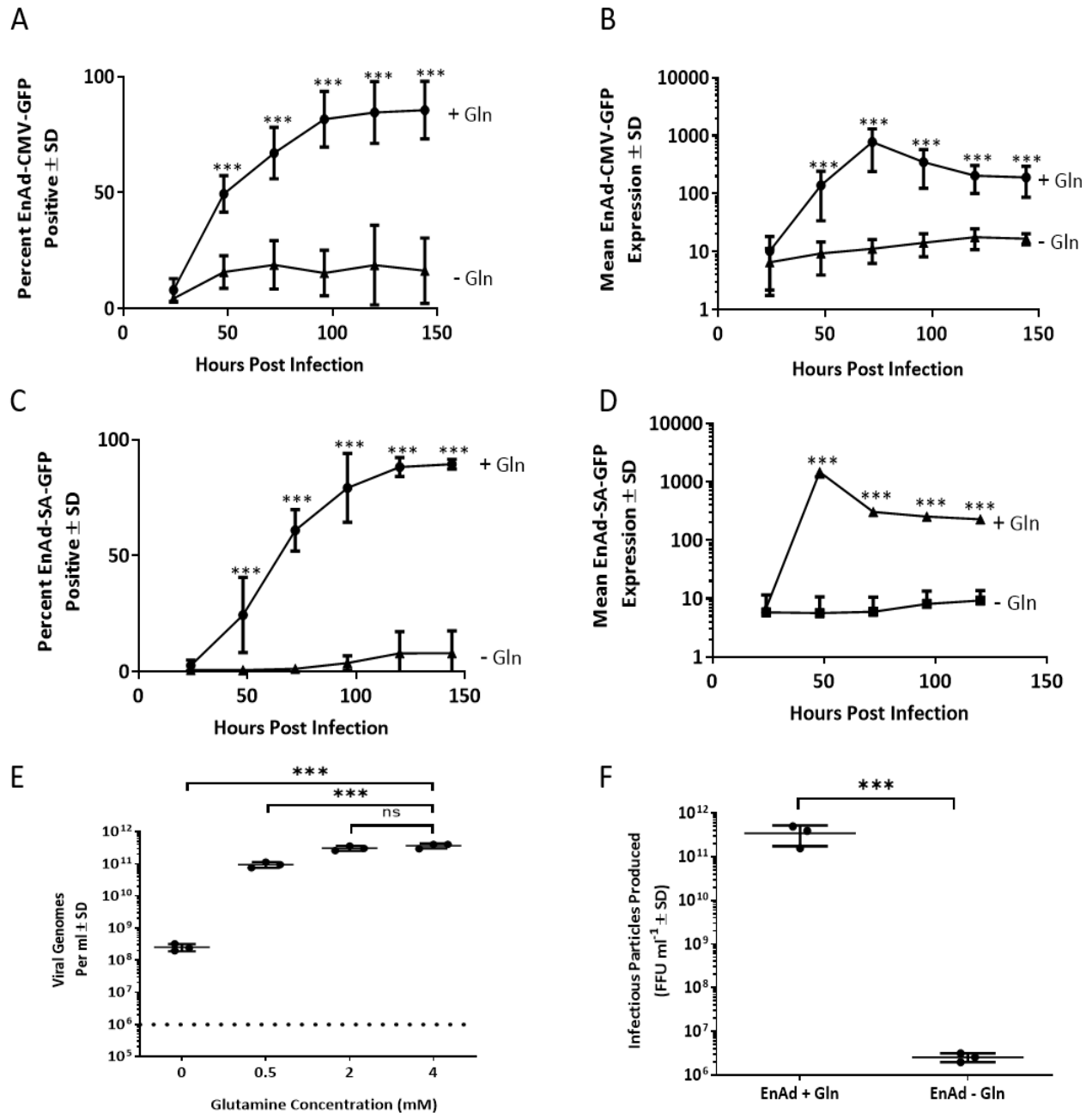


Figure 4.3 Removal of glutamine from A549 cells infected with EnAd inhibits the viral lifecycle.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4mM glutamine (+Gln) or without glutamine (-Gln). (A-D) Viral protein synthesis analysed by flow cytometry. (E) Quantitative real-time PCR analysis of virus genome levels in EnAd-infected A549 cells in the presence or absence of glutamine (96 hours post-infection). (F) TCID₅₀ analysis of EnAd-infected A549 cells in the presence or absence of glutamine (4 mM) from samples collected 96hpi. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

We next analysed the effect of limiting glutamine on viral genome replication using quantitative real-time polymerase chain reaction (qPCR) and on the production of infectious virus particles using the TCID₅₀ method. Removal of glutamine resulted in over a 1,000-fold decrease in viral genome production (**figure 4.3 E**) and a 100,000-fold reduction in infectious viral particles (**figure 4.3 F**). It is interesting to note that the observed reduction in viral genome copies is only significant at 0.5 mM glutamine and below suggesting some tolerance of alterations in the glutamine concentration. Considering, however, that glutamine is observed in the blood at concentrations from 0.5-1 mM and in tumours, vasculature and circulation is often reduced, these are the concentrations which the virus is most likely to encounter *in vivo* (Zhu *et al.* 2017; Liu *et al.* 2020). A similar effect was also observed using the parental virus Ad11p (**figure 4.4**). As with EnAd, there was no noticeable reduction in viral genome copy numbers until glutamine was at below 1mM glutamine.

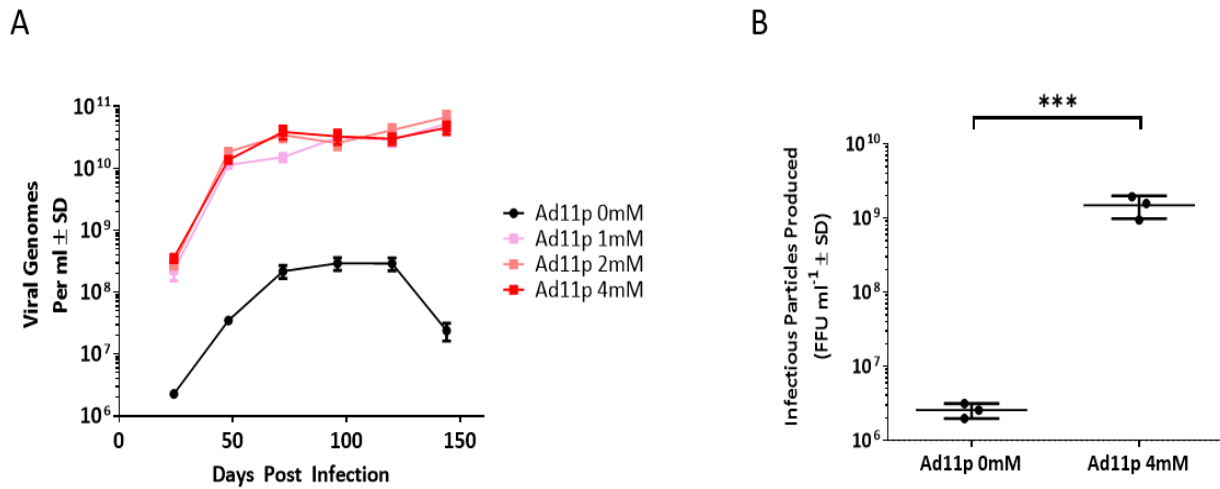


Figure 4.4 Removal of glutamine from A549 cells infected with Ad11p inhibits the viral lifecycle. A549 cells infected Ad11p at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing different glutamine concentrations (A) Quantitative real-time PCR analysis of virus genome levels in Ad11p-infected A549 cells in the presence or absence of glutamine. (B) TCID₅₀ analysis of Ad11p-infected A549 cells in the presence or absence of glutamine (4 mM) from samples collected 96 hpi. Data shown represent the mean ± SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

To further confirm that glutamine is crucial for adenovirus replication in cancer cells, we treated A549 cells with the commonly used glutaminase (GLS) inhibitor, CB-839. Previous research by Thai *et al.* in 2015 reported that treatment of the non-cancerous cell line NHBE with 1 μ M CB839 dramatically decreases wildtype Ad5 replication by 80-fold (Thai *et al.* 2015). We found that the number of viral genomes significantly declined in the presence of CB-839 for A549 cells infected with Ad5 as previously reported but also for cells infected with Ad11p and EnAd (figure 4.5). These results confirm that, like wildtype adenoviruses in normal cells, glutamine is critically required during the adenovirus lifecycle and that removal of glutamine or inhibition of glutamine metabolism is significantly deleterious to all stages of the viral lifecycle.

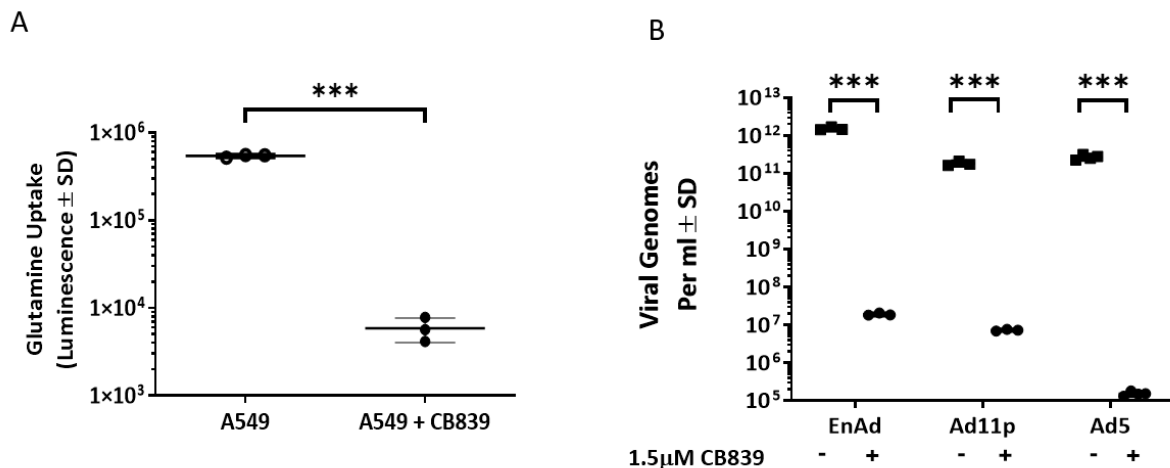


Figure 4.5 Treatment of A549 cells with the glutaminase inhibitor CB839 decreases glutamine uptake and viral genome copy numbers with wildtype adenoviruses Ad11p, Ad5 and the oncolytic adenovirus EnAd.

(A) A549 cells were treated with 1.5 μ M of the glutaminase inhibitor CB839 diluted in DMSO or mock-treated for 24 hours then glutamine uptake was analysed using Glutamine-Glo (Promega). (B) A549 cells were infected with Ad11p, Ad5 or EnAd, and 90 minutes post-infection, the inoculum was removed and replaced with DMEM containing 1.5 μ M of the glutaminase inhibitor CB839 diluted in DMSO or mock-treated. 5 days post-infection, cells were collected, and Quantitative real-time PCR analysis of virus genome levels was conducted. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

4.3.2 Enadenotucirev replication under glutamine starvation can be rescued through the addition of α -ketoglutarate in A549 cells.

Glutamine appears to be essential to support adenovirus replication in A549 cells.

We set out to determine whether this reflects glutamine's role as a component of macromolecular synthesis or as a metabolite for anaplerotic fuelling of the tricarboxylic acid (TCA) cycle. Many cancer cell types utilise exogenous glutamine, via its conversion to α -ketoglutarate (α -KG), as a TCA cycle intermediate (as depicted in **figure 1.3**). This anaplerotic role of glutamine allows the cell to use more glucose-derived carbon for alternative biosynthesis such as the pentose phosphate pathway instead of feeding into the TCA cycle. To assess the

importance of this pathway during adenovirus infection, we attempted to supplement the missing glutamine by adding TCA cycle intermediates to EnAd-infected cells.

A549 cells were infected with EnAd-CMV-GFP at an MOI of 1 in DMEM containing 10% FBS and 0mM glutamine. Ninety minutes post-infection, the inoculum was removed and replaced with glutamine free DMEM containing either alpha-ketoglutamine (α -KG), pyruvate or oxaloacetic acid (OAA) at concentrations that have been shown to rescue vaccinia virus under glutamine starvation (Fontaine, Camarda and Lagunoff 2014). Only α -KG, and not pyruvate or OAA, caused a rescue in the percentage of GFP-positive cells as analysed by flow cytometry (**figure 4.6 A**). Still, this rescue was only partial and was only apparent at 120 hours post-infection. We also examined the mean fluorescence of infected cells which indicates the level of infection of each cell as opposed to the number of cells infected. The mean fluorescence of EnAd-CMV-GFP infected A549 cells was partially restored in glutamine starved cells treated with α -KG at 120 hours post-infection. As with the analysis of the percentage of cells infected, the mean fluorescence was only partially restored with cells treated with α -KG, and this was still significantly lower than cells infected in replete media (**figure 4.6 B**).

The rescue of EnAd infection in glutamine-starved A549 cells supplemented with α -KG was more evident for cells infected with EnAd expressing GFP under the control of the major late promoter (EnAd-SA-GFP). Removal of glutamine from the

media decreased the percentage of A549 cells positive for GFP expression by 75% at 144 hours post-infection. The addition of OAA increased the percentage of cells positive for GFP to just below 50%. In contrast, the addition of α -KG increased the percentage of GFP positive cells to 75% of the cells grown in replete DMEM (**figure 4.6 C**).

Analysis of the mean fluorescence of glutamine-starved A549 cells infected with EnAd-SA-GFP at 120 hours post-infection revealed that glutamine starvation declined the mean fluorescence from 295 to 130. Addition of pyruvate did not rescue the mean fluorescence and addition of OAA mildly improved the mean fluorescence to 190. The addition of α -KG to glutamine starved A549 cells infected with EnAd-SA-GFP rescued the mean fluorescence and, in fact, significantly increased the mean fluorescence when compared to A549 cells infected in the presence of replete DMEM (**figure 4.6 D**) suggesting that α -KG may assist the virus moving through its life cycle or particularly benefit late-stage viral protein synthesis.

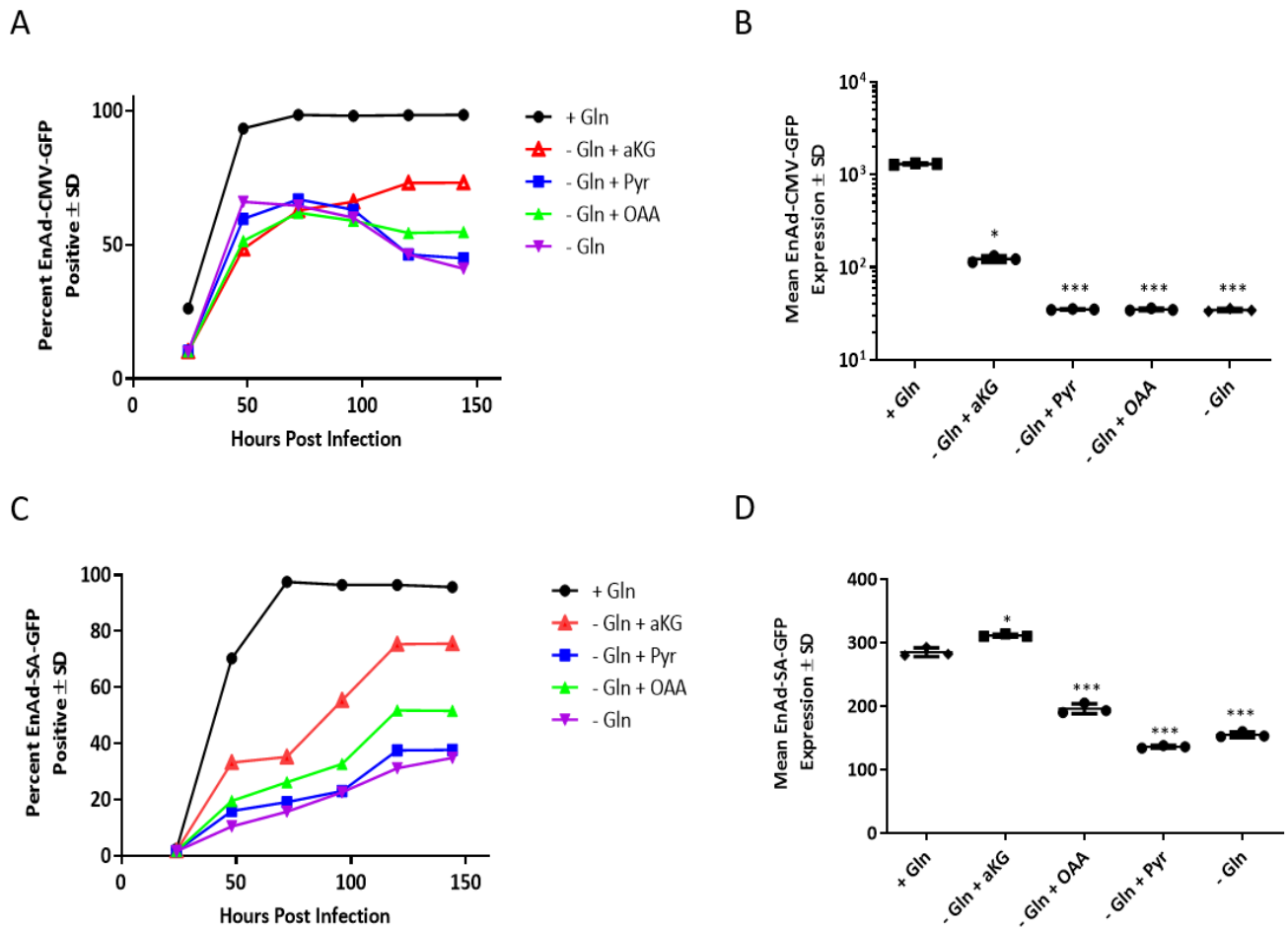


Figure 4.6 Addition of α -ketoglutarate and not oxaloacetate or pyruvate partially restores enadenotucirev viral protein synthesis under conditions of glutamine withdrawal in A549 cells. A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine (+ Gln, black), without glutamine (- Gln, purple) or in glutamine free medium supplemented with dimethyl- α -ketoglutarate (α KG, 7 mM, red), oxaloacetic acid (OAA, 4 mM, green) or pyruvate (4 mM, blue). Figures B and D represent results from 120 hours post-infection denoting mean GFP expression. Figures A and C represent the percentage of GFP positive cells. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

We then assessed the ability of anaplerotic TCA cycle intermediates to substitute for glutamine during virus genome synthesis in A549 cells. Glutamine-free cells showed relatively low levels of genome synthesis when analysed by real-time qPCR with a decline from 2×10^{10} viral genomes per mL to 5×10^8 viral genomes per mL (**figure 4.7 A**). This inhibition was only mildly alleviated following the addition of pyruvate or oxaloacetic acid with genomes per mL, rising to 2×10^9 and 3×10^9 , respectively (**figure 4.7 A**). The addition of α -KG to EnAd-infected A549 cells starved of glutamine, however, showed a complete recovery in viral genomes produced (**figure 4.7 A**). These findings were also mirrored in infectious viral progeny particle production with only α -KG supplementation rescuing levels (**figure 4.7 B**).

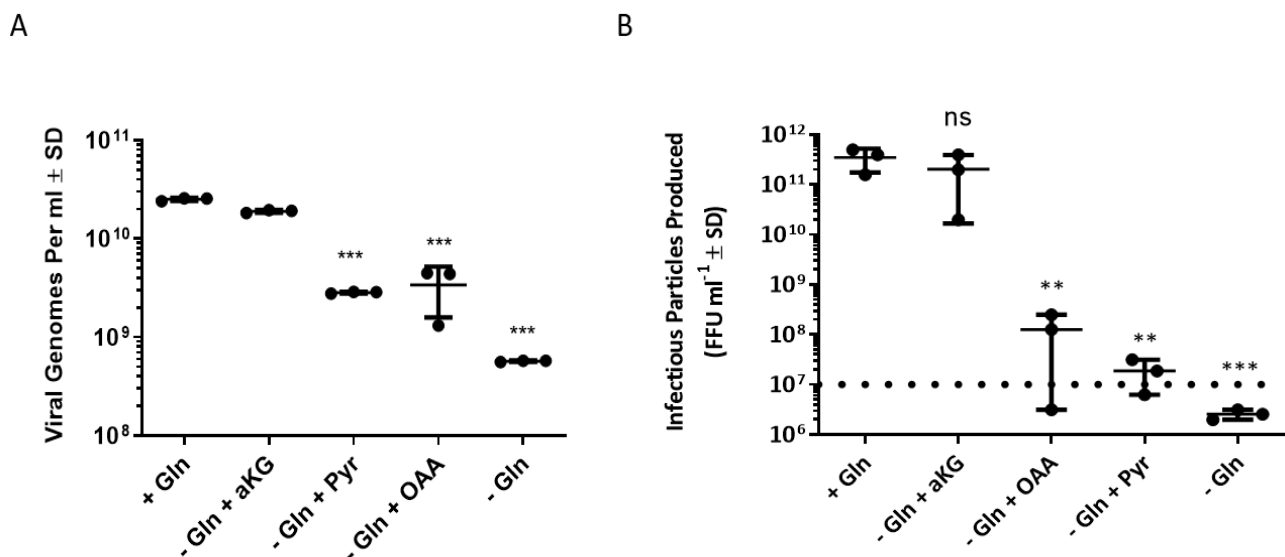


Figure 4.7 Addition of α -ketoglutarate and not oxaloacetate or pyruvate partially restores enadenotucirev viral genome production and infectious viral progeny production under conditions of glutamine withdrawal in A549 cells.

A549 cells were infected with at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine (+ Gln), without glutamine (- Gln) or in glutamine free medium supplemented with dimethyl- α -ketoglutarate (α KG, 7 mM), oxaloacetic acid (OAA, 4 mM) or pyruvate (4 mM). (A) Quantitative real-time PCR analysis of virus genome levels in EnAd-infected A549 cells from samples collected 96 hours post-infection. (B) TCID₅₀ analysis of EnAd-infected A549 cells from samples collected 96 hours post-infection. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

4.3.3 Enadenotucirev replication under glutamine starvation can be rescued through the addition of α -ketoglutarate in SKOV3 cells.

Having shown that α -KG can rescue EnAd infection in glutamine starved A549 cells, we next sought to see if a less glutamine addicted cell line would show similar results. As shown in chapter 3, A549 cells take up high levels of glucose which is mainly excreted as lactate meaning that any other metabolites required are sourced from glutamine, and other researchers have previously described A549 cells as being “glutamine addicted” (Hatzivassiliou et al. 2005). SKOV3 cells have a more normal metabolic phenotype with glycolysis linked to oxidative phosphorylation and less of a dependency on glutamine (**figure 3.2**).

As with A549 cells, SKOV3 cells were starved of glutamine after infection with EnAd-CMV-GFP in media supplemented with either pyruvate, oxaloacetate acid (OAA) or α -ketoglutarate (α -KG). In the absence of glutamine, the addition of only α -KG caused a restoration of the percentage of cells that were positive for GFP expression (**figure 4.8 A**) as well as the frequency of GFP-expression (**figure 4.8 B**) using the EnAd-CMV-GFP reporter virus. α -KG caused the peak CMV-GFP expression to occur approximately four days earlier (although reaching a lower average intensity) than in cells infected in the presence of complete medium (**figure 4.8 A & B**).

Addition of α -KG to EnAd-SA-GFP-infected SKOV3 cells starved of glutamine restored the percentage of GFP positive cells close to levels observed in complete

medium (figure 4.8 C). We observed a dramatic increase in the mean GFP expression that reached 4-times higher than levels seen even in complete medium (figure 4.8 D). These observed results mirror the results seen in A549 cells, again suggesting a particular benefit to late-stage viral protein synthesis with α -KG supplementation.

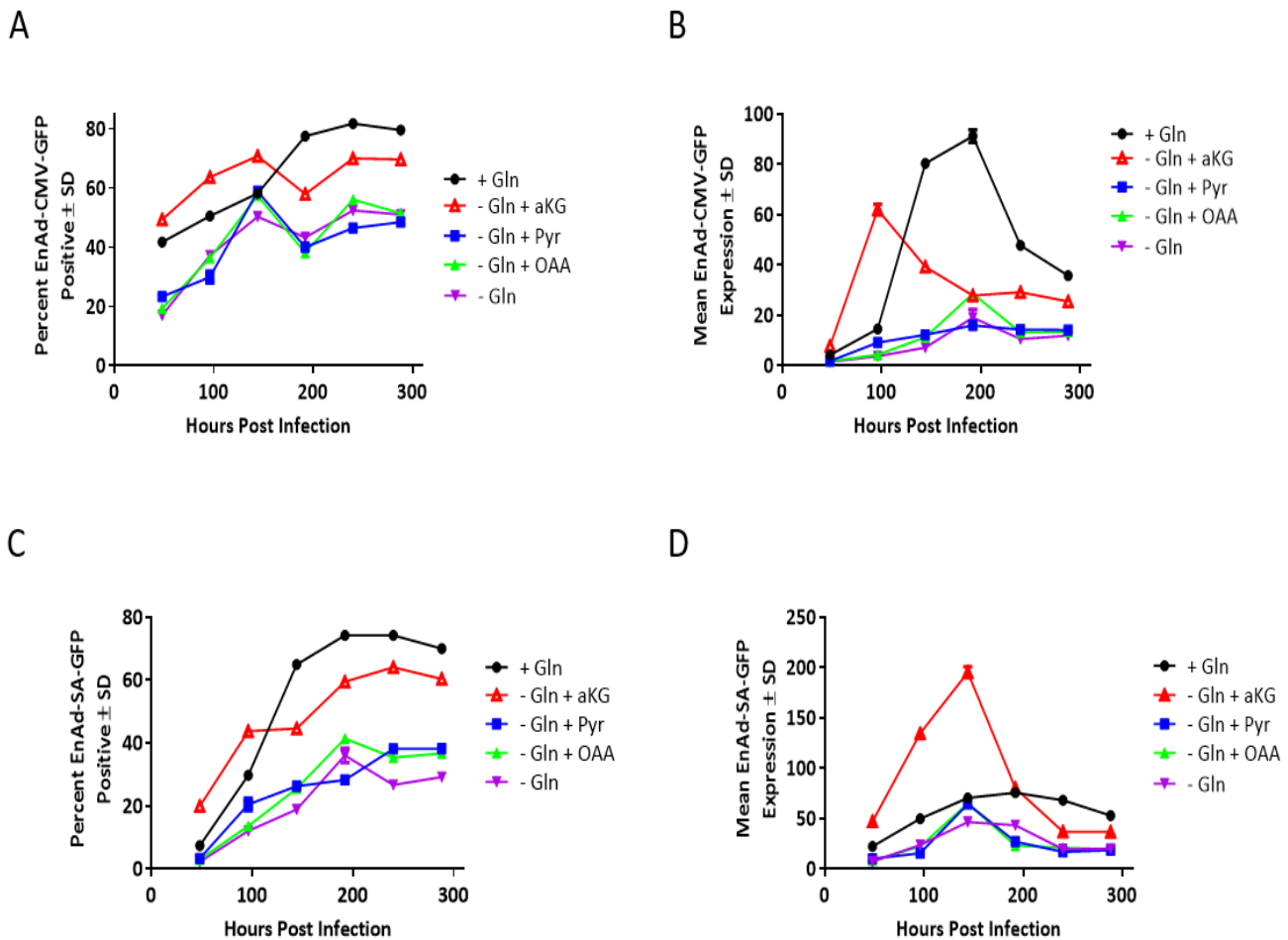


Figure 4.8 Addition of α -ketoglutarate and not oxaloacetate or pyruvate partially restores enadenotucirev viral protein synthesis under conditions of glutamine withdrawal in SKOV3 cells.

SKOV3 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine (+ Gln, black), without glutamine (- Gln, purple) or in glutamine free medium supplemented with dimethyl- α -ketoglutarate (α KG, 7 mM, red), oxaloacetic acid (OAA, 4 mM, green) or pyruvate (4 mM, blue). Figures B and D represent mean GFP expression. Figures A and C represent the percentage of GFP positive cells. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

4.3.4 Addition of α -ketoglutarate and not TCA intermediates improve the replication of EnAd in replete media

The findings presented above show that glutamine is critical for EnAd viral replication and host cell lysis. TCA intermediates, OAA and pyruvate, did not rescue viral replication suggesting that glutamine is being utilised in a fairly unique manner. The findings that α -KG supplementation can rescue (and even improve) viral replication and lysis under conditions of glutamine starvation suggest the possibility that reductive carboxylation of glutamine is the pathway through which glutamine is being used in EnAd-infected cancer cells in a manner similar to previous reports using Ad5 and non-cancerous cells (Thai et al. 2015, these pathways are discussed further in chapter 1).

We next aimed to investigate if inducing reductive carboxylation of glutamine in conditions with complete levels of glutamine might improve the virus lifecycle. Previous publications have shown that when there is a high ratio of α -KG to citrate, glutamine is metabolised preferentially via reductive carboxylation (Fendt *et al.* 2013). We, therefore, hypothesised that the addition of α -ketoglutarate to replete media would shift the ratio of α -ketoglutarate to citrate in the favour of reductive carboxylation of glutamine and thus favour EnAd oncolysis.

