EXPLORING TERT EXPRESSION AND REGULATION IN TUMORIGENESIS:

LESSONS FROM ALV

by

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A dissertation submitted to Johns Hopkins University in conformity with the
Requirements for the degree of Doctor of Philosophy

Baltimore, Maryland

April, 2018

ABSTRACT

Avian leukosis virus (ALV) is a simple retrovirus that infects chickens and causes cancer. Chickens exposed to ALV typically develop B-cell lymphomas within a few months after hatching. ALV-induced tumors have been shown to develop by insertional mutagenesis mediated by proviral integration. ALV proviral integration into the chicken genome disrupts the normal expression of neighboring host genes, commonly by promoter insertion and/or enhancer activation. Previous studies have identified several common proviral integration sites near genes that are now known to drive tumorigenesis. Notable genes include MYC, MYB, mir-155, and, more importantly, TERT.

Unique clonal integrations in the TERT promoter suggest that they are early events in ALV-induced B-cell lymphomas. In this thesis, I report the results of overexpressing TERT in early chicken embryos coinfected with ALV in new *in vivo* experiments. Although no definite conclusion could be made about TERT overexpression and tumor progression, two tumors in one chicken were identified to retain the recombinant virus used to overexpress TERT. High-throughput sequencing analysis of integration sites in one tumor implicated genes previously described. In contrast, the other tumor implicated an AT-rich interacting transcriptional coactivator, ARIDB5, as the top potential cooperating gene in TERT overexpressing cells.

In addition, we observed an unexpected prevalence of hemangiomas in our chickens infected with ALV-A, which is more commonly associated with ALV-J. Integration site analysis of multiple hemangiomas implicate an exclusive subset of genes that include FRK, PLAG1, and GLIS3.

In human cancers, the association between TERT promoter methylation and TERT expression is an area of active investigation. The relationship between the two factors has proven to be complex and controversial. Proviral integration adds another layer of complexity. Investigation of clonal TERT promoter integrations by bisulfite sequencing showed that ALV integration is associated with a decrease

in methylation of the flanking genomic DNA, suggesting that ALV may contribute to TERT expression by inhibiting methylation at the TERT promoter.

Lastly, we surveyed an extensive subset of human hematological malignancies for the prevalence of previously reported TERT promoter mutations by conventional Sanger sequencing. TERT promoter mutations have been recently identified as a highly conserved and ubiquitous somatic change in various cancers where TERT expression has been directly induced. We observed a lack of TERT promoter mutations in the human samples tested, suggesting that TERT promoter mutations are not a prevalent mechanism of TERT activation in hematological malignancies.

Reader: Dr. David Zappulla Advisor: Dr. Karen Beemon

Committee Members: Dr. David Zappulla, Dr. Kyle Cunningham, and Dr. Robin Morgan

ACKNOWLEDGEMENTS

First, I would like to thank Dr. Karen Beemon for her continued support and mentorship. My graduate study had a less than ideal start with a brief period with another investigator, which quickly devolved into a fruitless relationship. At the time, I was strongly encouraged by my advisor and committee members to consider alternative careers outside of science. Fortunately, Karen was willing to accept me as a graduate student, essentially saving my scientific career. For the next five years, I really appreciated the welcoming positive attitude, encouraging words, and, most importantly, trust she has given me regardless of whether our experiments are producing seminal discoveries or encountering some of the many roadblocks. Admittedly, the many technical hurdles and limited results from some of the major projects really presented mental challenges to my inherent lack of self-esteem and confidence. Karen's calm knowledgeable perspective and eagerness for scientific exploration provided the solace and inspiration for me to remain vigilant and ultimately complete my graduate studies.

I am not one to believe in luck, but my career in science may be the closest thing. I owe my success in my scientific career to the educators that I have been lucky enough to have in my life. Like my undergraduate advisor, Dr. Walter Patton, and Karen, I hope to become a pillar of inspiration and support for the next generation of prospective scientists as an educator myself. I would also like to thank my thesis committee members for their helpful suggestion and valuable input, Dr. David Zappulla, Dr. Kyle Cunningham, and Dr. Robin Morgan. In addition, I would like to thank Robin, Erin Bernberg, Amy Anderson for their help with the chicken experiments at the University of Delaware.

I am thankful for all the great people I have been fortunate to work with in the Beemon lab. First, I'd like to thank Lin Quek for opening the opportunity for me to join the Beemon lab. Next, I'd like to thank James Justice, Sanandan Malholtra, and Shelby Winans for the professional and personal relationships we developed over the years. Many thanks to YingYing Li for everything from the daily upkeep of the lab to the occasional candid conversations.

