

Georgia State University

ScholarWorks @ Georgia State University

Biology Dissertations

Department of Biology

12-14-2017

Adenovirus ?Hit-And-Run? Eviction From B-Cells Harboring Leukemic Runx1 Fusion Genes And Adp Controlled Latency In Lymphocytes

Hans T. Wilms
Georgia State University

Follow this and additional works at: https://scholarworks.gsu.edu/biology_diss

Recommended Citation

Wilms, Hans T., "Adenovirus ?Hit-And-Run? Eviction From B-Cells Harboring Leukemic Runx1 Fusion Genes And Adp Controlled Latency In Lymphocytes." Dissertation, Georgia State University, 2017.
doi: <https://doi.org/10.57709/11154614>

This Dissertation is brought to you for free and open access by the Department of Biology at ScholarWorks @ Georgia State University. It has been accepted for inclusion in Biology Dissertations by an authorized administrator of ScholarWorks @ Georgia State University. For more information, please contact scholarworks@gsu.edu.

ADENOVIRUS “HIT-AND-RUN” EVICTION FROM B-CELLS HARBORING LEUKEMIC
RUNX1 FUSION GENES AND ADP CONTROLLED LATENCY IN LYMPHOCYTES

by

HANS WILMS

BS, Kennesaw State University, 2010

MS, Georgia State University, 2015

Under the Direction of Charlie Garnett Benson, PhD

ABSTRACT

The common species C adenoviruses infect more than 80% of the human population early in life. The virus can establish an asymptomatic persistent infection in mucosal-associated lymphocytes. Adenovirus has long been classified as DNA tumor virus, however it still has no established associations with any specific cancer. This phenomenon may be explained by the model of hit-and-run viral oncogenesis, which proposes that a virus can infect a cell population, causing oncogenic mutations (hit) that in turn inhibit the virus from persisting in the cancerous cell progeny (run). One type of cancer in particular, childhood precursor B-cell acute lymphoblastic

leukemia, is suspected of having an infectious cause, yet no viral agent has been conclusively linked to the disease. Childhood acute lymphoblastic leukemia is associated with frequently occurring chromosomal translocations that encode fusion proteins. ETV6/RUNX1 is the most commonly found translocation and the only one to be associated with the infectious etiology of the disease. We hypothesized that adenovirus may be an agent capable of initiating childhood precursor B-cell acute lymphoblastic leukemia but that the virus is evicted from cancer cells by the activities of the ETV6/RUNX1 leukemic fusion gene. We found that adenovirus retention in a B-cell model of persistence was inhibited by direct binding of the ETV6/RUNX1 fusion gene to the viral genome. This loss of virus was dependent on histone deacetylation activities, but was not impacted by NF- κ B interference, selective cell death of infected cells, or known interactions of RUNX1 with viral protein. Further, epigenetic changes induced in cells following viral infection were still present after viral eviction from the cells, which demonstrates that viral retention is unnecessary for sustained repression of cellular genes. Because latency is likely related to viral retention in B-cells, we also studied how NF- κ B control of the adenovirus E3 region and expression of the contained adenovirus death protein (ADP) gene could effect viral latency in lymphocytes. We failed to find specific activation of E3 region genes with NF- κ B activation through PMA/Ionomycin. ADP was necessary for lytic infection in lymphocytes, but not epithelial cells, signifying differences in regulation of ADP between these cell types.

INDEX WORDS: Adenovirus, Hit-and-run viral oncogenesis, acute lymphoblastic leukemia, childhood leukemia, ETV6/RUNX1, ADP

ADENOVIRUS “HIT-AND-RUN” EVICTION FROM B-CELLS HARBORING LEUKEMIC
RUNX1 FUSION GENES AND ADP CONTROLLED LATENCY IN LMYPHOCYTES

by

HANS WILMS

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2017

Copyright by
Hans Trevor Wilms
2017

ADENOVIRUS “HIT-AND-RUN” EVICTION FROM B-CELLS HARBORING LEUKEMIC
RUNX1 FUSION GENES AND ADP CONTROLLED LATENCY IN LMYPHOCYTES

by

HANS WILMS

Committee Chair: Charlie Benson

Committee: Timothy Denning

Richard Plemper

Electronic Version Approved:

Office of Graduate Studies

College of Arts and Sciences

Georgia State University

December 2017

DEDICATION

To my Father, Ulrich Heinz Wilms, who supported me throughout everything in my life and never asked for anything in return.

To my Mother, Robyn Hayes Wilms, who taught me creativity and pushed me to be a better man.

I hope one day I can give back to the world what these two have given me.

ACKNOWLEDGEMENTS

This work would not have been possible without my advisor's insight and direction, Dr. Charlie Garnett-Benson. It would not have been completed without her ability to direct focus on the critical questions while still fostering an atmosphere of excited inquiry. I cannot imagine another mentor under which this project, or student, could have thrived so fully. She has my fullest appreciation, and my most heartfelt thanks.

I would like to also thank my committee members Dr. Timothy Denning and Dr. Richard Plemper for their valuable guidance and input through all the years of my research.

ACKNOWLEDGMENTS

This work was supported by R01 CA127621 from the National Cancer Institute and by the Children's Leukemia Research Association.

TABLE OF CONTENTS

1	TABLE OF CONTENTS	
	ACKNOWLEDGEMENTS	V
	LIST OF TABLES	IX
	LIST OF ABBREVIATIONS	XIII
1	BACKGROUND.....	1
1.1	SIGNIFICANCE OF THE STUDY.....	10
2	ADENOVIRUS ONCOGENIC MECHANISMS AND REVIEW OF INFECTIOUS RISK FACTORS CORRELATION WITH CHILDHOOD LEUKEMIA..	12
2.1	INTRODUCTION.....	13
2.2	GENOME INSTABILITY AND MUTATION	14
2.3	RESISTING CELL DEATH.....	15
2.4	DEREGULATING CELLULAR ENERGETICS	16
2.5	SUSTAINING PROLIFERATIVE SIGNALING.....	17
2.6	EVADING GROWTH SUPPRESSORS.....	17
2.7	AVOIDING IMMUNE DESTRUCTION.....	18
2.8	ENABLING REPLICATIVE IMMORTALITY	19
2.9	TUMOR PROMOTING INFLAMMATION.....	19
2.10	EVIDENCE OF ADENOVIRUS AND ALL CORRELATING	20
2.11	ADENOVIRUS AS THE 1ST HIT.....	26

2.12	ADENOVIRUS AS THE 2ND HIT.....	32
3	LEUKEMIC RUNX1 FUSION GENES EVICT ADENOVIRUS FROM B-CELL POPULATION VIA DIRECT BINDING TO VIRAL DNA	36
3.1	ABSTRACT.....	38
3.2	INTRODUCTION.....	39
3.3	MATERIALS AND METHODS	43
3.4	RESULTS.....	51
3.5	DISCUSSION	75
4	NO EVIDENCE FOR INDIRECT MECHANISMS OF ADENOVIRUS EVICTION FROM RUNX1 LEUKEMIC FUSION PROTEIN EXPRESSING B CELL POPULATIONS: EXPLORATION OF CELL DEATH, NF-KB INHIBITION, AND INTERFERENCE WITH VIRAL E4ORF6 PROTEIN FUNCTION.....	81
4.1	ABSTRACT.....	82
4.2	INTRODUCTION.....	82
4.3	MATERIALS AND METHODS	88
4.4	RESULTS.....	92
4.5	DISSCUSSION	103
5	THE ROLE OF ADENOVIRUS E3 REGION IN CONTROLLING VIRAL LATENCY IN LYMPHOCYTES	107
5.1	ABSTRACT.....	108
5.2	INTRODUCTION.....	108

5.3	MATERIALS AND METHODS	113
5.4	RESULTS.....	116
5.5	DISSCUSSION	134
6	DISCUSSION AND CONCLUSIONS.....	137
	REFERENCES.....	145
	APPENDICES.....	159
	Appendix A	159
	Appendix B	223

LIST OF TABLES

Table 3.1 Primers and Probes for RT-QPCR.....	46
Table 3.4 HDAC inhibition increases viral gene expression in persistently infected cells.	66
Table 3.5 Adenovirus-provoked downregulation of cellular genes is retained after departure of the virus.....	71
Table 4.1 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, low cell concentration.....	98
Table 4.2 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, high cell concentration.....	98
Table 4.3 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, Ad5wt infected cells.	99
Table 4.4 I κ B mRNA transcription after PMA/Ionomycin stimulation.	101
Table 5.1 Jurkat and BJAB cells infected with Ad2 and Ad5 display different patterns of viability.	129

LIST OF FIGURES

Figure 2.1 Adenovirus mechanisms of oncogenesis and the hallmarks of cancer.....	14
Figure 2.2 Adenovirus type 5 seroprevalence decreases with increasing HDI of a country.	23
Figure 2.3 Childhood leukemia incidence decreases with increasing adenovirus type 5 seroprevalence.....	23
Figure 2.4 Initiation and latency period of childhood ETV6/RUNX1 precursor B cell acute lymphoblastic leukemia.	24
Figure 3.1 Stable expression of the ETV6/RUNX1 and RUNX1/MTG8 leukemia fusion genes persists in B lymphocytic cells following transfection and selection.	53
Figure 3.2 Schematic representation of the RUNX1 and RUNX1-related leukemic fusion proteins and RUNX1-antibody recognition sites.....	54
Figure 3.3 The “acute” phase of adenovirus infection in B lymphocytes is not impacted by enforced expression of the leukemia-associated ETV6/RUNX1 or RUNX1/MTG8 fusion genes.	56
Figure 3.4 Expression of the late viral hexon gene declines in persistently infected B lymphocytes that express the leukemia-associated ETV6/RUNX1 or RUNX1/MTG8 fusion genes.	58
Figure 3.5 Expression of the early viral genes declines in persistently infected B lymphocytes that express the leukemia-associated ETV6/RUNX1 or RUNX1/MTG8 fusion genes.	59
Figure 3.7 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of persistently infected B lymphocytic cells.	60
Figure 3.8 Expression of ETV6/RUNX1 or RUNX1/MTG8 in persistently infected B lymphocytes promotes the loss of viral DNA.....	62

Figure 3.9 Expression of ETV6/RUNX1 or RUNX1/MTG8 during persistent significantly reduces amount of viral DNA retention in B cells.....	63
Figure 3.10 The leukemic associated fusion protein ETV6/RUNX1 binds the adenoviral genome.	65
Figure 3.11 Levels of the viral protein required for viral DNA replication decline in advance of the decline in viral DNA levels.....	69
Figure 3.12 Persistently infected BJAB cells transfected with RUNX1 fusion genes.	74
Figure 3.15 Persistently infected BJAB have low rates of transfection.	75
Figure 4.1 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of adenovirus dl309 persistently infected B lymphocytic cells (day 28 post infection).	94
Figure 4.2 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of adenovirus dl309 persistently infected B lymphocytic cells (day 40 post infection).	95
Figure 4.3 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of uninfected B lymphocytic cells.....	96
Figure 5.1 Alignment of human adenovirus type 5 genome to type 2, type 1, type 31 and simian adenovirus type 11 (NCBI BLAST).	112
Figure 5.2 Alignment of human adenovirus type 5 E3 region to type 1 and type 2 (NCBI BLAST).....	113
Figure 5.3 A549 Hexon expression.	118
Figure 5.4 A549 ADP expression.	119
Figure 5.5 A549 growth and viability.....	119
Figure 5.6 Jurkat Hexon expression.....	121
Figure 5.7 Jurkat ADP expression.	122

Figure 5.8 Jurkat concentration and viability.	122
Figure 5.9 KE37 Hexon expression.	123
Figure 5.10 KE37 ADP expression.	124
Figure 5.11 KE37 concentration and viability.	125
Figure 5.12 BJAB Hexon expression.	126
Figure 5.13 BJAB ADP expression.	126
Figure 5.14 Hexon protein expression in Jurkat cells infected with Ad2 and Ad5.	130
Figure 5.15 Viral transcript levels of PMA/Ionomycin treated persistently infected BJAB cells, 1hr (A, experiment 1, 28 days post infection) 4hrs (B, experiment 1, 28 days post infection) and 1hr (C, experiment2 33 days post infection).	132

LIST OF ABBREVIATIONS

ALL- Acute Lymphoblastic Leukemia

cALL- Childhood Acute Lymphoblastic Leukemia

pre-B ALL- Precursor B cell Acute Lymphoblastic Leukemia

Ad- Adenovirus

Ad2- Human Adenovirus Species C type 2

Ad5- Human Adenovirus Species C type 5

1 BACKGROUND

Human adenoviruses are non-enveloped, icosahedral shaped viruses that commonly infect the upper respiratory and gastrointestinal tract in young children (Avila, Carballal et al. 1989). Adenoviruses are ubiquitous in any human population and exhibit dozens of serotypes categorized into 7 species. Of these, human adenovirus species C infects over 80% of humans by adulthood. There are 4 known serotypes of species C Adenovirus, with serotypes 2 and 5 being the most commonly investigated (Krilov, 2005). Adenovirus genes are divided into “Early”, designated by an “E” at the beginning of each name, and “Late” genes. The early genes are expressed during the initial stages of infection and work primarily to control host cell functions to allow efficient viral replication. These include interfering with immune, intracellular antiviral, apoptosis and cell cycle host cell mechanisms. The late genes primarily encode viral capsid and assembly proteins. Disease caused by the acute infection with these serotypes is relatively mild in most cases, and occurs in epithelial cells. Adenovirus infection in lymphocytes occurs as a persistent infection. Species C Adenovirus persists with a significant amount of adenovirus genomes detectable in mucosal lymphocytes in a predominantly inactive form (Garnett, Erdman et al. 2002). In addition, live virus is shed in stool periodically, which may be how the virus remains ubiquitous in the population (Fox, Brandt et al. 1969). Although the molecular mechanisms behind reactivation from latency are not understood, it has been shown *in vitro* that infectious viral reactivation can occur from lymphocytes found in adenoid and tonsil tissue that harbor noninfectious adenovirus DNA (Garnett, Talekar et al. 2009).

Human adenovirus has been suspected to be oncogenic since its use to immortalize primary cell lines and human adenovirus is commonly listed amongst oncogenic DNA viruses (Graham, Smiley et al. 1977, Schreiner, Wimmer et al. 2011, Speiseder, Nevels et al. 2014).

Despite being highly oncogenic in animals and displaying a variety of mechanisms in its lifecycle that are linked to oncogenesis (Gallimore 1974, White 1998, Liu, Colosimo et al. 2000, Nevels, Tauber et al. 2001, Hart, Yannone et al. 2005, Zhang, Huang et al. 2010, Schreiner, Wimmer et al. 2011, Speiseder, Nevels et al. 2014); direct evidence of adenovirus oncogenicity in humans has yet to be documented. However, we see both of the common processes embedded within the adenovirus lifecycle, namely persistent infection and inhibition of normal tumor suppressor activities of p53 and Rb proteins (Whyte, Buchkovich et al. 1988, Yew and Berk 1992, Liu and Marmorstein 2007, Speiseder, Nevels et al. 2014). During tumor formation in animals, the complete absence of adenovirus genomes has been documented in cancerous cells after passaging *in vitro*, so clearly continued viral presence alone is not driving cellular growth and division, but is needed for cancer initiation (Kuhlmann, Achten et al. 1982). In the normal adenovirus lifecycle, early adenovirus protein E1A disrupts cellular Rb binding to cellular E2F, while adenovirus E1B protein inhibits p53 and caspase activity (Whyte, Buchkovich et al. 1988, Yew and Berk 1992, White 1998, Liu, Colosimo et al. 2000, Liu and Marmorstein 2007, Speiseder, Nevels et al. 2014). Additionally, adenovirus E4 protein reduces nuclear p53 levels while also inhibiting the DNA double strand break repair (DSBR) mechanism {Hart, 2005 #1400;Weiden, 1994 #1397;Querido, 1997 #2871;Querido, 1997 #2872;Dobner, 1996 #2875}. *As discussed below, inhibition of pRb and p53 occurs in almost all the other known oncogenic DNA viruses and they represent a major class of genes that must be modulated for cancer to develop.*

The process of viral oncogenesis is inefficient and typically takes years to progress from infection to clinical diagnosis of cancer, only occurring in a minority of infected individuals. Currently known human DNA oncogenic viruses include human papilloma viruses (HPV),

hepatitis B virus, some types of human herpes viruses (HHV) (zur Hausen 1991, Liao 2006) and merkel cell polyomavirus (MCV) (Feng, Shuda et al. 2008). Two common processes are seen during oncogenesis by these viruses. The first common process is that each virus is associated with the progression of particular types of cancer derived from cells that the viruses **persistently** infect (zur Hausen 1991, Liao 2006). This persistence can be due either to viral latency programs or infection of cell types in which viral gene expression is so limited that it hinders viral replication. For example, hepatitis B oncogenesis is restricted to hepatocytes, which are chronically infected, leading to chronic liver inflammation and liver cancer (Fattovich, Stroffolini et al. 2004, Bialecki and Di Bisceglie 2005). Epstein-Barr virus (HHV-4) causes cancer in B-lymphocytes during latency by promoting cell proliferation via LMP-1 and other viral genes (Young and Murray 2003, Thompson and Kurzrock 2004). The second common process that all of these viruses share is the disruption of normal tumor suppressor activity as part of their normal life cycle, mostly Retinoblastoma protein (Rb) and p53 (Wang, Forrester et al. 1994, Thomas, Massimi et al. 1996, Doniger, Muralidhar et al. 1999, Rivas, Thlick et al. 2001, Liu, Chang et al. 2005, Bittar, Shrivastava et al. 2013, Borchert, Czech-Sioli et al. 2014).

Given these observations, adenovirus would be most likely to cause cancer in the lymphocytic cells in which it persists; giving rise to a leukemia. Interestingly, adenovirus is known to interact with RUNX1 proteins, which are important regulatory proteins in lymphocytes {Ichikawa, 2004 #1778;Koh, 2013 #1819;Kurokawa, 2006 #2782;Lichtinger, 2013 #1947;Tober, 2013 #1810;Webber, 2013 #1803}. Moreover, RUNX1 associated mutations, chromosomal abnormalities resulting from dsDNA breaks, are commonly associated with/characterize childhood leukemias. For example, the translocation t(12;21), is found in about a quarter of cALL cases and results in the fusion protein ETV6/RUNX1 (also known as TEL/AML1)

(Romana, Poirel et al. 1995, Fears, Vignon et al. 1996, Zelent, Greaves et al. 2004). **These translocation mutations are not enough to cause overt ALL**, and thus are thought to be only the first “hit”, i.e. initiating mutation, in a multi-hit mechanism of oncogenesis (reviewed in (Greaves, Maia et al. 2003)).

Cancer results only after *multiple* mutations in genes with specific functions related to oncogeneses occur (Figure 2.1). ETV6/RUNX1 would be classified as an oncogene that confers sustained growth abilities. Yet this single mutation is not sufficient for full carcinogenesis and additional mutations are necessary (Figure 2.4). After the initial translocation mutation of ETV6/RUNX1 there would need to be subsequent mutations in tumor suppressors genes that result in their inactivation for cancer to develop. These genes have been shown to be inactivated by deletion (Hanahan and Weinberg 2011, Papaemmanuil, Rapado et al. 2014) or by epigenetic silencing (Flavahan, Gaskell et al. 2017). Classic and well-known tumor suppressors include p53 and pRb, which adenovirus is known to inhibit and manipulate (Querido, Blanchette et al. 2001, Ferrari, Pellegrini et al. 2008). In addition, 3 of the 6 genes we recently found downmodulated in B cells (Ornelles, Gooding et al. 2016) have also been reported to have tumor suppressor functions in other cancers and to be repressed by methylation (Bacon, Fox et al. 2007, Zoppoli, Regairaz et al. 2012, Cao, Wang et al. 2013, Xiang, Qiu et al. 2013). It takes decades for most cancers to develop the necessary mutations in genes in the pathways shown in Figure 2.1, and thus their incidence increases with age in the population, peaking late in life. Thus, for pre-B cell ALL to peak so early in life (2-5 year olds) suggests that this process, that normally takes decades, is somehow accelerated.

Further, childhood acute lymphoblastic leukemia (ALL) displays an infectious etiology. The common pre-B cell acute lymphoblastic leukemia (cALL) has a peak incidence around 2-5

years of age (Greaves, Pegram et al. 1985, Greaves and Alexander 1993, O'Connor and Boneva 2007), which is also the peak incidence of human species C adenovirus infections. There is also some molecular evidence that points towards a viral agent. One study found that interferon pathways were upregulated in the childhood population of ALL patients (Einav, Tabach et al. 2005). While many viruses have been investigated, no correlation between any in utero viruses and childhood ALL has emerged (Schlehofer, Blettner et al. 1996, Shiramizu, Yu et al. 2002, Priftakis, Dalianis et al. 2003, Bogdanovic, Jernberg et al. 2004, Isa, Priftakis et al. 2004). This may be due to viral cell tropism. For example, when Guthrie card blood spots from neonates were assayed via nested PCR for adenoviral DNA, no correlation between adenovirus and progression of childhood ALL emerged. However, Guthrie card blood spots are collected from a heel prick of peripheral blood, where adenovirus is rarely found (~1% frequency) (Flomenberg, Gutierrez et al. 1997). Yet persistent adenovirus infections have ~80% prevalence in the same population when mucosal lymphocytes from tonsils and adenoids are assayed, showing a clear discrepancy between peripheral and mucosal lymphocytes that harbor adenovirus, making correlation in Guthrie cards unreliable (Garnett, Talekar et al. 2009). In contrast, when cord blood lymphocytes are assayed, adenovirus is found much more frequently again (3.7% frequency) suggesting this would be a more reliable source for this type of evaluation (Ornelles, Gooding et al. 2015). Thus, adenovirus can be found in cord blood lymphocytes during the time frame that the leukemic chromosomal translocations have been detected.

While it is commonly speculated that leukemia is likely caused by a yet to be identified infectious agent, it is not agreed upon exactly where the agent imparts its influence on the disease, and there are several hypotheses in the field. Hypotheses from Kinlen and Smith suggest that the infectious etiology of childhood ALL is a rare response to viral infections near birth,

likely in utero, and that naïve populations can have clusters of ALL incidents (Kinlen 1995, Smith 1997). Later studies have supported this proposed pattern and linked the infectious pattern to both a time near the birth of the patient and suggest that the 2-5 year old peak is mainly due to an infectious agent (Gustafsson and Carstensen 2000, McNally, Alexander et al. 2002, Wartenberg, Schneider et al. 2004, Parslow, Law et al. 2005, Stiller, Kroll et al. 2008). These studies found that when rural, isolated communities experienced sudden population mixing, incidences of childhood leukemia cases rose in a manner correlating with time and space of the population mixing. These outbreaks were thought to be due to a rare response to viral infection of either the naïve, pregnant mothers and fetus or the child near birth. Because these communities were relatively isolated prior to the population mixing, it was thought that the community was mostly naïve to the oncogenic agent, resulting in the outbreaks of childhood leukemia. Despite this, one would still expect to find viral DNA in the cancerous progeny if the Kinlen and Smith hypotheses were true, as a viral oncogenic agent would be an intracellular pathogen. The absence of any viral nucleic acids can be explained by two possible hypotheses.

The first, termed the Greaves hypothesis, proposes that childhood ALL arises from an abnormal immune response to infection that occurs later than normal in life. No viral sequences would be detectable in the leukemic cells because the leukemia would be driven by inflammation, rather than molecular mechanisms of infection, allowing a broad range of pathogens as initiators of leukemia, including bacterial pathogens (Greaves 1988). In this hypothesis, the timing of infections during childhood is thought to be critical to driving immune dysregulation, but the infectious agent is not. More recently, large scale studies and the finding of CMV in the bone marrow/Guthrie cards bloodspots of childhood ALL patients have enticed a different version of this hypothesis, stating that an infection early in life predisposes these

children to developing leukemia, but there is still another later infection that sets it off at a particular point in childhood. These infections could be the first and second “hits”. The first hit would predispose the child to ALL, while the second would initiate the overt disease.

The second hypothesis, termed the “hit-and-run” hypothesis, states that a viral infection causes oncogenic genetic or epigenetic changes in the infected cells (hit), but that these changes also create an environment in which the virus can no longer persist (run). The mechanisms of viral loss could be due to dysregulated patterns of viral gene expression, sudden failure to evade the immune system, death of cells still containing the virus or simply inhibition of viral nucleic acid replication in these cells.

A key difference between the Greave and hit-and-run hypotheses is that in the former the pathogen does not enter the cell to initiate oncogenesis, but in the latter it does. While known oncogenic viruses integrate into the cellular DNA or somehow persist during oncogenesis, increasing evidence is showing that viral nucleic acids can be eradicated from the cancerous progeny (Kuhlmann, Achten et al. 1982, zur Hausen 1991, Nevels, Tauber et al. 2001, Liao 2006, Stevenson, May et al. 2010, Niller, Wolf et al. 2011). Vaccination against the hit-and-run model was recently supported when researchers infected a *Cre-lox* rat model with herpesvirus, which initiated an oncogenic transformation event in the infected cell. However, when the resulting tumors were tested for herpesvirus, almost all tumors lacked herpesvirus. Further, no tumors formed in rats receiving vaccination for gamma herpesvirus (Stevenson, May et al. 2010). A hit-and-run mechanism was also detected in adenovirus studies both *in vitro* using primary rat cells and *in vivo/vitro* using hamster models (Kuhlmann, Achten et al. 1982, Nevels, Tauber et al. 2001). Studies using different viruses and models have supported “hit and run” viral

oncogenesis (Ambinder 2000, Nevels, Tauber et al. 2001, Stevenson, May et al. 2010, Niller, Wolf et al. 2011), but the mechanisms of loss had remained unclear.

This dissertation presents a possible mechanism for viral loss from cancerous cells using the leukemic fusion gene ETV6/RUNX1 and an adenovirus/B-cell line model. In these experiments, cells containing the fusion gene inhibited persistence of the virus, yet retained epigenetic marks from viral infection.

The persistence of adenovirus infection in lymphocytes, inhibition of tumor suppressor genes, and high oncogenicity in animals make the oncogenic potential of adenovirus in lymphocytes likely, as these are traits of most known oncogenic DNA viruses. It has come under suspicion that it may cause oncogenic mutations in a hit-and-run manner. If this were true, it could in part be responsible for the peculiar epidemiology and infectious etiology of childhood cALL or the oncogenesis of a broad number of other cancers, but not leave behind any overt evidence. Increasing evidence is showing that viruses can inflict oncogenic mutations via epigenetic alterations in infected cells which carry on to the cellular progeny, even if the virus has been lost from the oncogenic population.

Almost certainly, adenovirus would not be the only cause of childhood ALL, given all the subtypes and different genetic abnormalities. But as one of many common childhood infections, studying it as a possible leukemic agent may help explain the childhood peak that is seen in cALL. Especially since this occurs during the same period in life as the known peak prevalence of adenovirus latently infected mucosal lymphocyte. In this dissertation, we focused on hit and run by adenovirus in ALL because of: 1) the ability of the virus to establish persistent/latent infections in human lymphocytes and to infect lymphocytes in utero, 2) the correlation between persistent adenovirus infections in lymphocytes during childhood (2-5 year olds) and the

incidence of childhood ALL disease (2-5 year olds), 3) the fact that adenovirus is frequently found in cord blood lymphocytes where the ALL initiating mutation (translocation) has been shown to occur, 4) **the well-known ability of the virus to transform cells in vitro through modulation of well characterized tumor suppressor genes, and further, the demonstrated ability of viral proteins to transform cells specifically in a hit-and-run mechanism (Liu, Colosimo et al. 2000, Nevels, Tauber et al. 2001, Stracker, Carson et al. 2002, Berk 2005, Ferrari, Pellegrini et al. 2008, Horwitz, Zhang et al. 2008, Schreiner, Wimmer et al. 2011, Singhal, Leo et al. 2013, Ferrari, Gou et al. 2014, Speiseder, Nevels et al. 2014, Berscheminski, Brun et al. 2016, Speiseder, Hofmann-Sieber et al. 2017)** , 5) the published evidence that lymphocyte lines naturally harboring the ETV6/RUNX1 translocation perturb adenovirus infection (Ornelles, Gooding et al. 2016), 6) the evidence that adenovirus proteins and endogenous RUNX1 interact in infected cells, and 7) the curious absence of this transforming DNA tumor virus in any studied cancer tissues. For other viruses to be plausible candidates they would, at minimum, need to chronically infect lymphocytes very early in life and be capable of modulating genes known to contribute to cancer development and demonstrated ability to transform cells (Figure 2.1). There are other viral candidates, like CMV, that also **commonly** infect children and may be able contribute to childhood ALL (Knowles, Pipkin et al. 2003, Cannon, Schmid et al. 2010, Stevenson, May et al. 2010, Francis, Wallace et al. 2016), but prevalence in lymphocytes with these viruses has not yet been shown to correlate with incidence of childhood leukemia (Gustafsson and Bogdanovic 2007 , Gustafsson, Honkaniemi et al. 2012). While there may be a link with *presence* of CMV and childhood leukemia (Francis, Wallace et al. 2016), no studies have thoroughly defined the transformative potential of CMV as has been shown in adenovirus (Michaelis, Doerr et al. 2009) To warrant preferential investigation over

adenovirus they would need to also have been shown to be able to transform cells in a hit and run manner. All of these criteria have not been met for any of the other viruses mentioned.

The initiating chromosomal translocations of childhood ALL occur in utero, but the disease peaks during the 2-5 year mark (McNeil, Cote et al. 2002). This indicates that there is something special about the biology of the disease, as other cancers tend to increase in frequency with age due to increasing somatic mutations, but also that the initiating chromosomal mutations are not enough to cause overt disease. Indeed, the additional mutations needed to convert pre-leukemic, ETV6/RUNX1 translocation containing cells to clinical disease do not seem to be due to somatic mutations but additional deletions/translocations, further supporting that the unique biology of this leukemia is not a factor of spontaneity and time (Papaemmanuil, Rapado et al. 2014). Finally, the infectious etiology during the peak prevalence of childhood precursor B cell ALL indicates that an infectious agent, not random chance, drives this cancer (Maia Rda and Wunsch Filho 2013).

1.1 SIGNIFICANCE OF THE STUDY

Evidence of viral eviction via naturally occurring oncogenic mutations makes conceivable that it is possible that cancers are initiated in a hit-and-run manner. The scientific significance of demonstrating that viruses are able to cause cancer in a hit-and-run manner is groundbreaking. This may mean that viruses cause many more cancers than what is estimated today.

Adenovirus latency is also poorly understood. Various serotypes of the species C human adenovirus group cause various infection phenotypes in different lymphocyte lines, ranging from overt, acute infection to silent latency. Adenovirus latency may play a part in the eviction of adenovirus in hit-and-run oncogenesis, in that factors that allow the virus to persist may be disrupted.

ALL accounts for a quarter of all childhood cancers and ~85% of childhood leukemias. Fortunately, the 5-year survival rate has improved to ~90%, with an overall cure rate of ~80% (Greaves 2003, Greaves, Maia et al. 2003). However, treatment entails intense financial and emotional support, and survivors must be closely monitored for months to years. For these reasons, prevention of childhood leukemia is a more desired alternative. If adenovirus is responsible for some percentage of leukemias, this would present a preventable mechanism for leukemogenesis, much as the HPV vaccine is administered to protect against cervical cancer. Investigating in this subject could additionally lead to the ability of detection of early risk factors for ALL. Adenovirus is detected in 1 of 20 pregnancies (Reddy, Baschat et al. 2005). Perhaps these pregnancies could be monitored more closely for the common chromosomal translocations found in childhood leukemias and allow early detection and treatment of developing leukemias. Additionally, use of adenoviruses as vaccine and gene therapy vectors has been increasingly popular {Tatsis, 2004 #2892}. A full understanding of the oncogenic potential of adenovirus could have a broad impact on the ubiquitous use of adenoviral vector based therapies.

2 ADENOVIRUS ONCOGENIC MECHANISMS AND REVIEW OF INFECTIOUS RISK FACTORS CORRELATION WITH CHILDHOOD LEUKEMIA

This chapter is an as of yet unpublished review that I researched and wrote for submission as a review of adenovirus oncogenic mechanisms and how they compare to the Hallmarks of Cancer (Hanahan and Weinberg 2011). Additionally, the last section (2.10) is a review of adenovirus infection risk factors and leukemia correlation. This chapter provides a detailed overview of the complex background of adenovirus, hit and run oncogenesis and the infectious etiology of childhood acute lymphoblastic leukemia.

2.1 INTRODUCTION

There are over 50 different serotypes of adenovirus divided into 7 species. The work presented in this dissertation focused on the most commonly found serotypes in the species C group, types 5 and 2. The adenovirus genome is separated into transcription units, that are categorized into either “Early” or “Late” genes. Viral Early genes are responsible for cellular control and immune evasion. Through these mechanisms, these genes can mutate and immortalize cells, but do not need to remain present (Nevels, Tauber et al. 2001). Adenovirus has many oncogenic mechanisms which span almost all the supported hallmarks of cancer (Hanahan and Weinberg 2011). These are summarized in (Figure 2.1). Because of the clear oncogenic traits of adenovirus, it is classically classified as a DNA tumor virus, despite not being associated with any cancers yet. This section reviews the known oncogenic mechanisms of adenovirus that relate to the hallmarks of cancer, and the evidence supporting a connection between the infectious etiology of ALL and adenovirus.

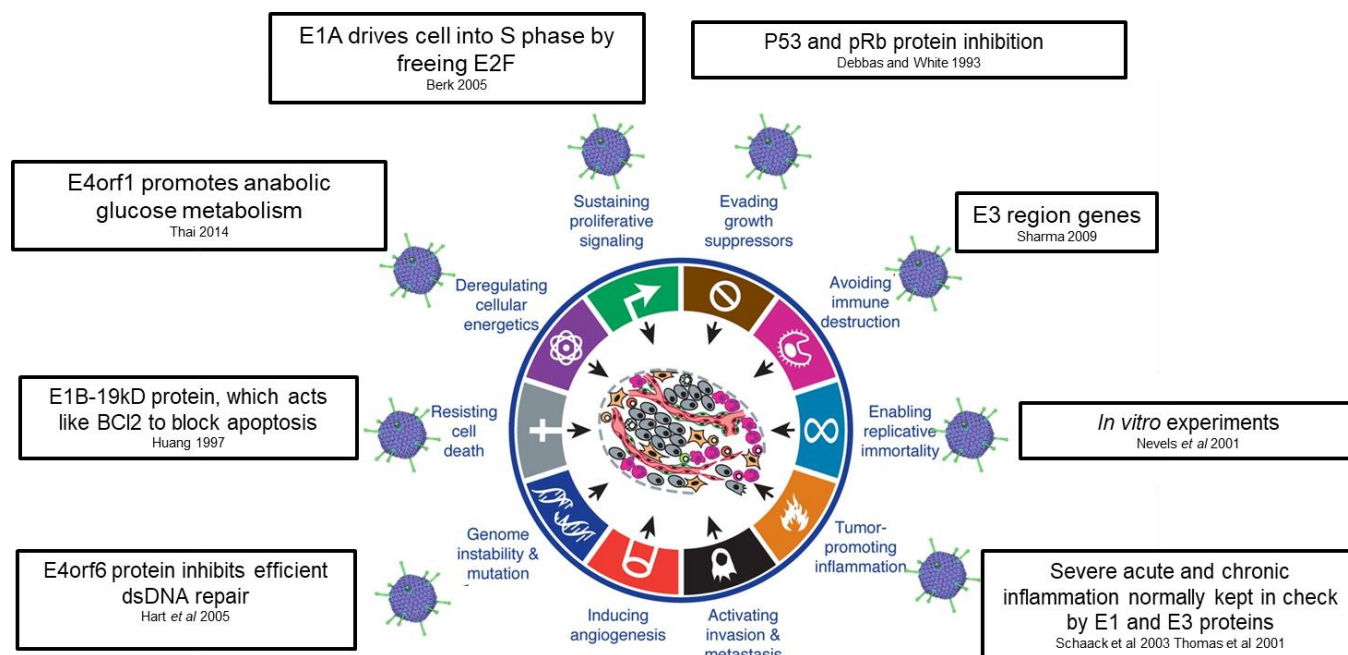


Figure 2.1 Adenovirus mechanisms of oncogenesis and the hallmarks of cancer.
 {Hart, 2005 #2663;Huang, 1997 #1645;Thai, 2014 #1958;Berk, 2005 #1968;Debbas, 1993 #1649;Sharma, 2009 #1977;Nevels, 2001 #507;Thomas, 2001 #2894;Schaack, 2004 #2897}(Hanahan and Weinberg 2011)

2.2 GENOME INSTABILITY AND MUTATION

Genomic instability is a driving force of oncogenesis. While adenovirus exhibits many mechanisms of oncogenesis, of particular note is the ability of species C adenovirus to suppress double stranded DNA break repair, creating a cellular environment of genome instability (Weiden and Ginsberg 1994, Weitzman, Carson et al. 2004, Hart, Yannone et al. 2005, Turnell and Grand 2012). Adenovirus genes E4orf6 and E4orf7 have been found to exhibit oncogenesis in a "hit and run" fashion when transfected in vitro alongside the adenovirus E1A oncogene (Nevels, Tauber et al. 2001). Additionally, the E4ORF4 adenovirus protein also acts to reduce the signaling of DNA damage by reducing the phosphorylation of proteins in the cellular DNA

damage response (Brestovitsky, Nebenzahl-Sharon et al. 2016). Additionally, the E4ORF4 protein expressed alone induced polyploidy and mitotic catastrophe in human lung carcinoma cells, a feature that other adenoviral proteins seem to counteract as discussed below (Li, Szymborski et al. 2009).

The viral E1B-55K and E4 proteins also inhibit function of critical dsDNA break repair protein complex, Mre11-Rad50-NBS1, during infection (Weiden and Ginsberg 1994, Hart, Yannone et al. 2005). This would otherwise result in concatemers of the viral genome which are too large to package. It seems plausible that the genomic instability caused by the E4 proteins could lead to inefficient and erroneous dsDNA repair in the form of chromosomal translocations, like the ETV6/RUNX1 fusion gene. While these chromosomal translocations would be unimportant during lytic infection, as the cell would soon lyse, cells undergoing persistent infection could obtain and harbor these chromosomal translocations in their cellular progeny.

2.3 RESISTING CELL DEATH

E1B proteins inhibit cell death signaling not only upstream, by interfering with p53 and NF- κ B functions, but also on downstream proteins in the caspase-dependent apoptosis pathway like CED-4 and Apaf-1 (reviewed in (Degenhardt, Perez et al. 2000, Burgert, Ruzsics et al. 2002)). The E1B-19KD adenovirus protein mimics the host Bcl-2 protein to block apoptosis in infected cells by associating with Bax and Bak and blocking their functions that release cytochrome c from mitochondria (Huang, Cory et al. 1997).

Another study showed that adenovirus E1B-55K and E4ORF3 proteins could independently inhibit poly(ADP-ribose) polymerase-1 dependent nuclear fragmentation and the nuclear translocation of cellular apoptosis inducing factor, which can lead to caspase-

independent cell death, though the mechanism remains unclear (Turner, Wilkinson et al. 2014). E1B proteins are an important counter to the activities of adenovirus E1A (discussed below), which would otherwise lead to apoptosis or cell arrest (reviewed in (Berk 2005)). E1B-55K not only binds to and inactivates the p53 tumor suppressor protein, but it induces the degradation of p53 protein by recruiting a cellular ubiquitin ligase alongside E4ORF6 to promote its proteasomal removal (Debbas and White 1993, Lowe and Ruley 1993, Querido, Blanchette et al. 2001, Harada, Shevchenko et al. 2002).

While these mechanisms could function to transiently block cell death to allow further mutations during viral infection, adenovirus can also leave epigenetic marks to inhibit p53-mediated cell death and drive oncogenesis in its absence. One study showed that E4ORF3 very selectively silences p53 targets via *de novo* histone methylation via SUV39H1/2 of H3k9 to produce H3k9me3 (Soria, Estermann et al. 2010). The H3k9 methylation is one of the best candidates for these heritable modifications (Huang, Xu et al. 2013). Interestingly, H3k9me3 recruits heterochromatin protein 1, which maintains the repressive transcriptional state and recruits DNA methyltransferase 3b to also facilitate DNA methylation to repress gene expression in a heritable manner (Okano, Bell et al. 1999, Lehnertz, Ueda et al. 2003).

2.4 DEREGULATING CELLULAR ENERGETICS

Adenovirus E4ORF1 induces glycolysis through interaction with host cell Myc protein. This E4ORF1 induced glycolysis did not decrease oxygen consumption, but adenovirus type 5 infection in breast epithelial cells did lead to decreased oxygen consumption alongside increased glucose consumption and lactic acid production (Thai, Graham et al. 2014). These activities

correlate with cancer progression, as reviewed in Hanahan and Weinberg 2011 (Hanahan and Weinberg 2011).

2.5 SUSTAINING PROLIFERATIVE SIGNALING

Host cell RAF, RAS and MYC oncogenes are all associated with oncogenesis via proliferative signaling (reviewed in (Hanahan and Weinberg 2011)). Adenovirus infection stimulates RAF/MAPK signaling pathway, which is modified in many cancers to be constitutively active (Bruder and Kovesdi 1997, Hanahan and Weinberg 2011). Chronic adenovirus infection may result in constitutive activation of this pathway. Further, adenovirus E1A protein transforms primary rat kidney cells through RAS oncogene associated functions (Ruley 1983, Zerler, Moran et al. 1986, Subramanian, La Regina et al. 1989). Additionally, as discussed above, adenovirus E4orf1 associates with the host MYC protein to drive proliferative signaling to increase glycolysis (Thai, Graham et al. 2014).

2.6 EVADING GROWTH SUPPRESSORS

E1A drives cell into S-phase by freeing E2F from RB, p107 and p130 host proteins, allowing it to activate transcription of late G1 and S-phase genes like CDK2, cyclin E and cyclin A (reviewed in (Berk 2005)). This E1A driven S-phase leads to extensive cellular DNA re-replication in a highly stressed replication dynamic, leading to hyperdiploidy in many of the resulting cells (Singhal, Leo et al. 2013). Adenovirus E1A protein also relocalizes p300/CBP to restrict H3k18 acetylation to genes that stimulate S-phase entry and p107 to inhibit cell differentiation genes (Ferrari, Pellegrini et al. 2008, Horwitz, Zhang et al. 2008, Ferrari, Gou et al. 2014).

The activities of E1B-55K and E4ORF3 prevent mitotic arrest of infected cells. The E1B-55K protein was shown to function in a p53-dependent manner to provide a “viral checkpoint” and may function to inhibit inappropriate entry into mitosis, which may otherwise result in mitotic arrest. E4ORF3 worked on the other side of this, facilitating exit from mitosis by functionally inactivating cyclin B1 (Turner, Groitl et al. 2015). At the same time, E1B-55K and E4ORF3 proteins, alongside E4ORF6, can inhibit ATM and ATR kinase pathways that would otherwise halt the cell cycle before mitosis (Stracker, Carson et al. 2002, Carson, Schwartz et al. 2003, Carson, Orazio et al. 2009). These host pathways are activated in response to DNA damage.

2.7 AVOIDING IMMUNE DESTRUCTION

Adenovirus E1A protein also relocalizes host proteins RB and p130 to promoters of anti-viral genes to inhibit their transcription (Ferrari, Pellegrini et al. 2008, Horwitz, Zhang et al. 2008). However, the true brunt of adenovirus immune evasion functions stems from the E3 region of the viral genome. E3 products function primarily to evade immune destruction of the host cell. E3-6.7K is involved in downregulation of TRAIL receptors. E3-10.4K and 14.5K proteins stop Fas and TRAIL receptor surface expression by promoting their degradation, inhibiting the cytotoxic effects of NK and T-cells and TRAIL-mediated apoptosis. E3-14.7K also blocks the antiviral and apoptosis functions of TNFs by interfering with downstream signaling. E3-GP19K blocks peptide digestion by tapasin, resulting in less peptide presentation by MHC I, while also retaining MHC I, as well as the NK cell activators MICA and MICB, in the endoplasmic reticulum (reviewed in (Sharma and Andersson 2009)).

2.8 ENABLING REPLICATIVE IMMORTALITY

Adenovirus proteins have long been known to immortalize primary cells in vitro, evading cell senescence primarily through the activities of E1A and E1B (reviewed in (Berk 2005)). E1A alone alongside activated RAS protein also seem to be enough to overcome cell senescence in vitro (Ruley 1983, Zerler, Moran et al. 1986, Subramanian, La Regina et al. 1989).

Curiously, transient E1A, E4ORF6 and E4ORF3 protein expression has led to hit and run transformation in primary rat cells (Nevels, Tauber et al. 2001). As described above, the E4 proteins function to inhibit DNA break repair, which may lead to chromosomal translocation events. While no specific mechanism of adenovirus and telomere extension has been shown, plenty of mechanisms exist that could explain how adenovirus infection could contribute to immortalization in absence of direct telomerase expression. The genomic instability caused by the E4, E1A and E1B proteins discussed above may lead to chromosomal breakage-fusion-bridge cycles. In the presence of viral proteins from the E1A and E1B region, cells would be more likely to survive such ordeals. Critically shortened telomeres may actually help induce the breakage-fusion-bridge cycle and lead to more rapid oncogenesis (reviewed in (Hanahan and Weinberg 2011)).

2.9 TUMOR PROMOTING INFLAMMATION

Inflammation is another enabling characteristic of cancer, supplying many of the signals needed for other hallmarks like survival factors, growth factors and proangiogenic factors (Hanahan and Weinberg 2011). As discussed above, adenovirus provides these other hallmarks in full. Nonetheless, severe acute and chronic inflammation is normally kept in check by the

adenovirus E1 and E3 proteins {Thomas, 2001 #2894;Russell, 2003 #5092}. Perhaps misregulation of these viral genes in particular cell environments or after certain host mutations allows further tumor promoting factors via inflammation. Our experiments showed that the ETV6/RUNX1 leukemic fusion gene was shown to bind to the E3 promoter region, and resulted in downregulation of viral mRNA transcripts in a B-cell line (Chapter 3). Perhaps shut down of the viral E3 region by ETV6/RUNX1 leads to inflammation and further oncogenesis in pre-leukemic cells.

2.10 EVIDENCE OF ADENOVIRUS AND ALL CORRELATING

Adenovirus and ALL epidemiology differ by global region and urban/rural settings (Nwanegbo, Vardas et al. 2004, Adelman, McLaughlin et al. 2005, Mast, Kierstead et al. 2010). Figure 2.2 and Figure 2.3 shows novel analyses (Wilms, unpublished) of combined data from different studies of human development index (HDI), childhood leukemia incidence, and adenovirus seroprevalence. HDI is a measure of life expectancy, per capita income, and education of a country. A country's cancer/childhood and leukemia incidence, adenovirus seroprevalence, and HDI were plotted when information for each was available. Some countries, like Malawi, were excluded because of the extremely high childhood mortality rates. It should be noted that most of the adenovirus seroprevalence data comes from individuals with high risk for HIV infections, which is estimated based on lifestyles of risk factors of those individuals (Mast, Kierstead et al. 2010). While this choice of population may skew the results, another study found that adenovirus seroprevalence varied very little between HIV positive and negative patients (Kostense, Koudstaal et al. 2004).

Unsurprisingly, HDI inversely correlates with adenovirus seroprevalence (Fig 2.1). This is likely because of less well-developed sanitation systems in countries with lower HDI. However,

it is curious that adenovirus seroprevalence is low in any country, since it has been documented in various studies that almost all children are infected with adenovirus at some point in developed countries where seroprevalence is low (Garnett, Erdman et al. 2002). This means that seropositivity likely fades with time after exposure to the virus, and in countries with low HDI a population's seroprevalence to adenovirus may be kept higher by constant repeated exposure to the virus, probably because of poor sanitation (Seder, Darrah et al. 2008). Data showing clear adenovirus infection in over 80% of children in the US (Garnett, Erdman et al. 2002) in comparison with the low seroprevalence (Mast, Kierstead et al. 2010) in adults argues heavily that there is constant adenovirus antigen exposure in low HDI populations. Strangely, overall cancer incidence does not correlate well with HDI in our analysis (not shown). On the other hand, childhood leukemia incidence can be very well predicted by a country's HDI (not shown). Childhood leukemia incidence is well predicted by adenovirus seroprevalence, which has an inverse correlation (Fig 2.2).

It is well known that childhood ALL differs by ethnicity as well as geography (Howlader N 2016). Increasing evidence shows that the subtypes and chromosomal abnormalities associated with childhood ALL also vary by ethnicity and geography. Specifically, the frequency of the ETV6/RUNX1 translocation has been shown to vary in leukemia patients with respect to ethnicity and geography, which may suggest an ethnic-specific difference in risk factors (Nakao, Yokota et al. 1996, Rubnitz and Look 1998, Garcia-Sanz, Alaejos et al. 1999, Pui, Sandlund et al. 2003, Woo, Kim et al. 2005, Aldrich, Zhang et al. 2006, Artigas, Cabrera et al. 2006, Emerenciano, Agudelo Arias et al. 2006, Ariffin, Chen et al. 2007, Jimenez-Morales, Miranda-Peralta et al. 2008, Siddiqui, Nancy et al. 2010). Non-Hispanic whites were shown to carry this common leukemic translocation almost twice as much as Hispanics (Jimenez-Morales, Miranda-

Peralta et al. 2008). Low numbers of racial variability in our Atlanta cord blood samples, in addition to low overall prevalence of adenovirus in cord blood samples, did not allow us to determine if in utero adenovirus infection also varies by ethnicity due to (Ornelles, Gooding et al. 2015). The prevalence of adenovirus in adults varying by location, however, is clear as discussed above (Figure 2.2, Figure 2.3). Interestingly, when cord blood samples were probed for adenovirus species C DNA and the ETV6/RUNX1 translocation simultaneously, only the sample with the highest adenovirus DNA content also contained an ETV6/RUNX1 translocation (Ornelles, Gooding et al. 2015). Recent evidence has shown that the ETV6/RUNX1 translocation was the only factor found to correlate with the clusters of childhood B-cell ALL outbreaks (Kreis, Lupatsch et al. 2017). This indicates that the ETV6/RUNX1 translocation could either be the outcome of an infectious agent as a first hit of leukemogenesis, or that an infectious agent collaborates best with the ETV6/RUNX1 fusion product as a second hit to drive overt leukemia. A model for adenovirus infection as either the first hit or the second hit is presented below (Figure 2.4), as well as a discussion for the feasibility of each mechanism.

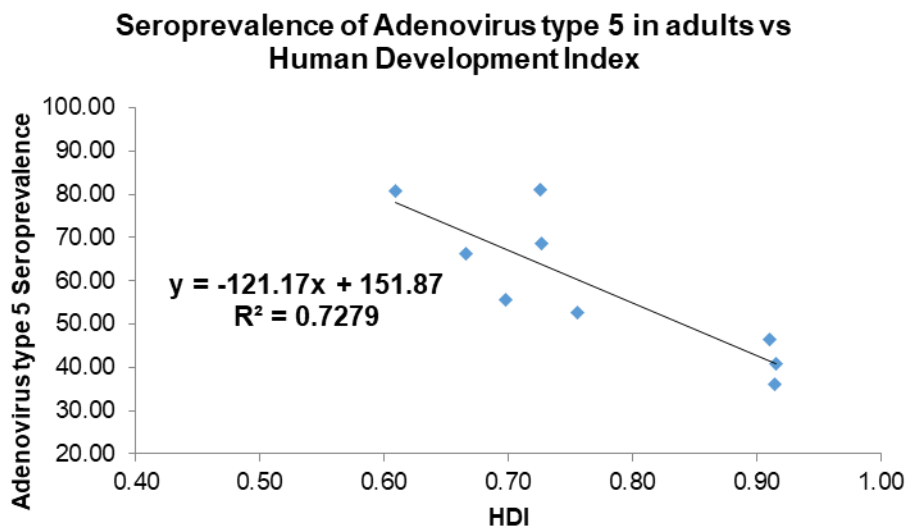


Figure 2.2 Adenovirus type 5 seroprevalence decreases with increasing HDI of a country.

{Mast, 2010 #1108;Yang, 2016 #1641;Yu, 2012 #2863;Sun, 2011 #2864;Pilankatta, 2010 #2865; 2016 #2869}

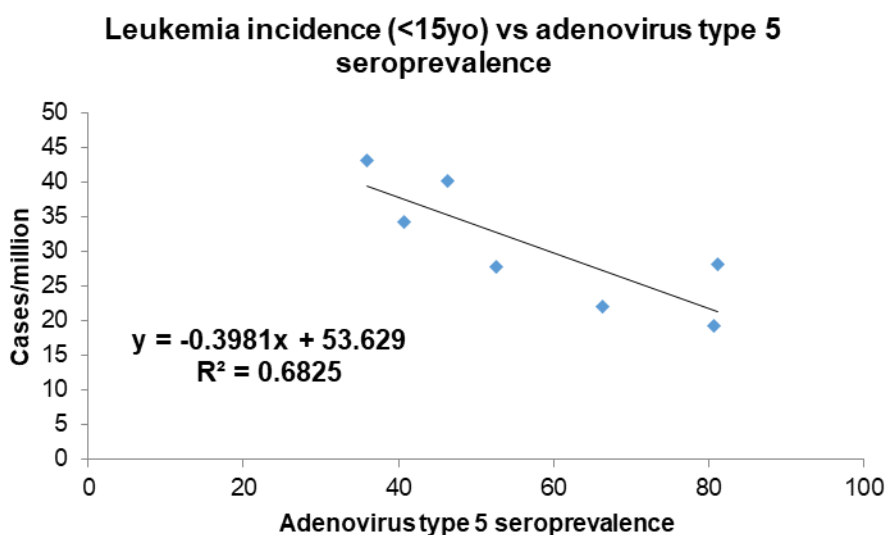


Figure 2.3 Childhood leukemia incidence decreases with increasing adenovirus type 5 seroprevalence.