For A549 cells infected with EnAd-CMV-GFP at an MOI of 1, the addition of pyruvate, OAA and α -KG to complete media showed no beneficial effects on the percentage of GFP-positive cells, nor the mean GFP expression (**figure 4.9 A & B**).

For A549 cells infected with EnAd-SA-GFP at an MOI of 1, all cell culture conditions showed 100% of cells positive for GFP expression by 72 hours post-infection (**figure 4.9 C**). Because these cells are highly permissive to EnAd, an increase in the percentage of cells positive for GFP is challenging to achieve. When analysing the mean fluorescence of GFP however, the addition of α -ketoglutarate to replete DMEM medium significantly increased the mean fluorescence of SA-GFP from 72 hours onwards. This increase in mean fluorescence suggests that even in the presence of high glucose and glutamine conditions, the addition of α -KG can enhance late-stage viral protein production (**figure 4.9 D**). The addition of α -ketoglutarate also resulted in a mild increase in the amount of infectious viral progeny produced, although none of the changes were statistically significant (**figure 4.10**).

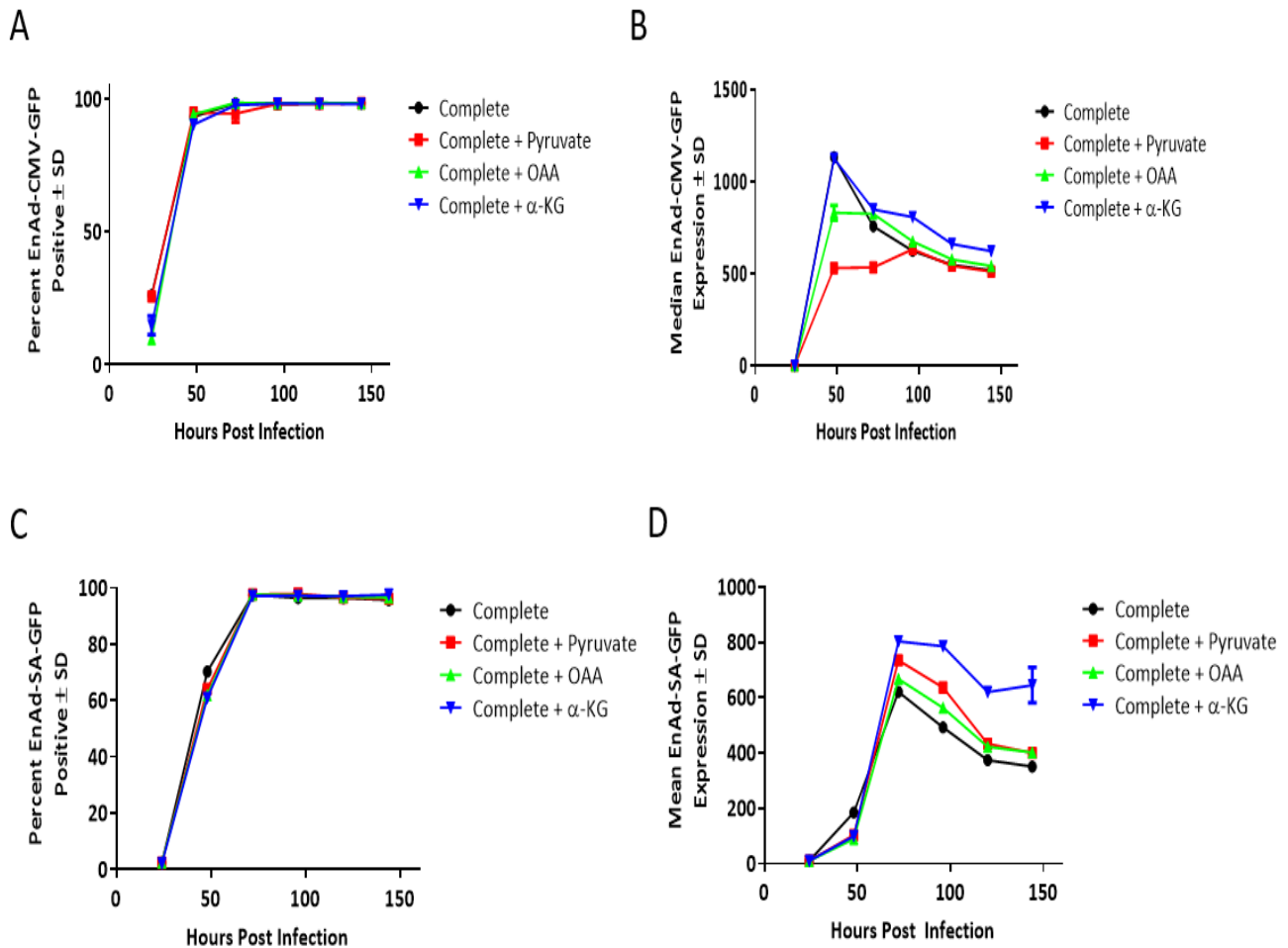


Figure 4.9 Addition of α -ketoglutarate and not oxaloacetate or pyruvate modestly improves late enadenotucirev viral protein synthesis under high glutamine concentrations in A549 cells.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine (black), or in glutamine containing medium supplemented with dimethyl- α -ketoglutarate (α KG, 7 mM, red), oxaloacetic acid (OAA, 4 mM, green) or pyruvate (4mM, blue). Figures **B** and **D** represent median GFP expression. Figures **A** and **C** represent the percentage of GFP positive cells. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

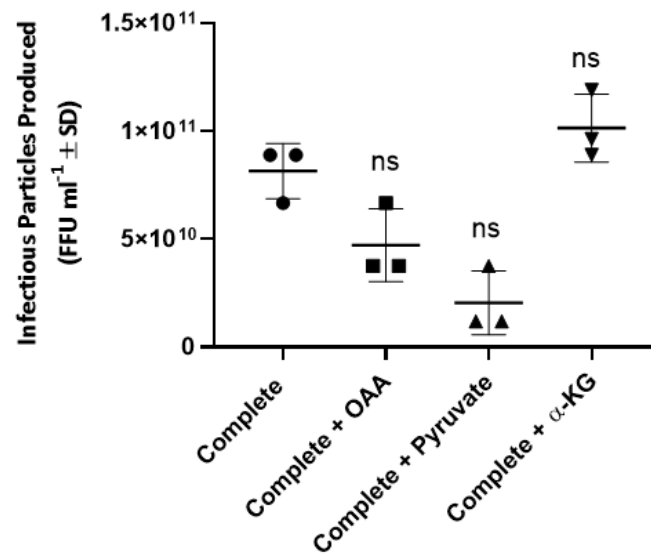


Figure 4.10 Effect of the addition of α -ketoglutarate, oxaloacetate or pyruvate on infectious viral progeny under high glutamine concentrations in A549 cells.

A549 cells infected with at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 4 mM glutamine, or in glutamine containing medium supplemented with dimethyl- α -ketoglutarate (α KG, 7 mM), oxaloacetic acid (OAA, 4 mM) or pyruvate (4 mM). Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

4.4 Chapter Discussion

In this chapter, we aimed to test our second hypothesis, that removal of glutamine from the media will inhibit EnAd oncolytic activity. In chapter 3, we investigated the metabolic response of cancer cells to infection with the oncolytic adenovirus enadenotucirev (EnAd). We showed that, in response to infection, cancer cells increase aerobic glycolysis alongside an increase in glutamine metabolism in agreement with previous work investigating wildtype adenoviruses in non-malignant cells (Thai *et al.* 2014, 2015; Prusinkiewicz and Mymryk 2019). We next aimed to understand if these metabolic alterations were critical to viral replication

through the removal of metabolites during EnAd infection. As previously mentioned, studies have shown that wildtype Ad5 infection results in increased glutamine metabolism and that this has the potential to be used as an antiviral target however there is a paucity of research investigating the role of glutamine metabolism in oncolytic viruses in the context of cancer cells. Because viruses are obligate intracellular pathogens, they are uniquely dependent upon the environment in which the host cells reside. For this reason, it is particularly surprising that most OV's are evaluated in conditions which contain nutrients in concentrations which are far above that found in healthy conditions *in vivo*, let alone in a tumour with poor vasculature and poor circulation. Usually, cells within our laboratory are cultured in 4 mM glutamine which is far in excess of physiological levels which are generally reported to be between 0.55 – 1 mM within blood (Bergstrom *et al.* 1974; Le Bacquer *et al.* 2001).

We first sought to understand the effect of removing glutamine from A549 cells infected with EnAd using two GFP reporter viruses with GFP promoted by CMV (EnAd-CMV-GFP) or using a splice acceptor under the virus major late promoter (MLP; EnAd-SA-GFP), allowing visualisation of immediate early viral protein synthesis or late protein synthesis respectively (the adenovirus lifecycle is discussed in more detail in chapter 1). Upon removal of glutamine from the media of EnAd-infected A549 cells, we saw a marked decline in all aspects of the viral lifecycle that were measured including viral protein synthesis (**figures 4.2 and 4.3**),

viral genome expression (**figure 4.3**), and viral infectious progeny production (**figure 4.3**), revealing that glutamine is required for optimal adenovirus replication.

The results in **figure 4.3 E** and **figure 4.4 A**, however, indicate that the tolerance for glutamine withdrawal is relatively wide as glutamine concentrations could fall to 0.5 mM before observing a substantial detrimental effect to viral genome numbers. Considering, however, that the physiological level of glutamine ranges from 0.5 mM to 1 mM in blood, it is concerning that this result suggests that EnAd is more suited to conditions found *in vitro* than *in vivo*. Further work remains to be done to fully understand the lowest concentrations of glutamine that can be used without seeing a substantial inhibition of the viral lifecycle in these cells. We also confirmed that the parental adenovirus Ad11p had a similar dependency upon the presence of glutamine in media when infecting A549 cells (**figure 4.4**), again with no detrimental effect observed until concentrations were below 1 mM glutamine.

We next sought to understand the effects of inhibiting glutaminolysis through the addition of a glutaminase inhibitor (CB839). This inhibitor was selected instead of the more widely used BPTES because BPTES has a relatively moderate potency, poor metabolic stability, and low solubility. CB839, however, is currently undergoing phase I studies for multiple cancer types, and has been shown to have

a nanomolar potency against both splice isoforms of glutaminase (Gross *et al.* 2014; Harding *et al.* 2015). Thai *et al.* showed that HSV-1, influenza and adenoviruses could be inhibited in normal human bronchial epithelial cells with treatment of 1 μ M CB839 (Thai *et al.* 2015). We, therefore, wanted to investigate if an oncolytic virus infecting cancer cells which have an already aberrant glutamine metabolism might also have a similar response to treatment with CB839. **Figure 4.5** confirms that CB839 can dramatically inhibit viral replication of both wildtype adenoviruses used and enadenotucirev. The results discussed thus far provide a compelling case for stating that enadenotucirev is dependent upon the presence of glutamine for adequate replication.

We next attempted to rescue virus replication under conditions of glutamine starvation to assess the metabolic fate of glutamine in EnAd-infected cells. A549 cells were fed with either replete medium (4 mM glutamine), glutamine free medium or medium supplemented with seven mM dimethyl- α -ketoglutarate (α -KG), 4 mM pyruvate or 4 mM oxaloacetic acid (OAA). These concentrations were chosen because previous work by Fontaine *et al.* published in 2014 showed that replication of vaccinia virus (an enveloped double-stranded DNA virus of the family Poxviridae) was able to be rescued by the addition of these compounds at these concentrations under conditions of glutamine starvation (Fontaine, Camarda and Lagunoff 2014). We had expected that, similarly to vaccinia virus, EnAd replication would be restored after addition of these compounds however only the addition

of α -KG and not OAA or pyruvate restored replication with particular benefit being observed for late-stage viral proteins (**figures 4.6 - 4.8**).

Given these surprising results, we had to consider why only α -KG was able to rescue viral replication. Thai *et al.* have shown that wildtype Ad5 infection of normal cells results in a unique metabolism of glutamine through metabolomics with radio-labelled glutamine highlighting reductive carboxylation of glutamine as being notably upregulated. One difference between these metabolites is that α -KG can be metabolised outside of the mitochondria and without a requirement for oxygen via reductive carboxylation, whereas OAA and pyruvate cannot (Mullen *et al.* 2012, 2014). Taking our rescue results in context with the results from Thai *et al.* provides a compelling argument for reductive carboxylation of glutamine being an important metabolic pathway during infection and the findings that α -KG addition can boost viral protein expression above levels seen in replete media was particularly promising as it suggests that altering metabolism can improve viral oncolysis.

The role of mitochondrial respiration during viral infection is a complex and understudied topic, however previous publications have shown that wildtype adenoviruses such as Ad5 trigger endolysosomal membrane disruption during cell entry which triggers cathepsin B release which in turn facilitates mitochondrial membrane disruption (McGuire *et al.* 2011). Many human adenoviruses have

been reported to localise proteins within and surrounding the mitochondria of host cells resulting in damage to the mitochondrial architecture (Matthews and Russell 1998; Degenhardt, Perez and White 2000; Lomonosova, Subramanian and Chinnadurai 2005; Alesci *et al.* 2007). More recent studies have posited that the interaction between adenoviruses and the mitochondria may act to inhibit mitochondrial function (Morrison *et al.* 2019) however, how EnAd affects and is affected by mitochondrial function remains to be fully elucidated. Some papers have shown a decrease in cellular respiration in adenovirus-infected cells, which will significantly restrict the oxidative TCA cycle making metabolic pathways such as reductive carboxylation all the more crucial (Thai *et al.* 2014; Prusinkiewicz *et al.* 2020). An interesting exception to these findings is human adenovirus 40 which lacks a number of early adenovirus genes including E4ORF1 and does not inhibit mitochondrial respiration (Davison *et al.* 1993; Guissoni *et al.* 2018). Considering that reductive carboxylation is considered vital in cancer cells with defective mitochondria, we can postulate a logical dependency of EnAd infected cells on this pathway for similar reasons (Mullen *et al.* 2012).

Given that we now hypothesised that glutamine is utilised during enadenotucirev infection in a reductive manner, we wanted to investigate whether artificially stimulating reductive carboxylation would improve the lifecycle of EnAd in replete media. In 2013, Fendt *et al.* reported that reductive carboxylation is a function of the α -KG to citrate ratio in cells and that artificially increasing the amount of α -KG

in culture conditions will trigger cells to reductively carboxylate glutamine (Fendt *et al.* 2013). We added dimethyl- α -ketoglutarate to cells cultured in replete medium to force cells to reductively metabolise glutamine (**figures 4.9 and 4.10**). We showed that the addition of α -KG, pyruvate or OAA does not increase the percentage of cells which are positive for GFP expression. This is perhaps unsurprising as A549 cells are highly susceptible to oncolysis with EnAd (**figure 3.9**) and so it is challenging to improve outcome in such cells. When considering the mean viral protein expression, the addition of α -KG to replete medium enhances the expression of GFP driven by the MLP to levels exceeding that seen in replete condition, again suggesting that reductive glutamine metabolism may be particularly important in later stages of the virus lifecycle. It is also interesting to note that the addition of TCA metabolites that favour oxidative phosphorylation appear to decrease viral protein synthesis and infectious particles produced. However, the latter is not to significant levels.

It is important to note that we have presented results here showing both the percentage of cells which are GFP positive and the mean fluorescence of the cells. The percentage of GFP positive cells is a readout for how many cells have been infected by the virus (in the case of EnAd-CMV-GFP) or how many cells have viruses in the late stage of the lifecycle (in the case of EnAd-SA-GFP). The mean fluorescence is an indication of the level of viral protein expression and, by proxy, the level of viral replication. In the case of highly permissive cells (such as A549

cells) all cells will have close to 100% positivity for GFP so it is crucial to probe the mean fluorescence as this will indicate an increased viral protein synthesis. In less permissive cells (such as SKOV3 cells), both readouts are important.

Considering the results from chapter 3 of this work where we presented a model for the metabolic alterations of cancer cells to infection with enadenotucirev (**figure 3.8**), we can now update these findings to reflect our current hypothesis that, alongside a general upregulation in aerobic glycolysis and glutaminolysis, EnAd-infected cells utilise glutamine in a reductive manner, likely outside of the mitochondria (**figure 4.11**). As noted in this figure, the fate of reductively carboxylated glutamine within these cells and the precise functional significance or advantages of enhanced reductive glutaminolysis is not yet clear, and further work is required to understand these points fully which includes metabolomics with radiolabelled glutamine. We can, however, accept the hypothesis that removal of glutamine from the media will inhibit EnAd oncolytic activity.

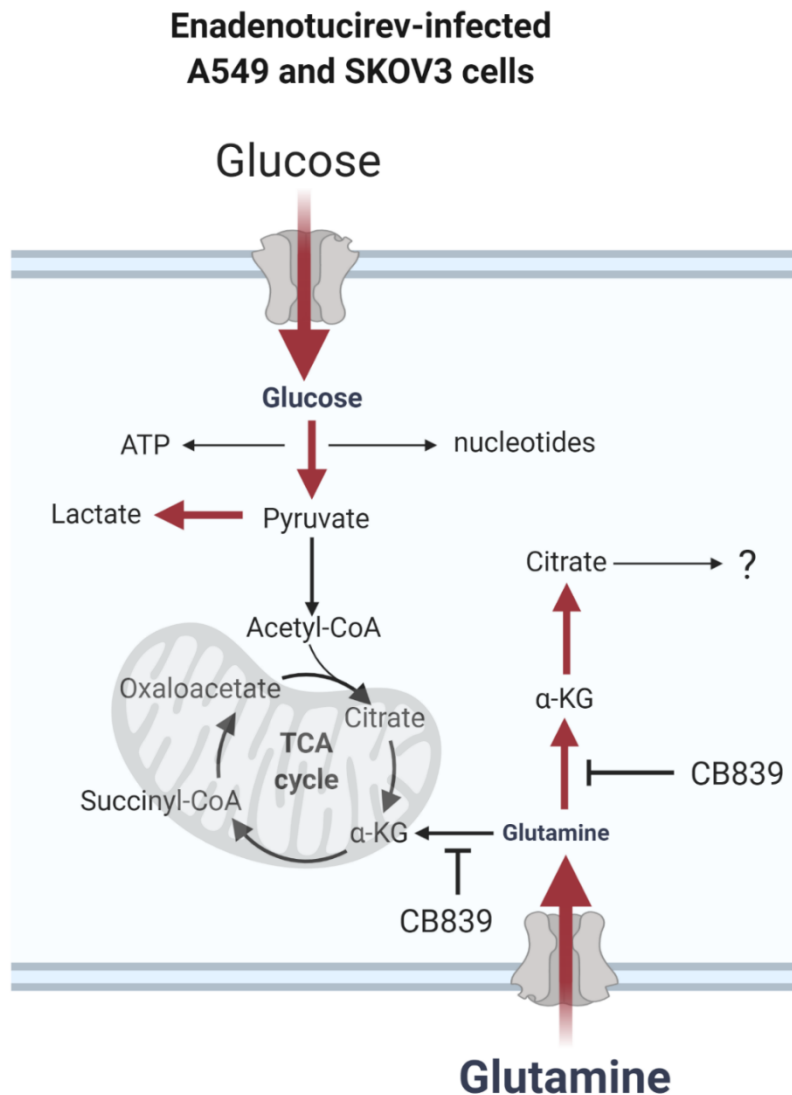


Figure 4.11 Metabolic alterations caused by infection of A549 or SKOV3 cells with Enadenotucirev.

Simplified schematic representation of metabolic phenotypes of A549 and SKOV3 cells post-infection with adenoviruses. The red arrows represent pathways that are increased under each circumstance. ATP, adenosine triphosphate; TCA, tricarboxylic acid cycle (Kreb's cycle); α -KG, alpha-ketoglutarate; CB839, glutaminase inhibitor. Created with Biorender.com.

4.5 Chapter Conclusions

1. Removal of glutamine after viral uptake in A549 cells is detrimental to adenovirus replication.
2. Inhibiting glutaminase using CB839 inhibits adenovirus replication in A549 cells.
3. Under conditions of glutamine starvation, the addition of α -ketoglutarate and not oxaloacetate acid or pyruvate rescue EnAd lifecycle.
4. In complete medium, the addition of α -ketoglutarate to EnAd-infected cells increases the mean expression of late viral proteins but not infectious viral progeny.

5 Altering glucose metabolism during adenovirus infection

5.1 Introduction

In the 1920s, Warburg and Cori documented that cancer consumes glucose at abnormally high levels and that this results in an increase in lactate excretion (Carl Cori and Cori 1925; Warburg, Wind and Negelein 1927). In normoxic conditions, non-cancerous mammalian cells typically use glucose to feed the TCA cycle in an oxidative manner, ultimately producing ATP and carbon dioxide and allowing cellular homeostasis. In the absence of oxygen however, these cells change their metabolism to anaerobic glycolysis in which glucose is converted to lactate. Otto Warburg first described the phenomenon whereby cancer cells, even in the presence of ample oxygen, preferentially convert glucose into lactate which is later termed the Warburg effect or aerobic glycolysis. Since the 1920s, many other cancer cells have been shown to rely on aerobic glycolysis, and the Warburg effect now forms the basis of PET imaging which underlies a significant pillar of cancer diagnosis (Pauls *et al.* 2007; Steinert 2011; McCarten *et al.* 2019; Zaucha *et al.* 2019). We now understand that non-malignant will not use oxidative phosphorylation (oxphos) alongside anaerobic glycolysis (sometimes referred to as the Crabtree effect). In contrast, tumour cells will preferentially rely upon aerobic glycolysis despite also being able to use glucose oxidation. This metabolic plasticity allows malignant cells to adapt to their surroundings, and when they

encounter hypoxia or when they have defective mitochondria, they can still survive without glucose oxidation.

Although at first, it may seem that this inefficient method of metabolising glucose may be a disadvantage to cancer cells, there are several key advantages provided to cells which exhibit the Warburg effect (these advantages are covered in more detail in chapter 1). In brief, however, the rate of metabolism through aerobic glycolysis occurs 10-100 times faster than glucose oxidation and oxphos such that the amount of ATP synthesised over a given period is comparable (Shestov *et al.* 2014). Meanwhile, the resulting production of lactate and other waste products of aerobic glycolysis can inhibit many immune cells functions, effectively creating an “immunologically cold” cancer (Romero-Garcia *et al.* 2016). The metabolic plasticity in most cancer cells also allows for alternative metabolites (such as glutamine) to be used for biosynthetic purposes which have less competitive demands.

Over the past few decades, our understanding of cellular metabolism as a scientific field, and cancer metabolism more specifically, has increased as new scientific methodology has been invented. We now know that not all cancer cells display the Warburg effect. Indeed, this was documented as early as 1967 when Weinhouse *et al.* reported that rat hepatoma cells that were relatively slow-growing were oxidative, whereas rapidly growing cancer cells were glycolytic

(Weinhouse 1967). In 2016 Pusapati *et al.* divided cancer cell lines grown into the lab into the useful characterisation of “glycolysis dependent cells” and “glycolysis independent cells” (Pusapati *et al.* 2016). They showed that cells which rely upon glucose to fill the TCA cycle (i.e. glycolysis dependent cells) are sensitive to treatment with inhibitors of glycolysis such as 2-deoxy-d-glucose (2DG) however cells which display the Warburg effect (i.e. glycolysis-independent cells) can metabolically rewire their metabolism as they are less dependent on glycolysis and so are not susceptible to metabolic-based monotherapies (Pusapati *et al.* 2016).

As viruses are obligate intracellular pathogens, they need to hijack cellular processes to create a favourable environment for efficient production of infectious progeny. Many viral infections increase aerobic glycolysis, however, whether this is a cellular response to the presence of the virus or an effect mediated by the expression of virally encoded proteins varies between cell type and virus type (Miyake-Stoner and O’Shea 2014; Thaker, Ch’ng and Christofk 2019; Dyer *et al.* 2020). Because of these differences, some viruses can replicate and kill in the absence of glucose, whereas others are critically dependent upon glucose. We will be focussing on adenoviruses for the remainder of this chapter.

Shortly after adenoviruses were discovered, it was observed that infection causes an increase in glycolysis and lactic acid production (Fisher and Ginsberg 1957; Rozee, Ottey and Rooyen 1957). Other members of the adenovirus family have

been shown to induce glycolysis through expression of an early viral gene, E4ORF1, which interacts with Myc (Thai *et al.* 2014, 2015). Interestingly, it has been shown that in cells that have a low glycolytic rate, knockdown of c-Myc decreases viral titres but not in cells with a high glycolytic rate (Thai *et al.* 2014; Prusinkiewicz and Mymryk 2019). Additionally, recent studies report that early wildtype adenovirus protein E1A also increases glycolysis and decreases cellular respiration through an unknown pathway (Prusinkiewicz *et al.* 2020). All of these studies focus on wildtype adenoviruses, and it is currently unclear how oncolytic adenoviruses such as EnAd alter glycolysis.

In all of the studies discussed above, cell lines were grown in cell culture media containing high levels of glucose at between 10 and 25 mM. This concentration of glucose is dramatically higher than the concentration of glucose in plasma which is around 6 mM. Solid tumours, which have poor vasculature, increased cell mass and an increased competition for glucose, can have glucose concentrations as low as 0.1 – 0.4 mM as well as increased concentrations of metabolic waste levels such as lactate (Hirayama *et al.* 2009). Additionally, glucose levels in tumours *in vivo* is not evenly distributed with concentrations being inversely correlated with distance from the capillary bed meaning glucose deprivation can occur in spatial and temporary manners as well as in more profound long-term cases (Skinner, Tutton and O'Brien 1990; Fukumura and Jain 2007). Understanding how any cancer treatment will affect cells exposed to more realistic conditions is vital to

the success of a treatment *in vivo*. It is of particular importance to metabolically reliant treatments such as oncolytic viruses.

Despite the apparent importance of trialling oncolytic viruses in realistic concentrations of metabolites such as glucose, there is a remarkable paucity of documented research in this area. In this chapter, we will aim to understand the effects of reducing glucose concentrations upon infection with EnAd in cancer cells that are dependent on glucose to fill the TCA cycle (e.g. SKOV3 cells) and cells which rely on aerobic glycolysis (e.g. A549 cells).

5.2 Chapter Aims

1. Analyse the effect of reducing glucose concentrations on the adenovirus lifecycle in cancer cells.
2. Analyse the effects of short-term versus long-term glucose starvation on the adenovirus lifecycle in cancer cells.
3. Analyse the effect of inhibiting glycolysis during adenovirus infection in cancer cell lines and *ex vivo* cancer cells.

5.3 Results

5.3.1 Removal of glucose in highly permissive A549 cells during adenovirus infection

The physiological average blood concentration of glucose in humans is around 6 mM. Solid cancers typically have poor circulation and leaky vasculature which, alongside an increased glucose uptake seen in many solid cancer cells, often means that the glucose concentration inside solid tumours can be as low as 0.1 - 0.4 mM (Hirayama *et al.* 2009). Typically, a blood glucose concentration of above 7 mM is considered to be a sign of hyperglycaemia (Giugliano, Ceriello and Esposito 2008; Association 2010). Given these concentrations, it is quite remarkable to note that a large proportion of cell culture medium preparations for experiments conducted *in vitro* contain glucose at a concentration of 25 mM.

We first aimed to understand how the removal of glucose from host cells infected with the oncolytic chimeric adenovirus enadenotucirev (EnAd) would affect the virus lifecycle. Initially, we chose to evaluate the expression of early and late viral gene constructs in media containing no glucose and in complete media containing 25 mM glucose, by using the reporter viruses harbouring green fluorescent protein (GFP) transgenes described in chapter 4.

A549 cells were infected with 100 PPC (approximately equating to an MOI of 1). At 90 minutes post-infection, virus inoculum was removed and replaced with medium containing either 25 mM glucose or 0 mM glucose for 48 hours. Analysis with a fluorescent microscope revealed that removal of glucose after infection markedly reduced the amount of fluorescence observed in A549 cells post-infection with both EnAd-CMV-GFP (**figure 5.1**).

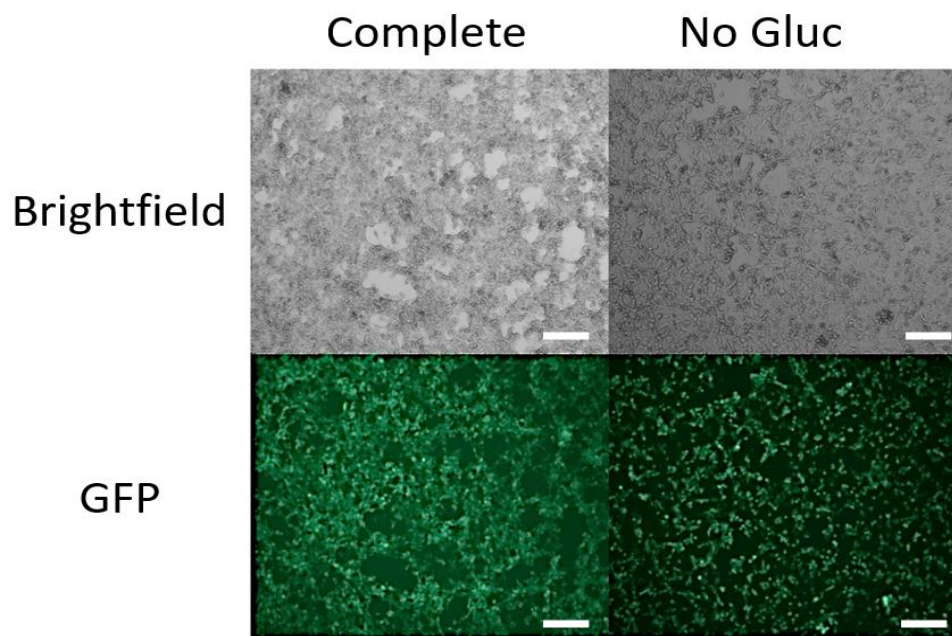


Figure 5.1 Removal of glucose from A549 cells infected with EnAd decreases viral protein synthesis.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 25 mM glucose (**complete – left-hand panel**) or without glucose (**No Gluc – right-hand panel**). 2 days post-infection, cells were imaged using a Nikon DS-U2 imaging microscope. The images are representative of biological triplicates. The scale bar represents 1000 μ M.

