Effect of adenovirus infection on the Hypoxia-inducible factor 1 pathway



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Abstract

Low oxygen (hypoxia) and stabilisation of the Hypoxia-Inducible Factor 1 alpha (HIF-1α) have been reported to be either beneficial or detrimental to the efficacy of candidate oncolytic viruses such as reovirus, vesicular stomatitis virus or Newcastle disease virus. We have found that Enadenotucirev (an oncolytic group B adenovirus) has increased activity in hypoxia compared with normoxia. In order to understand how hypoxia benefits EnAd, we studied the HIF-1 pathway during virus infection.

Here we have shown that EnAd infection leads to down-regulation of the HIF-1α protein in certain virus-permissive cell lines in hypoxia. We also report a similar effect after infection with wild type adenoviruses (Ad5 and Ad11). To determine the mechanisms underlying this down-regulation, we measured the mRNA levels of *HIF-1α* and its target genes by reverse transcription quantitative PCR and found that all three viruses down-regulate *HIF-1α* transcripts in both normoxia and hypoxia. We also found that this down-regulation is independent of the proteasome complex.

Our data has also shown that AD293 cells (which have Ad5 E1 gene) have lower basal levels of HIF-1α protein compared to other cell lines. Since several studies suggest that adenovirus E1A regulates many cellular genes, we speculate that adenovirus mediated down-regulation of *HIF-1α* mRNA is achieved by E1A. Future work will look at the role of E1A in regulating the HIF-1 pathway.

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List of abbreviations

Ad Adenovirus

bHLH Basic-helix-loop-helix

bp Base pairs

CAIX Carbonic anhydrase 9

CAR Coxsackievirus B and adenovirus receptor

CD46 Cluster of differentiation 46

cDNA Complementary DNA

CMV Cytomegalovirus

CRUK Cancer research United Kingdom

DMEM Dulbecco's Modified Eagle's Medium

DMSO Dimethyl sulfoxide

DNA Deoxyribonucleic acid

DPBS Dulbecco's phosphate-buffered saline

EMA European medicines agency

EnAd Enadenotucirev

FBS Foetal bovine serum

FDA food and drug administration

FIH Factor inhibiting HIF-1

GLUT1 Glucose transporter 1

GLUT3 Glucose transporter 3

GM-CSF Granulocyte-macrophage colony-stimulating factor

HIF Hypoxia inducible factors

HIF-1 Hypoxia inducible factor 1

HIF-1α Hypoxia inducible factor 1 alpha

HIF-1β Hypoxia inducible factor 1 beta

HIF-2 Hypoxia inducible factor 2

HIF-3 Hypoxia inducible factor 3

HRE Hypoxia response element

HSV Herpes Simplex virus

MLP Major late promoter

MOI Multiplicity of infection

mRNA messenger RNA

MV Measles virus

N/A Not applicable

NDV Newcastle disease virus

NPC Nuclear pore complex

ODDD Oxygen-dependent degradation domain

OV Oncolytic virus

PCNA Proliferating cell nuclear antigen

PCR Polymerase chain reaction

PD-1 Programmed cell death protein 1

PDGF Platelet derived growth factor

PDK Pyruvate dehydrogenase kinases

Pfu Plaque forming units

PHD Prolyl-4-hydroxylases

***p*O_2** Partial oxygen pressure

qPCR quantitative PCR

RIPA Radioimmunoprecipitation assay

RNA Ribonucleic acid

RT-qPCR Real time qPCR

SDS-PAGE Sodium dodecyl sulphate polyacrylamide gel

SP-1 Specificity protein 1

T-VEC Talimogene laherparepvec

TAD transactivation domains

TBP TATA-binding protein

TCA Tricarboxylic acid cycle

TGF-β Transforming growth factor beta

UV ultraviolet

VEGF Vascular endothelial growth factor

VHL von Hippel-Lindau

vp Virus particles

VSV Vesicular Stomatitis virus

WHO World Health organisation

wt Wild type

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Chapter 1: Introduction

1.1 Hallmarks of cancer

Cancer is a major health concern for humankind and has been one of the top five causes of death worldwide for about three decades now (Fitzmaurice et al., 2017). According to Cancer Research United Kingdom CRUK (2016), about 28% of all deaths in the UK are caused by cancer and a fifth of these deaths are attributed to lung cancer. This year on world cancer day, World Health Organization (WHO) further stressed that cancer still remains a threat to public health causing one in six deaths globally (World Health Organization, 2018). Therefore, despite an increase in our understanding of cancer and a wide range of therapies developed to "treat" cancer, there still remains an urgent need to develop more robust therapies in order to reduce the burden of mortalities caused by cancer.

Biologically, cancer is abnormal growth of cells marked by certain characteristics which were termed as "hallmarks of cancer" by Robert Allan Weinberg and Dough Hanahan. There are ten hallmarks of cancer: constitutive cell division, replicative immortality, evasion of growth suppressors, escaping immune system, tumour-promoting inflammation, metastasis, angiogenesis, genomic instability, avoiding cell death and deregulating cellular genes (Hanahan and Weinberg, 2011). Since these hallmarks are essential for tumour progression and metastasis, they are often the target of current therapies against cancer (Figure 1).

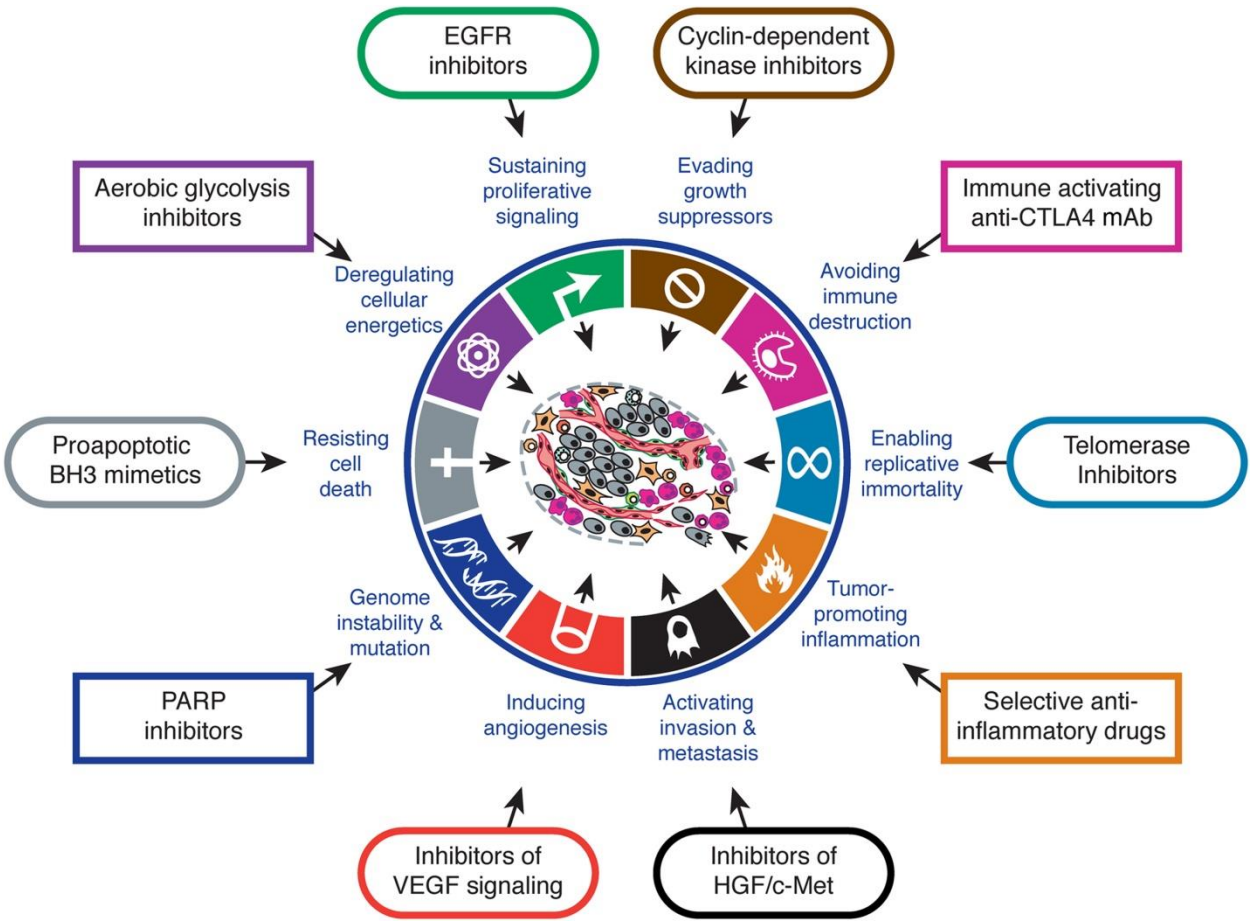

Figure 1. Hallmarks of cancer and current therapies targeting them. The ten hallmarks of cancers are the principle characteristics of cancer biology. Several therapies are designed to target one or more of these hallmarks. (Figure is adapted from Hanahan and Weinberg, 2011)

1.2 Cancer and hypoxia

As the hallmarks of cancer ensure the survival, growth and metastasis of tumour cells, they drastically change tumour microenvironment. One essential component of this microenvironment is oxygen tension. As tumour cells proliferate rapidly, they require more nutrients and oxygen. This high demand for nutrients and oxygen leads to the development of new blood vessels (angiogenesis). However, these blood vessels are either aberrant or develop at a rate lower than that of tumour cells (Folkman, 1995). This leads to a deprivation of oxygen

(hypoxia) in regions of the tumour that are far (>70µm) from vasculature or are in the vicinity of aberrant vessels (Thomlinson, 1977). In fact, it has been suggested that due to extreme deprivation of oxygen and nutrients most solid tumours tend to develop a necrotic core (Brahimi-Horn, Chiche and Pouysségur, 2007). However, oxygen tension varies among tumours and ranges from 0.3% to 4.2% pO_2 with most tumour having a median value of < 2% pO_2 (McKeown, 2014).

The role of hypoxia in cancer is mostly helpful and is often associated with aggressive cancer phenotype. Firstly, in order to alleviate the nutrient and oxygen deprivation, hypoxia upsurges angiogenesis to increase blood supply to the tumour mass. This is mediated by induction of pro-angiogenic genes like vascular endothelial growth factor (VEGF) (Steinbrech et al., 1999), aiding the migration of endothelial cells to tumour mass providing building blocks for blood vessels, and vessel maturation by inducing angiopoietin 1, platelet-derived growth factor (PDGF) and transforming growth factor beta (TGF-β) (Krock, Skul and Simon, 2011). Although these neovessels are often still aberrant and leaky, depending on the tumour, they either meet the oxygen demand or cause even more severe hypoxia (Carmeliet, 2005). Secondly, a hallmark of cancers is the shift in glucose metabolism to a glycolytic phenotype. Hypoxia forces cancer cells to adapt to this change by increasing glycolysis. During hypoxia, cells increase the expression of glucose transporters 1 and 3 (GLUT1 and GLUT3) to meet the glucose demand of cells. Hypoxia also induces pyruvate dehydrogenase kinases (PDK) which inactivate pyruvate dehydrogenase and reduce its flux into the tricarboxylic acid cycle (TCA) further facilitating glycolysis (Semenza, 2010). Thirdly, tumour hypoxia is strongly correlated with cell invasion and metastasis. Hypoxia facilitates metastasis by selecting for the most 'fit' cells which are capable

of surviving in extreme conditions and have an aggressive phenotype (Luoto, Kumareswaran and Bristow, 2013), by driving these 'fit' cells towards oxygenated regions (Chang and Erler, 2014) and by regulating extracellular matrix to support cell invasion and vascular permeability (Gilkes, Semenza and Wirtz, 2014). Fourthly, hypoxia causes resistance to most commonly used therapies for cancer (discussed in detail in section 1.4).

1.3 Hypoxia inducible factor 1 (HIF-1) pathway

There are three hypoxia inducible factors (HIF-1, HIF-2 and HIF-3) which modulate cellular gene expression during adaptation to hypoxia. All three of these share the common HIF1-β subunit which forms a dimer with HIF-1α, HIF-2α or HIF-3α to produce functional transcription factors, which then bind to DNA sequences called hypoxia response elements (HRE) in promoters of hypoxia induced genes (Bracken, Whitelaw and Peet, 2003). The HIF1-β subunit is oxygen-independent and constitutively produced in cells, whereas the HIF-α subunits are oxygen-dependent and stabilised at < 5% pO_2 depending on the type of HIF. HIF-1α protein is stabilised at < 2% pO_2 and is essential during early stages of hypoxia (Loboda, Jozkowicz, and Dulak, 2010). HIF-2α protein is stabilised at < 5% pO_2 during prolonged periods of hypoxia (Holmquist-Mengelbier et al., 2006). HIF-3α protein is stabilised at < 5% pO_2 and is known to compete with both HIF-1α and HIF-2α and antagonize their function (Yang et al., 2015). Even though all HIFs have distinct roles in hypoxia, the role of HIF-1 is the most understood or investigated in tumour hypoxia.

HIF-1 was first discovered by Professor Gregg L. Semenza in 1991 during the study of hypoxia regulated human erythropoietin gene (Semenza et al., 1991). The structure of the HIF-1 protein

was also elucidated by Semenza lab in 1995. They showed that both HIF-1α and HIF-1β subunits have basic-helix-loop-helix (bHLH) motif which allows heterodimer formation between subunits and also allows binding to HREs to regulate target gene expression (Wang et al., 1995). HIF-1α subunit has two transactivation domains (TAD) as well. The NH_2-terminal (N-TAD) domain is required for HIF-1α stability and COOH-terminal (C-TAD) domain is required to bind co-activators such as p300/CBP. In addition to these, HIF-1α also has an oxygen-dependent degradation domain (ODDD) which is responsible for oxygen mediated degradation of HIF-1α (Lee et al., 2004).

In normoxia, HIF-1α is inactivated via two pathways. The first pathway involves direct degradation of HIF-1α protein via proteasomal pathway. Two proline residues in the ODDD were found to be recognised by prolyl-4-hydroxylases (PHDs) which hydroxylate these residues in the presence of oxygen, iron, 2-oxoglutarate and ascorbate (Bruick and McKnight, 2001). This hydroxylated HIF-1α subunit is then recognised and ubiquitinated by von Hippel-Lindau protein (pVHL) which is an E3 ubiquitin protein ligase (Maxwell et al., 1999). The ubiquitinated HIF-1α subunit is recognised and degraded by the proteasome complex (Salceda and Caro, 1997). During hypoxia, PHDs fail to hydroxylate most HIF-1α protein due to decreased oxygen levels and the proteins are, thus, stabilised (Figure 2).

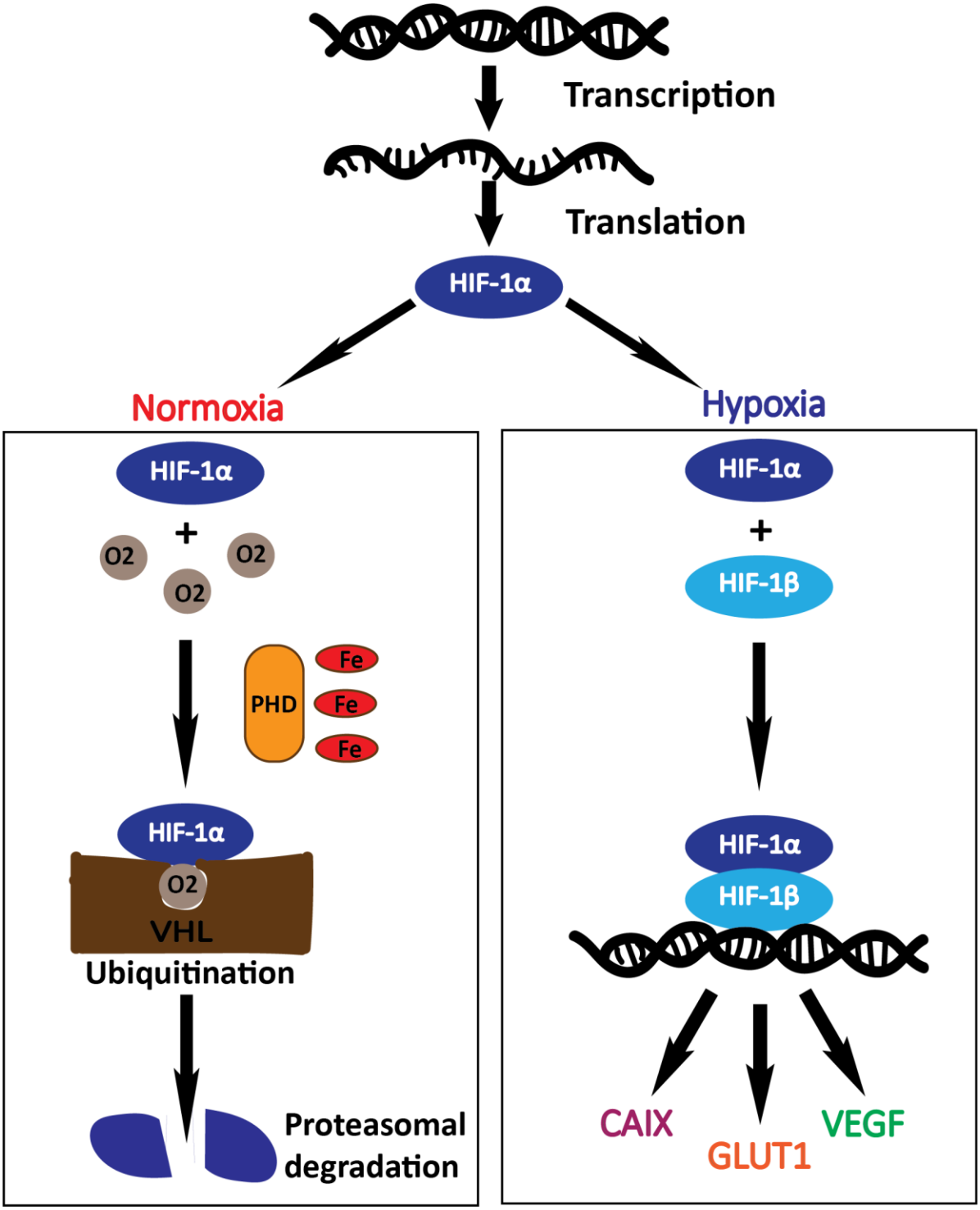

Figure 2. The HIF-1 pathway. *HIF-1α* gene is constitutively expressed in both hypoxia and normoxia but the protein is only stabilised in hypoxia. In normoxia HIF-1α protein is degraded via proteasomal pathway shown in the figure.