{Mast, 2010 #1108;Yang, 2016 #1641;Yu, 2012 #2863;Sun, 2011 #2864;Pilankatta, 2010 #2865;Howard, 2008 #1612}

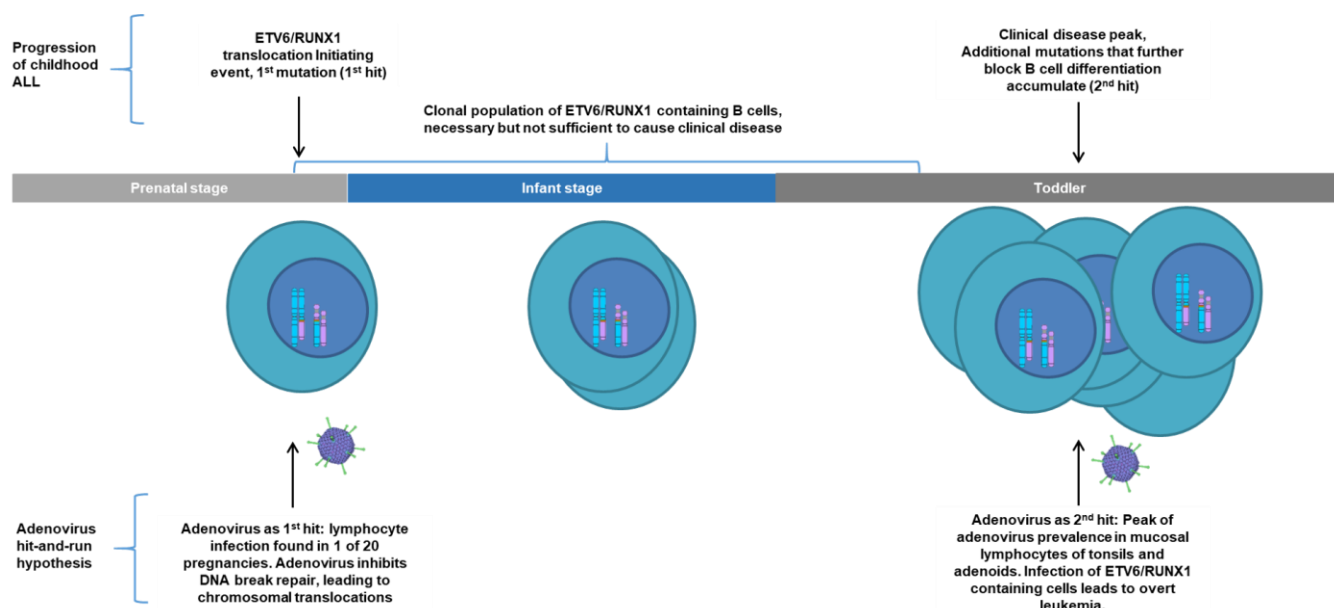


Figure 2.4 Initiation and latency period of childhood ETV6/RUNX1 precursor B cell acute lymphoblastic leukemia.

The ETV6/RUNX1 translocation is commonly found in utero, but is not enough to cause overt disease. A clonal population of cells containing the ETV6/RUNX1 translocation remains, but only develops into leukemia in a subset of children. A clear identification of the additional mutations needed to induce childhood ALL are still unclear {Bateman, 2010 #1082; Fischer, 2005 #1077; Greaves, 2003 #1081; Hemmeryckx, 2002 #1080; Nambiar, 2008 #1076; Schindler, 2009 #1075; Zelent, 2004 #1078; Zhang, 2011 #1079} (Papaemmanuil, Rapado et al. 2014). Chance/random accumulation of additional relevant cancer promoting mutations in an

increased proliferative pool would not be expected to peak in 2-5 year olds, nor would it result in a peak during childhood with an infectious etiology. Adenovirus may contribute to development of pre-leukemic clones as the first hit, in which adenovirus infection may lead to the initial development of chromosomal translocations (leukemia associated mutations). Adenovirus infection inhibits efficient double stranded DNA break repair {Hart, 2005 #1083}, which may lead to the chromosomal translocations that generate ETV6/RUNX1. Adenovirus is unique in that it is found at high frequency in cord blood lymphocytes of otherwise healthy neonates, putting this virus at the correct location to initiate leukemia {Ornelles, 2015 #650}. Alternatively, adenovirus could function as the second hit. The epidemiology of the viral prevalence and leukemia incidence both peak around the 4-year age mark (Garnett, Erdman et al. 2002, Howlader N 2016). In this scenario, adenovirus could support cell survival through transformation and permanently manipulate host cell gene regulation through DNA methylation, even after the virus has been lost as was shown by our results in the studies presented here.

2.11 ADENOVIRUS AS THE 1ST HIT

Studies show that common preleukemic chromosomal translocations frequently occur in utero {Greaves, 2005 #2889;O'Connor, 2007 #2725}. ETV6/RUNX1 is found in approximately 1% of healthy neonates. Of these, about 1 in 8000 will develop leukemia (reviewed in (Ma, Urayama et al. 2009)), resulting in about 25% of childhood ALL cases (Romana, Poirel et al. 1995, Shurtleff, Buijs et al. 1995, Borkhardt, Cazzaniga et al. 1997).

The Smith hypothesis states that an infectious in utero event could produce cells with preleukemic damage, like the chromosomal translocations associated with childhood ALL.

It seems likely that adenovirus could cause the initial step in leukemogenesis by causing the common preleukemic chromosomal translocations found in childhood leukemias. Adenovirus is ubiquitous, has the potential to cause chromosomal translocations, commonly infects in utero where common preleukemic chromosomal translocations occur, and has been shown to have a relation to one of the most commonly affected genes in these chromosomal translocations, RUNX1 (Hart, Yannone et al. 2005, Reddy, Baschat et al. 2005, Marshall, Moore et al. 2008). Further, adenovirus is persistent in lymphocytes and has a range of cellular deregulatory mechanisms associated with cancer (Figure 2.1). For these reasons it is plausible that adenovirus may cause the initial steps of leukemogenesis in the form of chromosomal translocations, but is then eliminated from the cancerous or precancerous progeny in “hit and run” fashion. This pattern of infection causing the pre-leukemic chromosomal abnormalities would be the initial event starting leukemogenesis, or the 1st hit in the proposed 2-hit mechanism of childhood ALL. While no viral nucleic acids have been found in the leukemias, this is predicted for a virus that caused cancer in a hit and run manner. Despite investigation by multiple studies, adenovirus peripheral blood detection in neonates has not been definitively associated with later

development of ALL (Gustafsson, Huang et al. 2007, Vasconcelos, Kang et al. 2008, Honkaniemi, Talekar et al. 2010). Adenovirus is found in ~5% of healthy pregnancies in the amniotic fluid and cord blood lymphocytes, is prevalent in over 80% of tonsil and adenoid mucosal lymphocyte samples from US children, but is rarely found in peripheral blood (Durepaire, Rogez et al. 1997, Flomenberg, Gutierrez et al. 1997, Garnett, Erdman et al. 2002, Baschat, Towbin et al. 2003, Ornelles, Gooding et al. 2015). Thus, given the tropism of the virus for mucosal lymphocytes this is not surprising (Garnett, Erdman et al. 2002, Garnett, Talekar et al. 2009).

The data showing that cancer/childhood leukemia and adenovirus seropositivity inversely correlate by country falls in line with childhood leukemia being associated with an infectious agent linked to hygiene (Smith, Simon et al. 1998). If constant, low level exposure to a viral agent confers better immunity, and if viral seroprevalence is a measure of level of immunity, then the average infection in countries with high viral seroprevalence would not be as severe as countries with low seroprevalence. This would mean that constant, low level exposure to pathogens would result in less overt infections. If a largely seronegative population exist, this may represent a more quasi-naïve population, in which any individual is more susceptible to infection.

Smith proposed that pregnant mothers with a primary infection would be more likely to pass on the virus in utero, setting the child up for increased risk of leukemia during the first 5 years of life. Indeed, the infectious events associated with childhood leukemia have been linked to a time near the birth of the patient in many studies (Gustafsson and Carstensen 2000, McNally, Alexander et al. 2002, Wartenberg, Schneider et al. 2004).

The timing of infection seems to be critical in childhood ALL. Monozygotic twin studies correlating ETV6/RUNX1 detection in utero suggest at the very least that the leukemic initiating events occur frequently in utero (Ford, Bennett et al. 1998). There is no reason to believe that this timing of chromosomal translocation event is completely limited to the prenatal period. If the ETV6/RUNX1 translocation was indeed the outcome of an infectious agent then a mother with a primary infection would also be likely to pass on the leukemic virus to her neonate as well as her fetus, setting the child up for ALL. This latter pattern of neonatal infectious has also been confirmed by large studies that show that leukemic patients had more clinically diagnosed infectious episodes than non-leukemic counterparts during the first year of life, with the most apparent difference during the first month of life (Roman, Simpson et al. 2007, Cardwell, McKinney et al. 2008).

If a serologically naïve pregnant or new mother came down with primary infection, she may be more likely to pass on the virus to the fetus than a constant low level infection or simply constant exposure to viral antigen. In fact, a constant low level of infection may reduce the risk of transmission during this critical time period. If the mother's immune system was constantly challenged and producing antibodies, these would be passed on to the fetus or breast-feeding neonate, protecting the child from overt, primary infection. Studies that have evaluated breast feeding have shown mixed results (discussed below).

If adenovirus initiated the first hit of childhood ALL, this could indicate that serologically naïve mothers who were exposed to adenovirus were more likely to come down with infection and pass it on to their fetus or neonate. At this critical time period the child would be predisposed to leukemia by generation of the chromosomal abnormalities, like the ETV6/RUNX1 translocation, either *in utero* or during the first year of life. Adenovirus inhibits the dsDNA break

repair response as part of its viral lifecycle (Weiden and Ginsberg 1994, Querido, Blanchette et al. 2001, Stracker, Carson et al. 2002, Hart, Yannone et al. 2005), which could feasibly lead to the generation of these pre-leukemic clones. Further, both adenovirus and ETV6/RUNX1 positive childhood leukemias differ by ethnicity and geography, and the ETV6/RUNX1 translocation is associated with the infectious etiology of ALL. These data suggest that adenovirus could be the causative agent of the chromosomal translocations associated with childhood ALL, especially ETV6/RUNX1. Unfortunately, the prevalence of the ETV6/RUNX1 translocation in the general population with respect to geography has not been studied. Thus the question remains on whether the infectious etiological agent is simply associated with the ETV6/RUNX1 fusion gene or whether it causes it.

Maternal infection, birth order, season of birth and socioeconomic status (SES) at birth have also been studied in regards to development of childhood leukemia as indicators of patient infection during infancy, some with conflicting results (Smith, Roman et al. 2006, Simpson, Smith et al. 2007, Feller, Adam et al. 2010). A number of maternal infections and infectious symptoms have evidence of correlating with development of childhood ALL (reviewed in (McNally and Eden 2004, Maia Rda and Wunsch Filho 2013)). Not all studies support maternal infections associating with childhood ALL. The methodologies of these studies vary widely in symptoms and pathogens assessed, which may explain the conflicting results. Maternal EBV infection/reactivation, sexually transmitted diseases, *Helicobacter pylori*, influenza, varicella, skin disease and lower genital tract infection have all been shown to significantly increase risk of childhood leukemia in at least one study. Whether these diseases are artifacts, cause the increased risk of childhood ALL, or are simply correlating factors with the actual infectious agent is still unknown. There have been no similar studies with adenovirus. In one study that

failed to identify maternal infections as a risk factor to developing childhood ALL, neonatal infections appeared as a protective factor against developing ALL. Notably, these excluded respiratory and gastrointestinal infections, the symptoms of primary adenovirus infection, as those were rarely found (McKinney, Juszczak et al. 1999).

As a mother has more children, the chances that she has been exposed to adenovirus increases. Evidence for increased risk, protective effect and no effect have all been shown for birth order (reviewed in (Maia Rda and Wunsch Filho 2013)). There are likely other effects at play here, like maternal age, which may confound this variable. There is some, albeit conflicting, evidence ALL correlating with season of birth (Meltzer, Annegers et al. 1996, Feltbower, Pearce et al. 2001, Higgins, dos-Santos-Silva et al. 2001, Sorensen, Pedersen et al. 2001, Kajtar, Fazekasne et al. 2003, McNally and Eden 2004, Nyari, Kajtar et al. 2008, Basta, James et al. 2010). A large UK study of 15,000 patients found only two seasonal peaks in the 1960s. The authors suggested that the results of the 60s time period may no longer be detectable as immunity to infectious agents becomes more widespread with more frequent population mixing as our world becomes more globalized. Interestingly, the studies that did not support a seasonal variation did not have data from before the 70s (Meltzer, Annegers et al. 1996, Kajtar, Fazekasne et al. 2003). Another study that supported a birth related seasonal variance found significant results in only the 1-6 year-old population (Basta, James et al. 2010). Studies on adenovirus have suggested that its prevalence varies seasonally (Schmitz, Wigand et al. 1983, Cooper, Hallett et al. 2000, Vetter, Staggemeier et al. 2015). In a study that looked at low-risk pregnancies in the second trimester, viral infection in the amniotic fluid was shown to vary seasonally, with adenovirus being the most frequently detected (Baschat, Towbin et al. 2003). Other common in

utero viruses suspect of being associated with childhood ALL, like CMV, are not known to vary by season (Gold 1976).

ALL development in regards to SES at birth has been conflicting, and is even more convoluted by different studies using different methodologies to measure SES. There are also probably problems with participation bias, and the authors suggested that the incidence of childhood leukemia varying over time and geography may also play a role, which again may be affected by increasing globalization (reviewed in (Poole, Greenland et al. 2006, Goujon-Bellec, Mollie et al. 2013)). Studies have shown positive and negative associations for ALL and higher SES, but a large, comprehensive UK showed no SES association with ALL (Smith, Roman et al. 2006). While the differences in adenovirus seropositivity and ALL incidence between countries of different SES levels based on HDI is clear (Figure 2.2, Figure 2.3), there may be some threshold SES level above which the differences in infection and ALL incidence are no longer apparent or is somehow saturated. One study in France showed that childhood ALL incidence in 7-14 year olds increased in the years 1990-2001, but the increase was less marked 2001-2007 (Goujon-Bellec, Mollie et al. 2013). This could indicate that such a threshold was reached. However, the same trend was not evident for 1-6 year olds.

Heavy birth weight has been shown to be an established risk factor in childhood ALL (summarized in meta-analysis (Caughey and Michels 2009) and review (Roman, Lightfoot et al. 2013)). While the causes behind the heavier birth weight remains somewhat of a mystery, the evidence further supports an at least partially *in utero* origin of childhood ALL. It has been suggested that increased growth factors and stem cells may work in unison with the chromosomal abnormalities of ALL and thus promote higher birth weight (Caughey and Michels 2009, Roman, Lightfoot et al. 2013), but no direct evidence on this exist. Birth weight is not

likely to be associated with infection near birth, and one study confirmed that there seemed to be no association with this factor, nor birth order, and the infectious clusters of childhood ALL (Kreis, Lupatsch et al. 2017).

While there is overwhelming evidence of an infectious agent instigating childhood ALL near birth of the patient, the data suggests that only one subtype is associated with the infectious etiology, ETV6/RUNX1 positive pre-B-cell ALL (Kreis, Lupatsch et al. 2017). It seems that these infection-associated cases may be linked to the 2-5 year-old peak of childhood ALL (Roman, Simpson et al. 2007, Stiller, Kroll et al. 2008, Basta, James et al. 2010).

2.12 ADENOVIRUS AS THE 2ND HIT

The peak incidence of pre-B-cell ALL and the peak prevalence of adenovirus in mucosal lymphocytes occur during the same age range (Garnett, Erdman et al. 2002, Howlader N 2016). The correlation of this time and cellular tropism in the diseases is striking. However, it is likely that many other pathogens are also prevalent during this time period when children are still relatively immunologically naïve but are simultaneously more frequently exposed to infections through greater social settings. This is the bases of the immune dysregulation hypothesis proposed by Greaves. In this model, children that are exposed to common pathogens later in life, like those in developed nations, are thought to exhibit a different, more vigorous immune response than children infected when they are younger. Younger age at infection would be more likely in developing nations with less stringent hygiene conditions, bringing the childhood leukemia rate down in these nations. In this model, the older children are thought to be more likely to develop ALL than infants, which is why the peak incidence associated with the disease is at the 2-5 year age range. Under this model, the infectious etiology of ALL associated with

patient date of birth and the higher number of clinical visits during the first year of life has been suggested to be due to a separate infection that causes the immune dysregulation as the first hit under the two-hit model of leukemia. This was purposed to predispose the patient to ALL, with the immune response to another infection causing the clinical manifestation of the disease, causing the peak incidence at 2-5 years of age as the second hit as the result of immunological stress (Francis, Wallace et al. 2016).

There have been many studies that have investigated infection at or before diagnosis of childhood ALL. These studies looked at different ages, infections/symptoms and time periods before or at disease using different methods (reviewed in (Maia Rda and Wunsch Filho 2013)). The results were conflicting, ranging from infections giving a protective effect to increasing risk, with some studies contradicting each other. For example, when specific viruses were probed, one study found an inverse correlation of EBV seropositivity and children with leukemia (Petridou, Dalamaga et al. 2001), while another found a direct correlation, but only when looking at particular EBV proteins (Mahjour, Ghaffarpasand et al. 2010).

Interestingly, tonsillitis 3-12 months before disease onset was associated with common ALL in one study (Chan, Lam et al. 2002). Another study associated acute leukemia, but not common precursor B-cell ALL, with tonsillectomy (Schuz, Kaletsch et al. 1999). Adenovirus infected mucosal lymphocytes reside in the tonsils and adenoids. In the same study, children were at significantly higher risk for having bronchitis or pneumonia less than a year before leukemia diagnosis, two symptoms of more severe adenovirus species C infections (Schuz, Kaletsch et al. 1999). This effect was even stronger for common precursor B-cell ALL.

The hit and run model of viral oncogenesis could also explain the infectious event near birth that is associated with ETV6/RUNX1 positive precursor B-cell ALL (Stiller, Kroll et al.

2008, Kreis, Lupatsch et al. 2017). Our research has shown that the ETV6/RUNX1 fusion gene inhibits adenovirus replication in a B-cell line. The infected cells retained viral regulatory effects on two genes associated with oncogenesis, SPARCL1 and CXADR, demonstrating that epigenetic effects could be retained after ejection of virus from the leukemic population (Chapter 2.11).

ETV6/RUNX1 was the only characteristic found to be associated with the infectious clusters of precursor B-cell ALL (Kreis, Lupatsch et al. 2017). Perhaps when adenovirus infects these preleukemic cells that originate naturally in 1% of human fetuses, the ETV6/RUNX1 containing cells are driven into a full blown leukemic disease. The presence of the ETV6/RUNX1 fusion gene does not inhibit initial adenovirus infection kinetics, so the virus would only be lost during the persistent infection after disrupting normal cellular epigenetics and functions (Figure 3.3). In this scenario, the ETV6/RUNX1 translocation would be the first hit and adenovirus would be the second hit, causing the overt leukemia. The simultaneous occurrence of adenovirus prevalence in mucosal lymphocytes with onset of the disease may indicate that the second hit with adenovirus occurs at that time of 2-5 years of age. However, this would fail to account for the space-time clustering associated with the birth of the patient, but there is also some evidence for infectious etiology around the time of diagnosis (discussed below). Another possibility is that the virus infects ETV6/RUNX1 preleukemic cells *in utero* or infancy and that the incubation period lags until clinical onset at 2-5 years, by which time the virus is ejected from the leukemic population. Such an infectious trend in place of birth and a consistent lag time to disease has been suggested with some evidence backing it (Birch, Alexander et al. 2000). However, when separating the ALLs by subtype, the same group later found space-time clustering at birth as the only significant association, and only for the peak of

precursor B-cell ALL (McNally, Alexander et al. 2002). These effects may be limited to the ETV6/RUNX1 fusion gene and adenovirus, causing only that subset of the disease. Alternatively, ETV6/RUNX1 or other leukemic mutations may also function similarly with adenovirus or other viruses to inhibit their persistence in leukemic populations by any number of various mechanisms.

There is evidence that the space-time clustering of ALL occurs around the diagnosis of the patient ((Alexander, Boyle et al. 1998, Birch, Alexander et al. 2000) and reviewed in (1999)). If adenovirus was the second hit, this would fall in line with the peak of adenovirus prevalence in mucosal lymphocytes. Additionally, most studies support a seasonal pattern of ALL diagnosis, which would also suggest an infectious agent that occurs seasonally, like adenovirus. However, the results are inconsistent with no clear peak time period. It was suggested that this is because the responsible agent or agents may vary with year and geography (reviewed in (McNally and Eden 2004, Goujon-Bellec, Mollie et al. 2013)). Conflicting evidence of space-time clustering and season at diagnosis may be due to difficulties in diagnosis periods of leukemia, as this may vary from patient to patient and the incubation period of ALL from initial onset until clinical symptoms manifest is unknown. If a common, mild infection like adenovirus was indeed the second hit, this would likely not result in a clinical visit and thus be extremely difficult to track. Birth dates are easy to identify, which may give them a bias when identifying infectious events.

Child social activity (daycare, nurseries, play groups, etc.) and birth order are generally thought to have a protective effect on development of childhood ALL, but is heavily conflicted (reviewed in (Urayama, Ma et al. 2008, Maia Rda and Wunsch Filho 2013)). The methodologies for data acquisition of child social activity relied on parental responses and assayed different ages, time periods, frequencies and geographic locations, which is possibly why the studies are

so conflicted. However, a large study carried out by the United Kingdom Childhood Cancer Study found that only children who developed ALL seemed to incur more clinically diagnosed infections during the first year of life when exposed to more possible infectious exposures (Simpson, Smith et al. 2007). At the same time, overall childhood social activity was a protective factor (Gilham, Peto et al. 2005). It has been proposed that this indicates that children who are predisposed to ALL are more susceptible to overt infection because of immune dysregulation perhaps by an early infection, leaving them predisposed to ALL (Chang, Zhou et al. 2011, Wiemels 2012). Perhaps this is the case, and adenovirus is a second hit that fully converts the malignant clones in susceptible children to ALL in a hit and run fashion during its peak prevalence in mucosal lymphocytes.

Breast feeding has been used as an indirect measure of both protection from and exposure to infection. Studies are inconsistent, with the majority finding no significant effect on ALL risk either way (reviewed in (McNally and Eden 2004, Maia Rda and Wunsch Filho 2013)). Two large UK studies found no significant effects, although one was borderline significant for a protective effect (Investigators 2001, Lancashire, Sorahan et al. 2003).

3 LEUKEMIC RUNX1 FUSION GENES EVICT ADENOVIRUS FROM B-CELL POPULATION VIA DIRECT BINDING TO VIRAL DNA

The work in this chapter represents the results of experiments I performed or directed for Aim 1 of my Dissertation Project: To explore whether that the leukemic chromosomal translocation ETV6/RUNX1 inhibits Ad Species C expression and retention, and that epigenetic effects of infection persist after virus loss.

I consider this chapter a complete study, detailing a “Hit and Run” model of viral eviction from a persistently infected B cell line by a common leukemic mutation, and evidence that epigenetic patterns of viral infection can be retained after loss of virus. To my knowledge, this work provides the first evidence of a mechanism of viral eviction in a “Hit and Run” fashion.

The manuscript for this work has been submitted for publishing. This chapter also includes additional related experiments that were not included in the submitted manuscript. Additionally, this work resulted in the generation of a provisional patent application, which is attached under Appendix A.

3.1 ABSTRACT

While cancer itself is not infectious, infectious agents like human papilloma virus have been identified as oncogenic agents. Epidemiological studies of one cancer in particular, common B cell precursor (BCP) acute lymphoblastic leukemia (ALL), point towards an infectious etiology (Greaves and Alexander 1993, Kinlen 1995, O'Connor and Boneva 2007). However, no study to date has detected an infectious agent in leukemic cells, causing speculation that the oncogenic agent has been lost in the cancerous progeny. In the “hit and run” model of viral oncogenesis, viral infection causes oncogenic mutations in the host cell genome (hit) that create an environment in which the virus is lost from the resulting transformed cells (run).

Specific chromosomal abnormalities resulting in mutated fusion proteins are detected in 60-85% of ALLs and are thought to be the initiating event for these cancers. The ETV6/RUNX1 fusion protein is present in ~25% of pre-B ALLs, and this initiating translocation can be detected in significantly higher frequency in neonatal blood of patients that later develop ALL {, 1983 #660;, 1996 #662;Fenaux, 1989 #663;Rieder, 1993 #664;Secker-Walker, 1997 #665}(Greaves 2003). Species C adenoviruses establish persistent infection in lymphocytes and can infect lymphocytes in utero. Thus, the virus can be detected within lymphocytes during the same time (pre-natally) that the leukemic translocations are detected (Ornelles, Gooding et al. 2015). As members of the DNA tumor virus family they have long been suspected of having oncogenic potential as they inhibit cellular DNA repair pathways and manipulate oncogenic cellular pathways such as p53 and pRB. While hit and run oncogenesis would be difficult to observe in nature, *in vivo*(Stevenson, May et al. 2010) and *in vitro*(Nevels, Tauber et al. 2001) experiments are starting to provide supporting evidence for the phenomenon. In this chapter, we attempted to model the “run” of the virus in a B-cell line by expression of RUNX1 fusion proteins associated

with leukemia to see if there is any evidence that these mutations create an environment that is refractory to adenovirus infection.

Here we show that expression of either of the two common leukemic fusion proteins, ETV6/RUNX1 or RUNX1/MTG8, impairs the ability of adenovirus to persist in a B-cell lymphocyte line, and that ETV6/RUNX1 protein can bind directly to the viral genome. Finally, we show that even after significant loss of the virus from infected cells virally silenced genes remain downregulated. These results provide support for how adenovirus could be lost from lymphocytes and potentially model the “run” of the “hit and run” model of adenovirus caused oncogenesis. They also provide the first evidence that the virus is able to leave a lasting imprint on cells previously infected in the form of an epigenetic echo. The results presented in this section have been submitted for publication to Proceedings of the National Academy of Sciences, and led to the creation of a provisional patent (Appendix A).

3.2 INTRODUCTION

Adenoviruses are ubiquitous non-enveloped, icosahedral viruses {Fields, 2007 #12}. The dozens of serotypes that infect humans are assigned to seven species, the most common of which include species C serotypes 1, 2, 5 and 6. These viruses establish lytic infections in epithelial cells of the upper respiratory and gastrointestinal tracts {Fields, 2007 #12} and latent infections in T and B lymphocytic cells {Garnett, 2002 #17;Assadian, 2016 #3}. The peak incidence of species C viruses in human mucosal lymphocytes occurs between two and five years of age {Garnett, 2002 #17;Garnett, 2009 #18}. Adenovirus infections also occur *in utero*; the species C viruses have been detected in the cord blood of one in 25 newborns {Ornelles, 2015 #47}.

Primary rodent cells are readily immortalized by the oncogenes of adenovirus {Schreiner, 2011 #53;Speiseder, 2014 #55}. Human mesenchymal stromal cells have been shown to be susceptible to transformation by the adenovirus E1 oncogenes {Speiseder, 2017 #135}. Although adenovirus is not known to cause any human cancer, an oncogenic role for this small DNA tumor virus is not unreasonable. Adenoviral gene products inactivate critical cell cycle checkpoints such as p53 and pRb, whose loss is known to contribute to tumor progression {Speiseder, 2014 #55;Liu, 2000 #32;White, 1998 #59;Yew, 1992 #62;Liu, 2007 #33;Whyte, 1988 #60;Hanahan, 2011 #22}. Adenovirus suppresses cellular DNA-break repair pathways in order to preserve the integrity of the linear double-stranded viral genome {Hart, 2005 #23;Weiden, 1994 #58}. The loss of DNA-break repair promotes genomic instability {Hart, 2005 #23} which is another hallmark of cancer {Hanahan, 2011 #22}. Additionally, adenovirus elicits re-replication of cellular DNA in quiescent cells, which can lead to hyperdiploidy {Singhal, 2013 #54}. Despite its oncogenic potential, adenovirus has yet to be implicated as the cause of any human cancer. One hypothesis for the absence of any connection between adenovirus and human cancers is that oncogenic mutations elicited by the virus preclude retention of the virus in the affected cell. This is the premise behind the “hit-and-run” model of viral oncogenesis {Nevels, 2001 #43;Niller, 2011 #44}. The loss of adenoviral genes {Nevels, 2001 #43} and the loss of herpes simplex virus gene products from rodent cells transformed *in vitro* {Stevenson, 2010 #139} by these viruses is experimental evidence that supports this possible mechanism of cellular transformation.

Viral genes are expressed in human cancers caused by Epstein-Barr virus (EBV) and human papillomavirus (HPV) {Thompson, 2004 #56;Walboomers, 1999 #57}. Cancers caused by these viruses emerge from tissue that harbors a persistent or latent infection. EBV is

responsible for tumors of B-cell origin including Hodgkin's lymphoma and Burkitt's lymphoma and tumors of the epithelial origin including nasopharyngeal carcinoma and gastric cancer {Thompson, 2004 #56}. All of these tumors are latently infected with EBV and express a subset of the viral genes {Young, 2003 #63;Thompson, 2004 #56}. HPV may establish quiescent, clinically latent infections in long-lived epithelial cell progenitors {Doorbar, 2013 #9}. Virtually all oropharyngeal cancers, as well as cancers of the uterine cervix, that are caused by HPV express the viral HPV E6 and E7 oncogenes {Münger, 2004 #140;Elrefaey, 2014 #141}. Because adenovirus can chronically infect B lymphocytes, we and others have speculated that this virus could initiate events that lead to the development of childhood acute lymphoblastic leukemia (ALL) {Honkaniemi, 2010 #25;Gustafsson, 2007 #21} by a mechanism that results in the loss of the viral genome from the transformed cell.

Epidemiological evidence supports an infectious contribution to development of childhood ALL. In particular, common precursor B cell ALL has been under intense investigation for an infectious cause due to the marked pattern of disease clustering {O'Connor, 2007 #45;Greaves, 1993 #20;Kinlen, 1995 #28}. Because no infectious agent has yet been identified within the leukemic cells themselves{MacKenzie, 2006 #37}, the viral contribution to transformation may be occurring through a hit-and-run mechanism. ALLs are typically associated with identifiable chromosomal translocation mutations that differ between childhood and adult leukemias {Pui, 2004 #51;Pui, 1998 #50}. Indeed, the best evidence for a multistep process leading to development of childhood ALL comes from the detection of leukemia-associated translocations in the blood of newborns many years before the development of overt leukemia {Zuna, 2011 #65;Mori, 2002 #41}. The most common translocation in childhood precursor B cell ALLs, t(12; 21) which produces the fusion protein ETV6/RUNX1, is found in

about one quarter of cases {Pui, 1998 #50}. Moreover, a recent study found that when only “clustered” cases of leukemia, those most likely to have been initiated by an infectious agent, are considered, the frequency of the *ETV6/RUNX1* translocation rises to 40% {Kreis, 2017 #30}.

While mutations in *RUNX1* frequently contribute to the initiation of leukemia {Yamagata, 2005 #61;Ichikawa, 2004 #26}, *RUNX1* it is an important transcription factor in normal B lymphocyte development {Okuda, 2001 #46;Ichikawa, 2004 #26;Kurokawa, 2006 #31;Lorsbach, 2004 #35;Yamagata, 2005 #61}. The *RUNX1* proteins show a distinct relationship to adenovirus. Human *RUNX1a* and *RUNX1b* isotype variants allowed nuclear localization of the normally cytoplasmic adenovirus E1B-55K protein in a mouse cell line where the *RUNX1* proteins localized to sites enriched for viral RNA processing factors. This association displaced the adenovirus E4orf6 protein from the complex. These results suggest that *RUNX1* may play a role in adenoviral RNA processing {Marshall, 2008 #38}. *RUNX1*, and *RUNX1* leukemic fusion proteins, are also known to bind to DNA and alter gene transcription {Yamagata, 2005 #61}. Human species C adenoviruses have canonical *RUNX1*-binding motifs in the promoter regions of early genes that control viral retention and replication {Brister, 2015 #6;Melnikova, 1993 #39;Chroboczek, 1992 #7}. *RUNX1* is reported to both activate and repress cellular gene expression by recruiting histone acetyl transferases (HATs) or histone deacetylases (HDACs), respectively. By contrast, *ETV6/RUNX1* primarily represses gene expression by HDAC recruitment {Yamagata, 2005 #61;Hiebert, 1996 #24}. Little is known about the influence of *RUNX1*, or *RUNX1* containing fusion proteins, on persistent or latent infections in B cells. We have previously shown that B cell lines naturally harboring the *ETV6/RUNX1* chromosomal translocation had reduced infectivity with species C adenovirus {Ornelles, 2016

#48}. Thus, we reasoned that the cells containing this translocation could be refractory to persistent infections with the virus.

If adenovirus contributes to childhood precursor-B cell leukemia, the absence of any viral product in these cancers would imply the existence of a hit-and-run mechanism. However, no study has yet shown a mechanism for viral loss from precancerous cells. Because of the difficulty of measuring the loss of a virus from the associated diseased tissue in humans, we sought to model this behavior experimentally. We asked if the transcriptionally repressive leukemic fusion proteins {Hiebert, 1996 #24; Yamagata, 2005 #61} could inhibit adenovirus retention in persistently infected B cells. Our results show for the first time that both viral gene expression and the viral genome are lost during a persistent infection of B cells expressing *RUNXI* leukemic fusion genes. The loss of viral gene expression and the viral genome appears to be mediated by histone deacetylation. Expression of the cellular genes *SPARCLI* and *CXADR*, which are epigenetically repressed in persistently infected cells {Ornelles, 2016 #48}, remains depressed even after loss of the virus. The *RUNXI* leukemic fusion products may expel adenovirus from persistently infected B cells while leaving an epigenetic echo of the infection.

3.3 MATERIALS AND METHODS

Cell lines

The human cell A549 lung carcinoma cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA). BJAB (EBV-negative Burkitt's lymphoma) cells were obtained from the ATCC {Klein, 1974 #29}. BJAB cells were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10 mM glutamine. A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 µg of glucose per ml, 10% FCS, and 10

mM glutamine. Cells were tested by Genetica to ensure the absence of mycoplasma and for cell line authentication.

Creation of stable cell lines

The original expression plasmids for the *ETV6/RUNX1* and *RUNX1/MTG8* were kindly provided by S. Hiebert (Vanderbilt University) and were subcloned into pTARGET (Promega) using the *EcoRI* restriction enzyme. A549 cells were transfected using Lipofectamine LTX with Plus reagent (ThermoFisher Scientific). BJAB cells were transfected using electroporation as described in Mchichi *et al* {El Mchichi, 2007 #10}, with minor modifications. Briefly, 4×10^6 cells in cold serum-free RPMI were transfected with 2 μ g of plasmid by electroporation at 120 V, 960 μ F using cuvettes with an inner width of 2 mm. After 20 min of recovery at 37°C, cells were plated into 6-well plates in RPMI supplemented with 10% FBS at 37°C for 48 hrs. Cell cultures were then supplemented with 1 mg per ml of G-418 (ThermoFisher Scientific) for at least 21 days before use in experiments. G-418 was maintained at 0.5 mg per ml throughout experiments to maintain stably transfected cell lines.

Adenoviruses

Wild-type species C Ad5 adenovirus was obtained from William S. Wold (St. Louis University). Similarly, the phenotypically wild-type mutant virus, Ad5dl309, was obtained from Tom Shenk (Princeton University, Princeton, NJ. Ad5dl309 is an Ad5 mutant that lacks the genes for the E3 RID α and RID β proteins as well as the 14,700-molecular-weight protein (14.7K protein) {Bett, 1995 #142}.

Infection of lymphocytes with adenovirus

Infection of lymphocyte cell lines with adenovirus was performed as described previously {McNees, 2004 #143} with minor modifications. Lymphocytes were collected and washed in serum-free (SF) RPMI medium. Cell density was adjusted to 10^7 cells per ml in SF-RPMI medium. Virus was added to the cell suspension at 50 PFU/cell, spun for 4 5min at 1000 x g at 25°C, resuspended by agitation. Cells were then incubated at 37°C for 1.5 hrs with gently flicking every 30 min. The infected cells were washed three times with RPMI complete medium and then resuspended in RPMI complete medium at 5×10^5 cells per ml. Cell concentration and viability were monitored throughout the infection.

Reverse transcription and quantitative PCR analysis of viral and cellular mRNA levels

RT-qPCR was performed as described previously {Ornelles, 2016 #48}, with minor modifications. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen Inc. Valencia, CA). RNA was treated with Rnase-free DNase (Qiagen) on isolation columns and quantified. Greater than 100 ng were reverse transcribed (RT) into cDNA, in 20 μ L reaction volumes, using Maxima First Strand cDNA Synthesis Kit (ThermoScientific). RT-enzyme negative controls were included for each reaction. Primers and probes were obtained from Integrated DNA Technologies (Coralville, IA). Viral primer and probe sequences are reported in the supplementary material (**Error! Reference source not found.**). Primer and probes for the cellular genes *CXADR* and *SPARCLI* were used as previously described {Ornelles, 2016 #48}. Probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) reporter molecule and contained dual ZEN and Iowa Black quenchers. Each sample was run in duplicate with at least 2 experimental repeats for each virus tested. All analyses were performed via the comparative

threshold cycle (Ct) method {Livak, 2001 #144}. Target Cts were normalized to the EIF1 housekeeping gene, and set relative to the lowest sample, which was set to 1. In some experiments, HDAC inhibition was performed by treating cells with 300 nM Trichostatin A (TSA) followed by quantifying transcript levels {Ornelles, 2016 #48}.

Table 3.1 Primers and Probes for RT-QPCR

Primer Name	Sequence
E1A	
Primer 2	CAG GCT CAG GTT CAG ACA C
Primer 1	GTT AGA TTA TGT GGA GCA SCC C
Probe	/56-FAM/ATG AGG ACC /ZEN/TGT GGC ATG TTT GTC T/3IABkFQ/
E2A	
Primer 2	ACA CGT TGC GAT ACT GGT G
Primer 1	GAA AAC TTC ACC GAG CTG C
Probe	/56-FAM/CGG ATG GTT /ZEN/GTG CCT GAG TTT AAG TG/3IABkFQ/
E4ORF6	
Primer 2	CTT AAG TTC ATG TTG CTG TCC AG
Primer 1	CTG CCC GAA TGT AAC ACT TTG
Probe	/56-FAM/ACT TGC GGT /ZEN/TGC TCA ACG GG/3IABkFQ/
E3GP19K	
Primer 2	GCA GCT TTT CAT GTT CTG TGG
Primer 1	TTT ACT CAC CCT TGC GTC AG
Probe	/56-FAM/CTG GCT CCT /ZEN/TAA AAT CCA CCT TTT GGG /3IABkFQ/
E1B55K	
Primer 2	CAT CAC AGG CTG GTT CCT AAT A
Primer 1	GGT GTT TGA CAT GAC CAT GAA G
Probe	/56-FAM/TGG AAG GTG CTG AGG TAC GAT GAG A /3IABkFQ/
TPL_HEXON	
Primer 2	CCC GAG ATG TGC ATG TAA GAC
Primer 1	AAA GGC GTC TAA CCA GTC AC
Probe	/56-FAM/ CGC TTT CCA AGA TGG CTA CCC CT /3IABkFQ/
EIF1	
Primer 2	GTA TCG TATGTC CGC TAT CCA G
Primer 1	GAT ATA ATC CTC AGT GCC AGC A
Probe	/56-FAM/CTC CAC TCT /ZEN/TTC GAC CCC TTT GCT /3IABkFQ/

Quantitative real time PCR analysis of viral DNA levels

Infected or uninfected control cells were washed in phosphate-buffered saline (PBS) and lysed in 100 μ l of NP-40–Tween buffer containing proteinase K, as described in Garnett et al {Garnett, 2002 #17}. Samples were tested by real-time PCR for a region of hexon gene that is conserved among species C adenovirus serotypes and the endogenous cellular gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Samples were run in duplicate for each independent experiment. Data was analyzed using the comparative threshold cycle (Ct) method, as described above. The amount of *hexon* gene DNA was normalized to the amount of GAPDH and then relative amounts were compared to the time point representing the peak of the acute infection. In some experiments, viral genome numbers were quantified using a standard curve, as previously described {Garnett, 2002 #17}.

RT-PCR detection of ETV6/RUNX1 and RUNX1/MTG8 transcripts

Total RNA was isolated from cells transfected with plasmids expressing either the *ETV6/RUNX1* or *RUNX1/MTG8* fusion genes and converted into cDNA as described above. The presence of *ETV6/RUNX1* and *RUNX1/MTG8* mRNA transcripts was tested by PCR amplification using indicated primers and PCR conditions from van Dongen *et al* and amplicons were run on a 2% agarose gel {van Dongen, 1999 #156}.

Flow cytometry

Intracellular staining for the viral capsid protein, hexon, was used to detect productively infected cells by flow cytometry as previously described {Zhang, 2010 #64}. Briefly, a mouse monoclonal antibody to adenovirus hexon protein (IgG1 κ , MAB8051, Chemicon International/Millipore) was used as a primary antibody. A mouse isotype IgG1, κ , antibody was

used as a negative control for primary antibody staining (BD Pharmingen). Cells were subsequently stained with a goat anti-mouse IgG-APC conjugated secondary antibody (Life Technologies). Results were analyzed on a LSR Fortessa flow cytometer using FACSDiva Software (BD Biosciences). Isotype control staining was used to define the hexon-positive staining cells and was 5% or less for all samples evaluated.

Immunoblots for protein detection

A total of $4-6 \times 10^6$ cells were collected and washed in cold PBS, the cell pellet was resuspended in 1 ml of cold RIPA buffer (R0278, Sigma) supplemented with 1 mM EDTA (161-0729, BioRad), protease/phosphatase inhibitor (1861281, Thermo Scientific), and incubated on ice for 30 min. Samples were then sonicated briefly, and boiled for 5 min with equal amounts of 2X Laemmli sample buffer (161-0737, Bio-Rad) before being run on a SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose membrane (BioRad). Immunoblotting of RUNX1 and RUNX1 fusion proteins was performed by using primary polyclonal rabbit anti-RUNX1 antibodies (ab23980, Abcam or PC285, CalBiochem) and secondary goat anti-rabbit IgG-HRP antibodies (sc-2004, Santa Cruz Biotechnology). Primary mouse antibody to actin (MAB1501, Chemicon) and secondary donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz Biotechnology) was blotted in parallel as a protein loading control. Proteins were visualized with HyGLO Reagent A/B (E2500, Denville Scientific Inc) and visualized with HyBlot ES Autoradiography Film (E3218, Denville Scientific Inc). The adenovirus E2A DNA-binding protein was visualized in the same manner using dilute hybridoma culture supernatant fluid from the mouse monoclonal antibody clone B6-8, kindly provided by A. Levine of Princeton

University {Reich, 1983 #52}. The exposed HyBlot ES Autoradiography film was scanned and the optical density of the specific signal quantified with the tools available in ImageJ.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay was performed as previously described {Maganti, 2014 #165} on cells approximately 1 month post infection. Briefly, cells were cross-linked with 1% formaldehyde for 8 min at room temperature. Crosslinking was stopped by the addition of 0.125 M glycine for 5 min at room temperature. Cells were lysed using cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 1% igepal) and protease inhibitors for 15 min on ice. The cell lysate was centrifuged at 2100 rpm for 5 min at 4°C. The supernatant was discarded and the pellet was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, dH₂O) and protease inhibitors for 25 min on ice followed by flash freezing in liquid nitrogen. Lysed nuclei were sonicated using a Bioruptor water bath sonicator for 15 sec “On” and 30 sec “Off” 3 times to generate an average of 800 bp of sheared DNA, from which the 15 ul input sample was taken. The sonicated lysates were pre-cleared with salmon-sperm coated agarose beads (Upstate) and lysates were divided equally. One half of the lysate was immunoprecipitated with 5 µg of ChIP-grade antibody to RUNX1 (Abcam ab23980) overnight at 4°C. The other half of the lysate was immunoprecipitated with isotype control antibody. Immunoprecipitated proteins were incubation with 60 µl of salmon-sperm coated agarose beads for 2 h and then washed for 3 min at 4°C with the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl, dH₂O), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl, dH₂O), LiCl buffer (0.25 M LiCl, 1% NP40, 1% DOC, 1 mM EDTA, 10 mM Tris pH 8.0, dH₂O) and 1X TE buffer. DNA was then eluted with SDS elution

buffer (1% SDS, 0.1 M NaHCO₃, dH₂O). After DNA elution, crosslinking was reversed overnight with 5 M NaCl at 65°C followed by treatment with proteinase K for 1 h at 45°C. Immunoprecipitated DNA was isolated using a phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer's instructions. QPCR reactions were run in triplicate on an ABI prism 7900 (Applied Biosystems, Foster City, CA). Isolated DNA was analyzed by real-time PCR using the following primers to E3, hexon, or GAPDH:

E3 promoter region: (Sense sequence, 5'-CCCGCTCCCACCACTGT-3', anti-sense sequence, 5'-TGCGCCCCTGAGTTAGTCA-3', probe sequence, 5'-56-FAM/CCCAGAGAC/ZEN/GCCCAGGCCG/3IABkFQ/-3').

Hexon gene region (Sense sequence, 5'-GCCATTACCTTTGACTCTTCTGT-3', anti-sense sequence, 5'-CCTGTTGGTAGTCCTTGTATTTAGTATC-3', probe sequence, 5'-56-FAM/AGAAACTTCCAGCCCATGAGCCG/36-TAMSp/-3').

GAPDH gene region (Sense sequence, 5'-AAATGAATGGGCAGCCGTTA-3', anti-sense sequence, 5'-TAGCCTCGCTCCACCTGACT-3', probe sequence, 5'-FAM-CCTGCCGGTGACTAACCCTGCGCTCCT-QSY7-3')

Values from real-time PCR reactions were analyzed using the $\Delta\Delta C_t$ method, using the gene of interest input values to normalize samples (which were run alongside the ChIP sample DNA), followed by normalizing to the negative control isotype antibody sample, which was set to 1.

3.4 RESULTS

The acute phase of adenovirus infection is unperturbed in B cells expressing *RUNX1* containing leukemic fusion genes

Several *RUNX1* proteins disrupt the organization of intranuclear sites of viral RNA synthesis {Marshall, 2008 #38}. We therefore postulated that *RUNX1*-related leukemic fusion proteins may inhibit adenovirus persistence in lymphocytes by interfering with viral replication. To evaluate the impact of the leukemic fusion genes on the course of an adenoviral infection, we created stable B cell lines expressing either the *RUNX1* fusion gene commonly associate with ALL (*ETV6/RUNX1*), or the *RUNX1* fusion gene commonly associated with acute myeloid leukemia (*RUNX1/MTG8*) {Miyoshi, 1993 #40; Erickson, 1992 #11}. While both fusion proteins can repress gene expression {Okuda, 2001 #46; Yamagata, 2005 #61; Ptasinska, 2012 #49}, the N-terminus of the *RUNX1/MTG8* fusion protein is derived from the *RUNX1* protein and differs from the N-terminus of the *ETV6/RUNX1* fusion. Because the N-terminus of *RUNX1* was shown to interact with adenoviral proteins and affect their localization {Marshall, 2008 #38}, we reasoned that the fusion products may have different effects on the virus infection. For these experiments, cells were transfected with the indicated fusion genes or an empty vector and selected for several weeks to create stable cell lines.

The *ETV6/RUNX1* and *RUNX1/MTG8* fusion product detected by RT-PCR in stably transfected B cell lines was indistinguishable from that detected in transiently transfected A549 epithelial cells (Figure 3.1). The patient-derived UoC-B4 cells served as a positive control for the *ETV6/RUNX1* translocation {Kim, 1996 #27}. KASUMI-1 cells, derived from an AML, served as a positive control for the expression of the *RUNX1/MTG8* fusion gene {Asou, 1991 #2}.

Continued presence of the fusion gene transcript was confirmed throughout the course of the experiments (data not shown). Western blotting confirmed expected presence of both ETV6/RUNX1 and RUNX1/MTG8 fusion proteins, although levels of the RUNX1/MTG8 fusion protein in stably expressed cells appeared substantially lower than that found in KASUMI-1 cells (Figure 3.2).

Cells expressing the fusion genes were infected with either wild-type serotype 5 adenovirus (Ad5wt) or a phenotypically wild-type mutant serotype 5 adenovirus (Ad5dl309). In four independent infections, synthesis of a representative viral structural protein (hexon) reached a maximum between day 8 and 14 post infection (Figure 3.3 **and data not shown**) as previously reported. A similar fraction of infected cells was found to have high levels of hexon protein whether transduced with the *ETV6/RUNX1*, *RUNX1/MTG8*, or an empty vector (Figure 3.3). The levels and pattern of hexon expression measured by flow cytometry in these lymphocytic cells resembled that measured in acutely infected epithelial cells {Murali, 2014 #42}. The progress of the infection over the first 20 days resembled that previously described for lymphocytic cell lines {Zhang, 2010 #64} and we term this period as the “acute” phase of the infection. After 20 days of infection, hexon protein levels diminished and became often undetectable by flow cytometry {Zhang, 2010 #64}. We designate this phase of the infection as the “persistent” phase because the viral genome persisted in several lymphocytic cell lines for over a year even though late gene expression was often undetectable {Zhang, 2010 #64}. Neither the ALL- or AML-associated translocation appeared to alter the synthesis of hexon protein the acute phase of the infection (Figure 3.3). By 21 days after infection, fewer than 15% of the cells contained hexon protein that could be detected by flow cytometry irrespective of the leukemic fusion gene (data not shown). The synthesis of hexon protein decayed in a comparable manner. Neither B cells containing the

empty vector, nor those expressing the translocations, contained high amounts of intracellular hexon protein by day 21 (<15%, data not shown).

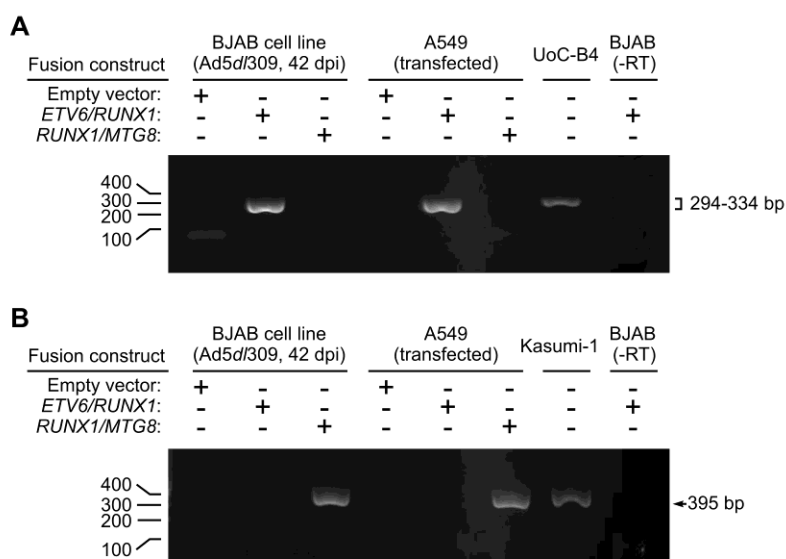
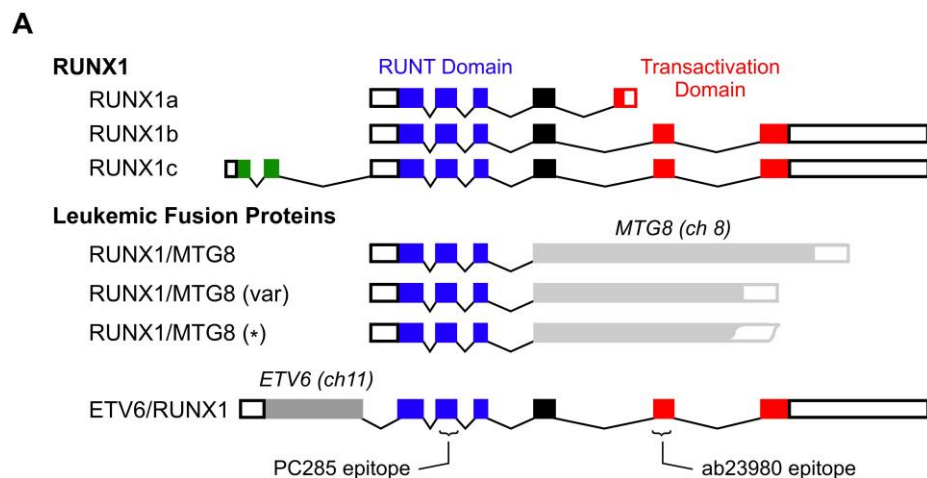
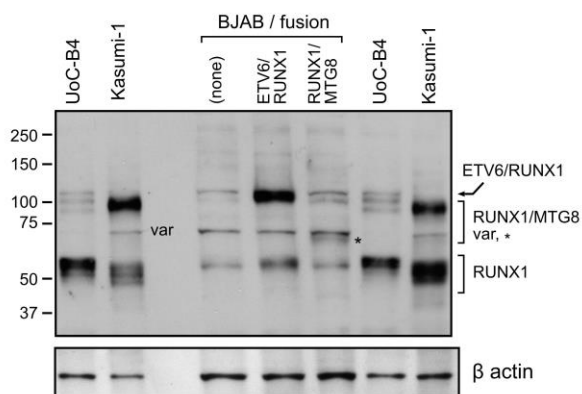


Figure 3.1 Stable expression of the ETV6/RUNX1 and RUNX1/MTG8 leukemia fusion genes persists in B lymphocytic cells following transfection and selection.

BJAB cells were transfected with the pTARGET expression vector (Empty vector) or the same vector expressing ETV6/RUNX1 or RUNX1/MTG8. Stable cell lines were established and selected as described in the Methods. mRNA was isolated from these cell lines 42 days after infection with Ad5dl309 and analyzed by PCR following reverse transcription. To serve as a control, RNA was isolated from A549 cells transiently expressing the same constructs as well as the leukemic cell lines UoC-B4 and Kasumi-1. (A) The TEL-H (ETV6) and AML1-G (RUNX1) primer pair described in (Guruprasad, Kavitha et al. 2014) yields ETV6/RUNX1 products of transcript 294-334 bp product (upper panel). (B) The AML1-A (RUNX1) and ETO-B (MTG8) primers described in (van Dongen, Macintyre et al. 1999) yields a 395 bp PCR product for the RUNX1/MTG8 fusion transcript. RNA from infected BJAB cells transduced with ETV6/RUNX1 served as a negative control by leaving out reverse transcriptase (-RT).



B α RUNX1 (PC285)



C α RUNX1 (ab23980)

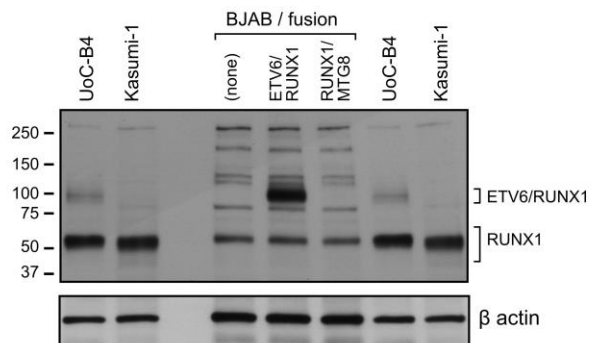


Figure 3.2 Schematic representation of the RUNX1 and RUNX1-related leukemic fusion proteins and RUNX1-antibody recognition sites.