To quantify this observed decrease in fluorescence, we analysed the percentage of cells positive for GFP and the mean fluorescence of the population using flow cytometry. We observed that this reduction in fluorescence corresponded with a statistical decrease in both the mean fluorescence (**figure 5.2 A**), and percentage of cells positive for GFP (**figure 5.2 B**) when A549 cells were infected with EnAd-CMV-GFP. In all these cases, however, the reduction was much less than observed when glutamine was removed in similar experiments documented in chapter 4 with a reduction of around half for mean fluorescence and approximately 10% for the percentage of cells which were EnAd-CMV-GFP positive. A similar trend of reduction was observed with A549 cells infected with EnAd-SA-GFP (**figures 5.2 C & D**) with statistically significant declines in fluorescence, although milder than observed in glutamine withdrawal (chapter 4).

To confirm if the reduction in viral protein expression also correlated with a decrease in infectious viral progeny, we conducted a TCID₅₀ experiment with the media of A549 cells infected with or without glucose for 72 hours. Removal of glucose after infection resulted in a fall in infectious viral progeny of over 10-fold (from 3×10^{12} to 3×10^{11} FFU per mL). This reduction was statistically significant but much less severe than when cells were starved of glutamine in chapter 4.

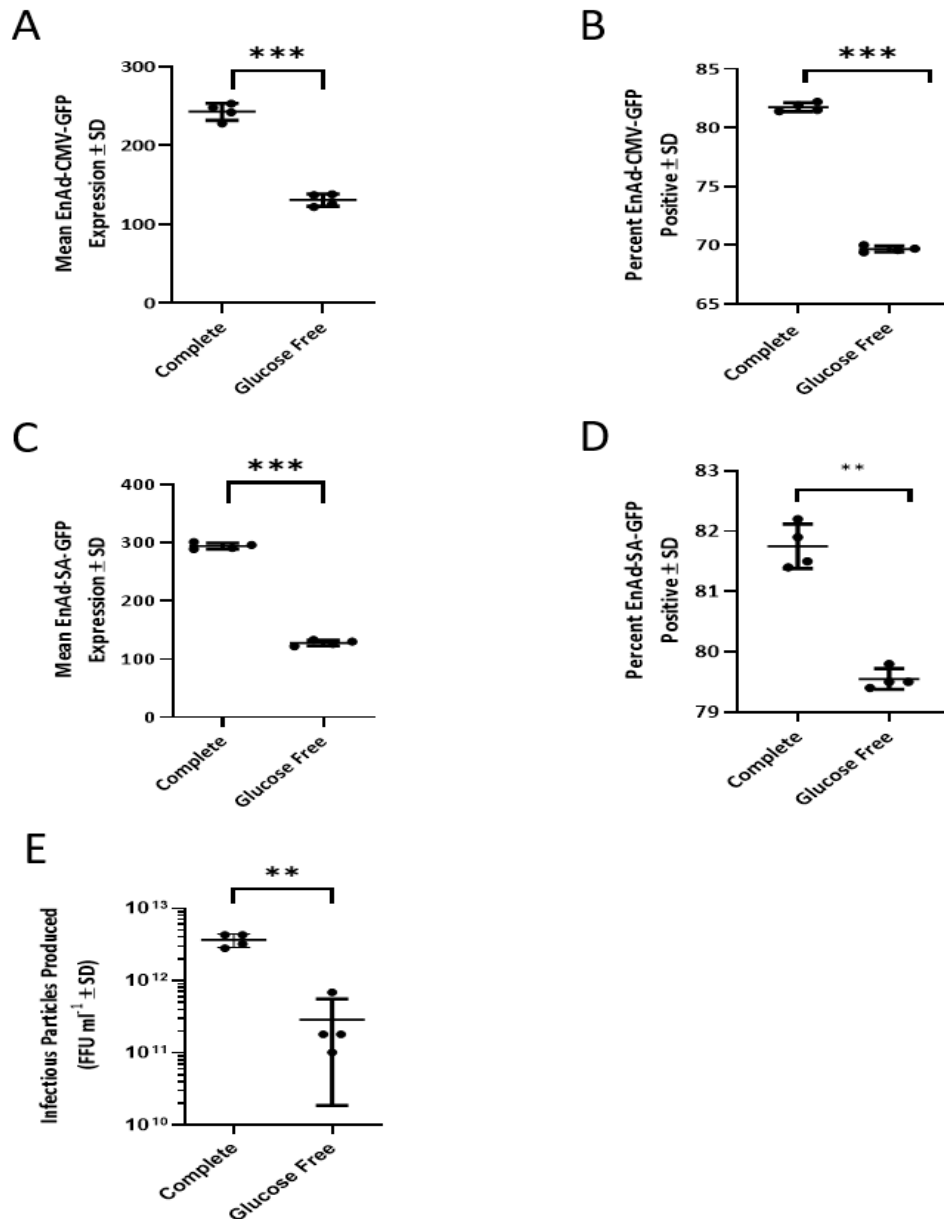


Figure 5.2 Removal of glucose from A549 cells infected with EnAd decreases viral protein synthesis and infectious viral progeny.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis – figures **A**, **B** and **E**) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis – figures **C** and **D**). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 25 mM glucose (Complete) or without glucose (Glucose Free). 2 days post-infection cells were detached and analysed for GFP expression via flow cytometry (**A-D**) or infectious particles were analysed via a TCID₅₀ assay (**E**). Figures **A** and **C** represent analysis of the mean GFP expression for each condition. Figures **B** and **D** represent the percentage of cells under each condition that are positive for GFP. Data shown represent the mean $\pm$ SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

5.3.2 Removal of glucose in highly permissive A549 cells before adenovirus infection

It has been reported that cancer cells are unable to respond to protective signals generated by fasting. Indeed, aberrant growth factor signalling is considered a key hallmark of cancer cells (Hanahan and Weinberg 2000, 2011). Therefore, malignant cells are overly vulnerable to removal of energy sources when compared to non-malignant cells. This phenomenon is referred to as the differential stress resistance (DSR). Short-term starvation (STS) is defined as fasting for 48 hours and is reported to cause a switch of normal cells to a protective mode, whereas tumour cells become particularly vulnerable to various anti-cancer treatments including chemotherapy and radiotherapy (Raffaghello *et al.* 2008; Lee *et al.* 2012).

There have been a limited number of studies investigating the effects of restricting nutrients or fasting on oncolytic virotherapy that have all shown promising results. The first such paper written by Esaki *et al.* showed that transient fasting improved the replication of oncolytic herpes virus in glioblastoma models with striking results shown both *in vitro* and *in vivo* (Esaki *et al.* 2016). In 2019, Scheubeck *et al.* reported that low-glucose, low serum starvation has the potential to synergistically enhance measles virus-mediated oncolysis efficacy in HT-29 cells (Scheubeck *et al.* 2019).

To investigate if a short-term starvation could improve the EnAd lifecycle and to mitigate the cellular shock of viral infection occurring at the same time as glucose withdrawal, we starved A549 cells of glucose for 48 hours prior to infection with EnAd. Surprisingly, A549 cells allowed to acclimatise to 0 mM glucose for 48 hours before infection showed elevated virus transgene expression as analysed via fluorescent microscopy (**figure 5.3**).

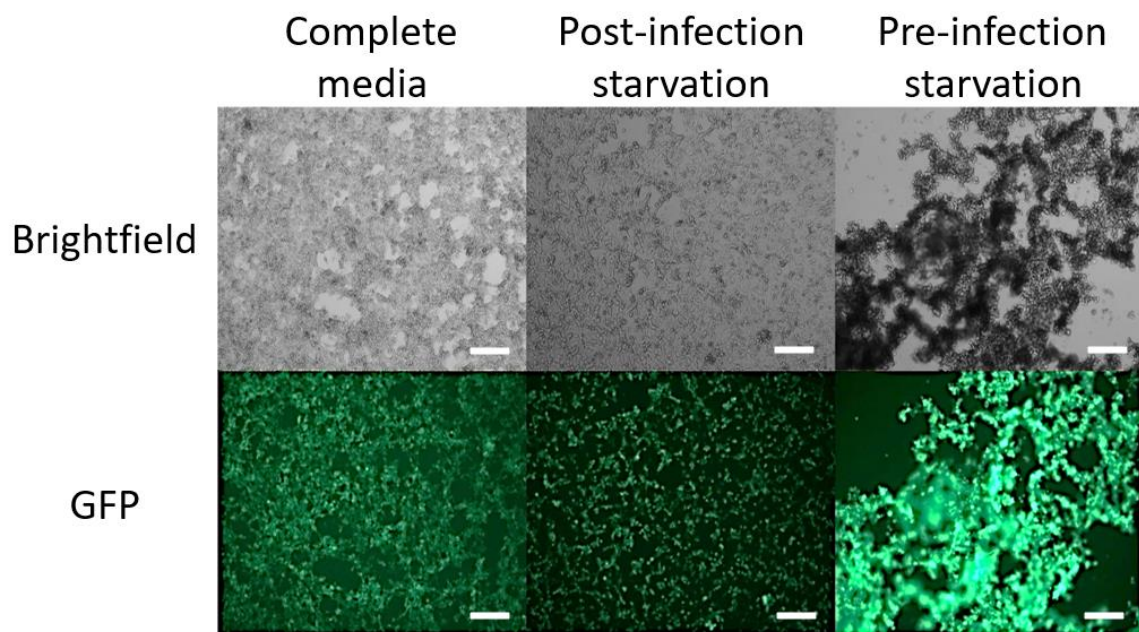


Figure 5.3 Long term removal of glucose from A549 cells infected with EnAd increases viral protein synthesis.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP, representing early viral protein synthesis). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 25 mM glucose (**Complete media – left-hand panel**) or without glucose (**Post-infection starvation – central panel**) For pre-infection starvation conditions, glucose was removed 48 hours before infection (**pre-infection starvation – right hand panel**). 2 days post-infection, cells were imaged using a Nikon DS-U2 imaging microscope. The images are representative of biological triplicates with the same cells pictured using brightfield (**top images**) and fluorescence (**bottom images**). The scale bar represents 1000 μ M.

We quantified the fluorescent signals using flow cytometry, removal of glucose for 48 hours before infection caused a significant increase in viral protein expression in A549 cells infected with EnAd-CMV-GFP when observing the mean fluorescence at 48 hours post-infection (**figure 5.4 A**). We did not observe an increase in the percentage of GFP positive cells, but the cells did not decrease their fluorescence in opposition to cells which are starved immediately after virus infection (**figure 5.4 B**). Similar results were observed for A549 cells infected with EnAd-SA-GFP following glucose starvation for 48 hours showing increased mean fluorescence (**figure 5.4 C**) and an increase in the percentage of cells expressing GFP (**figure 5.4 D**). The increase in viral protein expression was matched with a significant increase in infectious viral progeny particles (**figure 5.4 E**). These results are particularly interesting as A549 cells are very highly permissive to EnAd lysis, so any increase in viral protein expression is difficult to achieve.

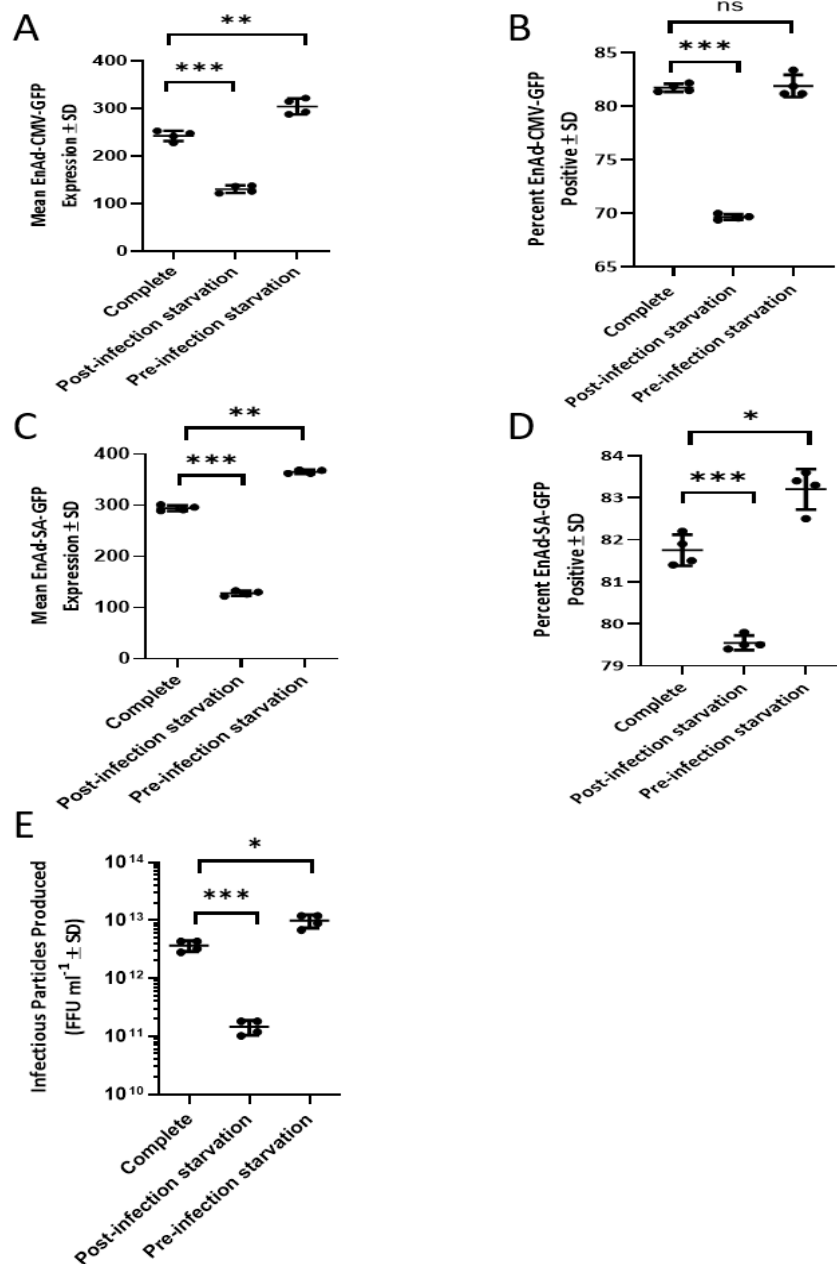


Figure 5.4 Long term removal of glucose from A549 cells infected with EnAd increases viral protein synthesis and infectious viral progeny.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis - figures **A** and **B**) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis – figure **C** and **D**). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 25 mM glucose (complete) or without glucose (Post-infection starvation). For cells labelled pre-infection starvation, glucose was removed 48 hours before infection. Two days post-infection cells were detached and analysed for GFP expression via flow cytometry (**A-D**) or infectious particles were analysed via a TCID₅₀ assay (**E**). Figures **A** and **C** represent analysis of the mean GFP expression for each condition. Figures **B** and **D** represent the percentage of cells under each condition that are positive for GFP. Data shown represent the mean $\pm$ SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Because *in vivo* concentrations of 25 mM or 0 mM glucose are unlikely to be encountered, we next sought to repeat the above data with varying concentrations of glucose between 0 mM and 25 mM. Post-infection removal of glucose (i.e. 90 minutes post-infection) caused a decline in mean viral protein synthesis only when concentrations reached 10 mM for GFP expressed under the control of CMV and 5 mM for EnAd-SA-GFP (**figures 5.5 A & B**). When cells were starved for 48 hours prior to infection, the inverse was seen with GFP expression increasing at the same concentrations of glucose starvation (**figures 5.5 C & D**). Interestingly, although both GFP-expressing viruses increased their GFP protein expression when starved for 48 hours, CMV-GFP expression peaked in 5 mM glucose. In contrast, SA-GFP increased up to 20 mM glucose. This suggests that early viral expression may rely somewhat upon glucose, although they can be expressed in the absence of glucose. However, late-stage viral proteins do not require glucose to be expressed and prosper when it is removed (**figure 5.5**). Again, because A549 cells are highly permissive to viral infection, any changes in the percentage of cells expressing GFP were modest.

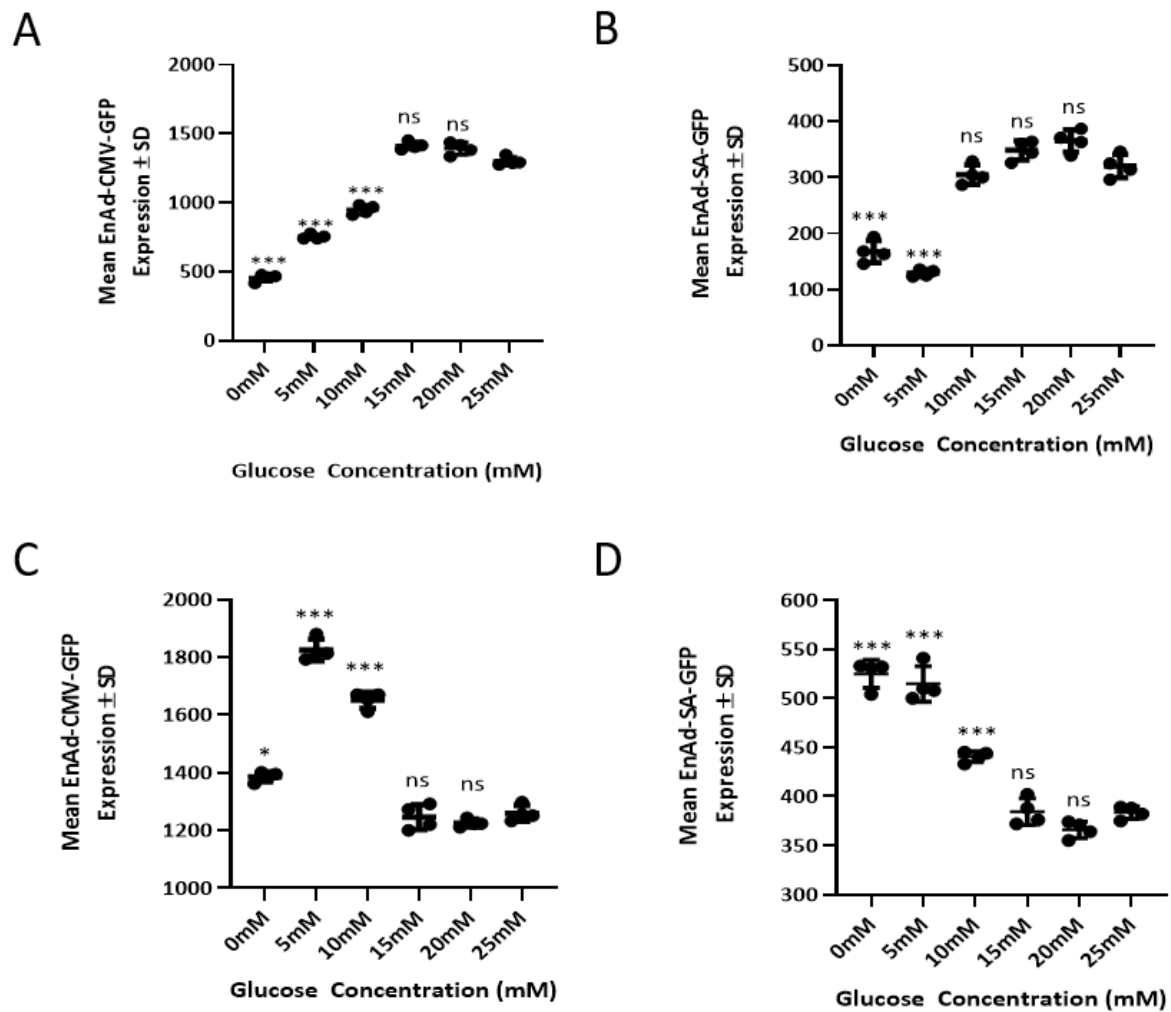


Figure 5.5 Removal of glucose from A549 cells 48 hours prior to infection with EnAd increases viral protein synthesis.

A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis – figures **A** and **C**) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis – figures **B** and **D**). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing various levels of glucose. 48 hours post-infection, cells were detached and analysed for fluorescence using flow cytometry. (**A** and **B**) represent cells starved 90 minutes post-infection. (**C** and **D**) represent cells treated with the stated concentration of glucose for 48 hours before infection until the point of measuring protein expression. All graphs denote the mean GFP expression. Data shown represent the mean ± SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

5.3.3 Inhibition of glycolysis in highly permissive A549 cells

Glucose is present in high levels within cell culture medium because, in static cell culture environments, maintaining lower (more realistic) levels of glucose would result in a need to frequently change the media, which is not practical. Likewise, restricting calories or specific metabolites from patients *in vivo*, whilst a promising option, is not always humane or easy to adhere to; and so a druggable target or a genetic target provide attractive alternatives when seeking to create a preferential metabolic microenvironment to support and improve viral oncolysis.

To further establish that glycolysis is non-essential to the successful EnAd life cycle, the competitive inhibitor of glucose-6-phosphate isomerase, 2-deoxy-D-glucose (2DG), was used to inhibit glycolysis. To initially analyse if the presence of 2DG inhibited glycolytic flux, we analysed lactate secretion, glucose uptake, and glutamine uptake in A549 cells treated with 2DG as previous research has stated that the presence of 2DG increases both glucose and glutamine uptake (Aft, Zhang and Gius 2002). The treatment of A549 cells with 2DG increased the basal glucose uptake cells as previously reported, decreased lactate secretion, and increased the basal glutamine uptake (**figure 5.6**) confirming that, at the doses used within this study, 2DG effectively inhibits glycolysis.

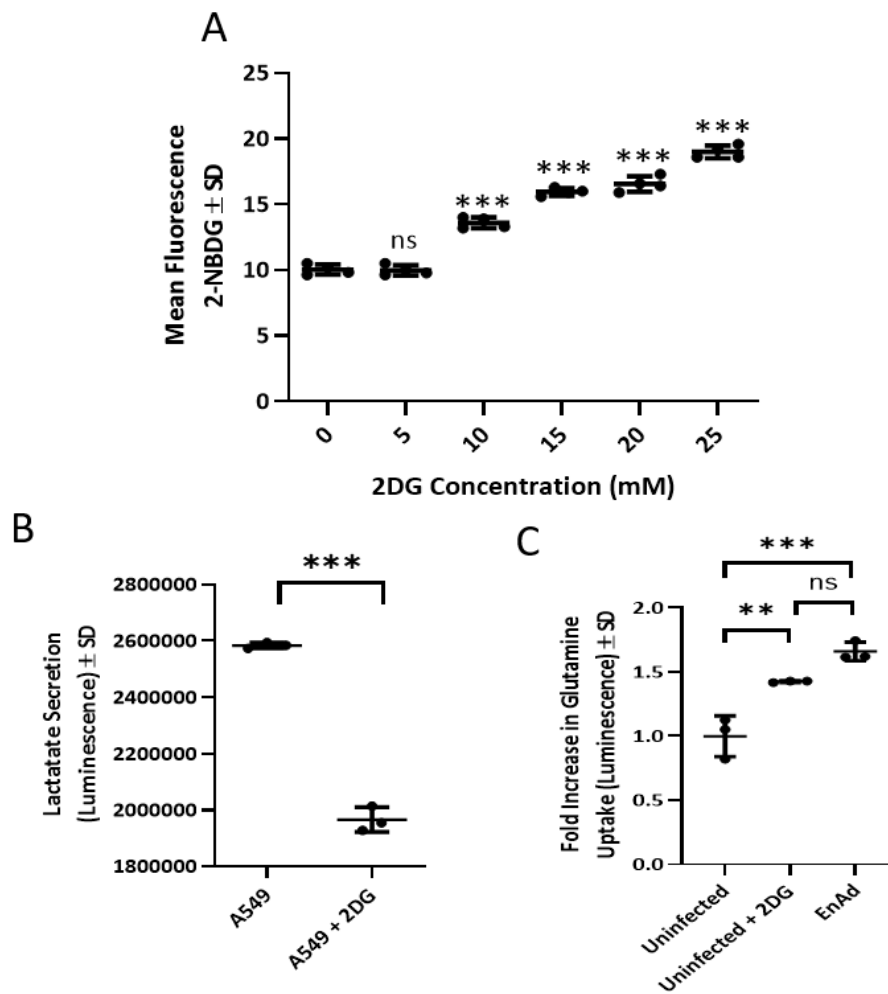


Figure 5.6 2-deoxy-D-glucose (2DG) inhibits glycolysis in A549 cells.

A549 cells were treated with 2DG for 48 hours, after which time metabolic responses were analysed. **(A)** Glucose uptake in A549 cells was analysed through exposure to 25 mM 2-NBDG during 2DG treatment and analysis via flow cytometry. **(B)** Lactate secretion of A549 cells treated with 15 mM 2DG for 48 hours, measured via lactate-Glo (Promega). **(C)** Fold changes in glutamine uptake of A549 cells 48 hours post-treatment with 15 mM 2DG or EnAd at an MOI of 1 measured using glutamine-Glo (Promega). Data shown represent the mean $\pm$ SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Virus activity in cells treated with 2DG was probed using EnAd-CMV-GFP and EnAd-SA-GFP. Low concentrations of 2DG (up to 10 mM) had little effect on the mean of GFP-positive cells (**figure 5.7 A**) or the percentage of GFP-positive cells using EnAd-CMV-GFP (**figure 5.7 B**). The highest concentration of 2DG used (20 mM) decreased both the percentage of cells positive for GFP and the signal intensity for EnAd-CMV-GFP infected A549 cells.

EnAd-SA-GFP showed similar effects of 2DG on the frequency of infection (**figure 5.7 D**); however, the intensity of GFP expression was enhanced, in a dose-dependent fashion, reaching at least twice the level of control cells at 5 mM 2DG and above (**figure 5.7 C**), again suggesting that antagonism of glycolysis might have a positive effect on the activity of the MLP.

We also assessed the effects of 2DG on virus genome replication and production of infectious viruses. The amount of daughter genomes produced by EnAd-infected A549 cells in the presence of 2DG decreased from 7×10^{11} to 2×10^{11} in a dose-dependent manner (**figure 5.7 E**). However, this decrease was not matched in the number of infectious virus progeny produced, which were largely unaffected (**figure 5.7 F**).

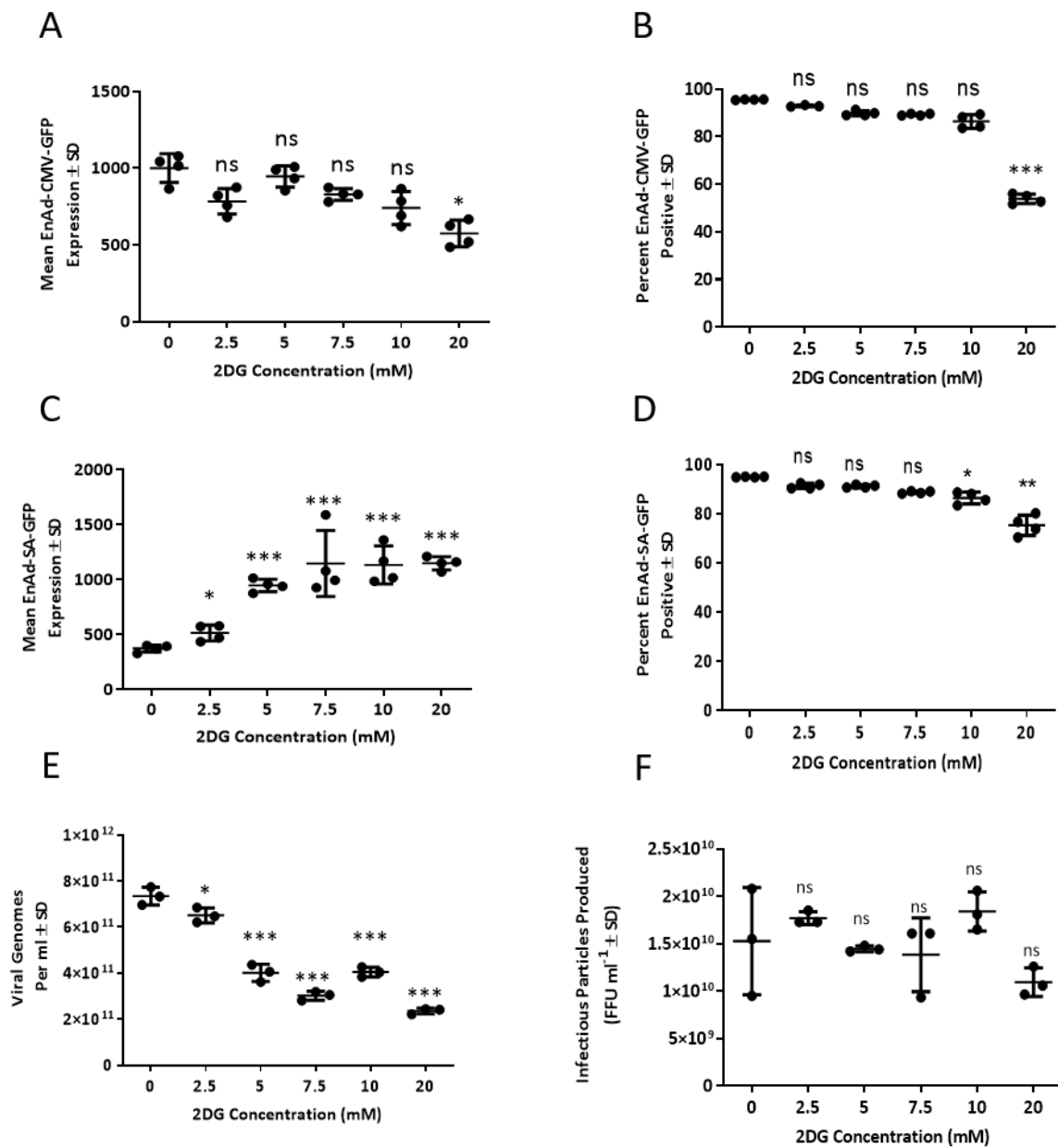


Figure 5.7 Inhibition of glycolysis during EnAd infection in highly permissive A549 cells. A549 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis – figures A and B) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis – figures C and D). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 2DG. 96 hours post-infection cells were detached and analysed for protein expression via flow cytometry (A–D), viral genomes via real-time qPCR (E), or infectious viral progeny via TCID₅₀ (F). Figures A and C represent mean GFP expression. Figures B and D represent the percentage of GFP positive cells. Data shown represent the mean $\pm$ SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

We next analysed the efficiency of virus genome packaging by comparing the amount of viral DNA to the number of infectious particles to give the ratio of viral genomes produced to infectious particles. If all the genomes produced are packaged then the ratio of viral genomes to infectious particles will be close to or equal to one, if however, an excess of viral genomes is produced compared to infectious particles then the ratio will increase to greater than 1. Increasing concentrations of 2DG gave fewer unpackaged virus genomes, suggesting greater efficiency of genome packaging (**figure 5.8**). These results show that, although viral genome numbers are reduced in EnAd-infected A549 cells treated with 2DG, the late-stage packaging protein expression is increased, justifying the lack of a statistical decline in infectious progeny produced.