Lastly, I'd like to thank my closest friends and family. I have been fortunate enough to have the support of families from dramatically different parts of the world, one of common blood, and another of spirit. These people continue to unconditionally support, respect, and trust me throughout life. I hope to continue to make them proud and remain deserving of their blessing.

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ALV	Avian Leukosis Virus
ARID5B	AT-Rich Interactive Domain-containing protein 5B
ASLV	Avian Sarcoma/Leukosis Viruses
CEF	Chicken Embryo Fibroblast
DAS	Replication Deficient ALV-derived vector
ERV	Endogenous Retroviruses
FRK	Fyn Related src family tyrosine Kinase
GLIS3	GLIS family zing finger 3
HIV	Human Immunodeficiency Virus
HTLV-1	Human T-lymphotropic Virus-1
LR-9	ALV mutant
LTR	Long Terminal Repeat
MLV	Murine Leukemia Virus
PLAG1	Pleiomorphic Adenoma Gene 1
RSV	Rous Sarcoma Virus
TAPAS	TERT Antisense Promoter Associated RNA
TERT	Telomerase Reverse Transcriptase
TSS	Transcriptional Start Site

Chapter 1. Introduction to Avian Retroviruses and Telomerase

Introduction

Retrovirology began with two seminal discoveries looking at neoplastic diseases in the chicken. In 1908, Vilhelm Ellermann and Oluf Bang demonstrated that chicken leukosis was caused by a virus (Vilhelm and Bang 1908). In 1911, Peyton Rous reported the cell free transmission of a sarcoma in chickens (Rous 1911). These agents turned out to be related and became known as avian leukosis virus (ALV) and Rous sarcoma virus (RSV), respectively. Together, these viruses are collectively referred to as avian sarcoma/leukosis viruses (ASLV), and their discovery provided the first evidence of virus-induced tumors in animals. Inevitably, the discovery of virus-induced tumors was extended to mammalian species. In 1957, Ludwik Gross reported one of the first isolations of a murine leukemia virus (MLV) (Gross, 1957). More than fifty years after the initial discoveries, using RSV, Howard Temin described evidence of DNA proviruses and, more importantly, viral reverse transcriptase, which are now considered a defining property of retroviruses (Mizutani, Boettiger, and Temin 1970). These observations were independently described at the same time by David Baltimore using other RNA containing viruses (Baltimore 1970). With this knowledge, the first human oncogenic retrovirus, human T-cell leukemia virus 1 (HTLV-1) was discovered as the causative agent of aggressive T-cell leukemia in humans (Poiesz et al. 1980). Around this time, the human immunodeficiency virus (HIV) was discovered as the retrovirus responsible for acquired immunodeficiency syndrome (Barré-Sinoussi et al. 1983). From their initial discoveries, ALSV, along with other retroviruses, continue to serve as excellent sources of information for the understanding of retroviral biology and cancer (Weiss and Vogt 2011).

ASLV Genome

ASLVs are members of the *Alpharetrovirus* genus of the family *Retroviridae* (International Committee on Taxonomy of Viruses. and King 2011). They are categorized as simple retroviruses, which are characterized by at most one additional coding region to the three essential genes: *gag*, *pol,* and *env*. These genes are found to be common among all retroviruses (Figure 1.1). In contrast, the complex

retroviruses include additional genes that encode for functional accessory proteins. The ASLV *gag* gene encodes the structural proteins capsid (CA), matrix (MA), nucleocapsid (NC), and viral protease (PR). The ASLV *pol* gene encodes the reverse transcriptase (RT) and integrase (IN) enzymes, and the *env* gene encodes the transmembrane (TM) and surface (SU) envelope glycoproteins. The 7.5 kb genome is flanked by two identical long terminal repeats (LTR) sequences that are approximately 350 bp (Figure 1.1). The LTR is divided into three notable segments (Figure 1.1). The unique 3' (U3) region contains the polyadenylation sequence, the transcriptional promoter, and enhancer sequences (Figure 1.1). The beginning of the repeat (R) region marks the start of transcription in the 5' LTR and the end of R is the site of polyadenylation in the 3' LTR (Figure 1.1).