(A) The predominant isoforms of human RUNX1, the ETV6/RUNX1, and the RUNX1/MTG8 leukemic fusion genes are represented by boxes for exons where filled boxes are coding regions and open boxes are non-translated regions. Splices in the RUNX1 gene are indicated by segmented lines connecting the exons. The DNA-binding (RUNT) domain is shown in blue, the transactivation domain in RUNX1 in red and the unique exon incorporated into RUNX1c is

green. The exon and intron structure of the fusion partners MTG8 and ETV6 are not shown. The predominant RUNX1/MTG8 fusion product is shown along with a naturally occurring variant (var). We used a RUNX1/MTG8 construct (*) which is similar in size to the truncated variant lacking the NHR3 and NHR4 regions of MTG8 as previously described (Yan, Burel et al. 2004, Yan, Kanbe et al. 2006). This variant protein associates with histone deacetylase complexes, but does not disrupt cell cycle (Burel, Harakawa et al. 2001, Yan, Burel et al. 2004, Yan, Kanbe et al. 2006). This variant has been detected in clinical isolates as well as Kasumi-1 cell line (Yan, Kanbe et al. 2006). The symbol “var” and “*” identify the likely RUNX1/MTG8 variant and variant construct in the corresponding immunoblot (**B**). Cellular lysates from UoC-B4, Kasumi-1 and stably transfected B lymphocytic cells expressing the RUNX1-related fusion genes were separated by electrophoresis through polyacrylamide with SDS, immobilized, and probed by immunoblotting with the RUNX1 antibody specific for the RUNT domain (clone PC285). All variants of the RUNX1 and RUNX-fusion proteins identified in (A) were detected although levels of the RUNX1/MTG8 fusion protein expressed the Kasumi-1 cells was significantly greater than that directed by the expression vector. (**C**) Immobilized cellular lysates were also probed with the ChIP-grade RUNX antibody clone ab23980 which detected the expected 100 kDa fusion protein in both UoC-B4 and transfected BJAB cells. Both antibodies detected the normal RUNX1 isoforms of 45-55 kDa while RUNX1a isoform lacks the epitope recognized by the ab23980 antibody. β -actin was used as a control for protein loading.

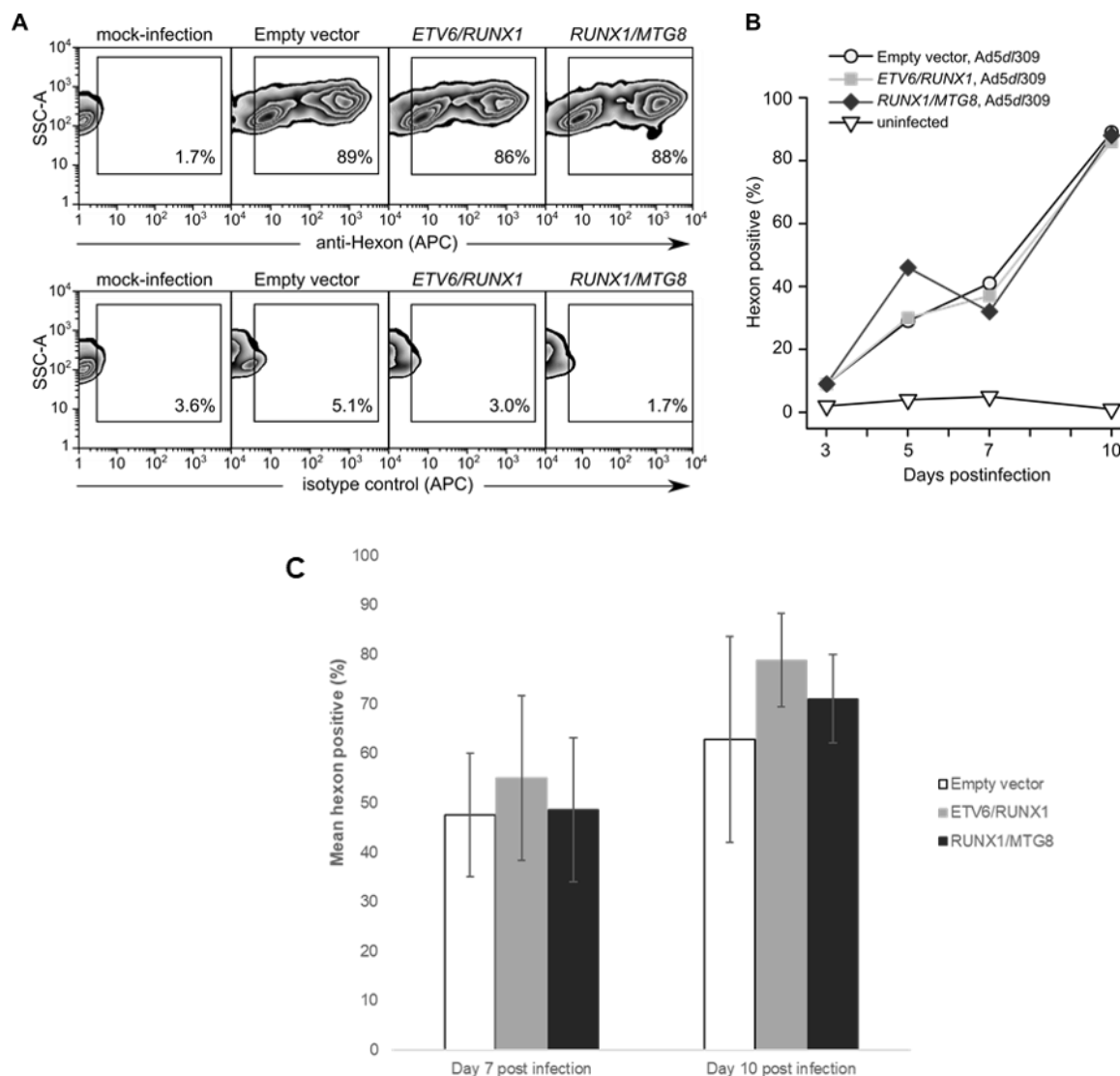


Figure 3.3 The “acute” phase of adenovirus infection in B lymphocytes is not impacted by enforced expression of the leukemia-associated ETV6/RUNX1 or RUNX1/MTG8 fusion genes.

BJAB cell lines transduced with the empty (pTARGET) vector or the indicated fusion gene were infected with Ad5dl309 virus and stained for the viral hexon protein at various days postinfection with a mouse monoclonal antibody to hexon or isotype control and an APC-conjugated goat-antibody specific for mouse IgG. (A) Representative flow cytometry plots showing infected cells stained for hexon (top panel) or with the isotype control (bottom panel) at 10 days postinfection. (B) The fraction of hexon-positive cells is shown as a function of days postinfection of a representative infection. (C) Frequency of hexon positive cells. Error bars are standard deviation of the mean of multiple independent Ad5 infections ($n=5$). No statistically significant difference in hexon protein expressing cells is detected between the empty vector and ETV6/RUNX1 ($p=0.1996$) or RUNX1/MTG8 ($p=0.4901$) fusion protein expressing cells at day 10 post infection.

RUNX1 containing fusion genes reduce adenoviral gene expression in infected B cells.

In contrast to the similar pattern of viral gene expression observed among the cell lines during the acute phase of the infection, the *RUNX1*-related fusion genes altered the course of the infection during the persistent phase. Although the hexon protein becomes undetectable during the persistent phase of the infection {Zhang, 2010 #64}, hexon transcripts are detectable at much later times post-infection. B cells transfected with the empty vector and infected with either Ad5dl309 or Ad5wt (Figure 3.4) contain similar levels of hexon mRNA throughout the 10- or 6-week period shown in Fig. 2. By contrast, levels of hexon mRNA declined over this period of time in cells expressing either the *ETV6/RUNX1* or *RUNX1/MTG8* fusion gene. In one experiment, hexon mRNA was undetectable in *ETV6/RUNX1*-expressing B cells after 10 weeks. To determine the extent of this loss of viral gene expression, the levels of representative early transcripts from five transcription units were measured. Levels of mRNA for E1A, E1B-55K, E2A, E3-GP19K and E4ORF6 were reduced in cells expressing either *ETV6/RUNX1* or *RUNX1/MTG8* compared to cells transduced with the empty vector (Figure 3.5). These results suggest that the presence of the *RUNX1*-related fusion genes reduced expression of both early and late adenovirus genes in a manner that is independent of the sequences at the N-terminus or C-terminus of the *RUNX1* protein. Moreover, this effect is likely to be independent of the *RUNX1* transactivation domain, which is absent in the *RUNX1/MTG8* fusion protein. Notably, the decrease in viral gene expression was not due to changes in the viability or growth of the infected cells which was unaffected by the presence of the *RUNX1*-related fusion gene (Figure 3.6**Error! Reference source not found.**).

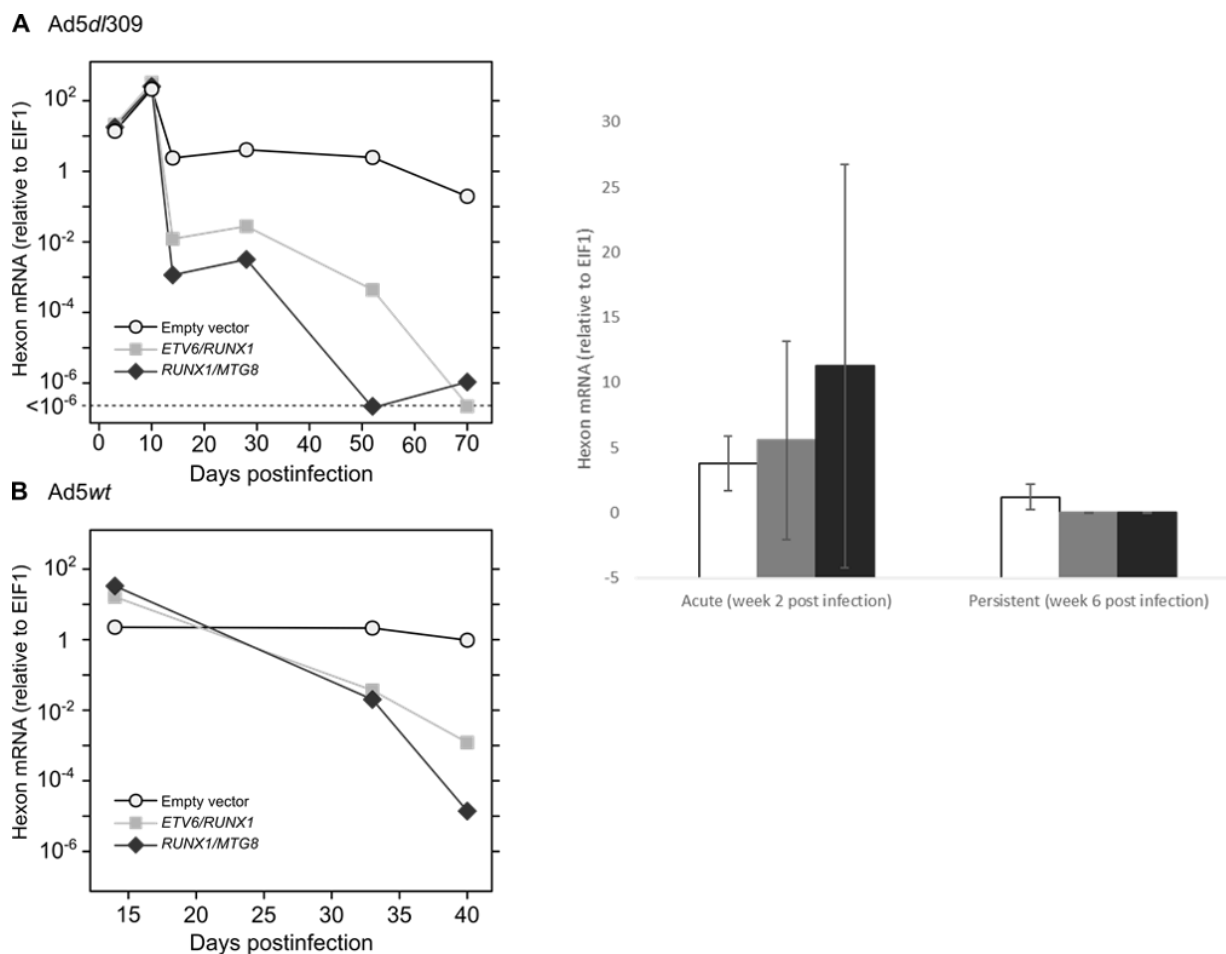


Figure 3.4 Expression of the late viral hexon gene declines in persistently infected B lymphocytes that express the leukemia-associated ETV6/RUNX1 or RUNX1/MTG8 fusion genes.

BJAB cells stably expressing either the empty vector, ETV6/RUNX1 or RUNX1/MTG8 were infected with either the (A) phenotypically wild-type virus Ad5dl309 or (B) true wild-type Ad5wt virus. At the indicated times, the level of hexon mRNA was measured by qPCR after reverse transcription and normalized to the level of the cellular EIF1 mRNA. Values presented in (A) are the average of two independent experiments. The dotted line in (A) represents the limit of detection. The Ad5dl309 (A) infected cells lost 6 log fold in mRNA transcript levels in the ETV6/RUNX1 or RUNX1/MTG8 fusion gene expressing cells compared to the empty vector, while the Ad5wt infection (B) lost around 3 log fold in RUNX1 fusion gene expressing cells compared to empty vector. (C) The mean normalized hexon mRNA values of 3 independent

infections using adenovirus type 5 was calculated. Sample values were pooled at 2 and 6 weeks post infection. Though the independent infections displayed great variability, the same trend was seen in every infection evaluated for hexon mRNA ($n=3$), with p -values approaching significance for differences between empty vector and *RUNX1* fusion gene cultures at 6 weeks post infection (0.0908 for *ETV6/RUNX1*, and 0.0907 for *RUNX1/MTG8*).

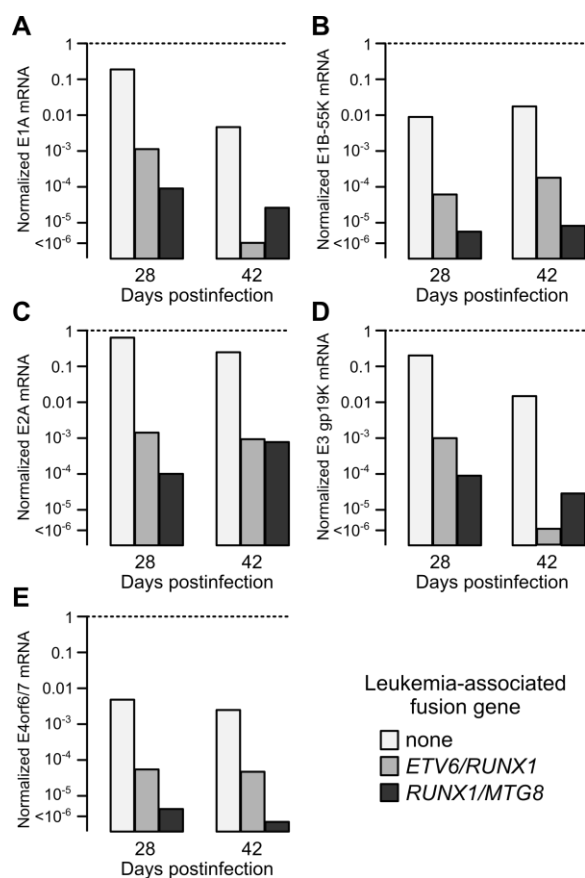


Figure 3.5 Expression of the early viral genes declines in persistently infected *B* lymphocytes that express the leukemia-associated *ETV6/RUNX1* or *RUNX1/MTG8* fusion genes.

BJAB cell lines stably transfected with constructs to express the indicated leukemia-associated genes were infected with Ad5dl309 and the levels of the indicated adenovirus early genes (A: E1A, B: E1B-55K, C: E2A DBP, D: E3 gp19K, and E: E4orf6/7) measured by qPCR after reverse transcription on days 28 and 42 postinfection.

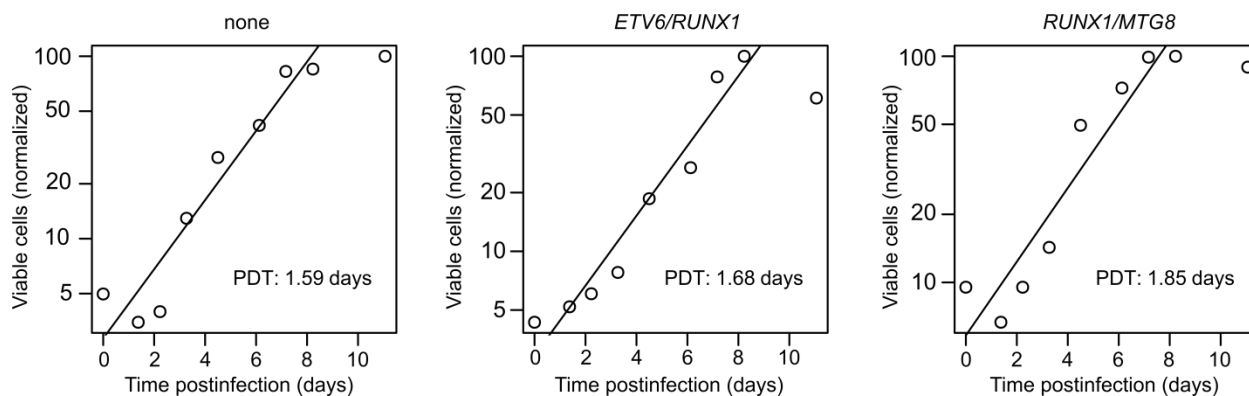


Figure 3.6 Stable expression of *ETV6/RUNX1* or *RUNX1/MTG8* does not affect the growth of persistently infected B lymphocytic cells.

A persistent infection of Ad5dl309 was established in BJAB cells stably transduced with the empty vector or expression vectors for the indicated leukemic fusion genes. At 28 days post infection, cell cultures were established at 1×10^5 cells per ml. The number of viable cells was determined daily for 12 days without supplementing the growth medium. The population doubling time (PDT) was calculated by a log-linear regression for the values up to 10 days. Expression of the leukemic fusion transcripts was confirmed in these same cultures by the results presented in Figure 3.1.

Adenovirus is not retained in persistently infected BJAB cells that express the *ETV6/RUNX1* or *RUNX1/MTG8* leukemic fusion genes.

The loss of viral transcripts could reflect the inhibition of transcription or loss of the viral genome. The amount of viral DNA in persistently infected BJAB cells stably transfected with the *RUNX1*-related fusion genes or empty vector was measured over the course of several weeks. Results reported in Figure 3.7 show the changes in viral DNA expressed as a fraction of amount present at the initial peak of infection (10 to 14 d post-infection) for two independent experiments. Levels of Ad5dl309 viral DNA dropped but remained relatively stable for 40 days after infection of cells transduced with the empty vector (Figure 3.7). By contrast, loss of the viral Ad5dl309 genome was greatly accelerated in cells expressing either the *ETV6/RUNX1* or *RUNX1/MTG8* fusion gene. B cells containing either fusion gene exhibit a 6- to 8-log reduction in viral genome levels. In a separate set of experiments, cells transduced with the empty vector

and infected with Ad5wt contained a constant level of the viral genome over the entire five-week observation period (Figure 3.7). However, Ad5wt-infected cells expressing *ETV6/RUNX1* suffered as much as a 4-log loss of viral DNA over this same period. Curiously, *RUNX1/MTG8*-expressing cells showed a minimal (1-log) reduction in viral DNA in one experiment, but mirrored the *ETV6/RUNX1* cells in another experiment by experiencing a 4-log loss. Although we are unable to explain the variable nature of the results measured in *RUNX1/MTG8*-expressing cells, this could be related to the low levels of *RUNX1/MTG8* fusion protein present in this cell line (see Figure 3.2). Nonetheless, in seven of eight independent infections, expression of a *RUNX1*-related leukemic fusion greatly reduced the levels of adenovirus genome in persistently infected B-cells. Overall acute infection showed no significant difference in viral DNA. However, persistently infected cells contained significantly less viral DNA in the *ETV6/RUNX1* and *RUNX1/MTG8* expressing B cells (Figure 3.8). Furthermore, these reveal an unexpected difference between the true wild-type Ad5 and phenotypically wild-type Ad5dl309 virus; the rate of genome loss for Ad5dl309 was substantially and significantly greater ($p < 0.04$ by log-linear regression) than the loss of the Ad5wt genome in cells containing the fusion gene.

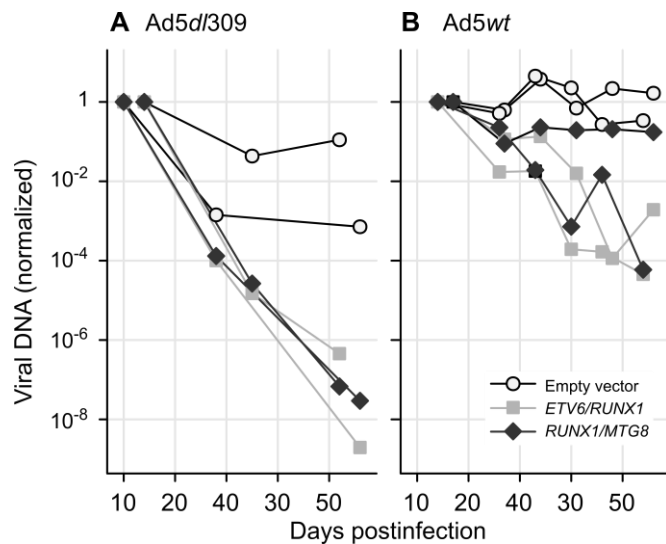


Figure 3.7 Expression of ETV6/RUNX1 or RUNX1/MTG8 in persistently infected B lymphocytes promotes the loss of viral DNA.

BJAB cells stably expressing either an empty vector or the indicated leukemia-associated fusion gene ETV6/RUNX1 or RUNX1/MTG8 were infected with the phenotypically wild-type virus Ad5dl309 or true wild-type Ad5wt virus. At the indicated days postinfection, viral DNA levels were measured by qPCR and normalized to the level of the cellular gene GAPDH. Viral DNA levels are presented after normalizing to levels measured at the start of each experiment. Results are shown for two independent experiments using each Ad5dl309 (A) or Ad5wt (B).

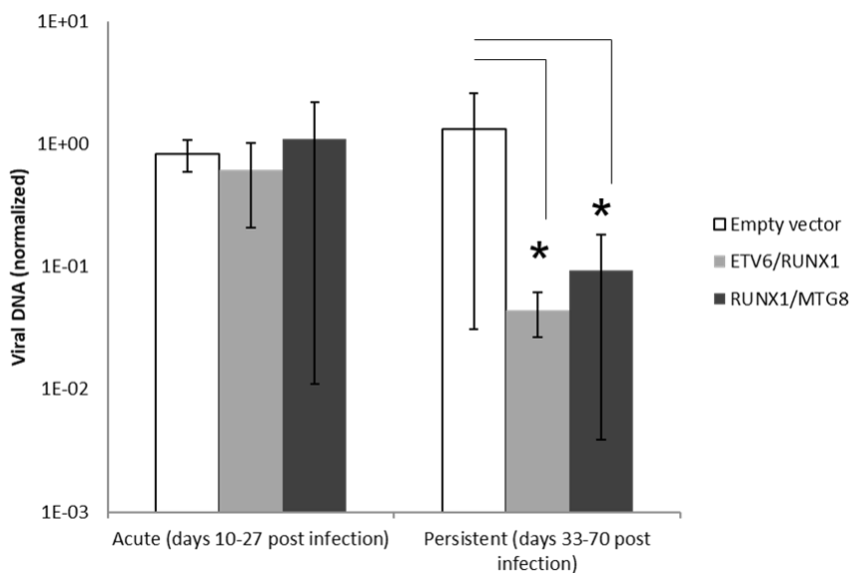


Figure 3.8 Expression of ETV6/RUNX1 or RUNX1/MTG8 during persistent significantly reduces amount of viral DNA retention in B cells.

*BJAB cells stably expressing either an empty vector or the indicated leukemia-associated fusion gene ETV6/RUNX1 or RUNX1/MTG8 were infected with adenovirus type 5. At the indicated days post-infection, viral DNA levels were measured by qPCR and normalized to the level of the cellular gene GAPDH. Viral DNA levels are presented after normalizing to levels measured at the start of each experiment. Results are shown for the means of 5 independent experiments, with time points pooled during acute (10-27) and persistent (33-70) phases of infection. Difference in virus DNA was **not** significant during **acute** infection for either ETV6/RUNX1 ($p=0.2896$) or RUNX1/MTG8 ($p=0.5780$), but was significant during **persistent** infection for ETV6/RUNX1 ($p=0.0091$) and RUNX1/MTG8 ($p=0.0116$). * $p \leq 0.05$.*

The ETV6/RUNX1 fusion protein binds adenovirus DNA.

Although the decline in viral gene expression in cells with *RUNX1*-related fusion genes can be explained by the loss of viral DNA, *RUNX1* fusion proteins repress gene expression by

directly binding chromatin and recruiting transcriptional repressors {Yamagata, 2005 #61}. To determine if the RUNX1-fusion proteins bound the viral genome, two canonical RUNX1 binding sites in the E3 promoter region and the *hexon* coding region were queried by a chromatin immunoprecipitation (ChIP) assay. B cell lines stably expressing the *ETV6/RUNX1* fusion gene or the empty vector were persistently infected with Ad5wt virus and evaluated for RUNX1 binding to viral DNA at 24 and 31 days post-infection (Figure 3.9). The *RUNX1/MTG8* expressing cells were not analyzed because the RUNX1/MTG8 fusion protein lacks the RUNX1 epitope recognized by the ChIP-grade antibody used for these experiments (Figure 3.2).

More viral DNA at both RUNX1 binding sites was immunoprecipitated from cells expressing the *ETV6/RUNX1* fusion gene than from cells transduced with the empty vector (Figure 3.9). Immunoprecipitation of the DNA required a canonical RUNX1-binding site because a region of the cellular *GAPDH* gene devoid of RUNX1-binding sites within 800 nucleotides of the target was not recovered in excess from *ETV6/RUNX1*-expressing cells (Figure 3.9). Because both empty vector-transduced and *ETV6/RUNX1*-transduced cell lines express similar amounts of endogenous RUNX1 protein (Figure 3.2) the additional RUNX1-related protein recovered by chromatin immunoprecipitation is most likely the *ETV6/RUNX1* fusion protein.

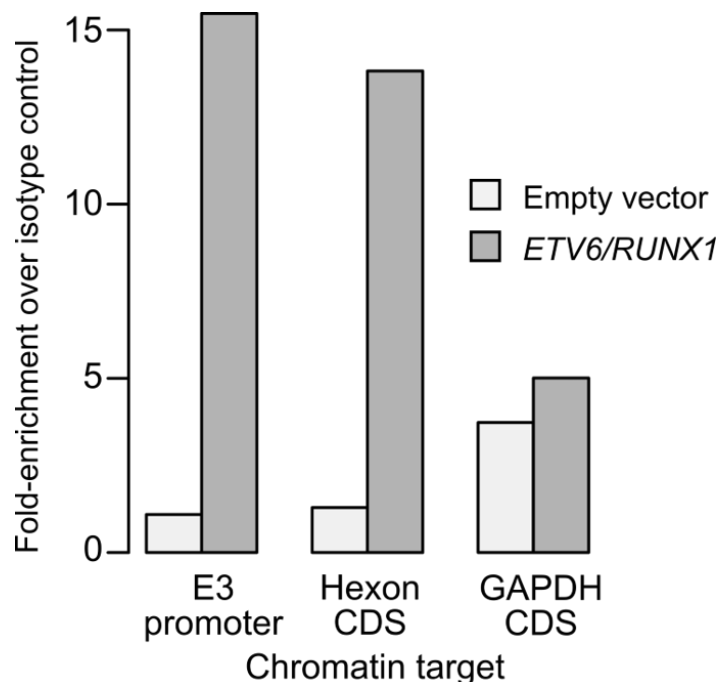


Figure 3.9 *The leukemic associated fusion protein ETV6/RUNX1 binds the adenoviral genome.*

BJAB cells stably expressing either an empty vector or ETV6/RUNX1 were infected with Ad5wt. On days 24 and 31 post-infection, cells were fixed and chromatin immunoprecipitation was performed with either a non-specific isotype-matched antibody as a control or an antibody specific for the RUNX1 protein. DNA that immunoprecipitated with the antibodies was measured by qPCR with primers specific for the adenovirus E3 promoter region, the adenovirus hexon gene coding region, or the coding region of the cellular GAPDH gene. The total amount of DNA (input) used for the immunoprecipitation was determined using 3% of the total lysate and used to normalize the amount of specific DNA recovered. Samples were further normalized to the amount of DNA immunoprecipitated with the isotype control antibody. Bars represent the average of 2 independent experiments each run in triplicate.

HDAC inhibitors increase viral gene expression in ETV6/RUNX1-expressing cells.

Because the chromatin-bound ETV6/RUNX1 protein may recruit HDACs to repress transcription {Yamagata, 2005 #61}, we asked if HDAC inhibition with TSA could increase viral mRNA in persistently infected B cells expressing ETV6/RUNX1. Treatment with TSA

increased viral gene expression in all cells evaluated after 40 days of infection (Table 3.2). *EIA* mRNA increased 8-fold in TSA-treated cells infected with Ad5wt and 6-fold in TSA-treated cells infected with Ad5dl309. *E3GP19K* and *hexon* mRNA increased after TSA treatment of infected cells with the empty vector. Expression of both of these genes, however, was greater in TSA-treated cells expressing *ETV6/RUNX1* than in cells containing the empty vector. TSA treatment increased *E3GP19K* mRNA levels 13-fold (24-fold versus 315-fold) and *hexon* mRNA levels by 94-fold (14-fold versus 1319-fold) in cells expressing *ETV6/RUNX1* over cells containing the empty vector. By contrast, *EIA* mRNA levels showed less variability between cell lines with an 8- and 9-fold increase following TSA treatment in empty vector-transduced and *ETV6/RUNX1*-transduced cells, respectively. Similar changes were observed in Ad5dl309-infected cells where the relative increase in *E3GP19K* and *hexon* mRNA was greater than the relative increase in *EIA* mRNA in *ETV6/RUNX1*-transduced cells. These results indicate that some viral genes are more strongly repressed by HDACs than others, particularly in presence of the ETV6/RUNX1 fusion protein. Interestingly, TSA increased the level of Ad5dl309 viral DNA 10-fold in cells expressing the *ETV6/RUNX1* fusion over that of cells transduced with the empty vector although changes in the adenovirus DNA level were not as substantially different than changes observed in mRNA between the translocation-containing translocation negative samples.

Table 3.2 *HDAC inhibition increases viral gene expression in persistently infected cells.*

B cells stably expressing either the empty vector or the leukemic fusion gene ETV6/RUNX1 were mock-infected or infected with the indicated virus. After 40 days cells were treated with the

300 nM TSA or vehicle control. After 48 h, viral genomic DNA was quantified by qPCR and the indicated viral mRNAs were quantified by qPCR after reverse transcription. Values shown represent the fold increase after TSA treatment normalized to mock-treated cells.

Leukemic fusion gene	Virus	Viral mRNA			Viral DNA
		E1A	E3gp19K	Hexon	
none	<i>dl309</i>	6	13	13	7
	Ad5wt	8	24	14	3
<i>ETV6/RUNX1</i>	<i>dl309</i>	22	1152	1063	72
	Ad5wt	9	315	1319	5

The amount of viral DNA-binding protein declines before the decline in the viral DNA genome *ETV6/RUNX1* expressing cells.

ETV6/RUNX1 most likely represses E3 expression by binding the viral genome and recruiting cellular histone deacetylases. Since the *RUNX1*-binding site at the E3 promoter (Figure 3.9) is near the E2A promoter, it seems plausible that any *ETV6/RUNX1* bound to this site could also repress transcription from the E2A promoter located on the reverse strand. Abundant levels of the viral DNA-binding protein (DBP) are required for viral DNA replication {de Jong, 2003 #8}. Consequently, a decline in E2A-DBP level would be expected to cause a corresponding decline in viral DNA levels. We therefore compared the amount of E2A-DBP measured by immunoblotting with levels of viral DNA measure by quantitative PCR at various times after infection with the wild-type Ad5wt virus. For cell lines transduced with the empty vector or an *ETV6/RUNX1*-expression vector, E2A-DBP levels at the onset of the persistent phase was about 30% of that measured in the middle of the acute phase (compare day 12 with

day 21 in Figure 3.10). This level of E2A-DBP remained stable over the course of the infection in cells transduced with the empty vector. By sharp contrast, levels of E2A-DBP declined at an exponential rate in cells that expressed the *ETV6/RUNX1* fusion gene. For both cell lines, the decline in E2A-DBP levels preceded the decline in viral DNA levels. Although a less extensive time course was evaluated for Ad5dl309-infected cells, E2A-DBP became undetectable in *ETV6/RUNX1*-expressing cells before becoming undetectable in cells transduced with the empty vector (day 21 in Figure 3.10). It seems reasonable that diminished level of E2A-DBP lead to a decline in viral genome levels, and that the decline in viral genome levels is accelerated by the presence of the *ETV6/RUNX1* translocation.

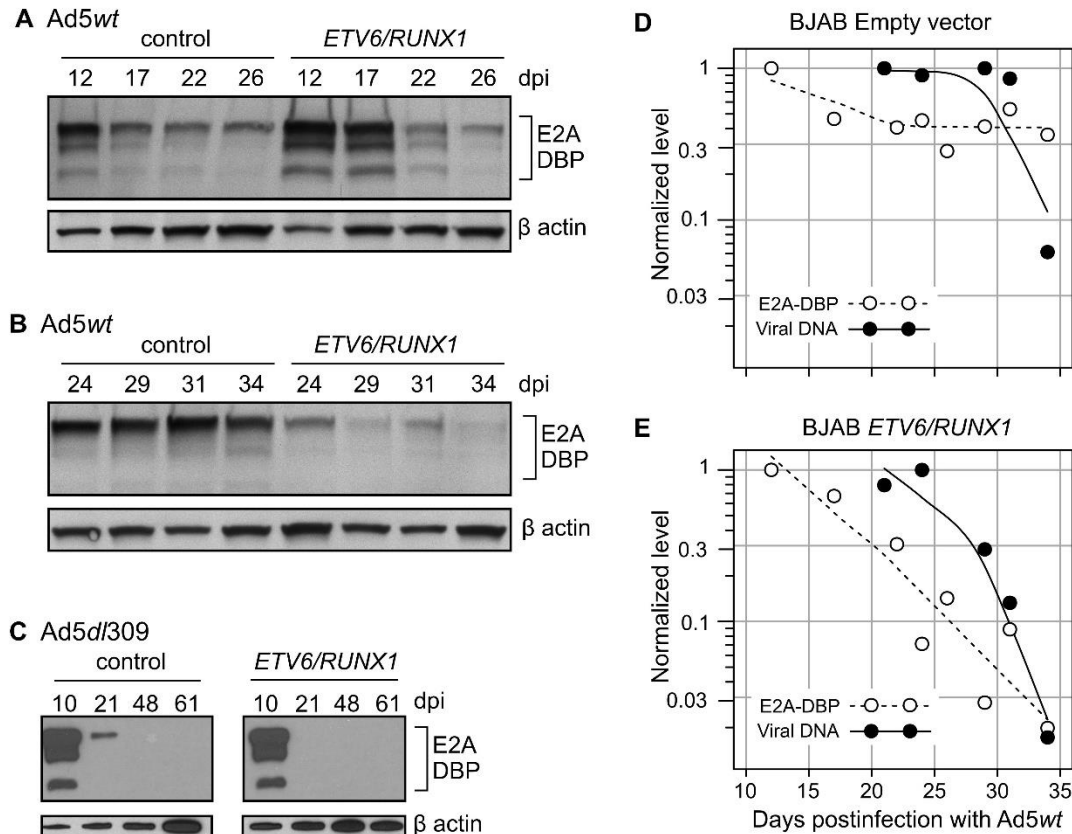


Figure 3.10 Levels of the viral protein required for viral DNA replication decline in advance of the decline in viral DNA levels.

The reduction of both viral protein and viral DNA is accelerated in cells expressing the ETV6/RUNX1 leukemic fusion gene. BJAB cells stably expressing the empty vector or ETV6/RUNX1 were infected with either Ad5wt or Ad5dl309 and evaluated by immunoblot for the viral E2A DNA-binding protein (DBP) and by qPCR for the viral DNA genome. (A, B) Cellular lysates from cells infected with Ad5wt were prepared on the days indicated and probe by antibodies for the viral E2A-DBP and the cellular β -actin protein. Levels of the protein were detected by chemiluminescent imaging. (C) Cellular lysates from cells infected with Ad5dl309 were prepared and analyzed as in panels A and B. (D) The levels of E2A-DBP and β -actin in BJAB cells transduced with the empty vector was quantified from the optical density of the chemiluminescent images shown in panels A and B with ImageJ. The level of E2A-DBP staining (open symbols) was normalized to that of β -actin then further normalized by setting the maximum value to 1. Levels of viral DNA (closed symbols) from the corresponding infection shown in Fig. 4 were normalized to the level of the cellular GAPDH gene and further normalized by setting the maximum value to 1. (E) An analysis similar to that described in panel D was performed for lysates and viral DNA from BJAB cells that were stably expressing the ETV6/RUNX1 fusion gene. Local polynomial smoothing curves were generated from the entire span of data with the loess function in R.

Virus-induced repression of the cellular genes *CXADR* and *SPARCLI* is retained after loss of the virus from *RUNXI* fusion gene expressing cells

Expression of the cellular genes *CXADR* and *SPARCLI* is downregulated by epigenetic means during the persistent phase of infection {Ornelles, 2016 #48}. Because of the durable nature of epigenetic changes, we wondered if these changes remained in cells that no longer harbored the virus. Cells expressing the *RUNXI* fusion genes or cells stably transfected with the empty vector were infected with Ad5dl309. After 56 days of infection, the number of viral genomes was determined and the relative level of mRNA for *CXADR* and *SPARCLI* was measured in infected and mock-infected cells. As expected, viral DNA was retained in the persistently infected B cells, which contained an average of 5,490 genomes per cell (Table 3.3). As previously shown (see Figure 3.7), the viral genome was lost from infected B cells that harbored the leukemic translocation. Cells expressing the *ETV6/RUNXI* translocation retained fewer than 6 genomes per 100 cells. Similarly, at least one-half of the cells expressed the *RUNXI/MTG8* translocation failed to retain the viral genome (46 genomes per 100 cells).

In agreement with previously reported results, infection reduced *CXADR* and *SPARCLI* expression to 13% of the level of non-infected cells transduced with the empty vector. Expression of both *CXADR* and *SPARCLI* was downregulated even further in cells expressing the *RUNXI*-related fusion genes to levels between 1 to 4% of that in non-infected cells. This reduction is especially striking in *ETV6/RUNXI*-expressing cells because at least 94% of these B cells no longer contained the viral genome. Cells expressing the *RUNXI/MTG8* fusion contained similarly low levels of *CXADR* and *SPARCLI* mRNA while retaining 12,000-fold less DNA than infected cells transduced with the empty expression vector. Because these cells contained only

46 genomes per 100 cells, it seems likely that the expression *CXADR* and *SPARCL1* remained low in a subset of cells that lost the viral genome. Expression of *CXADR* or *SPARCL1* was not downregulated in presence of the *RUNX1* fusion in the absence of a viral infection (data not shown). These results indicate that adenovirus may leave epigenetic echoes of its infection in the form of durable epigenetic repression of certain cellular genes after the virus is expelled from the infected cell. *The results of this section led to the filing of a provision patent, the draft of which can be found in Appendix A.*

Table 3.3 Adenovirus-provoked downregulation of cellular genes is retained after departure of the virus.

Levels of CXADR and SPARCL1 mRNA were measured 56 days after infection with Ad5dl309 and normalized to the housekeeping gene EIF1. The relative level of gene expression in infected B cells expressing the empty vector, ETV6/RUNX1 or RUNX1/MTG8 is expressed as a percentage of the level in uninfected cells. At the same time, the number of viral genomes was determined by qPCR.

Leukemic fusion gene	Cellular gene		Viral genomes per 100 cells
	CXADR	SPARCL1	
none	13%	13%	549,000
<i>ETV6/RUNX1</i>	1%	3%	5.54
<i>RUNX1/MTG8</i>	4%	1%	45.5

Transfection of the RUNX1 fusion genes into B cells already persistently infected, with adenovirus is technically difficult to evaluate with low reproducibility

Our results demonstrate that translocation containing cells can become efficiently infected with adenovirus but that the ability of the virus to be retained is lost. We next wanted to see if we could test the reverse scenario, experimentally, by introducing the translocation *after* the cells were already persistently infected. BJAB cells persistently infected with Ad5dl309 were transfected with empty vector, ETV6/RUNX1, or RUNX1/MTG8. This was attempted at different times post infection (122, 59 and 78 days post infection) from three different infections. To optimize transfection of the plasmids both Lipofectamine3000 and electroporation were tested. Neither transfection mechanism gave demonstrably better results. Transfected cells were selected for in G-418 supplemented medium for 30 days post infection to create stable cells line and maintained in G-418. Expression of RUNX1 fusion genes was confirmed periodically throughout experiments via RT-PCR. To ensure there was no reactivation of adenovirus, viral Hexon and ADP protein levels were assayed via flow cytometry. No protein expression was detected in any of the cells lines 21 days post transfection (data not shown). Viral DNA was measured by qPCR periodically for up to 72 days post transfection. In 2 of 3 experiments, RUNX1 fusion gene transfected cultures demonstrated more rapid viral DNA loss than empty vector for about 30 days post transfection (**Error! Reference source not found.**, Figure 3.11). However, in a third experiment, viral DNA remained high in ETV6/RUNX1 expressing cells but fell to undetectable levels in empty vector and RUNX1/MTG8 (**Error! Reference source not found.**). In another experiment, the trend of viral DNA being retained in empty vector cells

reversed after 30 days and cells expressing RUNX1 fusion genes retained higher amounts of viral DNA (**Error! Reference source not found.**). To determine the transfection efficiency of these infected cells, BJAB cells were transfected with a plasmid expressing GFP from the synthetic CAG promoter, and gated on MOCK transfected cells. Transfection efficiency was very low (14%) when using persistently infected cells (Figure 3.12). This may have formed a “bottle neck”, allowing only a very small percentage of cells with varying amounts of virus to survive, which may account for the highly variable results that were seen.

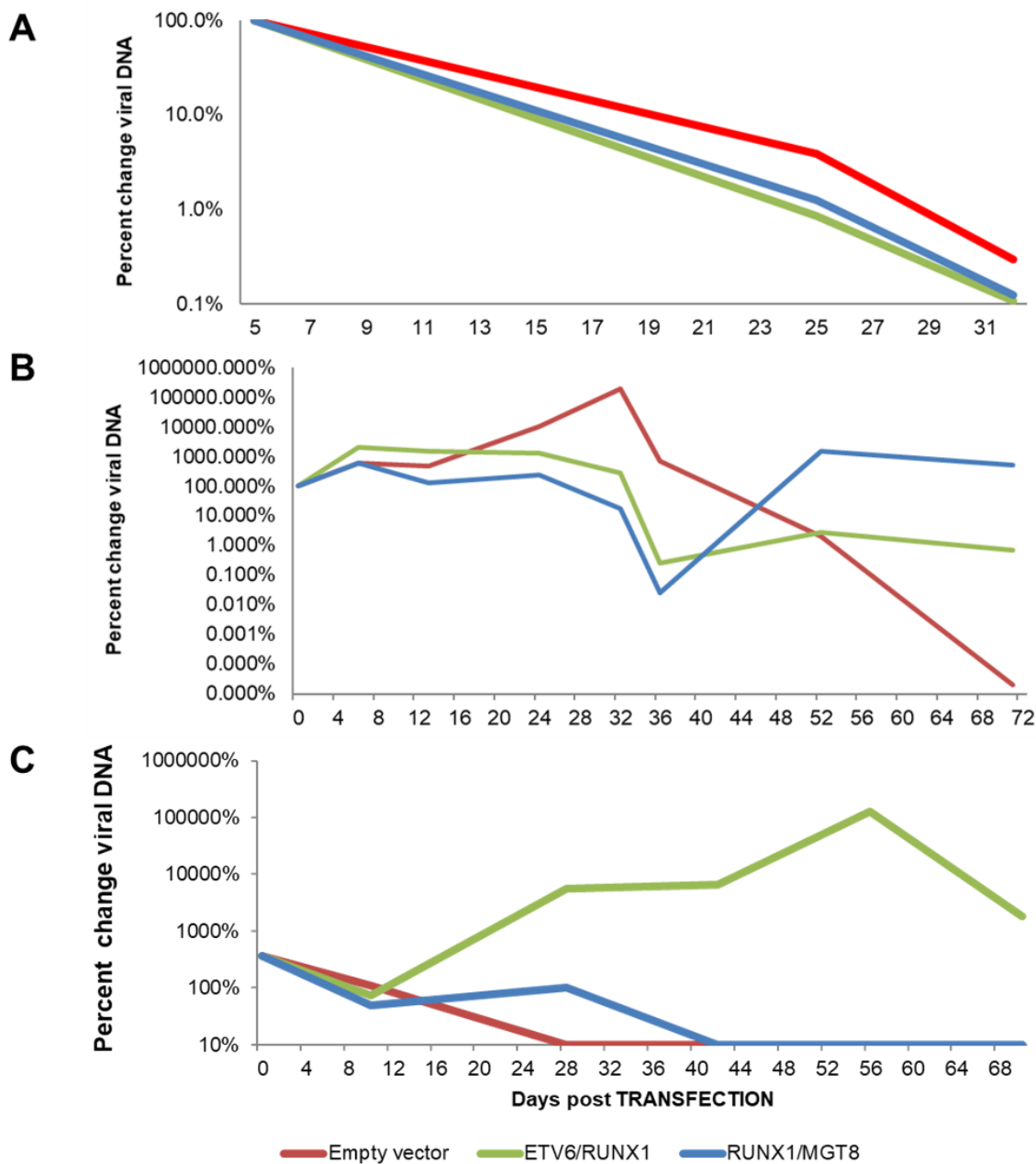


Figure 3.11 Persistently infected BJAB cells transfected with RUNX1 fusion genes. BJAB cells at 78 (A), 122 (B) and 59 (C) days post infection were transfected with empty vector (red line), ETV6/RUNX1 (green line) and RUNX1/MGT8 (green line) and put under drug selection (G-418). Viral DNA was measured by QPCR in duplicate wells after transfection, and set relative to starting viral DNA quantity. Data shown are from 3 independent experiments.

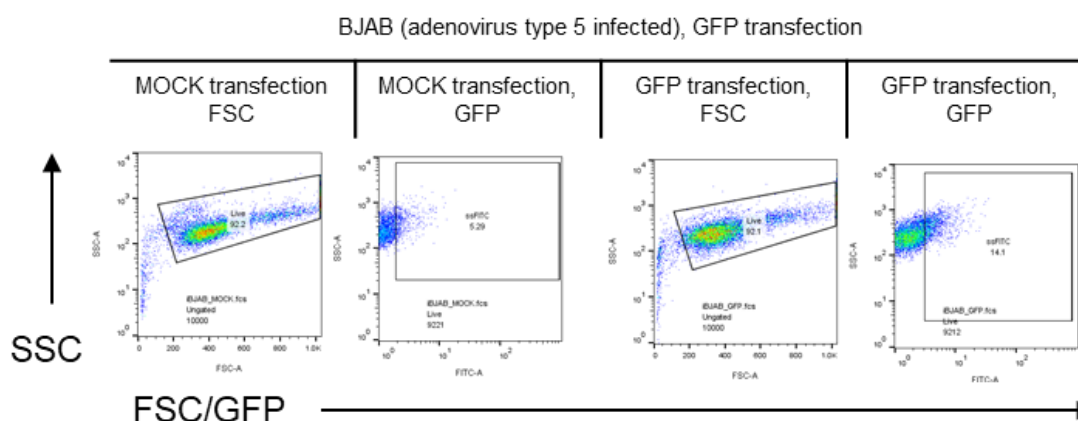


Figure 3.12 Persistently infected BJAB have low rates of transfection.

Persistently infected BJAB cells were transfected with a GFP plasmid with GFP under a CAG promoter using Lipofectamine3000 according to protocol, then tested for GFP expression 24hrs after transfection. Cells were gated on MOCK transfected cells and measured for GFP fluorescence. Only low levels of GFP expressing cells were detected in the GFP transfects (14%). Data are shown from 1 representative experiment of 2 such independent experiments.

3.5 DISCUSSION

Viruses are responsible for over twenty types of cancer and as much as 15% of the world-wide cancer burden {recently reviewed in Chang, 2017 #1; Lunn, 2017 #60}. An infectious etiology for ALL has long been postulated {Smith, 1997 #65; McNally, 2004 #20; Heath, 2005 #12; O'Connor, 2007 #24} and although a recent study suggests that congenital cytomegalovirus infection may be a risk factor for ALL {Francis, 2017 #63} no microorganism has definitively been linked to this disease. One hypothesis that accounts for the absence of any link is that the common virus responsible for ALL initiates mutations in the leukemic progenitor cell that preclude retention of the virus. The DNA tumor viruses JC virus {Smith, 1997 #65} and adenovirus {Honkaniemi, 2010 #66; Gustafsson, 2007 #67} have been suggested to be good

candidates for this common virus. In this study, we sought to evaluate the potential for adenovirus to serve this role by examining the impact of RUNX1 leukemic fusion genes on adenovirus retention in B lymphocytes already harboring a persistent infection with adenovirus.

The program of adenovirus gene expression differs in lymphocytic cells during the acute phase, which is characterized by robust viral gene expression, and the persistent phase in which viral gene expression is gradually dampened. The common RUNX1 leukemic fusion genes ETV6/RUNX1 and RUNX1/MTG8 had no impact on viral gene expression during the acute phase but accelerated the decrease in viral gene expression and viral DNA replication during the persistent phase after 21 days of infection. This decrease in viral gene expression was measured as a decrease in the levels of representative early and late viral mRNAs. The decrease in viral DNA levels was preceded by an accelerated loss of E2A DNA-binding protein. Since the E2A DNA-binding protein is essential for viral DNA replication {Dekker, 1997 #6; Hoeben, 2013 #48}, reduced levels of this protein would logically lead to diminish viral DNA replication. We infer that viral DNA replication was affected because without viral DNA replication, viral genomes would have been diluted below the limit of detection (5-orders of magnitude) after less than four weeks (Figure 3.6). We also cannot exclude the possibility that the stability of viral DNA was further decreased by the RUNX1 leukemic fusion genes.

An unexpected finding was that viral genome levels declined at a greater rate in cells infected with the E3-deleted virus Ad5dl309 than the wild-type virus ($p < 0.04$ by log-linear regression). E3 products promote immune evasion, confer resistance to death-promoting cytokines, and promote cell death at late times of a productive infection {Tollefson, 1996 #75; Burgert, 2002 #4; Lichtenstein, 2004 #71}. Although these activities would impact the persistence of infected cells, it seems unlikely that the E3 14.7K, 14.5K and 10.4K proteins,

which are missing in Ad5dl309-infected cells, directly control viral DNA replication. Nonetheless, expression of the E3 and E2A genes may be linked. The E3 14.5K and 10.4K protein were reported to affect E1A expression {Zhang, 1994 #72}, which would in turn affect E2A transcription. The E2A and E3 promoters direct transcription in opposite directions from a common position on the viral chromosome. Because the E3 promoter can respond to activation signals independently of E1A {Deryckere, 1996 #7; Mahr, 2003 #18}, co-stimulation of the E3 and E2 promoter may promote adenovirus persistence in lymphocytes where E2 products direct viral DNA replication while E3 products allow for immune evasion. These results may hint at additional roles for the E3 region in persistence of viral DNA even in the absence of immune pressure.

Viral gene expression appears to be repressed by epigenetic changes in persistently infected cells. The adenovirus chromosome acquires histones shortly after entering the nucleus in absence of DNA replication {Komatsu, 2012 #47; Giberson, 2012 #84}. This would render it susceptible to regulation by histone modifications. Accordingly, viral mRNA levels increased after exposure to an HDAC inhibitor. Additionally, since the inhibitor increased levels of E3gp19K and hexon mRNA as much as 100-fold more in ETV6/RUNX1-expressing than in vector-transduced cells (Table 3.2), the ETV6/RUNX1 fusion protein appears to strongly repress adenoviral gene expression. Both ETV6 and the ETV6/RUNX1 fusion proteins are site-specific DNA-binding proteins that recruit chromatin remodeling proteins to repress target gene transcription {Yamagata, 2005 #38; Brettingham-Moore, 2015 #76; Hock, 2017 #77}. The ETV6/RUNX1 protein was enriched at a site in the E3 promoter region as well as at a site distant from any promoter in the hexon coding region. Curiously, ChIP analysis showed no enrichment of endogenous RUNX1 on the viral chromosome. Since the amount of endogenous protein and

fusion protein was similar (Figure 3.9), endogenous RUNX1 may not bind the viral genome with the same avidity as the ETV6/RUNX1 fusion protein. Alternatively, interactions between other adenovirus proteins and the RUNX1 proteins {Marshall, 2008 #19} may limit access of the endogenous RUNX1 protein to the viral genome. These results suggest that RUNX1 fusion proteins can evict adenovirus in this B-cell model by histone deacetylation, and the subsequent loss of viral gene expression, that is followed by a decline in viral DNA replication.

We previously identified a small number of cellular genes that are downregulated in B-cells persistently infected with adenovirus {Ornelles, 2016 #25}. Here, we found that expression of two of these genes, *SPARCLI* and *CXADR*, remained repressed after the virus was evicted by ETV6/RUNX1 (Table 3.3). These results indicate that epigenetic remodeling of the cellular genome by adenovirus persists in the absence of the virus. This epigenetic echo could be part of the mechanism that contributes to hit-and-run transformation. We previously reported that *SPARCLI* and *CXADR* are downregulated in cell lines that harbor translocations commonly associated with childhood leukemia; expression of these genes was de-repressed by inhibitors of repressive chromatin modifiers {Ornelles, 2016 #25}. Collectively, these data make it tempting to speculate that the leukemic cells under study were once infected with adenovirus and have acquired a persistent epigenetic signature that has been predicted to be a hallmark of cells transformed by a hit-and-run mechanism {Niller, 2011 #23}. A greater understanding of these changes in patterns of methylation and acetylation may inform the development of therapeutic and diagnostic targets for the treatment or detection of leukemia with a potential viral etiology.

Compelling evidence indicates that most chromosomal translocations found in pediatric leukemias occur before birth. This was suggested by the frequent concordance of clonally identical leukemia in monozygotic twins and confirmed by retrospective analysis of archived

neonatal blood samples of children who later developed leukemia {Greaves, 2005 #11; Greaves, 2003 #10; Teuffel, 2004 #35}. Most of the common translocations of pediatric leukemia have been identified in cord or neonatal blood samples {Smith, 2005 #29; Taub, 2004 #34}. Although chromosomal translocations are often the initiating event, they are usually not sufficient for full-blown malignancy. A genetic analysis of random cord blood samples found the ETV6/RUNX1 translocation in 1%, a frequency 100-fold greater than the frequency of overt leukemia with that fusion gene in the population at large {Mori, 2002 #22}, indicating that 99% of the time these expanded clones fail to develop into leukemia. If indeed a virus is responsible for creating these clones of translocation-containing cells, that virus must be present in at least 1% of otherwise normal newborns. By this criterion adenoviruses alone qualify. Studies looking by PCR for between 6 and 8 viruses in amniotic fluids from abnormal pregnancies found that the vast majority (77% and 84% and 64% and 77%) of virus-positive samples were adenovirus {Reddy, 2005 #27; Baschat, 2003 #2; Wenstrom, 1998 #37; Adams, 2012 #41}. In addition, while most studies suggest that the percentage of adenovirus-positive samples is larger among phenotypically abnormal pregnancies, adenovirus DNA is found in the amniotic fluid of 6% {Baschat, 2003 #3}, 5.4% {Baschat, 2003 #2}, 2.6% {Van den Veyver, 1998 #36}, and 5.1% {Miller, 2009 #21} of otherwise normal pregnancies as well. These numbers compare well with our finding of adenovirus DNA in 3.7% of cord blood samples from normal pregnancies {Ornelles, 2015 #26}.