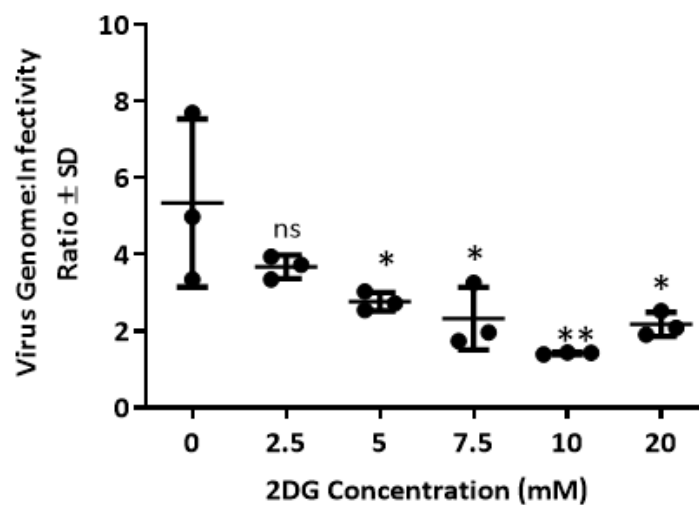


Figure 5.8 Inhibition of glycolysis during EnAd infection lowers the viral genome to infectious particle ration in A549 cells.

A549 cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 2DG. 96 hours post-infection cells were detached and analysed for viral genomes via real-time qPCR (**figure 5.6 E**), or infectious viral progeny via TCID₅₀ (**figure 5.6 F**) and a ratio of the two was calculated. Data shown represent the mean ± SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Light microscopy images of EnAd-infected A549 cells at the point of death (4 days post-infection) show that, in the presence of 2DG, cells exhibited the characteristic formation of single-membrane blisters that are typically observed when cells are killed by EnAd-mediated oncosis as previously reported by our group (Dyer *et al.* 2017). The presence of this unique form of cell death indicates that cell death in the presence of 2DG is not due to any toxicity of 2DG but is likely due to active killing by the virus (**figure 5.9**).

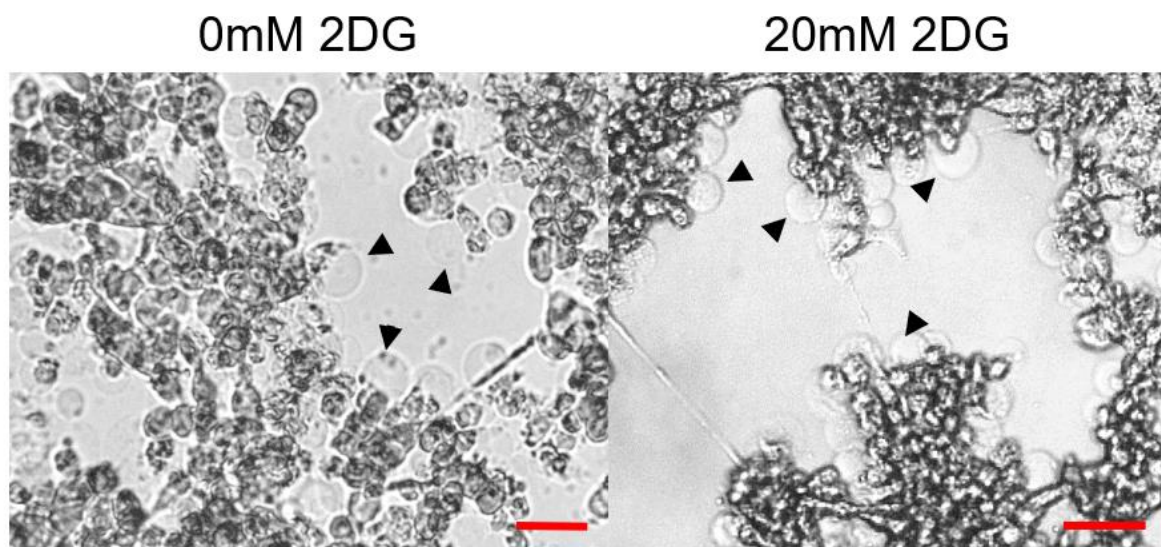


Figure 5.9 A549 cell morphology during infection with EnAd with or without the presence of 2DG. A549 cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing mock treatment (**0mM 2DG left-hand panel**) or 2DG (**20mM 2DG right-hand panel**). 96 hours post-infection cells were imaged using a Nikon DS-U2 imaging microscope. The black arrowheads are examples of EnAd-associated single membrane blistering. The images are representative of biological triplicates. The scale bar represents 100 μ M.

The effects of glycolysis inhibition on virus cytotoxicity were measured in real-time in A549 cells using the xCELLigence system, giving a real-time analysis of cell viability via measuring impedance. 2DG treatment alone was not cytotoxic up to 25 mM and, in fact, increased the cell index (a measurement for cell viability) in a dose-dependent manner (**figure 5.10**). A549 cells infected with EnAd at an MOI of 1 showed the onset of cytotoxicity from 60 hours after seeding, which equated to 36 hours post-infection and showed 100% toxicity at 72 hours post-infection (**figure 5.10**). EnAd infected A549 cells treated with 2DG showed the onset of viral cytotoxicity at approximately 10 hours earlier than cells infected with EnAd alone and reached 100% cytotoxicity at the same point as untreated A549 cells infected with EnAd (**figure 5.10**). These results when considered together indicate that the viral lifecycle including protein synthesis, genome replication and cell lysis mechanisms are not largely altered by restricting glycolysis and, in keeping with the results presented with short-term starvation, these results indicate that glycolysis is not an essential pathway for viral replication in A549 cells.

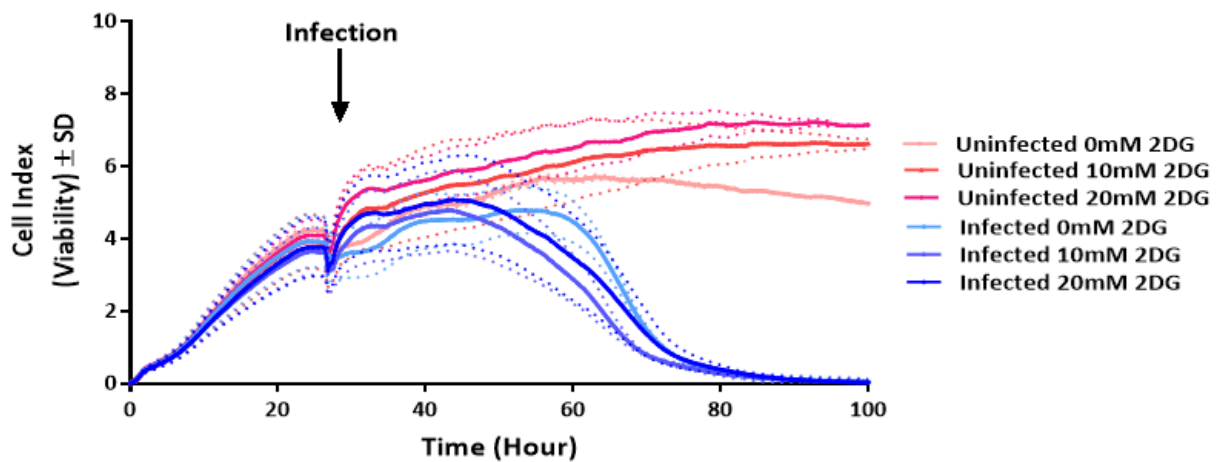


Figure 5.10 Real-time cytotoxicity of A549 cells during infection with EnAd with or without the presence of 2DG.

xCELLigence analysis of A549 cells infected with EnAd at an MOI of 1. Cell index (a measure of cell viability) was measured every 10 minutes for 100 hours. Cells were plated at 0 hours and were infected at approximately 24 hours post-seeding as indicated on the graph. Data shown represent the mean $\pm$ SD of biological triplicates.

5.3.4 Inhibition of glycolysis in less permissive SKOV3 cells

In chapter 3, we show that the IC_{50} of A549 infected with EnAd is relatively low (0.3 viral particles per cell), meaning that these cells are already highly susceptible to lysis by EnAd. Because of this high viral permissivity, any improvement in viral oncolysis will be modest. Also, because in A549 cells glucose is utilised via aerobic glycolysis and not the fuel the TCA cycle, inhibiting glycolysis will have a markedly different effect than if one were to inhibit glycolysis in a glucose-dependent cell line. For these reasons, we next examined the impact of inhibiting glycolysis in the relatively poorly permissive cell line SKOV3.

The presence of 2DG showed a statistically significant increase in the percentage of EnAd-CMV-GFP positive SKOV3 cells only at the highest dose at 144 hours post-infection (**figure 5.11 A**). The peak of mean GFP expression occurred at 144 hours post-infection followed by a decline as opposed to a gradual rise in mean GFP expression in untreated conditions (**figure 5.11 B**). The mean fluorescence at the highest point recorded is over two times higher than untreated cells in cells treated with 20 mM 2DG (**figure 5.11 B**).

SKOV3 cells infected with EnAd-SA-GFP showed a significant increase in the percentage of cells expressing GFP with treatment of 2DG (**figure 5.11 C**). We again observed an accelerated peak of mean GFP expression (**figure 5.11 D**).

To investigate whether the virus life cycle is shortened in SKOV3 cells by 2DG, cell growth and death profiles were assessed in real-time using the xCELLigence system. In mock-infected cells (**figure 5.11 E**), cell growth was slowed by increasing doses of 2DG, although no toxicity was observed even at the highest dose. In cells infected with EnAd without 2DG, cytotoxicity began at roughly 170 h post-seeding (approximately five days post-infection), and the presence of 2DG accelerated the onset of cytotoxicity, with 5 mM 2DG causing cytotoxicity at just three days post-infection (**figure 5.11 F**). These data, therefore, indicate that inhibition of glycolysis using 2DG can accelerate production of late virus proteins, shorten the virus life cycle, and accelerate the onset of cytotoxicity.

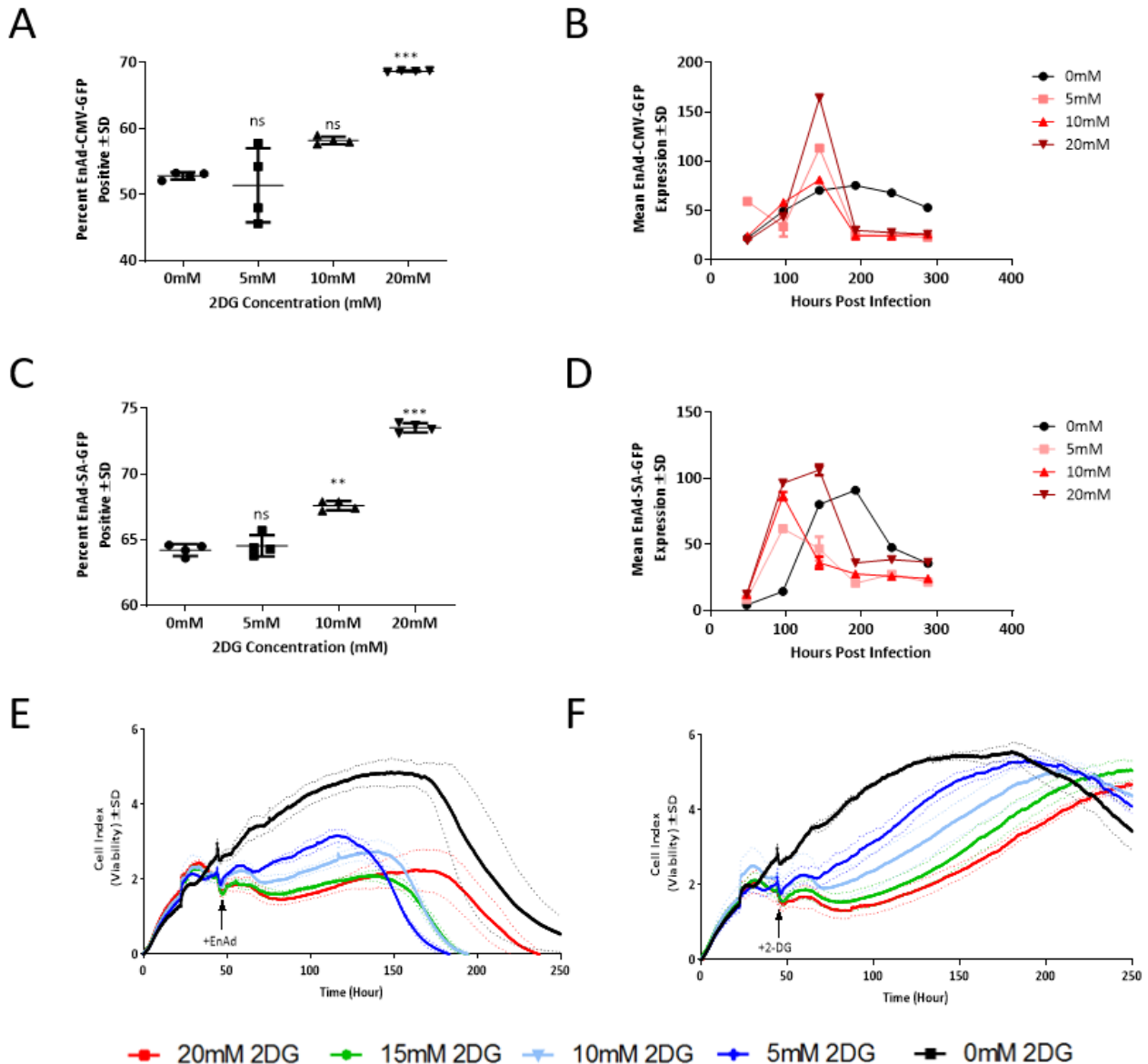


Figure 5.11 Inhibition of glycolysis during EnAd infection in less permissive SKOV3 cells.

(A-D) SKOV3 cells infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis - figures A and B) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis - figures C and D). Cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 2DG. (A) represents cells at 144 hours post-infection denoting the percentage of cells which are GFP positive and (C) represents cells at 192 hours post-infection, again denoting the percentage of GFP positive cells. Figures B and D represent mean GFP expression. (E and F) xCELLigence analysis of SKOV3 cells infected with EnAd at an MOI of 1. Cell index (a measure of cell viability) was measured every 10 minutes for 250 hours. Cells were plated at 0 hours and were infected at approximately 48 hours post-seeding as indicated on the graph with or without 2DG. (E) Represents infected cells, and (F) represents uninfected cells. Data shown represent the mean $\pm$ SD of biological quadruplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

Measured at five days post-infection, analysis of infectious viral progeny production revealed an increase of 3-fold when 2DG was present in any dose (**figure 5.12 A**). The total number of EnAd genomes (measured by real-time qPCR) increased from 1×10^{11} genomes per mL to around 1×10^{12} genomes per mL with the greatest effect observed after treatment with 5 mM 2DG (**figure 5.12 B**), similar to the impact on accelerating cytotoxicity.

These data show that in A549 cells, where viral lysis and genome production occurs rapidly, inhibition of glycolysis causes a modest increase in the speed of kill, the number of viral genomes and the number of infectious particles produced. However, in SKOV3 cells, where viral lysis and replication of viral genomes occurs slowly, inhibition of glycolysis dramatically shortens the virus life cycle, accelerates cytotoxicity, and greatly increases the number of infectious particles produced.

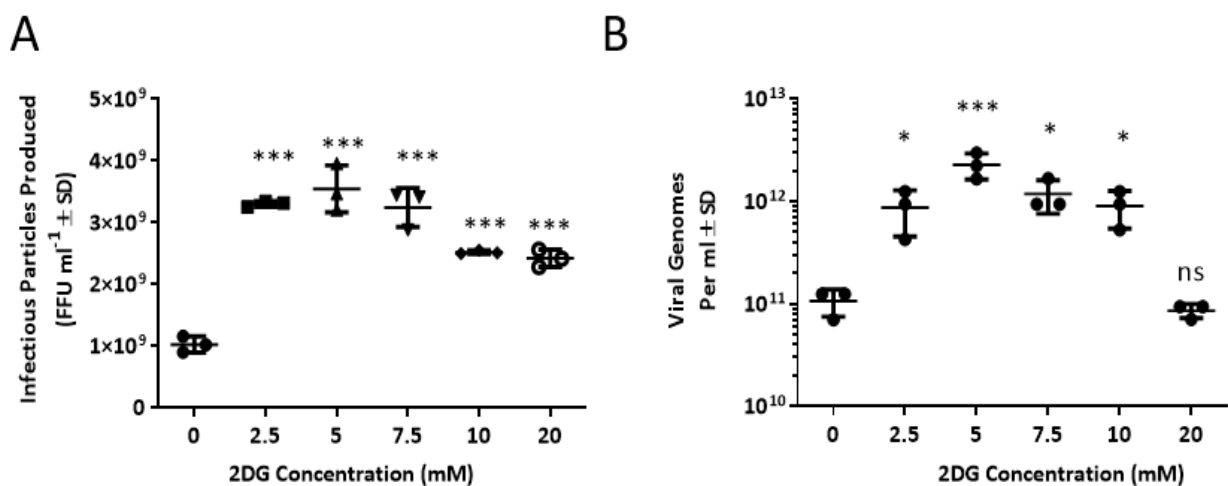


Figure 5.12 Inhibition of glycolysis during EnAd infection in less permissive SKOV3 cells. SKOV3 cells were infected with EnAd at an MOI of 1 and, 90 minutes post-infection, the inoculum was removed and replaced with DMEM medium containing 2DG at the listed concentrations. 120 hours post-infection cells were collected and analysed for viral genomes via real-time qPCR (A) or infectious viral progeny via TCID₅₀ (B). Data shown represent the mean ± SD of biological triplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

5.3.5 Assessing the activity of enadenotucirev in *Ex vivo* samples treated with 2-deoxy-d-glucose

Malignant ascites samples were acquired from the Churchill Hospital, Oxford, UK, following routine drainage of patients with advanced metastatic cancer and after informed consent and ethical approval. Ascites cells were separated from the fluid fraction by centrifugation, then subjected to red blood cell lysis. Characterisation of the cell types present within each sample was performed by flow cytometry, staining with antibodies recognising T cells (via CD4 and CD8), tumour cells (via EpCAM), fibroblasts (via FAP) and myeloid cells (via CD11b (Scott *et al.* 2019)). Since we are interested in studying replication and oncolysis within cancer cells, we selected a sample with the highest positivity for EpCAM (69% EpCAM positive).

Ascites samples were infected with 500 EnAd-SA-GFP particles per cell (roughly an MOI of 5) in DMEM media, and 90 minutes later, the inoculum was removed and replaced with DMEM containing either 0, 5, 10, or 20 mM of 2DG. Seven days post-infection, late viral protein synthesis was analysed using flow cytometry. In agreement with previous results using cancer cell lines, treatment of ascites cells with 2DG resulted in a significant increase in the percentage of GFP positive cells (**figure 5.13**). RT-qPCR analysis of viral genome replication at seven days post-infection shows a doubling in viral genomes produced in ascites cells treated with 20 mM 2DG. These results suggest that inhibition of glycolysis boosts virus activity in primary cancer cells as well as in cell lines.

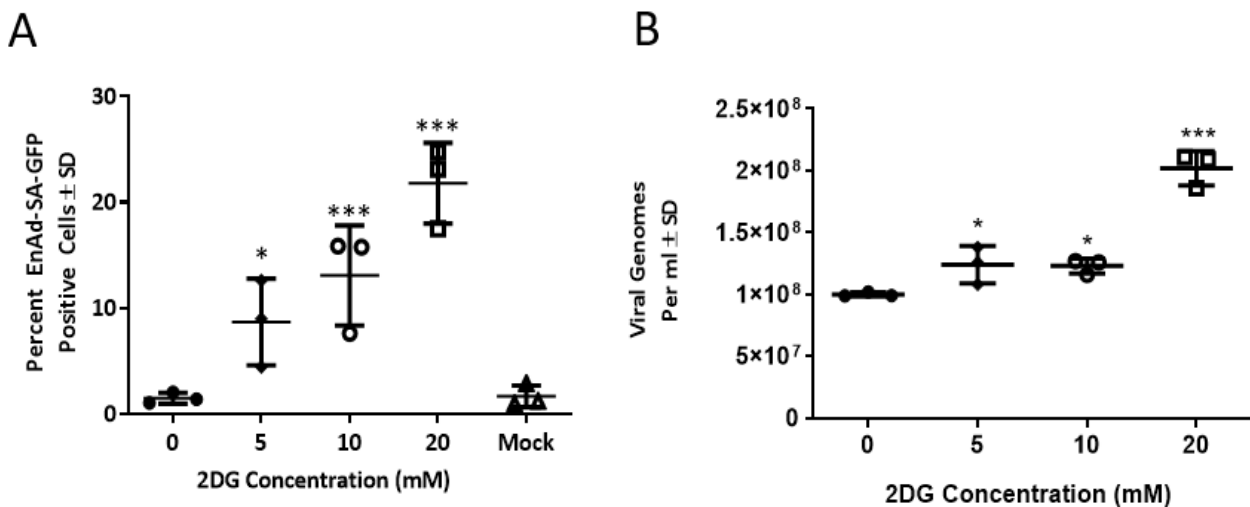


Figure 5.13 Inhibition of glycolysis during EnAd infection *ex vivo* malignant ascites cells.

Malignant ascites samples were collected, and cells were separated from the ascites fluid. Cells were left to adhere overnight, and any dead or floating cells were removed, leaving only adherent cells (mainly cancer cells). These cells were infected with EnAd expressing GFP under the control of the major late promoter (EnAd-SA-GFP) at an MOI of 5 and, 90 minutes post-infection, the inoculum was removed and replaced with RPMI medium containing 2DG at the listed concentrations. Seven days post-infection, cells were collected and analysed for viral protein synthesis (**A**) via flow cytometry or viral genomes via real-time qPCR (**B**). Data shown represent the mean ± SD of biological triplets. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

5.4 Chapter Discussion

In this chapter, we sought to probe the third of our stated hypotheses from section 1.8.2, namely that removal of glucose from the media will inhibit EnAd oncolytic activity. As viruses are obligate intracellular pathogens, they need to hijack cellular processes to create a favourable environment for efficient production of infectious progeny. In many ways, their demands for macromolecular synthesis appear similar to those of a cancer cell. In chapter three, we showed that cancer cells infected with the oncolytic adenovirus EnAd show a metabolic phenotype of increased aerobic glycolysis with an increased glucose uptake alongside an

increased glutamine uptake. We therefore hypothesised that glucose was required for optimal virus replication. We assessed this by removing glucose from A549 cells infected with EnAd, and observed a diminishing viral protein expression alongside decreased infectious viral protein progeny which bolstered our hypothesis (**figures 5.1 and 5.2**).

There is an increasing body of scientific literature in both pre-clinical and clinical settings showing that fasting can sensitise malignant cells to cancer treatments whilst simultaneously protecting non-malignant cells in a phenomenon that is referred to as the differential stress response (DSR; (Lee *et al.* 2010)). Early reports indicate that triggering the DSR can be beneficial to numerous oncolytic viruses (Esaki *et al.* 2016; Scheubeck *et al.* 2019). We aimed to see if a similar effect could be seen in the case of our oncolytic virus by starving cells for 48 hours prior to infection with EnAd in keeping with protocols established by Esaki *et al.* (Esaki *et al.* 2016). We found a surprising result that short-term glucose starvation prior to infection with EnAd increased viral protein synthesis, genome production and infectious progeny production in A549 cells (**figures 5.3 – 5.5**).

Whilst removing glucose all together is a beneficial experimental model; we consider it unlikely that most cells in a solid tumour will experience 0 mM glucose for extended periods. To address this issue, we used a variety of concentrations of glucose from 25 mM (the concentration present in standard DMEM) to 0 mM

glucose. As can be seen in **figure 5.5**, removal of glucose 90 minutes post-infection is deleterious to the virus when glucose is in the range of 0 – 10 mM. This is particularly concerning as the normal concentration of glucose in the blood of healthy individuals is around 6 mM. In solid tumours, this concentration can fall to as low as 0.1 – 0.4 mM (Hirayama *et al.* 2009). Because our ultimate aim is in improving the outcome of treatment with EnAd, we have not performed glucose withdrawal experiments on wildtype viruses such as Ad11p and Ad5 during this thesis. The experiments addressing the withdrawal of glucose during or prior to wildtype adenovirus infection have not yet been conducted by us, nor by others in the field. For these reasons, it is not possible to assess whether this response is a result of EnAd being bioselected in highly glycolytic cells in high glucose medium (Kuhn *et al.* 2008) or if this is a feature of all adenoviruses and we hope that future research addresses this question but for our purposes.

In a clinical setting, although utilising the DSR is an attractive proposal, it would be difficult to ensure that cancer patients would restrict calories or completely avoid glucose for an extended period, and so we, therefore, sought to use a druggable target. 2-deoxy-D-glucose (2DG) is a glucose analogue that inhibits glycolysis by acting as a competitive inhibitor of glucose-6-phosphate isomerase. We treated A549 cells with 2DG and saw that even at high concentrations there was an increase in late-stage viral protein synthesis accompanied by a decrease in early protein synthesis only at the highest concentrations of 2DG (**figure 5.7**). There is

also a decline in viral genome synthesis seen in EnAd-infected A549 cells (**figure 5.7**). One of the alterations gained by EnAd during its creation is a chimeric E2B region which encodes for the viral DNA polymerase, these repeated chimeric alterations coupled with a smaller genome size lead to an increase in viral genome creation compared to wildtype adenoviruses (Kuhn *et al.* 2008) meaning that EnAd often creates up a tenfold increase in unpackaged viral genomes compared to those which are packaged. The increase in viral protein synthesis accompanied by the decrease in viral genome synthesis seen during treatment of EnAd-infected A549 cells with 2DG results in no significant change in infectious viral progeny (**figure 5.7**) and a decrease in the amount of unpackaged genomes produced (**figure 5.8**).

As we have previously shown, A549 cells are very permissive to viral oncolysis with EnAd; therefore, any improvement seen in the viral lifecycle will be minimal. Furthermore, since these cells are not using glucose to fill their TCA cycle, they are considered not to be “addicted” to glucose, and so altering glycolysis may not have as dramatic an effect upon these cells. Given this, we chose to investigate the impact of treating poorly permissive and glucose “addicted” SKOV3 cells with 2DG. 2DG treatment in EnAd-infected SKOV3 cells resulted in a more dramatic alteration to permissivity to EnAd in all measures (**figures 5.11 and 5.12**). These findings were further corroborated using an *ex vivo* model of malignant ascites cells. Under normal conditions, EnAd could infect ascites cells but did not produce late viral proteins as has been previously reported (Pokrovska *et al.* 2020). Upon

2DG treatment, EnAd achieved enhanced late viral protein and genome synthesis (**figure 5.13**). It is interesting to note that in papers published by other groups, altering c-Myc (often considered a “master regulator of cellular metabolism”) can affect wildtype Ad5 replication differently depending on how glycolytic the cell is bolstering our findings that EnAd behaves differently in highly glycolytic cells (Thai *et al.* 2014; Prusinkiewicz *et al.* 2020). We would encourage future studies to be conducted in a broader panel of cells using 2DG to truly analyse the effects of glycolysis inhibition upon viral infection with EnAd.