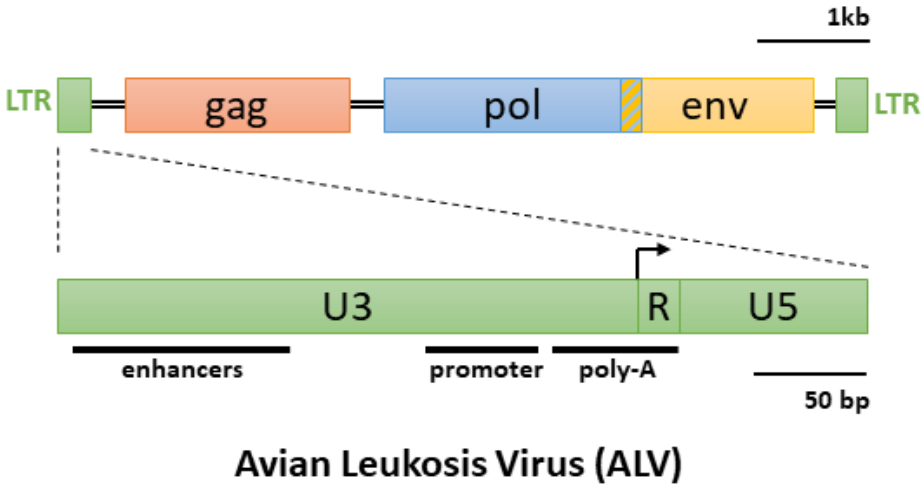


Figure 1.1. Schematic of the avian leukosis virus (ALV) proviral genome. (Top) Integrated ALV genomes are composed of three genes: *gag, pol,* and *env*, which are flanked by two identical sequences called the long terminal repeats (LTRs). (Bottom) The 5' LTR of ALV is composed of a U3, R, and U5 region.

ASLVs are further classified into 11 distinct subgroups (A-K) based on the properties of the viral envelope glycoproteins (Fadly and Nair 2008; Coffin 1992). Subgroups A, B, C, D, and J are exclusively exogenous ALSVs while subgroup E ALSVs are endogenous. The chicken genome encodes many endogenous retroviruses (ERVs). The majority of the these ERVs do not share any significant homology with known exogenous ALSVs (Bolisetty et al. 2012). Most are related to beta- and gammaretroviruses,

suggesting that these exogenous viruses were once a dominant type of exogenous avian retrovirus (Bolisetty et al. 2012).

Viral Entry

Key concepts for viral entry were established using ASLV as a model. Like all known retroviruses, ASLV virions, are coated with Env proteins. Env proteins are transmembrane proteins that bind to specific cellular receptors on the target cell surface and facilitate viral entry into the host cell. As previously mentioned, ASLVs are classified into distinct subgroups partly based on the cellular receptor specificity of their Env proteins. Genetic analyses revealed genes involved with susceptibility to infection. The *tva* and *tvc* genes confer susceptibility to ASLV infection by subgroup A and C, respectively, while the *tvb* locus confers susceptibility to subgroups B, D, and E (Crittenden et al. 1967; Duff and Vogt 1969; Hanafusa 1965; Payne and Biggs 1966; Payne and Biggs 1964; Payne and Pani 1971; Rubin 1965; Vogt and Ishizaki 1965). In the case of *tvb*, two functional alleles were discovered. The tvb^{S1} allele conferred susceptibility to infection by all three viral subgroups, and tvb^{S3} conferred susceptibility for subgroups B and D only (Adkins, Brojatsch, and Young 2000; Barnard and Young 2003; Brojatsch et al. 1996; Crittenden and Motta 1975; Crittenden, Wendel, and Motta 1973). The *tva* gene encodes TVA, a low-density lipoprotein (LDL) receptor-related protein (Bates, Young, and Varmus 1993; Young, Bates, and Varmus 1993). The *tvb* alleles encodes TVB, members of the tumor necrosis factor receptor (TNFR) family (Adkins, Brojatsch, and Young 2000; Barnard and Young 2003; Brojatsch et al. 1996). The *tvc* gene encodes for TVC, a member of the immunoglobulin superfamily (Elleder et al. 2005). More recently, the chicken Na^+/H^+ exchanger type 1 (chNHE1) was identified as a cellular receptor protein for subgroup J (Chai and Bates 2006). ASLV was shown to have a broad range of cell targets, being able to infect and replicate in many types of chicken tissues and organs (Dougherty and Di Stefano 1967; S. M. Williams et al. 2004).