Virtually 100% of children in the United States contain persistent group C adenoviruses in their tonsils by 4-years of age {Garnett, 2009 #9}. If adenoviruses are responsible for the development of translocation-containing clones of pre-malignant lymphoid cells, why isn't this phenomenon much more widespread? The answer may reside either in the unique route of

infection seen in the fetus, or in a unique progenitor cell target that is present during gestation but not after birth, or both. Infection in utero can occur from virus in the mother's lower genital tract or via a hematogenous route. Several studies have detected replication competent adenoviruses in the adult female genital tract {Kulcsar, 1975 #15; Parkin, 1999 #40; Swenson, 1995 #42}. The adenovirus cellular receptor CAR is expressed on both extravillous and villous syncytiotrophoblast cells in the first trimester and on extravillous trophoblast cells thereafter {Koi, 2001 #14}, providing a potential route for transplacental movement of the virus. In any case, the virus will be presented to the developing fetus as a blood-borne infection, a site entirely different from that of a small child exposed probably via a fecal-oral route and mucosal infection. Although the precise cell of origin of childhood acute leukemias has not been definitely identified, it is certainly possible that either the relevant cell is not exposed to the virus during normal mucosal infection or the relevant state of differentiation of the target cell is no longer present after the child is born. In either case, infection in utero with a common virus can lead to a rare outcome not encountered after the child is born.

4 NO EVIDENCE FOR INDIRECT MECHANISMS OF ADENOVIRUS EVICTION FROM RUNX1 LEUKEMIC FUSION PROTEIN EXPRESSING B CELL POPULATIONS: EXPLORATION OF CELL DEATH, NF-KB INHIBITION, AND INTERFERENCE WITH VIRAL E4ORF6 PROTEIN FUNCTION

The work in this chapter represents the results of experiments I performed as part of Aim 2 of my Dissertation Project: To determine if ETV6/RUNX1 inhibits Ad5 retention in lymphocytes via indirect methods that do not involve direct binding to viral DNA. Chronologically, these experiments were performed before the results of the experiments in Chapter 3 were apparent. Specifically, I explored if there was any evidence for perturbations in cell death/growth dynamics, inhibition of NF- κ B activity, or disruption in E4orf6 protein function that could contribute to the loss of virus from translocation positive B cells.

Results generated in this chapter were exploratory and remain unpublished. However, they may be useful as a starting point for those who choose to continue work in this area and were written and discussed with this purpose in mind.

4.1 ABSTRACT

In Chapter 3 we showed that the most common fusion protein found in childhood acute lymphoblastic leukemia, ETV6/RUNX1, binds directly to the adenovirus genome, which correlated with downregulation of viral genes and inhibition of viral persistence in B cells. RUNX1 is a transcription regulatory protein, but also interacts with many other proteins both inside and outside the nucleus, including cellular regulators of the NF- κ B pathway as well as adenovirus proteins E4ORF6 and E1B-55K. Fusion of RUNX1 to ETV6 and MTG8 changes many of the transcriptional and protein interaction functions of RUNX1, and confers higher proliferation rates to cells. We hypothesized that the activities of the ETV6/RUNX1 and RUNX1/MTG8 fusion genes could indirectly lead to adenovirus loss from a B cell population in a manner that does not involve direct binding to the genome as was found in Chapter 3. The impact of ETV6/RUNX1 and RUNX1/MTG8 expression on cell viability and growth, NF- κ B activation, and E4ORF6 interaction with RUNX1 were evaluated to determine if adenovirus could be lost from persistently infected B cells through indirect mechanisms that do not involve direct binding of the RUNX1 fusion genes to the genome. Overall, no evidence of such indirect mechanisms could be reliably demonstrated.

4.2 INTRODUCTION

In Chapter 2.11, we showed that expression of the ETV6/RUNX1 and RUNX1/MTG8 fusion genes inhibited adenovirus transcription and retention in B cells. We concluded that ETV6/RUNX1 inhibited adenovirus persistence by *directly* binding to the viral genome and shutting down transcription of viral genes via HDAC recruitment. However, the ETV6/RUNX1

and RUNX1/MTG8 leukemic fusion genes may inhibit adenovirus retention via indirect methods in addition to binding directly to the viral genome described in Chapter 2.11.

ETV6/RUNX1 and RUNX1/MTG8 have both been shown to be necessary for cell proliferation and survival, with inhibition of their functions leading to cell death and senescence (Martinez, Drescher et al. 2004, Hong, Gupta et al. 2008, Fuka, Kantner et al. 2012). While most studies explore the transcription factor properties of RUNX1 (Yamagata, Maki et al. 2005), this protein has also been shown modulate the NF- κ B pathway in the cytoplasm (Nakagawa, Shimabe et al. 2011) and direct adenovirus proteins to the nucleus (Marshall, Moore et al. 2008). All these traits of RUNX1 and the RUNX1 fusion genes represent possible alternative mechanisms that could *indirectly* inhibit viral retention in B lymphocytes instead of directly binding to the viral genome as shown in Chapter 2.11. This chapter summarizes the *exploratory* experiments conducted to discover other possible mechanisms of inhibition of viral retention in B cells by the RUNX1 fusion genes. The indirect mechanisms studied and the rationale behind their testing is summarized below.

First, the RUNX1 fusion proteins may interfere with normal cell growth and death (Martinez, Drescher et al. 2004, Hong, Gupta et al. 2008, Fuka, Kantner et al. 2012). Faster cell growth may result in a dilution of viral genome in a cell population if viral replication remains constant. If the RUNX1 fusion genes conferred any kind of faster cell growth and division, this may lead to loss of virus from that population. On the other hand, persistently infected cells expressing the RUNX1 fusion proteins may have higher rates of death due to some unknown mechanism of instability between adenovirus and the RUNX1 fusion genes. If the RUNX1 fusion genes sensitized cells to apoptosis or cell death, this would create a selective pressure to select uninfected cells, resulting in loss of virus from the RUNX1 fusion expressing population.

Two other indirect mechanisms that could inhibit viral persistence in lymphocytes would rely on RUNX1 fusion protein interference with the NF- κ B pathway. Cytoplasmic RUNX1 has been shown to directly interact with I κ B α -kinase (IKK), a kinase that frees active NF- κ B by phosphorylating I κ B α . This interaction between IKK and RUNX1 inhibited IKK's function and kept NF- κ B bound to I κ B α , thereby attenuating NF- κ B activation (Nakagawa, Shimabe et al. 2011). Another group observed that ETV6/RUNX1 fusion gene was *mostly* localized to the cytoplasm, while the unfused genes were not (Rho, Kim et al. 2002). This may mean that ETV6/RUNX1 binds to and inhibits IKK in the cytoplasm to a greater extent than RUNX1 alone, in turn also inhibiting downstream NF- κ B activity to a greater extent.

This possible interaction between the RUNX1 fusion proteins and NF- κ B may influence cell growth or viability as described above, but only occur during activation of the NF- κ B pathway. This presents a second possible mechanism that could indirectly inhibit viral persistence in lymphocytes: via NF- κ B controlled cell growth and viability. NF- κ B complexes can induce antiapoptotic or proapoptotic factors, depending on the context of the cellular environment. In T lymphocytes it was shown that PMA plus Ionomycin (P/I) treatment induced NF- κ B activity to upregulate Fas ligand and induced apoptosis, however NF- κ B also functioned in an anti-apoptotic manner by blocking apoptosis induced by glucocorticoid treatment in the same cell type {Lin, 1999 #3974}. The complex nature of NF- κ B activity is due in part to the various proteins (RelA, RelB, c-Rel, p100/p52, and p105/p50) that combine in homo- or heterodimers to form the NF- κ B complexes (Hayden and Ghosh 2012). If RUNX1 fusion genes interfere with activation of some NF- κ B complexes but not others, this could impact the viability or growth dynamics of fusion gene expressing cells by disrupting either pro- or anti-apoptotic inducing NF- κ B complexes. For example, inhibition of anti-apoptotic NF- κ B complexes by

RUNX1 fusion genes may allow uninfected cells to survive, but not persistently infected cells due to the additional stressors of infection, much like discussed above in this section regarding cell death causing viral loss from RUNX1 fusion gene expressing cells. However, unlike above, this mechanism would rely on activation of the NF- κ B pathways and only be present upon induction of those pathways.

Alternatively, apoptosis of persistently infected cells could also be due to dysregulation of viral control over the NF- κ B pathway. As part of the many ways adenovirus controls cell growth and death (discussed in Chapter 2.3, 2.5, and 2.6), adenovirus E1B 19KDA protein is known to inhibit generation of active NF- κ B complexes in the nucleus by targeting a common, but unidentified, upstream component (Limbourg, Stadtler et al. 1996, Schmitz, Indorf et al. 1996, Degenhardt, Perez et al. 2000). Because there are multiple different NF- κ B complexes with different gene targets and resulting functions (Hayden and Ghosh 2012), simultaneous interference of different NF- κ B complexes by both the RUNX1 fusion genes and adenovirus E1B 19KDA could feasibly lead to dysregulation of pro- or anti-apoptotic factors induced by NF- κ B complexes. This could result in higher cell death in persistently infected cells that express the RUNX1 fusion proteins, again creating a selective pressure against RUNX1 fusion expressing cells that continued to harbor adenovirus.

As a third possible mechanism, the RUNX1 fusion genes may interfere with adenovirus immune evasion mechanisms through the NF- κ B pathway. The virus E3 region expresses important immune evasion genes. The viral E3 14.7K protein retains MHCI in the cytoplasm, inhibiting viral peptide exposure to immune cells (Wold and Tollefson 1998). The E3 10.4K-14.5K proteins also protect persistently infected cells from immune induced apoptosis by down modulating Fas expression (Elsing and Burgert 1998, Liu, Govindarajan et al. 2000, Burgert,

Ruzsics et al. 2002). If the virus E3 region was dysregulated, this would not lead to direct loss of virus *in vitro*, but may allow the immune system to target virus infected cells *in vivo* leaving behind only the translocation positive cells that had lost the virus. Such a model was supported with a herpes hit and run system, in which a Cre recombinase expressing murine herpes virus was used to initiate pre-programed oncogenesis in a Cre-*lox* recombination mouse model. Most of the resulting induced cancers lacked herpes virus genome. This lack of virus seemed to be due to viral clearance by the immune system (Stevenson, May et al. 2010). Loss of E3 activity in adenovirus infected cells expressing the RUNX1 fusion proteins could occur in an NF- κ B dependent manner. During viral persistence in lymphocytes, expression of the E3 region is controlled by NF- κ B binding motifs (Williams, Garcia et al. 1990, Mahr, Boss et al. 2003). This indicates that continued persistence *in vivo* is regulated by NF- κ B, and if E3 were dysregulated it could expose the virus to the immune system and result in viral clearance. Additionally, the viral E2 region, which codes for viral proteins necessary for DNA replication, shares this promoter (Machitani, Sakurai et al. 2016) and thus may rely on NF- κ B activity. The role of the E2/E3 region and its relation to persistence in lymphocytes is studied more in Chapter 5. If NF- κ B activity were inhibited by RUNX1 fusion proteins, this may be a way that the RUNX1 fusion genes shut down adenovirus E3 expression in persistently infected lymphocytes and expose the virus infected cell to the immune system. This would pressure the cell progeny to lose the virus or die.

A final potential indirect mechanism is that adenovirus could be lost if RUNX1 fusion proteins interact with the adenovirus E4ORF6 protein and thereby inhibit late gene expression. The interaction of normal RUNX1 and E4ORF6 has previously been shown to allow E1B-55K localization to the cell nucleus, which is critical for adenovirus infection (Marshall, Moore et al.

2008), and the presence of the q terminus of human chromosome 21, which contains RUNX1, in infected mouse cells allowed for more viral late gene expression in mouse cells (Chastain-Moore, Roberts et al. 2003). Proper localization of E4ORF6 and E1B-55K proteins are needed for expression of viral late gene products (Halbert, Cutt et al. 1985, Huang and Hearing 1989, Sandler and Ketner 1989). Together, these data suggest that proper RUNX1 interaction with E4ORF6 may be necessary for viral late gene expression. The ETV6/RUNX1 fusion protein interacts with normal RUNX1 (Yamagata, Maki et al. 2005) and could interfere with normal RUNX1 activity during adenovirus infection. By mislocalizing RUNX1, and thereby E4ORF6 and E1B-55K, to the cytoplasm where ETV6/RUNX1 has been shown to accumulate (Rho, Kim et al. 2002), ETV6/RUNX1 may hinder the normal localization of E4ORF6 and E1B-55K. Because proper localization of E4ORF6 and E1B-55K proteins are needed for expression of viral late gene products (Halbert, Cutt et al. 1985, Huang and Hearing 1989, Sandler and Ketner 1989), this could inhibit viral persistence in lymphocytes. This would be a way that RUNX1 fusion genes could inhibit adenoviral retention in a cell population, without binding directly to the viral genome as a transcription factor.

The aim of this chapter was to determine whether mechanisms that did not involve direct binding of the RUNX1 fusion genes to the viral DNA could contribute to adenovirus loss from B-cells. We hypothesized that adenovirus could be 'kicked out' of lymphocytic cells due to RUNX1 fusion protein induced changes in cell growth or death, RUNX1 fusion gene induced NF- κ B dysregulation of apoptotic genes and resulting sensitization to apoptosis, RUNX1 fusion protein inhibition of NF- κ B regulation of the adenovirus E3 region, or inhibition E4orf6 activity. In our studies, we found no evidence to support such indirect mechanisms of adenovirus eviction from B lymphocytes. Cell growth and viability remained the same between cells with empty

vector and those expressing the RUNX1 fusion genes. There was some indication that activity was affected in RUNX1 fusion gene expressing cells. NF- κ B upregulation of I κ B α mRNA was inhibited in the RUNX fusion gene expressing cells in some experiments, however, there was no effect seen in viability after NF- κ B stimulation, and results of I κ B α transcription after NF- κ B induction were small and varied between repeats. Unfortunately, no conclusions could be drawn related to RUNX1 fusion protein interactions with the adenovirus E4ORF6 or normal RUNX1 due to technical failure to detect the E4ORF6 protein by immunoblotting. E4ORF6 protein could not be detected in infected B cells, nor positive control infected epithelial cells, despite previous reports of detection using the same hybridoma supernatant (Marshall, Moore et al. 2008). Thus it remains unclear if this mechanism is at work in infected B cells that harbor RUNX1 leukemic fusions genes.

4.3 MATERIALS AND METHODS

Cell lines

The human cell lines A549 (lung carcinoma) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). BJAB (EBV-negative Burkitt's lymphoma) cells were obtained from ATCC {Klein, 1974 #2857}. BJAB were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10 mM glutamine. A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 μ g of glucose/ml, 10% FCS, and 10 mM glutamine.

Creation of stable cell lines

The original expression plasmids for the *ETV6/RUNX1* and *RUNX1/MTG8* were kindly provided by S. Hiebert (Vanderbilt University) and were subcloned into pTARGET plasmid (Promega) using EcoRI restriction enzyme. BJAB cells were transfected using electroporation as described in Mchichi *et al* (El Mchichi, Hadji et al. 2007), with minor modifications. Briefly, 4×10^6 cells in cold serum-free RPMI were transfected with 2 μ g of plasmid by electroporation at 120V, 960 μ F using cuvettes with an inner width of 2mm. After 20min of recovery at 37°C, cells were plated into 6-well plates in RPMI supplemented with 10% FBS at 37°C for 48hrs. Cell cultures were then supplemented with 1mg/ml of G-418 (ThermoFisher Scientific) for at least 21 days before use in experiments. G-418 was maintained at 0.5mg/ml throughout experiments to maintain stably transfected cell lines.

Adenoviruses

Phenotypically wild-type mutant virus, Ad5dl309, was obtained from Tom Shenk (Princeton University, Princeton, NJ (Attarbaschi, Mann et al. 2007, Arbogast, Raveau et al. 2015). Ad5dl309 is an Ad5 mutant that lacks the genes for E3 RID α and RID β proteins as well as the 14,700-molecular-weight protein (14.7K protein).

Infection of lymphocytes with adenovirus

Infection of lymphocyte cell lines with adenovirus was performed as described previously (Flomenberg, Gutierrez et al. 1997) with minor modifications. Lymphocytes were collected and washed in serum-free (SF) RPMI medium. Cell density was adjusted to 10^7 cells/ml in SF-RPMI medium. Virus was added to the cell suspension at 50 PFU/cell, spun for 45min at 1000G at 25°C, resuspended by agitation. Cells were then incubated at 37°C for 1.5hrs

with gently flicking every 30min. The infected cells were washed three times with RPMI complete medium and then resuspended in RPMI complete medium at 5×10^5 cells/ml. Cell concentration and viability were monitored throughout the infection.

Reverse transcription and quantitative PCR analysis of viral and cellular mRNA levels

RT-qPCR was performed as described previously (Chroboczek, Bieber et al. 1992), with minor modifications. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen Inc. Valencia, CA). RNA was treated with Rnase-free DNase (Qiagen) on isolation columns and quantified. Greater than 100ng were reverse transcribed (RT) into cDNA, in 20 μ L reaction volumes, using Maxima First Strand cDNA Synthesis Kit (ThermoScientific). RT-enzyme negative controls were included for each reaction. Primers and probes were obtained from Integrated DNA Technologies (Coralville, IA). Viral primer and probe sequences are reported in our previous publications (Garnett, Talekar et al. 2009, Murali, Ornelles et al. 2014). I κ B α and GAPDH primers for SYBR green RT-qPCR are described in {Bottero, 2003 #5106}. Probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) reporter molecule and contained dual ZEN and Iowa Black quenchers. Each sample was run in duplicate with at least 2 experimental repeats for each virus tested. All analyses were performed via the comparative threshold cycle (Ct) method as previously described (Chroboczek, Bieber et al. 1992). Target Cts were normalized to the EIF1 housekeeping gene, and set relative to the lowest sample, which was set to 1. In some experiments, HDAC inhibition was performed by treating cells with 300nM Trichostatin A (TSA) followed by quantifying transcript levels (Ornelles, Gooding et al. 2016).

Flow cytometry

Surface staining for FasR (CD95) occurred as described in {McNees, 2002 #5107}. Cells were subsequently stained with a goat anti-mouse IgG-APC conjugated secondary antibody (Life Technologies). Results were analyzed on a LSR Fortessa flow cytometer using FACSDiva Software (BD Biosciences). Isotype control staining was used to define the FasR-positive staining cells and was 5% or less for all samples evaluated.

Immunoblots for protein detection

A total of $4-6 \times 10^6$ cells were collected and washed in cold PBS, the cell pellet was resuspended in 1ml of cold RIPA buffer (R0278, Sigma) supplemented with 1mM EDTA (161-0729, BioRad), protease/phosphatase inhibitor (1861281, Thermo Scientific), and incubated on ice for 30min. Samples were then sonicated briefly, and boiled for 5min with equal amounts of 2X Laemmli sample buffer (161-0737, Bio-Rad) before being run on a SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose membrane (BioRad). Immunoblotting of E4ORF6 was performed by using primary anti-E4ORF6 hybridoma supernatant as described in (Marshall, Moore et al. 2008) and secondary goat anti-rabbit IgG-HRP antibodies (sc-2004, Santa Cruz Biotechnology). Primary mouse antibody to actin (MAB1501, Chemicon) and secondary donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz Biotechnology) was blotted in parallel as a protein loading control. Proteins were visualized with HyGLO Reagent A/B (E2500, Denville Scientific Inc) according to the manufacturer's instructions.

PMA/Ionomycin treatments

Lymphocytes were cultured either alone or with phorbol myristate acetate (PMA) (50 ng per ml; Sigma) and ionomycin (1 M; Calbiochem) for 48 h as described before (Garnett, Talekar et al. 2009) and samples were tested for adenovirus RNA by RT-qPCR as described above.

4.4 RESULTS

The presence of RUNX1 fusion genes does not affect BJAB cell growth or viability of either uninfected or infected cultures.

Death of RUNX1 fusion expressing cells harboring adenovirus would create a selective pressure against retaining the virus, and may be seen in lower viability of cells expressing the RUNX1 fusion proteins. On the other hand, if cells harboring the RUNX1 fusion genes grew more quickly than empty vector cells, this could dilute adenovirus from RUNX1 fusion expressing cells if the virus replication remained constant. This effect would be seen in higher growth rates of cells expressing the RUNX1 fusion proteins. To determine if the RUNX1 fusion genes had any impact on cell growth or viability, growth curves of cells expressing the RUNX1 fusion proteins or empty vector were evaluated. Infected cells were seeded at $1-5 \times 10^5$ cells/ml and allowed to grow for the indicated number of days. Samples were taken daily and both total cell numbers and viability were measured. Figure 4.1 shows the growth curve of BJAB cells, 28 days post infection, that expressed either the empty vector, ETV6/RUNX1, or RUNX1/MTG8. The growth rates during the log phase of growth were plotted and the slope was calculated for each culture. The RUNX1/MTG8 expressing culture did not reach the same concentration in this particular experiment. However, the growth rate during log phase remained similar to the other cultures, and in other experiments (Figure 4.2, Figure 4.3) this cell line reached the same concentration as the other cultures. The Figure 4.2 are replicate results but at 40 days post

infection. Similar results were obtained in uninfected cells (Figure 4.3). No difference in growth rate or viability was seen between empty vector, ETV6/RUNX1, or RUNX1/MTG8 containing samples. The cultures had different growth rates between experiments highlighting some of the variability between infections as well as uninfected cells. In Chapter 2.11, cell concentration and viability in BJAB transfect cultures was measured during feeding and sampling during infections, which lasted up to 70 days (data not shown). These measurements did not show any obvious differences during persistent infection either, supporting the results of the growth curves in this chapter. Overall, there was no significant effect on growth or viability of BJAB cells by the RUNX1 fusion proteins suggesting that these factors do not account for the loss of virus from cells expressing the RUNX1 fusion proteins.

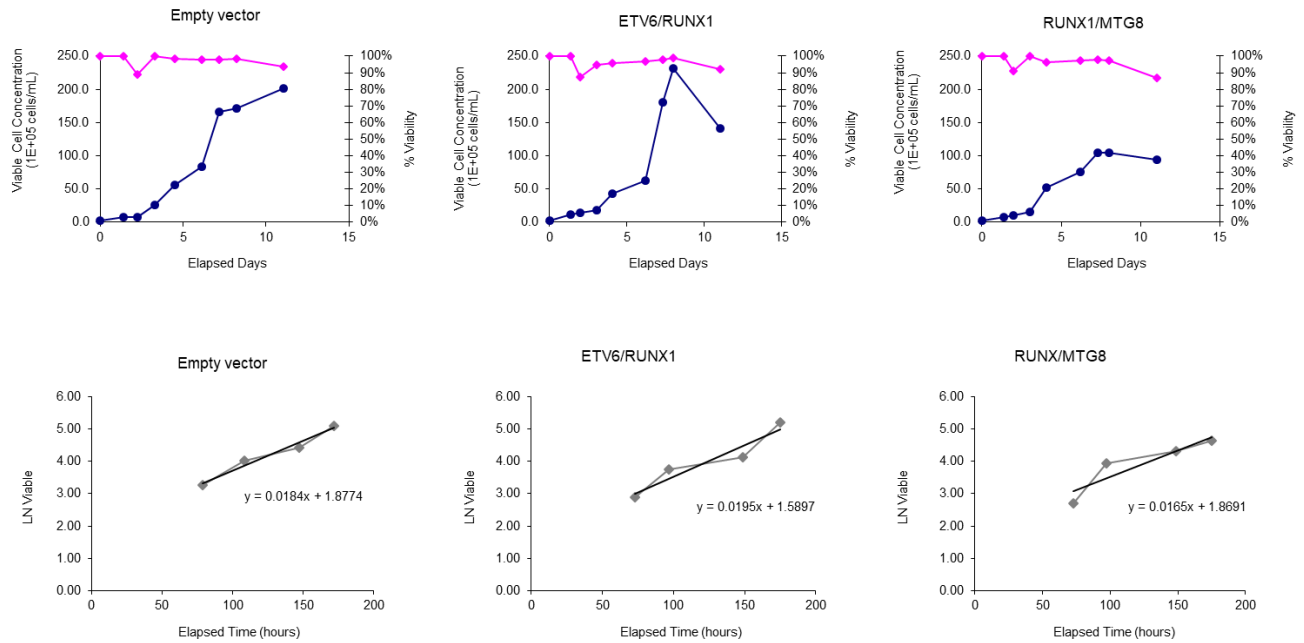


Figure 4.1 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of adenovirus dl309 persistently infected B lymphocytic cells (day 28 post infection). A persistent infection of adenovirus dl309 was established in BJAB cells stably transduced with the empty vector or expression vectors for the indicated leukemic fusion genes. At 28 days post infection, cell cultures were established at 1×10^5 cells per ml. The number of viable and inviable cells was determined daily for 12 days without supplementing the growth medium. Viable cell concentration (top graphs, blue circles) and percent viability (pink diamonds) are plotted by day post seeding. Log phase growth rates (bottom graphs, gray diamonds) were calculated by natural log calculation for the values between 3-7 days.

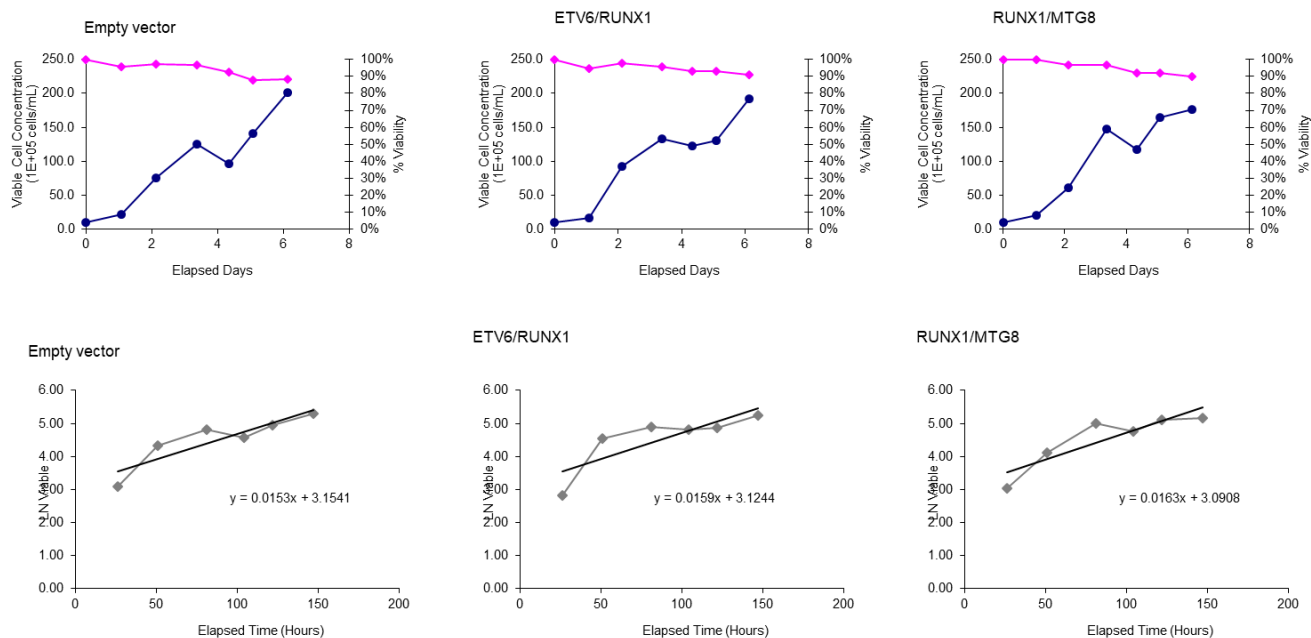


Figure 4.2 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of adenovirus dl309 persistently infected B lymphocytic cells (day 40 post infection). A persistent infection of adenovirus dl309 was established in BJAB cells stably transduced with the empty vector or expression vectors for the indicated leukemic fusion genes. At 40 days post infection, cell cultures were established at 1×10^5 cells per ml. The number of viable and nonviable cells was determined daily for 7 days without supplementing the growth medium. Viable cell concentration (top figures, blue circles) and percent viability (pink diamonds) are plotted by day post seeding. Log phase growth rates (bottom graphs, gray diamonds) were calculated by natural log calculation for the values between 1-7 days.

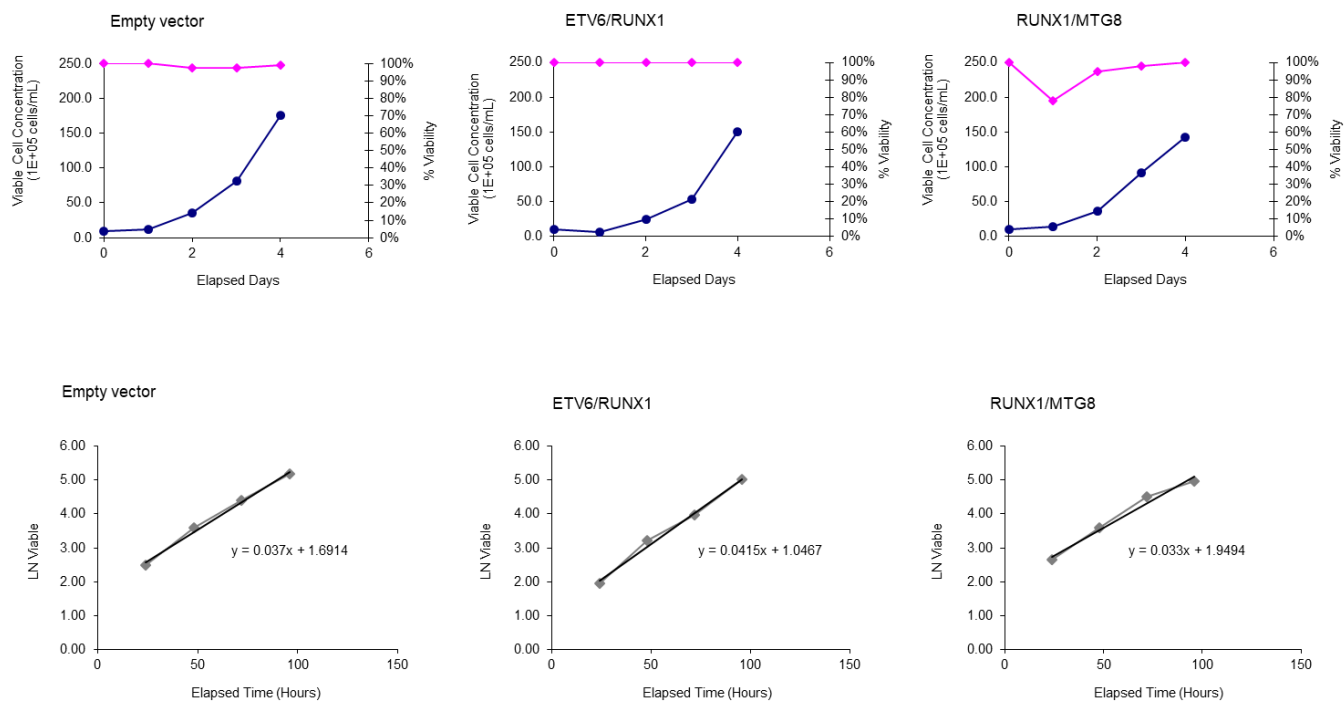


Figure 4.3 Stable expression of ETV6/RUNX1 or RUNX1/MTG8 does not affect the growth of uninfected B lymphocytic cells.

Cell cultures of BJAB cells stably transduced with the empty vector or expression vectors for the indicated leukemic fusion genes were established at 1×10^5 cells per ml. The number of viable and inviable cells was determined daily for 12 days without supplementing the growth medium. Viable cell concentration (top figures, blue circles) and percent viability (pink diamonds) are plotted by day post seeding. Log phase growth rates (bottom graphs, gray diamonds) were calculated by natural log calculation for the values between 3-10 days.

ETV6/RUNX1 and RUNX1/MTG8 leukemic fusion proteins have no effect on NF- κ B mediated apoptosis in BJAB cells.

Another indirect mechanism of viral loss due to RUNX1 fusion gene expression could be via interference with NF- κ B pathways. As described above, NF- κ B can induce anti-apoptotic or pro-apoptotic factors in the same cell type (Lin, Williams-Skipp et al. 1999) due to the multiple proteins that comprise NF- κ B complexes and their activation by either the canonical pathways or non-canonical pathways (Hayden and Ghosh 2012). Adenovirus inhibits NF- κ B activation to avoid cell apoptosis, but exactly which complexes it inhibits and how is unknown (how is this known then?). We reasoned that if the RUNX1 fusion genes interfere with the ability of the virus to inhibit NF- κ B activation, this may decrease viability as compared to empty vector containing cells because of dysregulation of some NF- κ B complexes in persistently infected cells {Lin, 1999 #3974}. If this was true, it could decrease cell viability in persistently infected cells by interfering with normal adenovirus control of apoptosis via the NF- κ B pathway (Limbourg, Stadtler et al. 1996, Schmitz, Indorf et al. 1996, Degenhardt, Perez et al. 2000), which would induce selective pressure for RUNX1 fusion expressing cells to lose the virus. In BJAB cells, the NF- κ B pathway is relatively inactive, but can be stimulated with PMA plus ionomycin (P/I) treatment (Lam, Davis et al. 2008). We tried to test if NF- κ B stimulation of Ad5wt infected BJAB cells with P/I treatment decreased viability or cell number in RUNX1 fusion gene expressing cells. Uninfected cells were included as a control to determine the contribution of the virus on NF- κ B activity. There was no discernable change in viability or cell number of BJAB cells after P/I treatment between the RUNX1 fusion gene expressing cultures and the empty vector control (Table 4.1, Table 4.2, Table 4.3). Initial cell concentrations varied with each

experiment (2E5 viable cells/ml for Table 4.1 or 1E6 viable cells/ml for Table 4.2, Table 4.3), but this had no effect on viability after 24hrs of P/I treatment, which remained unchanged. Inclusion of an untransfected control indicated that the vector itself had no influence either (Table 4.2). Strangely, most cultures showed a decrease in live cell concentration with P/I treatment, for which the viability failed to account for. This indicates that the cells may have died earlier and are no longer detectable after 24hrs. However, evaluation of samples at an earlier time point (4 hours) also failed to show a decrease in viability, suggesting that the cells simply didn't grow following treatment (data not shown).

Table 4.1 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, low cell concentration.

BJAB cells stably transfected with empty vector or the RUNX1 fusion genes were treated with PMA/Ionomycin for 24 hrs (bottom row), and then assayed for cell concentration (live cells/ml) and percent viability.

Leukemic fusion gene:	Empty vector		ETV6/RUNX1		RUNX1/MTG8	
none	3.30x10 ⁵	92%	3.10x10 ⁵	86%	3.30x10 ⁵	85%
<i>PMA/Ionomycin</i>	4.20x10 ⁵	90%	2.90x10 ⁵	98%	3.00x10 ⁵	92%

Table 4.2 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, high cell concentration.

BJAB cells stably transfected with empty vector or the RUNX1 fusion genes were treated with PMA/Ionomycin for 24 hrs (bottom row), and then assayed for cell concentration (live cells/ml) and percent viability.

Leukemic fusion gene:	No vector		Empty vector		ETV6/RUNX1		RUNX1/MTG8	
none	2.05x10 ⁶	96%	2.05x10 ⁶	98%	1.89x10 ⁶	90%	1.82x10 ⁶	95%
<i>PMA/Ionomycin</i>	1.30x10 ⁶	94%	1.09x10 ⁶	94%	1.57x10 ⁶	96%	1.28x10 ⁶	97%

Table 4.3 Cell concentration and viability 24hrs after PMA/Ionomycin stimulation, Ad5wt infected cells.

BJAB cells stably transfected with empty vector or the RUNX1 fusion genes and infected with Ad5wt (40 days post infection) were treated with PMA/Ionomycin for 24 hrs (bottom row), and then assayed for cell concentration (live cells/ml) and percent viability.

Leukemic fusion gene:	Empty vector, Ad5wt		ETV6/RUNX1, Ad5wt		RUNX1/MTG8, Ad5wt	
none	1.30x10 ⁶	98%	1.10x10 ⁶	98%	9.30x10 ⁵	95%
<i>PMA/Ionomycin</i>	5.90x10 ⁵	96%	6.70x10 ⁵	96%	5.90x10 ⁵	94%

ETV6/RUNX1 and RUNX1/MTG8 leukemic fusion proteins may reduce I κ B α expression in NF- κ B stimulated cells

While there was no evidence that the RUNX1 fusion genes disrupted NF- κ B induced apoptosis in infected nor uninfected cells (Table 4.1, Table 4.2, Table 4.3), this did not necessarily mean that no NF- κ B pathways were disrupted at all, since multiple pathways and NF- κ B complexes exist (Hayden and Ghosh 2012). If the leukemic RUNX1 fusion genes interfere with normal NF- κ B signaling, this may in turn disrupt normal viral gene expression, especially from the E2/E3 region. As discussed above, these regions code for genes needed for viral replication and immune evasion, making them critical for persistence in lymphocytes. Their loss via RUNX1 fusion gene interference with NF- κ B pathways would both inhibit viral E2 expression and DNA replication in lymphocytes, as was seen in Chapter 3 (Figure 3.5, Figure 3.7, Figure 3.10), and force a selective pressure against RUNX1 fusion expressing cells that still retained the virus *in vivo* by inhibiting E3 expression.

I κ B α protein inhibits NF- κ B activity and P/I induced activation of NF- κ B has been shown to upregulate I κ B α mRNA {Brown, 1993 #4867}. If the RUNX1 leukemic fusion genes interfere with normal NF- κ B activation, we reasoned that I κ B α mRNA upregulation following P/I treatment would be inhibited in cultures expressing the RUNX1 fusion genes. BJAB cells expressing empty vector, ETV6/RUNX1 or RUNX1/MTG8 were treated with P/I for 1hr and then assayed for I κ B α mRNA levels. Two separate experiments showed that BJAB cells expressing the RUNX1 fusion genes exhibited lower levels of I κ B α mRNA after P/I treatment than the empty vector control samples (Table 4.4). However, the experimental repeats displayed considerable variability in that I κ B α mRNA levels were detectable after P/I treatment in the RUNX1 fusion cells in the second experiment whereas they became undetected in the first

experiment. Regardless, the presence of the RUNX1 fusion genes did appear to result in lower I κ B α mRNA levels following P/I stimulation as compared to fusion gene negative samples. There did not appear to be an impact of RUNX1 fusion genes on basal levels of I κ B α mRNA. These results suggest that the RUNX1 fusion genes may disrupt normal NF- κ B activation, but further investigation needs to occur to draw any conclusive results. If true, this would mean that the RUNX1 fusion genes could also *indirectly* inhibit viral persistence in B cells by inhibiting activation of NF- κ B complexes needed for activation of viral genes of the E2/E3 region.

Table 4.4 I κ B mRNA transcription after PMA/Ionomycin stimulation.

BJAB cells stably transfected with empty vector or the RUNX1 fusion genes were treated with PMA/Ionomycin for 1 hr, and then sampled for total mRNA. All samples were compared to empty vector untreated. Untreated cells displayed little variability between empty vector and RUNX1 fusion cultures. Empty vector cells treated with PMA/Ionomycin increased I κ B mRNA transcription, while RUNX1 fusion cells I κ B mRNA could not be detected or did not increase as much.

Leukemic fusion gene:	Empty vector	ETV6/RUNX1	RUNX1/MTG8
Experiment 1			
none	1.0	1.7	1.2
<i>PMA/Ionomycin</i>	3.5	undetected	undetected
Experiment 2			
none	1.0	0.9	1.3
<i>PMA/Ionomycin</i>	3.0	2.1	2.4

The E4orf6 protein was not detected in infected BJAB cells

Previous research has shown that localization of the adenovirus protein complex of E1B-55k by E4ORF6 to the nucleus seemed to depend on the interaction between normal human RUNX1 with adenovirus E4ORF6 in infected mouse fibroblast cells (Marshall, Moore et al. 2008). The E1B-55K and E4ORF6 complex regulates late adenovirus mRNA nuclear export {Babiss, 1985 #5010;Halbert, 1985 #4369;Pilder, 1986 #3601}, and the localization of E4ORF6 by RUNX1 may be necessary for viral late gene expression (Chastain-Moore, Roberts et al. 2003).

The RUNX1 fusion proteins still retain the runt domain, which allows dimerization with normal RUNX1 protein (Yamagata, Maki et al. 2005). If the RUNX1 fusion proteins interacted with E4ORF6 either directly or through normal RUNX1, they could mislocalize or disable it. This could inhibit viral late gene expression and lead to loss of virus from cells expressing the RUNX1 fusion proteins. Such a loss of viral late gene transcripts was shown in Chapter 2.11 (Figure 3.4). To investigate whether the RUNX1 fusion proteins could inhibit viral late gene expression by interacting with E4ORF6, we attempted to co-immunoprecipitate E4ORF6 with the RUNX1 fusion proteins in persistently infected lymphocytes to assess whether mislocalization of E4ORF6 via the RUNX1 fusion proteins was possible.

Unfortunately, E4orf6 protein could not be detected during initial or persistent adenovirus infection in BJAB cell transfects even though actin was easily detected in these samples (data not shown). However, E4ORF6 protein detection also failed repeatedly in lytically infected epithelial cells revealing a technical difficulty in detection of this viral protein. In contrast, viral E2A DNA binding protein was easily detected via immunoblot in persistently infected lymphocytes up to

day 34 post infection (Figure 3.10), again suggesting that failure to detect E4ORF6 was specific to that protein and/or antibody. Further consideration revealed that failure to detect E4ORF6 was likely the result of crosslinking between E4ORF6 protein molecules (Ornelles, 2016 #3648 and personal communication). During immunoblot sample generation, samples are treated with a denaturation buffer at high temperature (95°C). This may cause cysteine residues to crosslink between E4ORF6 molecules, resulting in structures far too large to be pulled through an SDS-PAGE gel. Subsequent work should lyse cells and denature proteins at no more than 65°C.

4.5 DISCUSSION

Because of the multiple functions of the RUNX1 protein, Chapter 4 focused on indirect mechanisms through which the RUNX1 fusion genes could inhibit adenovirus retention in B cells. These mechanisms would not be the result of direct binding to the viral genome that was demonstrated in Chapter 3. No difference in cell growth or viability was detected between empty vector and RUNX1 fusion cultures, suggesting that dilution through faster cell growth or death of infected cells do not account for the loss of adenovirus from RUNX1 fusion gene expressing cultures (Figure 4.1, Figure 4.2, Figure 4.3).

RUNX1 has been shown to bind to and inhibit IKK, effectively regulating NF- κ B activation by inhibiting its activation. We reasoned that the RUNX1 fusion proteins may inhibit NF- κ B to a greater extent than normal RUNX1, interfering with normal regulation of NF- κ B

induced pro- and anti-apoptotic factors. Such interference could sensitize RUNX1 fusion expressing cells to apoptosis, causing cells with additional stressors like persistent adenovirus infection to die more rapidly upon NF- κ B stimulation. However, no change was seen in viability of RUNX1 fusion expressing cells treated with P/I to induce NF- κ B activity (Table 4.1, Table 4.2, Table 4.3).

Even though no change in viability was seen, RUNX1 fusion protein interference with IKK may still influence NF- κ B activity. The I κ B α gene, a target of NF- κ B, showed lower transcriptional activity after NF- κ B stimulation by P/I treatment in RUNX1 fusion gene expressing B cells than empty vector cells in two separate experiments (Table 4.4). This indicates that the RUNX1 fusion genes interfere with NF- κ B activation, but further work is needed to confirm these results. If true, this means that the RUNX1 fusion proteins may inhibit proper activation of the adenovirus E2/E3 region indirectly by inhibiting NF- κ B activity.

Last, we reasoned that the RUNX1 fusion genes may interact with the viral E4ORF6 protein and misregulate its function, inhibiting viral late gene expression as was seen in Chapter 2.11. However, the E4orf6 adenovirus protein could not be detected in BJAB or A549 cultures in our experiments, despite previous publications using the same hybridoma supernatant (**Error! Reference source not found.****Error! Reference source not found.**) (Marshall, Moore et al. 2008).

In summary, our data do not provide strong evidence that the indirect mechanisms investigated in this chapter inhibit adenovirus retention in leukemic RUNX1 fusion gene expressing B cells. Future work in this area could expand on the positive results of the P/I induced I κ B α transcription experiments. The low increase in I κ B α mRNA may have led to the variability between repeats. Other studies showed that P/I treatment of BJAB cells resulted in the

10-fold increase of CD83 expression, another NF- κ B target gene (Lam, Davis et al. 2008). The greater increase in gene expression of CD83 would hopefully decrease variability between experiments, as was seen with I κ B α transcription. Additionally, the technical failure to detect E4ORF6 protein by immunoblot was likely due to crosslinking between proteins due to high temperature during sample processing (**Error! Reference source not found.**). Viral early E2A DNA binding protein was expressed until at least 34 days post initial infection with Ad5wt in BJAB cells in Chapter 2.11 (Figure 3.10). The chromatin immunoprecipitation experiment in Chapter 2.11 (Figure 3.9) showed that ETV6/RUNX1 binds to the viral genome, but normal RUNX1 did *not*. This could indicate that endogenous RUNX1 is being prevented from binding to the viral genome by a protein it interacts with like E4ORF6. Marshall *et al* found that RUNX1 proteins were localized to regions around the *periphery* of viral replication centers, but did not actually associate with them during infection (Marshall, Moore et al. 2008), supporting the idea that viral proteins interact with normal RUNX1 and prevent its association with the viral genome. It seems fusion of ETV6 to RUNX1 inhibits adenovirus's ability to keep RUNX1 from binding to its genome. Perhaps RUNX1 binding to the viral genome inhibits gene expression, whether or not RUNX1 is fused to ETV6/RUNX1. Further studies using co-immunoprecipitation of RUNX1 and RUNX1 fusion proteins with viral proteins it associates with may elude such interactions with RUNX1, or lack of them with ETV6/RUNX1.

Overall, the work in this chapter indicates that there *may* be some inhibition of NF- κ B activity by the RUNX1 fusion genes (Table 4.4), but further research is necessary to demonstrate that this has any relation to adenovirus infection or inhibition of adenovirus retention in B cells. There does not seem to be any differences in viability conferred by the RUNX1 fusion proteins in adenovirus infected cells, either during normal growth or when NF- κ B activity is stimulated.

Future research in this area should concentrate on NF- κ B activity effects on adenovirus transcriptional activity, particularly from the E2/E3 regions, and RUNX1 fusion gene interaction, or lack thereof, with adenovirus proteins.

5 THE ROLE OF ADENOVIRUS E3 REGION IN CONTROLLING VIRAL LATENCY IN LYMPHOCYTES

The work in this chapter represents the results of experiments I performed as part of Aim 3 of my Dissertation Project: To demonstrate the role of the E3 protein, Adenovirus Death Protein (ADP), in control of latent vs lytic infection in lymphocyte cells, and how differences in E3 promoter regulation by NF- κ B may control infection states.

Some of the results generated in this chapter related to ADP resulted in an authorship on a manuscript published in The Journal of Virology (Murali, Ornelles et al. 2014). Other results are from *exploratory* experiments examining infection patterns between adenovirus type 2 and 5 (which differ most significantly in their E3 region), and these findings remain unpublished. As with Chapter 4, the experiments and results were written in such a way that they may prove useful as a starting point for anyone who wishes to continue work in this area.

5.1 ABSTRACT

Adenovirus undergoes latent infection in lymphocytes infected naturally *in vivo*. However, adenovirus latency in lymphocytes is still poorly understood. While some lymphocyte cell lines undergo lytic infection, others support a long-term persistent infection which retains virus for hundreds of days. Viral ADP is expressed late during lytic infections, facilitating cell death and cell lysis. The work in this chapter helped demonstrate that ADP expression is required for lytic infection in lymphocytes. The work done in this chapter contributed to a published article on which I am an author (Murali, Ornelles et al. 2014). There also appears to be serotype dependent differences that contribute to establishment of lytic versus persistent infections. Specifically, adenovirus type 2 (Ad2) does not undergo lytic infection in Jurkat cells while adenovirus type 5 (Ad5) does. Moreover, the expression patterns of the late structural protein, hexon, differ between infections with Ad2 versus Ad5. We reasoned that the differences between the E3 regions in Ad2 and Ad5 may point to mechanisms that regulate latent vs lytic infections in lymphocytes. Because Ad2 and Ad5 differ primarily in their E3 regulatory region, where NF- κ B transcription factor binding sites are located, we hypothesized that the observed differences between these two serotypes may be indicative of the importance of the E3 regulatory region and NF- κ B regulation of this region to establish latent infections.

5.2 INTRODUCTION

Understanding the mechanisms that control latent infection could relate to mechanisms of adenovirus loss in cancerous cells transformed in a hit-and-run manner. Since the virus remains episomal in infected cells, mechanisms that too strongly repress viral expression and replication could lead to subsequent loss of latent/persistent virus from the cellular population. Mechanisms

that control latent adenovirus infection or reactivation to lytic infection remain elusive. Adenovirus death protein (ADP) is expressed during late lytic infection of adenovirus species C and is responsible for cell lysis, facilitating the release and spread of virus progeny (Tollefson, Ryerse et al. 1996). ADP is found in the E3 region of adenovirus and is known to be expressed through the adenovirus major late promoter (MLP) late in infection {Tollefson, 1992 #2862}. Because ADP lysis forces host cells to release the virus, we reasoned that high ADP expression would be inhibited during viral persistence but would be needed for lytic infections in lymphocytes. The initial experiments performed under this chapter support this hypothesis, which contributed to a publication (Murali, Ornelles et al. 2014).

We, and others, also noted differences in lymphocytes infected with species C adenovirus type 2 (Ad2) versus adenovirus type 5 (Ad5) ((Markel, Lam et al. 2014) and unpublished observations). Notably, while Ad2 forms persistent infections in the Jurkat T cell line in which cells continue to proliferate, infection with Ad5 leads to a lytic infection. While Ad2 and Ad5 share a high degree of homology, they differ the most in the *hexon* region and the *E3* region (Figure 5.1). The *hexon* coding region would be expected, as this forms the capsid of the two different serotypes, which are exposed to the host immune system (and would therefore induce distinct neutralizing antibodies which classify the serotypes). The *E3* region however was not expected, particularly since most of the differences are in non-coding regions (Figure 5.2). Curiously, Ad5 has more NF- κ B sites than Ad2 within the *E3* promoter region, which may allow differences in regulation of that region (Ad5 accession number AC_000008.1, Ad2 accession number AC_000007.1) {Brister, 2015 #731;Coordinators, 2016 #2861}. Additionally, the ADP gene, which we found is expressed at high amounts in lytic infections ((Murali, Ornelles et al. 2014) and the results below), is contained within the *E3* region. We reasoned that the differences

in infection dynamics between Ad2 and Ad5 in lymphocytes could make these suitable candidates for studying latency in lymphocytes. Because of the unique regulation of the E3 region in lymphocytes, we hypothesized that differences in regulation of E3 genes by NF- κ B between Ad2 and Ad5 may control whether lytic infection occurs in lymphocytes. It may also be possible that activation of this region may be linked to the ADP gene expression, which is contained within this region. If true, studying the infection kinetics between Ad2 and Ad5 and activation of the E3 region could help shed light on E3 or ADP-dependent mechanisms of reactivation of the virus in latently infected cells.

The adenovirus E3 region contains genes necessary for control of inflammatory mechanisms and immune system evasion (Burgert, Ruzsics et al. 2002, Sharma and Andersson 2009). The E3 region seems to be controlled by NF- κ B in lymphocytes, and may be independent of E1A transcriptional control. Though E1A is classically thought of as the master regulator of virus transactivation, the adenovirus E3 promoter has NF- κ B binding sites and has been shown to be heavily upregulated by NF- κ B activation (Mahr, Boss et al. 2003, Machitani, Sakurai et al. 2016). Indeed, one study showed that E1A and NF- κ B mediated transcription of E3 may compete with each other {Mahr, 2003 #3882}. Alone, E1A also *mildly* upregulates E3 transcription, but reduces transcription from NF- κ B activation in a dose-dependent manner when both transcription factors are present in luciferase assays studying the E3 promoter. More recently, Machitani et al., reported that NF- κ B promotes leaky transcription of *all* viral genes, but *most strongly* from the E2/E3 promoter where NF- κ B was shown to bind {Machitani, 2016 #2859}. Studies treating naturally infected lymphocytes from donors with agents known to activate the NF- κ B pathway also suggest that NF- κ B activation may play a role in viral activation by initiating viral gene transcription. PMA plus ionomycin (P/I) stimulation of

primary T lymphocytes harboring natural latent adenovirus infections induced transcription of viral genes (Garnett, Talekar et al. 2009). This same study saw that when P/I, IL-2, and antibodies to CD3 and CD28, were used to stimulate infected primary lymphocyte cells that infectious virus was produced (Garnett, Talekar et al. 2009). Many of these molecules can activate the NF- κ B pathway, making it plausible that NF- κ B activation could influence viral activation by initiating viral gene transcription. The NF- κ B binding sites in the E3 promoter may allow for independent control of the E3 region while other viral genes remain silent. Such a mechanism would be biologically relevant and allow for immune evasion during latency, since the E3 region harbors viral immune evasion genes (Burgert, Ruzsics et al. 2002, Sharma and Andersson 2009). This scenario would also fit with true definitions of latency where only genes absolutely necessary to maintain that state are expressed while other viral genes remain silent until full viral reactivation.

The aim of this chapter was to 1) characterize the role of ADP expression in lytic versus latent infections, 2) determine if differences in E3 region regulation between Ad2 and Ad5 could contribute to the different infection patterns observed, and explore if E3 region regulation by NF- κ B controlled these differences in infection dynamics. Hypothesis 1) was that high ADP expression would correlate with lytic infection, and that no ADP expression would result in persistent infection. Hypothesis 2) was that expression of the viral E3 region depended on NF- κ B activation and could facilitate induction of lytic infection from latency.

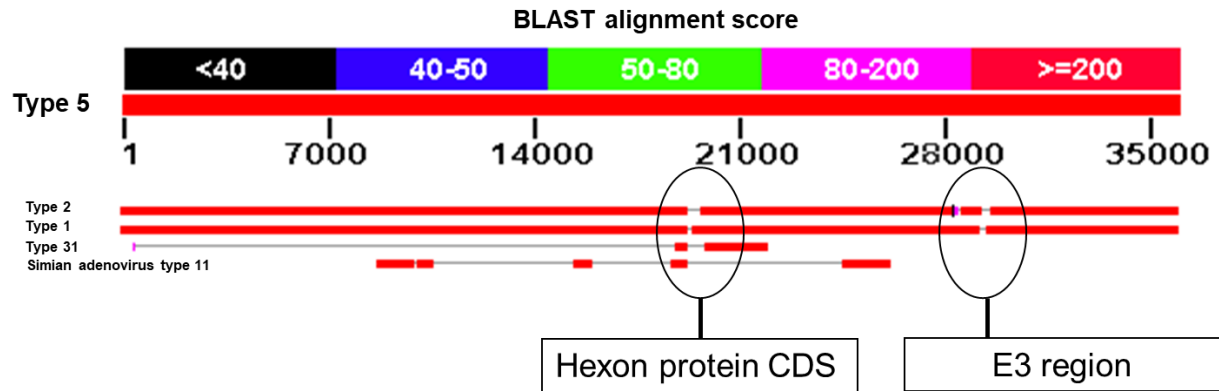


Figure 5.1 Alignment of human adenovirus type 5 genome to type 2, type 1, type 31 and simian adenovirus type 11 (NCBI BLAST).