The second pathway involves inactivation of HIF-1α protein to inhibit its transactivation ability. HIF-1α needs to bind p300/CBP for transcriptional regulation of its targets. In normoxia, an asparagine residue is hydroxylated by asparaginyl hydroxylase called factor inhibiting HIF-1 (FIH-1). This hydroxylation prevents the binding of p300/CBP to C-TAD abrogating the transactivation function of HIF-1 (Lando et al., 2002; Mahon, Hirota and Semenza, 2001). During hypoxia, FIH-1 fails to hydroxylate HIF-1α restoring its transcriptional activity.

HIF-1 protein controls the regulation of over hundred genes in hypoxia. In tumour hypoxia, HIF-1 is found to be the major cause of hypoxia-associated aggressiveness of tumours (Majeed and Amir, 2007). It is the main regulator of genes involved in angiogenesis (Manola et al., 2005) and metabolic responses in hypoxia (Semenza et al., 2013). HIF-1 is also shown to directly regulate twist transcription factor which has a critical role in mediating metastasis (Yang and Wu, 2008). In fact, some reports suggest that HIF-1 plays an essential role in hypoxia mediated drug resistance in cancer (Lv et al., 2015; Warfel and El-Deiry, 2014). Therefore, considering HIF-1 signaling is crucial when developing cancer therapeutics.

1.4 Effect of hypoxia on current cancer therapies

Chemotherapy is one of the most commonly used therapies for cancer. It has a broad range of mechanisms which vary from drug to drug. However, most chemotherapy drugs fail to eradicate hypoxic areas of tumours. It is proposed that hypoxia might be directly or indirectly involved in this event. Hypoxia directly causes resistance to drugs that are dependent on oxygen or oxygen-mediated microenvironment. For example, Bleomycin is a drug used for testicular, ovarian and cervical cancer. It relies on oxygen species to produce free radicals which

cause DNA damage. In low oxygen conditions, the drug fails to form free radicals and thus its activity is significantly reduced in hypoxia (Teicher, Lazo and Sartorelli, 1981). Hypoxia causes indirect resistance to drugs by providing a physical barrier for drugs to reach hypoxic regions due to leaky blood vessels or increased distances from the vasculature, by altering pH which affects the toxicity of certain pH dependent drugs, and by producing proteins that inhibit drugs (Shannon, Aoife M., et al., 2003).

Radiotherapy which is another conventional therapeutic platform used for most solid tumours is oxygen-dependent (Teicher, 1994). Although the mechanism is not fully understood, there are at least two explanations for how hypoxia causes radio-resistance. Firstly, it is proposed that radiation causes DNA damage by producing radicals on DNA strands. These radicals can either be oxidised by oxygen (which makes the damage permanent) or reduced primarily by sulfhydryl-containing compounds (which can reverse the damage). In hypoxia, there is more reduction and thus DNA damage is not as effective (Brown, J. Martin, and William R. Wilson, 2004). Secondly, hypoxic tumour cells are well-equipped to deal with severe stresses like apoptosis (Calderwood et al., 2006) and DNA damage (Bristow and Hill, 2008). Therefore, even if radiation produces significant DNA damage, there will be hypoxic cells capable of evading the damage (Harrison and Blackwell, 2004).

Immunotherapy for cancer treatment is a relatively new therapy. It relies on three principles: 1) anti-cancer vaccines which stimulate immune system to recognise and destroy tumour cells, 2) checkpoint inhibitors which block the signals that prevent T-cells from killing tumour cells thereby facilitating tumour-killing by T-cells and, 3) facilitated adoptive immunotherapy which

involves engineering a patient's own tumour reactive T-cells in vitro and then their delivery into the same patient. There is not much known about the effect of immunotherapy on hypoxic regions of tumours. However, it is proposed that hypoxia supports an immunosuppressive microenvironment which can be attributed to abnormal vasculature and presence of immunosuppressive factors (Huang et al., 2013). Doedens et al. (2010) showed that expression of HIF-1α in tumour-associated macrophages (TAM) led to suppression of tumour infiltrating T-cells. Some studies have suggested that alleviating hypoxia improves the outcome of immunotherapy in hypoxic regions (Noman et al., 2014; Sun et al., 2001). However, further research on the impact of hypoxia is required to understand the full potential of immunotherapy in targeting hypoxic regions of tumours.

1.5 Oncolytic virotherapy

Oncolytic virotherapy is an emerging therapy which relies on the principle that viruses can selectively target and kill tumour cells and evoke anti-tumour immune response. The potential of viruses as oncolytic agents was realised in 1950s, but it is only in the recent decades that this therapy has been taken seriously (Kelly and Russell, 2007). Oncolytic viruses (OV) fall into two groups on the basis of their ability to selectively kill cancer cells. First group consists of viruses like reovirus, Newcastle disease virus (NDV) and mumps virus which have intrinsic ability to grow in tumour cells (Southam, 1960). The second group consists of viruses like measles, vaccinia virus, adenovirus, Vesicular Stomatitis virus (VSV) and Herpes Simplex virus (HSV) which can be engineered to selectively kill cancer cells (Liu and Krin, 2008). Regardless of which group OVs belong to, they are a great candidate to kill cancer cells. Several hallmarks of cancer, like constitutive cell division, evasion of growth suppressors, escaping immune system, genomic

instability, avoiding cell death and deregulating cellular genes, are indeed found in virus infection as well. In fact, it can be said that most hallmarks of cancer are similar to “hallmarks of virus infection”. This makes it possible to engineer OVs that lack one or more of these hallmarks in order to ensure that they selectively replicate in tumour mass and cause oncolysis.

Currently, there are many ongoing trials with different oncolytic viruses (Pol et al., 2014; Russell, Peng and Bell, 2012); however, there are only three oncolytic viruses that have been approved so far. Rigvir, a nonpathogenic ECHO-7 picornavirus, was the first-ever virus to be approved as oncolytic virotherapy against melanoma in Latvia in 2004. It was later approved in Georgia in 2015 (Donina et al., 2015). Despite promising pre-clinical and clinical data, Rigvir has not become a mainstream treatment due to lack of sufficient trials required for US food and drug administration (FDA) or European medicines agency (EMA) (Babiker et al., 2017). Shortly after Rigvir approval in Latvia, Oncorine or H101 (an E1B-deleted adenovirus) was approved in China as oncolytic virotherapy against head and neck cancer in 2005 (Yu and Fang, 2007). Unfortunately, the clinical data and use of Oncorine remains limited to China due to inadequate phase III clinical trials required for US FDA (Liang, 2012). Talimogene laherparepvec (T-VEC), an attenuated herpes simplex virus 1 (HSV-1) expressing granulocyte-macrophage colony-stimulating factor (GM-CSF), was the first oncolytic virus to be approved by US FDA in 2015 and then in Europe and Australia in 2016 (Pol, Kroemer and Galluzzi, 2016). The approval of T-VEC at such large scale for treatment of melanoma revolutionised the field of oncolytic virotherapy and revived the interest in the development of more robust OVs.

Even though there is great promise in oncolytic virotherapy, it has its fair share of challenges. Firstly, the route of virus delivery is still largely limited to intratumoural injection which is not ideal for targeting metastatic tumours. While there are several studies to suggest that systemic delivery of different OVs like measles virus (MV-NIS), adenovirus (EnAd) and Newcastle disease virus (PV701) manage to achieve the destruction of the disseminated tumour (Gil et al., 2014; Pecora et al., 2002; Russell et al., 2014), there is no consensus about the efficiency of intravenously delivered OVs and the role of pre-existing neutralising antibodies in systemic delivery (Ferguson, Lemoine and Wang, 2012). Secondly, whether or not immune system is beneficial to virotherapy is still poorly established and under debate (Prestwich et al., 2009). While initial trends in the field were to engineer OVs to evade host immune responses to benefit direct and efficient oncolysis in tumours (Fulci et al., 2006), there recent trends have taken a shift towards provoking immune system as a result of OVs infection providing an interface between virotherapy and immunotherapy (Melcher et al., 2011). Thirdly, the efficiency of OVs to target hypoxic tumours is poorly understood and varies from virus to virus (Morinet et al., 2015; Vassilaki and Frakolaki, 2017).

1.6 Adenoviruses as oncolytic viruses

Adenoviruses are DNA viruses which were first discovered by Rowe and colleagues while studying human adenoid tissues, hence the name adenovirus (Rowe et al., 1953). Since then adenoviruses have been discovered in many different mammalian and avian species. There are over 50 distinct serotypes of human adenoviruses that have been isolated. These have been divided into seven groups (A-G) based on their genetic, biological and physicochemical properties (Xu, McDonough and Erdman, 2000). Even though there are a great number of

adenoviruses infecting humans, they have been shown to cause only mild respiratory, ocular or gastrointestinal diseases, especially in children (Ghebremedhin, 2014).

Adenoviruses are 70-90 nm non-enveloped icosahedral particles. Virus particles consist of an outer protein capsid, an inner nucleoprotein core and approximately 10 copies of adenovirus protease. The capsid is made of trimers of the hexon proteins (which are trimeric proteins forming hexagonally packed arrays) with the penton bases (pentameric structural proteins) sitting at the vertices and providing a base for fiber proteins to bind (Figure 3). There are also several other proteins like pIIIa, pVI, pVIII and pIX which hold hexons and pentons in place. The core consists of viral DNA associated with protein pV, mu and pVII. (Jörnvall et. al, 1981; Vellinga, Heijdt and Hoeben, 2005; Zubieta et al., 2005).

Adenovirus genome is about 36 kb long, double stranded and linear DNA with terminal proteins covalently bound to both ends (Lichy, Horwitz and Hurwitz, 1981). The genome is organized into early and late genes. Early genes are transcribed immediately after infection and consist of E1A, E1B, E2A, E2B, E3 and E4 transcription units. E1A is the most important gene in adenovirus as it regulates the expression of both viral and cellular genes to facilitate virus replication (Nevins, 1981). E1B proteins have anti-apoptotic functions (Yew, Liu and Berk, 1994). E2 genes encode proteins involved in viral replication like terminal proteins and DNA polymerase (Gorziglia et al., 1996). E3 region proteins are involved in anti-immune responses to facilitate persistent infection (Wold and Gooding, 1991). E4 region proteins have diverse roles in cell cycle regulation and anti-apoptotic signaling (Halbert, Cutt and Shenk, 1985). Late genes (L1 to L5), which are mainly under the control of the major late promoter (MLP), are responsible for

producing structural proteins which later form viral capsids (Young, 2003). Another product of late genes are virus associated RNAs (VA RNA I and II) which are involved in inhibiting interferon response and influencing cellular gene expression via micro-RNA processing (O'Malley et al., 1986; Xu et al., 2007).

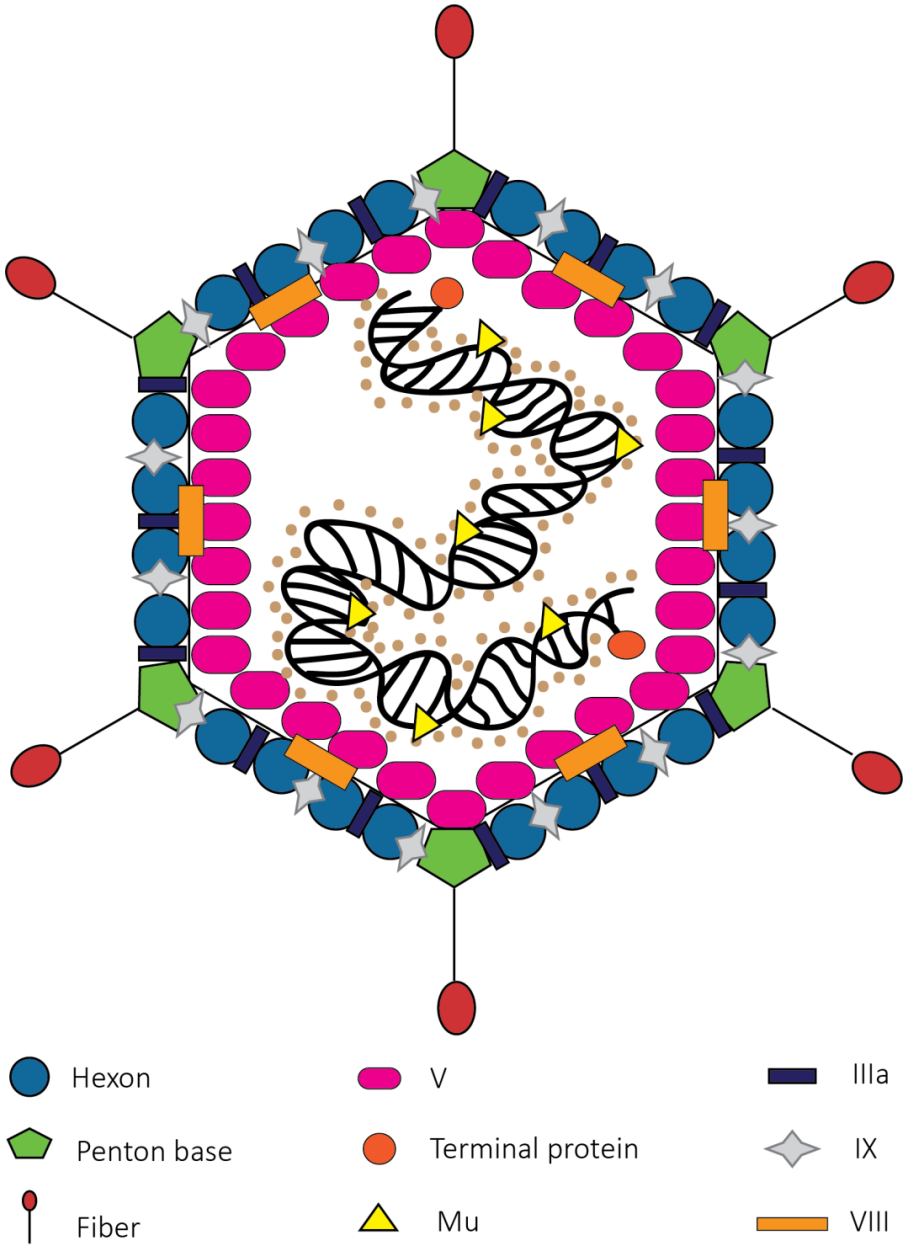

Figure 3. Human adenovirus particle structure. The figure shows a schematic cross-section of adenovirus core and capsid.

Adenovirus lifecycle consists of four main events: a) virus attachment and uptake, b) virus replication, c) virion assembly and d) virus release. Adenovirus attachment to cells is mediated by interaction of fiber protein with coxsackievirus B and adenovirus receptor (CAR) for all subgroups except for group B viruses which require CD46 receptor for attachment (Bergelson et al., 1997; Gaggar, Shayakhmetov and Lieber, 2003). The attachment of fiber protein to respective cellular receptor leads to clathrin-dependent endocytosis of virus particle (Meier et al., 2002). The acidic environment of the endosome facilitates release of virion in the cytoplasm which is then transported to nucleus via microtubules in a dynein-dependent process (Bremner et al., 2009). Once virus reaches the nucleus, it docks on the nuclear pore complex (NPC) where capsid is disassembled and genome is transported inside the nucleus and transcribed (Greber, et al., 1997). Viral early then carry out host cell cycle modulation, antiapoptotic response and viral replication while late genes form structural proteins. Virus assembly takes place inside the nucleus. Even though hexon, penton and fiber proteins are translated in the cytoplasm, hexon trimers are transported to nucleus via viral pVI protein (Snijder et al., 2014). Inside the nucleus, hexon proteins are associated with penton, fiber and other minor proteins to form capsid shells. These empty capsids are then filled with virus genome with the help of virus proteins and a packaging sequence present in viral DNA (Wodrich et al., 2003). Once capsids are formed, adenovirus protease cleaves certain proteins to from mature virus particles (Pérez-Berná e al., 2012). These virus particles are then released from cell after E3 11.6K protein mediated cell lysis (Tollefson, Ann E., et al., 1996). Released viruses can infect the neighboring cells and restart the entire virus lifecycle. (Figure 4)

Adenoviruses are a great candidate for oncolytic virotherapy for several reasons. Firstly, adenoviruses are very easy to engineer which can be exploited to increase their tumor-specificity (He et al., 1998). Secondly, adenoviruses are self-replicating agents and should be able to destroy the entire tumour mass. Thirdly, adenovirus genome allows the addition of a reasonable number of transgenes which have been successfully shown to replicate and express along with viral genes (Benihoud, Yeh and Perricaudet, 1999). Fourthly, the mass production of oncolytic adenoviruses for clinical use is very feasible and robust. In fact, 99% pure adenovirus production has been shown from 20-L cell suspensions using ion and size-exclusion chromatography (Ehrke-Schulz et al., 2016; Kamen and Henry, 2004).