Once bound to its receptor on the target cell, viral entry is achieved through a two-step fusion activation mechanism (Mothes et al. 2000). The first step involves receptor-mediated conformational changes in Env at neutral pH, exposing the fusion peptide of Env, which allows the insertion of this peptide into the cell surface membrane. Next, low pH activation facilitates completion of the fusion reaction in an acidic endosomal compartment after virus uptake and endosomal trafficking (Barnard and Young 2003). Once in the cytoplasm, reverse transcription of the viral RNA genome occurs through a complex multi-step process in which cellular nucleotides and packaged tryptophan tRNAs are utilized by the viral reverse transcriptase to convert viral RNA into a double-stranded DNA provirus. A more detailed description of these steps is available in *Retroviruses* (Coffin, Hughes, and Varmus 1997).

Nuclear Entry and Proviral Integration

From studies of HIV, the DNA copy of the viral genome associates with viral integrase (IN) and other viral and cellular proteins to form the preintegration complex (PIC) prior to nuclear entry (Piller, Caly, and Jans 2003). The ability of the PIC to gain access to nuclear DNA varies among retroviruses. Some retroviruses, like the gammaretroviruses, are able to integrate only during mitosis, following nuclear envelope disassembly. In contrast, lentiviruses, like HIV, circumvent the need for actively dividing host cells by utilizing a form of active transport across the nuclear envelope (Lewis and Emerman 1994). Initially, alpharetroviruses were believed to share the same restriction of the nuclear envelope (Temin 1967). However, more recent work from multiple groups demonstrated the successful infection of non-cycling cells by ALSV (Hatziioannou and Goff 2001; Richard A. Katz, Greger, and Skalka 2005; Richard A Katz et al. 2002; Greger et al. 2004). These observations were further supported by the characterization of a nuclear localization signal (NLS) that is sufficient for active transport of the ASV integrase through the nuclear pore (Andrake et al. 2008). Once in the nucleus, the PIC mediates integration of the provirus into the host genomic DNA. Integration is achieved through a two-step mechanism. First, the 3’ thymidine dinucleotide of the proviral genome is cleaved by IN; subsequently,

the PIC binds the host cell DNA and allows IN to catalyze a strand transfer reaction with the opposite strands of the host DNA (Bushman and Craigie 1990; Bushman, Fujiwara, and Craigie 1990; Engelman, Mizuuchi, and Craigie 1991). Following proviral integration, the host cell repair machinery fills any gaps at the ends of the integration site, which results in a six-nucleotide repeat sequence at both ends of the provirus (Hughes et al. 1981).

Integration site selection varies significantly among retroviruses. Site preferences are influenced by targeting factors that interact with the PIC and the viral genome during integration. Retroviruses like HIV-1 preferentially integrate into actively transcribed and spliced genes (Schröder et al. 2002; Singh et al. 2015). The lens epithelial derived growth factor (LEDGF), a general transcriptional co-activator, was reported to play a role in facilitating HIV-1 integration into gene regions (Maertens et al. 2003). In comparison, MLV preferentially integrates close to transcriptional start sites (TSS) and CpG islands (Mitchell et al. 2004; X. Wu et al. 2003). This preference is mediated by the interaction with host bromodomain and extraterminal domain (BET) proteins and the MLV integrase (Sharma et al. 2013).

In contrast to HIV and MLV, ALV was initially reported to integrate relatively randomly with slight preferences for transcribed genes (Barr et al. 2005; Mitchell et al. 2004; Narezkina et al. 2004; Withers-Ward et al. 1994). Further investigation from our lab provided a more comprehensive and improved analysis of ALV integration preferences (Malhotra, Winans, et al. 2017). Analysis of ALV integration sites in cultured cells infected with ALV revealed that ALV integration was relatively random, which is consistent with previous reports; however, slight preferences for genes, transcriptional start sites, and CpG islands were also observed (Malhotra, Winans, et al. 2017). In addition, a preference for integrations in and near expressed and spliced genes was observed (Malhotra, Winans, et al. 2017). This preference is likely in part influenced by the facilitates chromatin transcription (FACT) complex, which is demonstrated to be a specific putative binding partner of ALV integrase (Winans et al. 2017).

Transcription/Splicing/Nuclear Export

The process of transcription is directed by the viral LTRs. ASLVs do not encode any transcriptional activators; consequently, they rely entirely on host transcription factors for transcription. The host transcription factors bind the U3 region of the LTR which drives RNA Polymerase II transcription of the provirus. All viral transcripts undergo m^7G capping at the 5' end and 3' end cleavage and polyadenylation by cellular machinery prior to export from the nucleus.