The Species C adenoviruses (types 1, 2 and 5) share high sequence homology in all but the hexon and E3 regions of the genome. Human type 31 and Simian type 11 are given for reference.



Figure 5.2 Alignment of human adenovirus type 5 E3 region to type 1 and type 2 (NCBI BLAST).

The Species C adenoviruses type 5 (top gray bar), type 1 (second to bottom gray bar) and type 2 (bottom gray bar) differ vastly in the E3 non-coding regions (highlighted) which may indicate different regulation patterns. Red bars indicate protein coding sequences. Red marks on type 1 and 2 indicate differences in sequence from type 5.

5.3 MATERIALS AND METHODS

Cell lines

The human cell lines A549 (lung carcinoma) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). BJAB (EBV-negative Burkitt's lymphoma) and Jurkat (acute T cell leukemia) cells were obtained from ATCC {Klein, 1974 #2857}. BJAB and Jurkat were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10 mM glutamine. A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 μg of glucose/ml, 10% FCS, and 10 mM glutamine. Cells were tested by Genetica to ensure the absence of mycoplasma and for cell line authentication.

Adenoviruses

Wild-type species C Ad5 adenovirus was obtained by Linda Gooding from William S. Wold. Similarly, phenotypically wild-type mutant virus, Ad5dl309, was obtained from Tom Shenk (Princeton University, Princeton, NJ (Attarbaschi, Mann et al. 2007, Arbogast, Raveau et

al. 2015). Ad5dl309 is an Ad5 mutant that lacks the genes for E3 RID α and RID β proteins as well as the 14,700-molecular-weight protein (14.7K protein). Adenovirus type 2 was obtained from Linda Gooding.

Infection of lymphocytes with adenovirus

Infection of lymphocyte cell lines with adenovirus was performed as described previously (Flomenberg, Gutierrez et al. 1997) with minor modifications. Lymphocytes were collected and washed in serum-free (SF) RPMI medium. Cell density was adjusted to 10^7 cells/ml in SF-RPMI medium. Virus was added to the cell suspension at 50 PFU/cell, spun for 45min at 1000G at 25°C, resuspended by agitation. Cells were then incubated at 37°C for 1.5hrs with gently flicking every 30min. The infected cells were washed three times with RPMI complete medium and then resuspended in RPMI complete medium at 5×10^5 cells/ml. Cell concentration and viability were monitored throughout the infection.

Reverse transcription and quantitative PCR analysis of viral and cellular mRNA levels

RT-qPCR was performed as described previously (Chroboczek, Bieber et al. 1992), with minor modifications. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (Qiagen Inc. Valencia, CA). RNA was treated with Rnase-free DNase (Qiagen) on isolation columns and quantified. Greater than 100ng were reverse transcribed (RT) into cDNA, in 20 μ L reaction volumes, using Maxima First Strand cDNA Synthesis Kit (ThermoScientific). RT-enzyme negative controls were included for each reaction. Primers and probes were obtained from Integrated DNA Technologies (Coralville, IA). Viral primer and probe sequences are reported in our previous publications (Garnett, Talekar et al. 2009, Murali, Ornelles et al. 2014).

Probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) reporter molecule and contained dual ZEN and Iowa Black quenchers. Each sample was run in duplicate with at least 2 experimental repeats for each virus tested. All analyses were performed via the comparative threshold cycle (Ct) method as previously described (Chroboczek, Bieber et al. 1992). Target Cts were normalized to the EIF1 housekeeping gene, and set relative to the lowest sample, which was set to 1.

Flow cytometry

Intracellular staining for hexon protein and ADP was used to detect productively infected cells by flow cytometry as previously described using the same antibodies (Zhang, Huang et al. 2010, Murali, Ornelles et al. 2014). Briefly, a mouse monoclonal antibody to adenovirus protein was used as a primary antibody. A mouse isotype IgG1, κ , antibody was used as a negative control for primary antibody staining (BD Pharmingen). Cells were subsequently stained with a goat anti-mouse IgG-APC conjugated secondary antibody (Life Technologies). Results were analyzed on a LSR Fortessa flow cytometer using FACSDiva Software (BD Biosciences). Isotype control staining was used to define the adenovirus protein-positive staining cells and was 5% or less for all samples evaluated.

PMA/Ionomycin treatments

Lymphocytes were cultured either alone or with phorbol myristate acetate (PMA) (50 ng per ml; Sigma) and ionomycin (1 M; Calbiochem) for 48 h as described before (Garnett, Talekar et al. 2009) and samples were tested for adenovirus RNA by RT-PCR as described above.

5.4 RESULTS

ADP expression is required for lytic infection in lymphocytes and its expression is repressed in persistently infected lymphocytes

Mutant viruses with variable amounts of ADP expression were utilized for these experiments. The three viruses used throughout include adenovirus dl309 (which expresses normal amounts of ADP; red in graphs below), pm534 (an ADP deletion mutant virus; green in graphs below), and VRX021 (an ADP-overexpressing virus mutant; red in graphs below). To confirm that the viral mutants were behaving as expected, we first infected epithelial cells with the viruses and evaluated expression of hexon and ADP protein. Adenovirus hexon protein levels were measured by intracellular flow cytometry of cells 2 days post infection (peak of infection in epithelial cells). Infected cells showed >80% hexon expression, confirming that most cells were infected and, further, that there was no obvious alteration to this in either of the ADP mutant viruses (Figure 5.3). As expected, cells infected with dl309 and VRX021 showed robust ($\geq 79\%$) ADP expression. In contrast, the ADP-deletion mutant, pm534, showed no expression of ADP (Figure 5.4). The effect of ADP on cell growth and viability in these epithelial cells was monitored up to 10 days post infection to determine if higher levels of ADP correlated with more robust cell death. A549 cells infected with the ADP-overexpressing VRX021 died more rapidly than the ADP wildtype containing virus (dl309) and the ADP-deletion mutant virus (pm534) (Figure 5.5). Thus the three viruses behaved as expected in the lung epithelial cell line evaluated here.

We reasoned that a switch from latent to lytic infection would require cell lysis for virus release and that, conversely, cells in which the virus is latent/persistent would not have high expression of ADP. The same virus mutants were used in 3 separate lymphocyte cell lines to test

whether ADP overexpression resulted in lytic infections and if the loss of ADP could promote viral persistence. Two lymphocyte cell lines that establish persistent infections with adenovirus were tested (BJAB B-cell line and KE37 T-cell line) and one lymphocyte cell line that undergoes a lytic infection with the virus (Jurkat T cell line) were evaluated. All cell lines were robustly infected with the viruses as determined by hexon protein levels (>70%) (Figure 5.6, Figure 5.9, Figure 5.12). However, ADP levels were low in cell lines that support persistent infection (BJAB and KE37) even when the ADP-overexpressing virus (VRX021) was used for infection (

Figure 5.10, Figure 5.13). In contrast, Jurkat cells, which support a lytic infection, demonstrated expression of ADP 3dpi (Figure 5.7) and increased cell death when infected with the ADP over-expressing virus (Fig 5.8). They also expressed detectable ADP protein when infected with the ADP-WT viral strain (dl309) (Fig 5.7). Thus, lymphocyte cell lines that support persistent infections with adenovirus are characterized by low levels of ADP following infection with either WT-ADP viruses or ADP-overexpressing viral mutants. *These results were included in a published manuscript* ((Murali, Ornelles et al. 2014) Figure 3 repeats, Figure 4, Figure 5 repeats). Further, results from this study demonstrated that this occurred despite the fact that ADP mRNA levels were elevated in VRX021 (ADP overexpressing mutant) infected cells compared to the dl309 (ADP WT virus). The absence of a corresponding increase in ADP at the protein level suggests that a mechanism other than transcriptional repression controls ADP protein levels during persistent lymphocyte infection (Murali, Ornelles et al. 2014). Viability was also not significantly altered in cells that support persistent infection when infected with the ADP overexpressor (**Error! Reference source not found.** and Figure 5 in (Murali, Ornelles et al. 2014)). Overall, the results indicated that higher ADP mRNA expression alone is not enough to push persistently infected lymphocytes into lytic infection, and that post-transcriptional

reduction of ADP transcripts or protein levels occurs in cells that support persistent infections (Murali, Ornelles et al. 2014).

As expected, the pm534 ADP-deletion mutant showed no ADP expression in lymphocyte cell lines (Figure 5.7,

Figure 5.10, Figure 5.13). When infected with this viral mutant, Jurkat cells remained viable (Fig 5.8; green line) indicating that ADP expression contributes to cell death in these cells. Studies performed by other students showed that virus persisted in Jurkat cells infected with pm534 and remained viable for over a month (Murali, Ornelles et al. 2014). These findings support the idea that reduced ADP expression allows the virus to persist in lymphocytes.

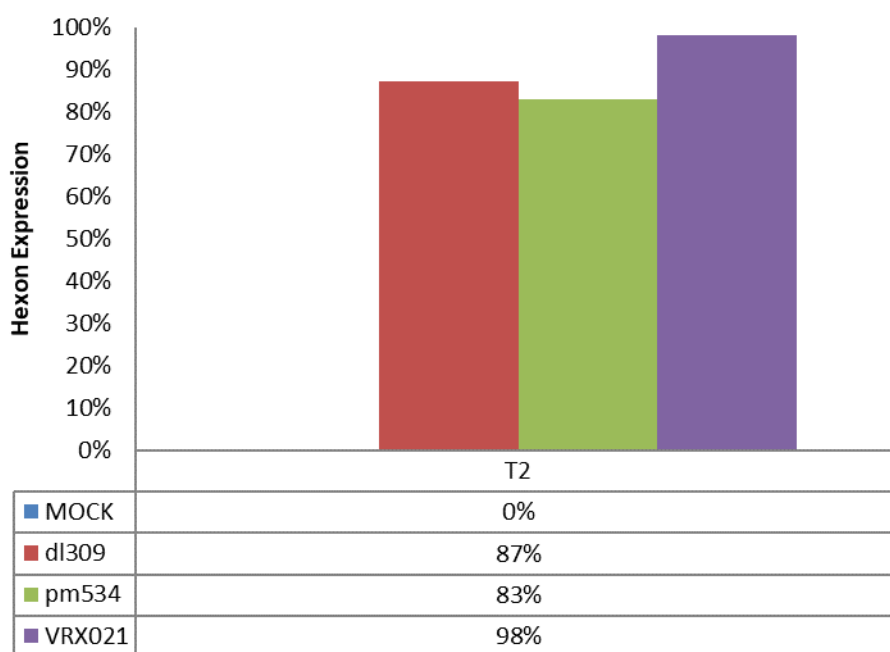


Figure 5.3 A549 Hexon expression.

A549 cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular hexon protein. Samples were assayed by flow cytometry, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

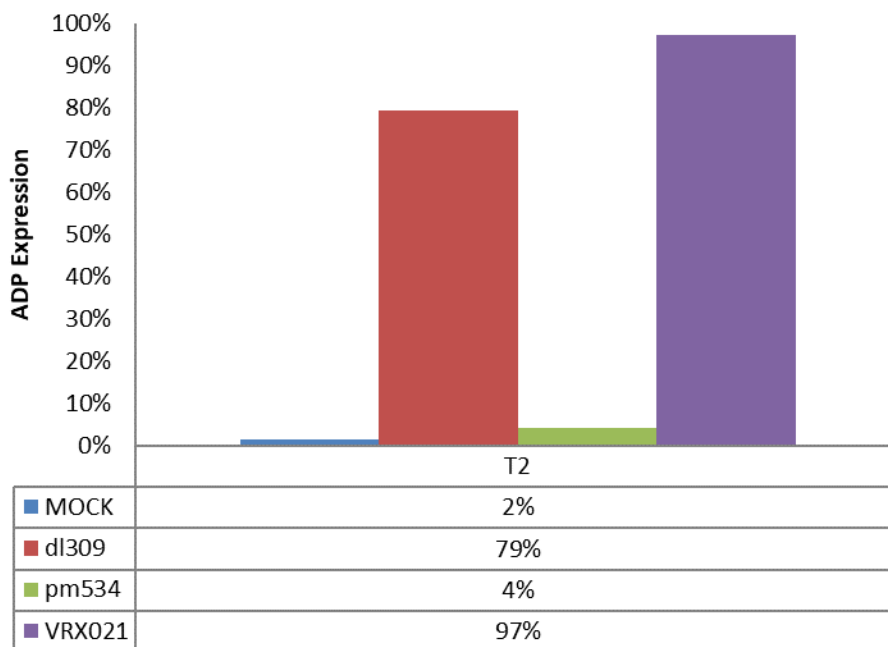


Figure 5.4 A549 ADP expression.

A549 cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular ADP protein. Samples were assayed by FACS, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

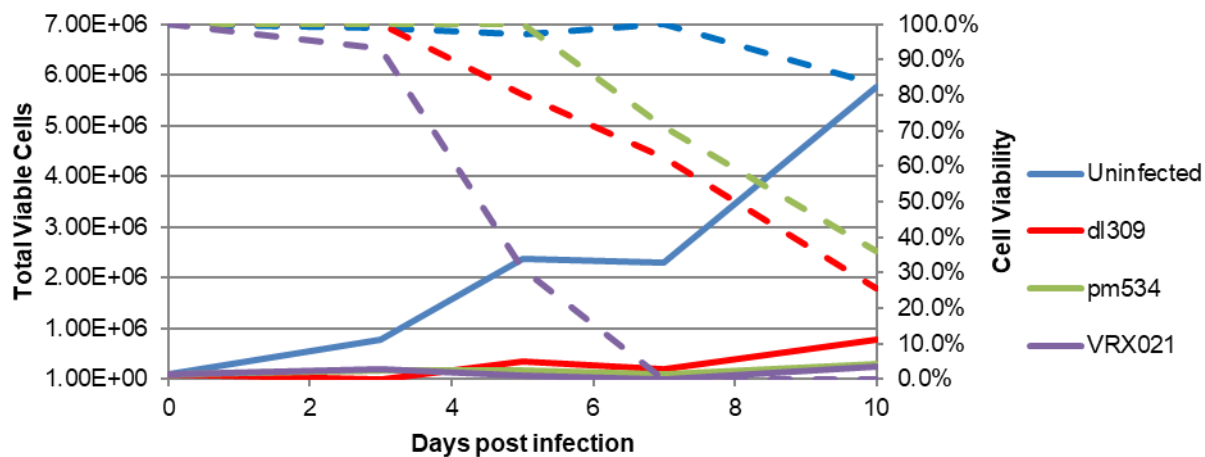


Figure 5.5 A549 growth and viability.

A549 cells were infected with indicated adenovirus mutant. Samples were collected on indicated days post infection and assayed for live cell number (solid lines) and viability (dotted lines).

Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

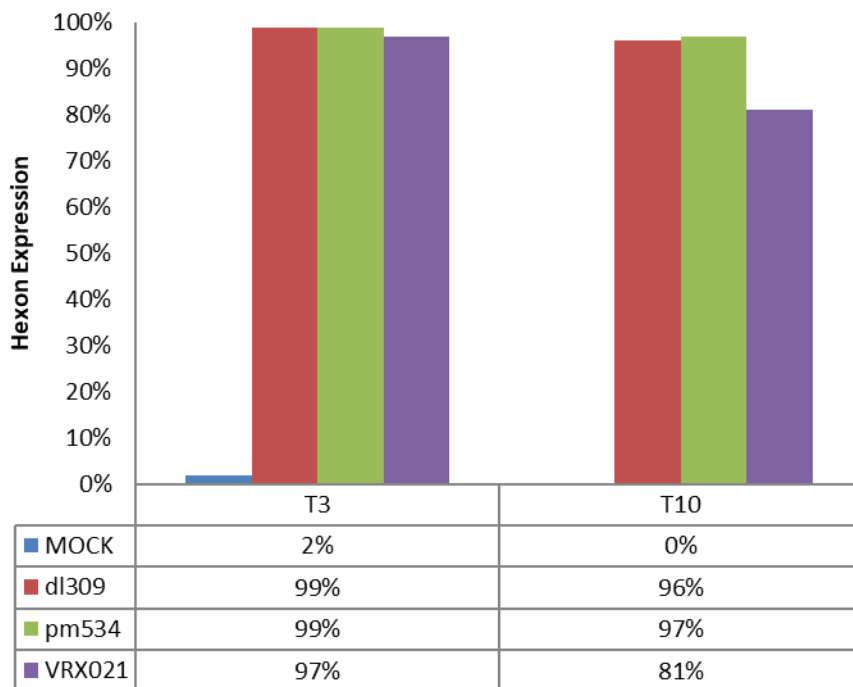


Figure 5.6 Jurkat Hexon expression.

Jurkat cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular hexon protein. Samples were assayed by flow cytometry, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. DL309 is a wild-type expression ADP virus.

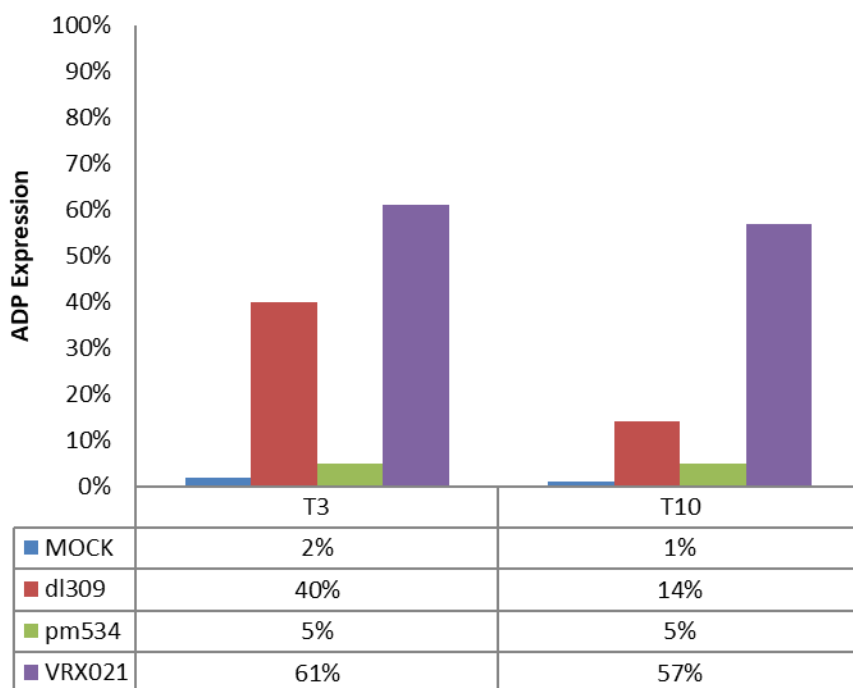


Figure 5.7 Jurkat ADP expression.

Jurkat cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular ADP protein. The peak of infection in Jurkat cells typically occurs around day 2-4. Samples were assayed by flow cytometry, using an isotype control antibody for background control and gating on the uninfected samples. Technically difficulties prevented use of day 7 values. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus.

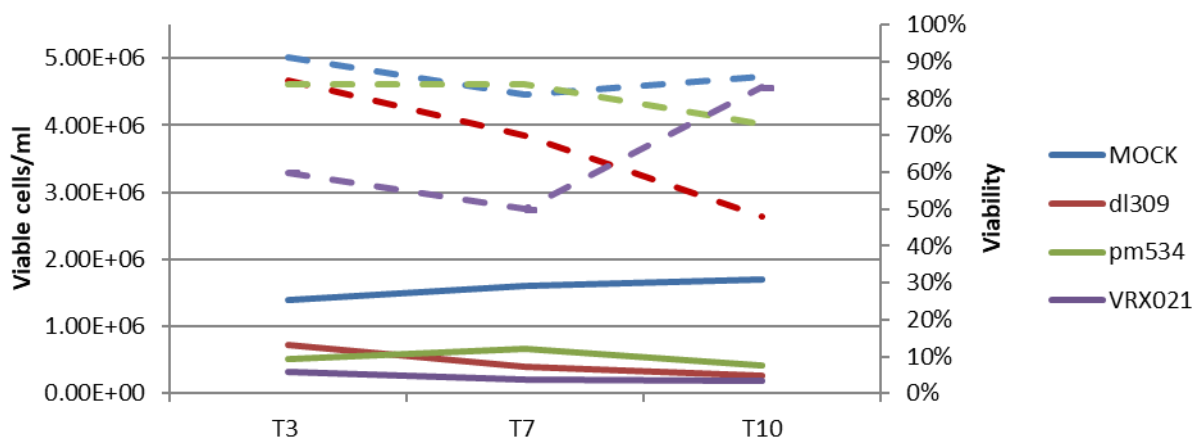


Figure 5.8 Jurkat concentration and viability.

Jurkat cells were infected with indicated adenovirus mutant. Samples were collected on indicated days post infection and assayed for live cell concentration (solid lines) and viability (dotted lines). Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus.

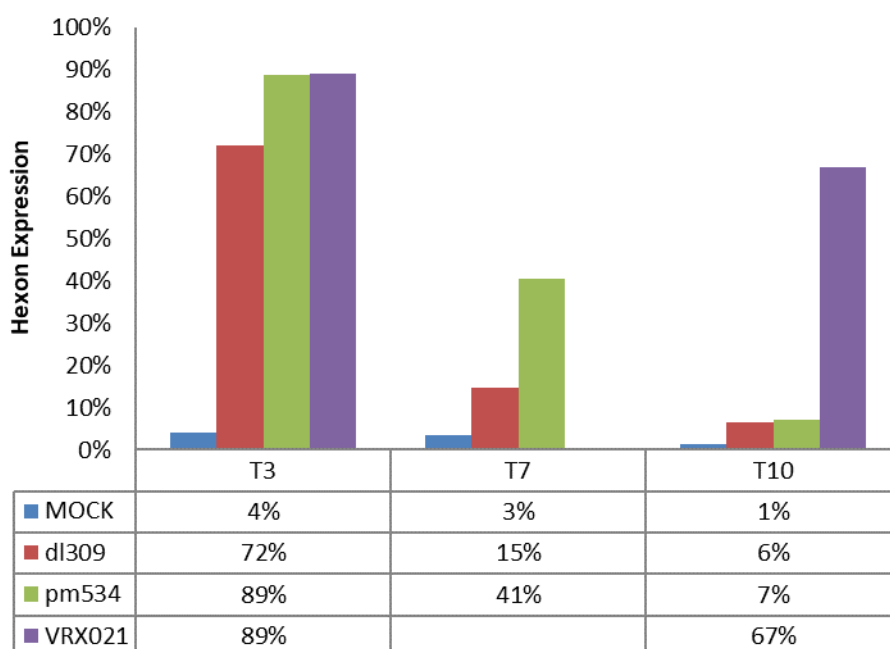


Figure 5.9 KE37 Hexon expression.

KE37 cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular hexon protein. The peak of infection in KE37 cells typically occurs between 3-5 days post-infection. Samples were assayed by flow cytometry, using an isotype control antibody for background control and gating on the uninfected samples. VRX021 day 7 was not recorded due to technical problems. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

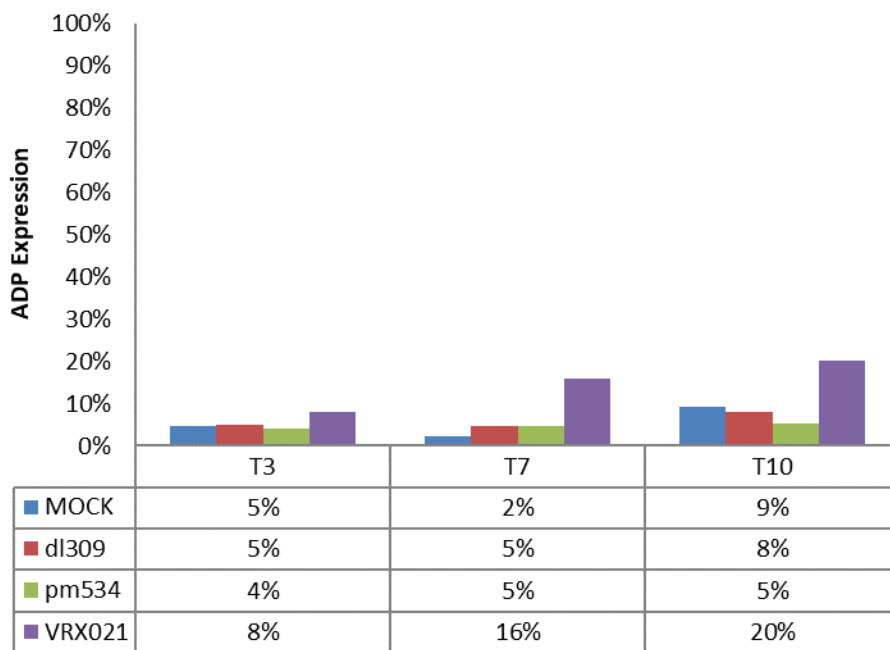


Figure 5.10 KE37 ADP expression.

KE37 cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular ADP protein. Samples were assayed by flow cytometry, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

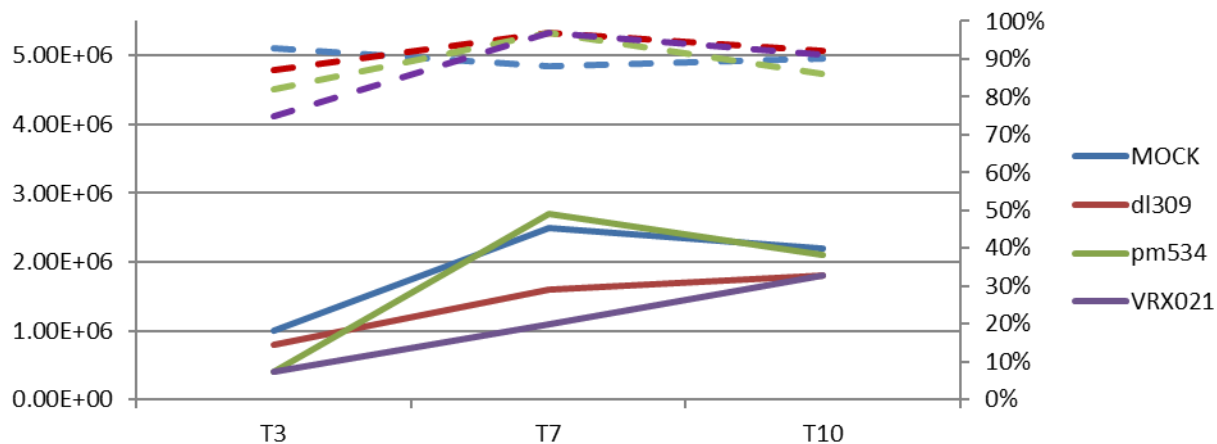


Figure 5.11 KE37 concentration and viability.

KE37 cells were infected with indicated adenovirus mutant. Samples were collected on indicated days post infection and assayed for live cell concentration (solid lines) and viability (dotted lines). Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus. (Murali 2014)

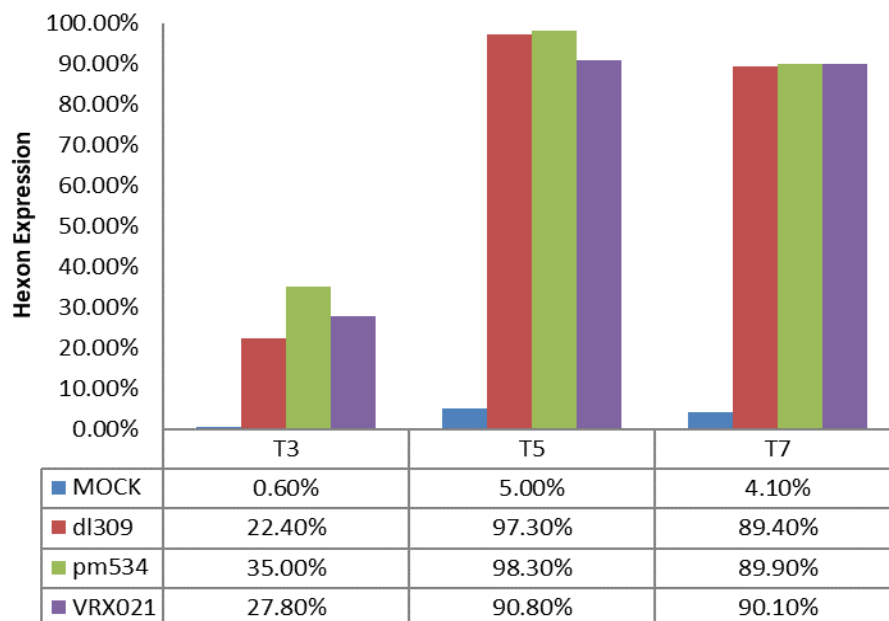


Figure 5.12 BJAB Hexon expression.

BJAB cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular hexon protein. Peak of infection in BJAB cells is typically 7-10 days post-infection. Samples were assayed by FACS, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. Dl309 is a wild-type expression ADP virus.

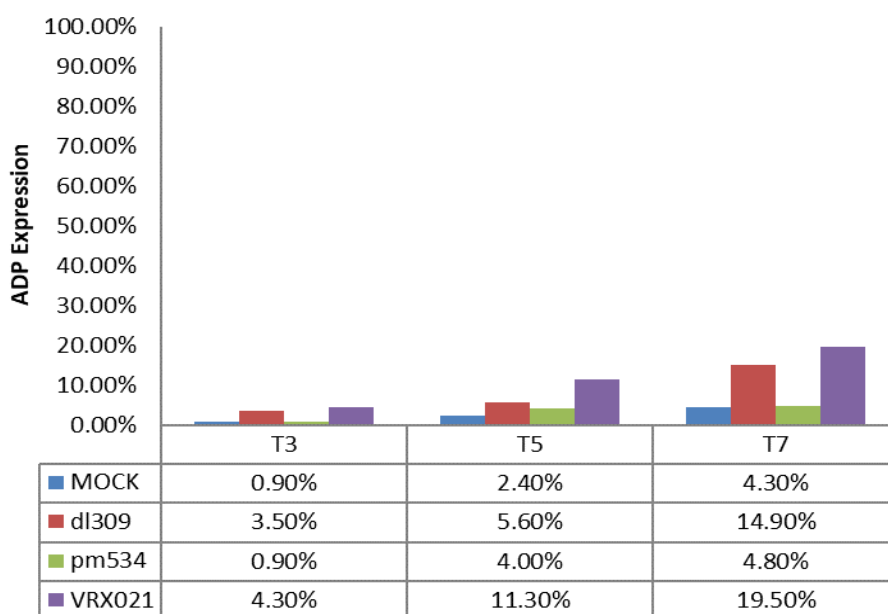


Figure 5.13 BJAB ADP expression.

BJAB cells were infected with indicated adenovirus mutant and allowed to progress through infection. Samples were taken on indicated days post infection and stained for intracellular ADP protein. Samples were assayed by FACS, using an isotype control antibody for background control and gating on the uninfected samples. Pm534 is an ADP deletion mutant virus and VRX021 is an ADP overexpressing virus. DL309 is a wild-type expression ADP virus.

Role for specific control of Ad5 E3 region following NF- κ B activation using PMA and ionomycin (P/I) in infected B cells remains unclear

Exploratory experiments infecting B and T lymphocytes using Ad2 and Ad5 showed a persistent infection pattern with Ad2, but a lytic infection using Ad5, confirming observations by Markel *et al* (Markel, Lam et al. 2014). Both cell lines were assayed for cell concentration and viability during peak on infection. Clear differences in viability in both B (BJAB) and T lymphocytes (Jurkats) were observed (Table 5.1). While Ad5 infection drastically decreased viability in both Jurkat cells (50%) and BJAB (73%), Ad2 infections showed much less effect on viability in Jurkat cells (71%) and BJAB cell viability was completely unaffected by Ad2 infection (96%) (Table 5.1). In line with previously reported observations, Jurkat cells infected with Ad2 remained viable and continued to grow while Ad5 infected cell cultures were no longer viable 7 days post infection (data not shown). Intracellular staining of Jurkat T cells revealed differences in hexon protein expression dynamics. While both Ad2 and Ad5 infections had relatively similar amounts of hexon expressing positive cells, 51% and 62% respectively, the Ad2 culture lacked the “high hexon” population of cells that is characteristic of a typical Ad5 infection in lymphocytes (Figure 5.14) ((Zhang, Huang et al. 2010) and unpublished observations). While virtually all hexon positive cells expressed low amounts of hexon in the

Ad2 infected Jurkat cells (99%), only 33% of Ad5 hexon positive Jurkat cells expressed low amounts of hexon, with the other 66% expressing high amounts of hexon (Figure 5.14, gates not shown). The novel hexon expression and previously demonstrated cell viability results (Markel, Lam et al. 2014), as well as other unpublished observations showing over 80% of Jurkat cells expressing hexon protein, indicated that while infection levels between Jurkat cells were similar, gene regulation between adenovirus serotype was different. The E3 promoter region is vastly different between these two strains (Figure 5.2) and ADP, a viral gene necessary for lytic infection in lymphocytes, is contained within this region. We reasoned that differences in the E3 promoter region may be control viral gene expression in lymphocytes, and through that latent infection.

The adenovirus E3 region has been reported to be regulated by NF- κ B in the absence of E1A, and this seems to be specialized for lymphoid cells {Mahr, 2003 #3882;Deryckere, 1996 #4692;Williams, 1990 #2860}. The NF- κ B pathway is relatively inactive in BJAB cells, but can be stimulated with PMA plus ionomycin (P/I) treatment (Lam, Davis et al. 2008). There is no hexon protein expression in BJAB cells during initial infection using Ad2, making confirmation of robust infection of all cells difficult (unpublished observations). However, hexon protein expression during initial Ad5 infection to confirm infection of all cells is readily measurable by intracellular staining (Figure 3.3, Figure 5.12). We tested whether activation of NF- κ B through P/I treatment would result in specific upregulation of E3 mRNA in BJAB cell persistently infected with Ad5. Figure 5.15 and **Error! Reference source not found.** show one experiment measuring viral hexon, E3gp19k, and E1A mRNA levels of Ad5 infected BJAB cells at 28 days post infection, at 1 and 4 hrs respectively after mock and P/I treatment. Hexon and E3gp19k mRNA are robustly upregulated compared to E1A mRNA at 1hr after treatment with P/I (Figure

5.15). After 4hrs, the mRNA of all genes had returned to almost basal levels for the first experiment (**Error! Reference source not found.**).

This experiment was repeated a second time (**Error! Reference source not found.**) but with cells at 33 days post infection. In this repeat, only moderate upregulation of all 3 genes was observed (Fig 5.20). Further, these results failed to show selective response of the E3 region in response to P/I induced NF- κ B activation. This area of study was not further evaluated because of the discrepancies between initial experiments. This may highlight problems with use of P/I as a treatment for specific NF- κ B activation. The primary target of P/I treatment is protein kinase C, which may activate a broad number of pathways confounding results (Castagna, Takai et al. 1982). Most NF- κ B activators function via broad or unknown mechanisms (Sun and Andersson 2002), so it may be wise to instead combine P/I treatment with a IKK inhibitor like TPCA 1 (Rauert-Wunderlich, Siegmund et al. 2013, Dondelinger, Jouan-Lanhouet et al. 2015) in future studies to single out effects of viral induction via NF- κ B complexes.

Table 5.1 Jurkat and BJAB cells infected with Ad2 and Ad5 display different patterns of viability.

Jurkat and BJAB cells were infected with Ad2 and Ad5, then measured for concentration and viability at day 2 (Jurkat) and day 10 (BJAB) post infection.

	Live/ml	Dead/ml	Viability
BJAB	1.5×10^6	5.0×10^4	97%
BJAB Ad2	1.6×10^6	6.0×10^4	96%
BJAB Ad5	6.0×10^4	2.0×10^4	73%
Jurkat	1.0×10^5	1.0×10^4	91%
Jurkat Ad2	8.0×10^4	3.0×10^4	71%
Jurkat Ad5	3.0×10^4	3.0×10^4	50%

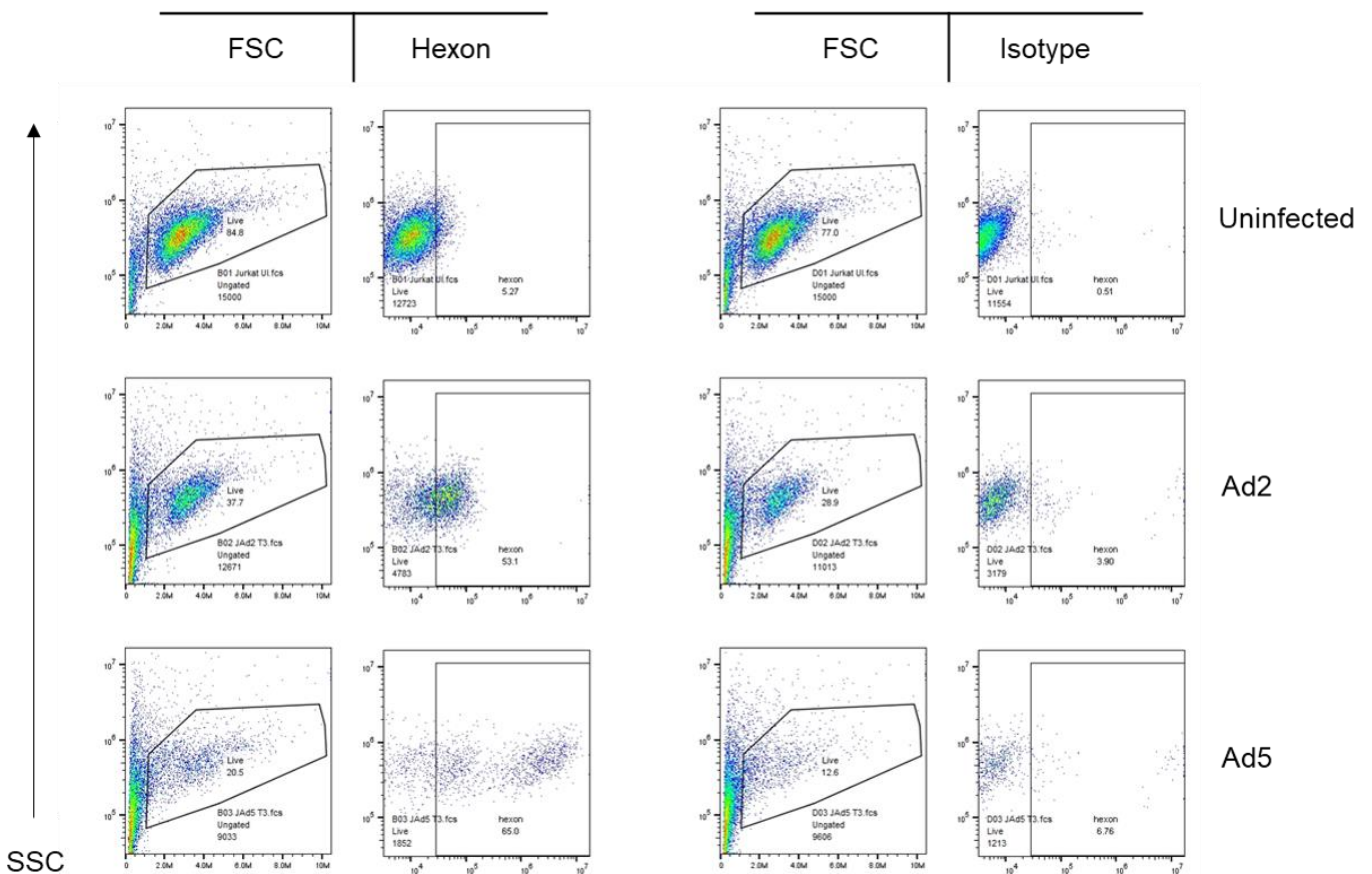


Figure 5.14 Hexon protein expression in Jurkat cells infected with Ad2 and Ad5.

Jurkat cells were infected with Ad2 and Ad5 at 50MOI, then measured for intracellular hexon expression via flow cytometry at day 2 (Jurkat) post infection. Cultures were gated on uninfected cells and isotype antibody was used as a control for background staining.

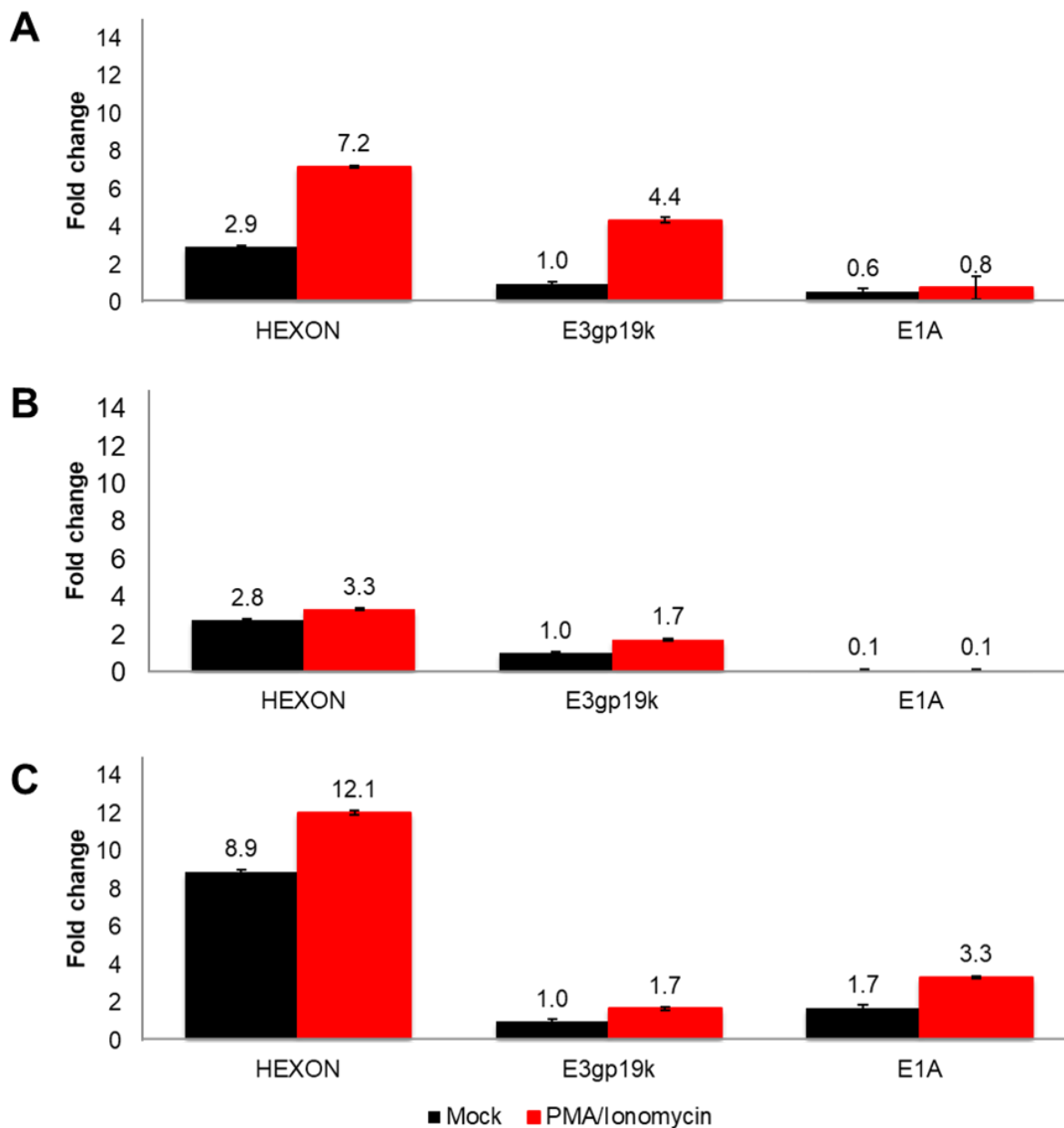


Figure 5.15 *Viral transcript levels of PMA/Ionomycin treated persistently infected BJAB cells, 1hr (A, experiment 1, 28 days post infection) 4hrs (B, experiment 1, 28 days post infection) and 1hr (C, experiment2 33 days post infection).*

BJAB cells were stimulated with PMA/Ionomycin cocktail (red bars) or mock treatment (black bars) for 1-4 hr before sampling for RNA. Indicated gene transcripts were compared to the mock treated E3gp19k sample. PMA/Ionomycin treatment increased viral transcription in all genes tested. Data shown are from 2 independent experiments.

5.5 DISCUSSION

The mechanisms that control adenovirus latency or lytic infection in lymphocytes remain elusive. The results of these studies, and the studies of the associated manuscript (Murali, Ornelles et al. 2014) reveal that ADP does not seem to be *required* for epithelial cell death, but its overexpression leads to more *rapid* cell death. However, ADP expression *is* required for lytic infection in lymphocytes that normally undergo a lytic infection, as the loss of ADP converted this to a persistent infection. In contrast, overexpression of ADP protein, using an ADP overexpressing virus, could not be achieved in lymphocyte cell lines that normally undergo persistent infection (

Figure 5.10, Figure 5.13)(Murali, Ornelles et al. 2014). ADP protein was low despite the increase in ADP mRNA in these cells. This indicates that post-transcriptional factors likely contribute to the expression and stability of ADP in lymphocytes. The ADP coding region is in the E3 region, which is curious since it is expressed during late infection with viral capsid proteins {Tollefson, 1992 #2862}. These data support the idea that there is some unique regulation of genes within the E3 region and that this may be important for the latent/persistent phase of the virus life cycle in lymphocytes.

Preliminary observations showed that the percent of cells expressing hexon protein in Jurkat cells did not change between Ad2 and Ad5 infection, yet the Ad2 infection did not have the same “high hexon expressing” cell population that is characteristic of the Ad5 infection. Measurement of ADP levels was not possible, as we lacked an Ad2 ADP specific antibody

(Figure 5.14). The forward and side scatter of the infected Jurkat plots show vast morphology differences between the Ad2 and Ad5 infected cells, probably related to viability and general infection dynamics. While the Ad5 infected cells were much more heterogeneous in size and granularity, the Ad2 infected cells resembled the uninfected forward and side scatter much more. The Ad5 infection also showed much more cell debris (Figure 5.14). These results correlated with the lower viability in Jurkat cells infected with Ad5 that viability of Ad2 infected cells Table 5.1. The different hexon protein expressing cultures in the Ad5 infected cells likely represent important infection dynamics. In the Jurkat Ad2 infection, which supports a persistent infection, there is no high hexon expressing population. These differences may elude to a mechanism in persistent and lytic infections. Maybe these high hexon expressing cells are also the ADP expressing cells that drive lytic infection? During Ad5 infection in BJAB cells, viability decreases during the peak of infection, but recovers past 18 days post infection ((Zhang, Huang et al. 2010) and unpublished observations). Perhaps these are a high hexon, high ADP expressing population that dies off and leaves only the persistently infected cells behind.

Further experiments are required in this project to make additional conclusions about ADP's role in lymphocyte infections. Demonstrating the effect of ADP on viability of Jurkat cells using Ad2 mutants Ad2 dl801 (ADP-) and Ad2 dl732 (ADP++) may reveal whether ADP overexpression in a persistent T cell infection can drive lytic infection. Additionally, it may be interesting to separate the high and low hexon populations in Ad5 infected BJAB cells by FACS to examine differences in viability and persistence. This may be technically challenging as intracellular staining is required to detect hexon which kill the cells as a result of permeabilization and fixing the cells.

It was also attempted to demonstrate the selective expression of the viral E3 region following NF- κ B induction, as previous reports have demonstrated that this region may be under NF- κ B regulation in lymphocytes {Mahr, 2003 #3882}. The results from two separate experiments showed contrasting results related to specific transcriptional activity from the adenovirus E3 region (Figure 5.15, **Error! Reference source not found., Error! Reference source not found.**). However, both experiments did reveal increased expression of viral genes following P/I. The adenovirus genome is small (~36kbp) transcription factors could feasibly impact the entire genome. This idea is supported by a paper that was published during my studies which showed that activated NF- κ B promoted global expression of adenovirus genes from all early gene regions {Machitani, 2016 #2859}. Using luciferase reporter plasmids, with promoters from each viral early gene region, only the E2/E3 shared promoter region showed activity with TNF- α treatment, and NF- κ B binding in this region was confirmed. Surprisingly, treatment with TNF- α upregulated the E3 region genes only *slightly* more than the other early genes. In light of this, it may be very difficult to detect specific activation of the E3 gene region if all genes are upregulated with NF- κ B binding to the E3 region, which may explain the inconsistency between Figure 5.15 and **Error! Reference source not found.** However, it is interesting to note that my experiments differed in the ratio of E1A and E3 transcripts initially expressed without treatment, with Figure 5.15 transcribing less E1A than E3 in untreated cells and vice versa in **Error! Reference source not found.** As noted before, E1A protein seems to compete with NF- κ B for E3 transcription promotion {Williams, 1990 #2860}. If the higher E1A to E3 ratio (**Error! Reference source not found.**) was some indication that the infection was more active than the other infection with the higher E3 to E1A ratio (Figure 5.15), then this could mean that the two experiments were in different stages of infection, thus giving different results when treated with

PMA/Ionomycin. This difference of activities in infection at the time of the assay may have had an impact on gene regulation by NF- κ B and would require further study by others.

6 DISCUSSION AND CONCLUSIONS

Adenovirus has long been suspected of oncogenesis, but viral nucleic acids have suspiciously not been found in any cancers. Our data in Chapter 3 showed for the first time that the common leukemic fusion protein ETV6/RUNX1 bound to the viral genome, which was associated with deacetylation of the viral genome. Subsequent loss of viral nucleic acids in our experimental model supported the feasibility of viral hit and run oncogenesis. Furthermore, viral induced epigenetic gene repression was retained in cells that had evicted the virus, showing that these *specific* changes induced by the virus can persist in the absence of the virus. This observation could have important medical applications if the virus is indeed a causative agent of leukemia. The genes repressed by the virus, even after viral loss, could serve as early biomarkers of virally induced childhood leukemia, which is discussed in detail in the related provisional patent (Appendix A). Findings in Chapter 4 suggest that indirect mechanisms of viral eviction from B cells expressing the RUNX1 fusion genes were not involved in this model. Cell growth and viability remained the same with or without the RUNX1 fusion genes, and our experiments failed to show any clear associations with E4ORF6 or NF- κ B. Chapter 5 revealed unclear selective expression of the E3 region after PMA/Ionomycin stimulation of NF- κ B in persistently infected lymphocytes.

The epidemiological evidence overwhelmingly supports an infectious event associated with precursor B-cell ALL, and the most recent large studies indicate that this event occurs at a time and place near the birth of the patient. The Smith hypothesis predicts an in utero agent that infects immunologically naïve mothers, predisposing the fetus to developing leukemia. The most common chromosomal translocation associated with precursor B-cell ALL creates the ETV6/RUNX1 fusion gene, which was the only characteristic significantly associated with the

clusters of precursor B-cell ALL in one study and is found in 1% of newborns (Mori, Colman et al. 2002, Kreis, Lupatsch et al. 2017).

Adenovirus has been shown to be in 5% of amniotic fluid samples and 4% of cord blood lymphocyte samples in healthy pregnancies. It inhibits efficient repair of dsDNA breaks as part of its lifecycle, the resulting chromosomal instability could feasibly lead to chromosomal translocations in the host cell. Transient expression of adenovirus genes have been shown to transform primary cells in vitro in a hit and run fashion, and we have shown that adenovirus is ejected from cell populations with expression of the ETV6/RUNX1 fusion gene. If the chromosomal instability produced by adenovirus caused the first hit in lymphocytes, presumably a fusion gene like ETV6/RUNX1, then the virus would be lost from the resulting pre-leukemic population by the activities of the new mutation.

Adenovirus peak prevalence in lymphocytes also correlates with the peak incidence of precursor B-cell ALL, which may indicate adenovirus as the second hit in ALL. In this model, adenovirus infection would infect pre-leukemic cells, like those harboring the ETV6/RUNX1 translocation, driving the pre-leukemic cells into full blown precursor B-cell ALL. Promotion and progression to full disease by a viral agent could also explain the early onset of this cancer which stands in contrast to the vast majority of all other cancers that require decades for the development of multiple carcinogenic mutations.

Both childhood ALL incidence and adenovirus seropositivity have been studied with respect to hygiene and SES factors or associated factors {Smith, 1998 #1387;Yang, 2016 #1641;Maia Rda, 2013 #1404;Stiller, 2008 #855;Smith, 2006 #1403;Dockerty, 2001 #5098}. The data supporting both hygiene and SES association for childhood ALL is conflicted. This could be in part because we don't see the effects of hygiene and SES in the developed countries

(like France (Goujon-Bellec, Mollie et al. 2013) and UK(Smith, Roman et al. 2006)) anymore because those countries reached a threshold related to these factors in which incidence has stabilized. Adenovirus is almost ubiquitous worldwide, making an association with childhood ALL between countries and viral prevalence in mucosal lymphocytes difficult to study. While adenovirus serotypes differ geographically, it is unknown whether specific serotypes could contribute differentially to oncogenicity. Adenovirus prevalence in lymphocytes at birth is less common, but still frequent enough (5% of newborns) to feasibly study. If adenovirus was the first hit, facilitating leukemic translocations, association between adenovirus and the translocations in otherwise healthy children may be a good target for future studies. The studies to date have looked almost exclusively in ALL diagnosed samples.

Various herpes viruses have been studied and supported to be one of the infectious agent responsible based on ELISA (Lehtinen, Koskela et al. 2003) in one study and ddPCR (Francis, Wallace et al. 2016) in another. A recent study by Francis et al showed that CMV nucleic acid was found in the bone marrow of ALL patients, but not AML patients (Francis, Wallace et al. 2016). In the same study, neonatal blood spots analyzed by highly sensitive ddPCR found CMV significantly more frequently in cases that later developed ALL than controls. While a small study conducted found an association of adenovirus present in neonatal blood spots from children who later developed leukemia using nPCR (Gustafsson, Huang et al. 2007), a later larger study by the same group not only failed to find the same association with adenovirus, but failed to find it in at least 5% of samples, as predicted by the amniotic fluid and cord blood studies (Baschat, Towbin et al. 2003, Honkaniemi, Talekar et al. 2010, Ornelles, Gooding et al. 2015). It seems clear that there is a problem with choice of tissue samples in these experiments. Adenovirus has not been tested by ddPCR.