The findings that viral infection increases glycolysis, whereas inhibiting glycolysis improves the viral lifecycle, seem to be incongruous at first. However, we are not the first to report this surprising dichotomy. 2DG has been shown to enhance the viral lifecycle of many viruses including Newcastle disease virus, Kaposi’s sarcoma-associated herpesvirus, influenza, vaccinia virus, and reovirus, even though all these viruses are associated with an increase in glycolysis (Delgado *et al.* 2010; Fontaine, Camarda and Lagunoff 2014; Smallwood *et al.* 2017; Al-Shammari *et al.* 2019; Kennedy *et al.* 2019). Although all of the viruses stated above do benefit from glycolysis inhibition, this is not a universally observed benefit. 2DG can significantly hamper the replication and lifecycles of viruses such as rhinovirus, herpesviruses, papillomavirus, semliki forest virus, and dengue virus (Schmidt, Schwarz and Scholtissek 1976; Maehama *et al.* 1998; Leung *et al.* 2012; Fontaine *et al.* 2015; Gualdoni *et al.* 2018). For most of these cases, other than dengue virus and rhinovirus, the inhibition of viral replication is due to 2DG triggering

endoplasmic reticulum stress (ER stress). However, we have previously shown that ER stress can be beneficial to EnAd replication in a calcium-dependent manner and which we show can be overcome through treatment with intracellular calcium agonists and not through glycolysis altering treatments (Taverner *et al.* 2019).

Of particular note amongst the examples listed above is that of Newcastle Disease Virus (NDV). NDV is a zoonotic paramyxovirus which are enveloped viruses containing a single strand of negative-sense RNA. This virus is a promising candidate for oncolytic virotherapy and is being investigated by many groups. Upon infection of cells, NDV triggers an increase in glycolysis, glutaminolysis, the pentose phosphate pathway and lactate secretion (Liu *et al.* 2019a; Meng *et al.* 2020). Intriguingly, despite the observed increase in lactate secretion and glycolysis, multiple strategies to inhibit glycolysis have been reported to increase viral replication and progeny production whilst decreasing the acidosis usually associated with viral infection (Li *et al.* 2015a; Al-Shammari *et al.* 2019; Al-Ziaydi *et al.* 2020; Meng *et al.* 2020).

When considering an explanation for why inhibiting glycolysis with 2DG may improve EnAd oncolysis in SKOV3 cells we believe that a likely reason is that by inhibiting glycolysis we are increasing the cells dependency upon glutamine anapleurosis (**figure 5.6 C**), a phenomenon that has been documented by others in the past (Pusapati *et al.* 2016). We have shown that EnAd infection is critically reliant upon glutamine metabolism (chapter 4), that artificially increasing

glutamine metabolism can improve oncolysis (chapter 4), and that cells which do not rely upon glucose to fill their TCA cycle trend towards being more susceptible to oncolysis by EnAd (chapter 3). We believe that, taken in context, these results provide a rationale as to why 2DG improves oncolysis with EnAd. To further confirm these results, we believe that future work could be conducted using labelled glutamine and glucose to track the fate of both of these metabolites, although this is known for wildtype adenoviruses in normal cells (Thai *et al.* 2014, 2015). Additionally, whilst considering the role of metabolism within the context of conventional laboratory cell culture conditions might provide key insights into treatment targets, there is mounting evidence that culturing cells in more life-like conditions (such as hypoxic cultures, spheroid cultures, co-cultures with cancer-associated fibroblasts, or culturing in ascites media) can dramatically alter cancer cell behaviours, including metabolism (Potter, Newport and Morten 2016). For these reasons, we hope that future work investigating the role of metabolism in viral oncolysis will include investigations in these more challenging models.

Based on the results presented above, we can reject the hypothesis that removal of glucose from the media will inhibit EnAd oncolytic activity. We now propose a new hypothesis that EnAd can be armed with glycolytic inhibitors which will alter host cell metabolism and improve its oncolytic potential.

5.5 Chapter Conclusions

1. Removal of glucose after viral uptake in A549 cells inhibits EnAd protein expression and genome replication.
2. Short term starvation of glucose for 48 hours improves EnAd lifecycle A549 cells.
3. Treatment with 2DG does not significantly inhibit infection of EnAd in highly permissive A549 cells and improves late stage protein expression.
4. Treatment with 2DG highly improves the viral lifecycle of EnAd in poorly permissive SKOV3 cells and primary *ex vivo* ascites cancer cells.

6 Optimisation of metabolic environments that favour enadenotucirev replication.

6.1 Introduction

We have established in previous chapters that, contrary to our initial hypotheses, EnAd preferentially requires glutamine and not glucose for efficient replication within cancer cells. In addition, we have demonstrated that cancer cell lines which do not metabolise glucose oxidatively tend to be more permissive to EnAd oncolysis, and that inhibition of glycolysis can improve oncolysis with EnAd. In this final chapter, we will aim to unify the knowledge gained so far in this thesis to bolster these findings further, to improve treatment with EnAd in an *in vivo* setting and to create a next-generation oncolytic adenovirus that will alter the tumour microenvironment to favour replication of the virus and killing of cancer cells.

In chapter 5, we demonstrated that treatment of EnAd-infected SKOV3 cells with the glycolysis inhibitor 2DG could improve the amount of viral protein expressed, viral genome numbers, infectious viral particles produced and the speed of cytotoxicity. Treatment with 2DG in non-infected SKOV3 cells, however, is associated with some side effects, including a decrease in doubling time (**figure 5.11 F**), which could potentially be explained as a consequence of slowing the cell cycle. Because adenovirus infection is also known to deregulate the cell cycle

(perhaps by rerouting biosynthetic intermediates from cell growth to virus production), it was feasible that 2DG treatment or glucose restriction might boost virus activity through a similar mechanism (Ben-Israel and Kleinberger 2002). To determine if alterations to cell cycle represent a fundamental factor underpinning the improved virus activity, we built on a technique first developed by Pusapati *et al.* in 2016 (Pusapati *et al.* 2016).

Pusapati *et al.* show that cancer cell lines which rely upon oxidative glucose metabolism can become glucose independent through repeated culturing in increasing doses of 2DG. In many ways, the metabolism of these newly created cells is similar to the metabolic phenotype of an EnAd-infected cell. These newly created glycolysis independent cells showed over a 10-fold reduced 2DG sensitivity compared to the parental cell lines whilst displaying a comparable growth rate (Pusapati *et al.* 2016). This method of repeated culturing in 2DG to produce two cell lines with the same parental origin but differing metabolism provides a promising experimental model to test many of the hypotheses raised in this thesis so far whilst avoiding issues such as toxicity.

One benefit of using oncolytic viruses as a cancer platform is the concept of “arming” these viruses, meaning using the oncolytic virus to express a genetically engineered therapeutic transgene from the virus (Hermiston and Kuhn 2002; Seymour and Fisher 2016). The tactic of arming viruses is a particularly attractive

one as it allows us to provide a self-amplifying and highly selective expression of a chosen biologic for localised production, which maximises the local activity whilst allowing minimal systemic toxicity because the virus replicates solely in cancer cells. The main virus platform that our laboratory uses (enadenotucirev, EnAd) contains several deletions within its genome, allowing the insertion of transgenes without affecting viral replication. Also, because the virus life cycle of adenovirus is well understood, we can control when in the viral lifecycle, these transgenes are expressed. The linking of transgene expression to stages of the viral lifecycle is best displayed in the GFP-expression experiments conducted previously in this thesis.

To date, our laboratory has had a high level of success in expressing transgenes within this platform such as immunomodulatory biologicals such as bispecific antibodies or cytokines, imaging agents such as GFP or NIS, or microenvironmental-modulating agents such as DNases (Tedcastle *et al.* 2016; Freedman *et al.* 2017, 2018; Lei *et al.* 2018; Scott *et al.* 2019) some of which are beginning clinical trials. To date, however, we are only aware of limited research on oncolytic viruses that alter host cell metabolism. The most successful of which aims to improve immune cell responses to OV therapy through expression of leptin (Rivadeneira *et al.* 2019).

Because controlling metabolism is crucial to all living organisms, there is a wide choice of transcription factors, receptors, and enzymes which could be chosen to

adapt metabolism to the preferential phenotype that we desire (namely one where glucose is not used to fuel the TCA cycle, and glutaminolysis is increased). We have identified several possible genetic targets to control metabolism in **figure 6.1**. Despite their potential for improving viral replication, a number of promising options (such as P53, c-Myc, HIF1 α , AMPK and mTOR) have to be discounted due to off-target effects owing to a lack of specificity for altering glycolysis alone. With this in mind, we identified TP53-inducible glycolysis and apoptosis regulator (TIGAR) as being the most suitable candidate for expression within EnAd.

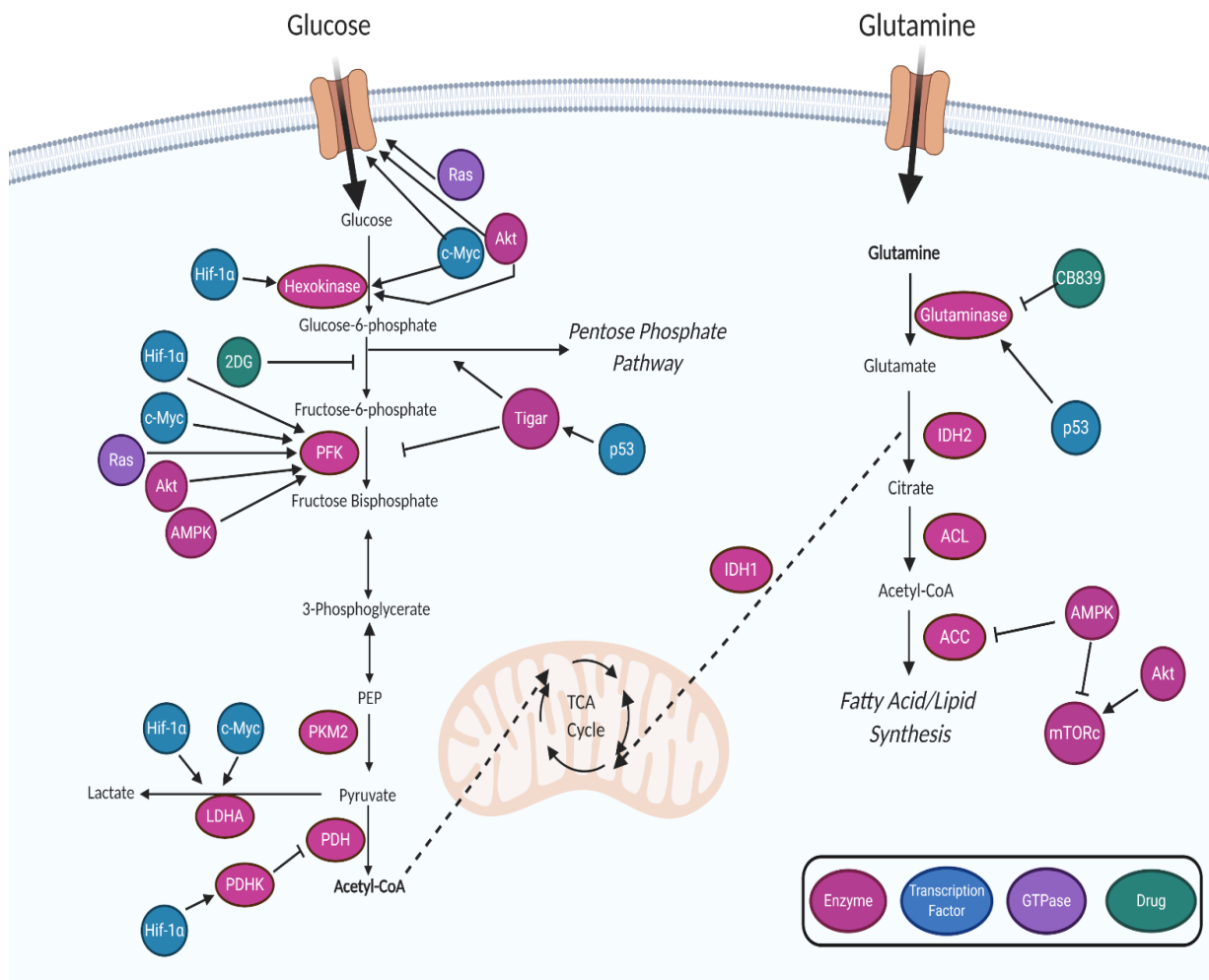


Figure 6.1 Cellular control of the Warburg effect. Simplified schematic representation of cellular factors that control the Warburg effect in mammalian cells.

Many cancer cells show an increase in aerobic glycolysis coupled with a dependency upon glutamine for anapleurosis (termed the Warburg effect). This effect is controlled through a variety of cellular factors, some of which are pictured above. TCA = tricarboxylic acid cycle (Kreb's cycle); 2DG = 2-deoxy-d-glucose; PFK = phosphofructokinase; PKM2 = pyruvate kinase M2; LDHA = lactate dehydrogenase A; PDH = pyruvate dehydrogenase; PDHK = pyruvate dehydrogenase kinase; IDH1/IDH2 = isocitrate dehydrogenase 1/2; ACL = ATP citrate lyase; ACC = Acetyl-CoA-carboxylase. Created with Biorender.com.

TIGAR was discovered by Jen and Cheung in 2005, and documented by Professor Karen Vousden's laboratory in 2006 as a regulator of glycolysis with particular importance in cancer (Jen and Cheung 2005; Bensaad *et al.* 2006). Since then, TIGAR has been characterised as a p53 inducible protein that is increasingly being

shown to be overexpressed in a wide range of cancers (Bensaad *et al.* 2006; Green and Chipuk 2006; Li and Jøgl 2009; Madan *et al.* 2011; Chu *et al.* 2020). When expressed in cells, TIGAR acts as a specific fructose-2,6-biphosphatase reducing levels of fructose-2-6-biphosphate (F-2,6-2P), leading to an inhibition of glycolysis. In cancer cells, expression of TIGAR also has the effect of redirecting glucose metabolism away from glycolysis whilst promoting the pentose phosphate pathways (PPP) which provides such cells with metabolic intermediates, nucleotides, and reductive power through the production of NADPH. Recent studies have shown that murine cancer models that overexpress TIGAR have an increased dependency upon glutamine metabolism and, because of the enforced lack of metabolic plasticity, are susceptible to dual treatments with glutaminase alongside other drugs such as chemotherapy (Chu *et al.* 2020). The finding that cells which have a forced dependency on glutaminolysis are susceptible to dual treatments in a way that glycolysis-dependent cancer cells are not, is mirrored by other researchers who found similar results with 2DG and provides a compelling rationale for expression of TIGAR from enadenotucirev (Pusapati *et al.* 2016).

6.2 Chapter Aims

1. Establish glycolysis independent SKOV3 lines.
2. Analyse the lifecycle of enadenotucirev in wildtype SKOV3 cells compared to glycolysis independent SKOV3 cells.

3. Analyse how treating xenograft mouse models with 2DG will affect viral genome replication and protein synthesis.
4. Generate enadenotucirev expressing TP53-inducible glycolysis and apoptosis regulator (TIGAR).
5. Establish that EnAd-CMV-TIGAR is functional, expresses TIGAR and is capable of lysing cells without modulating culture pH.

6.3 Results

6.3.1 Establishing glycolysis independent SKOV3 cells

SKOV3 cells have previously been described as showing a glycolysis dependent phenotype where they couple glycolysis to oxidative phosphorylation, shedding less lactate than A549 cells (Hatzivassiliou *et al.* 2005). Our data confirms this, with SKOV3 cells showing a lower glucose uptake, glutamine uptake and lactate secretion than A549 cells. Additionally, we have demonstrated that SKOV3 cells have an oxygen consumption rate and extracellular acidification rate that denote a more “normal” metabolic phenotype (chapter 3).

We first wanted to create glycolysis independent SKOV3 cells through repeated propagation of wildtype SKOV3 cells in increasing doses of 2DG. After several passages in cell culture, we established a cell line which was able to survive and grow in high levels of 2DG (**figure 6.2**). When we compared the viability of wildtype SKOV3 (WT SKOV3) cells and glycolysis independent SKOV3 (GI SKOV3) to treatment with 2DG, we found that the IC_{50} of the newly established glycolysis independent (GI) SKOV3 cells is 17 mM compared to 1.4mM for wildtype SKOV3 cells (**figure 6.3**). Previous publications have defined that cells which have an IC_{50} of ≥ 3 mM can be defined as being glycolysis independent whereas cells which have a mean IC_{50} can be defined as being glycolysis addicted (Pusapati *et al.* 2016) meaning that, compared to wildtype SKOV3 cells, GI SKOV3 cells can be defined as being glycolysis independent.

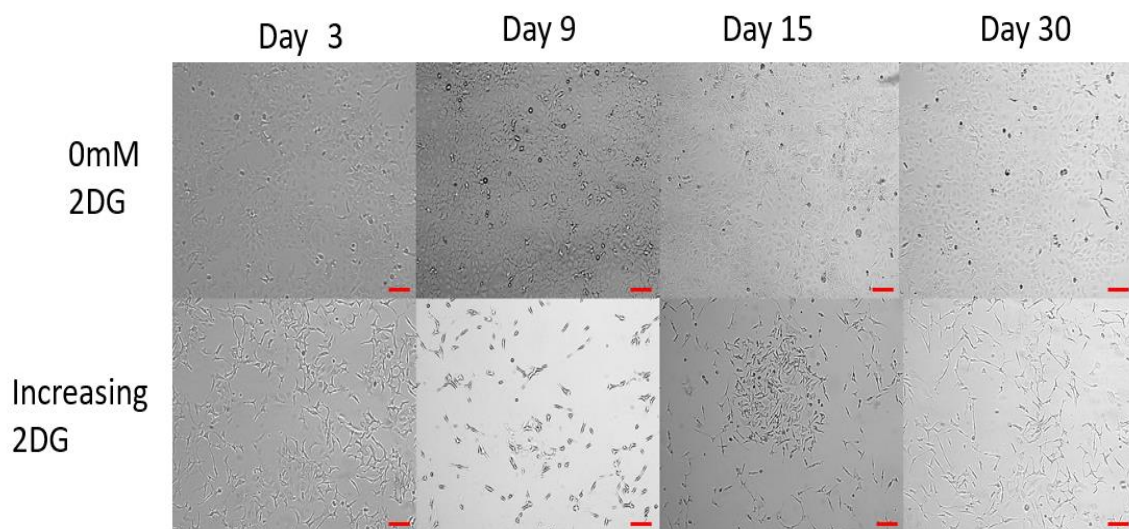


Figure 6.2 Establishment of glycolysis independent SKOV3 cells.

Brightfield microscopy images of SKOV3 cells growing in DMEM supplemented with 10 % Foetal Calf Serum or with DMEM supplemented with 10 % Foetal Calf Serum and increasing doses of 2DG (2,6,10 and 15 mM 2DG). Cells were imaged using a Nikon DS-U2 imaging microscope. The images are representative of biological triplicates. The scale bar represents 500 μ M.

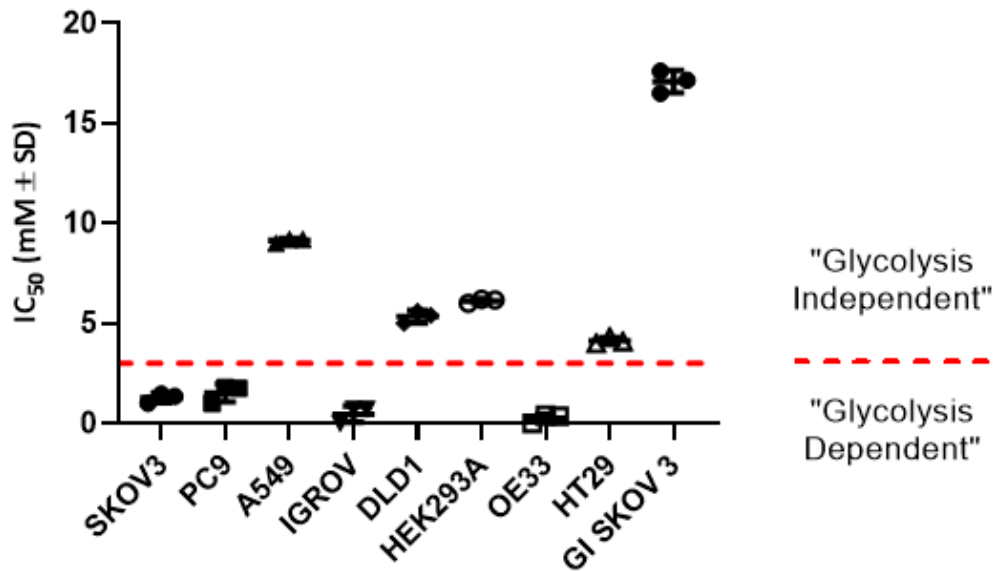


Figure 6.3 Quantification of the susceptibility of various cancer cell lines to treatment with the glycolysis inhibitor 2-deoxy-d-glucose (2DG).

Analysis of the IC₅₀ of various cancer cell lines when treated with the glycolysis inhibitor 2-deoxy-d-glucose (2DG). Cell viability was measured 72 hours post-treatment to create cell viability curves, and the IC₅₀ was determined in Prism. The broken line represents a “dependency threshold” of 3 mM 2DG. GI SKOV3 cells are glycolysis independent SKOV3 cells after multiple passages in 2DG. Data shown represent the mean ± SD of biological triplicates.

6.3.2 Metabolic profiling of glycolysis independent SKOV3 cells

We next attempted to compare the metabolic phenotype of GI SKOV3 cells with wildtype SKOV3 cells and the glycolysis independent cell line A549 using methods established in chapter 3. Analysis of oxygen consumption rate (OCR, a measurement of mitochondrial respiration) and extracellular acidification rates (ECAR, a measurement of glycolysis) were performed using the Seahorse XF analyser and plotted against each other. GI SKOV3 cells showed a relatively low oxygen consumption rate coupled with a very low extracellular acidification rate. These results indicate that GI SKOV3 cells have a similar ECAR to WT SKOV3 cells,

signifying a lack of aerobic glycolysis alongside a low OCR, signifying a lack of mitochondrial respiration (**figure 6.4**).

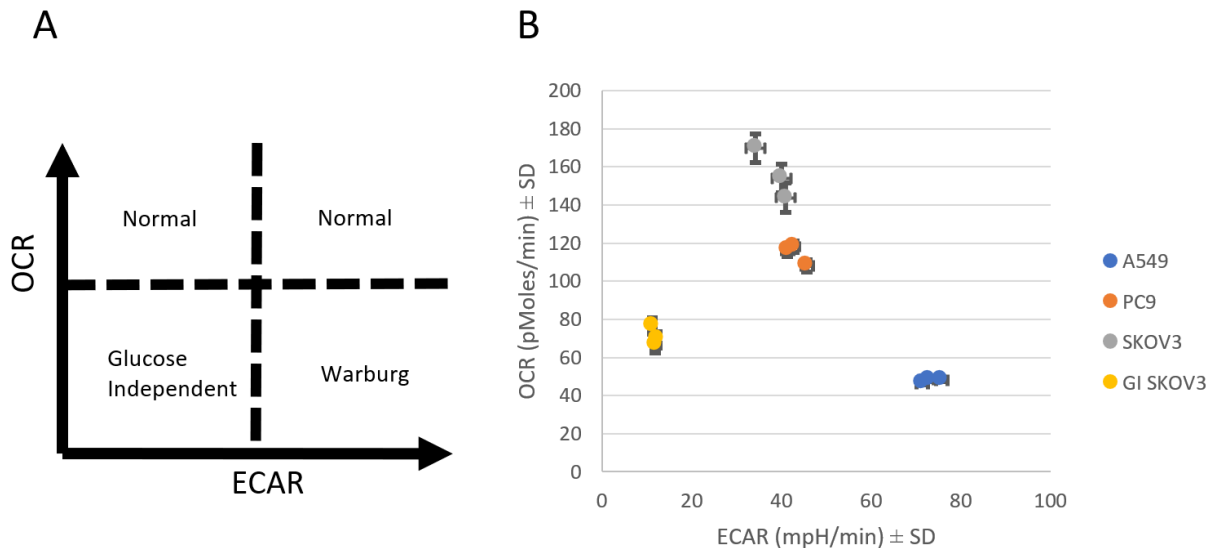


Figure 6.4 Bioenergetic profiles of PC9, SKOV3, A549, and glycolysis independent SKOV3 (GI SKOV3) cancer cell lines.

Oxygen consumption rate (OCR – a measurement of mitochondrial respiration) and extracellular acidification rate (ECAR – a measurement of glycolysis) were measured using a Seahorse XF analyser and normalised to cell number. (A) A simplified diagram representing the different bioenergetic profiles of cells and what they represent and (B) the metabolic profiles of PC9, A549 and SKOV3 cells. Data shown represent the mean $\pm$ SD of biological triplicates.

We next wanted to establish if repeated culturing in increasing doses of 2DG altered glutamine uptake and lactate secretion using protocols established in chapter 3. A549 cells show a high level of lactate secretion, which is significantly decreased upon addition of 15 mM 2DG. In contrast, WT SKOV3 cells show a lower lactate secretion than A549 cells that declined significantly upon treatment with 15mM 2DG. GI SKOV3 cells have a markedly lower lactate secretion compared to both wildtype A549 and SKOV3 cells which was not significantly altered upon addition of 15mM 2DG (**figure 6.5 A**). Upon comparison of glutamine uptake, A549 cells show a high level of glutamine uptake which is significantly higher than

wildtype SKOV3 (**figure 6.5 B**), GI SKOV3 cells showed a statistically similar glutamine uptake when compared to A549 cells.

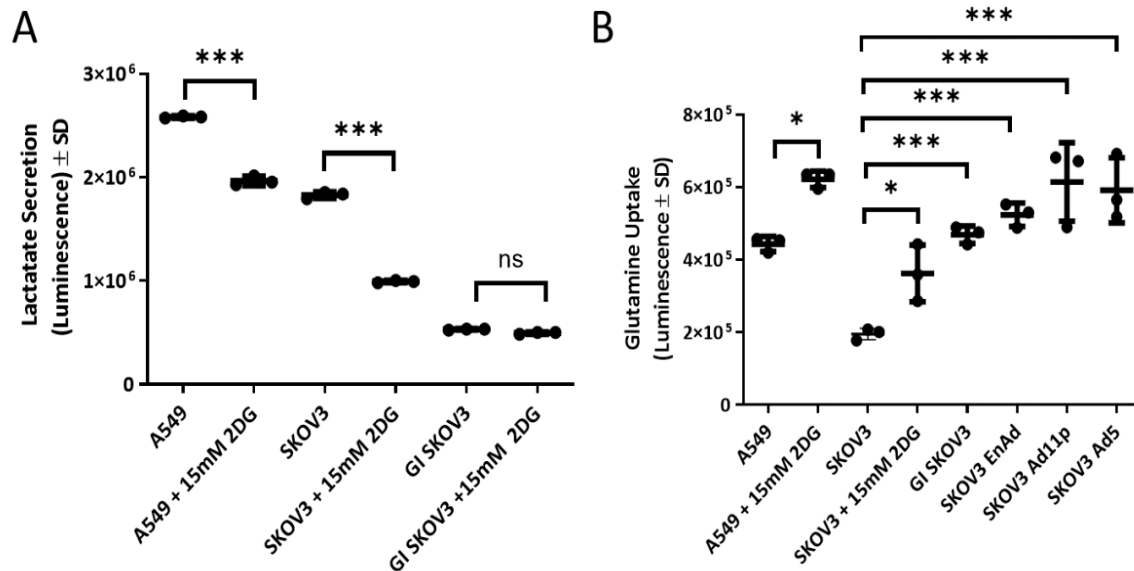


Figure 6.5 Lactate secretion and glutamine uptake of selected cancer cell lines.

Analysis of Lactate Secretion (**A**), and Glutamine Uptake (**B**) in A549, SKOV3, or GI SKOV3 cells. Cells were treated with viruses at an MOI of 1 or with 15 mM 2DG then 48 hours later, and media was removed and analysed for lactate secretion using Lactate Glo (Promega) or Glutamine-Glo (Promega). Data shown represent the mean ± SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

6.3.3 Viral oncolysis is enhanced in glycolysis independent SKOV3 cells compared to wildtype SKOV3 cells

We next wanted to test our hypothesis that permanently inhibiting glycolysis in cells that are usually glycolysis addicted will create a cell line that is more permissive to oncolysis via enadenotucirev. Wildtype SKOV3 cells and glycolysis independent SKOV3 cells were infected with EnAd-CMV-GFP or EnAd-SA-GFP at an MOI of 1. 96 hours post-infection, fluorescence was analysed using a Celigo imaging cytometer which provides a quantifiable fluorescent image of an entire well. GI SKOV3 cells, in comparison to WT SKOV3 cells, showed much higher levels of fluorescence at 96 hours post-infection (**figure 6.6**).