All replication-competent ASLVs produce a single primary RNA transcript. This full-length, unspliced viral RNA serves as a mRNA for translation of *gag* and *pol* genes, as well as the genomic material to be packaged into new virions. A fraction of these primary transcripts is also spliced to generate *env* mRNA. In contrast, replication-deficient ASLVs such as myelocytomatosis virus (MC29) generally produces a single unspliced *gag-onc* fusion transcript due to the lack of an *env* gene. For future infection and replication, replication-deficient viruses like MC29 require the presence of a helper virus that provides the means for viral entry into a host cell. Additionally, avian retroviruses like RSV are able to produce a second spliced transcript as a result of acquiring new cellular genes. In the case of RSV, a second spliced transcript that encodes the *v-src* oncogene can be generated.

The ~7-9kb ASLV full-length viral RNA transcript is a foreign element in the host cell that harbors various characteristics that would be considered a target of host cellular restriction factors. To ensure successful replication, ASLV has evolved various properties that aid in necessary transcription, export, and translation of its genome. For example, while most RNA transcripts would undergo splicing, ASLV has several elements that protect these transcripts from splicing. These elements include suboptimal 3' splice sites (McNally and Beemon 1992) and cis-acting RNA elements.

The efficiency of splicing is significantly dependent on the architecture of the transcripts. The consensus sequence for 5' splice sites is AG/GURAGU, where the exon/intron junction is denoted by the slash. This sequence is recognized by the small nuclear ribonucleoprotein (snRNP), U1. The consensus

sequence for the 3' splice site is YAG/G, and the branch point sequence (YNYURAC) is optimally located ~30 nt upstream of the 3' splice site (Burge, Tuschl, and Sharp 1999). The intervening sequence between the splice sites contain a polypyrimidine tract that binds U2 auxiliary factor (U2AF) and facilitates the recruitment of U2 snRNP to the branch point. Following U2 binding, the two exons are brought together, and splicing is achieved through the coordinated action of U2, U5, and U6 snRNPs. The two exons are joined, and any intervening intron, in the form of a lariat, is debranched and degraded (Burge, Tuschl, and Sharp 1999). In contrast, the ASLV *env* splice acceptor region is suboptimally located 16 nts upstream of the 3' splice site, which ultimately reduces splicing efficiency (R A Katz and Skalka 1990).

An example of a cis-acting element is the negative regulator of splicing (NRS). A 230 nts RNA element located in the *gag* coding sequence (Arrigo and Beemon 1988), the NRS is located ~300 nts downstream of the 5' splice site and has been shown to be functional as long as it is located in close proximity to the 5' splice site (McNally, Gontarek, and Beemon 1991). The NRS has been proposed to act in a competitive manner as a pseudo 5' splice site that recruits components of the spliceosome by interacting with the 3' splice site. This interaction sequesters the 3' splice site away from the bonafide 5' splice site, reducing the efficiency of proper splicing of the viral transcripts in addition to the suboptimal 3' splice site (McNally and Beemon 1992; Cook and McNally 1999; Giles and Beemon 2005).

In contrast to other complex retroviruses that encode additional accessory proteins that mediate nuclear export, ASLV contains a 100-nt direct repeat (DR) RNA element that facilitates nuclear export. The number of DRs may vary between retroviruses where ALSV may utilize a single DR located in the 3' untranslated region (UTR). RSV has two DRs flanking the *src* oncogene. One DR element was shown to be sufficient to form a highly stable stem loop structure that mediates nuclear export. This method of nuclear export was shown to be dependent on the nuclear export factor, Tap (LeBlanc et al. 2007; Paca et al. 2000).

The RNA stability element (RSE) is another example that aids the proper life cycle of ASLVs. In contrast to cellular mRNA, the ASLV RNA viral transcript is polygenic. More specifically, the viral RNA has a stop codon at the end of the *gag* gene. To the cellular host factors, this would be considered a premature stop codon, and the region downstream of the *gag* stop would appear to be a long 3'UTR, which often targets cellular transcripts for degradation by the nonsense mediated decay (NMD) machinery. In order to circumvent degradation, ASLV has evolved this 400 nt element, RSE (Weil, Hadjithomas, and Beemon 2009). When positioned near a premature stop codon like the *gag* stop codon in ASLV, the RSE protects the full length viral RNA from NMD-mediated decay (Ge et al. 2016).