Finally, it could be a mix of the Greaves, Kinlen and Smith hypotheses. Perhaps there are multiple steps or situations to the infectious etiology of cALL, which would be supported by the infectious etiology found associated both with birth and diagnosis in various studies. If populations of serologically naïve mothers became infected by a virus that could cross the placental barrier they may give birth to a susceptible population of pre-leukemic ETV6/RUNX1 positive children in that area. Additionally, or alternatively, an infection with human species C adenovirus early in life could possibly cause children to harbor the ETV6/RUNX1. Either scenario would sub sequentially eject the virus from the pre-leukemic cells due to activities of ETV6/RUNX1. The children may be more susceptible to other subsequent infections due to immune dysregulation caused by the first infection. If these susceptible children were infected relatively late in life with another virus, like CMV, this late infection could result in aberrant immune stimulation, leading to the onset of leukemia. Or maybe the process is a reverse of what was described above, and CMV is the in utero agent as the data in Francis et al suggests (Francis, Wallace et al. 2016). Such a model would satisfy all the results supporting the Greaves, Kinlen, and Smith hypotheses and the findings that the infectious etiology is associated with time and place of both birth and diagnosis.

Interestingly, adenovirus was shown to induce lower IL-10 levels than respiratory syncytial virus (Diaz, Calhoun et al. 1999). IL-10 is an important cytokine in suppressing the immune system in order to avoid over-reactions. This cytokine was found to be lower in neonates who later developed ALL and low IL-10 seems to be needed for persistent viral clearance (Brooks, Ha et al. 2008, Chang, Zhou et al. 2011). Perhaps low IL-10 levels are a sign that the child was once infected at a critical time period when their immune system was still

developing, or perhaps it has another cause and these children's immune systems are simply poor at removing chronic infections.

Adenovirus and CMV genes can also work in unison to transform cells in vitro in a hit and run fashion (Shen, Zhu et al. 1997). Strangely, CMV proteins stops cell replication, but is thought to “oncomodulate” them (Michaelis, Doerr et al. 2009). Adenovirus forces cells into the S cycle, driving replication and inhibiting cell death (Chapter 2). Even more interesting, both CMV and species C adenovirus have a high number of RUNX1 binding sites when compared to other DNA viruses (independent observation). Perhaps both play a role in the development of precursor B-cell ALL, but are both rejected from the leukemic population by inhibition of viral replication by the leukemic fusion proteins, particularly ETV6/RUNX1. Curiously, CMV was found in the bone marrow of child ALL patients (Francis, Wallace et al. 2016), but is not found on ALL cells circulating in the blood. The childhood leukemia fusion genes are thought to primarily function in similar ways by inhibiting target gene expression resulting in inhibition of cell maturation (reviewed in (Guidez and Zelent 2001, Greaves 2002)). RUNX1 is a master regulator of hematopoiesis and functions primarily by recruiting other effector proteins to the promoter regions of genes (Ichikawa, Asai et al. 2004, Lichtinger, Obier et al. 2013). To think that normal RUNX1 may be involved in recruiting other fusion proteins to viral DNA is not implausible. Perhaps the two viruses work together, resulting in an increased risk of leukemia in children, much like coinfection with the hepatitis B together with C or D virus can greatly increase risk of hepatocellular carcinoma (Fattovich, Stroffolini et al. 2004). Given the vast complexities of oncogenesis, to have both the Greaves and hit and run hypothesis to be correct or correct in particular scenarios would not be all to surprising.

The clustering etiology of precursor B-cell ALL may be dwindling with time as our world becomes increasingly globalized. Yet, childhood ALL continues to rise in most countries where quality of life continues to increase. Perhaps this trend is because population mixing has become a norm in many developed countries, where hygiene is also better. The more immunologically naïve population in the countries with better hygiene would more frequently be exposed to new viruses to which they were not immune. As a consequence, these populations would experience a greater childhood ALL peak, as we see in developed countries.

Adenovirus latency in lymphocytes, its relation to ADP expression and RUNX1 interaction likely play a role in adenovirus hit and run. The differences in infection pattern between Ad2 and Ad5, and their vast differences in the E3 promoter region, may help elude the mechanisms of viral latency and lytic infection. The results discussed in this dissertation on adenovirus latency and ADP are inconclusive. Hopefully future research may be able to definitively link ADP expression to adenovirus latency and E2/E3 regulatory mechanisms, if such mechanisms exist.

One surprising finding in Chapter 3, was that the ChIP experiment (Figure 3.9) failed to demonstrate any endogenous RUNX1 binding to the adenovirus genome. Though RUNX1 has been shown to associate with adenovirus proteins, I fully expected this known lymphocyte specific transcription factor to also bind to viral DNA in lymphocytes. However, I saw no evidence of this. This may have been due to timing, or signify other mechanisms at play. During viral infection, RUNX1 protein locates around the peripheries of viral DNA replication and displace viral protein E4ORF6, but RUNX1 does not localize with the viral DNA replication centers themselves (Marshall, Moore et al. 2008). Perhaps RUNX1 binding occurred during the initial acute phase of infection which wasn't evaluated here. Alternatively, it may be that the interactions with E4ORF6 keep RUNX1 from binding to the viral genome. This could indicate

that these proteins do have some relationship with viral latency that are not associated directly with DNA.

Future directions of linking adenovirus to leukemia or other cancers should focus on identifying common methylation patterns between adenovirus and cancers. Our results in Chapter 2.11 showed that adenovirus left an epigenetic mark of SPARCL1 and CXADR downregulation that was likely DNA methylation induced. As discussed in section 2.3, adenovirus E4ORF3 selectively silences p53 targets in a manner that allows heritable downregulation. If methylation patterns between persistently or latently infected adenovirus cells and cancer could be matched, this would provide strong evidence that the cancer's methylation pattern was a shadow left by a past infection. Based on our findings, experiments screening B lymphocytes from patients with ETV6/RUNX1 initiated ALL could be screened for epigenetic repression of these genes. This could provide additional evidence linking adenoviral induced cellular changes to ALL diseased patients.

To provide further epidemiological evidence to correlate adenovirus with childhood leukemia, newborn cord blood samples could be assayed for correlation between common leukemic translocations and presence of adenovirus DNA or anti-adenovirus antibodies. While this would assume adenovirus as the first hit for leukemia, detection of anti-viral antibodies could indicate infection in either mother or child. The epidemiological evidence in 2.10 is suggestive of maternal adenovirus infection susceptibility correlating with ALL in children. Finally, humanized mouse models for persistent adenovirus infection in lymphocytes have recently been established (Rodríguez, Ip et al. 2017). These show adenovirus persistence in bone marrow is feasible, and seem to recapitulate adenovirus persistence in humans. Studies using ETV6/RUNX1 containing lymphocytes in persistent adenovirus mouse models could provide

evidence in support for the virus's role in promoting ALL disease development post-translocation as a 2nd hit.

REFERENCES

- (1999). "Epidemiology of childhood cancer." IARC Sci Publ(149): 1-386.
- Adelman, A. S., C. C. McLaughlin, X. C. Wu, V. W. Chen and F. D. Groves (2005). "Urbanisation and incidence of acute lymphocytic leukaemia among United States children aged 0-4." Br J Cancer **92**(11): 2084-2088.
- Aldrich, M. C., L. Zhang, J. L. Wiemels, X. Ma, M. L. Loh, C. Metayer, S. Selvin, J. Feusner, M. T. Smith and P. A. Buffler (2006). "Cytogenetics of Hispanic and White children with acute lymphoblastic leukemia in California." Cancer Epidemiol Biomarkers Prev **15**(3): 578-581.
- Alexander, F. E., P. Boyle, P. M. Carli, J. W. Coebergh, G. J. Draper, A. Ekbom, F. Levi, P. A. McKinney, W. McWhirter, C. Magnani, J. Michaelis, J. H. Olsen, R. Peris-Bonet, E. Petridou, E. Pukkala and L. Vatten (1998). "Spatial temporal patterns in childhood leukaemia: further evidence for an infectious origin. EUROCLUS project." Br J Cancer **77**(5): 812-817.
- Ambinder, R. F. (2000). "Gammaherpesviruses and "Hit-and-Run" oncogenesis." Am J Pathol **156**(1): 1-3.
- Arbogast, T., M. Raveau, C. Chevalier, V. Nalesso, D. Dembele, H. Jacobs, O. Wendling, M. Roux, A. Duchon and Y. Herault (2015). "Deletion of the App-Runx1 region in mice models human partial monosomy 21." Disease Models & Mechanisms **8**(6): 623-634.
- Ariffin, H., S. P. Chen, C. S. Kwok, T. C. Quah, H. P. Lin and A. E. Yeoh (2007). "Ethnic differences in the frequency of subtypes of childhood acute lymphoblastic leukemia: results of the Malaysia-Singapore Leukemia Study Group." J Pediatr Hematol Oncol **29**(1): 27-31.
- Artigas, A. C., C. M. Cabrera, A. A. Melo, F. E. Paez, M. M. Arriagada, A. C. Astete, E. I. Roa and S. J. Roa (2006). "[Frequency of TEL/AML1 and BCR/ABL fusion genes in children with acute lymphoblastic leukemia]." Rev Med Chil **134**(11): 1367-1376.
- Attarbaschi, A., G. Mann, S. Strehl, M. Konig, M. Steiner, V. Jeitler, T. Lion, M. N. Dworzak, H. Gadner and O. A. Haas (2007). "Deletion of 11q23 is a highly specific nonrandom secondary genetic abnormality of ETV6/RUNX1-rearranged childhood acute lymphoblastic leukaemia (vol 21, pg 584, 2007)." Leukemia **21**(5): 1137-1137.
- Avila, M. M., G. Carballal, H. Rovaletti, B. Ebekian, M. Cusminsky and M. Weissenbacher (1989). "Viral etiology in acute lower respiratory infections in children from a closed community." Am Rev Respir Dis **140**(3): 634-637.
- Bacon, A. L., S. Fox, H. Turley and A. L. Harris (2007). "Selective silencing of the hypoxia-inducible factor 1 target gene BNIP3 by histone deacetylation and methylation in colorectal cancer." Oncogene **26**(1): 132-141.
- Baschat, A. A., J. Towbin, N. E. Bowles, C. R. Harman and C. P. Weiner (2003). "Prevalence of viral DNA in amniotic fluid of low-risk pregnancies in the second trimester." J Matern Fetal Neonatal Med **13**(6): 381-384.
- Basta, N. O., P. W. James, A. W. Craft and R. J. McNally (2010). "Season of birth and diagnosis for childhood cancer in Northern England, 1968-2005." Paediatr Perinat Epidemiol **24**(3): 309-318.
- Berk, A. J. (2005). "Recent lessons in gene expression, cell cycle control, and cell biology from adenovirus." Oncogene **24**(52): 7673-7685.
- Berscheminski, J., J. Brun, T. Speiseder, P. Wimmer, W. H. Ip, M. Terzic, T. Dobner and S. Schreiner (2016). "Sp100A is a tumor suppressor that activates p53-dependent transcription and counteracts E1A/E1B-55K-mediated transformation." Oncogene **35**(24): 3178-3189.

- Bialecki, E. S. and A. M. Di Bisceglie (2005). "Clinical presentation and natural course of hepatocellular carcinoma." *Eur J Gastroenterol Hepatol* **17**(5): 485-489.
- Birch, J. M., F. E. Alexander, V. Blair, O. B. Eden, G. M. Taylor and R. J. McNally (2000). "Space-time clustering patterns in childhood leukaemia support a role for infection." *Br J Cancer* **82**(9): 1571-1576.
- Bittar, C., S. Shrivastava, J. Bhanja Chowdhury, P. Rahal and R. B. Ray (2013). "Hepatitis C virus NS2 protein inhibits DNA damage pathway by sequestering p53 to the cytoplasm." *PLoS One* **8**(4): e62581.
- Bogdanovic, G., A. G. Jernberg, P. Priftakis, L. Grillner and B. Gustafsson (2004). "Human herpes virus 6 or Epstein-Barr virus were not detected in Guthrie cards from children who later developed leukaemia." *Br J Cancer* **91**(5): 913-915.
- Borchert, S., M. Czech-Sioli, F. Neumann, C. Schmidt, P. Wimmer, T. Dobner, A. Grundhoff and N. Fischer (2014). "High-affinity Rb binding, p53 inhibition, subcellular localization, and transformation by wild-type or tumor-derived shortened Merkel cell polyomavirus large T antigens." *J Virol* **88**(6): 3144-3160.
- Borkhardt, A., G. Cazzaniga, S. Viehmann, M. G. Valsecchi, W. D. Ludwig, L. Burci, S. Mangioni, M. Schrappe, H. Riehm, F. Lampert, G. Basso, G. Masera, J. Harbott and A. Biondi (1997). "Incidence and clinical relevance of TEL/AML1 fusion genes in children with acute lymphoblastic leukemia enrolled in the German and Italian multicenter therapy trials. Associazione Italiana Ematologia Oncologia Pediatrica and the Berlin-Frankfurt-Munster Study Group." *Blood* **90**(2): 571-577.
- Brestovitsky, A., K. Nebenzahl-Sharon, P. Kechker, R. Sharf and T. Kleinberger (2016). "The Adenovirus E4orf4 Protein Provides a Novel Mechanism for Inhibition of the DNA Damage Response." *PLoS Pathog* **12**(2): e1005420.
- Brooks, D. G., S. J. Ha, H. Elsaesser, A. H. Sharpe, G. J. Freeman and M. B. Oldstone (2008). "IL-10 and PD-L1 operate through distinct pathways to suppress T-cell activity during persistent viral infection." *Proc Natl Acad Sci U S A* **105**(51): 20428-20433.
- Bruder, J. T. and I. Kovesdi (1997). "Adenovirus infection stimulates the Raf/MAPK signaling pathway and induces interleukin-8 expression." *J Virol* **71**(1): 398-404.
- Burel, S. A., N. Harakawa, L. Zhou, T. Pabst, D. G. Tenen and D. E. Zhang (2001). "Dichotomy of AML1-ETO functions: growth arrest versus block of differentiation." *Mol Cell Biol* **21**(16): 5577-5590.
- Burgert, H. G., Z. Ruzsics, S. Obermeier, A. Hilgendorf, M. Windheim and A. Elsing (2002). "Subversion of host defense mechanisms by adenoviruses." *Curr Top Microbiol Immunol* **269**: 273-318.
- Cannon, M. J., D. S. Schmid and T. B. Hyde (2010). "Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection." *Rev Med Virol* **20**(4): 202-213.
- Cao, F., K. Wang, R. Zhu, Y. W. Hu, W. Z. Fang and H. Z. Ding (2013). "Clinicopathological significance of reduced SPARCL1 expression in human breast cancer." *Asian Pac J Cancer Prev* **14**(1): 195-200.
- Cardwell, C. R., P. A. McKinney, C. C. Patterson and L. J. Murray (2008). "Infections in early life and childhood leukaemia risk: a UK case-control study of general practitioner records." *Br J Cancer* **99**(9): 1529-1533.
- Carson, C. T., N. I. Orazio, D. V. Lee, J. Suh, S. Bekker-Jensen, F. D. Araujo, S. S. Lakdawala, C. E. Lilley, J. Bartek, J. Lukas and M. D. Weitzman (2009). "Mislocalization of the MRN complex prevents ATR signaling during adenovirus infection." *EMBO J* **28**(6): 652-662.

- Carson, C. T., R. A. Schwartz, T. H. Stracker, C. E. Lilley, D. V. Lee and M. D. Weitzman (2003). "The Mre11 complex is required for ATM activation and the G2/M checkpoint." EMBO J **22**(24): 6610-6620.
- Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa and Y. Nishizuka (1982). "Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters." J Biol Chem **257**(13): 7847-7851.
- Caughey, R. W. and K. B. Michels (2009). "Birth weight and childhood leukemia: a meta-analysis and review of the current evidence." Int J Cancer **124**(11): 2658-2670.
- Chan, L. C., T. H. Lam, C. K. Li, Y. L. Lau, C. K. Li, H. L. Yuen, C. W. Lee, S. Y. Ha, P. M. Yuen, N. K. Leung, S. L. Patheal, M. F. Greaves and F. E. Alexander (2002). "Is the timing of exposure to infection a major determinant of acute lymphoblastic leukaemia in Hong Kong?" Paediatr Perinat Epidemiol **16**(2): 154-165.
- Chang, J. S., M. Zhou, P. A. Buffler, A. P. Chokkalingam, C. Metayer and J. L. Wiemels (2011). "Profound deficit of IL10 at birth in children who develop childhood acute lymphoblastic leukemia." Cancer Epidemiol Biomarkers Prev **20**(8): 1736-1740.
- Chastain-Moore, A. M., T. Roberts, D. A. Trott, R. F. Newbold and D. A. Ornelles (2003). "An activity associated with human chromosome 21 permits nuclear colocalization of the adenovirus E1B-55K and E4orf6 proteins and promotes viral late gene expression." J Virol **77**(14): 8087-8098.
- Chroboczek, J., F. Bieber and B. Jacrot (1992). "The sequence of the genome of adenovirus type 5 and its comparison with the genome of adenovirus type 2." Virology **186**(1): 280-285.
- Cooper, R. J., R. Hallett, A. B. Tullo and P. E. Klapper (2000). "The epidemiology of adenovirus infections in Greater Manchester, UK 1982-96." Epidemiol Infect **125**(2): 333-345.
- Debbas, M. and E. White (1993). "Wild-type p53 mediates apoptosis by E1A, which is inhibited by E1B." Genes Dev **7**(4): 546-554.
- Degenhardt, K., D. Perez and E. White (2000). "Pathways used by adenovirus E1B 19K to inhibit apoptosis." Symp Soc Exp Biol **52**: 241-251.
- Diaz, P. V., W. J. Calhoun, K. L. Hinton, L. F. Avendano, A. Gaggero, V. Simon, S. M. Arredondo, R. Pinto and A. Diaz (1999). "Differential effects of respiratory syncytial virus and adenovirus on mononuclear cell cytokine responses." Am J Respir Crit Care Med **160**(4): 1157-1164.
- Dondelinger, Y., S. Jouan-Lanhouet, T. Divert, E. Theatre, J. Bertin, P. J. Gough, P. Giansanti, A. J. Heck, E. Dejardin, P. Vandenabeele and M. J. Bertrand (2015). "NF-kappaB-Independent Role of IKKalpha/IKKbeta in Preventing RIPK1 Kinase-Dependent Apoptotic and Necroptotic Cell Death during TNF Signaling." Mol Cell **60**(1): 63-76.
- Doniger, J., S. Muralidhar and L. J. Rosenthal (1999). "Human cytomegalovirus and human herpesvirus 6 genes that transform and transactivate." Clin Microbiol Rev **12**(3): 367-382.
- Durepaire, N., J. P. Rogez, M. Verdier, S. Rogez, P. Weinbreck and F. Denis (1997). "Detection of adenovirus DNA by polymerase chain reaction in peripheral blood lymphocytes from HIV-infected patients and a control group: preliminary results." J Acquir Immune Defic Syndr Hum Retrovirol **14**(2): 189-190.
- Einav, U., Y. Tabach, G. Getz, A. Yitzhaky, U. Ozbek, N. Amariglio, S. Izraeli, G. Rechavi and E. Domany (2005). "Gene expression analysis reveals a strong signature of an interferon-induced pathway in childhood lymphoblastic leukemia as well as in breast and ovarian cancer." Oncogene **24**(42): 6367-6375.

- El Mchichi, B., A. Hadji, A. Vazquez and G. Leca (2007). "p38 MAPK and MSK1 mediate caspase-8 activation in manganese-induced mitochondria-dependent cell death." Cell Death Differ **14**(10): 1826-1836.
- Elsing, A. and H.-G. Burgert (1998). "The adenovirus E3/10.4K–14.5K proteins down-modulate the apoptosis receptor Fas/Apo-1 by inducing its internalization." Proceedings of the National Academy of Sciences of the United States of America **95**(17): 10072-10077.
- Emerenciano, M., D. P. Agudelo Arias, V. M. Coser, G. D. de Brito, M. L. Macedo Silva, M. S. Pombo-de-Oliveira and L. Brazilian Collaborative Study Group of Infant Acute (2006). "Molecular cytogenetic findings of acute leukemia included in the Brazilian Collaborative Study Group of Infant acute leukemia." Pediatr Blood Cancer **47**(5): 549-554.
- Fattovich, G., T. Stroffolini, I. Zagni and F. Donato (2004). "Hepatocellular carcinoma in cirrhosis: incidence and risk factors." Gastroenterology **127**(5 Suppl 1): S35-50.
- Fears, S., C. Vignon, S. K. Bohlander, S. Smith, J. D. Rowley and G. Nucifora (1996). "Correlation between the ETV6/CBFA2 (TEL/AML1) fusion gene and karyotypic abnormalities in children with B-cell precursor acute lymphoblastic leukemia." Genes Chromosomes Cancer **17**(2): 127-135.
- Feller, M., M. Adam, M. Zwahlen, P. Brazzola, F. Niggli, C. Kuehni, G. Swiss Pediatric Oncology and C. Swiss National (2010). "Family characteristics as risk factors for childhood acute lymphoblastic leukemia: a population-based case-control study." PLoS One **5**(10).
- Feltbower, R. G., M. S. Pearce, H. O. Dickinson, L. Parker and P. A. McKinney (2001). "Seasonality of birth for cancer in Northern England, UK." Paediatr Perinat Epidemiol **15**(4): 338-345.
- Feng, H., M. Shuda, Y. Chang and P. S. Moore (2008). "Clonal integration of a polyomavirus in human Merkel cell carcinoma." Science **319**(5866): 1096-1100.
- Ferrari, R., D. Gou, G. Jawdekar, S. A. Johnson, M. Nava, T. Su, A. F. Yousef, N. R. Zemke, M. Pellegrini, S. K. Kurdistani and A. J. Berk (2014). "Adenovirus small E1A employs the lysine acetylases p300/CBP and tumor suppressor Rb to repress select host genes and promote productive virus infection." Cell Host Microbe **16**(5): 663-676.
- Ferrari, R., M. Pellegrini, G. A. Horwitz, W. Xie, A. J. Berk and S. K. Kurdistani (2008). "Epigenetic reprogramming by adenovirus e1a." Science **321**(5892): 1086-1088.
- Flavahan, W. A., E. Gaskell and B. E. Bernstein (2017). "Epigenetic plasticity and the hallmarks of cancer." Science **357**(6348).
- Flomenberg, P., E. Gutierrez, V. Piaskowski and J. T. Casper (1997). "Detection of adenovirus DNA in peripheral blood mononuclear cells by polymerase chain reaction assay." J Med Virol **51**(3): 182-188.
- Ford, A. M., C. A. Bennett, C. M. Price, M. C. Bruin, E. R. Van Wering and M. Greaves (1998). "Fetal origins of the TEL-AML1 fusion gene in identical twins with leukemia." Proc Natl Acad Sci U S A **95**(8): 4584-4588.
- Fox, J. P., C. D. Brandt, F. E. Wassermann, C. E. Hall, I. Spigland, A. Kogon and L. R. Elveback (1969). "The virus watch program: a continuing surveillance of viral infections in metropolitan New York families. VI. Observations of adenovirus infections: virus excretion patterns, antibody response, efficiency of surveillance, patterns of infections, and relation to illness." Am J Epidemiol **89**(1): 25-50.
- Francis, S. S., A. D. Wallace, G. A. Wendt, L. Li, F. Liu, L. W. Riley, S. Kogan, K. M. Walsh, A. J. de Smith, G. V. Dahl, X. Ma, E. Delwart, C. Metayer and J. L. Wiemels (2016). "In utero cytomegalovirus infection and development of childhood acute lymphoblastic leukemia." Blood.

- Fuka, G., H. P. Kantner, R. Grausenburger, A. Inthal, E. Bauer, G. Krapf, U. Kaindl, M. Kauer, M. N. Dworzak, D. Stoiber, O. A. Haas and R. Panzer-Grumayer (2012). "Silencing of ETV6/RUNX1 abrogates PI3K/AKT/mTOR signaling and impairs reconstitution of leukemia in xenografts." Leukemia **26**(5): 927-933.
- Gallimore, P. H. (1974). "Viral DNA in transformed cells. II. A study of the sequences of adenovirus 2 DNA IN NINE LINES OF TRANSFORMED RAT CELLS USING SPECIFIC FRAGMENTS OF THE VIRAL GENOME." J Mol Biol **89**(1): 49-72.
- Garcia-Sanz, R., I. Alaejos, A. Orfao, A. Rasillo, M. C. Chillon, M. D. Tabernero, M. V. Mateos, R. Lopez-Perez, D. Gonzalez, A. Balanzategui, M. Gonzalez, J. F. San Miguel and A. Bortolucci (1999). "Low frequency of the TEL/AML1 fusion gene in acute lymphoblastic leukaemia in Spain." Br J Haematol **107**(3): 667-669.
- Garnett, C. T., D. Erdman, W. Xu and L. R. Gooding (2002). "Prevalence and quantitation of species C adenovirus DNA in human mucosal lymphocytes." J Virol **76**(21): 10608-10616.
- Garnett, C. T., G. Talekar, J. A. Mahr, W. Huang, Y. Zhang, D. A. Ornelles and L. R. Gooding (2009). "Latent species C adenoviruses in human tonsil tissues." J Virol **83**(6): 2417-2428.
- Gilham, C., J. Peto, J. Simpson, E. Roman, T. O. Eden, M. F. Greaves, F. E. Alexander and U. Investigators (2005). "Day care in infancy and risk of childhood acute lymphoblastic leukaemia: findings from UK case-control study." BMJ **330**(7503): 1294.
- Gold, E. N., G. A. (1976). In viral infections of humans. Cytomegalovirus. New York, Plenum Press: 143-161.
- Goujon-Bellec, S., A. Mollie, J. Rudant, A. Guyot-Goubin and J. Clavel (2013). "Time trends and seasonal variations in the diagnosis of childhood acute lymphoblastic leukaemia in France." Cancer Epidemiol **37**(3): 255-261.
- Graham, F. L., J. Smiley, W. C. Russell and R. Nairn (1977). "Characteristics of a human cell line transformed by DNA from human adenovirus type 5." J Gen Virol **36**(1): 59-74.
- Greaves, M. (2002). "Childhood leukaemia." BMJ **324**(7332): 283-287.
- Greaves, M. (2003). "Pre-natal origins of childhood leukemia." Rev Clin Exp Hematol **7**(3): 233-245.
- Greaves, M. F. (1988). "Speculations on the cause of childhood acute lymphoblastic leukemia." Leukemia **2**(2): 120-125.
- Greaves, M. F. and F. E. Alexander (1993). "An infectious etiology for common acute lymphoblastic leukemia in childhood?" Leukemia **7**(3): 349-360.
- Greaves, M. F., A. T. Maia, J. L. Wiemels and A. M. Ford (2003). "Leukemia in twins: lessons in natural history." Blood **102**(7): 2321-2333.
- Greaves, M. F., S. M. Pegram and L. C. Chan (1985). "Collaborative group study of the epidemiology of acute lymphoblastic leukaemia subtypes: background and first report." Leuk Res **9**(6): 715-733.
- Guidez, F. and A. Zelent (2001). "Role of nuclear receptor corepressors in leukemogenesis." Curr Top Microbiol Immunol **254**: 165-185.
- Guruprasad, B., S. Kavitha, B. S. Aruna Kumari, B. R. Vijaykumar, B. G. Sumati, S. Mahua, L. Appaji and R. S. Jayshree (2014). "Risk of hepatitis B infection in pediatric acute lymphoblastic leukemia in a tertiary care center from South India." Pediatr Blood Cancer **61**(9): 1616-1619.
- Gustafsson, B. and G. Bogdanovic (2007). "Specific viruses were not detected in Guthrie cards from children who later developed leukemia." Pediatr Hematol Oncol **24**(8): 607-613.
- Gustafsson, B. and J. Carstensen (2000). "Space-time clustering of childhood lymphatic leukaemias and non-Hodgkin's lymphomas in Sweden." Eur J Epidemiol **16**(12): 1111-1116.

- Gustafsson, B., E. Honkaniemi, S. Goh, G. Giraud, E. Forestier, U. von Döbeln, T. Allander, T. Dalianis and G. Bogdanovic (2012). "KI, WU, and Merkel cell polyomavirus DNA was not detected in Guthrie cards of children who later developed acute lymphoblastic leukemia." J Pediatr Hematol Oncol **34**(5): 364-367.
- Gustafsson, B., W. Huang, G. Bogdanovic, F. Gauffin, A. Nordgren, G. Talekar, D. A. Ornelles and L. R. Gooding (2007). "Adenovirus DNA is detected at increased frequency in Guthrie cards from children who develop acute lymphoblastic leukaemia." British Journal of Cancer **97**(7): 992-994.
- Halbert, D. N., J. R. Cutt and T. Shenk (1985). "Adenovirus early region 4 encodes functions required for efficient DNA replication, late gene expression, and host cell shutoff." J Virol **56**(1): 250-257.
- Hanahan, D. and R. A. Weinberg (2011). "Hallmarks of cancer: the next generation." Cell **144**(5): 646-674.
- Harada, J. N., A. Shevchenko, A. Shevchenko, D. C. Pallas and A. J. Berk (2002). "Analysis of the adenovirus E1B-55K-anchored proteome reveals its link to ubiquitination machinery." J Virol **76**(18): 9194-9206.
- Hart, L. S., S. M. Yannone, C. Naczki, J. S. Orlando, S. B. Waters, S. A. Akman, D. J. Chen, D. Ornelles and C. Koumenis (2005). "The adenovirus E4orf6 protein inhibits DNA double strand break repair and radiosensitizes human tumor cells in an E1B-55K-independent manner." J Biol Chem **280**(2): 1474-1481.
- Hayden, M. S. and S. Ghosh (2012). "NF-kappaB, the first quarter-century: remarkable progress and outstanding questions." Genes Dev **26**(3): 203-234.
- Higgins, C. D., I. dos-Santos-Silva, C. A. Stiller and A. J. Swerdlow (2001). "Season of birth and diagnosis of children with leukaemia: an analysis of over 15 000 UK cases occurring from 1953-95." Br J Cancer **84**(3): 406-412.
- Hong, D., R. Gupta, P. Ancliff, A. Atzberger, J. Brown, S. Soneji, J. Green, S. Colman, W. Piacibello, V. Buckle, S. Tsuzuki, M. Greaves and T. Enver (2008). "Initiating and Cancer-Propagating Cells in *TEL-AML1*-Associated Childhood Leukemia." Science **319**(5861): 336-339.
- Honkaniemi, E., G. Talekar, W. Huang, G. Bogdanovic, E. Forestier, U. von Döbeln, M. Engvall, D. A. Ornelles, L. R. Gooding and B. Gustafsson (2010). "Adenovirus DNA in Guthrie cards from children who develop acute lymphoblastic leukaemia (ALL)." Br J Cancer **102**(5): 796-798.
- Horwitz, G. A., K. Zhang, M. A. McBrien, M. Grunstein, S. K. Kurdistani and A. J. Berk (2008). "Adenovirus small e1a alters global patterns of histone modification." Science **321**(5892): 1084-1085.
- Howlander N, N. A., Krapcho M, Miller D, Bishop K, Kosary CL, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, . (2016). "SEER Cancer Statistics Review, 1975-2014." Retrieved April 2017, from https://seer.cancer.gov/csr/1975_2014/.
- Huang, C., M. Xu and B. Zhu (2013). "Epigenetic inheritance mediated by histone lysine methylation: maintaining transcriptional states without the precise restoration of marks?" Philos Trans R Soc Lond B Biol Sci **368**(1609): 20110332.
- Huang, D. C., S. Cory and A. Strasser (1997). "Bcl-2, Bcl-XL and adenovirus protein E1B19kD are functionally equivalent in their ability to inhibit cell death." Oncogene **14**(4): 405-414.
- Huang, M. M. and P. Hearing (1989). "Adenovirus early region 4 encodes two gene products with redundant effects in lytic infection." J Virol **63**(6): 2605-2615.

- Ichikawa, M., T. Asai, S. Chiba, M. Kurokawa and S. Ogawa (2004). "Runx1/AML-1 ranks as a master regulator of adult hematopoiesis." Cell Cycle **3**(6): 722-724.
- Investigators, U. K. C. C. S. (2001). "Breastfeeding and childhood cancer." Br J Cancer **85**(11): 1685-1694.
- Isa, A., P. Priftakis, K. Broliden and B. Gustafsson (2004). "Human parvovirus B19 DNA is not detected in Guthrie cards from children who have developed acute lymphoblastic leukemia." Pediatr Blood Cancer **42**(4): 357-360.
- Jimenez-Morales, S., E. Miranda-Peralta, Y. Saldana-Alvarez, P. Perez-Vera, R. Paredes-Aguilera, R. Rivera-Luna, R. Velazquez-Cruz, J. Ramirez-Bello, A. Carnevale and L. Orozco (2008). "BCR-ABL, ETV6-RUNX1 and E2A-PBX1: prevalence of the most common acute lymphoblastic leukemia fusion genes in Mexican patients." Leuk Res **32**(10): 1518-1522.
- Kajtar, P., K. M. Fazekasne and K. Mehes (2003). "[Month of birth in childhood acute lymphoblastic leukemia]." Orv Hetil **144**(38): 1869-1871.
- Kinlen, L. J. (1995). "Epidemiological evidence for an infective basis in childhood leukaemia." Br J Cancer **71**(1): 1-5.
- Knowles, W. A., P. Pipkin, N. Andrews, A. Vyse, P. Minor, D. W. Brown and E. Miller (2003). "Population-based study of antibody to the human polyomaviruses BKV and JCV and the simian polyomavirus SV40." J Med Virol **71**(1): 115-123.
- Kostense, S., W. Koudstaal, M. Sprangers, G. J. Weverling, G. Penders, N. Helmus, R. Vogels, M. Bakker, B. Berkhout, M. Havenga and J. Goudsmit (2004). "Adenovirus types 5 and 35 seroprevalence in AIDS risk groups supports type 35 as a vaccine vector." AIDS **18**(8): 1213-1216.
- Kreis, C., J. E. Lupatsch, F. Niggli, M. Egger, C. E. Kuehni, B. D. Spycher, G. Swiss Paediatric Oncology and G. the Swiss National Cohort Study (2017). "Space-Time Clustering of Childhood Leukemia: Evidence of an Association with ETV6-RUNX1 (TEL-AML1) Fusion." PLoS One **12**(1): e0170020.
- Kuhlmann, I., S. Achten, R. Rudolph and W. Doerfler (1982). "Tumor induction by human adenovirus type 12 in hamsters: loss of the viral genome from adenovirus type 12-induced tumor cells is compatible with tumor formation." EMBO J **1**(1): 79-86.
- Lam, L. T., R. E. Davis, V. N. Ngo, G. Lenz, G. Wright, W. Xu, H. Zhao, X. Yu, L. Dang and L. M. Staudt (2008). "Compensatory IKKalpha activation of classical NF-kappaB signaling during IKKbeta inhibition identified by an RNA interference sensitization screen." Proc Natl Acad Sci U S A **105**(52): 20798-20803.
- Lancashire, R. J., T. Sorahan and Oscc (2003). "Breastfeeding and childhood cancer risks: OSCC data." Br J Cancer **88**(7): 1035-1037.
- Lehnertz, B., Y. Ueda, A. A. Derijck, U. Braunschweig, L. Perez-Burgos, S. Kubicek, T. Chen, E. Li, T. Jenuwein and A. H. Peters (2003). "Suv39h-mediated histone H3 lysine 9 methylation directs DNA methylation to major satellite repeats at pericentric heterochromatin." Curr Biol **13**(14): 1192-1200.
- Lehtinen, M., P. Koskela, H. M. Ogmundsdottir, A. Bloigu, J. Dillner, M. Gudnadottir, T. Hakulinen, A. Kjartansdottir, M. Kvarnung, E. Pukkala, H. Tulinius and T. Lehtinen (2003). "Maternal herpesvirus infections and risk of acute lymphoblastic leukemia in the offspring." Am J Epidemiol **158**(3): 207-213.
- Li, S., A. Szymborski, M. J. Miron, R. Marcellus, O. Binda, J. N. Lavoie and P. E. Branton (2009). "The adenovirus E4orf4 protein induces growth arrest and mitotic catastrophe in H1299 human lung carcinoma cells." Oncogene **28**(3): 390-400.

- Liao, J. B. (2006). "Viruses and Human Cancer." The Yale Journal of Biology and Medicine **79**(3-4): 115-122.
- Lichtinger, M., N. Obier, R. Ingram, R. Hannah, M. Hoogenkamp, M. S. Vijayabaskar, M. Wu, S. A. Assi, D. G. Tenen, D. R. Westhead, V. Kouskoff, G. Lacaud, B. Göttgens and C. Bonifer (2013). "The hematopoietic master regulator RUNX1 reshapes the epigenetic landscape at the onset of hematopoiesis." Epigenetics & Chromatin **6**(Suppl 1): O18-O18.
- Limbourg, F. P., H. Stadtler, G. Chinnadurai, P. A. Baeuerle and M. L. Schmitz (1996). "A hydrophobic region within the adenovirus E1B 19 kDa protein is necessary for the transient inhibition of NF-kappaB activated by different stimuli." J Biol Chem **271**(34): 20392-20398.
- Lin, B., C. Williams-Skipp, Y. Tao, M. S. Schleicher, L. L. Cano, R. C. Duke and R. I. Scheinman (1999). "NF-kappaB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type." Cell Death Differ **6**(6): 570-582.
- Liu, M. T., Y. T. Chang, S. C. Chen, Y. C. Chuang, Y. R. Chen, C. S. Lin and J. Y. Chen (2005). "Epstein-Barr virus latent membrane protein 1 represses p53-mediated DNA repair and transcriptional activity." Oncogene **24**(16): 2635-2646.
- Liu, X. and R. Marmorstein (2007). "Structure of the retinoblastoma protein bound to adenovirus E1A reveals the molecular basis for viral oncoprotein inactivation of a tumor suppressor." Genes Dev **21**(21): 2711-2716.
- Liu, Y., A. L. Colosimo, X. J. Yang and D. Liao (2000). "Adenovirus E1B 55-kilodalton oncoprotein inhibits p53 acetylation by PCAF." Mol Cell Biol **20**(15): 5540-5553.
- Liu, Z. X., S. Govindarajan, S. Okamoto and G. Dennert (2000). "Fas- and tumor necrosis factor receptor 1-dependent but not perforin-dependent pathways cause injury in livers infected with an adenovirus construct in mice." Hepatology **31**(3): 665-673.
- Lowe, S. W. and H. E. Ruley (1993). "Stabilization of the p53 tumor suppressor is induced by adenovirus 5 E1A and accompanies apoptosis." Genes Dev **7**(4): 535-545.
- Ma, X., K. Urayama, J. Chang, J. L. Wiemels and P. A. Buffler (2009). "Infection and pediatric acute lymphoblastic leukemia." Blood Cells Mol Dis **42**(2): 117-120.
- Machitani, M., F. Sakurai, K. Wakabayashi, K. Nakatani, K. Shimizu, M. Tachibana and H. Mizuguchi (2016). "NF-κB promotes leaky expression of adenovirus genes in a replication-incompetent adenovirus vector." **6**: 19922.
- Mahjour, S. B., F. Ghaffarpasand, M. J. Fattahi, A. Ghaderi, A. Fotouhi Ghiam and M. Karimi (2010). "Seroprevalence of human herpes simplex, hepatitis B and epstein-barr viruses in children with acute lymphoblastic leukemia in southern iran." Pathol Oncol Res **16**(4): 579-582.
- Mahr, J. A., J. M. Boss and L. R. Gooding (2003). "The Adenovirus E3 Promoter Is Sensitive to Activation Signals in Human T Cells." Journal of Virology **77**(2): 1112-1119.
- Maia Rda, R. and V. Wunsch Filho (2013). "Infection and childhood leukemia: review of evidence." Rev Saude Publica **47**(6): 1172-1185.
- Markel, D., E. Lam, G. Harste, S. Darr, M. Ramke and A. Heim (2014). "Type dependent patterns of human adenovirus persistence in human T-lymphocyte cell lines." J Med Virol **86**(5): 785-794.
- Marshall, L. J., A. C. Moore, M. Ohki, I. Kitabayashi, D. Patterson and D. A. Ornelles (2008). "RUNX1 permits E4orf6-directed nuclear localization of the adenovirus E1B-55K protein and associates with centers of viral DNA and RNA synthesis." J Virol **82**(13): 6395-6408.
- Martinez, N., B. Drescher, H. Riehle, C. Cullmann, H.-P. Vornlocher, A. Ganser, G. Heil, A. Nordheim, J. Krauter and O. Heidenreich (2004). "The oncogenic fusion protein RUNX1-

- CBFA2T1 supports proliferation and inhibits senescence in t(8;21)-positive leukaemic cells." BMC Cancer **4**: 44-44.
- Mast, T. C., L. Kierstead, S. B. Gupta, A. A. Nikas, E. G. Kallas, V. Novitsky, B. Mbewe, P. Pitisuttithum, M. Schechter, E. Vardas, N. D. Wolfe, M. Aste-Amezaga, D. R. Casimiro, P. Coplan, W. L. Straus and J. W. Shiver (2010). "International epidemiology of human pre-existing adenovirus (Ad) type-5, type-6, type-26 and type-36 neutralizing antibodies: correlates of high Ad5 titers and implications for potential HIV vaccine trials." Vaccine **28**(4): 950-957.
- McKinney, P. A., E. Juszczak, E. Findlay, K. Smith and C. S. Thomson (1999). "Pre- and perinatal risk factors for childhood leukaemia and other malignancies: a Scottish case control study." Br J Cancer **80**(11): 1844-1851.
- McNally, R. J., F. E. Alexander and J. M. Birch (2002). "Space-time clustering analyses of childhood acute lymphoblastic leukaemia by immunophenotype." Br J Cancer **87**(5): 513-515.
- McNally, R. J. and T. O. Eden (2004). "An infectious aetiology for childhood acute leukaemia: a review of the evidence." Br J Haematol **127**(3): 243-263.
- McNeil, D. E., T. R. Cote, L. Clegg and A. Mauer (2002). "SEER update of incidence and trends in pediatric malignancies: acute lymphoblastic leukemia." Med Pediatr Oncol **39**(6): 554-557; discussion 552-553.
- Meltzer, A. A., J. F. Annegers and M. R. Spitz (1996). "Month-of-birth and incidence of acute lymphoblastic leukemia in children." Leuk Lymphoma **23**(1-2): 85-92.
- Michaelis, M., H. W. Doerr and J. Cinatl (2009). "The story of human cytomegalovirus and cancer: increasing evidence and open questions." Neoplasia **11**(1): 1-9.
- Mori, H., S. M. Colman, Z. Xiao, A. M. Ford, L. E. Healy, C. Donaldson, J. M. Hows, C. Navarrete and M. Greaves (2002). "Chromosome translocations and covert leukemic clones are generated during normal fetal development." Proc Natl Acad Sci U S A **99**(12): 8242-8247.
- Murali, V. K., D. A. Ornelles, L. R. Gooding, H. T. Wilms, W. Huang, A. E. Tollefson, W. S. Wold and C. Garnett-Benson (2014). "Adenovirus death protein (ADP) is required for lytic infection of human lymphocytes." J Virol **88**(2): 903-912.
- Nakagawa, M., M. Shimabe, N. Watanabe-Okochi, S. Arai, A. Yoshimi, A. Shinohara, N. Nishimoto, K. Kataoka, T. Sato, K. Kumano, Y. Nannya, M. Ichikawa, Y. Imai and M. Kurokawa (2011). "AML1/RUNX1 functions as a cytoplasmic attenuator of NF- κ B signaling in the repression of myeloid tumors." Blood **118**(25): 6626-6637.
- Nakao, M., S. Yokota, S. Horiike, M. Taniwaki, K. Kashima, Y. Sonoda, S. Koizumi, Y. Takaue, T. Matsushita, T. Fujimoto and S. Misawa (1996). "Detection and quantification of TEL/AML1 fusion transcripts by polymerase chain reaction in childhood acute lymphoblastic leukemia." Leukemia **10**(9): 1463-1470.
- Nevels, M., B. Tauber, T. Spruss, H. Wolf and T. Dobner (2001). "'Hit-and-run' transformation by adenovirus oncogenes." J Virol **75**(7): 3089-3094.
- Niller, H. H., H. Wolf and J. Minarovits (2011). "Viral hit and run-oncogenesis: genetic and epigenetic scenarios." Cancer Lett **305**(2): 200-217.
- Nwanegbo, E., E. Vardas, W. Gao, H. Whittle, H. Sun, D. Rowe, P. D. Robbins and A. Gambotto (2004). "Prevalence of neutralizing antibodies to adenoviral serotypes 5 and 35 in the adult populations of The Gambia, South Africa, and the United States." Clin Diagn Lab Immunol **11**(2): 351-357.
- Nyari, T. A., P. Kajtar, K. Bartyik, L. Thurzo, R. McNally and L. Parker (2008). "Seasonal variation of childhood acute lymphoblastic leukaemia is different between girls and boys." Pathol Oncol Res **14**(4): 423-428.

- O'Connor, S. M. and R. S. Boneva (2007). "Infectious etiologies of childhood leukemia: plausibility and challenges to proof." Environmental health perspectives: 146-150.
- Okano, M., D. W. Bell, D. A. Haber and E. Li (1999). "DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development." Cell **99**(3): 247-257.
- Ornelles, D. A., L. R. Gooding, M. L. Dickherber, M. Policard and C. Garnett-Benson (2016). "Limited but durable changes to cellular gene expression in a model of latent adenovirus infection are reflected in childhood leukemic cell lines." Virology **494**: 67-77.
- Ornelles, D. A., L. R. Gooding and C. Garnett-Benson (2015). "Neonatal infection with species C adenoviruses confirmed in viable cord blood lymphocytes." PLoS One **10**(3): e0119256.
- Papaemmanuil, E., I. Rapado, Y. Li, N. E. Potter, D. C. Wedge, J. Tubio, L. B. Alexandrov, P. Van Loo, S. L. Cooke, J. Marshall, I. Martincorena, J. Hinton, G. Gundem, F. W. van Delft, S. Nik-Zainal, D. R. Jones, M. Ramakrishna, I. Titley, L. Stebbings, C. Leroy, A. Menzies, J. Gamble, B. Robinson, L. Mudie, K. Raine, S. O'Meara, J. W. Teague, A. P. Butler, G. Cazzaniga, A. Biondi, J. Zuna, H. Kempinski, M. Muschen, A. M. Ford, M. R. Stratton, M. Greaves and P. J. Campbell (2014). "RAG-mediated recombination is the predominant driver of oncogenic rearrangement in ETV6-RUNX1 acute lymphoblastic leukemia." Nat Genet **46**(2): 116-125.
- Parslow, R. C., G. R. Law, R. G. Feltbower and P. A. McKinney (2005). "Childhood leukaemia incidence and the population mixing hypothesis in US SEER data." Br J Cancer **92**(5): 978; author reply 979-980.
- Petridou, E., M. Dalamaga, A. Mentis, A. Skalkidou, M. Moustaki, T. Karpathios, D. Trichopoulos and G. Childhood Haematologists-Oncologists (2001). "Evidence on the infectious etiology of childhood leukemia: the role of low herd immunity (Greece)." Cancer Causes Control **12**(7): 645-652.
- Poole, C., S. Greenland, C. Luetters, J. L. Kelsey and G. Mezei (2006). "Socioeconomic status and childhood leukaemia: a review." Int J Epidemiol **35**(2): 370-384.
- Priftakis, P., T. Dalianis, J. Carstensen, U. Samuelsson, I. Lewensohn-Fuchs, G. Bogdanovic, J. Winiarski and B. Gustafsson (2003). "Human polyomavirus DNA is not detected in Guthrie cards (dried blood spots) from children who developed acute lymphoblastic leukemia." Med Pediatr Oncol **40**(4): 219-223.
- Pui, C. H., J. T. Sandlund, D. Pei, G. K. Rivera, S. C. Howard, R. C. Ribeiro, J. E. Rubnitz, B. I. Razzouk, M. M. Hudson, C. Cheng, S. C. Raimondi, F. G. Behm, J. R. Downing, M. V. Relling and W. E. Evans (2003). "Results of therapy for acute lymphoblastic leukemia in black and white children." JAMA **290**(15): 2001-2007.
- Querido, E., P. Blanchette, Q. Yan, T. Kamura, M. Morrison, D. Boivin, W. G. Kaelin, R. C. Conaway, J. W. Conaway and P. E. Branton (2001). "Degradation of p53 by adenovirus E4orf6 and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex." Genes Dev **15**(23): 3104-3117.
- Rauert-Wunderlich, H., D. Siegmund, E. Maier, T. Giner, R. C. Bargou, H. Wajant and T. Stuhmer (2013). "The IKK inhibitor Bay 11-7082 induces cell death independent from inhibition of activation of NFkappaB transcription factors." PLoS One **8**(3): e59292.
- Reddy, U. M., A. A. Baschat, M. G. Zlatnik, J. A. Towbin, C. R. Harman and C. P. Weiner (2005). "Detection of viral deoxyribonucleic acid in amniotic fluid: association with fetal malformation and pregnancy abnormalities." Fetal Diagn Ther **20**(3): 203-207.

- Rho, J. K., J. H. Kim, J. Yu and S. Y. Choe (2002). "Correlation between cellular localization of TEL/AML1 fusion protein and repression of AML1-mediated transactivation of CR1 gene." Biochemical and biophysical research communications **297**(1): 91-95.
- Rivas, C., A. E. Thlick, C. Parravicini, P. S. Moore and Y. Chang (2001). "Kaposi's sarcoma-associated herpesvirus LANA2 is a B-cell-specific latent viral protein that inhibits p53." J Virol **75**(1): 429-438.
- Rodríguez, E., W. H. Ip, V. Kolbe, K. Hartmann, G. Pilnitz-Stolze, N. Tekin, S. Gómez-Medina, C. Muñoz-Fontela, S. Krasemann and T. Dobner (2017). "Humanized Mice Reproduce Acute and Persistent Human Adenovirus Infection." The Journal of Infectious Diseases **215**(1): 70-79.
- Roman, E., T. Lightfoot, A. G. Smith, M. R. Forman, M. S. Linet, L. Robison, J. Simpson, P. Kaatsch, K. Grell, K. Frederiksen and J. Schuz (2013). "Childhood acute lymphoblastic leukaemia and birthweight: insights from a pooled analysis of case-control data from Germany, the United Kingdom and the United States." Eur J Cancer **49**(6): 1437-1447.
- Roman, E., J. Simpson, P. Ansell, S. Kinsey, C. D. Mitchell, P. A. McKinney, J. M. Birch, M. Greaves, T. Eden and I. United Kingdom Childhood Cancer Study (2007). "Childhood acute lymphoblastic leukemia and infections in the first year of life: a report from the United Kingdom Childhood Cancer Study." Am J Epidemiol **165**(5): 496-504.
- Romana, S. P., H. Poirel, M. Leconiat, M. A. Flexor, M. Mauchauffe, P. Jonveaux, E. A. Macintyre, R. Berger and O. A. Bernard (1995). "High frequency of t(12;21) in childhood B-lineage acute lymphoblastic leukemia." Blood **86**(11): 4263-4269.
- Rubnitz, J. E. and A. T. Look (1998). "Molecular genetics of childhood leukemias." J Pediatr Hematol Oncol **20**(1): 1-11.
- Ruley, H. E. (1983). "Adenovirus early region 1A enables viral and cellular transforming genes to transform primary cells in culture." Nature **304**(5927): 602-606.
- Sandler, A. B. and G. Ketner (1989). "Adenovirus early region 4 is essential for normal stability of late nuclear RNAs." J Virol **63**(2): 624-630.
- Schlehofer, B., M. Blettner, K. Geletneky, H. G. Haaf, P. Kaatsch, J. Michaelis, N. Mueller-Lantzsch, D. Niehoff, B. Winkelspecht, J. Wahrendorf and J. R. Schlehofer (1996). "Sero-epidemiological analysis of the risk of virus infections for childhood leukaemia." Int J Cancer **65**(5): 584-590.
- Schmitz, H., R. Wigand and W. Heinrich (1983). "Worldwide epidemiology of human adenovirus infections." Am J Epidemiol **117**(4): 455-466.
- Schmitz, M. L., A. Indorf, F. P. Limbourg, H. Stadler, E. B. Traenckner and P. A. Baeuerle (1996). "The dual effect of adenovirus type 5 E1A 13S protein on NF-kappaB activation is antagonized by E1B 19K." Mol Cell Biol **16**(8): 4052-4063.
- Schreiner, S., P. Wimmer, P. Groitl, S. Y. Chen, P. Blanchette, P. E. Branton and T. Dobner (2011). "Adenovirus type 5 early region 1B 55K oncoprotein-dependent degradation of cellular factor Daxx is required for efficient transformation of primary rodent cells." J Virol **85**(17): 8752-8765.
- Schuz, J., U. Kaletsch, R. Meinert, P. Kaatsch and J. Michaelis (1999). "Association of childhood leukaemia with factors related to the immune system." Br J Cancer **80**(3-4): 585-590.
- Seder, R. A., P. A. Darrah and M. Roederer (2008). "T-cell quality in memory and protection: implications for vaccine design." Nat Rev Immunol **8**(4): 247-258.
- Sharma, S. and A. Andersson (2009). "Adenovirus E3 proteins help tumors to evade innate and adaptive immune responses." Cancer Biol Ther **8**(12): 1133-1135.