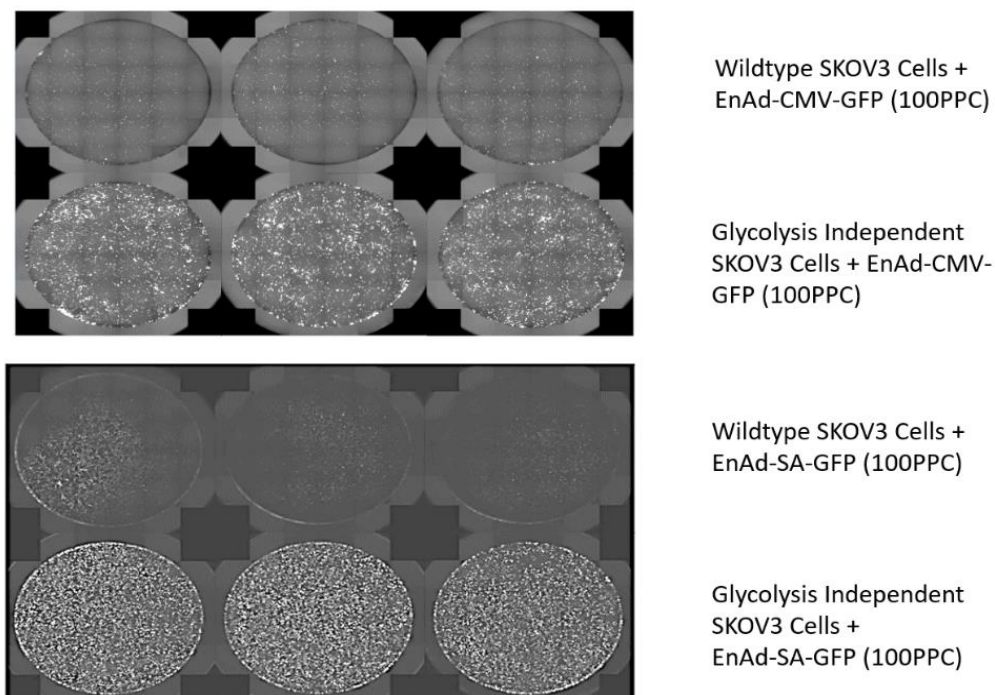


Figure 6.6 Fluorescent viral protein expression of wildtype and glycolysis independent SKOV3 cells.

SKOV3 cells and GI SKOV3 cells were infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis). Cells were infected with EnAd at an MOI of 1. 5 days post-infection cells were imaged using the Celigo imaging cytometer. The images are representative of experimental triplicates with exposure remaining consistent between plates.

When analysed by flow cytometry, GI SKOV3 cells infected with EnAd-CMV-GFP at an MOI of 1 showed a dramatically increased mean fluorescence with the peak of GFP expression achieving double the peak mean GFP expression of wildtype SKOV3 cells (**figure 6.7 A**) accompanied by a faster increase in the percentage of cells positive for GFP (**figure 6.7 B**). GI SKOV3 cells also showed a similar trend when infected with EnAd-SA-GFP at an MOI of 1 (**figures 6.7 C & D**). At 120 hours post-infection, treatment of wildtype SKOV3 cells with the glycolysis inhibitor 2DG significantly increased the number of viral genomes produced (**figure 6.7 F**). GI SKOV3 cells showed an even higher number of viral genomes produced which did not increase substantially in the presence of 2DG (**figure 6.7 F**). These findings were mirrored in results analysing infectious viral progeny production as analysed by the TCID₅₀ assay (**figure 6.7 E**).

The results showing that viral oncolysis was improved through the addition of 2DG only in wildtype SKOV3 cells and not in GI SKOV3 cells indicates that the presence of 2DG alone is not increasing permissivity but rather that the adaptation of the cells to inhibition of glycolysis both in the short term (through treatment of wildtype cells with 2DG) and in the long term (through the creation of GI SKOV3 cells) which creates an environment that favours enadenotucirev replication.

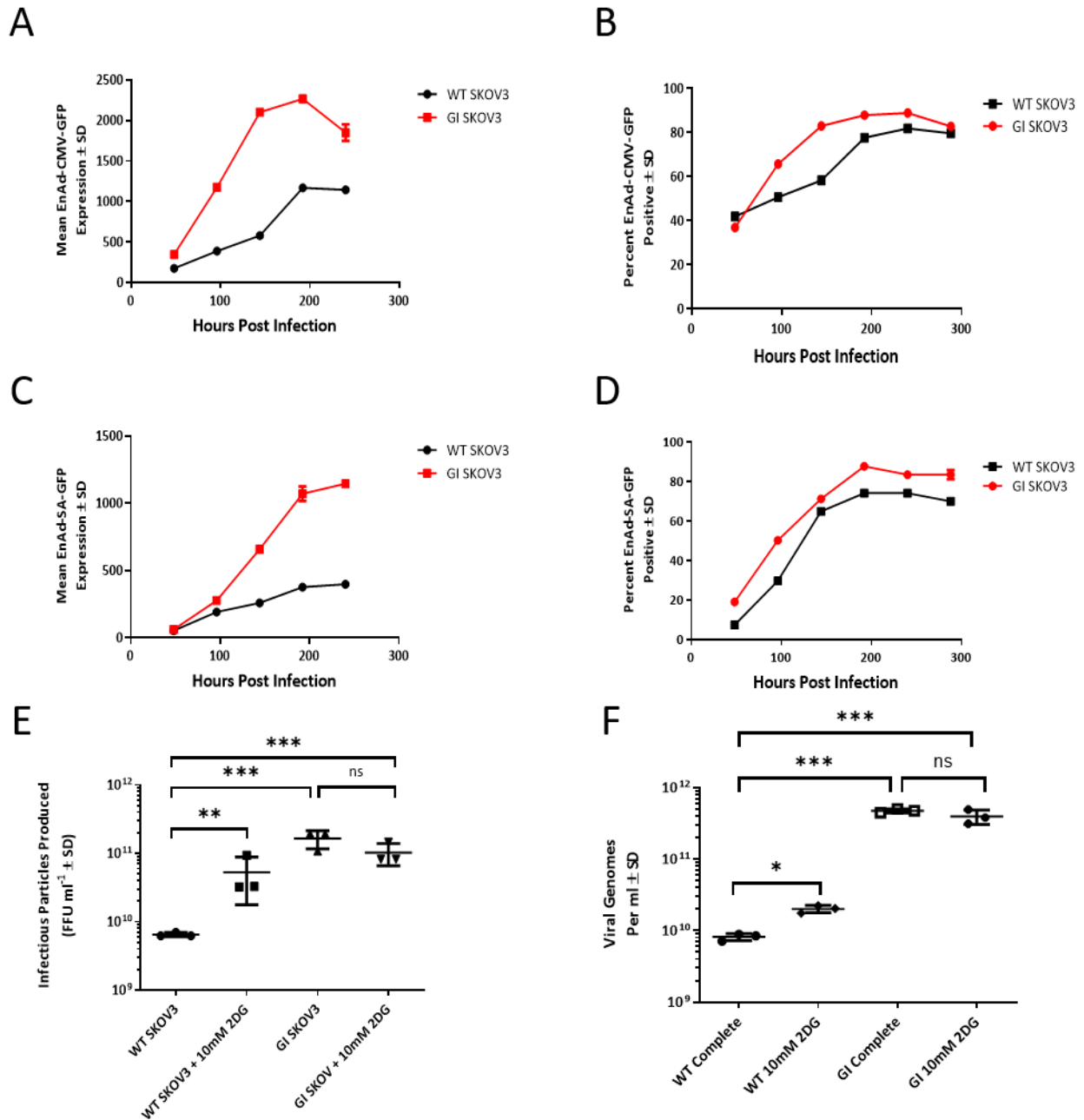


Figure 6.7 Fluorescent viral protein expression in wildtype and glycolysis independent SKOV3 cells.

SKOV3 cells and GI SKOV3 cells were infected with EnAd expressing green fluorescence protein (GFP) under control of the CMV promoter (EnAd-CMV-GFP; representing early viral protein synthesis; figures **A** and **B**) or the major late promoter (EnAd-SA-GFP; representing late viral protein synthesis; figures **C** and **D**). Cells were infected with EnAd at an MOI of 1 and every 48 hours post-infection, monitored for mean fluorescent signal (figures **A** and **C**) or the percentage of cells that are GFP positive (figures **B** and **D**). 120 hours post-infection, cells were detached and analysed for viral genomes via real-time qPCR (**E**), or infectious viral progeny via TCID₅₀ (**F**). Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

6.3.4 Inhibiting glycolysis using 2-deoxy-d-glucose in xenograft mouse models after infection with enadenotucirev.

We next wanted to understand how treatment of 2DG would affect viral replication in a pilot in vivo xenograft model. Athymic nude mice were first injected subcutaneously with 5×10^6 DLD-1 cells. Once xenografts developed, mice were treated with a single intratumoural dose of EnAd expressing firefly luciferase under the control of the splice acceptor (EnAd-Sa-FLuc) of 5×10^9 viral particles per mouse. Ninety-six hours post-infection, mice were treated with 500 mg per kg 2DG every other day for two weeks whereupon the mice were sacrificed, and the xenografts were removed. The timeline for this experiment is shown in **figure 6.8**

A.

Immediately before sacrifice, the mice were imaged using a murine in vivo imaging system, and representative images of mice are shown in **figure 6.8 B**. Treatment of mice with 2DG increased the luciferase expression when compared to mice treated with EnAd-SA-FLuc alone (**figure 6.8 B**). Mice treated with EnAd-SA-FLuc showed a mean luminescence of 1525 units per mg of protein and EnAdSA-FLuc-infected mice treated with intraperitoneal doses of 2DG showed a significant increase in viral protein expression with a mean luminescence of 15,728 units per mg of protein (**figure 6.8 C**). We also analysed the viral genome synthesis and saw that mice treated with EnAd-SA-FLuc had a mean value of 778 viral genomes per

DLD-1 cell. In contrast, treatment of mice with 2DG significantly increased the value of mean viral genomes per DLD-1 cell to 33,432 (**figure 6.8 D**).

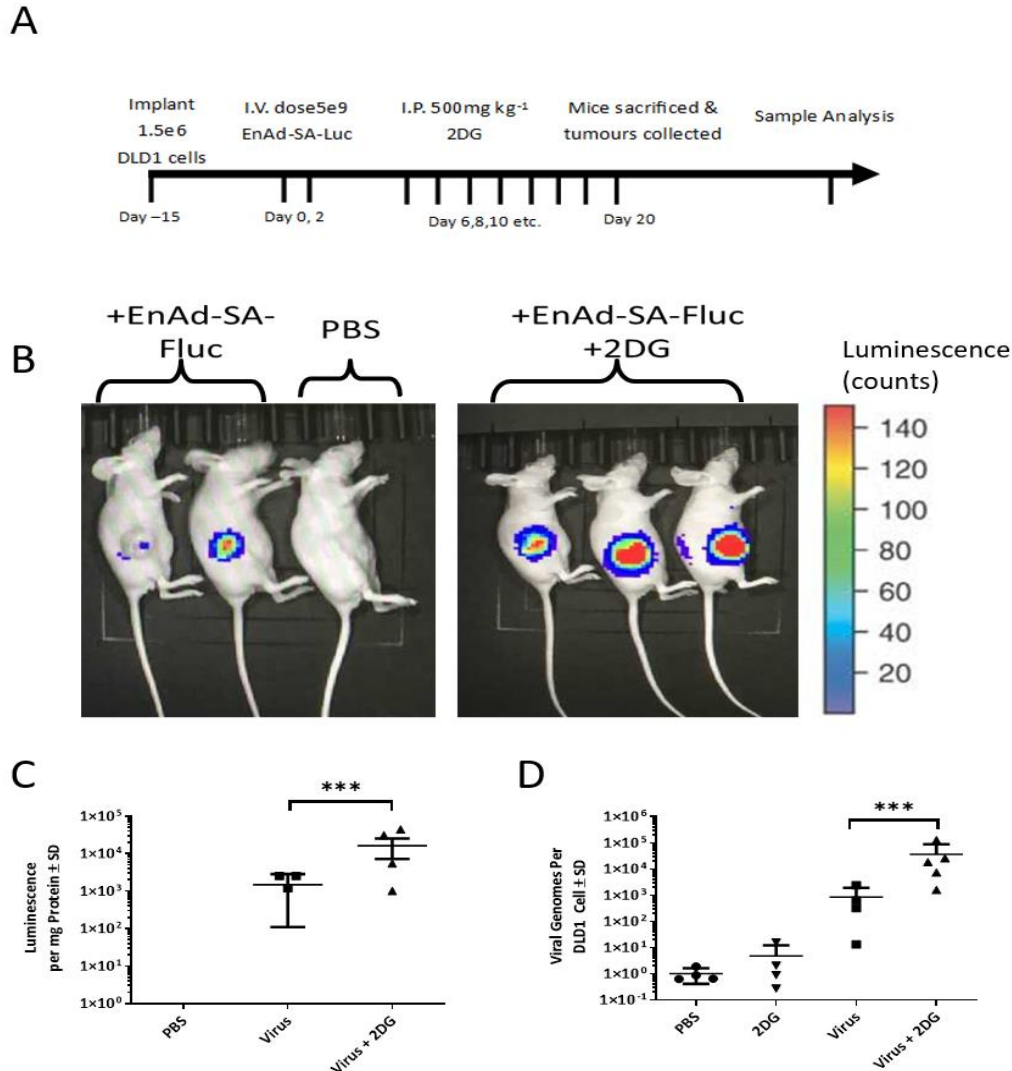


Figure 6.8 Antagonising glycolysis, using 2DG, boosts oncolytic virus replication and transgene expression in a murine xenograft model.

6-week old female athymic nude mice Crl:NU(NCr)-*Foxn1*^{nu} (Charles River, Margate, UK) were injected subcutaneously with 5×10^6 DLD1 cells. Once xenografts developed to $\sim 100 \text{ mm}^3$, mice were randomised and dosed intratumorally with $10 \mu\text{L}$ containing 5×10^9 particles of enadenotucirev or vehicle control intravenously. 96 hours post-infection mice received 500 mg kg^{-1} 2DG every other day for 2 weeks whereupon they were sacrificed, and the xenografts were removed. (**A**) a representative schematic timeline. (**B**) At the point of sacrificing, mice were injected with Luciferine (Promega) intraperitoneally, and imaged using the IVIS imaging system, and luminescence was recorded using Living Image™. All images were taken 15 minutes post-injection at the same exposure. (**C**) Luminescence was measured using the Luciferase Assay System (Promega) and normalised to protein concentrations using a BCA assay (Quantipro BCA Kit, Sigma). (**D**) Viral genomes were analysed using real-time quantitative PCR from the homogenates and normalised to quantities of human cells as measured by RT-qPCR. Data shown represent the mean $\pm$ SD of biological triplicates. Statistical analysis was performed by two-way ANOVA with Bonferroni posthoc tests comparing with the relevant (mock) condition (*, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.001$).

6.3.5 Cloning strategy to generate enadenotucirev expressing TP53-inducible glycolysis and apoptosis regulator.

Using the oncolytic adenovirus platform technology developed in our lab, the oncolytic adenovirus EnAd can be engineered to encode biologics that are expressed by infected tumour cells as the virus replicates, offering a multipronged and tumour-targeted therapeutic strategy that amplifies a high expression of the target directly within the tumour (Seymour and Fisher 2016; Freedman *et al.* 2018; Scott *et al.* 2019). As previously discussed, the TP53-inducible glycolysis and apoptosis regulator (TIGAR) is a recently discovered protein which functions as an inhibitor of glycolysis in human cells and is highly expressed in several cancer cells (Madan *et al.* 2011; Ko *et al.* 2016; Geng *et al.* 2018; Liu *et al.* 2019b; Chu *et al.* 2020). TIGAR, acts to degrade intracellular fructose-2,6-bisphosphate, which is a potent activator of phosphofructokinase-1 (PFK-1). PFK-1 catalyses the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate and in doing so, drives glycolysis (Bensaad *et al.* 2006; Li and Jogl 2009). Overexpression of TIGAR, therefore, will inhibit glycolysis in a similar manner to 2DG allowing for entry of glucose into the pentose phosphate pathway whilst reducing the amount of metabolised via glycolysis and thus provides an attractive target to clone into EnAd considering our previous results (**figure 6.1**).

To explore the feasibility of overexpressing TIGAR, we cloned TIGAR into a plasmid expressing GFP under a CMV promoter, creating pCMV-TIGAR-GFP (**figure 6.9 A**).

We then transfected this plasmid into A549 cells, and transfection efficiency was analysed through the transfection of pCMV-GFP plasmids into control wells. We observed that SKOV3 cells were not able to be efficiently transfected without significant cytotoxicity, and so were not analysed further at this stage. A549 were transfected at close to 80% efficiency (**figure 6.9 B**). A549 cells showed expression of TIGAR-GFP at the expected weight up to 5 days post-transfection (**figure 6.9 C**). Confirmation that the plasmid CMV-TIGAR-GFP can inhibit glycolysis was performed using phenol red, a known pH indicator present in cell media. A549 cells transfected with pCMV-TIGAR-GFP and not with pCMV-GFP showed a loss of acidification of the media at day two post-transfection (**figure 6.9 D**).

The plasmid CMV-TIGAR-GFP was then cloned into Sbfl-linearised EnAd viral backbone by Gibson assembly technology. The insert containing CMV-TIGAR-GFP was placed downstream of the viral fibre gene. A schematic representation of the plasmid EnAd-CMV-Tigar-GFP is shown in **figure 6.10 A**. Successful transgene insertion was confirmed by diagnostic restriction digest (**figure 6.10 B**) leaving a single colony (colony F) as a candidate for expansion.

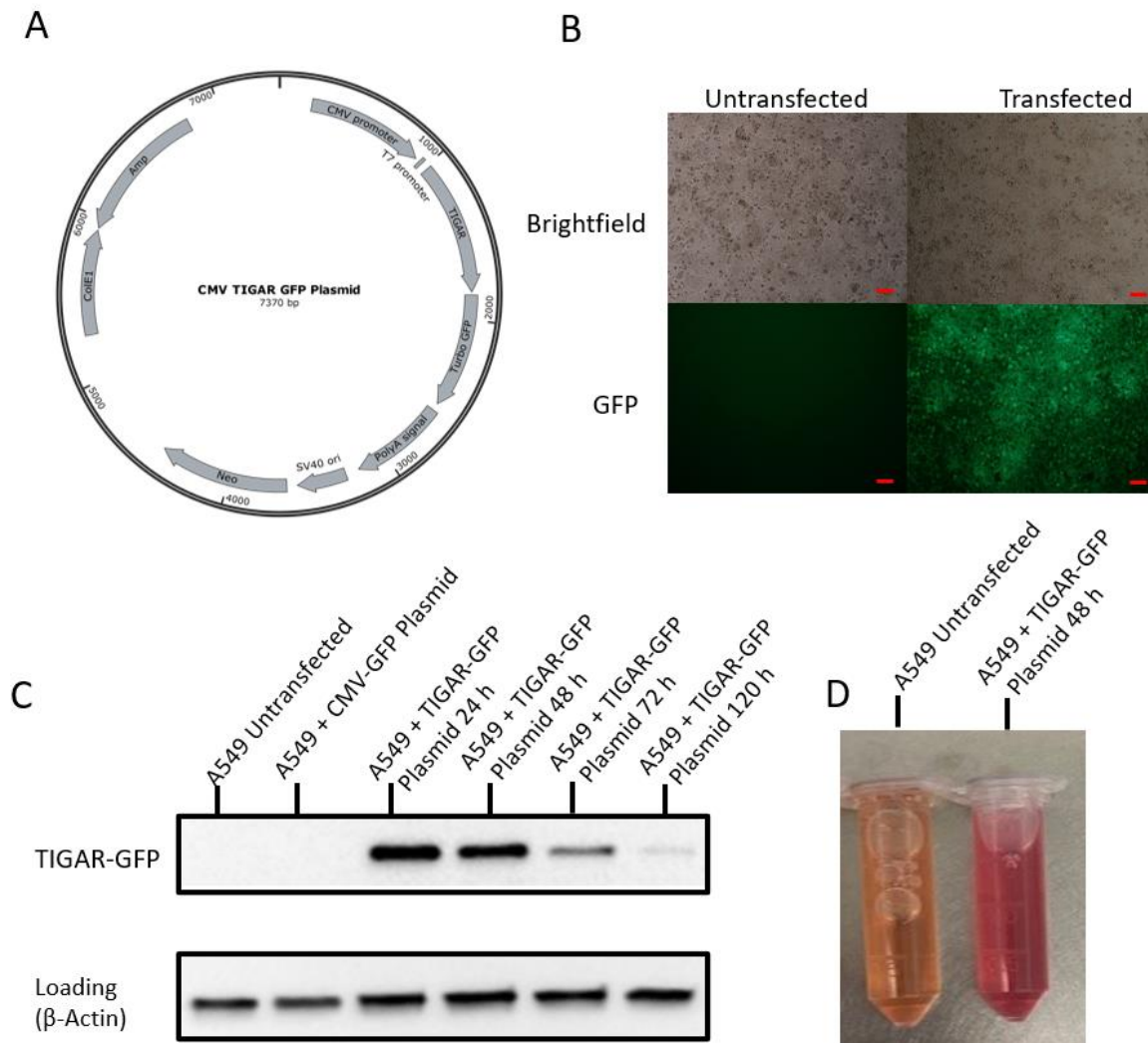
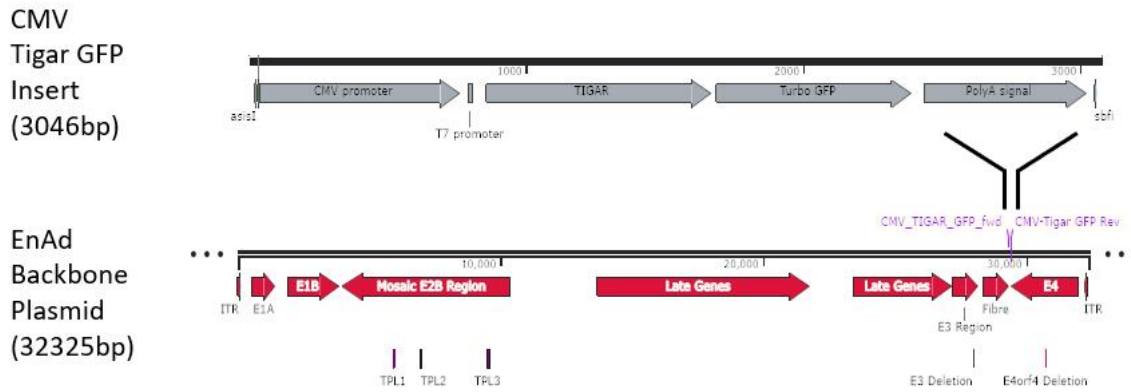


Figure 6.9 Engineering a TIGAR-GFP-expressing plasmid for later incorporation into EnAd. (A) A schematic representation of the plasmid expressing TIGAR-GFP under the control of the CMV promoter after Gibson assembly. (B) A549 cells were plated and transfected with CMV-TIGAR-GFP or mock-transfected using Lipofectamine 2000 (Thermo) and 48 hours post-transfection, microscopy images were obtained using a Nikon DS-U2 imaging microscope at the same exposure between conditions. The images are representative of biological triplicates. The scale bar represents 500 μ M. (C) Western Blot analysis of TIGAR expression and β -actin from A549 cells either untransfected (lane 1), transfected with CMV-GFP-expressing plasmid (lane 2), or transfected with CMV-TIGAR-GFP (lanes 3-6). (D) Visual representation of a loss of acidification of media 48 hours following transfection with CMV-TIGAR-GFP plasmid. CMV = Cytomegalovirus promoter; TIGAR = TP53-inducible glycolysis and apoptosis regulator; GFP = green fluorescent protein; SV40 ori = simian virus 40 origin of replication; Neo = neomycin resistance; ColE1 = E. coli origin; Amp = Ampicillin resistance.

A



B

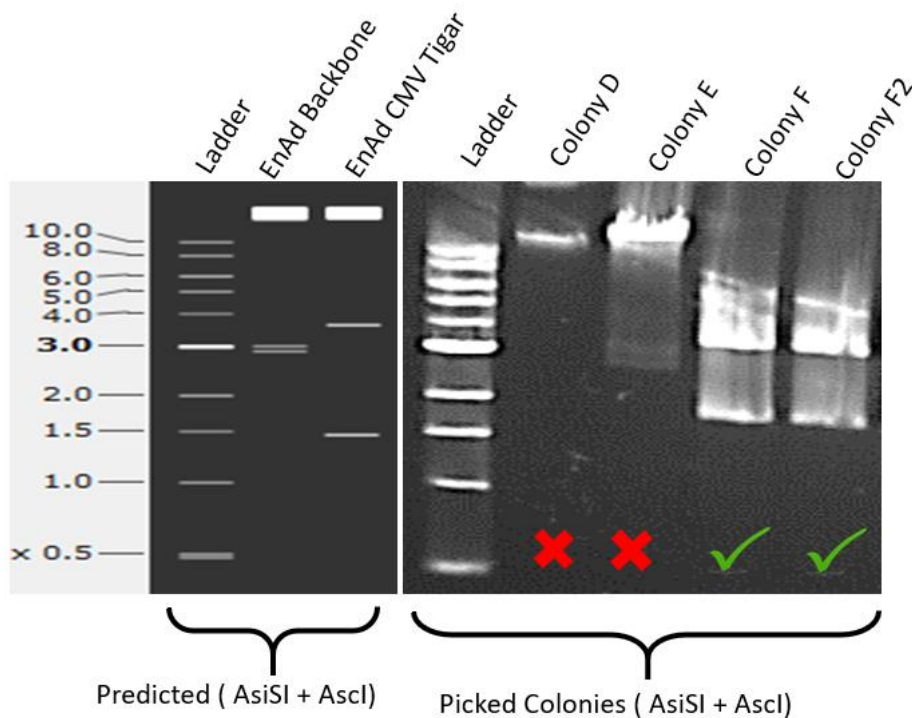


Figure 6.10 Engineering a CMV-TIGAR-GFP-expressing EnAd.

(A) A schematic representation of the EnAd plasmid backbone engineered to express TIGAR-GFP downstream of the CMV promoter through Gibson assembly technology. (B) A CMV-TIGAR-GFP plasmid was amplified by polymerase chain reaction and inserted into a linearised EnAd vector by Gibson assembly technology. An exemplary diagnostic restriction digest of DNA from a bacterial colony transformed with TIGAR-GFP-expressing EnAd are shown. Colonies with the predicted digest pattern are indicated with a tick. CMV = Cytomegalovirus promoter; TIGAR = TP53-inducible glycolysis and apoptosis regulator; GFP = green fluorescent protein.

CMV-TIGAR-GFP-armed EnAd constructs were linearised with *Ascl* prior to transfection into HEK293A cells, which were monitored closely for signs of cytopathic effect (CPE), indicating successful virus production (**figure 6.11 A**). The cell lysates and supernatants generated above represent “crude” virus preparations, possibly containing a mixture of transgene-armed and parental virus, as well as transfection reagents. We therefore isolated single clones of the EnAd-CMV-TIGAR_GFP viruses (i.e. deriving from single virus particles) by plaque purification. In brief, HEK293A cells were infected with serially diluted viruses, before being overlaid with 1% agarose. Two unique virus plaques were isolated and expanded, with cells and supernatant subsequently harvested following the observation of extensive CPE. The cell supernatants from viral preps that exhibited CPE alongside GFP expression were probed by western blotting for successful expression of TIGAR-GFP (**figure 6.11 B**). From this western blot analysis, one of the viral preparations (prep F1) was chosen for further purification using a plaque assay followed by infection in A549 cells to ensure purity of viral stocks and to monitor the ability for the virus to replicate in cells which were not transformed using adenoviral proteins.