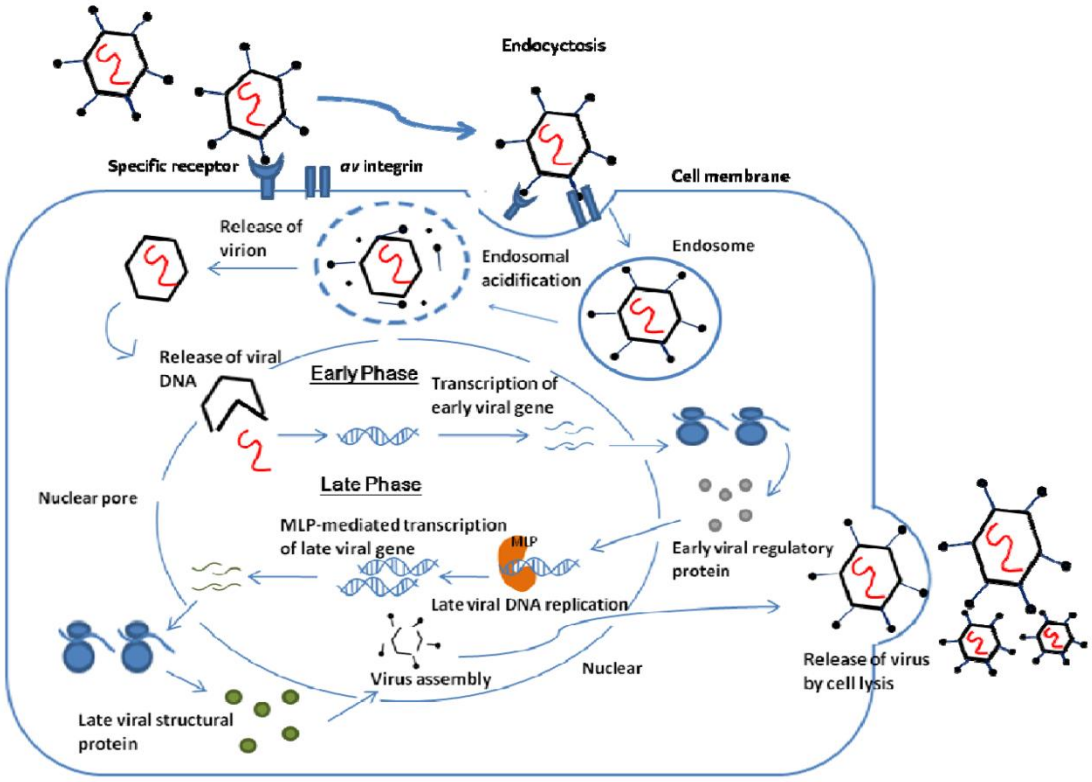

Figure 4. The lifecycle of human adenovirus. Adenovirus infection begins with virus attachment to its specific receptor followed by entry of virus into the cell via endocytosis. The endosome undergoes acidification and virus is release. It then docks on to the nucleus and releases its genome. Virus early genes mediate viral gene transcription and replication and late genes are involved in virus capsid

formation and release. Progeny viruses are assembled inside the nucleus and are then released from the cell by cell lysis. (Figure adapted from Waye and Sing, 2010)

There are several adenoviruses that have been engineered as potential oncolytic viruses and are in clinical trials (Pesonen, Kangasniemi and Hemminki, 2010). ONYX-015 was the first adenovirus to enter clinical trials. It is Ad5 adenovirus which is engineered to have an E1B-55k deletion which inhibits the ability of virus to block p53 mediated apoptosis (Heise et al., 1997). It was initially hypothesised that due to its inability to inhibit p53 pathway ONYX-015 will selectively replicate in tumour cells which usually have defective p53 pathway. However, later studies revealed that the selectively of ONYX-015 to tumour cells was because of effective late viral mRNA export in the absence of E1B-55k in tumour cells relative to normal cells (O'Shea et al., 2004). Even though ONYX-015 showed promising results in many phase I and II clinical trials, its phase III trial was terminated in early 2000s (Kirn, 2001; Kirn, Hermiston and McCormick, 1998). However, it formed the basis for the design of Oncorine, another E1B-deleted Ad5, which was approved in China in 2005. Oncorine also has E3 region deleted. This double deletion made Oncorine more selective to tumours (Zhang et al., 2009). Other oncolytic adenoviruses are also mostly based on group C adenoviruses with modifications like addition of tumour specific E1A promoters, transgene incorporation to promote anti-tumour activity, deletion of E3 region for tumour specificity and HRE derived promoters to target hypoxic regions (Choi et al., 2012).

Even though group C adenoviruses are used as gene therapy and oncolytic agents, they pose two major challenges in the success of this oncolytic virotherapy. Firstly, group C Ads receptor CAR has been shown to be down-regulated in certain tumours so they are not as effective in targeting all tumour cells (Li, Yingming, et al., 1999; Matsumoto et al., 2005). A strategy used to

overcome this is modifications in group C fiber to target receptors which are over expressed in tumour (Rivera et al., 2004). Secondly, there are many neutralising antibodies against group C adenoviruses which make their systemic delivery difficult (Sumida et al., 2005; Yang et al., 1995). One strategy used to overcome this is 'hiding' viruses from immune system by coating their capsid with non-immunogenic agents or using virus carrier cells (Komarova et al., 2006, O'Riordan, 1999).

1.7 Enadenotucirev (EnAd)

EnAd, also known as ColoAd1, is a chimeric group B (Ad11/Ad3) virus owned by PsiOxus Therapeutics. It was developed via a process, directed evolution, which involves serial passaging of different adenovirus serotypes on tumour cell lines to allow recombination of viruses and then selecting the most potent progeny virus. EnAd was found to be more potent than wild type adenoviruses (Ad5 and Ad11) and oncolytic adenovirus ONYX-015. (Khun et al., 2008)

EnAd was found to have about 98.6 % sequence homology with Ad11; however, it has three mutations that make it different from the parental virus. Firstly, the E2B region of EnAd is a chimera of Ad11 and Ad3 E2 regions. As seen in section 1.6, E2 genes are involved in viral replication and this mutation is hypothesised to enhance EnAd replication. Secondly, EnAd has a large deletion in E3 region which might make it tumour selective as E3 region counter attacks antiviral host responses. Thirdly, there is a 24bp deletion in E4 region open-reading frame 4 (E4orf4). E4orf4 is mostly studied in Ad5 and has been shown to down-regulate E1A induced AP-1 and junB transcription (Kleinberger and Shenk, 1993). It has also been reported that the

absence of E4orf4 increases cytotoxicity of group C adenoviruses (Müller, Kleinberger and Shenk, 1992). Our group believes that deletion of E4orf4 increases the ability of cells to produce intact virus particles and to selectively induce necrosis-like death in cancer cells (Seymour et al., 2017).

EnAd is a better oncolytic adenovirus than member of the group C as it overcomes the two major obstacles faced by group C viruses: loss of CAR expression in certain tumours and neutralising antibodies. Since EnAd is a group B adenovirus it requires CD46 for attachment and entry. In fact, some tumours have been shown to overexpress CD46 receptor (Cho et al., 2016). EnAd has an Ad11 capsid which has been shown to have very low titers of neutralising antibodies in the Western population (Holterman et al., 2004) which allows intravenous delivery of EnAd. In fact, EnAd was found to be effectively delivered to tumours systemically after a phase I trial study concluded in 2014 (Gil Martin et al., 2014). It is currently in phase I/II trials for ovarian cancer and phase I trial for combination therapy with programmed cell death protein 1 (PD-1) inhibitor (nivolumab) against metastatic or advanced epithelial tumours. Currently, our group is extensively studying the activity of EnAd in different components of the tumour microenvironments to design an even more robust oncolytic candidate.

1.8 Influence of hypoxia on oncolytic viruses

As previously discussed in section 1.2 and 1.4, hypoxia is an essential feature of solid tumours and the common cancer therapies which rely on either efficient drug perfusion for delivery or oxygen for drug activity fail to perform proficiently in hypoxia. Viruses are different from all other therapies because they don't necessarily rely on vasculature to travel from one cell to

another. Thus, theoretically, it should be possible to design OVs to disseminate in hypoxic regions of tumour. However, the role of hypoxia and HIF-1 pathway in oncolytic virus infection is still to be determined. Current studies in the field have reported both beneficial and detrimental roles of hypoxia and HIF-1α on OV infections depending on the type of virus.

Several studies have suggested improved activity of many OVs in hypoxia and HIF-1α expressing cells. Connor et al., (2004) showed similar viral burst in both hypoxia and normoxia after VSV infection. VSV was also able to disseminate to hypoxic regions of tumour in vivo after intravenously delivery. Ch'ng et al., (2013) indicated that NDV has improved cytotoxicity in hypoxia irrespective of VHL status of renal carcinoma cells. They also suggested that this increased cytotoxicity might be related to increased levels of HIF-2α. Reo virus was shown to have similar activity in normoxia and hypoxia in brain tumour-derived cell lines (Figova, Hrabeta and Eckschlager, 2013). Further, Hiley at el., (2010) reported that vaccinia virus also has comparable oncolytic activity and transgene expression in both normoxia and hypoxia. Our group has recently found that EnAd uptake and entry is not affected by hypoxia. We have also shown that EnAd replication and protein synthesis is, in fact, increased in hypoxia at 24hpi and 48hpi.

However, there are studies on certain oncolytic viruses that show that opposite. Contrary to what Connor et al., (2004) argued, Hwang et al., (2006) showed that excessive activity of HIF-1α and HIF-2α caused resistance to VSV infection at low MOI 0.1 hinting towards antiviral function of HIFs. Contrary to our findings with EnAd, Shen et al. (2006) showed that group B adenovirus (Ad11 and Ad3) replication may be reduced by hypoxia in a cell dependent manner. They also

showed that virus cytotoxicity was significantly attenuated in hypoxia in a cell line- and time point- dependent manner. For Ad5, there are two different reports that suggest that the virus has decreased replication and oncolysis efficiency in hypoxia (Pipiya et al., 2005; Shen and Hermiston, 2005)

Even though OVs behave differently in hypoxia, it appears that most OVs inherently perform better in hypoxia. Interestingly, most literature on oncolytic viruses in hypoxia suggests that there seems to be a trend of OVs-mediated HIF-1α down-regulation. NDV, VSV and reovirus have been shown to down-regulate HIF-1α protein. Vaccinia virus stabilises HIF-1α protein in normoxia during early phase of infection. However, there is no data on the effect of adenoviruses and herpes virus infection on HIF-1α pathway. Confusingly, there seems to be no correlation of virus infectivity in hypoxia to its effect on HIF-1 pathway as summarized in table 1. It is possible that HIF-1 can be either beneficial or detrimental to OVs depending on the virus.

Table 1: Activity of different OVs in hypoxia and their effect on HIF-1α.

Virus	Activity in hypoxia	Effect on HIF-1α
Newcastle disease virus	Increased oncolysis in RCC (Ch'ng et al., 2013)	Degradation via proteasome pathway independent of VHL and p53 (Abd-Aziz et al., 2016)
Vesicular stomatitis virus	Similar viral burst in glioblastoma (Connor et al., 2004) HIF mediated resistance to VSV infection in RCC (Hwang et al., 2006)	HIF-1α down-regulated at 12hpi (Connor et al., 2004)
Reovirus	Similar activity to normoxia in brain-derived tumour cells (Figova, Hrabeta and Eckschlager, 2013)	HIF-1α stabilised in normoxia in brain-derived tumour cells (Figova, Hrabeta and Eckschlager, 2013) HIF-1α down-regulated in adenocarcinoma cells and soft tissue sarcoma cells in a proteasome dependent manner (Carew et al., 2017; Cho et al., 2010)
Herpes simplex virus	Enhanced replication in hypoxia (Aghi et al., 2009)	Not available
Vaccinia virus	Similar activity and transgene expression to normoxia (Hiley et al., 2010)	HIF-1α stabilised in normoxia up to 8hpi by inhibiting PHDs (Mazzon et al., 2013)
Adenoviruses	Replication and oncolysis reduced in hypoxia (Pipiya et al., 2005; Shen and Hermiston, 2005; Shen at al., 2006)	Not available

1.9 Aims of the project

Currently, there are very few reports that elucidate the behavior of adenoviruses in hypoxia and none that have looked at the effect of adenoviruses on the HIF-1 pathway. Literature suggests that oncolytic viruses regulate HIF-1α (Table 1). We hypothesised that adenoviruses regulate

HIF-1 pathway.

The main aims of the project are as following:

1) Determine the effect of hypoxia on release of EnAd from colorectal cancer cell lines
2) Determine the effect of EnAd, Ad5 and Ad11 on HIF-1 pathway
3) Determine the mechanisms for regulation of HIF-1 pathway by EnAd, Ad5 and Ad11

Chapter 2: Materials and methods

2.1 Cell lines and reagents

DLD1, HCT116, A549, AD293 and SW480 cells were purchased from ATCC. RKO cells were a gift from Professor Esther Hammond's lab (University of Oxford). Proteasome inhibitor MG132 was purchased commercially (#M7449, Sigma-Aldrich) and stored at -20°C prior to use.

2.2 Cell culture

All cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM) (#D5796, Sigma-Aldrich) with 10% heat-inactivated foetal bovine serum (FBS) (#10500064, Gibco™). During routine passages, cells were washed twice with sterilised Dulbecco's phosphate-buffered saline (DPBS) (#14190094, Gibco™), incubated with trypsin-EDTA (#T3924, Sigma-Aldrich) for 5 to 10 minutes and then re-seeded into T175 flasks (#660160, CELLSTAR™) at 1:10 dilution. Cells were passaged every 4 to 5 days. For cell stocks, cells were resuspended in FBS with 10% dimethyl sulfoxide (DMSO) (#M81802, Sigma Aldrich), distributed into 1.8 mL cryogenic vials (Thermo Scientific™) in a volume of 1 mL and then frozen at a rate of 1°C/minute in a freezing container (Thermo Scientific™ Mr. Frosty™) filled with isopropanol.

2.3 Experiment conditions and timelines

For all experiments, cells were seeded at a density of 40000 to 50000 cells/well in 6-well plates (#CLS3516, Corning™) and at a density of 10000 to 20000 cells/well in 24-well plates (#CLS3526, Corning™) in DMEM with 10% FBS. The next day medium was replaced with fresh phenol red-free DMEM (#21063029, Gibco™) with 2% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin (#1514022, Gibco™) in cells maintained in normoxia. The cells that

needed hypoxic conditions were moved to hypoxia chamber where medium was replaced by hypoxic phenol red-free DMEM with 2% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin. The following day medium was aspirated from cells in both conditions. Cells were then infected with virus in 500 µL of serum-free DMEM. After two hours virus inoculum was removed and cells were topped up with 1 mL of DMEM with 2% FBS, 100 units/mL of penicillin and 100 µg/mL of streptomycin. Cells were then harvested between 6 to 96 hours depending on the experiment.

Note: For hypoxic cells, DMEM with 100 units/mL of penicillin and 100 µg/mL of streptomycin was brought into hypoxia chamber (Ruskinn Invivo 400 hypoxic workstation), maintained at 1% pO_2, the night before the start of each experiment and was kept inside the chamber throughout the experiment.

2.4 Virus titration by plaque assay

Plaque assay measures the ability of a single virus particle to form visible holes called 'plaques' in cell monolayer by infecting neighboring cells.

Titers for all the viruses used in this study, except Ad11, were calculated using plaque assay technique. A549 cells were seeded on a 6-well plate at a density of 8E5 cells per well. The following day, , wells were infected with 200 µL of 10-fold serial dilutions of virus suspensions in serum free DMEM for 2 hours at 37°C. Plates were rocked in a left-left/right-right movement after every 30 minutes to keep cells from drying out. After 2 hours, virus inoculum was aspirated and wells were topped-up with 2.5 mL of agarose overlay made by mixing 1:1 of 2x DMEM with 4% FBS and 1.4% UltraPure™ low melting point agarose (#16520100, Invitrogen™)

dissolved in water. Plates were gently swirled, left in tissue culture hood for 10 minutes to let agarose set and then moved to incubator.

Plaques were counted 5 to 10 days after infection, depending on the virus. Plaques were fixed by adding one volume of acetic acid and methanol (mixed in a ratio of 1:3) to agarose overlay for 30 minutes minimum. Overlay was removed by letting a gentle stream of tap water fall on the edge of the well. Wells were then stained with crystal violet for one minute, rinsed with water and left to dry overnight. The next day, plaques were counted by visual observation. Titers were calculated by dividing number of plaques by 200 (inoculum volume) and multiplying with 5 to get concentration as plaque forming units (pfu) per mL. Viruses used and their titers are summarised in table 2. Schematic diagrams of all viruses are summarised in figure 5.

Table 2: Viruses used in this study and their titers

Virus	**Titer**	**Comments**
ColoAd1 2.4-SSA-Fluc	5.35E10 pfu/mL	N/A
EnAd-CMV-tRFP	3.71E10 pfu/mL	N/A
EnAd-cflagtag-E1A	8.57E9 pfu/mL	N/A
Ad5-E1A-Fluc	1.42E10 pfu/mL	N/A
Ad11 wt	2.51E9 TCID50 1.73E9 pfu/mL (Day 7)	Virus obtained from Tzveta Pokrovska (University of Oxford). Virus titer was calculated by her using TCID50.



Figure 5. Schematics of the virus genomes used in this study.

2.5 Western blot

Western blot is a technique used to detect specific proteins from cell lysates. It consists of five main steps: a) protein harvesting, b) protein quantification, c) separation of proteins based on size, d) transfer of proteins to a membrane and e) detection of target proteins using primary and secondary antibodies.

2.5.1 Protein harvesting protocol

For strongly adherent cells in hypoxia or experiments that were performed up to 30 hpi, medium was aspirated from cell monolayers, which were then washed twice with PBS. Hypoxic

cells were harvested inside the chamber and were washed with PBS kept inside the chamber for 2 days before harvesting. Then 80 µL of radioimmunoprecipitation assay (RIPA) lysis buffer (Thermo Scientific™ Pierce™) with 3 x protease and phosphatase inhibitor (Thermo Scientific™ Halt™) was added on to each well. After one minute, cells along with RIPA were scraped and collected into 1.5 mL Eppendorf tubes which were then transferred to dry ice.