- Shen, Y., H. Zhu and T. Shenk (1997). "Human cytomegalovirus IE1 and IE2 proteins are mutagenic and mediate "hit-and-run" oncogenic transformation in cooperation with the adenovirus E1A proteins." *Proc Natl Acad Sci U S A* **94**(7): 3341-3345.
- Shiramizu, B., Q. Yu, N. Hu, R. Yanagihara and V. R. Nerurkar (2002). "Investigation of TT virus in the etiology of pediatric acute lymphoblastic leukemia." *Pediatr Hematol Oncol* **19**(8): 543-551.
- Shurtleff, S. A., A. Buijs, F. G. Behm, J. E. Rubnitz, S. C. Raimondi, M. L. Hancock, G. C. Chan, C. H. Pui, G. Grosveld and J. R. Downing (1995). "TEL/AML1 fusion resulting from a cryptic t(12;21) is the most common genetic lesion in pediatric ALL and defines a subgroup of patients with an excellent prognosis." *Leukemia* **9**(12): 1985-1989.
- Siddiqui, R., N. Nancy, W. P. Naing, S. Ali, L. Dar, B. K. Khan, R. A. Padua and R. Carr (2010). "Distribution of common genetic subgroups in childhood acute lymphoblastic leukemia in four developing countries." *Cancer Genet Cytogenet* **200**(2): 149-153.
- Simpson, J., A. Smith, P. Ansell and E. Roman (2007). "Childhood leukaemia and infectious exposure: a report from the United Kingdom Childhood Cancer Study (UKCCS)." *Eur J Cancer* **43**(16): 2396-2403.
- Singhal, G., E. Leo, S. K. Setty, Y. Pommier and B. Thimmapaya (2013). "Adenovirus E1A oncogene induces rereplication of cellular DNA and alters DNA replication dynamics." *J Virol* **87**(15): 8767-8778.
- Smith, A., E. Roman, J. Simpson, P. Ansell, N. T. Fear and T. Eden (2006). "Childhood leukaemia and socioeconomic status: fact or artefact? A report from the United Kingdom childhood cancer study (UKCCS)." *Int J Epidemiol* **35**(6): 1504-1513.
- Smith, M. (1997). "Considerations on a possible viral etiology for B-precursor acute lymphoblastic leukemia of childhood." *J Immunother* **20**(2): 89-100.
- Smith, M. A., R. Simon, H. D. Strickler, G. McQuillan, L. A. Ries and M. S. Linet (1998). "Evidence that childhood acute lymphoblastic leukemia is associated with an infectious agent linked to hygiene conditions." *Cancer Causes Control* **9**(3): 285-298.
- Sorensen, H. T., L. Pedersen, J. Olsen and K. Rothman (2001). "Seasonal variation in month of birth and diagnosis of early childhood acute lymphoblastic leukemia." *JAMA* **285**(2): 168-169.
- Soria, C., F. E. Estermann, K. C. Espantman and C. C. O'Shea (2010). "Heterochromatin silencing of p53 target genes by a small viral protein." *Nature* **466**(7310): 1076-1081.
- Speiseder, T., H. Hofmann-Sieber, E. Rodriguez, A. Schellenberg, N. Akyuz, J. Dierlamm, T. Spruss, C. Lange and T. Dobner (2017). "Efficient Transformation of Primary Human Mesenchymal Stromal Cells by Adenovirus Early Region 1 Oncogenes." *J Virol* **91**(1).
- Speiseder, T., M. Nevels and T. Dobner (2014). "Determination of the transforming activities of adenovirus oncogenes." *Methods Mol Biol* **1089**: 105-115.
- Stevenson, P. G., J. S. May, V. Connor and S. Efsthathiou (2010). "Vaccination against a hit-and-run viral cancer." *J Gen Virol* **91**(Pt 9): 2176-2185.
- Stiller, C. A., M. E. Kroll, P. J. Boyle and Z. Feng (2008). "Population mixing, socioeconomic status and incidence of childhood acute lymphoblastic leukaemia in England and Wales: analysis by census ward." *Br J Cancer* **98**(5): 1006-1011.
- Stracker, T. H., C. T. Carson and M. D. Weitzman (2002). "Adenovirus oncoproteins inactivate the Mre11-Rad50-NBS1 DNA repair complex." *Nature* **418**(6895): 348-352.
- Subramanian, T., M. La Regina and G. Chinnadurai (1989). "Enhanced ras oncogene mediated cell transformation and tumorigenesis by adenovirus 2 mutants lacking the C-terminal region of E1a protein." *Oncogene* **4**(4): 415-420.

- Sun, Z. and R. Andersson (2002). "NF-kappaB activation and inhibition: a review." Shock **18**(2): 99-106.
- Thai, M., N. A. Graham, D. Braas, M. Nehil, E. Komisopoulou, S. K. Kurdistani, F. McCormick, T. G. Graeber and H. R. Christofk (2014). "Adenovirus E4ORF1-induced MYC activation promotes host cell anabolic glucose metabolism and virus replication." Cell Metab **19**(4): 694-701.
- Thomas, M., P. Massimi and L. Banks (1996). "HPV-18 E6 inhibits p53 DNA binding activity regardless of the oligomeric state of p53 or the exact p53 recognition sequence." Oncogene **13**(3): 471-480.
- Thompson, M. P. and R. Kurzrock (2004). "Epstein-Barr virus and cancer." Clin Cancer Res **10**(3): 803-821.
- Tollefson, A. E., J. S. Ryerse, A. Scaria, T. W. Hermiston and W. S. Wold (1996). "The E3-11.6-kDa adenovirus death protein (ADP) is required for efficient cell death: characterization of cells infected with adp mutants." Virology **220**(1): 152-162.
- Turnell, A. S. and R. J. Grand (2012). "DNA viruses and the cellular DNA-damage response." J Gen Virol **93**(Pt 10): 2076-2097.
- Turner, R. L., P. Groitl, T. Dobner and D. A. Ornelles (2015). "Adenovirus replaces mitotic checkpoint controls." J Virol **89**(9): 5083-5096.
- Turner, R. L., J. C. Wilkinson and D. A. Ornelles (2014). "E1B and E4 oncoproteins of adenovirus antagonize the effect of apoptosis inducing factor." Virology **456-457**: 205-219.
- Urayama, K. Y., X. Ma and P. A. Buffler (2008). "Exposure to infections through day-care attendance and risk of childhood leukaemia." Radiat Prot Dosimetry **132**(2): 259-266.
- van Dongen, J. J., E. A. Macintyre, J. A. Gabert, E. Delabesse, V. Rossi, G. Saglio, E. Gottardi, A. Rambaldi, G. Dotti, F. Griesinger, A. Parreira, P. Gameiro, M. G. Diaz, M. Malec, A. W. Langerak, J. F. San Miguel and A. Biondi (1999). "Standardized RT-PCR analysis of fusion gene transcripts from chromosome aberrations in acute leukemia for detection of minimal residual disease. Report of the BIOMED-1 Concerted Action: investigation of minimal residual disease in acute leukemia." Leukemia **13**(12): 1901-1928.
- Vasconcelos, G. M., M. Kang, M. S. Pombo-de-Oliveira, J. D. Schiffman, F. Lorey, P. Buffler and J. L. Wiemels (2008). "Adenovirus detection in Guthrie cards from paediatric leukaemia cases and controls." Br J Cancer **99**(10): 1668-1672.
- Vetter, M. R., R. Staggemeier, A. Dalla Vecchia, A. Henzel, C. Rigotto and F. R. Spilki (2015). "Seasonal variation on the presence of adenoviruses in stools from non-diarrheic patients." Braz J Microbiol **46**(3): 749-752.
- Wang, X. W., K. Forrester, H. Yeh, M. A. Feitelson, J. R. Gu and C. C. Harris (1994). "Hepatitis B virus X protein inhibits p53 sequence-specific DNA binding, transcriptional activity, and association with transcription factor ERCC3." Proc Natl Acad Sci U S A **91**(6): 2230-2234.
- Wartenberg, D., D. Schneider and S. Brown (2004). "Childhood leukaemia incidence and the population mixing hypothesis in US SEER data." Br J Cancer **90**(9): 1771-1776.
- Weiden, M. D. and H. S. Ginsberg (1994). "Deletion of the E4 region of the genome produces adenovirus DNA concatemers." Proc Natl Acad Sci U S A **91**(1): 153-157.
- Weitzman, M. D., C. T. Carson, R. A. Schwartz and C. E. Lilley (2004). "Interactions of viruses with the cellular DNA repair machinery." DNA Repair (Amst) **3**(8-9): 1165-1173.
- White, E. (1998). Regulation of apoptosis by adenovirus E1A and E1B oncogenes. Seminars in VIROLOGY, Elsevier.

- Whyte, P., K. J. Buchkovich, J. M. Horowitz, S. H. Friend, M. Raybuck, R. A. Weinberg and E. Harlow (1988). "Association between an oncogene and an anti-oncogene: the adenovirus E1A proteins bind to the retinoblastoma gene product." Nature **334**(6178): 124-129.
- Wiemels, J. (2012). "Perspectives on the causes of childhood leukemia." Chem Biol Interact **196**(3): 59-67.
- Williams, J. L., J. Garcia, D. Harrich, L. Pearson, F. Wu and R. Gaynor (1990). "Lymphoid specific gene expression of the adenovirus early region 3 promoter is mediated by NF-kappa B binding motifs." Embo j **9**(13): 4435-4442.
- Wold, W. S. M. and A. E. Tollefson (1998). "Adenovirus E3 Proteins: 14.7K, RID, and gp19K Inhibit Immune-Induced Cell Death; Adenovirus Death Protein Promotes Cell Death." Seminars in Virology **8**(6): 515-523.
- Woo, H. Y., D. W. Kim, H. Park, K. W. Seong, H. H. Koo and S. H. Kim (2005). "Molecular cytogenetic analysis of gene rearrangements in childhood acute lymphoblastic leukemia." J Korean Med Sci **20**(1): 36-41.
- Xiang, Y., Q. Qiu, M. Jiang, R. Jin, B. D. Lehmann, D. W. Strand, B. Jovanovic, D. J. DeGraff, Y. Zheng, D. A. Yousif, C. Q. Simmons, T. C. Case, J. Yi, J. M. Cates, J. Virostko, X. He, X. Jin, S. W. Hayward, R. J. Matusik, A. L. George, Jr. and Y. Yi (2013). "SPARCL1 suppresses metastasis in prostate cancer." Mol Oncol **7**(6): 1019-1030.
- Yamagata, T., K. Maki and K. Mitani (2005). "Runx1/AML1 in normal and abnormal hematopoiesis." Int J Hematol **82**(1): 1-8.
- Yan, M., S. A. Burel, L. F. Peterson, E. Kanbe, H. Iwasaki, A. Boyapati, R. Hines, K. Akashi and D. E. Zhang (2004). "Deletion of an AML1-ETO C-terminal NcoR/SMRT-interacting region strongly induces leukemia development." Proc Natl Acad Sci U S A **101**(49): 17186-17191.
- Yan, M., E. Kanbe, L. F. Peterson, A. Boyapati, Y. Miao, Y. Wang, I. M. Chen, Z. Chen, J. D. Rowley, C. L. Willman and D. E. Zhang (2006). "A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis." Nat Med **12**(8): 945-949.
- Yew, P. R. and A. J. Berk (1992). "Inhibition of p53 transactivation required for transformation by adenovirus early 1B protein." Nature **357**(6373): 82.
- Young, L. S. and P. G. Murray (2003). "Epstein-Barr virus and oncogenesis: from latent genes to tumours." Oncogene **22**(33): 5108-5121.
- Zelent, A., M. Greaves and T. Enver (2004). "Role of the TEL-AML1 fusion gene in the molecular pathogenesis of childhood acute lymphoblastic leukaemia." Oncogene **23**(24): 4275-4283.
- Zerler, B., B. Moran, K. Maruyama, J. Moomaw, T. Grodzicker and H. E. Ruley (1986). "Adenovirus E1A coding sequences that enable ras and pmt oncogenes to transform cultured primary cells." Mol Cell Biol **6**(3): 887-899.
- Zhang, Y., W. Huang, D. A. Ornelles and L. R. Gooding (2010). "Modeling Adenovirus Latency in Human Lymphocyte Cell Lines." Journal of Virology **84**(17): 8799-8810.
- Zoppoli, G., M. Regairaz, E. Leo, W. C. Reinhold, S. Varma, A. Ballestrero, J. H. Doroshov and Y. Pommier (2012). "Putative DNA/RNA helicase Schlafen-11 (SLFN11) sensitizes cancer cells to DNA-damaging agents." Proc Natl Acad Sci U S A **109**(37): 15030-15035.
- zur Hausen, H. (1991). "Viruses in human cancers." Science **254**(5035): 1167-1173.

APPENDICES

Appendix A

Provisional patent: COMPOSITIONS AND METHODS FOR IMPROVED MONITORING AND TREATMENT OF LEUKEMIA

Received from email to Hans Wilms from Stacey Brandenburg of the Georgia State University Office of Legal Affairs, Apr 25, 2017. GSURF tech ID #2017-12PROV1.

COMPOSITIONS AND METHODS FOR IMPROVED MONITORING AND TREATMENT OF LEUKEMIA

FIELD OF THE INVENTION

The disclosed invention is generally in the field of diagnostic methods and kits related to leukemia and specifically in the area of precursor B-cell acute lymphoblastic leukemia in children.

BACKGROUND OF THE INVENTION

While cancer itself is not infectious, infectious agents like human papilloma virus have been identified as oncogenic agents. Known human oncogenic DNA viruses include human papilloma viruses, hepatitis B and C viruses, Merkel cell polyomavirus, and human herpes viruses-4 (HHV4, also known as Epstein-Barr virus), HHV6 and HHV8 (also known as Kaposi's

sarcoma human virus) (Liao, JB, Yale J Biol Med, 2006. 79(3-4): p. 115-22; zur Hausen, Z, Science, 1991. 254(5035): p. 1167-73; Feng, H et al., Science, 2008. 319(5866): p. 1096-100). Two common processes are seen in the mechanisms of oncogenesis by these viruses. The first common process is that each virus is associated with the progression of particular types of cancer derived from cells that the viruses persistently infect. This persistence can be due either to viral latency programs or infection of cell types in which viral gene expression is so limited that it hinders lytic viral replication or results in chronic infection. For example, hepatitis B and C oncogenesis is restricted to hepatocytes, which are chronically infected, leading to chronic liver inflammation and liver cancer (Bialecki, ES et al., Eur J Gastroenterol Hepatol, 2005. 17(5): p. 485-9; Fattovich, G et al., Gastroenterology, 2004. 127(5 Suppl 1): p. S35-50). HHV4 causes cancer in B-lymphocytes during latency by promoting cell proliferation via LMP-1 and other viral genes (Young, LS et al, Oncogene, 2003. 22(33): p. 5108-21; Thompson, MP et al., Clinical Cancer Research 2004. 10(3): p. 803-821). The second common process that all of these viruses share is the disruption of normal p53 or Rb tumor suppressor activity as part of their normal life cycle (Borchert, S et al., J Virol, 2014. 88(6): p. 3144-60; Thomas, M, et al., Oncogene, 1996. 13(3): p. 471-80; Wang, XW et al., Proc Natl Acad Sci U S A, 1994. 91(6): p. 2230-4; Bittar, C et al., PLoS One, 2013. 8(4): p. e62581; Liu, MT et al., Oncogene, 2005. 24(16): p. 2635-46; Doniger, J et al., Clin Microbiol Rev, 1999. 12(3): p. 367-82; Rivas, C et al., Journal of Virology, 2001. 75(1): p. 429-438).

Some cancers have well known infectious causes, such as the aforementioned liver cancer caused by hepatitis infection or cervical cancer caused by human papilloma virus. Others cancers with infectious etiologies have been identified, but still lack a culprit. For example, epidemiological evidence points towards an infectious basis of childhood acute lymphoblastic

leukemia (cALL), with common precursor B-cell ALL is particularly suspected of having an infectious causation, yet no infectious agent has been identified to date that is definitively associated with the disease (Greaves, MF et al., *Leukemia*, 1993. 7(3): p. 349-60; Kinlen, LJ *Br J Cancer*, 1995. 71(1): p. 1-5). ALLs are typically associated with distinct chromosomal abnormalities resulting from dsDNA breaks that characterize these leukemias. The most common translocation in these leukemias is t(12;21), which found in ~25% of B-cell ALL cases and results in the fusion protein ETV6/RUNX1 (TEL-AML1) (Romana, SP et al., *Blood*, 1995. 86(11): p. 4263-9; Fears, S et al., *Genes Chromosomes Cancer*, 1996. 17(2): p. 127-35; Zelent, A et al., *Oncogene*, 2004. 23(24): p. 4275-83). ETV6/RUNX1 is found in 1% of healthy neonates. Of these, about 1 in 8000 will develop leukemia (reviewed in Ma, X et al., *Blood Cells Mol Dis*, 2009. 42(2): p. 117-20).

Kinlen and Smith have hypothesized that the infectious etiology of childhood ALL is a rare response to viral infections near birth, likely in utero (Kinlen LJ *Cancer Causes & Control*, 1998. 9(3): p. 237-239; Smith M, J *Immunother*, 1997. 20(2): p. 89-100). Later studies have supported this proposed pattern. While many viruses have been investigated, no correlation between any in utero viruses and childhood ALL has emerged.

Thus, compositions and methods for early identification of subjects with a risk of developing acute lymphoblastic leukemia are needed.

It is an object of the present invention to provide compositions and methods for identifying subjects with a risk of developing leukemia, such as acute lymphoblastic leukemia, especially in children aged 12 and under.

It is a further object of the present invention to provide compositions and methods that help select and monitor treatment regimens for patients diagnosed with leukemia, such as ALL.

BRIEF SUMMARY OF THE INVENTION

Disclosed are methods and compositions for assessing the presence or risk of developing leukemia in a subject. The methods generally involve measuring the level of CXADR of B lymphocytes in a sample from a subject. It has been discovered that a reduced level of CXADR measured in the sample compared to the level of CXADR in a control indicates that the subject has, or is at elevated risk of developing, leukemia in cells containing chromosomal abnormalities or translocations. This indication is particularly relevant to the presence or risk of developing B-precursor acute lymphoblastic leukemia (ALL).

Thus, for example, disclosed are methods for identifying a subject having an elevated risk of developing B-precursor acute lymphoblastic leukemia (ALL) comprising the steps of (a) measuring the level of CXADR of B lymphocytes in a sample from a subject; (b) comparing the amount of CXADR in the sample to a control; and (c) determining whether the sample has a reduced level of CXADR compared to the control, and optionally producing a CXADR assay output.

The disclosed methods can further comprise the step of measuring the amount of SPARCL1 in the sample, and comparing the level of SPARCL1 in the sample to a control, determining whether the sample has a reduced level of SPARCL1 compared to the control, and optionally producing an assay output.

Optionally, the methods can further comprise the step of measuring the amount of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample, and comparing the level of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample to a control, and optionally producing an assay output.

The disclosed methods can identify subject as at risk of developing ALL, where the level of CXADR is less than the control, preferably at least 50%. The disclosed methods can identify subject as at risk of developing ALL with a confidence level of at least a 50%, 60%, 70%, 80%, 90%, 95%, 97%, or 99%.

Methods of treating a subject diagnosed with ALL are also described. Method of selecting a treatment strategy for subjects suffering from B-precursor acute lymphoblastic leukemia (ALL) comprise (a) measuring the level of CXADR and/or SPARCL1 of B lymphocytes in a sample from a subject; (b) comparing the amount of CXADR and/or SPARCL1 in the sample to a control; and (c) determining whether the sample has a reduced level of CXADR and/or SPARCL1 compared to the control; (d) treating the subject with a drug including one or more histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors.

The disclosed subjects can be a child less than 18 year of age, preferably less than 5 years of age. The disclosed sample can be a bone marrow sample.

The step of measuring can comprise measuring the amount of protein or nucleic acid in the sample. Levels can be measured by performing an ELISA assay or a hybridization assay, flow cytometry, RT, PCR, or qPCR assays.

Kits including reagents for assaying the levels of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 of B lymphocytes in a sample from a subject are also described. In some forms, the kits include a negative control.

Additional advantages of the disclosed method and compositions will be set forth in part in the description which follows, and in part will be understood from the description, or may be learned by practice of the disclosed method and compositions. The advantages of the

disclosed method and compositions will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention as claimed.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several embodiments of the disclosed method and compositions and together with the description, serve to explain the principles of the disclosed method and compositions.

[##INVENTORS: PLEASE PROVIDE FIGURES IF ANY##]

DETAILED DESCRIPTION OF THE INVENTION

The disclosed method and compositions may be understood more readily by reference to the following detailed description of particular embodiments and the Example included therein and to the Figures and their previous and following description.

It has been hypothesized that some childhood leukemia may be caused by a viral infection. But while many viruses have been investigated, no correlation between any in utero viruses and childhood ALL has emerged. It was realized that this may be due to viral cell tropism. For example, when Guthrie card blood spots from neonates were assayed via nested PCR for adenoviral DNA, no correlation between adenovirus and progression of childhood ALL emerged. However, Guthrie card blood spots are collected from a heel prick of peripheral blood, where, for example, adenovirus is only found at a ~1% prevalence in children. Yet it is known

that persistent adenovirus infections have ~80% prevalence in the same population when mucosal lymphocytes from tonsils and adenoids are assayed, showing a clear discrepancy between peripheral and mucosal lymphocytes that harbor adenovirus, making correlation in Guthrie cards unreliable (Garnett, CT et al., *J Virol*, 2009. 83(6): p. 2417-28).

Species C adenoviruses infect most people asymptotically in the first few years of life, and appear to be the most common prenatal viral infection in humans as well. These viruses establish long-term, perhaps life-long, latent infections of mucosal lymphocytes as evidenced by the observation of intermittent virus shedding in stool, and that activation of non-virus-producing tonsil lymphocytes *in vitro* results in infectious virus production from most individuals. Adenoviruses are the most frequently detected viruses in human cord blood. It has been speculated that these viruses are candidates for producing the initiating event leading to the development of childhood acute leukemias. However, following initial infection, some lymphocyte cell lines gradually lose expression of viral genes while maintaining the quiescent viral genome for weeks or months with minimal virus replication detected in the cultures, suggesting a latent state. Significantly, expression of the virus receptor (CAR) is lost on the cell surface at late times of infection. Loss of CAR late in infection of lymphocytes is due to absence of CAR mRNA expression, suggesting that adenovirus infection elicits long-lasting changes in cellular gene expression by an epigenetic mechanism.

Mucosal lymphocytes support latent infections of species C adenoviruses. Bardet-Biedl syndrome 9 (BBS9), BCL2/adenovirus E1B 19 kDa interacting protein 3 (BNIP3), BTG family member 3 (BTG3), coxsackie virus and adenovirus receptor (CXADR), schlafen family member 11 (SLFN11), and SPARC-like 1 (hevin) (SPARCL1) were differentially expressed between mock and persistently infected B cells (Ornelles DA et al., *Virology*. 2016 Jul; 494: 67–77).

Most of these genes are associated with oncogenesis or cancer progression. Childhood leukemic B-cell lines resist adenovirus infection and also show reduced expression of CXADR and SPARCL. Thus, in some embodiments, the disclosed methods involve steps of detecting and/or measuring one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1, compared to a control. Methods for determining transcript levels of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 using microarray or PCR are described in Ornelles DA et al., *Virology*. 2016 Jul; 494: 67–77, which is specifically incorporated by reference herein in their entirety.

The disclosed methods and compositions are for detecting cells, specifically, T- and B-lymphocytes, that had been infected by adenovirus, which may increase the likelihood of the cells becoming cancerous in the patients.

The methods, compositions, and kits disclosed herein accomplish this desire because particular cellular markers are now shown to be specifically associated with adenoviral infections even when the viral components are lost in the cells. Thus, the reduction in these targets indicates that an adenoviral infection was experienced in the past. The methods are not specific for any method of detection or assay as these are well known, but rather focus on the specific markers, disclosed herein, in combination or alone, and with the infections they indicate.

Disclosed are a variety of methods each of which can include assaying samples, such as bone marrow sample, and/or blood sample, from a subject, which can in turn produce an assay output, which can be used. The methods can also involve transmitting the assay output to a recipient. Typically the assays can be an in vitro assay, but under certain circumstances could be in vivo as well. Any type of assay for looking at amounts of molecules, such as hybridization assays, flow cytometry, RT, PCR, and qPCR assays involving probes and primers, as well as

ELISA assays and the like, looking at different expression of the molecules. In varying embodiments, the methods can include or utilize binding affinities and complexes, as well as a variety of components. The methods can involve contacting various reagents together, as well as using controls, such as positive controls, and they can involve normalizing as well as standards. In any embodiment disclosed, it is understood that other steps or embodiments can optionally be included or removed. In certain embodiments, the methods can utilize cells and can involve steps of comparing different results or molecules or materials or substances, or any disclosed aspect herein, by for example comparing whether they are higher, or inhibited, lower, reduced, or prevented, for example. The methods can also include the step of obtaining results or samples or the like. The methods can also include the step of determining and diagnosing, as well as looking at the confidence of a particular result or conclusion to determine its accuracy.

The methods typically revolve around acute lymphoblastic leukemia. The methods can also include prescribing treatments, such as a prescription, such as those provided by a physician. The methods can also include treatments and treatment options, of for example chemotherapeutic drugs alone or in co-application with other molecules such as pharmaceuticals or pro-drugs, having pharmacological activity. Treatments can also seek to provide a therapeutically effective amount of a drug.

Disclosed are methods and compositions for assessing the presence or risk of developing leukemia in a subject. The methods generally involve measuring the level of CXADR of B lymphocytes in a sample from a subject. It has been discovered that a reduced level of CXADR measured in the sample compared to the level of CXADR in a control indicates that the subject has, or is at elevated risk of developing, leukemia. This indication is particularly relevant to the presence or risk of developing B-precursor acute lymphoblastic leukemia (ALL).

The disclosed methods of measurement of the level of CXADR can be used in combination with a number of other steps and methods, thus applying the indication established by reduced levels of CXADR to different purposes and goals. In some forms, the method can further include testing the subject for the presence of leukemia (or the leukemic chromosomal abnormalities/translocations) if the subject has a reduced level of CXADR measured in the sample compared to the level of CXADR in the control. In some forms, the method can further include monitoring the subject for the development of leukemia if the subject has a reduced level of CXADR measured in the sample compared to the level of CXADR in the control. In some forms, the method can further include producing a CXADR assay output.

In some forms, the method can further include measuring the amount of SPARCL1 in the sample, comparing the level of SPARCL1 in the sample to a control, determining whether the sample has a reduced level of SPARCL1 compared to the control. In some forms, the method can further include measuring the amount of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample, and comparing the level of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample to a control.

In some forms, the method can further include treating the subject with one or more histone deacetylase (HDAC), one or more DNA methyltransferase (DNMT) inhibitors, or both, if the level of CXADR of one or more B lymphocytes of the subject is less than the control. In some forms, the method can further include discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is the same as the control. In some forms, the method can further include discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control.

In some forms, the method can further include, prior to, following, or both prior to and following the measuring, treating the subject for ALL if the subject has ALL. In some forms, the method can further include, following treating the subject for ALL, measuring the level of CXADR of B lymphocytes in another sample from the subject and discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control. In some forms, the method can further include, following treating the subject for ALL, monitoring the level of CXADR measured in samples from the subject.

Thus, for example, the method can involve treating a subject for leukemia and then measuring the level of CXADR of B lymphocytes in a sample from the subject during the course of treatment and then discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control. In some forms, the method can involve treating a subject for leukemia and then measuring the level of CXADR of B lymphocytes in a sample from the subject following the treatment to monitor the effectiveness of the treatment over time. In such a case, for example, a continued or renewed reduced level of CXADR can indicate relapse or incomplete effectiveness of the treatment. In some forms, the method can involve treating a subject for leukemia and then measuring the level of CXADR of B lymphocytes in a sample from the subject following a course of treatment and then, for example, either administering another course of treatment if the level of CXADR of B lymphocytes of the subject is reduced compared to the level of CXADR in a control or discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control.

As another example, the method can involve first measuring the level of CXADR of B lymphocytes in a sample from the subject, and then treating the subject in order to reduce the risk of the development of leukemia if the subject has a reduced level of CXADR measured

in the sample compared to the level of CXADR in a control, and then again measuring the level of CXADR of B lymphocytes in a sample from the subject during the course of treatment, and then discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control. In some forms, the method can involve treating a subject in order to reduce the risk of the development of leukemia and then measuring the level of CXADR of B lymphocytes in a sample from the subject following the treatment to monitor the effectiveness of the treatment over time. In such a case, for example, a continued or renewed reduced level of CXADR can indicate relapse or incomplete effectiveness of the treatment. In some forms, the method can involve treating a subject in order to reduce the risk of the development of leukemia and then measuring the level of CXADR of B lymphocytes in a sample from the subject following a course of treatment and then, for example, either administering another course of treatment if the level of CXADR of B lymphocytes of the subject remains reduced compared to the level of CXADR in a control or discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control.

In some forms, the method can further include, prior to, following, or both prior to and following the measuring, treating the subject for ALL if the subject has ALL. In some forms, the method can further include, following treating the subject for ALL, measuring the level of CXADR of B lymphocytes in another sample from the subject and discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control. In some forms, the method can further include, following treating the subject for ALL, monitoring the level of CXADR measured in samples from the subject.

In some forms, the method can be used to select a treatment strategy for subjects suffering from B-precursor acute lymphoblastic leukemia (ALL). In some forms, the method can

include measuring the level of CXADR of B lymphocytes in a sample from a subject and administering to the subject one or more histone deacetylase (HDAC), one or more DNA methyltransferase (DNMT) inhibitors, or both, if the measured level of CXADR measured in the sample is reduced compared to the level of CXADR in a control.

In some forms, the method can be used to identify a subject having an elevated risk of developing B-precursor acute lymphoblastic leukemia (ALL). In some forms, the method can include measuring the level of CXADR of B lymphocytes in a sample from a subject, comparing the amount of CXADR in the sample to a control, and determining whether the sample has a reduced level of CXADR compared to the control, and optionally producing a CXADR assay output.

In some forms, the method can be used to monitor a subject having B-precursor acute lymphoblastic leukemia (ALL). In some forms, the method can include treating the subject for ALL, and then performing the method of claim 1.

In some forms, the method can be used to selecting a treatment strategy for subjects suffering from B-precursor acute lymphoblastic leukemia (ALL). In some forms, the method can include measuring the level of CXADR of B lymphocytes in a sample from a subject, comparing the amount of CXADR in the sample to a control, determining whether the sample has a reduced level of CXADR compared to the control, and administering to the subject one or more histone deacetylase (HDAC), one or more DNA methyltransferase (DNMT) inhibitors, or both.

In some forms, the subject can be monitored for the development of leukemia by testing the subject for the presence of leukemia two or more times. In some forms, at least two of the two or more times at which the subject is tested for the presence of leukemia can be separated

by an interval of time. In some forms, the interval of time can be between three and twelve months, inclusive. In some forms, the interval of time can be between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 months and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or 110 weeks, inclusive. In some forms, the interval of time can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or 110 months. Different intervals can be the same length, different lengths, or a combination thereof.

In some forms, the reduced level of CXADR measured in the sample compared to the level of CXADR in a control can be determined by comparing the amount of CXADR measured in the sample to the control and determining whether the sample has a reduced level of CXADR compared to the control.

In some forms, the sample can be a bone marrow sample. In some forms, the measuring can involve measuring the level of CXADR protein in the sample. In some forms, the measuring can be accomplished by performing an enzyme-linked immunosorbent assay (ELISA) or flow cytometry. In some forms, the measuring can be measuring the level of CXADR mRNA in the sample. In some forms, the measuring can be accomplished by performing a hybridization assay or RT, PCR, or qPCR.

In some forms, the control can be a standard. In some forms, the control can be a subject sample wherein the subject does not have leukemia. In some forms, the control can be a subject sample wherein the subject does not have an ALL. In some forms, the subject can be a child less than 18 years of age. In some forms, the subject can be a child less than 5 years of age (such as cord blood collection).

In some forms, the reduced level of CXADR relative to the control indicates that the subject is at risk of developing ALL. In some forms, the level of CXADR can be at least 50% less than the control. In some forms, the subject can be identified as at risk of developing ALL with a confidence level of at least a 50%, 60%, 70%, 80%, 90%, 95%, 97%, or 99%. In some forms, the increased level of both of CXADR and SPARCL1 measured relative to the control indicates that the subject is at risk of developing ALL.

In some forms, the level of CXADR measured in samples from the subject can be monitored by measuring the level of CXADR of B lymphocytes in another sample from the subject two or more times. In some forms, at least two of the two or more times at which the level of CXADR of B lymphocytes is measured can be separated by an interval of time. In some forms, the interval of time can be between three and twelve weeks, inclusive. In some forms, the interval of time can be between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, or 100 weeks and 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or 110 weeks, inclusive. In some forms, the interval of time can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100 or 110 weeks. Different intervals can be the same length, different lengths, or a combination thereof.

Also disclosed are kits that can include, for example, reagents for assaying the level of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 of B lymphocytes in a sample from a subject.

In a variety of situations, ranges can be appropriate for a description of a concentration or the like.

It is to be understood that the disclosed method and compositions are not limited to specific synthetic methods, specific analytical techniques, or to particular reagents unless otherwise specified, and, as such, may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only and is not intended to be limiting.

A. Definitions

The use of the terms “a,” “an,” “the,” and similar referents in the context of describing the presently claimed invention (especially in the context of the claims) are to be construed to cover both the singular and the plural, unless otherwise indicated herein or clearly contradicted by context.

Recitation of ranges of values herein are merely intended to serve as a shorthand method of referring individually to each separate value falling within the range, unless otherwise indicated herein, and each separate value is incorporated into the specification as if it were individually recited herein.

Use of the term “about” is intended to describe values either above or below the stated value in a range of approx. +/- 10%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 5%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 2%; in other embodiments the values may range in value either above or below the stated value in a range of approx. +/- 1%. The preceding ranges are intended to be made clear by context, and no further limitation is implied. All methods described herein can be performed in any suitable order unless otherwise indicated herein or otherwise clearly contradicted by context. The use of any and all examples, or exemplary language (e.g., “such as”) provided herein, is intended

merely to better illuminate the invention and does not pose a limitation on the scope of the invention unless otherwise claimed. No language in the specification should be construed as indicating any non-claimed element as essential to the practice of the invention.

The term “subject” or “individual” refers to animals, especially mammals. For example, a subject can be a vertebrate, more specifically a mammal (e.g., a human, horse, pig, rabbit, dog, sheep, goat, non-human primate, cow, cat, guinea pig or rodent), a fish, a bird or a reptile or an amphibian. The term does not denote a particular age or sex. Thus, adult and newborn subjects, as well as fetuses, whether male or female, are intended to be covered. A patient refers to a subject afflicted with a disease or disorder. The term “patient” includes human and veterinary subjects. Preferred subjects are those in need of treatment with the disclosed compounds, compositions or methods.

"Acute lymphoblastic leukemia", abbreviated "ALL", when used herein encompasses B-precursor ALL or as is also called B-cell precursor ALL (both terms are also included by the abbreviation "ALL") as well as pediatric or childhood ALL as well as ALL in adults, i.e., adult ALL. B-precursor ALL is the most common type of ALL. Acute lymphoblastic leukemia (ALL) is a form of leukemia, or cancer of the white blood cells characterized by excess lymphoblasts. ALL is, inter alia, characterized by continuously multiplying malignant, immature white blood cells that are overproduced in the bone marrow. ALL causes damage and death by crowding out normal cells in the bone marrow, and by spreading (infiltrating) to other organs. ALL is most common in childhood with a peak incidence at 2-5 years of age, and another peak in old age. "Acute" refers to the relatively short time course of the disease (being fatal in as little as a few weeks if left untreated) to differentiate it from the very different disease of chronic lymphocytic leukemia, which has a potential time course of many years. It is interchangeably referred to as

lymphocytic or lymphoblastic. This refers to the cells that are involved, which if they were normal would be referred to as lymphocytes but are seen in this disease in a relatively immature (also termed "blast") state. B-precursor ALL is, in the context of the present invention, a preferred embodiment of ALL. Pediatric or childhood acute lymphoblastic leukemia (ALL) encompasses pediatric B-lineage ALL, preferably pediatric B-precursor acute lymphoblastic leukemia ALL, more preferably pediatric pro-B ALL, pre-B ALL, or common ALL (cALL). Even more preferred the pediatric B-precursor ALL is common ALL (cALL). Pediatric or childhood acute lymphoblastic leukemia (ALL) also encompasses minimal residual disease (MRD) in a pediatric patient with acute lymphoblastic leukemia (ALL).

The terms "prevent," "prevention," and "preventing" means to administer a composition to a subject or a system at risk for or having a predisposition for one or more symptoms caused by a disease or disorder to cause cessation of a particular symptom of the disease or disorder, a reduction or prevention of one or more symptoms of the disease or disorder, a reduction in the severity of the disease or disorder, the complete ablation of the disease or disorder, stabilization or delay of the development or progression of the disease or disorder. Preferably treatment for prevention would be prior to the onset of clinical symptoms of a disease or conditions so as to prevent a physical manifestation of aberrations associated with the disease or condition.

The term "inhibit," "suppress," "decrease," "interfere," and/or "reduce" (and like terms) generally refers to the act of reducing, either directly or indirectly, a function, activity, level, concentration, behavior, etc., relative to the natural, expected, or average or relative to current conditions. It is understood that this is typically in relation to some standard or expected value, in other words it is relative, but that it is not always necessary for the standard or relative

value to be referred to. This can be a complete inhibition, suppression, decrease, interference, and/or reduction of the function, activity, level, concentration, behavior, etc. Inhibition, suppression, decrease, interference, and/or reduction can be compared to a control or to a standard level. Inhibition, suppression, decrease, interference, and/or reduction can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100%.

The term “increase,” “enhance,” “stimulate,” “promote,” and/or “induce” (and like terms) generally refers to the act of improving or increasing, either directly or indirectly, a function, activity, level, concentration, behavior, etc., relative to the natural, expected, or average or relative to current conditions. It is understood that this is typically in relation to some standard or expected value, in other words it is relative, but that it is not always necessary for the standard or relative value to be referred to. Increase, enhancement, stimulation, promotion, and/or induction can be compared to a control or to a standard level. Increase, enhancement, stimulation, promotion, and/or induction can be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, 100, 105, 110, 115, 120, 125, 130, 135, 140, 145, 150, 155, 160, 165, 170, 175, 180, 185, 190, 195, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 320, 325, 340, 350, 360, 375, 380, 400, 425, 450, 475, 500, 550, 600, 650, 700, 750, 800, 850, 900, 103, 104, 105, 106, or 107%.

The terms “treatment” and "treating" mean the medical management of a subject with the intent to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder. This term includes active treatment, that is, treatment directed specifically toward the improvement of a disease, pathological condition, or disorder, and also includes causal treatment, that is, treatment directed toward removal of the cause of the associated disease, pathological condition, or disorder. In addition, this term includes palliative treatment, that is, treatment designed for the relief of symptoms rather than the curing of the disease, pathological condition, or disorder; preventative treatment, that is, treatment directed to minimizing or partially or completely inhibiting the development of the associated disease, pathological condition, or disorder; and supportive treatment, that is, treatment employed to supplement another specific therapy directed toward the improvement of the associated disease, pathological condition, or disorder. It is understood that treatment, while intended to cure, ameliorate, stabilize, or prevent a disease, pathological condition, or disorder, need not actually result in the cure, amelioration, stabilization or prevention. The effects of treatment can be measured or assessed as described herein and as known in the art as is suitable for the disease, pathological condition, or disorder involved. Such measurements and assessments can be made in qualitative and/or quantitative terms. Thus, for example, characteristics or features of a disease, pathological condition, or disorder and/or symptoms of a disease, pathological condition, or disorder can be reduced to any effect or to any amount.

The term “in need of treatment” as used herein refers to a judgment made by a caregiver (e.g. physician, nurse, nurse practitioner, or individual in the case of humans; veterinarian in the case of animals, including non-human mammals) that a subject requires or will benefit from treatment. This judgment is made based on a variety of factors that are in the

realm of a care giver's expertise, but include the knowledge that the subject is ill, or will be ill, as the result of a condition that is treatable by the compounds of the invention. For the disclosed treatment, subjects exhibiting or at risk for acute lymphoblastic leukemia are preferred subjects in need or treatment.

A cell can be in vitro. Alternatively, a cell can be in vivo and can be found in a subject. A "cell" can be a cell from any organism including, but not limited to, a bacterium.

By the term "effective amount" of a compound as provided herein is meant a nontoxic but sufficient amount of the compound to provide the desired result. As will be pointed out below, the exact amount required will vary from subject to subject, depending on the species, age, and general condition of the subject, the severity of the disease that is being treated, the particular compound used, its mode of administration, and the like. Thus, it is not possible to specify an exact "effective amount." However, an appropriate effective amount can be determined by one of ordinary skill in the art using only routine experimentation.

The dosages or amounts of the compounds described herein are large enough to produce the desired effect in the method by which delivery occurs. The dosage should not be so large as to cause adverse side effects, such as unwanted cross-reactions, anaphylactic reactions, and the like. Generally, the dosage will vary with the age, condition, sex and extent of the disease in the subject and can be determined by one of skill in the art. The dosage can be adjusted by the individual physician based on the clinical condition of the subject involved. The dose, schedule of doses and route of administration can be varied.

The efficacy of administration of a particular dose of the compounds or compositions according to the methods described herein can be determined by evaluating the particular aspects of the medical history, signs, symptoms, and objective laboratory tests that are

known to be useful in evaluating the status of a subject in need for the treatment of acute lymphoblastic leukemia or other diseases and/or conditions. These signs, symptoms, and objective laboratory tests will vary, depending upon the particular disease or condition being treated or prevented, as will be known to any clinician who treats such patients or a researcher conducting experimentation in this field. For example, if, based on a comparison with an appropriate control group and/or knowledge of the normal progression of the disease in the general population or the particular individual: (1) a subject's physical condition is shown to be improved (e.g., a tumor has partially or fully regressed), (2) the progression of the disease or condition is shown to be stabilized, or slowed, or reversed, or (3) the need for other medications for treating the disease or condition is lessened or obviated, then a particular treatment regimen will be considered efficacious.

The term "consisting essentially of" limits the referenced material or subject matter to the specified materials or steps "and [to] those that do not materially affect the basic and novel characteristic(s)" of the referenced material or subject matter. In re Herz, 537 F.2d 549, 551-52 (CCPA 1976).

The terms "relative" and "relative to" refer to a condition, measure, level, etc., that is described in terms of a reference, control, or comparator condition, measure, level, etc.

The terms "involved" and "involved in," in the context of a biological process, refer to a biological process that is caused by or that affects a referenced product, condition, or process.

The term "expression," in the context of genes and gene products, refers to the process by which information from a gene is used in the synthesis of a functional gene product.

Expression can be measured in a variety of ways, including, for example, by measuring the level of one or more of the products of expression of the gene.

The term "protein level" refers to the level (e.g., amount, concentration) of the referenced protein(s). The term "transcript level" refers to the level (e.g., amount, concentration) of the referenced transcript(s).

The term "monitoring" as used herein refers to any method in the art by which an activity can be measured.

The term "assaying", "assay", or like terms refers to an analysis to determine a characteristic of a substance, such as a protein, DNA, RNA, or a cell, such as for example, the presence, absence, quantity, extent, kinetics, dynamics, or binding.

The term "cell" as used herein also refers to individual cells, cell lines, or cultures derived from such cells. A "culture" refers to a composition comprising isolated cells of the same or a different type. The term co-culture is used to designate when more than one type of cell are cultured together in the same dish with either full or partial contact with each other.

Throughout the description and claims of this specification, the word "comprise" and variations of the word, such as "comprising" and "comprises," means "including but not limited to," and is not intended to exclude, for example, other additives, components, integers or steps.

Contacting or like terms means bringing into proximity such that a molecular interaction can take place, if a molecular interaction is possible between at least two things, such as molecules, cells, markers, at least a compound or composition, or at least two compositions, or any of these with an article(s) or with a machine. For example, contacting refers to bringing at least two compositions, molecules, articles, or things into contact, i.e. such that they are in proximity to mix or touch. For example, having a solution of composition A and cultured cell B

and pouring solution of composition A over cultured cell B would be bringing solution of composition A in contact with cell culture B.

It is understood that anything disclosed herein can be brought into contact with anything else. For example, a sample can be brought into contact with a reagent, such as an antibody that binds BBS9, BNIP3, BTG3, CXADR, SLFN11, SPARCL1, and so forth.

A “positive control” or like terms is a control that shows that the conditions for data collection can lead to data collection.

The terms control or "control levels" or "control cells" or like terms are defined as the standard by which a change is measured, for example, the controls are not subjected to the experiment, but are instead subjected to a defined set of parameters, or the controls are based on pre- or post-treatment levels. They can either be run in parallel with or before or after a test run, or they can be a pre-determined standard. For example, a control can refer to the results from an experiment in which the subjects or objects or reagents etc. are treated as in a parallel experiment except for omission of the procedure or agent or variable etc. under test and which is used as a standard of comparison in judging experimental effects. Thus, the control can be used to determine the effects related to the procedure or agent or variable etc. For example, if the effect of a test molecule on a cell was in question, one could a) simply record the characteristics of the cell in the presence of the molecule, b) perform a and then also record the effects of adding a control molecule with a known activity or lack of activity, or a control composition (e.g., the assay buffer solution (the vehicle)) and then compare effects of the test molecule to the control. In certain circumstances once a control is performed the control can be used as a standard, in which the control experiment does not have to be performed again and in other circumstances the control experiment should be run in parallel each time a comparison will be made.

“Comparing” or like words or other forms refers to the act of reviewing something in relation to something else.

“Determining” or like words or other forms refers to the act of settling or deciding by choice from different alternatives or possibilities.

The terms different expression and like terms can include any difference including at least a 1%, 5%, 10%, 15%, 20%, 30%, 40%, 50%, 75%, 100%, 300%, 500%, 750%, 1000%, 5000%, 10,000%, or 50,000% difference.

“Optional” or “optionally” means that the subsequently described event, circumstance, or material may or may not occur or be present, and that the description includes instances where the event, circumstance, or material occurs or is present and instances where it does not occur or is not present.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, also specifically contemplated and considered disclosed is the range from the one particular value and/or to the other particular value unless the context specifically indicates otherwise. Similarly, when values are expressed as approximations, by use of the antecedent “about,” it will be understood that the particular value forms another, specifically contemplated embodiment that should be considered disclosed unless the context specifically indicates otherwise. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint unless the context specifically indicates otherwise. It should be understood that all of the individual values and sub-ranges of values contained within an explicitly disclosed range are also specifically contemplated and should be considered disclosed unless the context specifically indicates otherwise. Finally, it should be understood that all ranges refer both to the

recited range as a range and as a collection of individual numbers from and including the first endpoint to and including the second endpoint. In the latter case, any of the individual numbers can be selected as one form of the quantity, value, or feature to which the range refers. In this way, a range describes a set of numbers or values from and including the first endpoint to and including the second endpoint from which a single member of the set (i.e. a single number) can be selected as the quantity, value, or feature to which the range refers. The foregoing applies regardless of whether in particular cases some or all of these embodiments are explicitly disclosed.

Methods

Disclosed are materials, compositions, and components that can be used for, can be used in conjunction with, can be used in preparation for, or are products of the disclosed method and compositions. These and other materials are disclosed herein, and it is understood that when combinations, subsets, interactions, groups, etc. of these materials are disclosed that while specific reference of each various individual and collective combinations and permutation of these compounds may not be explicitly disclosed, each is specifically contemplated and described herein. Thus, if a class of molecules A, B, and C are disclosed as well as a class of molecules D, E, and F and an example of a combination molecule, A-D is disclosed, then even if each is not individually recited, each is individually and collectively contemplated. Thus, in this example, each of the combinations A-E, A-F, B-D, B-E, B-F, C-D, C-E, and C-F are specifically contemplated and should be considered disclosed from disclosure of A, B, and C; D, E, and F; and the example combination A-D. Likewise, any subset or combination of these is also specifically contemplated and disclosed. Thus, for example, the sub-group of A-E, B-F, and C-E are specifically contemplated and should be considered disclosed from disclosure of A, B, and C;

D, E, and F; and the example combination A-D. Further, each of the materials, compositions, components, etc. contemplated and disclosed as above can also be specifically and independently included or excluded from any group, subgroup, list, set, etc. of such materials. These concepts apply to all aspects of this application including, but not limited to, steps in methods of making and using the disclosed compositions. Thus, if there are a variety of additional steps that can be performed it is understood that each of these additional steps can be performed with any specific embodiment or combination of embodiments of the disclosed methods, and that each such combination is specifically contemplated and should be considered disclosed.

A. Methods of Identifying Subjects Predisposed to Developing Acute Lymphoblastic Leukemia

The disclosed methods involve steps of detecting and/or measuring one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1. In some embodiments, the methods involve measuring protein quantities, mRNA levels, and/or epigenetic modifications of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1. The methods disclosed herein can be performed in any combination, or alone. The biomarkers can be measured in any order, or alone as well. In some embodiments, the methods are carried out on lymphocytes, particularly T and B lymphocytes. In some embodiments, the methods are carried out on mucosal lymphocytes from tonsils and adenoids.

In preferred embodiments, the disclosed methods involve steps of detecting and/or measuring CXADR, and/or SPARCL. B lymphocytes that are predisposed to becoming cancerous are therefore tested for their status of CXADR, and/or SPARCL1, including mRNA transcript levels, protein expression levels, and/or epigenetic modifications.

In some embodiments, the methods are performed on infants less than a year of age. In some embodiments, the methods are performed on children 1-10 years of age. In some embodiments, the methods are performed on children 10 or older.

The disclosed methods can comprise the steps of a) collect a sample (e.g., a bone marrow sample) from a subject (e.g., a child at risk of developing ALL); b) measuring the mRNA and/or protein level of CXADR in the sample; c) comparing the amount of CXADR mRNA and/or protein in the sample to a healthy control; and c) determining whether the sample has a reduced level of CXADR mRNA and/or protein compared to the control and/or producing an assay output. If the sample from the subject has reduced levels of CXADR mRNA and/or protein, then the subject is identified as having a risk of developing ALL.

The disclosed methods can further comprise the steps of measuring the amount of SPARCL1 mRNA and/or protein in the sample, comparing the amount of SPARCL1 mRNA and/or protein in the sample to a control, and determining whether the sample has a decreased level of SPARCL1 mRNA and/or protein compared to the control producing a SPARCL1 assay output.

The methods can further comprise the steps of measuring the amount of mRNA and/or protein of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample, comparing the amount of mRNA and/or protein of one or more of these targets in the sample to a control, and determining whether the sample has a decreased level of mRNA and/or protein of one or more of these targets compared to the control, producing an assay output.

The disclosed methods can involve steps of detecting and/or measuring one or more of chromosomal abnormalities. In some embodiments, the methods involve measuring protein quantities, mRNA levels, and/or epigenetic modifications of one or more chromosomal

abnormalities. Particular chromosomal abnormalities are detected in 60-85% of ALLs, like the ETV6/RUNX1 fusion present in ~25% of BCP-ALLs (Cancer Research, 1983. 43(2): p. 868-873; Blood, 1996. 87(8): p. 3135-42; Hematological oncology, 1989. 7(4): p. 307-317; Recent Results Cancer Res, 1993. 131: p. 133-48; Br J Haematol, 1997. 96(3): p. 601-10). Thus, in some embodiments, the disclosed methods involve steps of detecting and/or measuring ETV6/RUNX1 in a patient.

In one embodiment, the methods include the step of obtaining a subject sample. For example, this step could be performed by someone other than the person or machine measuring the levels of the targets. Obtaining the sample can include obtaining the sample directly from the subject or obtaining the sample from a storage area.

In certain embodiments, disclosed are methods that include the step of obtaining the assay output, and prescribing a treatment option for the subject in a prescription if the amount of the BBS9, BNIP3, BTG3, CXADR, SLFN11, SPARCL1, or any combination thereof is less than the control. Also disclosed are methods that comprise the step of obtaining the prescription and undertaking the treatment. In addition, methods are disclosed which include filling the prescription, so obtained, for a subject.

Regardless of which biomarker or combination of biomarkers is originally measured, the methods can further comprise the step of determining the amount of the CXADR is less than the control.

The levels of the biomarkers in the disclosed methods can be reduced by about 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or more than 90% compared to the control levels. In some forms the biomarker levels can be at least 1.1x, 1.5x, 2x, 2.5x, 3x, 4x, 5x, 6x, 7x, 8x, 9x, 10x, 20x, 30x, 40x, 50x, 60x, 70x, 80x, 90x, 100x, 200x, 300x, 400x, 500x, 1000x

fold reduction compared to the control levels. In one embodiment, the level of CXADR levels can be at least 6-fold decrease compared to the control levels.

The sample used in the disclosed methods can be a blood sample or bone marrow sample.

In some forms, the methods can comprise performing an assay. The assay can be done to measure the levels of the specific biomarker of interest. The assay can include but is not limited to an ELISA, radioimmunoassay (RIA), western blot, and dot blot.

The measuring step comprises measuring the amount of biomarker mRNA in the sample. The samples can be proteins or nucleic acids. The mRNA levels of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 can all be measured. In preferred embodiments, mRNA levels of CXADR, and SPARCL1 are measured. Measuring mRNA levels can comprise performing a hybridization assay or RT, PCR, or qPCR assay. Some exemplary qPCR primers/probes can be found in Ornelles DA et al., *Virology*. 2016 Jul; 494: 67–77 for detecting BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1.

The disclosed methods can comprise a control which can be a standard. The control can comprise a subject sample wherein the subject does not have an ALL. In some embodiments, the control can be a cell line with known genetic background, and/or are free of any adenoviral infection, for example, BJAB Burkitt lymphoma cell line.

In some forms, the methods further comprise the step of transmitting the assay output to a recipient.

Further disclosed are methods wherein decreased levels of BBS9, BNIP3, BTG3, CXADR, SLFN11, or SPARCL1 can provide at least 50%, 60%, 70%, 80%, 90%, or 95% confidence or accuracy of the diagnosis or identification of a subject with an ALL, or at risk for developing ALL. Further disclosed are methods wherein decreased levels of CXADR and

SPARCL1 can provide at least 70% confidence or accuracy of the diagnosis or identification of a subject with ALL, or at risk for developing ALL. Decreased levels of CXADR and SPARCL1 can provide at least 80% confidence or accuracy of diagnosis or identification of a subject with ALL, or at risk for developing ALL. Decreased levels of CXADR and SPARCL1 can provide at least 90% confidence or accuracy of diagnosis or identification of a subject with ALL, or at risk for developing ALL. The confidence or accuracy can be 50%, 60%, 70%, 80%, 90%, 95%, 98%, 99% or 100%.

B. Methods of Risk Classification And Treatment Strategies Subjects Identified as Having Acute Lymphoblastic Leukemia

The disclosed methods are suitable for help guide risk classification and treatment strategies subjects identified as having Acute Lymphoblastic Leukemia (ALL).

As insights are gained into host pharmacogenetics and the heterogeneity of response to chemotherapeutic agents in various leukemia subtypes, and therapy is guided by sensitive and specific methods of MRD monitoring, tailoring the type and intensity of chemotherapeutic agents becomes feasible to improve cure rates and decrease morbidity. The disclosed methods can thus improve event-free survival (EFS) or overall survival (OS) over 5-year or 10-year period in ALL patients by about 1%, 2%, 3%, 4%, 5%, 10%, 20%, or more than 20% compared to general therapy towards general ALL. The disclosed methods can help reduce short and long-term adverse events, for example, decreased the chances of secondary malignancies. In some embodiments, the disclosed methods help reduce the need of prophylactic cranial irradiation (PCI), and/or use of epipodophyllotoxins and alkylating agents.

The disclosed methods thus help guide treatment in patients identified with specific subtypes of ALL, especially those associated with changes in protein and/or gene expression of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1.

The disclosed method for risk-stratifying, and/or selecting treatment options for subjects suffering from B-precursor acute lymphoblastic leukemia (ALL), said subjects are intended for a therapy which comprises one or more histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors, comprising: (a) measuring the level of CXADR in a sample from a subject; (b) comparing the amount of CXADR in the sample to a control; and (c) determining whether the sample has a reduced level of CXADR compared to the control, optionally producing a CXADR assay output, and wherein the subject a reduced level of CXADR indicates the subject is suitable for the therapy. The disclosed method further comprises measuring the level of SPARCL1 in a sample from a subject; (b) comparing the amount of SPARCL1 in the sample to a control; and (c) determining whether the sample has a reduced level of SPARCL1 compared to the control, optionally producing an SPARCL1 assay output, and wherein the subject a reduced level of SPARCL1 indicates the subject is suitable for the therapy.