Translation

The full-length viral transcript serves as a template for the synthesis of two different polyproteins, Gag-Pro and Gag-Pro-Pol. In order to replicate efficiently, virions require a significantly greater expression of the Gag structural proteins than Pol proteins. All retroviruses evolved a strategy to achieve this requirement, despite being encoded on the same transcript. ASLV accomplishes this with a short A-U rich "slippery sequence" upstream of the *gag* termination codon next to a downstream RNA pseudoknot (Jacks et al. 1988). This sequence promotes ribosome pausing which leads to occasionally slipping a single nucleotide backwards before continuing forward approximately five percent of the time. This one nucleotide frameshift places the *gag* stop codon out of frame and allows the ribosome to read through to the *pol* termination codon, generating the Gag-Pro-Pol polyprotein. ASLV Gag is known to be additionally modified with low levels of N-terminal phosphorylation and acetylation (Swanstrom and Wills 1997; Palmiter et al. 1978).

The ASLV spliced proviral transcript serves as a template for the synthesis of Env polyproteins which are produced by splicing of the *env* splice donors located eighteen nucleotides downstream of the *gag* start codon. Consequently, ASLV Env polyproteins contains the first six amino acids of the Gag protein at the N-terminus (Ficht, Chang, and Stoltzfus 1984; Swanstrom and Wills 1997). The Env

polyproteins is post-translationally modified by terminal glycosylation and, subsequently, proteolytically cleaved into three peptides. These peptides oligomerize to form a trimer (Einfeld and Hunter 1988). Subsequently, the Env trimer is exported to the cell surface and may then interact with host cell receptors that are processed concurrently. This interaction forms the basis of resistance against additional infection by the virions using the same host cell receptor. This phenomenon is called super infection resistance.

Virion Assembly and Budding

Gag protein mediates most of the assembly and budding process. After synthesis, Gag is imported into the nucleus and interacts with a packaging sequence (Ψ) on the viral RNA genome through its NC domain (Gudleski et al. 2010; Scheifele, Ryan, and Parent 2005). Gag dimerizes and then nuclear export occurs, mediated by the nuclear export signal within the p10 domain of Gag. The export of the entire ribonucleoprotein (RNP) complex is mediated by the CRM1 Pathway (Gudleski et al. 2010; Scheifele, Ryan, and Parent 2005). Trafficking from the nucleus is a phosphoinositide-dependent process (Nadaraia-Hoke et al. 2013). Gag mediates stable association with the plasma membrane through its membrane-binding domain (MBD) (Verderame, Nelle, and Wills 1996). At the plasma membrane, processed Env proteins, viral polyproteins, two linked genomic RNAs, and viral tRNAs are assembled into viral particles, which is facilitated by Gag protein and different cellular proteins (Swanstrom and Wills 1997; Pincetic and Leis 2009). After budding, the viral PR mediates cleavage of the polyproteins, leading to the maturation of the virions for future infection.

ALV Insertional Mutagenesis as a Model for Tumorigenesis

Lacking any viral oncogene, ALV is considered a non-acute transforming virus. Non-acute transformation is characterized by a long latency period (4-8 months) between the time of infection to death from tumors (Fan and Johnson 2011). The major source of transformation occurs through ALV integration events. Proviral integration into the host genome can dysregulate the expression of

neighboring host genes mediated through the strong enhancer and promoter elements in the viral LTRs (Figure 1.2).