For cell lines that were less adhesive to culture plates or experiments that were performed beyond 30 hpi, cells were scraped inside the medium in each well and collected in 1.5 mL Eppendorf tubes which were immediately kept on ice. Plates in hypoxia were brought outside the chamber and kept immediately on a steel platform chilled with layers of ice and dry ice. Tubes were spun at 21000 x g for 15 sec and then kept on ice again. Supernatants were carefully aspirated. Empty wells were washed with chilled PBS which was collected in corresponding tubes with pellets. Tubes were spun again at 21000 x g for 15 sec. Chilled PBS wash was repeated once more. Pellets were resuspended in 80 uL of RIPA with 3 x protease and phosphatase inhibitor. Plates and tubes were kept on ice as much as possible.

2.5.2 Protein quantification

Cell lysates were sonicated for 10 seconds and then centrifuged at maximum speed for 15 minutes at 4°C. Supernatants were collected in fresh 1.5 mL Eppendorf tubes. All samples were diluted 5 fold in water before protein quantification which was carried out using Pierce™ BCA protein assay kit (#23227, Thermo Scientific™). Standards were prepared according to the manufacturer's protocol with the addition of 20% RIPA. Samples were prepared by diluting cell

lysates in 6 x Laemmli SDS sample buffer (#J61337, Alfa Aesar)) and RIPA to a final concentration of 25 to 50 µg / sample.

2.5.3 Electrophoretic separation by Sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE)

Mini-PROTEAN TGX™ precast protein gels (BIO-RAD) were commercially obtained and stored at 4°C. The gels were warmed at room temperature and combs were removed before moving the gels to electrode assembly module. Tetra vertical electrophoresis cell (BIO-RAD) was then filled with SDS running buffer (25 mM tris-base, 190 mM glycine and 0.1% SDS) and wells were washed with buffer before loading the prepared protein samples. The protein samples were boiled at 100°C for 15 minutes and loaded on the gel. The first lanes were filled with 6 to 8 µL of PageRuler™ prestained protein ladder (#26616, Thermo Scientific™) and any empty lanes were filled with 6x Laemmli buffer and RIPA. Electrophoresis cell was connected to the power supply and run at 80V until samples had moved through 1/4th of gels and then at 120V until desired separation of bands was achieved.

2.5.4 Transfer

After electrophoretical separation, proteins were transferred to 0.2 µm nitrocellulose membranes (#162013, BIO-RAD) via wet-tank transfer in electrophoretic transfer cell (BIO-RAD). Transfer cell was filled with pre-cooled Towbin buffer (25 mM tris-base and 190 mM glycine) with 20% methanol. An ice pack was placed in the tank to keep the buffer cool and a magnetic stirrer

was also used to ensure even temperature and ion distribution in the tank. Transfer was carried out at 320 mA for 90 minutes in the cold room.

2.5.5 Blocking and protein detection

Efficient transfer was confirmed by staining the membranes with Ponceau S solution (#P71701L, Sigma-Aldrich). Membranes were then washed with tris-buffered saline with 0.05% tween20 (TBST) to remove Ponceau residues before blocking with 5% skimmed milk (#70166500, Sigma-Aldrich) in TBST for 45 to 60 minutes. Blocked membranes were incubated with primary antibody, diluted in blocking solution, overnight at 4°C. During incubation membranes were kept on rockers to ensure the entire membrane was soaked with antibody mix. The next day, membranes were washed three times for 5 minutes each with TBST and then incubated with secondary antibody for about 2 hours. Membranes were washed three more times for 10 minutes each. SuperSignal™ West Dura extended duration substrate (#34076, Thermo Scientific™) was used to detect HRP signal. Blotted proteins were visualised using GE Healthcare Amersham™ Hyperfilm™ ECL (#1620213, Fisher scientific) in a dark room. Antibodies used are summarised in the following table.

Table 3: Antibodies used for Western blots

Target	Species	Type	Clonality and Isotype	Company	Catalogue number	Dilution
HIF-1α	Mouse	Primary	Monoclonal IgG1	Biosciences	610958	1:1000
CAIX	Mouse	Primary	Monoclonal IgG1	Biosciences Slovakia	M75	1:1000
β-Actin (HRP-linked)	Mouse	Primary	Monoclonal IgG1	Sigma-Aldrich	A3854	1:100000
Anti-Mouse IgG (HRP-linked)	Rabbit	Secondary	Polyclonal IgG	Dako	PO161	1:10000

Western blot band intensities were measured as mean of grey value using Image J software. Band densities normalised to reference gene beta actin were calculated using the following formula:

$$Adjusted\ density\ = \frac{Target_{control} - Target_{sample}}{Reference_{control} - Reference_{sample}}$$

2.6 Gene expression analysis by qPCR

2.6.1 RNA harvesting

For strongly adherent cells in hypoxia or experiments that were performed up to 30 hpi, medium was aspirated from cell monolayers, which were then washed twice with PBS. Hypoxic cells were harvested inside the chamber and were washed with PBS kept inside the chamber

for 2 days before harvesting. Then 350 µL buffer RLT (#74106, QIAGEN) with 3.5 µL of 2-Mercaptoethanol (#M6250, Sigma-Aldrich) was added onto each well. After one minute, cells along with RLT buffer were scraped and collected into 1.5 mL Eppendorf tubes which were then transferred to dry ice.

For cell lines that were less adhesive to culture plates or experiments that were performed beyond 30 hpi, cells were scraped inside the medium in each well and collected in 1.5 mL Eppendorf tubes which were immediately kept on ice. Plates in hypoxia were brought outside the chamber and kept immediately on a steel platform chilled with layers of ice and dry ice. Tubes were spun at 21000 x g for 15 sec and then kept on ice again. Supernatants were carefully aspirated. Empty wells were washed with chilled PBS which was collected in corresponding tubes with pellets. Tubes were spun again at 21000 x g for 15 sec. Chilled PBS wash was repeated once more. Pellets were lysed in 350 µL buffer RLT with 3.5 µL of 2-Mercaptoethanol. Cell lysates were then either stored at -70°C or directly used for RNA extraction.

2.6.2 RNA extraction

Lysates were thawed on ice and homogenized by passing the lysate at least 5 times through a blunt 21-guage needle (#ND434, BD Microlance) attached to 1ml RNase-free syringe (#SS-01T1, Terumo). RNA extraction was then performed using RNeasy Mini kit (#74106, QIAGEN) according to manufacturer's protocol with on-column DNA digest using RNase-Free DNase set (#79254, QIAGEN). RNA was eluted from RNeasy spin columns in 50 µL of RNase-free water. RNA was kept on ice before reverse transcription either stored at -20°C or kept on ice.

2.6.3 Reverse transcription

RNA samples were quantified three times using NanoDrop™ One/OneC Microvolume (Thermo Scientific™). Between 700 to 800ng of RNA was converted to cDNA using QuantiTech reverse transcription kit (#205313, QIAGEN) according to manufacturer's protocol.

2.6.4 Quantitative polymerase chain reaction (qPCR)

Primer pair sequences for 18S, HIF-1α, VEGF and GLUT1 were obtained from Ester Hammond´s lab (University of Oxford), TBP primer sequence was obtained from Egon Jacobus from Seymour lab (University of Oxford) and PCNA primer pair was designed using NM_002592.2 mRNA sequence from NCBI. All primer pairs were manufactured by Sigma-Aldrich and reconstituted in elution buffer to stock concentration of 100 µM. Final concentrations of primer pairs were 100 nM for 18S, 300 nM for HIF-1α, 500 nM for VEGF, 300 nM for GLUT1, 400 nM for PCNA and 400 nM for TBP.

Table 4. Primers used for gene expression

Primer		Sequence
18S (Human)	Forward	5'-GCCCGAAGCGTTTACTTTGA-3'
	Reverse	5'-TCCATTATTCCTAGCTGCGGTATC-3
HIF-1α (Human)	Forward	5'-TTC ACC TGA GCC TAA TAG TCC-3'
	Reverse	5'-CAA GTC TAA ATC TGT GTC CTG-3'
VEGF (Human)	Forward	5'-CTACCTCCACCATGCCAAGT-3'
	Reverse	5'-CTCGATTGGATGGCAGTAGC-3'
GLUT1 (Human)	Forward	5'-ATACTCATGACCATCGCGCTAG-3'
	Reverse	5'-AAAGAAGGCCACAAAGCCAAAG-3'
TBP (Human)	Forward	5'-CTCCCGGAATCCCTATCTT-3'
	Reverse	5'-GCCTTTGTTGCTCTTCCA-3'
PCNA (Human)	Forward	5'-GTGTTGGAGGCACTCAAGG-3'
	Reverse	5'-TGAGCTGCACCAAAGAGACG-3'

For each 20 µL reaction mix in MicroAmp™ fast optical 96-well plates (#4346907, Applied Biosystems™), 10 µL of qPCRBIO SyGreen Blue Mix Hi-ROX (#PB20.16-01, PCRBIOSYSTEMS), 2 µL of forward and reverse 10X concentrated primer, 1 µL of dUTP UDGase Additive (#PB20.51, PCRBIOSYSTEMS) and 5 µL of cDNA at a concentration of 2 ng/µL were added. Reaction plates were sealed with adhesive seals (#AB-0558, Thermo Scientific™) and centrifuged at 400 x g for 1 min. Polymerase chain reactions (PCR) were run on StepOnePlus™ Real-time PCR system (#4351107, Applied Biosystems™) at standard speed. Cycle conditions

used were: UDGase activation at 37°C for 10 minutes, denaturation at 95°C for 3 minutes, 40 cycles of denaturation at 95°C for 5 seconds and annealing at 60°C for 30 seconds, followed by melt curve stage. For each condition three reactions were performed and negative controls (without template) were used for each primer pair. Gene expression was determined using relative expression ratio (*R*) formula (Pfaffl, 2001):

$$R = \frac{E_{target}^{\Delta CPtarget\,(Control-Sample)}}{E_{ref}^{\Delta CPref\,(Control-Sample)}}$$

2.7 EnAd copy number quantification

EnAd genome can be measured from the virus particles released in the supernatant during infection. Here absolute genome has been measured which means that copy number represents both naked and encapsidated genome.

2.7.1 DNA extraction

48 hours after EnAd infection in DLD1 and HCT116, supernatants were collected in 1.5 mL Eppendorf tubes. Each tube was weighed to account for total supernatant volume and then 200 µL of supernatant for every sample was transferred to new 1.5 mL Eppendorf tubes. These were then either stored at -20°C or processed for DNA extraction. Before starting extraction, all equipment and DNA extraction hood were sprayed with DNAZap (#AM9890, Invitrogen™) to avoid contamination. Water bath was also set in a container treated with Distel™ surface disinfectant (Thermo Scientific™) and DNAZap containing ultraviolet (UV) treated distilled water. DNA extraction was carried out using PureLink™ genomic DNA mini kit (#K182002,

Thermo Scientific™) according to the manufacturer's protocol. DNA was eluted in 100 µL of elution buffer provided with the kit.

2.7.2 qPCR

Primers for Ad11 fiber gene were used to measure EnAd genome copy number.

Table 5. Primers used for Ad11 fiber

Primer		**Sequence**
Fiber (Ad11)	Forward	5'-ACCGAAGAGCAATAAATG-3'
	Reverse	5'-TCGTCTTCTCTGATGTAG-3'
	Probe	5'-TCGTATAACTTGGTCCTGGAACACA-3'

For each 15 µL reaction mix in MicroAmp™ fast optical 96-well plates, 7.5 µL of 2X qPCR Probe Mix Hi-ROX (#PB20.22, PCRBIOSYSTEMS), 0.8 µL of each primer with final concentration of 400nm, 0.4 µL of probe, 0.5 µL of water and 5 µL of DNA was added. Standards were made using 10-fold dilutions of ColoAd1 2.4 SSA-fluc (stock concentration of 2.00E9/ul) virus in lambda carrier DNA.

Table 6. Standard preparation for Ad11 fiber qPCR

	Virus volume	Diluent	Dilution factor	Virus Concentration in 5 µL
A	5 µL from Stock	25 µL of 20X lambda carrier + 470 µL of EB	100	1.00E8
B	5 µL from A	45 µL of 1X lambda carrier	10	1.00E7
C	5 µL from B	45 µL of 1X lambda carrier	10	1.00E6
D	5 µL from C	45 µL of 1X lambda carrier	10	1.00E5
E	5 µL from D	45 µL of 1X lambda carrier	10	1.00E4
F	5 µL from E	45 µL of 1X lambda carrier	10	1.00E3
G	5 µL from F	45 µL of 1X lambda carrier	10	1.00E2
H	5 µL from G	45 µL of 1X lambda carrier	10	1.00E1

2.8 Infectious virus particle quantification using luciferase assay

ColoAd1 2.4 SSA-Fuc is a reporter virus with luciferase under the control of the MLP. This assay exploits the ability of this reporter virus to produce luciferase in infected cells during late phase of infection.

2.8.1 Virus harvesting

Supernatants from DLD1 and HCT116 cells infected with ColoAd1 2.4 SSA-Fluc virus were collected in 1.5 mL Eppendorf tubes and weighed to later account for volume differences among samples. Tubes were then spun at 300 x g for 3 minutes to remove cellular debris and 200 µL of supernatants were transferred to V-bottom 96-well plates (#CLS3896, Corning™).

2.8.2 Virus quantification

1.5e4 A549 cells/well were seeded onto 96-well black plates (#CLS3916, Corning™) in 50uL Phenol red-free DMEM with 10% FBS. The following day, 25uL of supernatant and standard were pipetted onto the A549 cells. Plates were spun down for 5 minutes at 450 x g. After 22 to 24 hours post infection, luminescence was measured using the POLARStar plate reader in a kinetic mode for 15 cycles. Using an injector 25uL of 1.2mg/mL D-Luciferin (#LUK-100, Goldbio) in PBS was pipetted into each well during 2nd cycle. All measurements were corrected for background noise by subtracting the signal from 1st cycle. Virus particles were calculated by integrating 7 measurements from virus standard into 4-parameter fit. Virus standard was prepared using ColoAd 2.4-SSA-Fluc with titer 5.35E10 in the following way:

Table 7. Standard preparation for luciferase assay

	Virus volume	Medium	Dilution factor	Virus Concentration in 25 µL
A	1 µL from Stock	2.4 mL total	2400	5.57E5
B	500 µL from A	500 µL	2	2.78E5
C	250 µL from B	750 µL	4	6.96E4
D	250 µL from C	750 µL	4	1.74E4
E	250 µL from D	750 µL	4	4.35E3
F	250 µL from E	750 µL	4	1.08E3
G	250 µL from F	750 µL	4	2.72E2
BLANK		1000 µL		0.00E0

2.9 Statistics

Statistical analysis was carried out by GraphPad Prism 5.0 (GraphPad Software Inc.). Unpaired Student's t-tests were used for comparison between two groups. Data was plotted as means and error was presented as SD, SEM or 95% confidence interval depending on the experiment. Further information can be found in figure legends.

Chapter 3: Results

3.1 Experimental model

Throughout this study, we will represent virus dose as multiplicity of infection (MOI) which is pfu/cell. To determine what percent of seeded cells were infected with different MOI, DLD1 cells were infected with a CMV-tRFP reporter virus at different doses and virus infectivity was analysed at 24hpi (Figure 6). We found that at lower MOI 0.1 and 1 only 1.07% and 13.4%, of the cells were infected respectively, while at higher MOI 3 and 10 approximately 53.95% and 65.79% of the cells were infected.

3.2 Effect of hypoxia on EnAd release

EnAd release from infected cells was measured using two methods: virus genome release via qPCR and infectious virus particles via luciferase reporter assay.

Virus genome release was a measure of total virus DNA present in the supernatants of infected cells measured by qPCR. DLD1 cells were infected with different doses of EnAd and supernatants were harvested at 48hpi. We found a significant decrease in genome copy numbers in hypoxia at lower doses of virus; however, at higher doses genome copy numbers were not significantly different (Figure 7A).

For infectious virus particle release, DLD1 and HCT116 cells were infected with EnAd encoding a luciferase reporter gene under the MLP at MOI 3 for 24 hours. After 24 hours, supernatants were collected. The supernatants were used to infect A549 cells. We then measured luciferase signal from A549 at 22hpi and calculated virus particles by integrating 7 measurements from

virus standard into 4-parameter fit. We found that virus release was increased in hypoxia in both cell lines but only significantly in DLD1 (Figure 7B).