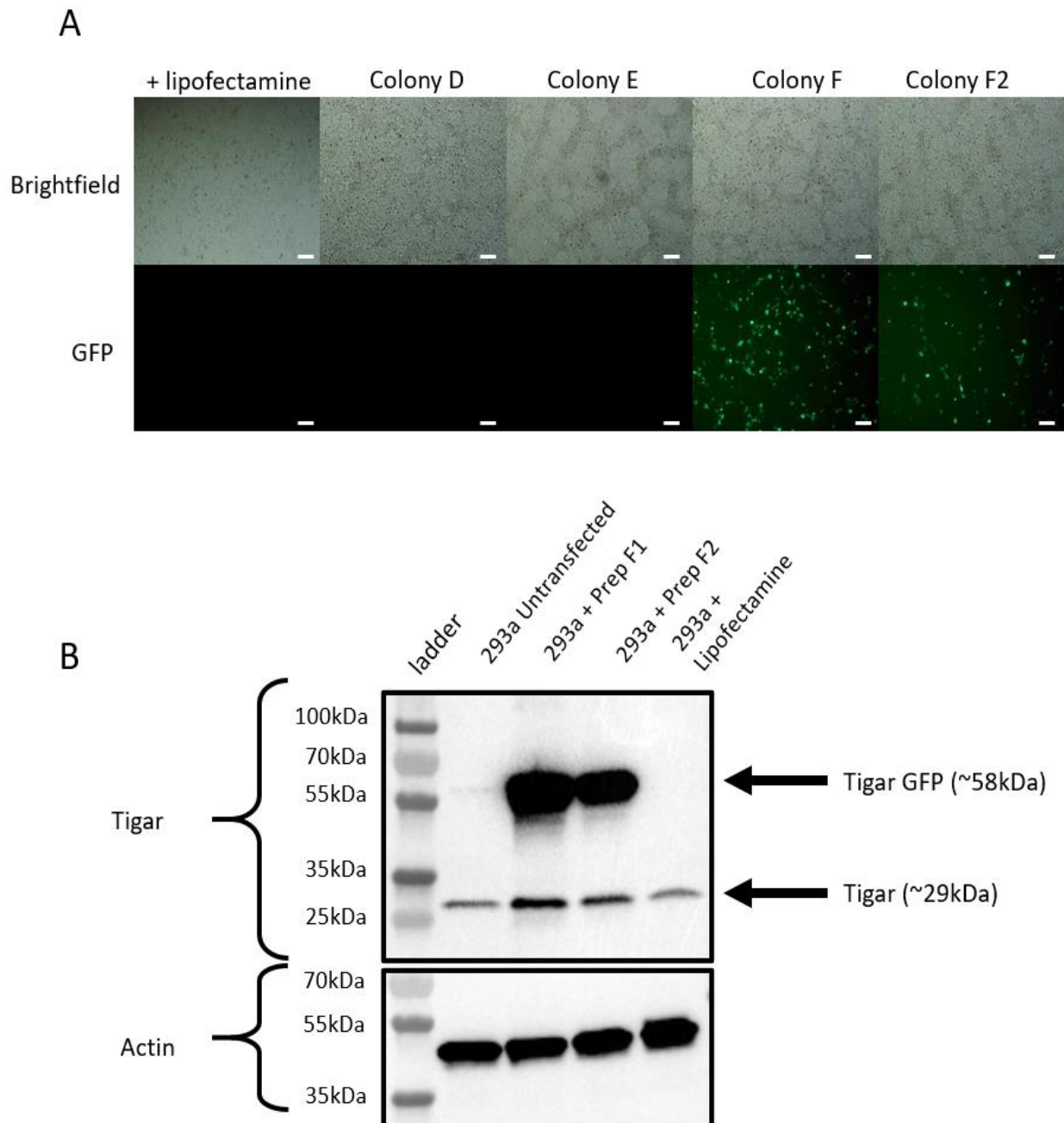


Figure 6.11 Transfection of 293A cells with EnAd-CMV-TIGAR-GFP plasmid construct. (A) HEK 293a cells were plated at 80 % confluency then transfected with plasmid constructs isolated in figure 6.10 after expansion in bacteria. Upon observation of cytopathic effects (CPE), cells were imaged using Nikon DS-U2 imaging microscope. The scale bar represents 500 μ M. (B) Upon observation of CPE alongside GFP expression from plasmid transfections in A, cell lysates and supernatant were collected, and plaque purified then analysed for TIGAR expression using western blot analysis with antibodies targeting TIGAR and β -Actin. Expected weights of TIGAR and TIGAR-GFP construct are labelled alongside the blot.

6.3.6 Enadenotucirev expressing TIGAR can replicate, express TIGAR, and is capable of lysing cells without modulating culture pH.

We next aimed to analyse the ability for the newly created virus to replicate, express early viral genes, and lyse cells. Firstly, the concentration of the virus was analysed using a plaque assay, and we assessed the concentration of the virus in the supernatant and found to be 1.45×10^8 plaque-forming units per mL. We next analysed the ability of the virus to express GFP and to lyse cells through fluorescent microscopy. To achieve this, A549 cells were infected with EnAd-CMV-TIGAR-GFP at an MOI of 1, and images were taken at 3, 5, and 6 days post-infection. We changed from 293A cells (which were immortalised using the adenovirus E1 gene) to A549 cells so that any viral replication is purely because of viable viruses and not from transcomplementation. At day three post-infection, the majority of the A549 cells were GFP positive but showed no apparent cytopathic effects. GFP-expression continued at day 5 and 6 post-infection but with plaques forming by five days post-infection (**figure 6.12 A**).

We next confirmed the expression of TIGAR from A549 cells infected with EnAd-CMV-TIGAR-GFP during infection. At days 3, and 5 post-infection TIGAR-GFP expression was confirmed via western blotting. TIGAR-GFP had an estimated weight of 58kDa, as opposed to TIGAR not bound to GFP, which has a weight of 29kDa, indicating that TIGAR-GFP is expressed throughout the EnAd infection lifecycle (**figure 6.12 B**).

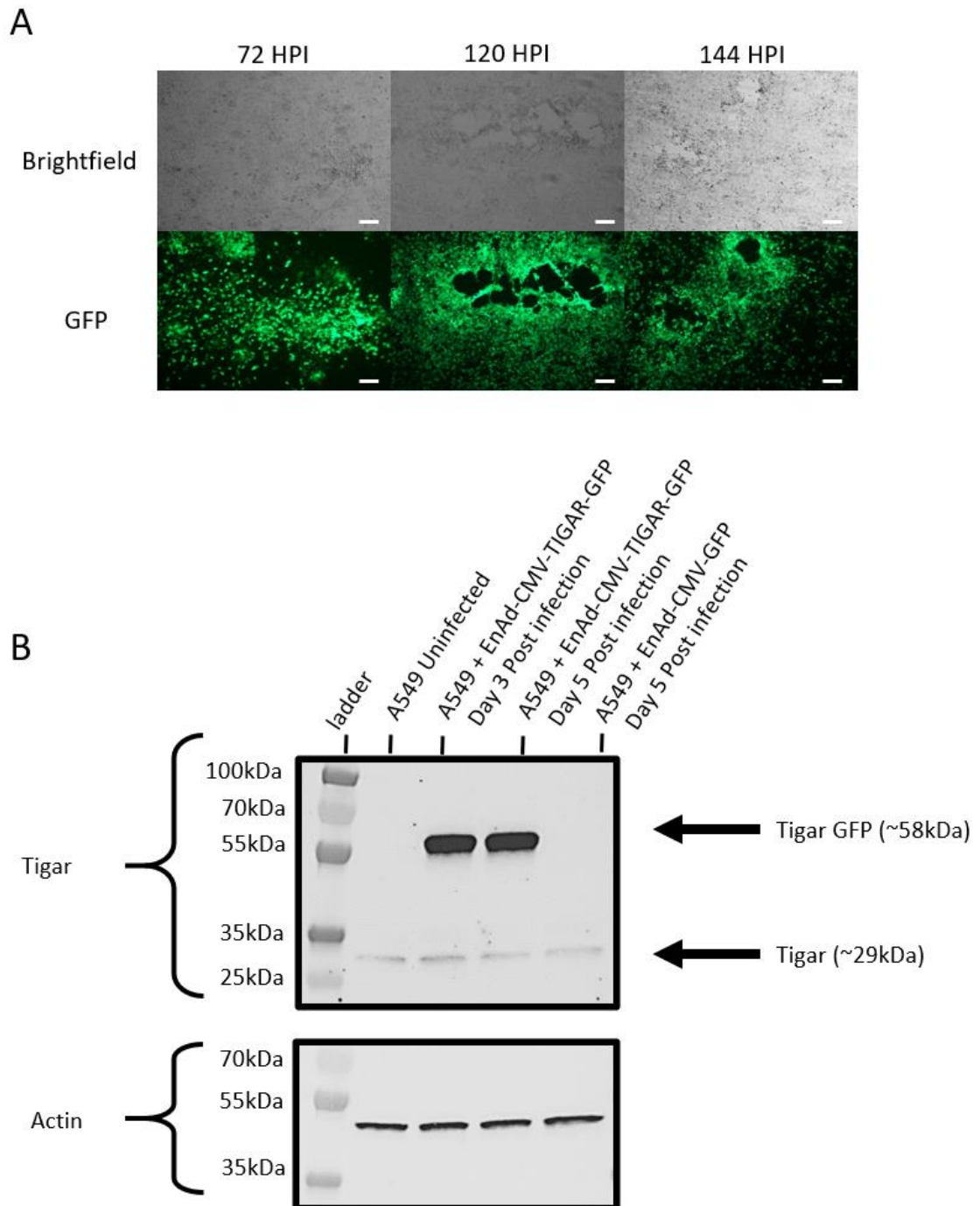


Figure 6.12 Infection of A549 cells with EnAd-CMV-TIGAR-GFP virus results in GFP expression, TIGAR expression, and viral plaque formation.

(A) A549 cells were infected with plaque purified EnAd-CMV-TIGAR-GFP at an MOI of 1 and assessed for the presence of cytopathic effects and GFP production. Cells were imaged using Nikon DS-U2 imaging microscope. The scale bar represents 500 μ m. (B) A549 cells were infected with EnAd-CMV-GFP or EnAd-CMV-TIGAR-GFP or mock-infected and at set points post-infection, protein samples were collected and analysed for TIGAR expression using western blot analysis with antibodies targeting TIGAR and β -Actin. Expected weights of TIGAR and TIGAR-GFP construct are labelled alongside the blot.

To analyse if TIGAR bound to GFP can successfully inhibit glycolysis, we next analysed the acidification of the media of A549 cells either uninfected or infected with EnAd-CMV-TIGAR-GFP at an MOI of 1 at five days post-infection. Cells infected with EnAd-CMV-TIGAR-GFP showed less acidification of the media than mock-infected A549 cells (**figure 6.13**). When this was analysed using a pH probe, mock-infected A549 cells had a pH of 7.6. In contrast, cells infected with EnAd-CMV-TIGAR-GFP had a pH of 8. As we have previously demonstrated that acidification of media of EnAd-infected cells is, at least in part, due to lactic acid formation, we can conclude that the lack of acidification in cells infected with EnAd-CMV-TIGAR-GFP could likely be explained lower lactic acid production because of inhibited glycolysis. The results above show that EnAd can be altered to express a transgene that directly inhibits glycolysis, and that this transgene does not inhibit viral replication, lysis or spread.

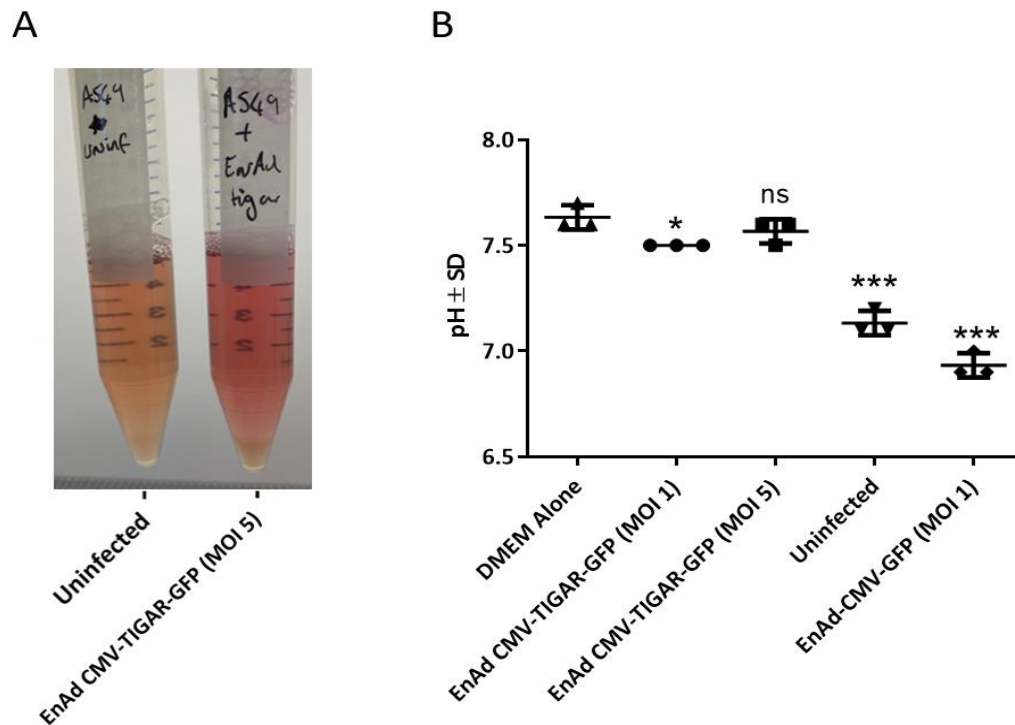


Figure 6.13 Infection of A549 cells with EnAd-CMV-TIGAR-GFP virus results in a decreased pH reduction in of the media.

(A) A549 cells were plated at 80% confluency then infected with EnAd-CMV-TIGAR-GFP at an MOI of 1 (on the right) or mock-infected (on the left). 3 days later the supernatant was collected and pictured, results are representative of biological triplets. (B) A549 cells were infected with EnAd-CMV-GFP, EnAd-CMV-TIGAR-GFP, or mock-infected and 3 days post-infection, media was removed, and pH was monitored using pH probe (Jenway). Data shown represent the mean $\pm$ SD of biological triplicates.

We recognise that further work is required to fully characterise the effects of the expression of TIGAR-GFP upon the outcomes of an oncolytic viral infection such as the speed of kill or tumour clearance. We are also cognisant that further confirmation of the impact of virally expressed TIGAR production on glycolysis is required. We have, however, provided a clear proof of principle that glucose is a non-essential metabolite for the lifecycle of EnAd. Additionally, we have provided evidence that inhibiting glycolysis through co-treatment with an agent such as 2DG, or by weaning cells away from using glucose can significantly improve the

outcome of oncolytic virotherapy using EnAd. We hypothesise that the expression of a genetic target to inhibit glycolysis (such as TIGAR) could enhance the outcome of oncolytic virotherapy, particularly in cancer cells that are poorly permissive to lysis by wildtype EnAd which tend to use glucose for aerobic respiration.

6.4 Chapter Discussion

In this chapter, we attempted to amalgamate the lessons learnt in the previous chapters, and apply this knowledge to produce favourable metabolic environments for oncolysis with the chimeric oncolytic adenovirus enadenotucirev (EnAd).

In chapter 5, we reported that EnAd does not require the presence of glucose to replicate in- and lyse cells and that the treatment of glycolysis dependent SKOV3 cells with 2DG increases viral oncolysis. As we show in **figure 5.11 E & F** however, these results can be hard to justify as being entirely the result of glycolysis inhibition and the metabolic demands this enforces. SKOV3 cells, once treated with 2DG, have a decreased growth rate and some toxicity at higher concentrations. We have also previously compared SKOV3 cells (which are derived from an ovarian epithelial adenocarcinoma) with A549 cells (which are derived from an alveolar epithelial adenocarcinoma). Because the cells are from different

origins, a true comparison of purely metabolic influences on the outcome of viral infection can be difficult.

The creation of a glycolysis independent SKOV3 cell line provides a highly compelling model with which to compare the effects of metabolism on the outcome of oncolytic virotherapy as these cells share a parental lineage with WT SKOV3 cells. Pusapati *et al.* reported that these newly created glycolysis independent cell lines show a consistent metabolic phenotype whereby glucose is preferentially shunted towards the pentose phosphate pathway alongside a high dependency on anaplerotic glutamine metabolism (Pusapati *et al.* 2016). It is precisely this metabolic phenotype that we have previously shown is advantageous for oncolysis with EnAd in cancer cells.

We show in **figures 6.3 - 6.5** that glycolysis independent SKOV3 cells (GI SKOV3) have very low lactate secretion but have a glutamine uptake rate that is similar to the basal rate of glutamine uptake in A549 cells and the glutamine uptake in adenovirus infected SKOV3 cells. These findings lead us to postulate that GI SKOV3 cells would be highly permissive to EnAd. We found in every output measured, that GI SKOV3 cells were significantly more permissive to EnAd (**figure 6.6 and 6.7**) when compared to wildtype SKOV3 cells. These results further confirm that cells which do not use glucose to fill the TCA cycle but rely upon glutamine for anapleurosis are more susceptible to viral lysis with EnAd.

We next wanted to investigate the effect of glycolysis inhibition in a murine xenograft cancer model. We considered the following three tactics: short-term starvation of glucose, delivery of alpha-ketoglutarate (α KG), or inhibition of glycolysis. Previous papers have reported some promising effects with short-term starvation of mouse models improving the amount of viral protein expressed and increasing survival in xenograft models (Esaki *et al.* 2016; Scheubeck *et al.* 2019), perhaps owing to a phenomenon known as the differential stress response (DSR). Whilst the DSR and short-term starvation is appealing, it provides challenges as restriction of diets in cancer patients could prove difficult to enforce. Additionally, in murine models, it can be difficult to ensure a strict starvation regime.

The addition of dimethyl-ketoglutarate (the cell-permeable form of α KG) is promising as an *in vivo* treatment. Previous papers have reported that, when given as a monotherapy, dimethyl-ketoglutarate is non-toxic to cancer cells however in combination with an Oxphos inhibitor (BAY87-2243) it is lethal to cancer cells (Sica *et al.* 2019). This is an attractive tactic which we hope to investigate further in the future. We are, however, cognisant of the inherent complexity of this approach. Cancer cells which rely upon oxidative glucose metabolism (and tend to be poorly permissive to EnAd) do not metabolise high amounts of glutamine substrates. In such cells, the addition of α KG may have a limited effect. For cells that already rely upon glutamine metabolism (which tend to be highly permissive to EnAd), addition

of α KG will likely exaggerate reductive carboxylation. We believe that rather than providing excess glutamine products, inhibiting the ability to use glucose is a more direct approach to ensure all cells within the tumour rely upon glutaminolysis.

In our pilot *in vivo* study, the mice which were treated with 2DG showed an increase in viral protein synthesis and genome copies when compared to mice treated with EnAd alone (**figure 6.8**). These results provide a promising first step in translating our findings into a more clinical setting; however, because this is a pilot experiment, the sample numbers between variables are low. A larger-scale experiment needs to be conducted to comprehensively analyse the impact of combining glycolysis inhibitors and treatment with EnAd. Additionally, this experiment was a fixed end-point experiment where mice were sacrificed 20 days post-infection and thus survival length, tumour volume and weight were not recorded for a long enough duration to monitor if 2DG co-treatment would improve these outcomes.

It is tempting to postulate that because glucose metabolism is often elevated or unusual in cancer cells (allowing us to visualise them with PET imaging), that we can exploit this selectively to eradicate them. Tumour cells, however, mostly use the same glycolytic enzymes as non-malignant cells. This is partly because metabolism is so fundamental to homeostasis that it is highly evolutionarily conserved. This means that the inhibition of glycolytic enzymes as a cancer

treatment may increase the risk of off-target effects. It is, therefore, particularly compelling to provide glycolytic inhibition in the context of a cancer-restricted oncolytic virus as this allows for expression at high concentrations specifically within the tumour. We attempted to clone a metabolic enzyme within the EnAd backbone to effectively “arm” the virus with a transgene that will create the desired metabolic environment within cancer cells specifically, potentially bolstering EnAd replication. We chose to express TP53-inducible glycolysis and apoptosis regulator (TIGAR) as this should potentially inhibit oxidative glucose metabolism whilst promoting nucleotide synthesis via the PPP and increase glutaminolysis (all of which we have previously shown increase viral oncolysis).

The fact that we were able to successfully rescue a replicating virus capable expressing a glycolytic inhibitor which can inhibit media acidification creates a highly compelling proof of the hypothesis that viral oncolysis with EnAd is not reliant upon functioning glycolysis. Further work remains to be conducted to compare the effect of TIGAR expression upon the oncolytic activity of EnAd when compared to the parental virus and to analyse the impact of this virus on metabolism within virally infected cells both in vitro and in vivo.

Together, these results provide a proof-of-concept that understanding preferential metabolic microenvironments for oncolytic viruses can be utilised to create increased susceptibility to oncolysis from previously poorly permissive cells,

and to increase expression of viral proteins and replication of the virus both *in vitro* and, *in vivo*. Additionally, deeper understanding of viral demands can help to create the next generation of oncolytic viruses which have the potential to combine direct anti-cancer activity with a reduction in immunosuppressive metabolic waste products such as lactic acid. These results also help to emphasise the ways in which cancer treatments behave is affected by the conditions in which the tumour cells are cultured and the importance of trying to bridge the gap between the laboratory bench and the patient bedside with a more appropriate culture conditions.

6.5 Chapter Conclusions

1. Repeated culturing of glycolysis dependent SKOV3 cells in 2DG results in the creation of a cell line (Glycolysis independent SKOV3 Cells) which are not reliant on glycolysis for survival but with have same parental lineage as SKOV3 cells.
2. Glycolysis independent SKOV3 cells are significantly more permissive to EnAd oncolysis than wildtype glycolysis dependent SKOV3 cells.
3. Antagonising glycolysis, using 2DG, boosts oncolytic virus replication and transgene expression in a murine xenograft model.

4. EnAd expressing TP53-inducible glycolysis and apoptosis regulator (TIGAR)
can be rescued and can lyse cells.
5. EnAd-CMV-TIGAR-GFP is functional, expresses TIGAR and is capable of
lysing cells without modulating culture pH.

7 Final Discussion

7.1 Thesis Overview

The central concept behind oncolytic virotherapy is that a single treatment of a virus, given intravenously, will self-amplify *in situ*, allowing targeted cancer toxicity with the possibility of treating metastatic disease. Once in the tumour, oncolytic viruses (OVs) can replicate in- and lyse cancer cells, then spread to neighbouring cells. Once they have lysed cells, OVs can create a pro-inflammatory environment that has the potential to trigger, or improve, antitumour immunogenic responses (Seymour and Fisher 2016). This simple concept is aided by the apparent inherent oncotropism of a wide array of viruses which can then be made even more tumour specific through attenuation or redirection of receptors (see chapter 2). However, despite the promise of oncolytic virotherapy, few viruses alone are potent enough to overcome the many challenges faced in a tumour environment. This has led prominent voices within the field to state this is impossible for an oncolytic virus to fully lyse all tumour cells within an individual after a single dose of systemically delivered virus (Matzinger 1994, 2001; Melcher 2018).

The potential lack of efficacy of oncolytic viruses has led some to argue for repeat dosing (Russell 2018), however this is not ideal for systemic viral therapy because multiple doses will trigger an antiviral immune response that could hamper delivery and treatment. Many groups have taken the approach of

“arming” oncolytic viruses, thereby allowing cancer-specific expression of encoded biologics in a self-amplifying manner whilst minimising off-target effects that would be present if the biological were administered systemically. The arming of viruses is a particularly important tactic as often OV are reduced in their potency for safety reasons or to limit their replication to tumour cells. Perhaps the most successful example of an armed OV is that of the herpes simplex virus Imlygic, the first FDA approved OV for treatment in cancer patients, which encodes granulocyte-macrophage colony-stimulating factor (GM-CSF, Andtbacka *et al.* 2015). Our research group has had particular success with encoding bispecific T cell engagers (BiTEs) that retarget endogenous T cell cytotoxicity towards tumour cells, or towards tumour-associated fibroblasts, or even towards the tumour-supportive immune system. We have also expressed DNase to degrade nucleic acids present in the interstitial cellular matrix and improve virus convection and spread (Tedcastle *et al.* 2016; Freedman *et al.* 2017, 2018).

Our ability to identify and exploit pathways with the potential to improve OV activity relies upon a thorough understanding of tumour cell biology and the interactions of viruses with their host cells. This is one of the reasons that, in the past two decades, vast improvements have been made in OV therapies as our scientific understanding has dramatically increased. One such area where our collective scientific knowledge has grown is that of metabolism, and it has been

observed by many that the metabolic phenotypes of tumour cells and virally-infected cells share some common overlapping features (Hanahan and Weinberg 2011; Miyake-Stoner and O'Shea 2014; Seymour and Fisher 2016; Thaker, Ch'ng and Christofk 2019; Dyer *et al.* 2020). Some tumour cells exhibit increased aerobic glycolysis (the Warburg effect) resulting in increased glucose consumption, increased lactate production and increased glutamine anapleurosis, and the most typical metabolic phenotype of virally infected cells is broadly similar.

The conditions used to study and develop OV_s *in vitro* are often suboptimal in mimicking the clinical challenge they will face. For example, many OV_s undergo initial evaluation in culture conditions which are optimised for the best cell growth with high levels of essential nutrients in growth media and with buffers that prevent development of an acidic extracellular microenvironment. We believe that creating more realistic culture conditions, for example with lower levels of essential nutrients, should allow for a narrowing of the gap between bench and bedside where many OV_s currently falter.

Understanding crucial metabolites for an OV platform may also allow productive innovation, for example by allowing identification of patients who are likely to respond through methods such as fluoro-deoxyglucose or fluoro-deoxyglutamine PET imaging. Additionally, altering the metabolic landscape within the tumour

through tactics such as dietary supplementation, the use of metabolic inhibitors, triggering the differential stress response through short-term starvation, or arming OV's with metabolic-modulating transgenes.

All viruses are obligate intracellular pathogens. For this reason, they are highly dependent upon the conditions in which their host cells reside. Oncolytic viruses are designed to replicate within cancer cells specifically and, as such, can only replicate in cells which often have highly unusual metabolism. Solid tumours contain hypoxic regions, regions with many necrotic cells, poor vasculature, areas of very low metabolite concentrations, and areas with high concentrations of metabolic waste. We must understand how any anti-cancer treatment will cope under such conditions, but it is particularly pertinent to viruses which are restricted to only infect and kill cells in these conditions. Because EnAd was selected in highly glycolytic cell lines and optimal culture conditions (Kuhn *et al.* 2008), it is unclear how EnAd will cope in more physiologically relevant metabolic conditions and if the metabolic requirements for wildtype adenoviruses in non-cancerous cells are the same as EnAd.

In this thesis, we have analysed the metabolic alterations that occur in two different cancer cell lines, one highly glycolytic (A549) and one with a more normal metabolic phenotype (SKOV3), upon infection with EnAd, with the wildtype viruses Ad11p (a parental virus for EnAd), and Ad5 (a widely studied adenovirus).

We observed an increase in glucose uptake, glutamine uptake and lactate secretion upon infection with each of the viruses. We also identified a correlation between cells which are dependent upon oxidative glucose metabolism and cells which are poorly permissive to oncolysis with EnAd. We then removed glutamine from the media of infected cells which severely inhibited the viral lifecycle, and which was only restored through the addition of α KG and not oxidative TCA cycle intermediates. Glucose withdrawal or inhibition of glycolysis, surprisingly, increased viral genome packaging of EnAd (particularly late cell protein synthesis) in highly glycolytic cells and, in glucose-dependent SKOV3 cells, and dramatically increased the speed of host cell lysis as well as viral progeny production.

These findings allowed us to postulate that an increased glutamine anapleurosis and increased flux of glucose into the pentose phosphate pathway was beneficial to EnAd lifecycle in cancer cells, particularly benefiting late-stage viral protein synthesis. This hypothesis was further bolstered through the creation of glycolysis-independent SKOV3 cells which became highly permissive to oncolysis. These findings lead us to investigate the co-administration of the glycolysis inhibitor 2DG alongside EnAd in *ex vivo* malignant ascites cancer cells and in a murine xenograft model, both of which showed increased late-stage viral protein synthesis although lysis was not measured. Finally, we cloned a genetic inhibitor of glycolysis TP53 inducible glycolysis and apoptosis regulator (TIGAR) into EnAd. We were able to clone successfully, and rescue, this virus (EnAd-CMV-TIGAR-GFP), and show that

the virus was able to replicate and produce infectious viral progeny without modulating culture pH.

7.2 Metabolic Assays

To measure basal levels of metabolism, we opted to first use the Seahorse metabolic analyser alongside analysis of glucose uptake, glutamine uptake and lactate secretion. We chose to use these systems as they are well established metabolic assays which provided an effective way to test numerous samples without lengthy sample preparations such as those involved with radioisotope assays with labelled glucose or glutamine (Potter, Newport and Morten 2016). These assays were effective and provided clear basal metabolic phenotypes with notable differences between cells, in keeping with previously reported work. Indeed, Hatzivassiliou *et al.* reported in 2005 that A549 cells take up high levels of glucose that is mainly secreted as lactate without entering the TCA cycle and SKOV3 cells route virtually all of their glucose into the TCA cycle, secreting little lactate (Hatzivassiliou *et al.* 2005). We do recognise that metabolomics and metabolic flux analysis would be powerful tools to further study the metabolic fate of glucose and glutamine and would undoubtedly highlight further unknown metabolic requirements of oncolytic viruses however we were unable to conduct such studies at this time.

In gaining a simple readout for basal metabolism, the precedent set by Pusapati *et al.* was particularly compelling and useful. Through exposing cells to the glycolysis inhibitor 2DG and analysing the IC_{50} of each cell line, they were able to analyse which cell lines easily could be classed as “glycolysis dependent” (an IC_{50} of $\leq 3\text{mM}$) or glycolysis independent (and IC_{50} above 3mM). This tactic allowed us to notice the apparent correlation between cells using glucose to fill the TCA cycle and cells which were poorly permissive to adenovirus oncolysis. This finding underpins a large amount of the remaining thesis.

We chose two cell lines (A549 and SKOV3) to continue analysing these findings as they represent the two polar metabolic phenotypes which are of interest to us. We also considered using PC9 cells as these have a similar cellular origin to A549 cells (lung adenocarcinoma) however these cells were so poorly permissive to oncolysis with EnAd that it would require either using 1000-fold more virus to lyse them at the same time as A549 cells or up to 300 additional hours of culturing to achieve lysis if they were infected with the same dose as A549 cells, both of which are variables that are hard to justify. Whilst we acknowledge that these two cell lines have some differences that make it difficult to point to metabolism as a sole independent variable, particular effort has been made to find multiple ways of verifying the results presented in this thesis. In particular, the creation of glycolysis-independent SKOV3 cells through repeated culturing in 2DG provides a compelling model with which to compare two cell lines with a single underlying

difference of metabolism. In considering future work to strengthen some of the findings discussed in this thesis, we would encourage the creation of glycolysis independent cells in some cell lines which are poorly permissive to EnAd lysis and are glycolysis dependent (such as PC9, IGROV and OVCAR8 cells) to see if the same pattern of improved oncolysis was observed.