The disclosed methods involve steps of detecting and/or measuring one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1. The disclosed methods can involve steps of detecting and/or measuring one or more of chromosomal abnormalities such as ETV6/RUNX1. ETV6-RUNX1-positive ALL cells are reported to have an increased in vitro sensitivity to anti-leukemic drugs such as L-asparaginase, doxorubicin, etoposide, and dexamethasone compared to leukemic cells of other cytogenetic subtypes (Frost BM et al., Blood. 2004 Oct 15;104(8):2452–2457 ; Ramakers-van Woerden NL et al, Blood. 2000 Aug 1;96(3):1094–1099.). Asparaginase, dexamethasone, and vincristine are known to be

preferentially cytotoxic to blasts bearing the ETV6-RUNX1 fusion (Krishna Narla R et al., *Leuk Lymphoma*. 2001 May;41(5-6):615–623). Thus, in some embodiments, the ETV6-RUNX1-positive ALL patients are given optimized amount of asparaginase, dexamethasone, etoposide, methotrexate, and/or vincristine.

1. Treatment Strategies

All existing cancer therapies, especially therapies for ALL are suitable. Although the effective dosage may be optimized for ETV6-RUNX1-positive ALL patients, and for ALL patients with reduced expression of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1.

BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 were differentially expressed between mock and persistently infected B cells (Ornelles DA et al., *Virology*. 2016 Jul; 494: 67–77). Histone deacetylase and DNA methyltransferase inhibitors released the repression of some of these genes. Thus, in some embodiments, ALL patients with reduction in one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 are subject to treatment with one or more histone deacetylase (HDAC) and DNA methyltransferase (DNMT) inhibitors.

Exemplary HDAC inhibitors suitable for treatment include hydroxamic acids (or hydroxamates), such as trichostatin A, cyclic tetrapeptides (such as trapoxin B), and the depsipeptides, benzamides, electrophilic ketones, and aliphatic acid compounds such as phenylbutyrate and valproic acid. Additional HDAC inhibitors suitable for treatment include hydroxamic acids vorinostat (SAHA), belinostat (PXD101), LAQ824, and panobinostat (LBH589); and the benzamides: entinostat (MS-275), CI994, and mocetinostat (MGCD0103). The sirtuin Class III HDACs are dependent on NAD⁺ and are, therefore, inhibited by

nicotinamide, as well derivatives of NAD, dihydrocoumarin, naphthopyranone, and 2-hydroxynaphthaldehydes.

Vorinostat (rINN) or suberoylanilide hydroxamic acid (SAHA) is a member of a larger class of compounds that inhibit histone deacetylases (HDAC). Histone deacetylase inhibitors (HDAC inhibitors) have a broad spectrum of epigenetic activities. Vorinostat has been shown to bind to the active site of histone deacetylases and act as a chelator for Zinc ions also found in the active site of histone deacetylases. Vorinostat's inhibition of histone deacetylases results in the accumulation of acetylated histones and acetylated proteins, including transcription factors crucial for the expression of genes needed to induce cell differentiation.

Panobinostat (LBH-589) (Novartis) is an experimental drug developed by Novartis for the treatment of various cancers. It is a hydroxamic acid and acts as a non-selective histone deacetylase inhibitor (HDAC inhibitor). Panobinostat inhibits multiple histone deacetylase enzymes, a mechanism leading to apoptosis of malignant cells via multiple pathways.

Romidepsin (RMD), a histone deacetylase inhibitor approved in the United States for the treatment of T-cell lymphomas.

Belinostat (PXD101) is a histone deacetylase inhibitor for the treatment of hematological malignancies and solid tumors. Belinostat is a HDAC inhibitor affecting class I and II HDACs. Belinostat is administered orally and IV. IV is infused at 400 mg/m² per day. Belinostat is administered orally at 500 mg/m² or 1000 mg/m² once or twice daily.

Aclarubicin (INN) or Aclacinomycin A is an anthracycline drug that is used in the treatment of cancer. Soil bacteria *Streptomyces galilaeus* can produce aclarubicin. The iv dosage initially is 175-300 mg/m², divided over 3-7 consecutive days, with a maintenance dose of 25-100 mg/m² 3-4 weekly.

Apicidin is a HDAC inhibitor affecting class I HDACs. Apicidin is administered orally daily at 10 mg/kg.

BIX-01294, a diazepin-quinazolinamine derivative, is a histone-lysine methyltransferase (HMTase) inhibitor that modulates the epigenetic status of chromatin. BIX-01294 inhibits the G9aHMTase dependent levels of histone-3 lysine (9) methylation (H3K9me).

BML-210 is a histone deacetylase inhibitor. Treatment of A549 cells with BML-210 results in a dose-dependent increase in acetylated histone levels ($EC_{50} = 36 \mu\text{M}$). In HeLa extracts, the IC_{50} for inhibition of HDAC activity is $80 \mu\text{M}$.

CG05/CG06 is a HDAC inhibitor. CG05/CG06 is administered at $0.15 \mu\text{M}$ or $0.3 \mu\text{M}$.

Dihydrocoumarin is a compound found in *Melilotus officinalis* (sweet clover) that is commonly added to food and cosmetics. Dihydrocoumarin is an HDAC inhibitor that disrupts heterochromatic silencing. Dihydrocoumarin is administered orally.

Droxinostat is a HDAC inhibitor affecting class III HDACs. Droxinostat selectively inhibits HDAC3, 6, and 8, with IC_{50} values of $16.9 \mu\text{M}$, $2.47 \mu\text{M}$, and $1.46 \mu\text{M}$, respectively, without inhibiting other HDAC members ($IC_{50} > 20 \mu\text{M}$). Droxinostat is administered IV or IM at 20 or $40 \mu\text{M}$.

Entinostat (MS-275) is an inhibitor of HDAC (histone deacetylase) that preferentially inhibits HDAC1 ($IC_{50} = 300 \text{ nM}$) over HDAC3 ($IC_{50} = 8 \mu\text{M}$). However, MS-275 does not inhibit HDAC8 ($IC_{50} > 100 \mu\text{M}$). Entinostat is administered orally at 10 mg or 15 mg once per day.

Givinostat (ITF2357) is a PAN HDAC inhibitor. Givinostat is administered orally once or twice daily at 50 mg or 100 mg (Rowinsky, et al. JCO December 1986 4 (12):1835-1844).

Oxamflatin is a HDAC inhibitor affecting class I HDACs. Romidepsin (Celgene) is a HDAC inhibitor that affects class I HDACs.

Scriptaid is a PAN HDAC inhibitor. Sodium butyrate is a HDAC inhibitor affecting class I and IIa HDACs.

Suberohydroxamic acid (SBHA) is a competitive HDAC inhibitor that affects HDAC classes I and III. SBHA has been shown to cause cell differentiation, cell cycle arrest, and apoptosis. SBHA inhibits HDAC1 with an $IC_{50} = 0.25 \mu M$ and HDAC3 with an $IC_{50} = 0.3 \mu M$.

Trichostatin A (TsA) is a PAN HDAC inhibitor. Valproic acid (VPA) is a PAN HDAC inhibitor.

Exemplary DNMT inhibitors suitable for treatment include azacytidine (i.e., VIDAZA®), 5-aza-2'-deoxycytidine (i.e., decitabine), and zebularine. Zebularine is more stable than 5-azacytidine or decitabine and may also be less toxic. Some nonnucleoside compounds can also inhibit DNA methyltransferase activity. These substances directly block DNA methyltransferase activity and therefore do not appear to have the inherent toxicity caused by the covalent trapping of the enzyme. One nonnucleoside DNA methyltransferase inhibitor is epigallocatechin-3-gallate (EGCG), the main polyphenol compound in green tea. EGCG is administered orally once daily at 800 mg. Further nonnucleoside DNA methyltransferase inhibitor includes RG108, a small-molecule inhibitor of human DNA methyltransferases. The group of nonnucleoside DNA methyltransferase inhibitors also contains three additional classes of less well-characterized compounds: 1) 4-Aminobenzoic acid derivatives, such as the antiarrhythmic drug procainamide and the local anesthetic procaine; 2) The psammaplins which also inhibit histone deacetylase activity; 3) The third class of DNA methyltransferase inhibitors are oligonucleotides, including hairpin loops and specific antisense oligonucleotides, such as MG98.

C. Methods of Monitoring Acute Lymphoblastic Leukemia

The disclosed methods include methods of monitoring a subject having acute lymphoblastic leukemia comprising treating the subject for acute lymphoblastic leukemia, and then performing any of the disclosed methods to monitor progress of ALL treatment, and/or to detect any relapse following the treatment.

D. Methods of Determining the Cause of Acute Lymphoblastic Leukemia

The disclosed are methods of determining that acute lymphoblastic leukemia is caused by, or associated with an adenoviral infection. In some embodiments, acute lymphoblastic leukemia is caused by, or associated with an infection by species C adenovirus.

E. Kits

The materials described above as well as other materials can be packaged together in any suitable combination as a kit useful for performing, or aiding in the performance of, the disclosed method. It is useful if the kit components in a given kit are designed and adapted for use together in the disclosed method. For example disclosed are kits for identifying individuals at risk of developing Acute Lymphoblastic Leukemia, the kit comprising antibodies, and/or PCR probes for one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1. The kits also can contain a control.

The disclosed kits can also include instructions on how to use the reagents included in the kits. The kits may be in a form of a test strip that have a spot for a positive reading for one or more of the target genes/proteins together and/or individual spots for positive readings of each gene/protein separately.

Examples

Example 1: ETV6/RUNX1 and RUNX1/MGT8 reduce adenovirus both late and early gene expression in infected B cells

2. Methods and Materials

Cell lines

The human cell lines A549 (lung carcinoma) was purchased from the American Type Culture Collection (ATCC, Manassas, VA). BJAB (EBV-negative Burkitt's lymphoma) cells were obtained by Linda Gooding from Carl Ware (La Jolla Institute for Allergy and Immunology). BJAB were grown in RPMI medium supplemented with 10% fetal calf serum (FCS) and 10 mM glutamine. A549 cells were grown in Dulbecco's modified Eagle medium (DMEM) with 4.5 µg of glucose/ml, 10% FCS, and 10 mM glutamine. Cells were tested by Genetica to ensure the absence of mycoplasma and for cell line authentication.

Generating stable cell lines

The cDNA ETV6/RUNX1 and RUNX1/MGT8 fusion genes were subcloned from their original pCMV backbone into the pTARGET plasmid (Promega) using EcoRI restriction enzyme to obtain the neomycin resistance gene for selection of cells expressing the plasmid genes. A549 cells were transfected using Lipofectamine LTX with Plus reagent (ThermoFisher Scientific). Due to poor transfection efficiencies when using Lipofectamine reagents in lymphocyte lines, BJAB cells were transfected using electroporation as described in Mchichi et al (El Mchichi, B., et al., *Cell Death Differ*, 2007. 14(10): p. 1826-36), with minor modifications. Briefly, 4×10^6 cells in cold serum-free RPMI were transfected with 2 µg of plasmid by electroporation at 120V, 960 µF using cuvettes with an inner width of 2mm. After 20min of recovery at 37°C, cells were plated into 6-well plates in RPMI supplemented with 10% FBS at 37°C for 48hrs. Cell cultures were then supplemented with 1mg/ml of G-418 (ThermoFisher

Scientific) for over 21 days, a time point when non-transfected cells lost all viable cells. G-418 was maintained at 0.5mg/ml throughout experiments to prevent loss of transfected genes.

Adenoviruses

Wild-type (WT) species C Ad5 adenoviruses was originally obtained from William S. Wold. Mutant virus Ad5dl309 was obtained from Tom Shenk (Princeton University, Princeton, NJ) (Arbogast, T et al., *Disease Models & Mechanisms*, 2015. 8(6): p. 623-634; Attarbaschi, A et al., *Leukemia*, 2007. 21(5): p. 1137-1137). Ad5dl309 is an Ad5 mutant that lacks the genes for E3 RID α and RID β proteins and for the 14,700-molecular-weight protein (14.7K protein).

Infection of lymphocytes with adenovirus

Infection of lymphocyte cell lines with adenovirus was performed as described previously (Flomenberg, P et al., *J Med Virol*, 1997. 51(3): p. 182-8) with minor modifications. Lymphocytes were collected and washed in serum-free (SF) RPMI medium. Cell density was adjusted to 10⁷ cells/ml in SF-RPMI medium. Virus was added to the cell suspension at 50 PFU/cell, spun for 45min at 1000G at 25°C, re-suspended by agitation, and then incubated at 37°C for 1.5hrs while gently flicking the container every 30min. The infected cells were washed three times with RPMI complete medium and then re-suspended in RPMI complete medium at 5x10⁵ cells/ml.

RT-quantitative PCR analysis of viral mRNA expression

RT-qPCR was performed as described previously, with minor modifications. Briefly, total RNA was isolated from cells using the RNeasy Mini Kit (74104, QIAGEN). RNA was treated with Rnase-free DNase (Qiagen Inc. Valencia, CA) on isolation columns, quantified and >100ng were reverse transcribed(RT) into cDNA in 20 μ L reaction volumes using Maxima First Strand cDNA Synthesis Kit for RT-qPCR (ThermoScientific).RT-enzyme negative controls were

included for each reaction. 20µl of MG water were added to each sample cDNA. Predesigned primers and probes (PrimeTime) were obtained from Integrated DNA Technologies (Coralville, IA). Primer and probe sequences are reported in the supplementary material (Table 1). Probes were labeled at the 5' end with 6-carboxyfluorescein (FAM) reporter molecule and contained dual ZEN and Iowa Black quenchers. Each sample was run in duplicate with at least 2 experimental repeats for each virus tested. All analyses were performed via the comparative threshold cycle (Ct) method as previously described (Chroboczek, J et al., *Virology*, 1992. 186(1): p. 280-5). All targets were normalized to the EIF1 housekeeping gene, and set relative to the lowest target expressing sample, which was set to 1.

Quantitative real time PCR analysis of viral DNA maintenance

Infected or control uninfected cells were washed in phosphate-buffered saline (PBS) and lysed in 100 µl of NP-40–Tween lysis buffer containing proteinase K, as described in Garnett et al (Garnett, CT et al., *J Virol*, 2002. 76(21): p. 10608-16). Samples were tested by real-time PCR for a region of hexon gene that is conserved among species C adenovirus serotypes and the endogenous gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH), using the same primers, probes and run method as described in Garnett et al. Each sample was run in duplicate from 2-3 biological repeats. Data was analyzed according to the comparative threshold cycle (Ct) method, as described above. The amount of hexon gene DNA was normalized to the amount of GAPDH and set relative to the first time point measured past the initial viral infection peak.

RT-PCR detection of ETV6/RUNX1 and RUNX1/MGT8 expression

Total RNA was isolated from BJAB cells transfected with pTARGET expressing the ETV6/RUNX1 or RUNX1/MGT8 fusion genes and converted into cDNA as described above. The presence of ETV6/RUNX1 and RUNX1/MGT8 mRNA transcripts was tested by PCR

amplification and analysis by running the products on a 2% agarose gel. The primers used for detection of the ETV6/RUNX1 fusion gene transcript were the TEL-H and AML1-G primers described in (Guruprasad, B et al., *Pediatr Blood Cancer*, 2014. 61(9): p. 1616-9), yielding a product of 294-334bp. The primers used for detection of RUNX1/MGT8 fusion gene transcripts were AML1-A and ETO-B described in Dongen et al (Kwon, H et al., *Blood Res*, 2016. 51(4): p. 281-285), yielding a product of 395bp. RNA extracted from UOCB4 cells, which naturally express the ETV6/RUNX1 fusion protein (Prakash, G et al., *Indian J Med Paediatr Oncol*, 2012. 33(4): p. 236-8); KASUMI-1, which naturally express the RUNX1/MGT8 fusion gene (Heath, JL et al., *J Pediatr Hematol Oncol*, 2012. 34(4): p. e161-3); or A549 cells transfected with the same plasmid was used as positive controls.

Flow cytometry

Mouse monoclonal IgG1, κ antibody to the adenovirus hexon protein (MAB8051, Chemicon International/Millipore) was used as a primary antibody. Mouse isotype was used as a primary IgG1, κ , antibody control (557273, BD Pharmingen). The secondary antibody was goat anti-mouse IgG-APC conjugate (A865, Life Technologies). Intracellular staining for hexon was performed as described previously (Zhang, Y et al., *Journal of Virology*, 2010. 84(17): p. 8799-8810), with minor modifications. Results were analyzed on a LRS Fortessa flow cytometer (fluorescence-activated cell sorter [FACS]) with FACSDiva Software (BD Biosciences). Hexon-positive cells were defined by cells tabulated in the region defined APC+ based on gating on a mock infected culture (Fig. 2).

Western Blot detection of ETV6/RUNX1 and RUNX1/MGT8 expression

A total of 4-6x10⁶ uninfected or infected BJAB cells were collected and washed in cold PBS, the cell pellet was resuspended in 1ml of cold RIPA buffer (R0278, Sigma) supplemented

with 1mM EDTA (161-0729, BioRad) and protease/phosphatase inhibitor (1861281, Thermo Scientific), incubated on ice for 30min, sonicated briefly, and then boiled for 5min with equal amounts of 2XLaemmli sample buffer (161-0737, Bio-Rad) before electrophoresis through an SDS-PAGE gel. The separated proteins were transferred to a nitrocellulose membrane (456-1024, BioRad). Immunoblotting of RUNX1 and RUNX1 fusion proteins was carried out by using primary polyclonal rabbit anti-RUNX1 antibody (ab23980, Abcam) and secondary goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz Biotechnology). Primary mouse antibody to actin (MAB1501, Chemicon) and secondary donkey anti-mouse IgG-HRP (sc-2314, Santa Cruz Biotechnology) was used as a loading control. Proteins were visualized with HyGLO Reagent A/B (E2500, Denville Scientific Inc) used in accordance with the manufacturer's instructions.

3. Results

While most oncogenic DNA viruses integrate at least some portion of their genome into the cellular genome during oncogenesis, increasing evidence is showing that viral genes can be eradicated from the cancerous progeny, either by the host immune system or by a change in the intracellular environment (Stevenson, PG et al., *J Gen Virol*, 2010. 91(Pt 9): p. 2176-85; Nevels, M et al., *Journal of Virology*, 2001. 75(7): p. 3089-3094; Kuhlmann, I et al., *EMBO J*, 1982. 1(1): p. 79-86). This is termed the "hit and run" model, in which an infectious agent causes oncogenic damage in the host cell and is then lost in the resulting cellular progeny. This model of viral oncogenesis was supported using a Cre-lox recombination system in a mouse/herpes virus-4 model. In this study, cancer progression was triggered by virus infection in that particular cell, supporting the Kinlen and Smith hypotheses. Other papers using different viruses and models have supported "hit and run" viral oncogenesis, but it was unclear whether the virus initiated cancer in only infected cells or if the cancers were driven by inflammation or some kind of

immune dysregulation. The latter would be in line with a hypothesis proposed by Greaves, which states that the cancer progression is driven by some kind of immune dysregulation. In this hypothesis, the timing of infections during childhood is thought to be critical to driving immune dysregulation, but the infectious agent is not.

The mechanism behind loss of viral nucleic acids from cancerous cells has not been studied, but is thought to either be from immune intolerance of virus infected cells or by creation of an intracellular environment that does not allow viral replication. Thus, it was a goal to investigate whether childhood leukemic mutations could inhibit viral replication in a persistently infected cell line model, which would support plausibility of the “hit and run” model in childhood leukemia. While there are many good virus candidates (Niller HH et al., *Cancer Lett*, 2011. 305(2): p. 200-17), it was shown previously that childhood leukemic B cell lines naturally harboring chromosomal translocations common in childhood ALL were refractory to adenovirus infection and that these cells showed reduced expression of genes that were also reduced in B cells persistently infected with adenovirus. Lymphocytes that have previously been infected with adenovirus are refractory to new adenovirus infection, so the observation these B cell lines harboring childhood-like chromosomal translocations were also refractory could indicate that they have previously been infected with adenovirus. Alternatively, the childhood-like chromosomal translocations themselves may have inhibited the viral infection by altering the cellular environment.

A functional relationship between RUNX1 and adenovirus proteins E1B-55K and E4orf6 has been shown (Marshall, LJ et al., *J Virol*, 2008. 82(13): p. 6395-408). Additionally, human adenovirus species C has canonical RUNX1-binding motifs in promoter regions of several of its early genes, which control viral expression and replication. Because the leukemic fusion protein

ETV6/RUNX1 is thought to convert RUNX1 from an activator of transcription to a repressor, we reasoned that this common leukemic fusion protein could inhibit adenovirus retention in a persistently infected B-cell model by interfering with normal virus transcription and replication (Hiebert, SW et al., *Mol Cell Biol*, 1996. 16(4): p. 1349-55). A well-studied fusion protein in acute myeloid leukemia, RUNX1/MGT8, was tested in tandem as it is thought to function in the same manner as ETV6/RUNX1 (Okuda, T et al., *Blood*, 1995. 86(10): p. 2372-2372; Miyoshi, H et al., *Embo Journal*, 1993. 12(7): p. 2715-2721; Erickson, P et al., *Blood*, 1992. 80(7): p. 1825-1831; Ptasinska, A et al., *Leukemia*, 2012. 26(8): p. 1829-1841).

Earlier studies have shown BJAB cells are capable of harboring a persistent adenovirus species C infection for over 300 days post initial infection (Zhang, Y et al., *Journal of Virology*, 2010. 84(17): p. 8799-8810). To evaluate if the gene translocation most commonly associate with ALL could alter viral persistence within infected B cells, stable lines were created from BJAB cells expressing empty pTARGET vector, the ETV6/RUNX1 fusion gene, or the RUNX1/MGT8 fusion gene. For the RUNX1/MGT8, an alternative isoform that results in a truncated protein lacking the NHR3 and NHR4 regions of MGT8 was used. This truncated form still associates with histone deacetylase complexes, but does not obstruct cell cycle machinery, which could indirectly interfere with adenovirus replication and thus shadow any direct effects of the RUNX1/MGT8 protein on adenovirus transcription (Yan, M., et al., *Proc Natl Acad Sci U S A*, 2004. 101(49): p. 17186-91; Yan, M., et al., *Nat Med*, 2006. 12(8): p. 945-9; Burel, S.A., et al., *Mol Cell Biol*, 2001. 21(16): p. 5577-90).

Cells were infected with adenovirus type 5 Wild-type (WT) or type 5 mutant strain dl309 and the infection continued for up to 70 days post infection. Five such independent experiments were completed. For these experiments, the infection is classified as “persistent” after 23 days

post infection, after which the high levels of the viral capsid protein, HEXON, observed during the acute phase of the infection abruptly drops and is no longer detectable. Infected BJAB cells expressing empty vector, retained some level of HEXON mRNA expression throughout the persistent infection (Fig 3). In contrast, BJAB cells expressing the ETV6/RUNX1 or RUNX1/MGT8 fusion proteins exhibited significantly decreased expression of HEXON beginning at 4 weeks post infection. In one independent experiment, HEXON mRNA was undetectable in ETV6/RUNX1 or RUNX1/MGT8 expressing BJAB cells by week ten post-infection.

The “Early” adenovirus gene mRNA transcripts were also assayed via RT-PCR in the same manner as the “Late” HEXON gene transcript, and found to also be significantly less expressed in the BJAB cells expressing ETV6/RUNX1 and RUNX1/MGT8 (Fig. 4). Expression of viral transcripts of E1A, E1B-55K, E2A, E3GP19 and E4ORF6 all decreased during persistent infection in a pattern similar to HEXON mRNA. The E3GP19 transcript was reduced in the ETV6/RUNX1 expressing BJAB culture more rapidly than other early transcripts tested. No significant difference existed between the ETV6/RUNX1 and RUNX1/MGT8 expressing BJAB cultures.

Expression of the ETV6/RUNX1 and RUNX1/MGT8 fusion gene was confirmed by RT-PCR of transfected B cells and lung epithelial cells (Fig. 5). UoC-B4 cells, which naturally harbor the translocation, were used as a positive control for ETV6/RUNX1 expression and KASUMI-1 cells, which naturally harbor the RUNX1/MGT8 translocation, were used as a positive control for the expression of that fusion gene. The amplicon generated by the UoC-B4 control was slightly larger than the amplicon generated by the BJAB and A549 cells expressing engineered ETV6/RUNX1. This is likely due to different break points between the recombinant

and naturally occurring UOC-B4 fusion genes which results in alternative splicing patterns in the ETV6/RUNX1 transcript, as described by van Dongen et al (van Dongen, J.J., et al., *Leukemia*, 1999. 13(12): p. 1901-28). Western blotting confirmed that the ETV6/RUNX1 gene insert was producing the fusion protein. ETV6/RUNX1 protein expression was confirmed by WB. Cells that naturally express the ETV6/RUNX1 and RUNX1/MGT8 fusion proteins were used as positive controls (Fig. 5).

Example 2: ETV6/RUNX1 and RUNX1/MGT8 inhibit adenovirus DNA retention in persistently infected BJAB cells

4. Methods and Materials

Methods are as described in Example 1.

5. Results

To investigate whether viral genomic DNA was also affected by expression of the RUNX1 fusion genes, the same 3 BJAB cell lines expressing empty pTARGET vector, ETV6/RUNX1, or RUNX1MGT8 were cultured, and periodically sampled for total DNA over a period of up to 70 days post adenovirus infection as described above. To assess viral gDNA loss over the course of infection, adenovirus HEXON gene normalized to cellular GAPDH housekeeping gene was measured via QPCR. Previous studies have shown that the viral genomic DNA (gDNA) peaks around 10 to 14 days post infection in BJAB cells. Fold change in HEXON gene over time was established by comparing samples to the first time point taken after the viral gDNA peak of the initial infection, which was set to 1, to indicate 100% initial viral gDNA level. No observable pattern in the amount of viral gDNA per cell was seen during the initial infection period between cultures (data not shown).

As expected, BJAB cells expressing empty vector maintained a stable level of viral genomes for 5 weeks post the peak of infection. In contrast, retention of adenovirus genome was significantly inhibited in BJAB cells expressing the RUNX1 fusion proteins, as compared to the empty vector controls. Figure 6 shows the pooled results from 2 independent Ad5 WT infections. The mean percent loss of viral DNA in BJAB cells expressing ETV6/RUNX1 or RUNX1/MGT8 over 4 weeks of persistent infection was significantly more than BJAB cells expressing empty vector.

Example 3: RUNX1 binds to the adenovirus E3 promoter region

6. Methods and Materials

Methods are as described in Example 1.

Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed as previously described (Ma, X et al., *Blood Cells Mol Dis*, 2009. 42(2): p. 117-20) using the persistently infected BJAB cell lines generated above at 25-31 days post infection. Briefly, cells were crosslinked with 1% formaldehyde for 8 minutes at room temperature; crosslinking was stopped by the addition of 0.125 M glycine for 5 minutes at room temperature. Cell nuclei were isolated and concentrated by lysing in cell lysis buffer (5 mM PIPES pH 8, 85 mM KCl, 1% igepal) and protease inhibitors for 15 minutes on ice. The cell lysate was centrifuged at 2100 rpm for 5 minutes at 4°C. The supernatant was discarded and the pellet was resuspended in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.0, dH2O) and protease inhibitors for 25 minutes on ice followed by flash freezing in liquid nitrogen. Lysed nuclei were sonicated using a Bioruptor water bath sonicator for 15 sec “On” and 30 sec “Off” 3 times to generate an average of 800 bp of sheared DNA. The sonicated lysates were pre-cleared with salmon-sperm coated agarose beads (Upstate) and lysates were divided equally. One half of

the lysate was immunoprecipitated with 5 µg of ChIP-grade antibody to RUNX1 (Abcam ab23980) overnight at 4°C. The other half of the lysate was immunoprecipitated with control antibody. Immunoprecipitated proteins were isolated during 2 h incubation with 60 µl of salmon-sperm coated agarose beads. Immunoprecipitated samples were washed for 3 minutes at 4°C with the following buffers: low salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 150 mM NaCl, dH₂O), high salt buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris pH 8.0, 500 mM NaCl, dH₂O), LiCl buffer (0.25 M LiCl, 1% NP40, 1% DOC, 1 mM EDTA, 10 mM Tris pH 8.0, dH₂O) and 1X TE buffer; DNA was then eluted with SDS elution buffer (1% SDS, 0.1 M NaHCO₃, dH₂O). After DNA elution, crosslinks were reversed overnight with 5 M NaCl at 65°C followed by treatment with proteinase K for 1 hr at 45°C and immunoprecipitated DNA was isolated using a phenol:chloroform:isopropanol mix (Invitrogen) as per the manufacturer's instructions. QPCR reactions were carried out on an ABI prism 7900 (Applied Biosystems, Foster City, CA). Isolated DNA was analyzed by real-time PCR using primers spanning:

E3 promoter region: (Sense sequence, 5'-CCCGCTCCCACCACTGT-3', anti-sense sequence, 5'-TGCGCCCCTGAGTTAGTCA-3', probe sequence, 5'-56-FAM/CCCAGAGAC/ZEN/GCCCAGGCCG/3IABkFQ/-3').

Hexon gene region (Sense sequence, 5'-GCCATTACCTTTGACTCTTCTGT-3', anti-sense sequence, 5'-CCTGTTGGTAGTCCTTGTATTTAGTATC-3' probe sequence, 5'-56-FAM/AGAAACTTCCAGCCCATGAGCCG/36-TAMSp/-3').

Values from real-time PCR reactions were calculated and graphed based on standard curves generated, were run in triplicate reactions, and were analyzed using the SDS 2.0 program.

7. Results

To assess whether the RUNX1 and/or ETV6/RUNX1 proteins bound to the E3 promoter of the Ad5 genome, the BJAB transfects expressing empty vector or ETV6/RUNX1 that were persistently infected with the Ad5 WT virus were assayed by ChIP at 31 days post infection. RUNX1/MGT8 was not assayed because it lacked the RUNX1 epitope to which the ChIP-grade antibody used in this experiment was raised. More E3 promoter region gDNA was immunoprecipitated in the BJAB culture expressing ETV6/RUNX1 (Fig. 7). This indicates that the RUNX1 and/or ETV6/RUNX1 are likely binding to the E3 promoter region of adenovirus. The assay was performed in tandem with isotype antibody to control for non-specific binding, which showed very little non-specific binding. The HEXON gene coding region of adenovirus was similarly assayed. This region has a canonical RUNX1 binding motif in <1kb proximity to the amplified region, but was not a promoter region. HEXON gene showed only a small increase in RUNX1 binding in the culture expressing ETV6/RUNX1, indicating that RUNX1 does not bind to this region as frequently.

Example 4: Decreased expression of viral proteins precedes loss of adenovirus gDNA in cultures expressing ETV6/RUNX1

8. Methods and Materials

Methods are as described in Example 1.

9. Results

ETV6/RUNX1 protein typically induces strong repression of target genes. If ETV6/RUNX1 was binding to viral promoters, we would expect to see a decrease in viral transcripts which would precede loss of viral gDNA.

To ascertain whether viral genes were shut down first or whether viral gDNA replication was directly somehow inhibited, RT-QPCR and Western blotting analysis were performed. The

mRNA of adenovirus E1A and HEXON was slightly more decreased at 4 weeks post infection in the RUNX1 fusion protein expressing cultures than the adenovirus gDNA (Table 8), insinuating that the viral transcripts were decreased before the gDNA. However these effects were not very clear in the viral transcripts.

The Western blotting analysis further supported this, as the difference in viral gDNA at day 24 post infection fails to account for the stark decrease of E2A-DBP (Fig 9). By day 34 post infection, adenovirus gDNA levels were very low in both samples. However, this drastic difference in viral gDNA levels did not have a large effect on adenovirus protein levels in the empty vector, strengthening the argument that the ETV6/RUNX1 fusion protein inhibits viral mRNA synthesis and protein production and doesn't affect viral gDNA replication directly.

Example 4: Lasting epigenetic effects on infected BJAB cultures

10. Methods and Materials

Methods are as described in Example 1.

11. Results

It was previously shown that both persistently infected BJAB cultures and ALL cell lines with childhood-like chromosomal abnormalities have downregulated SPARCL and CXADR mRNA. This downregulation on SPARCL and CXADR was shown to likely be due to DNA methylation in both persistently infected cells and childhood leukemia cells. This indicated that the childhood leukemia cells may have once been infected. To investigate whether this trend of downregulation continued even after adenovirus was mostly lost from the BJAB cultures expressing the RUNX1 fusion proteins, RT-QPCR for SPARCL and CXADR mRNA was performed (Table 10). Even though the BJAB cells expressing the RUNX1 fusion proteins had almost entirely lost the adenovirus gDNA, both these cells and the empty vector control

continued to exhibit decreased expression of SPARCL and CXADR compared to the uninfected cells. These results indicate that adenovirus can leave epigenetic echoes of its infection, even when the virus is lost from the cellular progeny.

Multiple experiments confirmed that expression of the RUNX1 fusion proteins alone had no effect on the expression of SPARCL and CXADR (Table 10). However, when cells had been infected with adenovirus, SPARCL and CXADR mRNA expression was more decreased than the infected empty vector.

The results show that a B-cell line carrying either the ETV6/RUNX1 or RUNX1/MGT8 fusion gene exhibit a loss in both viral gene expression and viral genome retention during persistent infection. Loss of viral nucleic acids seemed to be due to direct binding of RUNX1 to the viral genome. Further, expression of cellular genes that are epigenetically reduced by viral infection, SPARCL1 and CXADR, remained decreased even after the virus had been lost from most of those cells. This suggests that the most common translocation of acute lymphoblastic leukemia, ETV6/RUNX1, as well as RUNX1/MGT8, can inhibit virus replication in infected B-cells while leaving the epigenetic marks of viral infection, presenting a plausible mechanism for the “run” of the “hit and run” hypothesis of viral oncogenesis that can be modeled in vitro.

Common precursor B-cell ALL has been hypothesized to be initiated by an infectious agent (O'Connor, SM et al., *Environmental Health Perspectives*, 2007. 115(1): p. 146-150). However no virus has been identified in cells obtained from ALL patients. Increasing evidence shows that “hit and run” oncogenesis is possible, which may explain why previous studies failed to find any viral DNA associated with leukemic cells. One possibility is that the virus causes disease by inducing the initiating mutations, but that these mutations then subsequently eject the virus from the cell progeny.

Alternatively, these mutations may already be present before infection, but disrupt the viral lifecycle in such a way that the virus cannot persist in that pre-leukemic population. In this case, the virus could act as a second hit in cells that already harbor the leukemic mutations that predispose one to developing leukemia, like ETV6/RUNX1. This infection could leave an epigenetic echo that drives the already mutated cell into overt leukemogenesis.

The observation that BJAB cells persistently infected adenovirus type 5 which expressed either the ETV6/RUNX1 or RUNX1/MGT8 fusion protein failed to retain adenovirus gDNA and express viral mRNA compared to BJAB cells expressing empty vector indicated that the ETV6/RUNX1 or RUNX1/MGT8 fusion proteins inhibit viral gDNA replication. This shows that leukemic mutations could plausibly inhibit viral lifecycles. This gives plausibility to the hit and run theory of viral oncogenesis.

Because both viral mRNA and gDNA is lost from the BJAB cells expressing the RUNX1 fusion proteins, it was unclear if expression of the fusion proteins may be downregulating viral genes needed for viral gDNA retention directly, and thus subsequently leading to loss of viral DNA, or if some other mechanism was causing loss of viral gDNA and thus loss of viral mRNA.

The most likely possibility was that ETV6/RUNX1 and RUNX1/MGT8 down regulate viral transcription from adenovirus genomes and so inhibit replication of the viral genome, as adenovirus encodes its own polymerase and DNA binding protein critical for virus replication. This seems likely as both fusion proteins are known to function in the same method of localizing histone deacetylases (HDACs) to promoters, guided by the RUNX1 portions to RUNX1 canonical binding motif to act as strong repressors of transcription due to the fused presence of ETV6 or MGT8. There are many RUNX1 canonical binding motif sequences on the adenovirus genome and RUNX1 was even originally shown to bind to viral genomes (Chroboczek, J et al.,

Virology, 1992. 186(1): p. 280-5; Thornell, A et al., J Virol, 1991. 65(1): p. 42-50). Because of the nature of RUNX1, which has been shown to act via enhancers 37kb away from target genes (Guo, H et al., Blood, 2012. 119(19): p. 4408-4418), and the small size of adenovirus genomes (~36kb), any binding of RUNX1 fusion genes would likely have a global effect on adenovirus genes. It is possible that the RUNX1 protein and its oncogenic fusion gene derivatives bind to the viral genome and various locations, which would decrease transcription from all of these regions in the case of ETV6/RUNX1 or RUNX1/MGT8.

The E3 and E2 promoter lie in close proximity and the E3 promoter contains a RUNX1 canonical binding motif, indicating that transcription factors that bind to one promoter or the other likely influence the expression of the other gene. Finally, the E3 promoter has been shown to function independently of the adenovirus E1A transcription factor protein, which was thought to be a master regulator that activate subsequent transcription of all other early viral genes, and may even be hindered by it in the lymphocyte environment (Graham-Pole, J et al., Bone Marrow Transplant, 1988. 3(6): p. 559-66). Therefore, the E3 promoter region was chosen for the ChIP assay to investigate if RUNX1 or the RUNX1 fusion genes bind to that area more frequently.

The ChIP assay revealed that RUNX1 was indeed enriched in the E3 promoter region. Strikingly, in ETV6/RUNX1 expressing cultures, the E3 promoter region was precipitated in even higher amounts than in cultures that expressed only RUNX1. This is likely indicative of the more frequent amount of binding of the target proteins in the culture that already expresses RUNX1 and was modified to also overexpress ETV6/RUNX1. The higher amount of target proteins could simply lead to higher binding frequency at the E3 region. However, this in no way invalidates the results of RUNX1 enrichment in the E3 promoter region of adenovirus, and

indeed seems to indicate that the ETV6/RUNX1 fusion protein is also capable of binding to this region.

It has been shown that RUNX1a and RUNX1b isotypes can direct adenovirus E1B-55K protein via viral E4ORF6 protein to the nucleus and even associates with centers of viral DNA and RNA synthesis in mouse A9 cells. It was reasoned that the fusion of the RUNX1 proteins could somehow interfere with this natural interaction and this could be the mechanism behind the loss of the viral nucleic acids in the BJAB cells expressing the ETV6/RUNX1 and RUNX1/MGT8 fusion proteins. However, western blots on co-IP assay products using the anti-RUNX1 antibody describe before (ab23980, Abcam) to pull down E4ORF6 and even direct cell lysate failed to detect the E4ORF6 protein in the BJAB cell lines during persistent infection (data not shown). Failure to detect viral proteins known to associate with RUNX1 protein was not unexpected. Because viral protein expression during persistent infection is so low as to be undetectable, it seems unlikely that a direct cellular/viral protein interaction was the mechanism behind the loss of viral nucleic acid in the ETV6/RUNX1 and RUNX1/MGT8 expressing BJAB cultures.

An alternative method of viral gDNA and mRNA loss could be through displacement of E2A-DBP. The E2A-DBP binds to the viral genome and helps it separate into ssDNA during viral genome replication. While the viral genome is known to interact with histones during its lifecycle, this phenomena is not well studied (REF). Perhaps histones on the viral genome are being deacetylated due to the activities of the RUNX1 fusion proteins, and the stronger binding of the histones to viral gDNA is inhibiting E2A-DBP from binding.

Finally, we showed that host cell gene expression of SPARCL1 and CXADR was decreased in cells that had been infected with adenovirus and lost almost all of the viral genome ,

but not affected by expression of the RUNX1 fusion proteins. This finding showed that the epigenetic remodeling of viral infection could have lasting effects on the cell once the virus was kicked out. Thus, presence of viral nucleic is not required for their epigenetic echoes to persist in the cancerous progeny of the cells.

The chromosomal translocations associated with ALL are found in healthy neonatal blood, indicating that the presence of these translocations alone is not enough to convey overt disease (Wiemels, JL et al., *Lancet*, 1999. 354(9189): p. 1499-1503). Recently, a correlation between neonatal cytomegalovirus (CMV) detection and progression of ALL has been shown using highly sensitive digital droplet PCR (Francis, SS et al., *Blood*, 2016), where before no CMV was detected in any patients (Gustafsson, B et al., *Pediatr Hematol Oncol*, 2006. 23(3): p. 199-205). Francis et al 2016 also found that CMV and other unclassified dsDNA virus nucleic acids were found more often in pre-treatment bone marrow samples of ALL patients than of acute myeloid leukemia (AML) patients, but found no such correlation with adenovirus. This is a very interesting finding, as CMV nucleic acids, like other viral nucleic acids, are not found in leukemic cells (MacKenzie, J et al., *Haematologica*, 2006. 91(2): p. 240-3). The authors suggested this supported Greaves hypothesis of aberrant immune function due to a common infection during a critical time period of development. It could also be an indication that the patients are immunocompromised, as is often a complication in ALL.

Alternatively, it could also indicate hit and run oncogenesis. If these patients had higher non-specific viral invasion of the bone marrow, they may be more susceptible to infection of hematopoietic cells and subsequent viral oncogenesis and loss of the virus from the cancerous progeny in a hit and run manner. It is important to note that while the hit and run model we

presented in this paper utilized adenovirus, there is no reason to suspect that CMV or other dsDNA viruses are not agents of hit and run oncogenesis in ALL.

Finally, the differences in patient ages could have also played an important role in viral detection in bone marrow samples. The ALL patients had a mean age of 3.51 years, while the control patients suffering from AML, had a mean age of 8.77 years. Specific tissue infection dynamics can change drastically during such differences in age. For example, adenovirus is almost always found in mucosal lymphocytes at 4 years of age, but is only found at <60% prevalence in mucosal lymphocytes from 8 year olds (Garnett, CT et al., *J Virol*, 2009. 83(6): p. 2417-28). Another example is seroprevalence to CMV steadily increasing with age (Colugnati, FA, et al., *BMC Infect Dis*, 2007. 7: p. 71). To think that other viruses may adhere to such trends in age of host is not farfetched.

A model of hit and run oncogenesis in a B-cell line has been presented that utilized the most commonly found ALL fusion gene, ETV6/RUNX1, and RUNX1/MGT8. We emphasize that this is only a working model. While it does give credence to adenovirus as an agent of ALL, it in no way suggests that other viruses like CMV are not the culprit or could not also be a culprit of the infectious etiology of ALL. No conclusive correlation between detection of adenovirus in utero and the leukemic chromosomal translocations has yet been shown to exist, albeit the methods utilized did not target the tissues in which the virus resides and were recently shown to not be sensitive enough with nested PCR, as discussed above. In the evidence generated in our presented model, initial adenovirus infection would not be affected by the presence of the fusion gene, but later persistence of adenovirus infection would be hindered. However, the expression of adenovirus genes during initial infection could leave a cancerous, epigenetic mark on the cell which could push the pre-oncogenic cell into a full blown state of cancer (El-Mahallawy, HA et

al., *J Pediatr Hematol Oncol*, 2004. 26(7): p. 403-6; Soria, C et al., *Nature*, 2010. 466(7310): p. 1076-81). Our model also supported this, as viral target genes SPARCL1 and CXADR expression remained low even after loss of viral gDNA from most cells. Finally, the prevalence of adenovirus in mucosal lymphocytes by age and its correlation with incidence of ALL is not only striking, but would further suggest that adenovirus would be a second hit. Further investigation is warranted into viral hit and run oncogenesis in ALL.

It is understood that the disclosed method and compositions are not limited to the particular methodology, protocols, and reagents described as these may vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to limit the scope of the present invention which will be limited only by the appended claims.

Unless defined otherwise, all technical and scientific terms used herein have the same meanings as commonly understood by one of skill in the art to which the disclosed method and compositions belong. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present method and compositions, the particularly useful methods, devices, and materials are as described. Publications cited herein and the material for which they are cited are hereby specifically incorporated by reference. Nothing herein is to be construed as an admission that the present invention is not entitled to antedate such disclosure by virtue of prior invention. No admission is made that any reference constitutes prior art. The discussion of references states what their authors assert, and applicants reserve the right to challenge the accuracy and pertinency of the cited documents. It will be clearly understood that, although a number of publications are referred to herein, such reference

does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the method and compositions described herein. Such equivalents are intended to be encompassed by the following claims.

CLAIMS

We claim:

1. A method comprising measuring the level of CXADR of B lymphocytes in a sample from a subject, wherein a reduced level of CXADR measured in the sample compared to the level of CXADR in a control identifies the subject as having an elevated risk of developing B-precursor acute lymphoblastic leukemia (ALL) if the samples are also translocation positive.
2. The method of claim 1 further comprising producing a CXADR assay output.
3. The method of claim 1 or 2 further comprising testing the subject for the presence of leukemia if the subject has a reduced level of CXADR measured in the sample compared to the level of CXADR in the control.
4. The method of any one of claims 1-3 further comprising monitoring the subject for the development of leukemia if the subject has a reduced level of CXADR measured in the sample compared to the level of CXADR in the control.
5. The method of claim 4, wherein the subject is monitored for the development of leukemia by testing the subject for the presence of leukemia two or more times.

6. The method of claim 5, wherein at least two of the two or more times at which the subject is tested for the presence of leukemia are separated by an interval of time.

7. The method of claim 6, wherein the interval of time is between three and twelve months, inclusive.

8. The method of any one of claims 1-7, wherein the reduced level of CXADR measured in the sample compared to the level of CXADR in a control is determined by comparing the amount of CXADR measured in the sample to the control and determining whether the sample has a reduced level of CXADR compared to the control.

9. The method of any one of claims 1-8, further comprising measuring the amount of SPARCL1 in the sample, comparing the level of SPARCL1 in the sample to a control, determining whether the sample has a reduced level of SPARCL1 compared to the control, and optionally producing an assay output.

10. The method of any one of claims 1-9 further comprising measuring the amount of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample, and comparing the level of one or more of BBS9, BNIP3, BTG3, and SLFN11 in the sample to a control, and optionally producing an assay output.

11. The method of any one of claims 10, wherein the sample is a bone marrow or blood sample.

12. The method of any one of claims 1-11, wherein the measuring comprises measuring the level of CXADR protein in the sample.

13. The method of any one of claims 1-12, wherein the measuring is accomplished by performing an enzyme-linked immunosorbent assay (ELISA).

14. The method of any one of claims 1-13, wherein the measuring comprises measuring the level of CXADR mRNA in the sample.

15. The method of any one of claims 1-11 and 14, wherein the measuring is accomplished by performing a hybridization assay or RT, PCR, or qPCR.

16. The method of any one of claims 1-15, wherein the control comprises a standard.

17. The method of any one of claims 1-15, wherein the control comprises a subject sample wherein the subject does not have leukemia.

18. The method of any one of claims 1-15, wherein the control comprises a subject sample wherein the subject does not have an ALL.

19. The method of any one of claims 1-18, wherein the subject is a child less than 18 years of age.

20. The method of any one of claims 1-19, wherein the subject is a child less than 5 years of age.

21. The method of any one of claims 1-20, wherein the reduced level of CXADR relative to the control indicates that the subject is at risk of developing ALL.

22. The method of any one of claims 1-21, wherein the level of CXADR is at least 50% less than the control.

23. The method of any one of claims 1-22, wherein the subject is identified as at risk of developing ALL with a confidence level of at least a 50%, 60%, 70%, 80%, 90%, 95%, 97%, or 99%.

24. The method of any one of claims 9-23, wherein the increased level of both of CXADR and SPARCL1 measured relative to the control indicates that the subject is at risk of developing ALL.

25. The method of any one of claims 1-24 further comprising treating the subject with one or more histone deacetylase (HDAC), one or more DNA methyltransferase (DNMT) inhibitors, or both, if the level of CXADR of one or more B lymphocytes of the subject is less than the control.

26. The method of claim 25 further comprising discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is the same as the control.

27. The method of claim 25 further comprising discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control.

28. The method of any one of claims 1-27 further comprising, prior to, following, or both prior to and following the measuring, treating the subject for ALL if the subject has ALL.

29. The method of claim 28 further comprising, following treating the subject for ALL, measuring the level of CXADR of B lymphocytes in another sample from the subject and discontinuing treatment of the subject if the level of CXADR of B lymphocytes of the subject is as high as the control.

30. The method of claim 28 or 29 further comprising, following treating the subject for ALL, monitoring the level of CXADR measured in samples from the subject.

31. The method of claim 30, wherein the level of CXADR measured in samples from the subject is monitored by measuring the level of CXADR of B lymphocytes in another sample from the subject two or more times.

32. The method of claim 31, wherein at least two of the two or more times at which the level of CXADR of B lymphocytes is measured are separated by an interval of time.

33. The method of claim 32, wherein the interval of time is between three and twelve weeks, inclusive.

34. A method of selecting a treatment strategy for subjects suffering from B-precursor acute lymphoblastic leukemia (ALL), the method comprising:

(a) measuring the level of CXADR of B lymphocytes in a sample from a subject;
and

(b) administering to the subject one or more histone deacetylase (HDAC), one or more DNA methyltransferase (DNMT) inhibitors, or both, if the measured level of CXADR measured in the sample is reduced compared to the level of CXADR in a control.

35. The method of claim 34, further comprising the measuring the amount of SPARCL1 in the sample, and comparing the level of SPARCL1 in the sample to a control, and determining whether the sample has a reduced level of SPARCL1 compared to the control.

36. A kit comprising reagents for assaying the level of one or more of BBS9, BNIP3, BTG3, CXADR, SLFN11, and SPARCL1 of B lymphocytes in a sample from a subject.

COMPOSITIONS AND METHODS FOR IMPROVED MONITORING AND TREATMENT OF LEUKEMIA

ABSTRACT

Compositions and methods related to identifying individuals predisposed to developing acute lymphoblastic leukemia (ALL), guiding risk classification and treatment

strategies of subjects identified as having ALL, and monitoring progress, and relapse in ALL patients after therapy. Kits containing compositions for use in the disclosed methods are also described.

[##INVENTORS: PLEASE PROVIDE FIGURES IF ANY##]

Table 1

Sequence Name	Sequence
---------------	----------

E1A13S.1.pt

PrimeTime Primer 2	CAG GCT CAG GTT CAG ACA C
--------------------	---------------------------

PrimeTime Primer 1	GTT AGA TTA TGT GGA GCA SCC C
--------------------	-------------------------------

PrimeTime Probe	/56-FAM/ATG AGG ACC /ZEN/TGT GGC ATG TTT GTC
-----------------	--

T/3IABkFQ/

E2A.1.pt

PrimeTime Primer 2	ACA CGT TGC GAT ACT GGT G
--------------------	---------------------------

PrimeTime Primer 1	GAA AAC TTC ACC GAG CTG C
--------------------	---------------------------

PrimeTime Probe	/56-FAM/CGG ATG GTT /ZEN/GTG CCT GAG TTT AAG
-----------------	--

TG/3IABkFQ/

5e4orf67.1.pt

REV.5e4orf67.1.pt	CTT AAG TTC ATG TTG CTG TCC AG
-------------------	--------------------------------

FOR.5e4orf67.1.pt	CTG CCC GAA TGT AAC ACT TTG
-------------------	-----------------------------

PRB.5e4orf67.1.pt	/56-FAM/ACT TGC GGT /ZEN/TGC TCA ACG GG/3IABkFQ/
-------------------	--

E3gp19.1.pt

REV.E3gp19.1.pt GCA GCT TTT CAT GTT CTG TGG

FOR.E3gp19.1.pt TTT ACT CAC CCT TGC GTC AG

PRB.E3gp19.1.pt /56-FAM/CTG GCT CCT /ZEN/TAA AAT CCA CCT TTT GGG

/3IABkFQ/

E1B-55k

PrimeTime Primer 2 CATCACAGGCTGGTTCCTAATA

PrimeTime Primer 1 GGTGTTTGACATGACCATGAAG

PrimeTime Probe TGGAAGGTGCTGAGGTACGATGAGA

TPL_HEXON

PrimeTime Primer 2 CCC GAG ATG TFC ATF TAA GAC

PrimeTime Primer 1 AAA GGC GTC TAA CCA GTC AC

PrimeTime Probe /56-FAM/CGC TTT CCA AGA TGG CTA CCC CT /3IABkFQ/

EIF1

PrimeTime Primer 2 GTA TCG TATGTC CGC TAT CCA G

PrimeTime Primer 1 GAT ATA ATC CTC AGT GCC AGC A

PrimeTime Probe /56-FAM/CTC CAC TCT /ZEN/TTC GAC CCC TTT GCT

/3IABkFQ/

Appendix B

Sanger sequencing results of the pMSCV-ADP sequencing

ADP sequence inserted twice, in the correct orientation in the Phoenix-AMPHO plasmid (pMSCV). Raw data received Aug 23, 2013 from GSU sequencing Core Facility Lab.

ADP gene was cloned into pMSCV plasmid using restriction enzyme digestion. EcoRI sites were inserted into primers specific for ADP. Adenovirus dl309 total RNA was collected, converted into cDNA, digested with EcoRI, ligated into pMSCV digested with EcoRI and then sent to the sequencing lab with M13 For primer.

```
TTAAGGGTGTCTCCTTTATCAGCCCTCACTCCTTCTCTAGGCGCCGGAATTAGATCT
CTCGAGGTTAACGAATTCGGCTTTACAGCGACCCACCCTAACAGAGATGACCAACA
CAACCAACGCGGCCGCGCTACCGGACTTACATCTACCACAAATACACCCCAAGTTT
CTGCCTTTGTCAATAACTGGGATAACTTGGGCATGTGGTGGTTCTCCATAGCGCTTAT
GTTTGTATGCCTTATTATTATGTGGCTCATCTGCTGCCTAAAGCGCAAACGCGCCCG
ACCACCCATCTATAGTCCCATCATTGTGCTACACCCAAACAATGATGGAATCCATAG
ATTGGACGGACTGAAACACATGTTCTTTTCTCTTACAGTATGATTAAATGAGACATG
ATTCCTCGAGTTTAAGCCGAATTCGGCTTTACAGCGACCCACCCTAACAGAGATGAC
CAACACAACCAACGCGGCCGCGCTACCGGACTTACATCTACCACAAATACACCCC
AAGTTTCTGCCTTTGTCAATAACTGGGATAACTTGGGCATGTGGTGGTTCTCCATAG
CGCTTATGTTTGTATGCCTTATTATTATGTGGCTCATCTGCTGCCTAAAGCGCAAACG
CGCCCGACCACCCATCTATAGTCCCATCATTGTGCTACACCCAAACAATGATGGAAT
CCATAGATTGGACGGACTGAAACACATGTTCTTTTCTCTTACAGTATGATTAAATGA
GACATGATTCCTCGAGTTTAAGCCGAATTCTACCGGGTAGGGGAGGCGCTTTTCCCA
```


AGGCAGTCTGGAGCATGCGCTTTAGCAGCCCCGCTGGGCACTTGGCGCTACACAAG
TGGCCTCTGGCCTCGCACACATTCCACATCCACCGGTAGGCGCCAACCGGCTCCGTT
CTTTGGTGGCCCCCTTCGCGCCACCTTCTACTCCTCCCCTAGTCAGGAAGTTCCCCCCC
GCCCCGAGCTCGCGTCGTGCAGGACGTGACAATGG_aAGTAGCACGTCTCACTAGTCT
CGTGCAGATGGACAGCACGCTGAGCATGGTAGCGGGTAGCTTTGGGGCAGCGCATA
GCAGCTGCTCTCGCTTCTGTCAAGTGGAAGGGGGGGTTCCGGGCGGGACTCAAGGC
GGTCTCAGGGCGGGGCGGGCCCCGAAGTCTCGAAGCCGGCATTGACGCTCACGCCAG
TGCCGCGATCCCTCCTATTCAGCCTTCGAACTGTA