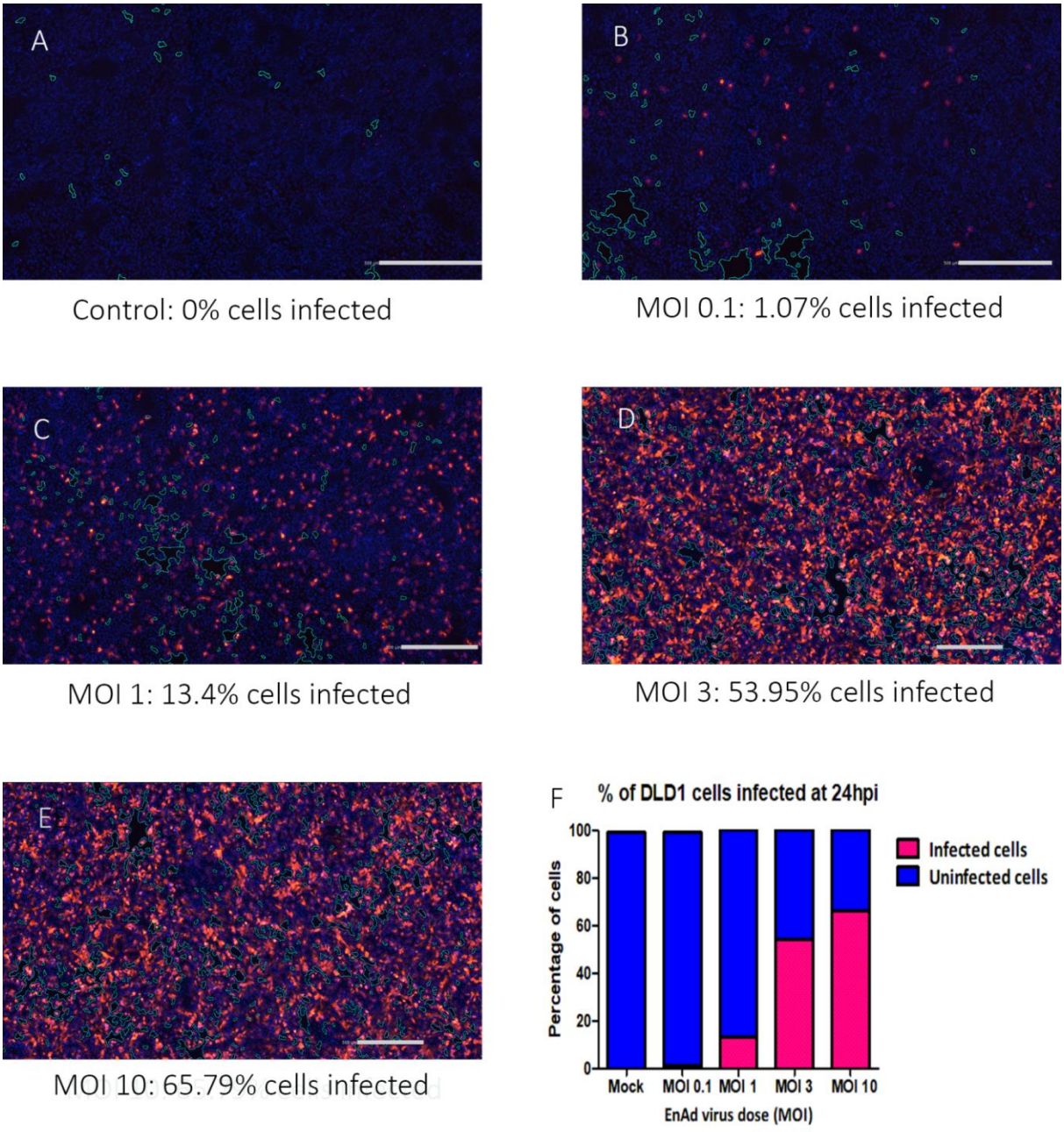

Figure 6. Percentage of cells infected with different EnAd doses. CMV-tRFP EnAd was used to infect DLD1 cells at different MOI. Images were taken and analysed using Celigo at 24hpi (A-F).

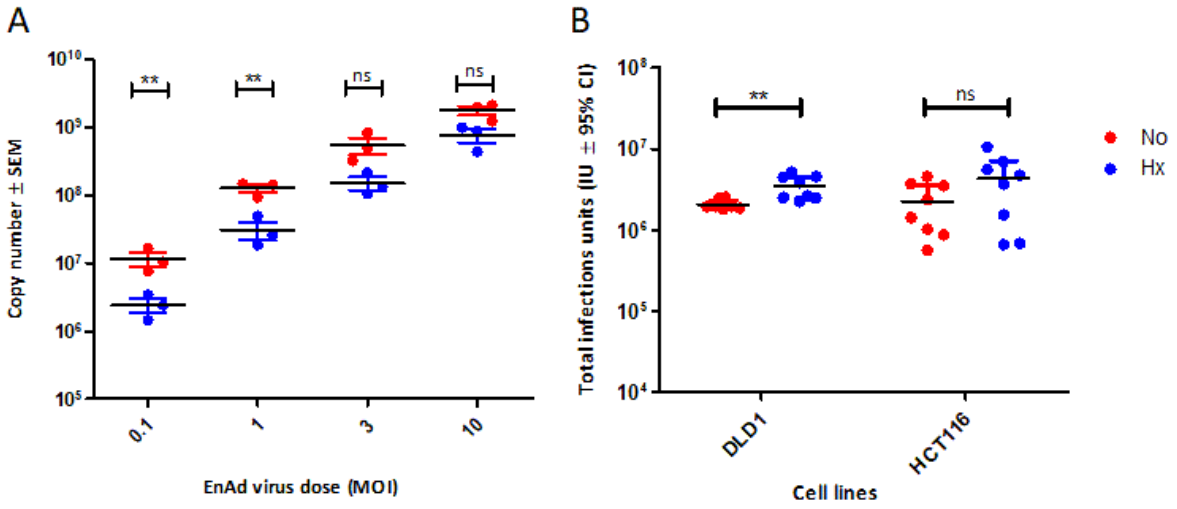

Figure 7. EnAd virus production in hypoxia is dependent on virus dose and cell line. Cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in respective condition during the infection. EnAd genome copy number from supernatants from DLD1 cells was quantified using qPCR at 48hpi. Error bars represent SEM from three biological replicates. (A). EnAd luciferase reporter virus from supernatants from DLD1 and HCT116 cells at 24hpi was calculated using luciferase reporter assay. Error bars represent 95% confidence interval from six biological replicates (B). (*p*-values were calculated using t test: * p<0.05, ** p<0.01, *** p<0.001, ns means non-significant, Hx means hypoxia, No means normoxia)

3.3 Effect of adenovirus infection on HIF-1α pathway

Since HIF-1 protein dominates the hypoxic phenotype and many OVs have been shown to alter HIF-1α, we studied the effect of Ad5, Ad11 and EnAd infection on HIF-1 pathway.

3.3.1 EnAd infection leads to down-regulation of HIF-1α protein in DLD1 cells

The effect of different doses of EnAd virus on HIF1α protein was studied at 24hpi and 48hpi in DLD1 cells. We observed a virus dose-dependent decrease in HIF-1α protein and its direct target carbonic anhydrase 9 (CAIX) at 24hpi and 48hpi (Figure 8).

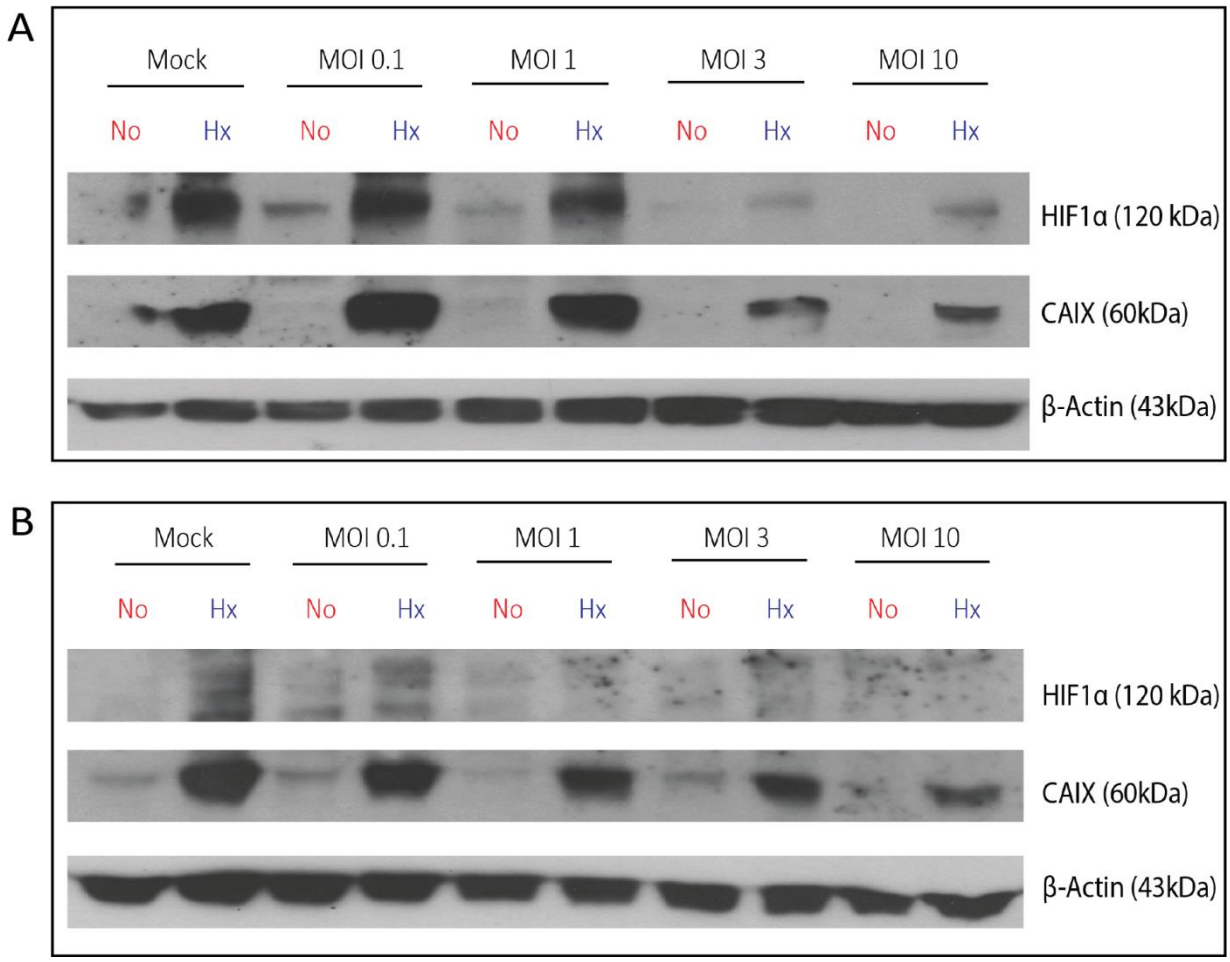

Figure 8. EnAd down-regulates HIF-1α protein and its target CAIX in single-and multi-step infection. Protein levels of HIF-1α and CAIX in DLD1 cells at 24hpi (A) and 48hpi (B) are shown via Western blot. Band intensity analysis was carried out using ImageJ and normalised to actin. Cells were pre-exposed to hypoxia (1% pO_2) or normoxia for 18 hours and maintained in respective condition during infection. Western blots were repeated minimum two times and typical results are shown. (No = normoxia, Hx = hypoxia)

3.3.2 Down-regulation of HIF-1α protein by EnAd is cell line dependent

We further wanted to determine if EnAd affects HIF-1 signaling in other adenocarcinoma cell lines. It was found that HCT116, A549 and SW480 cells show similar results to DLD1. AD293 cells which have Ad5 E1 region showed decreased basal levels of HIF-1α but these levels didn't decrease further with EnAd infection. CAIX was not detected in AD293 cells. RKO cells had similar levels of HIF-1α in both infected and uninfected samples. (Figure 9)

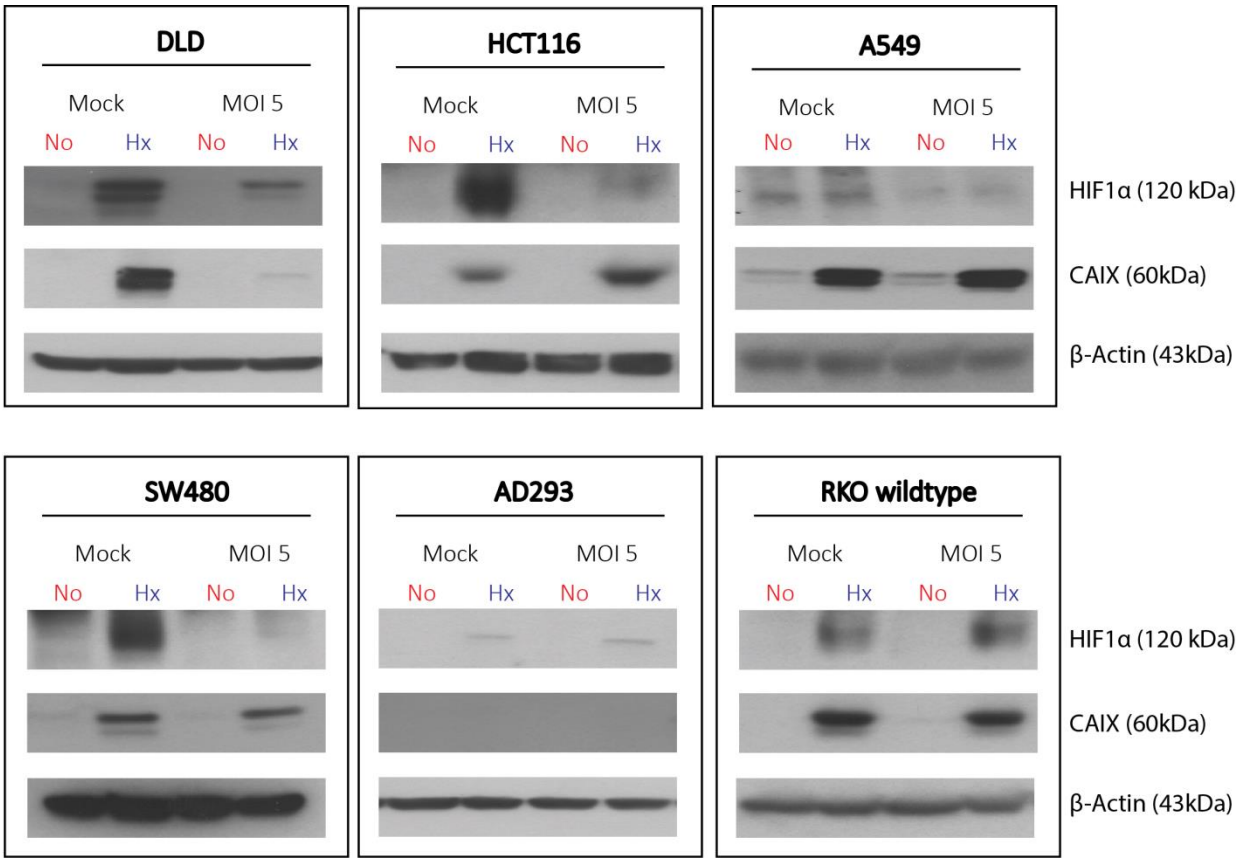

Figure 9. Effect of EnAd infection on HIF-1α expression in different EnAd permissive cell lines. HIF-1α and CAIX protein levels were measured at 24hpi in different cell lines via Western blot and ImageJ analysis. All cells were pre-exposed to hypoxia (1% pO_2) or normoxia for 18 hours and maintained in respective condition during infection.

3.3.3 Ad5 and A11 also down-regulate HIF-1α protein

Next we studied the effect of Ad5 and Ad11 infection on HIF-1 pathway. Both HIF-1α and CAIX protein levels in DLD1 cells were found to be down-regulated after infection with Ad5 and Ad11 infection (Figure 10).

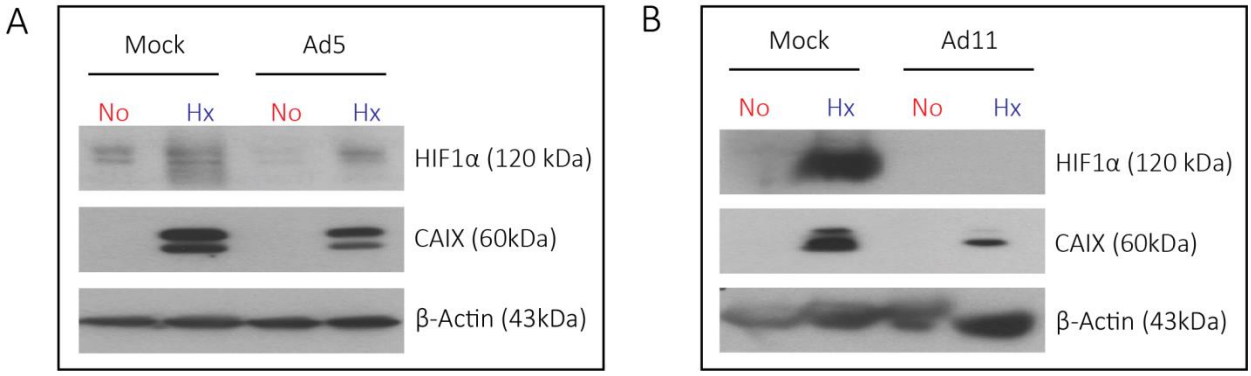

Figure 10. Down-regulation of HIF-1α and CAIX protein after Ad5 and Ad11 infection. Western blot showing the protein levels of HIF-1α and CAIX in DLD1 infected with Ad5 and Ad11 at MOI 3 for 24 hpi. Cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in respective condition during infection.

3.4 Adenoviruses down-regulate HIF-1α protein independent of proteasome complex

As discussed in section 1.8 that some oncolytic viruses down-regulate HIF-1α in a proteasomal dependent manner, therefore, to determine if adenoviruses also achieve degradation of HIF-1α protein via this pathway, we inhibited proteasome complex using MG132. Inhibition of proteasome complex leads to stabilisation of HIF-1α in normoxia. If adenoviruses were degrading HIF-1α post-translationally via proteasome pathway, inhibiting proteasome would restore levels of HIF-1α in virus infected cells. However, adenoviruses still showed down-regulation of HIF-1α protein suggesting that the mechanism for this down-regulation is independent of proteasome complex (Figure 11).