7.3 Adenoviruses and glucose

In a seminal paper, Thai *et al.* reported that adenovirus early region E4ORF1 can bind to host cell MYC in the nucleus of infected cells and preferentially activate glycolytic targets that in turn promote a Warburg-like shift to anaerobic glycolysis causing an increase in lactic acid as well as an increased shift of glucose into the pentose phosphate pathways allowing increased nucleotides for viral replication (Thai *et al.* 2014). Thai *et al.* also reported that E4ORF1 decreases cellular respiration which, significantly hampers the oxidative TCA cycle and oxidative phosphorylation. Recent reports have also shown that the E1A protein of wildtype Ad5 can have a similar effect in A549 cells, increasing aerobic glycolysis and decreasing cellular respiration (Prusinkiewicz *et al.* 2020).

Another example of the interaction between adenoviruses and glucose is that of Ad36, a group D adenovirus. There is a correlation between the presence of antibodies for Ad36 and the presence of excessive body fat (Atkinson *et al.* 2005;

Ponterio and Gnessi 2015). Further research conducted in murine and avian models showed that infection with Ad36 resulted in a statistically significant weight gain (Dhurandhar *et al.* 2002) alongside an improved glycaemic control (Dhurandhar *et al.* 2000; Krishnapuram *et al.* 2011; Lin *et al.* 2013). These alterations were later found to be attributable to the E4ORF1 gene and its interactions with phosphatidylinositol 3-kinase (PI3K) and with MYC leading some research groups to suggest the potential of E4ORF1 as a template for developing antidiabetic therapies because of its ability to encourage glucose uptake in host cells (Dhurandhar *et al.* 2011, 2012; Krishnapuram *et al.* 2013; McMurphy *et al.* 2017).

These results help to contextualise our findings that cells which have increased glucose uptake due to a Warburg phenotype (such as A549 cells) could perhaps be a favourable environment that fosters viral replication. We postulate that this environment being present before early gene expression allows the virus to accelerate its replication cycle. Likewise, cells which have slower glucose uptake such as SKOV3 cells will require the creation of this glycolytic environment and so will slow down viral replication.

Given this hypothesis and the findings that adenoviruses increase glycolysis, our conclusion that inhibiting glycolysis improves oncolysis may at first seem to be incongruous. When considering why restricting glycolysis may be beneficial to the

virus, it is helpful to consider first what effect 2DG has upon glycolysis. As demonstrated in **figure 6.1**, 2DG is a competitive inhibitor of hexokinase. However, multiple research groups have found that 2DG encourages the shunt of glucose towards the pentose phosphate pathway (PPP) because of the inhibition of hexokinase stopping glycolysis (Ralser *et al.* 2008; Li *et al.* 2015b; Pusapati *et al.* 2016). We believe that, through treatment with 2DG, we are increasing glucose flux into the PPP, facilitating an increase in nucleotide synthesis, which is vital for adenovirus genome replication. This conclusion is bolstered by our findings in **figure 5.6A**, showing increased glucose uptake in the presence of 2DG. Additionally, we also believe that cells which are permissive to EnAd and cells treated with 2DG do not use glucose to create TCA metabolites but instead rely upon glutamine. Encouraging this metabolic phenotype of increased glucose shunted to the PPP and increased glutamine anapleurosis appears to be highly beneficial to EnAd.

As with 2DG treatment, a growing body of work investigating the metabolic alterations of tumour cells when treated with short-term starvation of glucose also presents a similar image. Several research papers now report that, upon starvation, tumour cells increase their nucleotide synthesis through the PPP which is thought to allow for a generation of NADPH to scavenge and protect from reactive oxygen species as well as allowing for a small amount of ATP production

and a large amount of nucleotide synthesis (Dong *et al.* 2013; Li *et al.* 2015b; Zhang *et al.* 2016; Cho *et al.* 2018).

We, therefore, propose that EnAd requires glucose flux through the PPP and does not require glucose to be used either through aerobic glycolysis or through the TCA cycle and that this preferential metabolic phenotype is created through 2DG treatment, through short-term glucose starvation or the expression of TIGAR in EnAd. To further confirm this, we would encourage the use of radio-labelled glucose which would elucidate the fate of glucose under each of the interventions mentioned above and, indeed, we aim to conduct these experiments in the future. Thai *et al.* provide an insight into this theory by showing that Ad5 infected non-cancerous cells increase glucose flux into the PPP, but we hope to further prove this for an OV in the context of tumour cells (Thai *et al.* 2014).

7.4 Adenoviruses and glutamine

Glutamine is a critical nutrient for a wide array of cellular biosynthetic processes. Many cancer cells rely on increased glutamine metabolism to a greater extent than normal cells, in part because altered aerobic glycolysis leaves a demand for TCA intermediates from an alternative source, which is often glutamine due to its abundance. Because viruses place a heavy demand upon host cells for anabolic metabolism to support proliferation, many viruses similarly require glutamine

anapleurosis.

We have shown that glutamine is crucial for EnAd replication and that, under conditions of glutamine starvation, EnAd can be rescued through the addition of α KG but not through the addition of TCA intermediates OAA or pyruvate. In 2015, Thai *et al.* reported that E4ORF1, as well as increasing glycolytic gene expression through MYC activation, also increases glutamine metabolism (Thai *et al.* 2015). Thai *et al.* reported that glutaminase inhibitors were very highly detrimental to the replication of Ad5 as well as HSV-1 and influenza type A in cultured primary human cells and suggested further research into the use of glutaminase inhibitors as a therapeutic strategy for antivirals. In the case of oncolytic viruses, we do not wish to inhibit viruses but instead increase their replication. We wanted to understand if the same weakness was present in EnAd, which is restricted to replicate in cells which already have aberrant glutamine metabolism.

To analyse the glutamine dependency of EnAd, we removed glutamine from the media. We found a severe impairment of the viral lifecycle, which was then rescued by the addition of α KG and not through the addition of pyruvate or OAA (**figures 4.6-4.8**). The concentrations of OAA, pyruvate and α KG used in the rescue experiments were taken from a previous study conducted by Fontaine *et al.* in 2014 who found that Vaccinia virus (VACV) did not require glucose for optimal replication but had a strong dependency upon glutamine metabolism (Fontaine,

Camarda and Lagunoff 2014). The paper reported that, upon conditions of glutamine starvation, OAA, pyruvate and α KG all could rescue VACV replication and that this defined a major metabolic requirement for the replication of poxviruses with a view to the discovery of novel antiviral therapies.

To understand the findings that α KG alone could rescue viral replication, we looked at the Thai *et al.* paper which utilised radio-labelled glutamine as a tool for understanding the metabolic fate of glutamine upon infection with wildtype Ad5 in normal primary human bronchial epithelial cells (Thai *et al.* 2015). This paper reported a unique pattern of radiolabelled glutamine after infection of cells with Ad5 suggesting that glutamine was being converted to α KG, then citrate and then malate in a manner that goes “backwards” from the usual oxidative cycle of the TCA. The paper concluded that the significance and advantages for adenovirus replication provided by reductive glutamine metabolism remain uncertain.

Previous work has shown that, particularly in cancer cells, increasing the α KG to citrate ratio in favour of α KG causes initiation of reductive glutamine metabolism (Mullen *et al.* 2012; Fendt *et al.* 2013). We believe that this could explain the improvement in viral protein synthesis observed in EnAd infected cells in replete media when excess α KG is added to the media (**figures 4.9 and 4.10**). Metabolomic analysis using U- $^{13}\text{C}_5$ -glutamine is a recommended future step, and one that our group aims to conduct. Likewise, the measurement of critical enzymes involved in

reductive carboxylation such as isocitrate 1 and 2 (IDH1 + 2), nicotinamide nucleotide (NNT), pyruvate dehydrogenase kinase 1 (PDK1) and aspartate transaminase (AST) during adenovirus infection will be a focus of future work. We believe that measuring these outcomes could garner insight into other targets to improve oncolysis as well as possibly being used as a marker of susceptibility before treatment.

Many papers find that reductive carboxylation of glutamine is advantageous for cancer for several reasons: the ability to generate acetyl-CoA; aiding acetylation and cell signalling; providing metabolites needed in lipid acid synthesis; anapleurosis to adapt to the lack of glucose entering the TCA cycle; and to produce TCA intermediates without requiring oxygen (particularly in conditions of defective mitochondria or hypoxia). When considering the benefits of reductive carboxylation to adenoviruses, it seems unlikely that lipids are needed for a non-enveloped virus unless these metabolites are feeding into another metabolic network, we believe it is likely that through E1A and E4ORF1, the virus upregulates a wide array of metabolic pathways including both glycolysis and glutaminolysis which in turn provides a highly anabolic environment allowing the virus to replicate easily. Adenovirus-induced increase in glycolysis is not required for viral replication, and may be a redundant consequence of MYC activation, which allows the increase in glutaminolysis necessary for viral replication. It may be that because the E4ORF1 interaction with MYC also decreases cellular respiration

(oxygen consumption rate), it could effectively inhibit oxidative metabolism leaving a particular reliance non-oxidative glutamine metabolism.

Despite the lack of clarity regarding the benefits of reductive carboxylation to the virus, it is evident that artificially creating an environment which promotes the use of glutamine benefits the virus. This was particularly apparent when we added excess α KG to replete media (**figures 4.9 and 4.10**). Likewise, in **figure 3.9**, we note that two cell lines that differ only in their Von Hippel-Lindau (VHL) status have noticeably different susceptibilities to viral oncolysis. The VHL^{-/-} cells have a mutated VHL meaning that they express hypoxia-inducible factor 1 α (HIF1 α) even in normoxic conditions because of a failure to degrade it via the ubiquitination pathway. The VHL mutations effectively mean that hypoxia-inducible genes are also expressed in normoxia. It has become apparent in recent years VHL^{-/-} cells have an increased glycolysis and PPP pathways (Kim *et al.* 2006; Papandreou *et al.* 2006; Lum *et al.* 2007; Gordan *et al.* 2008; Meléndez-Rodríguez *et al.* 2018) as well as recent reports that these cells display, and are reliant upon, reductive carboxylation of glutamine (Kaushik *et al.* 2020).

Considering our results in the context of the findings above, it is clear that, as with wildtype viruses in normal cells, EnAd has a high dependency upon glutamine. Further work remains to track the fate of glutamine during EnAd infection. We believe that creating an environment whereby cancer cells become more

dependent upon glutamine metabolism will bolster EnAd replication, and we have attempted to show that using an array of experiments from glucose starvation, to α KG addition. We also used more challenging models such as *ex vivo* and *in vivo* xenografts.

7.5 Arming EnAd with TIGAR

As previously discussed, encoding a transgene within an oncolytic virus (“arming the virus”) provides an attractive model for delivery, and production of high levels of a therapeutic directly within a cancer whilst minimising off-target toxicities. EnAd provides a good platform for arming an OV because, owing to the deletions in the E3 and E4 regions, the genome of EnAd is 2470 bp shorter than Ad11p. Additionally, because the life cycle of the virus is relatively well studied, we can control when transgenes are expressed allowing for the restriction of transgene expression to cells harbouring a productive infection through placing expression of a transgene under the control of the endogenous late viral promoter (MLP), via a splice-acceptor (SA) site.

As discussed in chapter 6, although increased glucose metabolism is a common feature of most cancers, which has allowed for powerful techniques such as the detection of [^{18} F] fluoro-2-deoxyglucose (FDG) using PET scanning, targeting this increased glycolysis can be difficult. Malignant cells largely use the same enzymes as normal cells to control this metabolism, and so targeting these enzymes can

have off-target effects. Using an oncolytic virus to express a transgene, therefore becomes a particularly compelling model as these viruses are already restricted to replicate solely within cancer cells.

Having shown that promoting glutaminolysis and inhibiting glycolysis (whilst promoting the PPP) can improve oncolysis, we sought to arm EnAd with a transgene that would create this metabolic environment and thus potentially increase oncolysis within tumour cells which are particularly resistant to EnAd (such as SKOV3 cells) which have an oxidative glucose metabolism. Many targets could be used to create this metabolic landscape in cells such as HIF1 α , PDHK, LDHA or IDH1 however we opted for TIGAR as this will have the most similar effect to 2DG treatment, in that it inhibits glycolysis whilst increasing the PPP and increases glutamine dependency. We successfully rescued a virus expressing TIGAR, which further confirms that glycolysis is not required during EnAd replication. Whilst we were unable to assess the effect of expressing TIGAR within tumour cells upon the virus especially in less permissive cells, we were able to show that expression of TIGAR did not inhibit viral propagation nor killing of A549 cells. Infection with EnAd-CMV-TIGAR-GFP did result in a loss of acidification of the media alongside measurable production of TIGAR-GFP.

7.6 Future Directions

In considering cancer as a whole, it would be remiss not to consider the effects of altering metabolism on the immune status of tumours. Little work is currently conducted on the impacts of oncolytic viruses upon the immune system focussed explicitly on the effects of altered metabolism however it remains clear that a large number of the platforms that oncolytic viruses are based upon will increase both nutrient competition and metabolic waste accumulation. Whilst it is true that the presence of viruses within the tumour environment has been shown increase the presence of immunostimulatory markers such as DAMPs and PAMPs and can cause immunogenic cell death of host cells, it remains unclear if this can outcompete the immunosuppressive environment that the presence of the virus will encourage because of the exaggeration of the Warburg effect (Fisher and Ginsberg 1957; Levy and Baron 1957; Ritter *et al.* 2010; Fontaine *et al.* 2015; Yogev *et al.* 2017; Gualdoni *et al.* 2018; Prusinkiewicz and Mymryk 2019).

Accumulation of lactic acid within tumours is a widely studied area. Many reports have claimed that tumour acidosis can promote radiotherapy- and chemotherapy-resistance and that this resistance can be alleviated or reduced through blocking glycolysis or blocking lactate production (Shimura *et al.* 2014; Wagner, Ciszewski and Kania 2015; Allen *et al.* 2016; Hao *et al.* 2016; Baltazar *et al.* 2020). There is a growing body of research showing that tumour-resident immune cells display an anergic, suppressive, senescent, or exhausted phenotype and that this immune environment is due, in part, to the low pH encountered in many solid tumours

(Calcinotto *et al.* 2012; Mendler *et al.* 2012; Huber *et al.* 2017; Erra Díaz, Dantas and Geffner 2018; Baltazar *et al.* 2020). Indeed, altering glycolysis or inhibiting lactate secretion has been shown to increase the effects of tumour immunotherapies (Calcinotto *et al.* 2012; Wu, Ying and Hu 2016; Damgaci *et al.* 2018).

Exciting research in this field was published earlier last year, highlighting the possibility of using oncolytic viruses as a vector to alter metabolism in favour of viral replication and improved immune cell phenotypes. Rivadeneira *et al.* report that immune responses to oncolytic viruses were incomplete due to metabolic insufficiencies induced by the tumour microenvironment and exacerbated by the presence of the virus (Rivadeneira *et al.* 2019). The authors then show that the adipokine leptin can metabolically reprogramme T cells and that engineering oncolytic viruses to express leptin in tumour cells induced complete responses in tumour-bearing mice and supported memory development in the tumour infiltrate. Meng *et al.* also reported that infection with oncolytic Newcastle disease virus caused an increase in immunosuppressive lactate alongside other suppressive factors which dampened the immune response (Meng *et al.* 2020). Through targeting aerobic glycolysis, Meng *et al.* showed a decrease in lactate accumulation in the tumour leading to improved antitumour efficacy and prolonged survival in mice models (Meng *et al.* 2020).

There is research currently being conducted in our group focussing around

reducing lactate accumulation in the tumour microenvironment using OVs, including the EnAd-CMV-TIGAR-GFP virus described in this thesis. We believe that, as well as having the potential to increase oncolysis in less permissive cancer cells, the newly created the EnAd-CMV-TIGAR-GFP could have the potential to improve oncolytic immunotherapy by decreasing tumour acidosis when compared to the parental EnAd.

There is a possibility that, by placing TIGAR under the control of the CMV promoter, the cancer specificity of EnAd could be lost. If EnAd can enter a normal cell and express the immediate early genes, it would result in the expression of TIGAR which may be enough to break specificity, and this is something we aim to analyse in future work. If this is the case, however, we will instead place the expression of TIGAR under the control of the major late promoter, thus ensuring that viral replication is achieved before TIGAR expression. Given that, in many of our assays, inhibiting glycolysis or increasing reductive carboxylation seemed to benefit late proteins, in particular, we believe that this would still be beneficial to the virus however it may diminish the effects of lactate and pH alterations. Additionally, as discussed in the introduction chapter, placing TIGAR under the control of a tumour-specific promoter or including a Tet-inducible repressor can also be used if the expression of the transgene is found to be leaky.

We recognise that the removal of a single metabolite is a somewhat simplistic tool for studying metabolism and we would strongly encourage future work to consider

a metabolomic analysis of infected, and uninfected cells to uncover the precise metabolic demands placed upon host cells after OV infection. Through the use of these methods, however, we have revealed previously unknown strengths, and weaknesses of EnAd which can be exploited, with the potential to improve the outcome of patients in the clinic.

7.7 Concluding remarks

This thesis had two overarching aims. Firstly, to understand how the EnAd lifecycle would be affected by reducing metabolites to physiologically relevant levels. The second aim was to characterise the relationship between host cell metabolism and adenoviral infection and identify strategies which could be employed to potentiate the activity of the OV, EnAd.

The approach taken built upon previous reports showing an increase in aerobic glycolysis and glutaminolysis in normal cells following wildtype adenovirus infection. Wildtype adenovirus Ad5, the parental adenovirus Ad11p, and EnAd all increased glucose and glutamine uptake as well as lactate secretion in highly glycolytic A549 cells as well as in oxidative SKOV3 cells. When comparing metabolism and susceptibility to EnAd oncolysis, a correlation emerged showing highly glycolytic cells are more likely to be susceptible to oncolysis.

EnAd and Ad11p replication cycles were significantly inhibited with removal of glutamine from the media of infected cells, and EnAd replication was only rescued through the addition of α K) and not oxaloacetate, or pyruvate to the media suggesting that the oxidative TCA cycle is not required for EnAd replication. Removal of glucose after virus entry inhibited viral replication, however cells that were starved of glucose for 48 hours to acclimatise and adapt to this novel condition were more permissive to viral lysis suggesting a beneficial enhancement to oncolysis when glycolysis is restricted.

Through treatment of highly permissive A549 cells with 2DG, we identified a decrease in unpackaged genomes and, in less permissive SKOV3 cells we saw a significant increase in viral protein synthesis, viral genome replication and speed of oncolysis, a finding that was repeated in *ex vivo* malignant ascites cells and a murine xenograft model when co-treated with EnAd and 2DG. Creation of glycolysis independent SKOV3 cells through repeated culturing of less permissive SKOV3 cells in increasing concentrations of 2DG resulted in the creation of a cell line that was highly permissive to EnAd. Finally, we cloned the glycolysis inhibitor TIGAR into EnAd. We observed expression of TIGAR alongside oncolysis without observing the modulation of culture pH. Although we were unable to assess if arming EnAd with TIGAR improved oncolysis (particularly in less permissive cell lines), the creation of this virus presents a novel platform for oncolytic virotherapy.

Through understanding the role of metabolism (and indeed other environmental stresses) in both viral and tumour biology, we believe there are insights to be gained to potentiate OV therapy and any other cancer treatment. Understanding tumour metabolism can allow us to target vulnerabilities in tumours and, ultimately, improve the outcome of treatment for patients. Whilst this work represents a small progress towards these grand aims, it also highlights the need to carefully consider the culture conditions in which pre-clinical evaluation of all cancer treatments are conducted.

Whilst precisely modelling the environment encountered *in vivo* can seem daunting, or even impossible, some methods can be adapted to attempt to bridge the gap between current culture conditions and those encountered in a tumour. Doing so will allow insight into previously unforeseen targets such as reducing glucose concentrations. Although metabolic requirements differ based on which virus is used, each OV requires a specific, yet definable, intracellular metabolic environment and they provide a prominent and, as yet, an underexplored platform with which to produce these conditions through the expression of metabolic reprogramming agents. OVs which can alter the tumour microenvironment to favour both virus infection and to create a more immune-stimulating environment represents an exciting step forward in the field of viro-immunotherapy with some promising results beginning to emerge to support this

theory (Li *et al.* 2015a; Esaki *et al.* 2016; Dyer *et al.* 2018; Al-Shammari *et al.* 2019; Scheubeck *et al.* 2019; Meng *et al.* 2020).

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Appendix I: Materials

Buffer	Application	Recipe
TAE buffer (50x)	Agarose gel electrophoresis	242 g Tris, 100 mL 0.5 M EDTA and 57.1 mL Glacial Acetic Acid in 1 L H ₂ O
Running buffer (10x)	Western blotting	19.8 g Tris, 86.4 g glycine, 6 g SDS, deionised H ₂ O to 1 L
Transfer buffer (10x)	Western blotting	30.29 g Tris, 144 g glycine, deionised H ₂ O to 1 L
MACS buffer	Flow cytometry	0.5% BSA, 2 mM EDTA in PBS
1.32p Caesium Chloride	Adenovirus banding	192 g CsCl, 367.2 mL of distilled H ₂ O, 40.8 mL of 0.5 M Tris-HCl (pH 7.9).
1.45p Caesium Chloride	Adenovirus banding	307.5g CsCl, 387 mL of distilled H ₂ O, 43.5 mL of 0.5 M Tris-HCl (pH 7.9).
40% glycerol	Adenovirus banding	165 mL glycerol, 10 mL of 0.5M Tris-HCl (pH 7.9), 2.5 mL of 0.2 M EDTA (pH 8.0), made up to 500 mL using dH ₂ O.
n-butanol	Adenovirus banding	20 mL of n-butanol is added to 20mls of distilled H ₂ O; once the solution has settled, the top layer is water-saturated n-butanol
TE buffer	Picogreen assay	5 mL of 1 M Tris-HCl pH 8, 1 mL of 0.5 M EDTA (pH 8.0), made up to 500 mL using dH ₂ O

BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; PBS, phosphate buffered saline

Appendix 2: DNA Sequences

CMV-TIGAR:

TTGAATCAATATTGGCAATTAGCCATATTAGTCATTGGTTATATAGCATAAATCAATATT
 GGCTATTGGCCATTGCATACGTTGTATCTATATCATAATATGTACATTTATATTGGCTCA
 TGTCCAATATGACCGCCATGTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATT
 ACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCGCGGTTACATAACTTACGGTAAA
 TGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCATTGACGTCAATAATGACGTATG
 TTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGG
 TAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTCCGCCCCCTATTGAC
 GTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTACGGGACTT
 TCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTTTTG
 GCAGTACACCAATGGGCGTGGATAGCGGTTTGACTCACGGGGATTTCCAAGTCTCCAC
 CCCATTGACGTCAATGGGAGTTTGTGTTTGGCACCAAATCAACGGGACTTTCCAAAATG
 TCGTAATAACCCCGCCCCGTTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTC
 TATATAAGCAGAGCTCGTTTAGTGAACCGTCAGAATTTTGTAAACGACTCACTATAGG
 GCGGCCGGGAATTCGTCGACTGGATCCGGTACCGAGGAGATCTGCCGCCGCGATCGCC
 ATGGCTCGCTTCGCTCTGACTGTTGTCCGGCATGGAGAAACAAGATTTAACAAGGAGA
 AAATAATCCAAGGACAAGGAGTAGATGAACCTCTTTCAGAACTGGATTTAAACAAGC
 AGCAGCTGCTGGTATATTTCTGAATAATGTGAAGTTTACTCATGCTTTCTCCAGTGATCT
 CATGAGGACAAAGCAGACCATGCATGGAATTTTGGAGAGAAGCAAATTTTGCAAAGAT
 ATGACGGTAAAGTATGACTCAAGACTTCGGGAAAGGAAATACGGGGTTGTAGAAGGC
 AAAGCGCTAAGTGAGCTGAGGGCCATGGCCAAAGCAGCCAGGGAAGAGTGCCCTGTG
 TTTACACCGCCCGGAGGAGAGACGCTGGACCAGGTGAAAATGCGTGGAATAGACTTTT
 TTGAATTTCTTTGTCAACTAATCCTGAAAGAAGCGGATCAAAAAGAACAGTTTTCCCAA
 GGATCTCCAAGCAACTGTCTGGAACTTCTTTGGCAGAGATATTTCTTTAGGAAAAAA
 TCACAGCTCTAAAGTTAATTCAGACAGCGGTATTCCAGGATTAGCAGCCAGTGTCTTAG
 TTGTGAGTCACGGTGCTTACATGAGAAGTCTGTTTGATTATTTCTGACTGACCTTAAGT
 GTTCCTTACCAGCCACTCTGAGCAGATCTGAACCTTATGTCAGTCACTCCCAATACAGGG
 ATGAGTCTCTTTATCATAAACTTTGAGGAAGGAAGAGAAGTTAAACCAACGGTTCAGTG
 TATTTGTATGAACCTACAGGATCATCTAAATGGACTGACTG

GFP-PolyA:

ATGGAGAGCGACGAGAGCGGCCTGCCCCCATGGAGATCGAGTGCCGCATCACCGGC
ACCCTGAACGGCGTGGAGTTCGAGCTGGTGGGCGGCGGAGAGGGCACCCCCGAGCAG
GGCCGCATGACCAACAAGATGAAGAGCACCAAAGGCGCCCTGACCTTCAGCCCCCTACC
TGCTGAGCCACGTGATGGGCTACGGCTTCTACCACTTCGGCACCTACCCCAGCGGCTAC
GAGAACCCCTTCCTGCACGCCATCAACAACGGCGGCTACACCAACACCCGCATCGAGA
AGTACGAGGACGGCGGCGTGCTGCACGTGAGCTTCAGCTACCGCTACGAGGCCGGCC
GCGTGATCGGCGACTTCAAGGTGATGGGCACCGGCTTCCCCGAGGACAGCGTGATCTT
CACCGACAAGATCATCCGCAGCAACGCCACCGTGGAGCACCTGCACCCCATGGGCGAT
AACGATCTGGATGGCAGCTTCACCCGCACCTTCAGCCTGCGCGACGGCGGCTACTACA
GCTCCGTGGTGGACAGCCACATGCACTTCAAGAGCGCCATCCACCCCAGCATCCTGCAG
AACGGGGGGCCCCATGTTCCGCTTCCGCCGCGTGGAGGAGGATCACAGCAACACCGAGC
TGGGCATCGTGGAGTACCAGCACGCCTTCAAGACCCCGGATGCAGATGCCGGTGAAGA
AAGAGTTTAAACGGCCGGCCGCGGTCATAGCTGTTTCCTGAACAGATCCCGGGTGGCA
TCCCTGTGACCCCTCCCCAGTGCCTCTCCTGGCCCTGGAAGTTGCCACTCCAGTGCCAC
CAGCCTTGTCTAATAAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCTATAAT
ATTATGGGGTGGAGGGGGGTGGTATGGAGCAAGGGGCAAGTTGGGAAGACAACCTG
TAGGGCCTGCGGGGTCTATTGGGAACCAAGCTGGAGTGCAGTGGCACAATCTTGGCTC
ACTGCAATCTCCGCCTCCTGGGTTCAAGCGATTCTCCTGCCTCAGCCTCCCGAGTTGTTG
GGATTCCAGGCATGCATGACCAGGCTCAGCTAATTTTGTGTTTTTGGTAGAGACGGGG
TTTACCATATTGGCCAGGCTGGTCTCCAACCTCTAATCTCAGGTGATCTACCCACCTTG
GCCTCCCAAATTGCTGGGATTACAGGCGTGAACCACTGCTCCCTTCCCTGTCCTTCTGAT
TTTAAATAACTATACCAGCAGGAGGACGTCCAGACACAGCATAGGCTACCTGGCCAT
GCCCAACCGGTGGGACATTTGAGTTGCTTGCTTGGCACTGTCC

Appendix 3: Primers

Ad11p/EnAd forward primer: TACATGCACATCGCCGGA

Ad11p/EnAd reverse primer: CGGGCGAACTGCACCA

Ad11p/EnAd probe:

[6FAM]CCGGACTCAGGTACTCCGAAGCATCCT[TAM]

DLD1 human beta-2-microglobulin forward primer:

ATCCGACATTGAAGTTGA

DLD1 human beta-2-microglobulin reverse primer:

CCCACTTAACTATCTTGGG

DLD1 human beta-2-microglobulin probe: TCACACGGCAGGCATACTCA

CMV TIGAR Gibson forward primer:

TTTGCGATCGCTACCCTGCAACATTGAATCAATATTGGCAATTAGCC

CMV TIGAR Gibson reverse primer:

CTAAATGGACTGACTGAACTCGCACGCGTACGCGGCCGCTCGAG

GFP PolyA Gibson Forward primer:

AAACTCGCACGCGTACGCGGCCGCTCGAGATGGAGAGCGACGAGA

GFP PolyA Gibson Reverse Primer:

CAAATAAACAAGTTCCTGCATCTACTGAGTGGACCCAAC