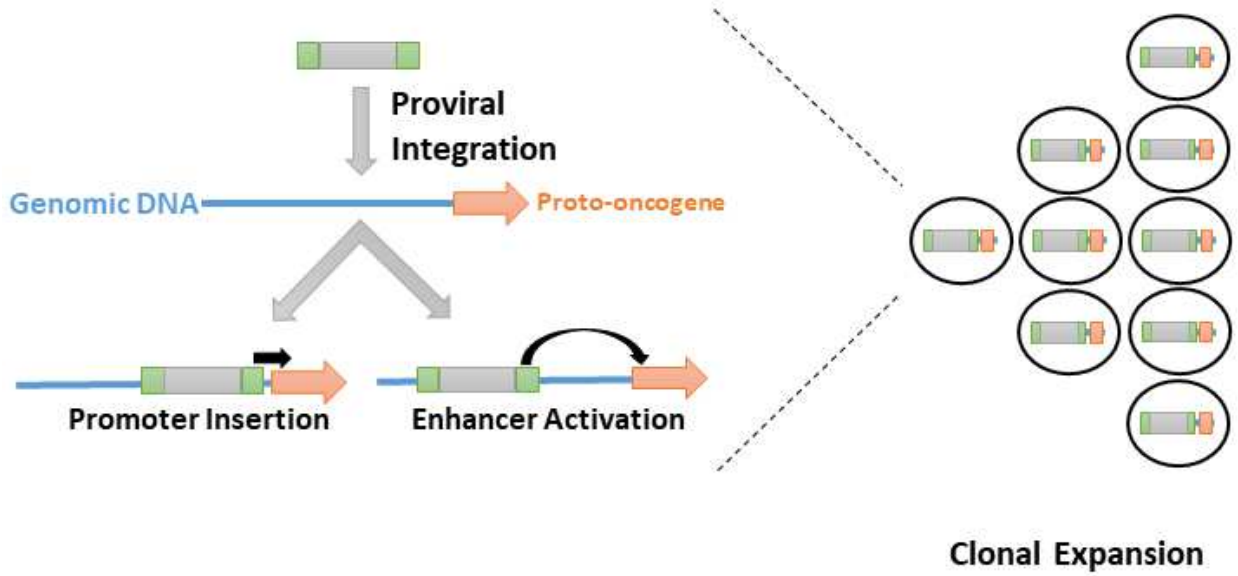


Figure 1.2. Schematic of proviral insertional mutagenesis. (Left) Proviral integration activating neighboring cellular proto-oncogene by promoter insertion or enhancer activation. (Right) Clonal expansion of transformed precursor cell.

Integration near or within specific host genes like pro-survival and proliferation genes can upregulate their expression, leading to tumor formation. The viral genome could also induce the expression of truncated gene products or impact post-transcriptional processes (Jiang et al. 1997). ALV enhancer elements can have long range effects on the expression of host genes (Y. Li et al. 2014). These characteristics of proviral integration are referred to as viral insertional mutagenesis (Uren et al. 2005).

Since the initial discovery and association of avian retroviruses with tumorigenesis, there are various properties that poise ALV as an attractive insertional mutagenesis model for the study of tumorigenesis. Like other integrating retroviruses, proviral integration is an efficient and naturally occurring event that researchers can exploit for cancer gene discovery. If ALV integration occurs near or within an oncogenic element, the host cell can undergo oncogenic transformation and proliferation, leading to tumor formation that is populated by a clonal expansion of the initial infected cell (Figure 1.2). Identification of these sites can be easily achieved by sequencing the junction between the proviral

and host genome. Initial studies mapping integration sites made use of low-throughput techniques to identity hotspots of proviral integrations to identify genes that may drive oncogenesis. In chapter 2, our work shows an example of a more efficient method to study hotspots of ALV integration in tumors by the application of high throughput sequencing techniques previously described (Justice, Morgan, and Beemon 2015; J. Justice et al. 2015; Malhotra, Winans, et al. 2017).

Clonal expansion in cancer is the concept that a tumor is derived from a small number of precursor cells that originally transformed and proliferated into expanded clones. In the context of retroviral infections, one can easily demonstrate clonality through quantitation of proviral integrations by various techniques. Initially using Southern blots, ALV tumors were shown to be clonal (Neiman et al. 2003; Yang et al. 2007). An empirical method of measuring the degree of clonality and extent of clonal expansion in different stages of tumorigenesis through the application of sequencing technology has been previously defined using HTLV-1 and HTLV-2 induced tumors (Gillet et al. 2011; Berry et al. 2012). This method was later applied in the analysis of ALV induced B-cell lymphoma tumors in our lab (Malhotra, Winans, et al. 2017). The study revealed a change in integration pattern when comparing ALV infection in cultured cells and tumors. In cell culture, observed integrations are relatively random with slight preferences for genes, TSS, and CpG islands. There is also a preference for integrations in or near expressed and spliced genes. In comparison, tumors show more selection for ALV integrations near TSS. There is also a significant selection of ALV integrations away from CpG islands in clonally expanded cells in tumors.

As previously mentioned, ALV integration occurs in a quasi-random fashion and exhibits minimal discrimination for specific integration sites, in contrast, to other retroviruses like MLV and HIV. Consequently, using ALSV as an insertional mutagen allows forward genetic studies of tumorigenesis in a more unbiased manner. The minimal integration site bias allows for the discovery of functional non-

coding elements like long non-coding RNA (lncRNA) (Nehyba et al. 2016) and micro-RNA (miR) (Clurman and Hayward 1989).