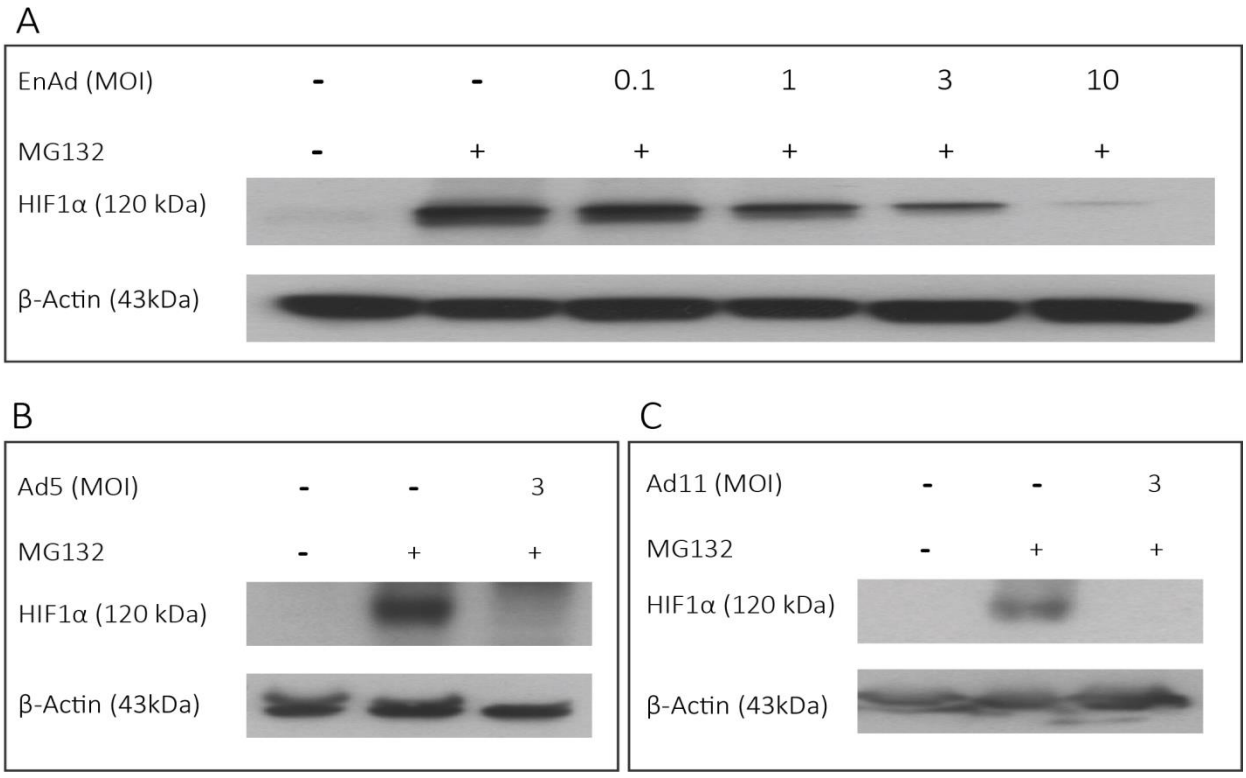

Figure 11. EnAd, Ad5 and Ad11 down-regulate HIF-1α protein independent of the proteasomal pathway. DLD1 cells were infected with EnAd in a virus dose dependent manner (A) and with Ad5 and Ad11 at MOI3 for 24 hours (B,C). Cells were treated with 5μM of proteasome inhibitor MG132 6 hours before harvesting.

3.5 Adenoviruses down-regulate HIF-1α at mRNA level

Before starting the mRNA expression analysis, we checked different primer combinations to select the ones with highest CT values and amplicon specificity. Using the selected primer concentrations for all primer pairs used in this study, we calculated amplification efficiency to ensure a robust assay. We plotted CT values against log DNA concentration values. The slope of the linear regression curve was then used to measure the amplification efficiency of primer pairs using the formula: $E = 10^{[-\frac{1}{slope}]}$ (Witter, 2001). Linear regression plots are shown in figure 12 and amplification efficiencies of all primers are summarised in table 8.

Table 8: Summary of amplification efficiency of primers

Gene	R^2	Slope	Efficiency
18S	0.9999	-3.480	1.938011
HIF-1α	0.9996	-3.465	1.94357
VEGF	0.9994	-3.502	1.929972
GLUT1	0.9991	-3.307	2.006268
PCNA pair 1	0.9991	-3.208	2.049843
TBP	0.9949	-3.184	2.060964

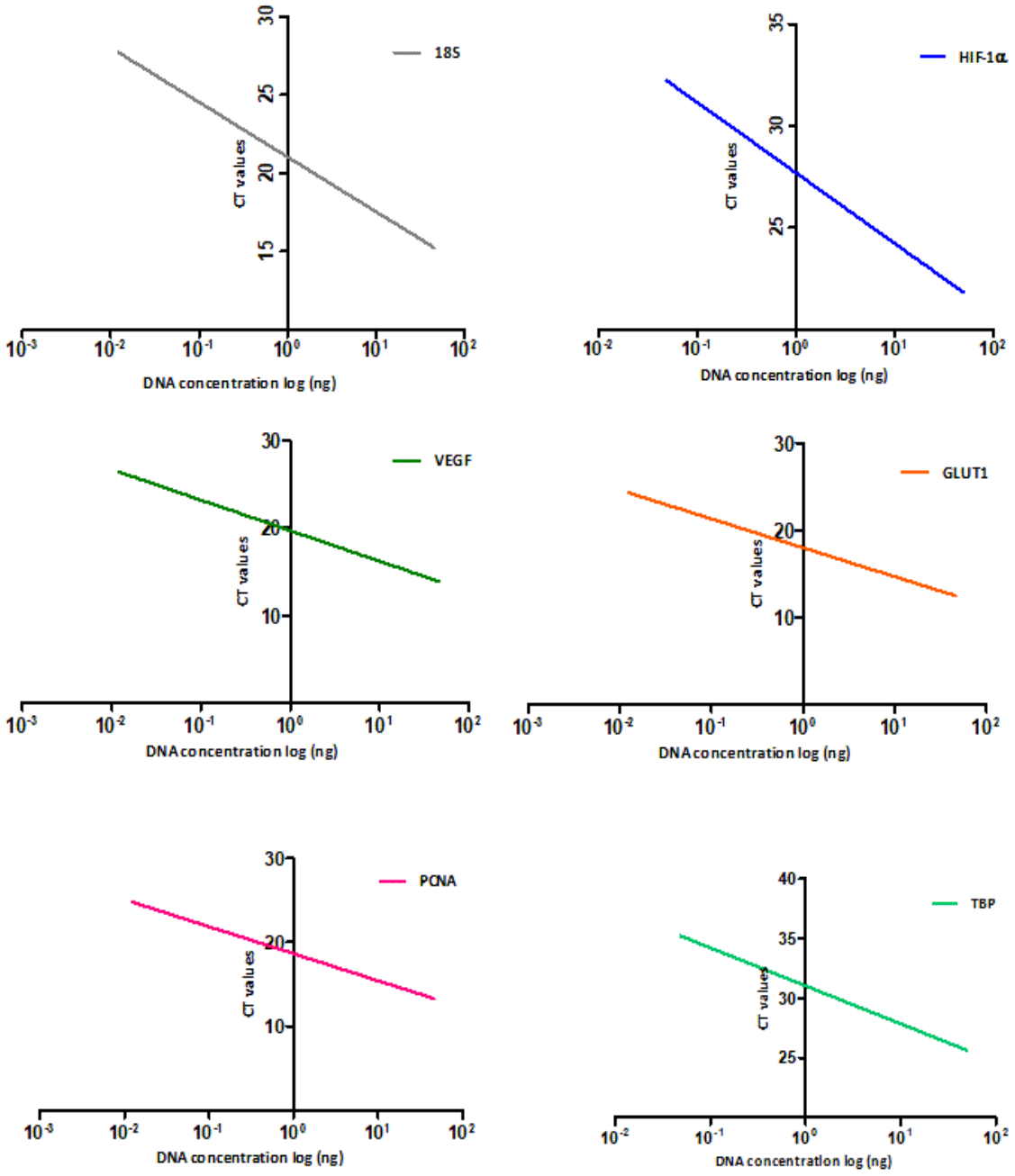

Figure 12. Linear regression plots of primers pairs used for qPCR (A-F). Seven 4-fold cDNA serial dilutions were made starting with 50ng of cDNA from normoxia control sample. Primer concentrations were: 100nM 18S, 300nM HIF-1α, 500nM VEGF, 300nM GLUT1, 400nM PCNA and 400nM TBP. Slopes were calculated using linear regression fit on Graphpad Prism.

3.5.1 EnAd down-regulates HIF-1α at mRNA level during late phase of infection

Since HIF-1α protein was found to be down-regulated independent of proteasome complex, we tested the possibility that EnAd down-regulates HIF-1α at transcriptional level. We performed RT-qPCR to check levels of *HIF-1α* and its target genes (*GLUT1* and *VEGF*) at 24 and 48 hpi in a virus dose dependent manner. We found that EnAd infection down-regulates *HIF-1α* and targets mRNA significantly at all MOI except 0.1 at 24hpi (Figure 14). At 48hpi, a virus dose-dependent down-regulation of *HIF-1α* and target genes was observed but only significantly at higher MOI (Figure 15). Levels of reference gene *18S* were not significantly changed with oxygen tension or virus infection at 24hpi and 48hpi (Figure 13).

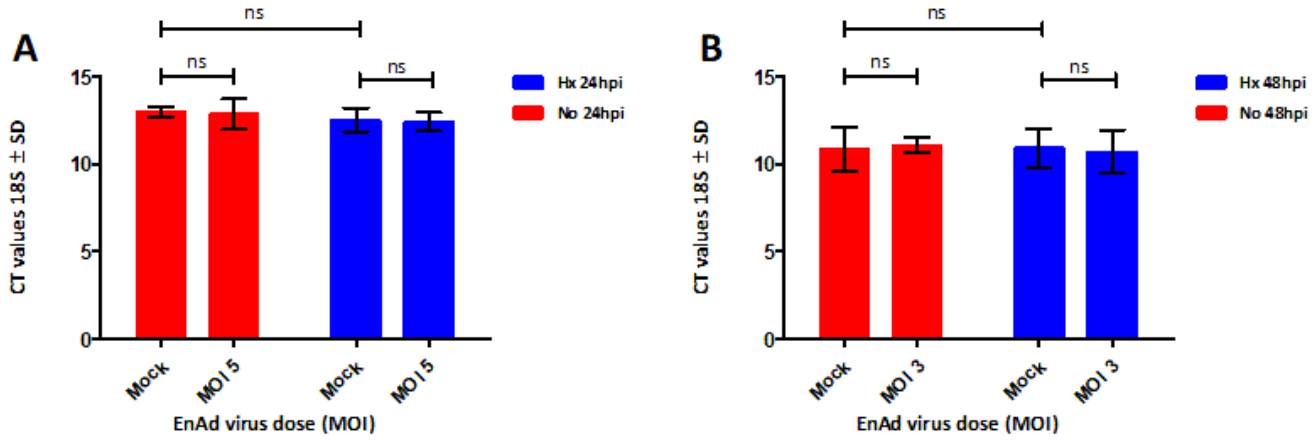

Figure 13. Reference gene 18S expression is not altered significantly by hypoxia or virus infection. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia 18 hours before infection and then maintained in respective condition during the infection. *18S* mRNA was quantified using RT-qPCR. Error bars represent SD from 4 independent experiments for 24hpi and 2 independent experiments for 48hpi, each with three technical replicates. (*p*-values were calculated using T test: * p<0.05, ** p<0.01, *** p<0.001, ns means non-significant, Hx means hypoxia, No means normoxia)

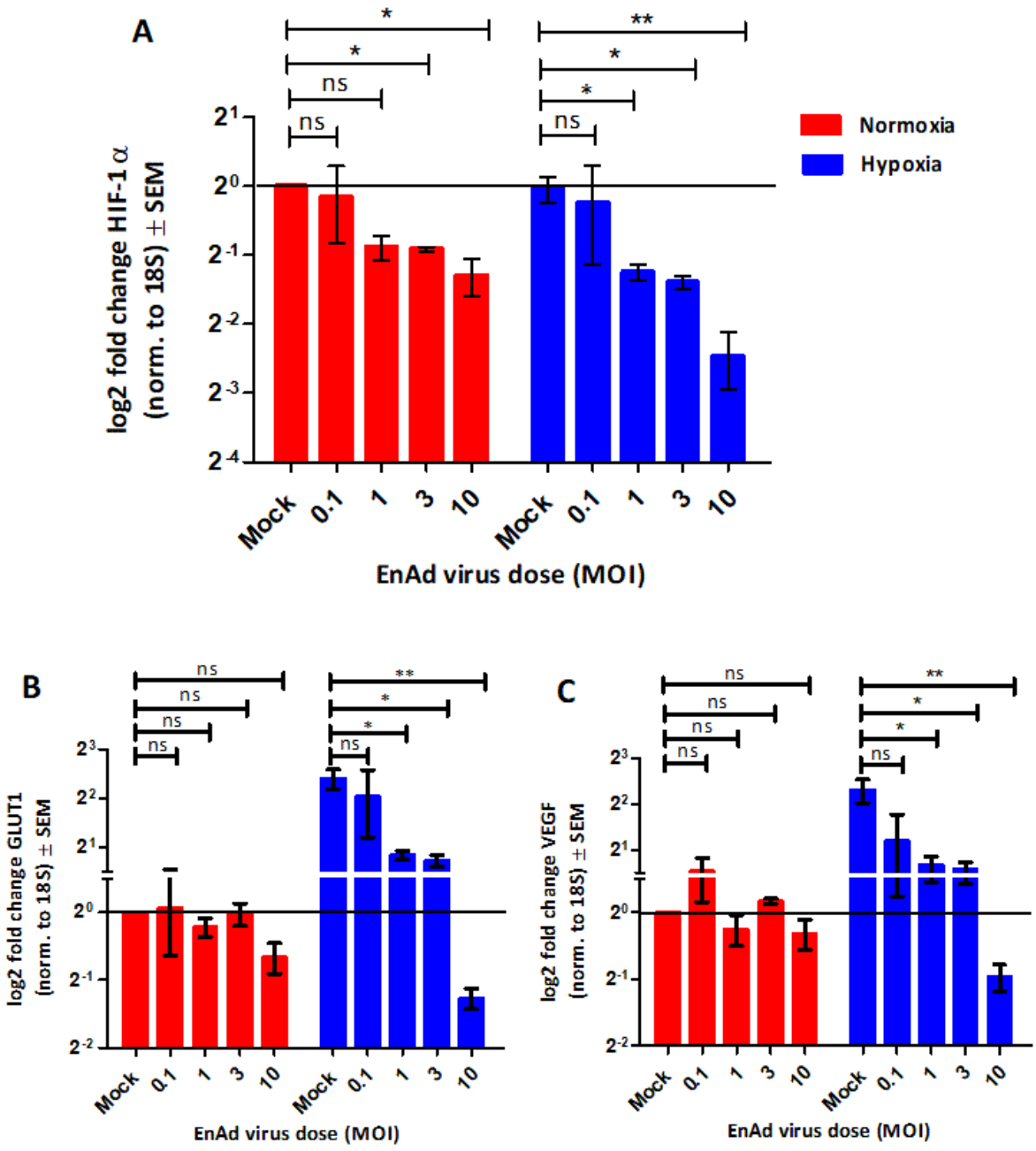

Figure 14. EnAd infection leads to down-regulation of HIF-1α and target genes (VEGF and GLUT1) mRNA at 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia 18 hours before infection and then maintained in respective condition during the infection. *HIF-1α* and its target genes (*GLUT1* and *VEGF*) mRNA was quantified using RT-qPCR. Pfaffl method was used to normalize gene expression to *18S* and normoxia control sample. Error bars represent SEM from 2 independent experiments, each with three technical replicates. (*p*-values were calculated using T test: * p<0.05, ** p<0.01, *** p<0.001, ns means non-significant)

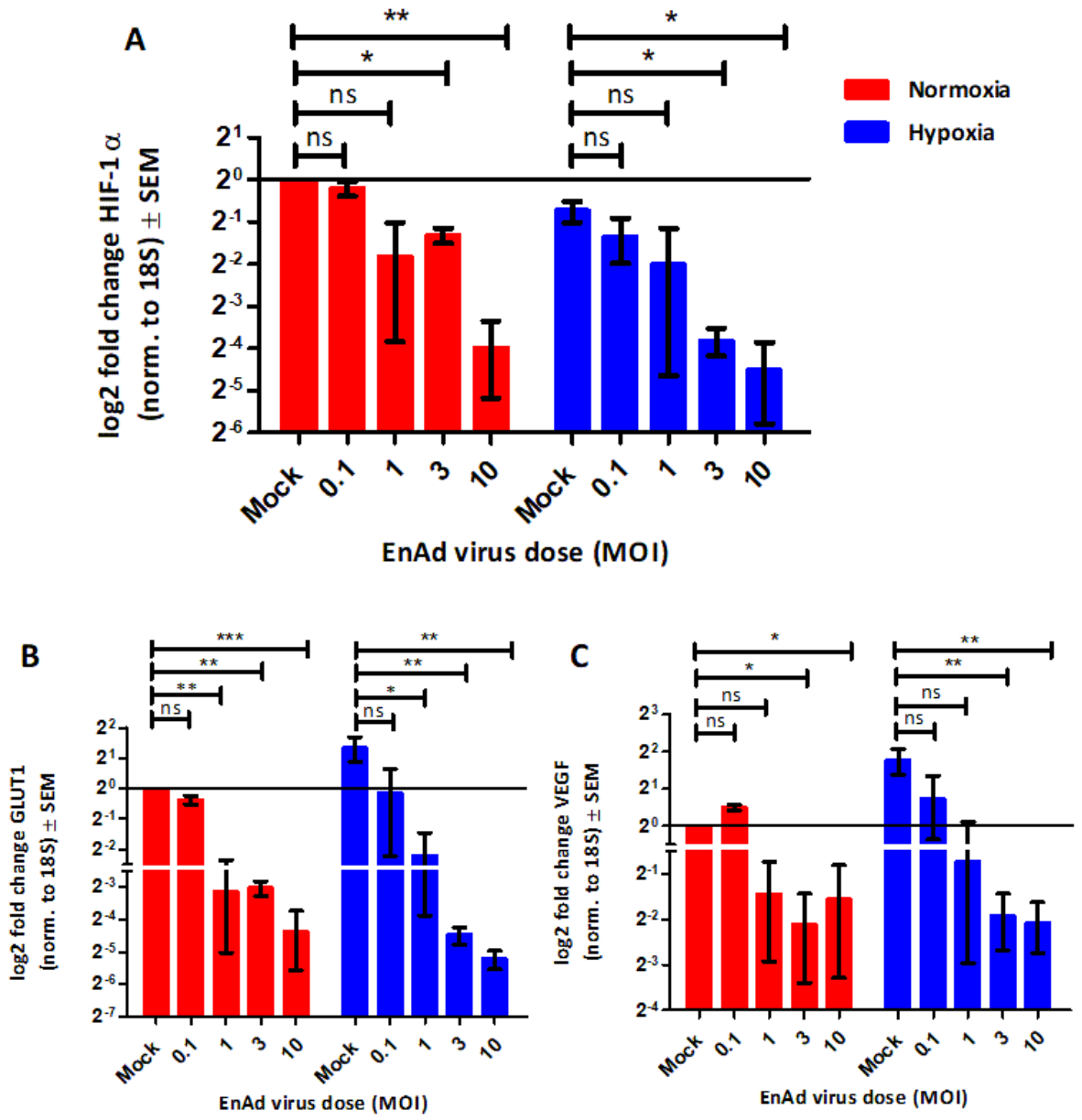

Figure 15. EnAd infection leads to down-regulation of HIF-1α and target genes (VEGF and GLUT1) mRNA at 48hpi. Hypoxic DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours before infection and then maintained in respective condition during the infection. *HIF-1α* and its target genes (*GLUT1* and *VEGF*) mRNA was quantified using RT-qPCR. Pfaffl method was used to normalize gene expression to *18S* and normoxia control sample. Error bars represent SEM from 2 independent experiments, each with three technical replicates. (*p*-values were calculated using T test: * p<0.05, ** p<0.01, *** p<0.001, ns means non-significant)

3.5.2 EnAd does not alter HIF-1α mRNA during early phase of infection

Mazzon et al. (2013) found that vaccinia virus stabilizes HIF-1α protein during early phase of infection (up to 8hpi) and suggested that post 8 hours of infection HIF-1α protein levels start to decrease due to virus induced shutdown of transcription. To determine if EnAd also up-regulates HIF-1α early during infection, we measured levels of HIF-1α mRNA and HIF-1 target genes (GLUT1 and VEGF) at 6 hpi (14). We found no significant change in the levels of genes suggesting that down-regulation of HIF-1 pathway is a late event in the virus life cycle (Figure 16).

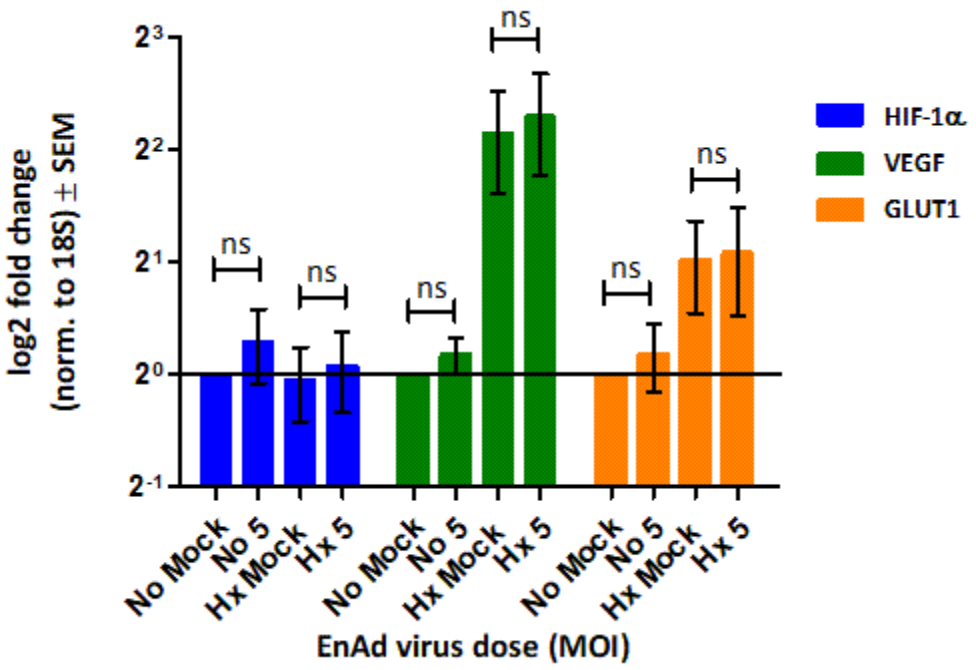

Figure 16. HIF-1α and target genes are not down-regulated during early phase of EnAd infection. DLD1 cells were pre-exposed to hypoxia (1% pO_2) or normoxia and maintained in respective condition during the infection. *HIF1-α*, *GLUT1* and *VEGF* mRNA was quantified at 6hpi using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control sample. Error bars represent SEM from 3 independent experiments (*p*-values were calculated using T test: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, ns = non-significant, No = normoxia, Hx = hypoxia)

3.5.3 Ad5 and Ad11 down-regulate *HIF-1α* mRNA during late phase of infection

To test if this mechanism is specific to EnAd or general feature of adenoviruses, we performed RT-qPCR to check levels of *HIF-1α* and its target genes (*GLUT1* and *VEGF*) at 24hpi after Ad5 and Ad11 infection at MOI3. We found that both Ad5 (Figure 17) and Ad11 (Figure 18) significantly down-regulate *HIF-1α* mRNA.

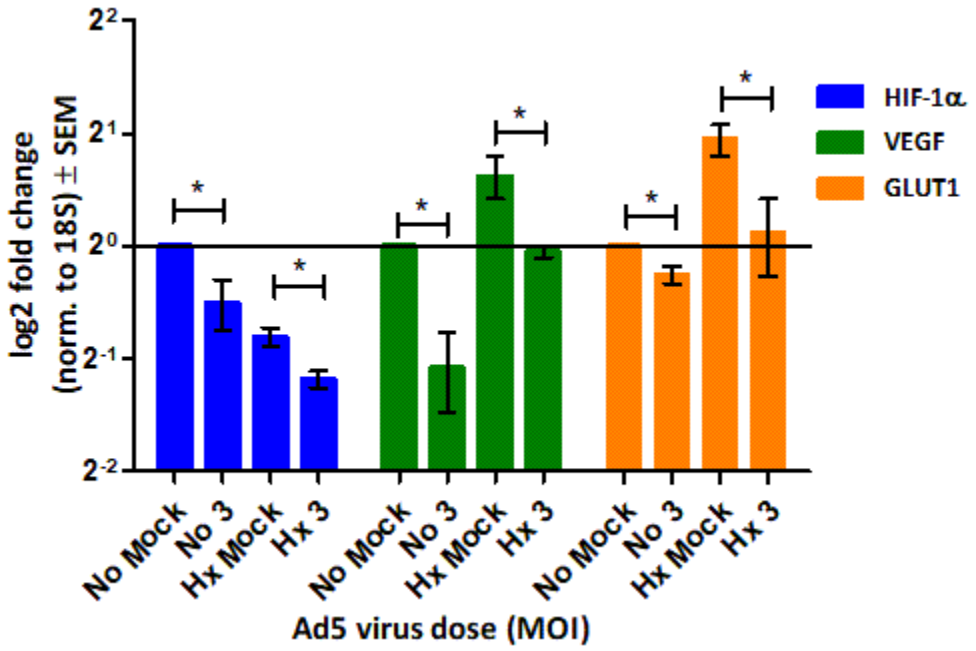

Figure 17. Ad5 infection leads to down-regulation of HIF-1α and target genes at 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in respective condition during infection. *HIF-1α* and its target genes (*GLUT1* and *VEGF*) mRNA was quantified using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control sample. Error bars represent SEM from 3 independent experiments (*p*-values were calculated using T test: * $p < 0.05$, ** $p < 0.01$,
*** $p < 0.001$, ns = non-significant, No = normoxia, Hx = hypoxia)

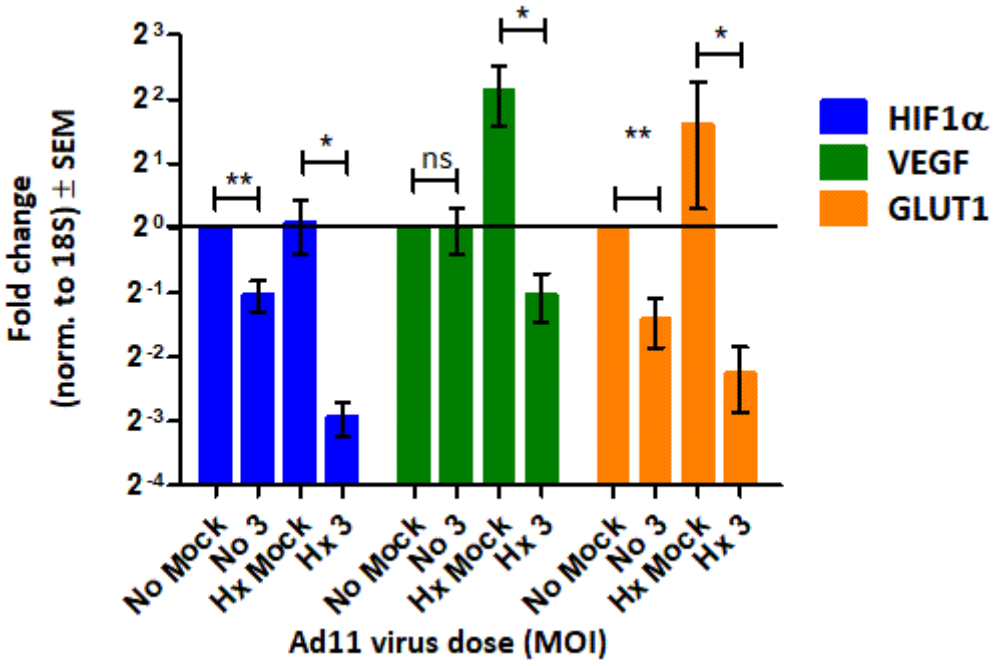

Figure 18. Ad11 infection leads to down-regulation of HIF-1α and target genes at 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in the respective condition during the infection. *HIF-1α* and its target genes (*GLUT1* and *VEGF*) mRNA was quantified using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control sample. Error bars represent SEM from 3 independent experiments (*p*-values were calculated using T test: * p< 0.05, ** p < 0.01, *** p < 0.001, ns = non-significant, No = normoxia, Hx = hypoxia)

3.6 EnAd regulates cellular genes differentially during early and late phase of infection

To further investigate whether EnAd was down-regulating HIF-1α mRNA specifically, we measured mRNA levels of proliferating cell nuclear antigen (PCNA) and TATA box-binding protein (TBP). These genes were chosen because PCNA was found to be up-regulated by Ad5 E1A (Lee, Liu and Mathews 1998) and TBP is a gene that is rarely regulated at the RNA levels and commonly used as a reference gene in qPCR studies (Ledderose et al., 2011; Ohl et al., 2006). We found that at 6 hpi PCNA was significantly up-regulated in normoxia only whereas

the levels of TBP were increased in both conditions but not significantly. At 24 hpi, PCNA levels remained unchanged after EnAd infection while TBP levels were significantly down-regulated in hypoxia and remained unchanged in normoxia (Figure 19).

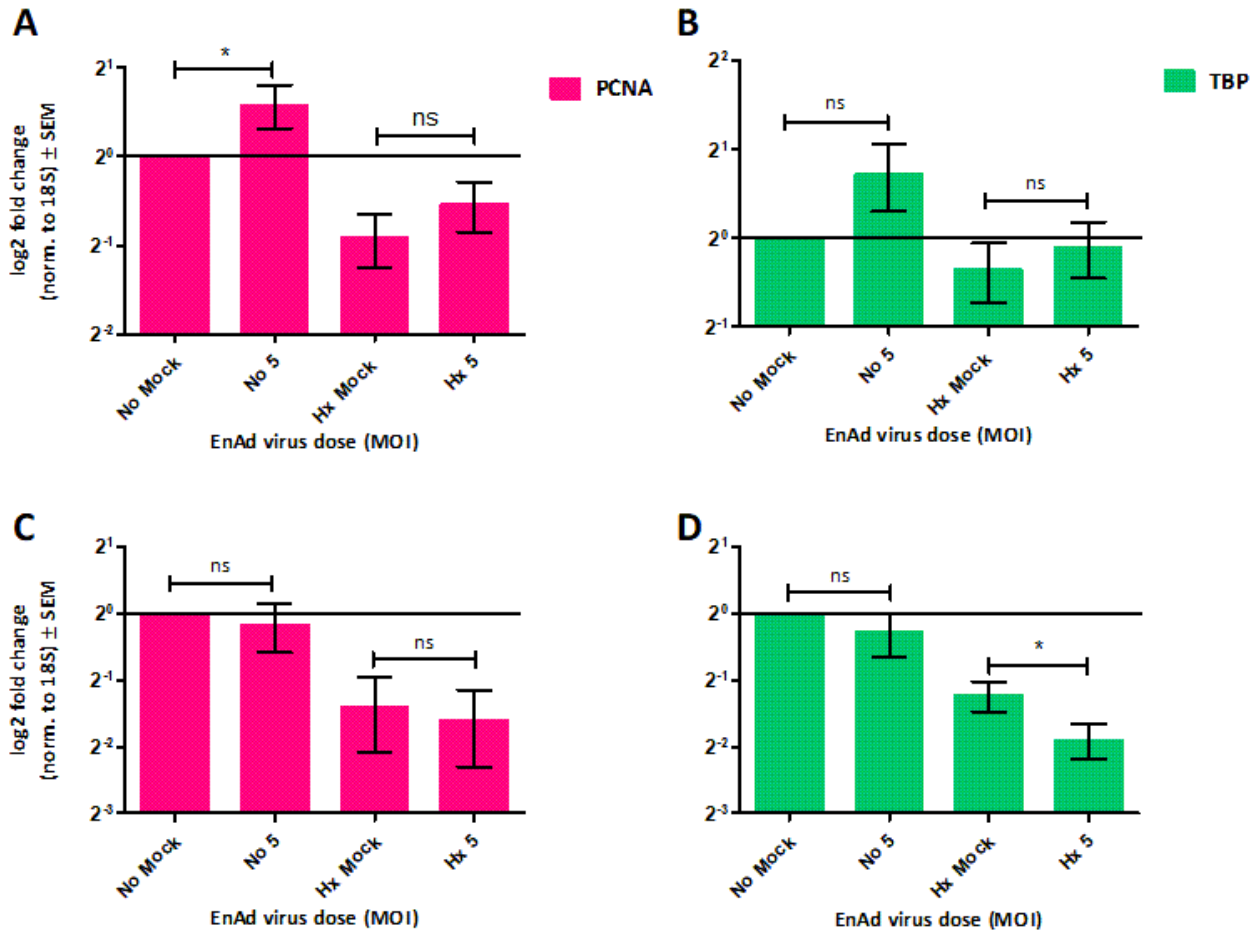

Figure 19. EnAd infection alters PCNA and TBP genes expression differently at 6hpi and 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in the respective consdition during the infection. PCNA and TBP mRNA was quantified at 6hpi (A,B) and 24hpi (C,D) using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control samples. Error bars represent SEM from 3 independent experiments (*p*-values were calculated using T test: * p< 0.05, ** p < 0.01, *** p < 0.001, ns = non-significant, No = normoxia, Hx = hypoxia)

3.7 Ad5 regulates cellular genes differentially during late phase of infection

We measured the transcript levels of PCNA and TBP 24 hours after infection with Ad5 and found that PCNA was significantly upregulated after virus infection in hypoxia but not in normoxia and the levels of TBP remained unchanged in both normoxia and hypoxia (Figure 20).

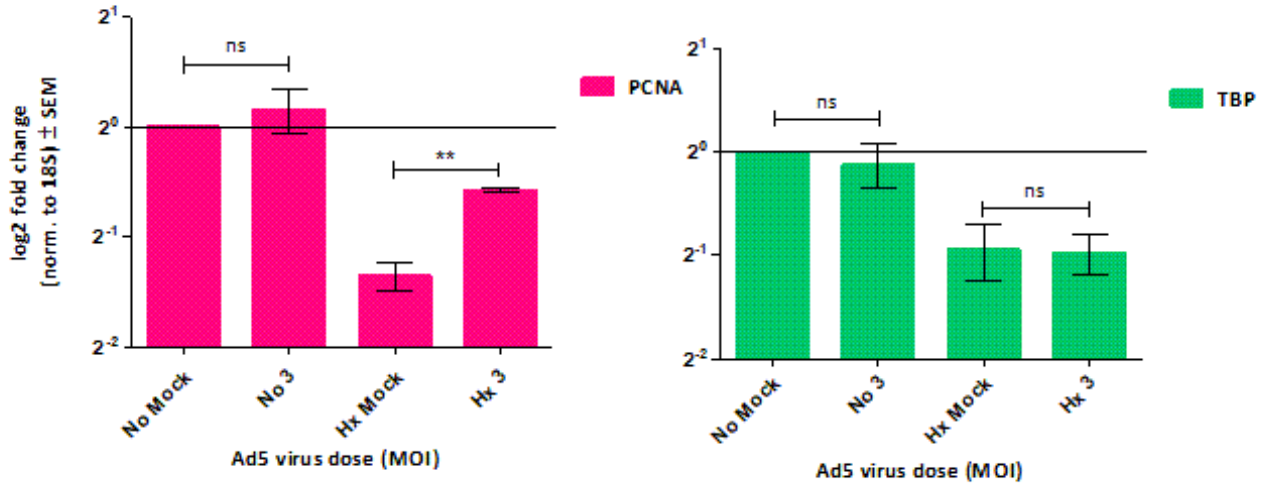

Figure 20. Ad5 regulates cellular genes differently at 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO_2) or normoxia for 18 hours and maintained in the respective condition during infection. PCNA and TBP mRNA was quantified at 6hpi (A,B) using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control samples. Error bars represent SEM from 3 independent experiments (*p*-values were calculated using T test: * p< 0.05, ** p < 0.01, *** p < 0.001, ns = non-significant, No = normoxia, Hx = hypoxia)

3.8 Ad11 infection leads to down-regulation of PCNA and TBP during late phase of infection

PCNA and TBP transcript levels were measured after Ad11 infection at 24hpi. We found that the levels of both PCNA and TBP were significantly decreased in both hypoxia and normoxia during the late phase of virus infection (Figure 21).

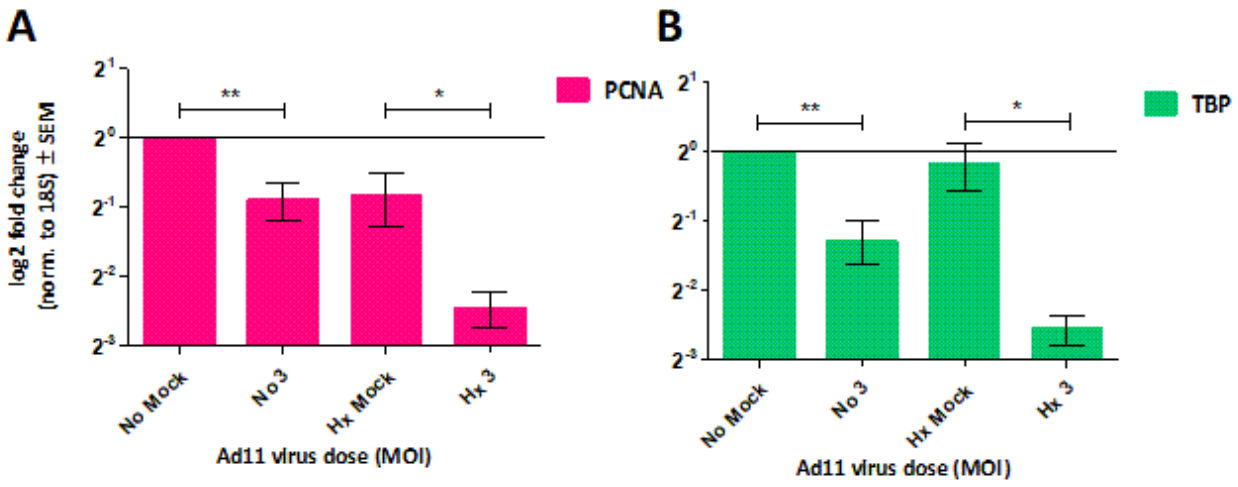

Figure 21. Ad11 down-regulates PCNA and TBP at 24hpi. DLD1 cells were pre-treated with hypoxia (1% pO2) or normoxia for 18 hours and maintained in the respective condition during the infection. PCNA and TBP mRNA was quantified at 24hpi using RT-qPCR. Pfaffl method was used to normalize gene expression to 18S and normoxia control samples. Error bars represent SEM from 3 independent experiments

(*p*-values were calculated using T test: * p< 0.05, ** p < 0.01, *** p < 0.001, ns = non-significant, No = normoxia, Hx = hypoxia)

Chapter 5: Discussion

5.1 General discussion

The data presented in this report shows that hypoxia affects the release of EnAd in virus dose- and cell line-dependent manner. EnAd leads to down-regulation of HIF-1α protein during single- and multi-step infection and this down-regulation is cell line specific. Ad5 and Ad11 also down-regulate HIF-1α protein during single step infection. The mechanism used by all three adenoviruses to down-regulate HIF-1α protein is independent of proteasome complex and relies on the transcriptional down-regulation of *HIF-1α*. While EnAd and Ad5 were found to down-regulate *HIF-1α* specifically, Ad11 led to down-regulation of all tested genes.

We found that where virus genome copy release upon EnAd infection at higher MOI (3 and 10) was not significantly decreased, there at lower MOI (0.1 and 1) it was significantly decreased. This behaviour has been observed previously in the case of VSV. Connor et al. (2004) showed that in hypoxic HeLa cells, VSV infection at lower MOIs resulted in decreased virus release compared with cells cultured in normoxia. Hwang et al. (2006) showed that induction of HIF-1α caused resistance to VSV infection at MOI of 0.1 but not at higher MOI. It is possible that cellular adaptation to hypoxia is associated with the increased antiviral activity, which hinders virus infection in conditions where the cell-to-cell spread is required, such as low MOI. However, since the data in our study is from only three measurements, it is possible that the study may not reflect a biological effect.

Even though hypoxia didn't alter virus genome release at higher MOI, we found that infectious particle production was significantly increased in hypoxic DLD1 cells, but unchanged in HCT116

cells. Our group has previously shown that EnAd has increased replication and protein synthesis in hypoxia. This suggests that overall EnAd activity is not attenuated by hypoxia and is even enhanced depending on the cell line. Shen et al. (2006) previously showed that Ad3 and Ad11 replication and lytic activity in hypoxia was affected in cell line-and time point- dependent manner. While there was no significant reduction in replication and cytolysis at 48hpi in most cell lines, there prolonged infection (up to 8 days) significantly attenuated virus activity in hypoxia. However, studies with Ad5 have reported a significant reduction in replication and lytic activity in hypoxia compared with normoxia (Pipiya et al., 2005; Shen and Hermiston, 2005). As summarised in table 1, most oncolytic viruses have either similar or increased activity in hypoxia so it is not surprising that EnAd has enhanced activity in hypoxia, especially as it is more potent that Ad5 and Ad11. Since HIF-1 dominates the hypoxia phenotype in tumours and many OVs have been shown to alter HIF-1α (Lu and Kang, 2010; Gordan and Celeste Simon, 2007), we hypothesised that adenoviruses regulate the HIF-1pathway.

We measured the protein levels of HIF-1α after single-step (24hpi) and multi-step (48hpi) infection with EnAd in DLD1 cells. We found increased depletion of HIF-1α protein with increasing virus doses at 24hpi and 48hpi. This led us to conclude that HIF-1α protein down-regulation is EnAd mediated. Since MOI 0.1 and 1 lead to infection in only 1% and 13.4% cells respectively, HIF-1α protein depletion at lower MOI was minimal compared to that at higher MOI. This hinted that HIF-1α might be involved in less progeny production in hypoxia compared with normoxia at lower MOI. This further supports Hwang et al., (2006) study with VSV, as discussed earlier, and reinforces the antiviral role of HIF-1α.

In order to ensure that we were observing the effect of EnAd infection in infected cells only, we decided to use high MOIs (≥ 3) for further studies to aim for ≥ 60 % cells infected population. We then measured the levels of HIF-1α protein after Ad5 and Ad11 infection in DLD1. We found that, similar to EnAd, both viruses down-regulated HIF-1α at MOI 3 at 24hpi. Interestingly, both EnAd and wild type adenoviruses regulated HIF-1α similarly despite the observation that wildtype Ads display attenuated activity in hypoxia, whilst EnAd activity is improved. It is tempting to deduce that the efficiency of adenoviruses in hypoxia might be independent of the HIF-1 pathway. However, there are some major methodology differences in the reports published about Ad5 and Ad11 (Shen et al., 2005; Shen et al., 2006) and our data with EnAd. While we have used MOI as a measure of virus doses, both reports have used virus particles per cell (vp/cell). Since EnAd activity has been shown to be attenuated by hypoxia at lower MOI, it is possible that the vp/cell used in reports on Ad5 and Ad11 are comparable to the lower MOI used in our study. Furthermore, while we have looked at the effect of hypoxia up to 48 hours, studies with Ad5 and Ad11 have measured virus activity from 3 days to 8 days. These factors can explain why published literature suggests that Ad5 and Ad11 have reduced activity while our data shows that EnAd has similar or improved activity in hypoxia. However, further experiments with HIF-1α knockout and overexpression need to be performed to actually establish if HIF-1α has an effect on adenovirus efficiency in hypoxia.

Since we had observed down-regulation of HIF-1α in only DLD1 cells so far, we studied whether HIF-1α down-regulation was a cell line specific effect or not. HIF-1α protein levels were measured in different adenocarcinoma cell lines (HCT116, A549, RKO, SW480) and AD293 (HEK293 cells with Ad5 E1 gene) after infection with EnAd at MOI5 at 24hpi. We found that all

colorectal cell lines except RKO showed down-regulation of HIF-1α and CAIX protein upon adenovirus infection. The lung adenocarcinoma cell line A549 also showed down-regulation and the immortalized cell line AD293 cells showed low basal levels of HIF-1α protein but no further decrease in protein levels after EnAd infection. Since infections were performed at MOI 5 for all cell lines, varying infectivity among these cell lines, viral entry and availability of the viral high affinity receptor CD46 could also explain why EnAd infection in RKO doesn't down-regulate HIF-1α.

Unlike DLD1, HCT116 and SW480, RKO cells encode wildtype *KRAS* and mutant $BRAF^{V600E}$, as summarised in table 9 (Ahmed et al., 2013). Interestingly, two studies suggest that mutant KRAS (G12V) increases the translation of HIF-1α protein in hypoxia, whereas mutant BRAF (V600E) increases the mRNA expression of both HIF-1α and HIF-2α in colon cancer cells (Kikuchi et al., 2009; Kumar et al., 2007). Unfortunately, neither study elucidates the mechanism for $BRAF^{V600E}$ mediated up-regulation of HIF-1α, but this suggests that induction of *HIF-1α* in the presence of mutant $BRAF^{V600E}$ abrogates the ability of EnAd to down-regulate *HIF-1α* mRNA in RKO cells. Future work will determine the effect of adenovirus infection on HIF-1α in other colorectal cancer cell lines with wt *KRAS* and mutated $BRAF^{V600E}$. To accomplish this, HIF-1α levels will be measured in RKO cells with overexpression and knockdown of $BRAF^{V600E}$ after virus infection to establish whether this mutation actually determines the phenomenon observed with RKO cell lines. The viral uptake and infectivity of RKO cell lines will also be determined.

Further, AD293 showed lower basal levels of HIF-1α protein compared to other cell lines without induction of CAIX, even in mock conditions. This led us to suspect that E1 proteins might be involved in down-regulation of HIF-1α protein.

Table 9: Mutations in selected oncogenes in different colorectal cancer cell lines (wt = wild type)

Cell line	Cell type	*KRAS*	*BRAF*	*TP53*	*PIK3CA*	*PTEN*
DLD1	Colorectal cancer	G13D	wt	S241F	E545K;D549N	Wt
HCT116	Colorectal cancer	G13D	wt	wt	H1047R	Wt
SW480	Colorectal cancer	G12V	wt	R273H;P309S	wt	Wt
RKO	Colorectal cancer	wt	V600E	wt	H1047R	Wt

Nonetheless, once it was established that HIF-1α protein is being down-regulated by all three adenoviruses in cell lines with wild type *BRAF* and mutant $KRAS^{G13D/G12V}$, the next step was to elucidate the mechanism used by adenoviruses to achieve this. Since proteasome complex is the main mechanism of HIF-1α degradation and other OVs have been shown to exploit this pathway to down-regulated HIF-1α (Cho et al., 2010; Abd-Aziz, Stanbridge and Shafee, 2006), we hypothesised that adenoviruses down-regulate HIF-1α at post-translational level. The stabilization of HIF-1α in normoxia upon proteasomal inhibition was lost after infection with EnAd, Ad5 or Ad11, indicating that virus-mediated HIF-1α down-regulation is upstream of the regulation by proteasomal degradation.

Adenoviruses have been widely shown to regulate many cellular genes at the transcriptional level. E1A, which is one of the main transcription factors of adenoviruses, has been found to both directly and indirectly regulate cellular genes. In fact, Ad2 E1A interacts with p300/CBP

which are co-factors for many transcription factors and leads to reduced transcription of cellular genes (Ferrari et al., 2014). Ad5 E1A is reported to sequester specificity protein 1 (SP1) for activation of the major late promoter of Ad5 (Parks and Shenk, 1997). Interestingly, SP1 is required for constitutive HIF-1α expression (Minet et al., 1999). We hypothesised that EnAd, Ad5 and Ad11 regulate HIF-1α at the transcriptional level. We found that *HIF-1α* and target transcripts (*GLUT1* and *VEGF*) levels were significantly down-regulated by EnAd in agreement with results observed at the protein level. Similar results were observed with Ad5 and Ad11 at 24hpi. To our knowledge, we are the first to report the transcriptional regulation of *HIF-1α* by Ad5 and Ad11.

It has been reported for Ad2 that about 70% of cellular genes are down-regulated during the late phase of infection (>20 hpi) (Zhao et al., 2007). In order to figure out whether *HIF-1α* mRNA down-regulation by EnAd, Ad5 and Ad11 is specific or a result of virus-mediated down-regulation of cellular genes, we studied the levels of two more genes. PCNA was chosen because it has been found to be up-regulated by Ad5 E1A (Lee, Liu and Mathews 1998) and TBP was chosen because it is a commonly used reference gene and has not yet been shown to be down-regulated after adenovirus infection. It was hypothesised that the levels of PCNA will be up-regulated and TBP levels will remain unchanged after adenovirus infection.

We found that PCNA was not differentially regulated during EnAd infection, was significantly up-regulated by Ad5 and significantly down-regulated by Ad11. TBP was significantly down-regulated by EnAd only in hypoxia, not regulated by Ad5 and significantly down-regulated by Ad11. The fact that PCNA is not down-regulated during EnAd and Ad5 infections indicates

that the down-regulation of HIF-1α is not a result of the cellular transcriptional down-regulation by virus. For Ad11, it is possible that virus induces cellular transcriptional shut down. However, with a panel of three genes, it is unreasonable to draw such a conclusion. It was quite surprising though that EnAd and Ad11 behave differently in regulating PCNA and TBP since EnAd has 98.6% per cent sequence homology with Ad11 (Kuhn, Irene, et al., 2008). We also studied that the levels of *HIF-1α, VEGF, GLUT1, PCNA and TBP* during the early phase of virus life cycle (6hpi) and found that they were all up-regulated by EnAd, but only PCNA was up-regulated significantly. This suggested that the down-regulation of HIF-1α specifically occurs during the late phase of the virus lifecycle. Taken together it is tempting to conclude that group B and group C adenoviruses decrease HIF-1α levels by down-regulation of *HIF-1α* mRNA. Since adenovirus E1A gene regulates many cellular genes as discussed earlier and our data has shown lower basal levels of HIF-1α protein in AD293 cells (which have adenovirus E1 gene) compared to other cell lines, we predict that E1A might be involved in virus mediated down-regulation of *HIF-1α*. An expansion of this work will, thus, investigate the role of E1A in the HIF-1 pathway regulation. There are two possible hypotheses to explore this.

Firstly, SP1 protein is a transcription factor which is required for the constitutive expression of HIF-1α (Minet et al., 1999). Interestingly, a few studies suggest that Ad5 major late promoter requires SP1 for transcription. Further, it has also been found hat Ad5 E1A and SP1 form stable complex in vitro (Parks and Shenk, 1997). During infection, it is plausible that the sequestration of SP1 by E1A leads to down-regulation of *HIF-1α* transcription. An analysis of SP1 binding sites in Ad11 and EnAd genome revealed two binding sites in MLP in Ad11 and several binding sites in E2B region and one in MLP in EnAd. However, since Ad11 and EnAd E1A differ in structure to

Ad5 E1A it is possible that group B viruses employ another mechanism to down-regulate *HIF-1α* transcription. This hypothesis can be tested by measuring the promoter (MLP and *HIF-1α* promoter) occupancy by SP1 using Chromatin immunoprecipitation analysis.

Secondly, there are some studies that have shown that NF-κB is one of the regulators of *HIF-1α* expression, especially in hypoxia, and HIF-1α in turn regulates NF-κB as well (Görlach and Bonello, 2008). In fact, inhibition of different NF-κB subunits leads to a drastic reduction in basal levels of HIF-1α (Van, Kenneth, and Rocha, 2008). NF-κB is involved in innate immunity and is activated by a wide range of agents including Ad5 E1A and adenovirus E3/19k (Pahl, 1999). In fact, it has been shown that E1A in turn represses NF-κB-mediated gene activation (Cook et al., 2002). It is very likely that E1A-mediated repression of NF-κB leads to a concomitant decrease in *HIF-1α* mRNA levels. To this end, NF-κB inhibition can be used to eventually mimic the effects of the virus on *HIF-1α* mRNA. Alternatively, NFkB overexpression during infection in hypoxia may abrogate virus-mediated *HIF-1α* down-regulation.

5.2 Conclusion

In conclusion, our data depicts that EnAd has a similar or improved release in hypoxia during the first virus replication cycle. Group B (Ad11 and EnAd) and group C (Ad5) adenoviruses lead to down-regulation of *HIF-1α* mRNA expression during the late phase of virus infection. This effect is specific to *HIF-1α*, at least in the case of EnAd and Ad5 and might be mediated by adenovirus E1A protein. Down-regulation of HIF-1α is cell line specific and might be dependent on *KRAS* and *BRAF* status of colorectal cancer cell lines.

Our findings are important in the field of virotherapy because they help us understand how tumour microenvironment can change after EnAd infection and how that will affect the final outcome of therapy. Since HIF-1α is very essential for tumour growth, angiogenesis and metabolism in hypoxia (Semenza, 2002); its down-regulation by EnAd might have consequences that can facilitate EnAd in killing cancer cells. For example, VEGF, a direct target of HIF-1, causes angiogenesis which is linked to drug resistance and even metastasis. In fact, VEGF inhibitors have been used in cancer therapy for decades now (Sukhramani and Suthar, 2010). Down-regulation of HIF-1α by EnAd will lead to down-regulation of VEGF causing vascular shutdown and cancer cell death.

Further, our results also provide insights into designing better adenoviruses for both oncolytic virotherapy and gene therapy. In the past, there are several groups that have engineered oncolytic adenoviruses with major late promoter under the control of HIF proteins to make them hypoxia specific (Cho et al., 2004; Guse et al., 2009; Kwon et al., 2009; Post and Van Meir, 2006). Since adenoviruses down-regulate HIF-1α during the late phase of the lifecycle, it might not be the best strategy to have late promoters under the control of HIF-1. In addition, this also highlights the significance of studying the effect of virus infection on other HIF proteins before designing any OVs with HREs.

Lastly, our findings have shown that oncolytic adenovirus activity in hypoxia is cell line-or tumour- dependent which further stresses the need to study the behaviour of OVs in hypoxia in order to create a more robust therapy for tumours. Future expansion of this work will likely

develop oncolytic adenoviruses capable of targeting both hypoxic and normoxic regions of tumours.

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