While ALSV can infect different bird species like ducks and turkeys (Payne et al. 1992), chickens are the natural hosts of ALSV infections. ALSV infections predominantly spread horizontally through physical contact, but may be transmitted vertically from the chicken to egg (Justice and Beemon 2013). Although chickens are more phylogenetically different from humans compared to mice as model organisms, many studies have demonstrated that discoveries in chickens are translatable to humans and may even serve as more appropriate tumorigenesis models than mice.

ALSV integration events may induce a wide range of tumors, the most common being lymphoid leukosis. Lymphoid leukosis is a type of B-cell lymphoma that originates in the bursa of Fabricius, an avian specific organ that serves as a site for B-cell development. ALSV infection initially causes transformation of B-cells in the bursa that eventually metastasize to distant organs like the liver, spleen, and kidney (Fadly and Nair 2008). Other types of ALSV induced tumors include hemangiomas, myeloid leukosis and erythroblastomas (Beemon and Rosenberg 2012; Justice and Beemon 2013; Justice, Morgan, and Beemon 2015). The spectrum of ALSV induced tumors is ultimately dependent on many factors including the type of viral strain, the age of the bird at the time of infection, as well as the genetic background of the birds.

ALV insertional mutagenesis studies have previously identified several cancer-associated genes like MYC, MYB, BIC (the gene locus for miR 155), and TERT (Baba and Humphries 1986; Clurman and Hayward 1989; Hayward, Neel, and Astrin 1981; Justice, Morgan, and Beemon 2015; Yang et al. 2007). In 1981, mapping of ALV integrations in long latency B-cell lymphomas identified *MYC* as an integration hotspot with the majority of the tumors (approximately 80%) having integrations in intron 1 of *MYC* (Neel et al. 1981; Hayward, Neel, and Astrin 1981). The integration led to the overexpression of MYC through the upregulation of a viral fusion transcript driven by the inserted strong promoter element in

the 3' LTR of the virus. Tumors were identified in chickens infected at 2-7 days after hatching and development of B-cell lymphomas were observed 4-6 months after infection. This seminal discovery demonstrates that oncogenic transformation could be caused by the activation of a cellular gene (Wasylishen and Penn 2010). Later, *c-bic* was shown to be a common integration site that often coincided with tumors that had MYC integrations (Clurman and Hayward 1989). Much later, *c-bic* was identified to be an oncogenic microRNA, now recognized as mir-155 (Tam, Ben-Yehuda, and Hayward 1997).

Further investigation into the time of infection revealed that ALV could also cause short-latency lymphomas by insertional mutagenesis. Approximately 14% of ten day-old chick embryos infected with ALV develop short-latency B-cell lymphomas within weeks (Pizer, Baba, and Humphries 1992). Furthermore, a recombinant strain of ALV, EU-8, was discovered later to have higher tumor incidence (40%-80%) under similar conditions (Kanter, Smith, and Hayward 1988; Simon et al. 1987). Integration site analysis of these tumors revealed a common integration site at the MYB locus, suggesting that MYB expression contributes to the short-latency lymphomas (Kanter, Smith, and Hayward 1988). EU-8 is a recombinant between two related viruses the ring-necked pheasant virus (RPV) and UR2 associated virus (UR2AV) (Simon et al. 1987). RPV is a subgroup F ALV, originally isolated from pheasant cells, and UR2AV is subgroup A. As characteristic of ALV, RPV lacks any viral oncogenes and contains endogenous retroviral sequences related to pheasant endogenous retroviruses. When RPV infects 10 day-old chick embryos, a high frequency of angiosarcomas is observed (Carter, Proctor, and Smith 1983).

In an effort to identify the genetic determinant for angiosarcomas, recombinants between RPV and UR2AV were created. Serendipitously, one of the recombinants, EU-8, was discovered to induce short-latency B-cell lymphomas. EU-8 is comprised mostly of RPV except for the *env* of UR2AV. In contrast, another recombinant, LR-9, which has *gag, pol,* and *env* of UR2AV, is unable to induce short-latency B-cell lymphomas. By sequence comparison, the genetic determinant was narrowed down to a

42-nucleotide deletion in the *gag* gene of RPV. Deletion of this 42-nucleotide element in LR-9, named ΔLR-9, enabled the virus to induce short-latency lymphomas at comparable levels to EU-8 (Smith et al. 1997). Later, the deletion was determined to be localized to the region of *gag* that corresponded to the matrix protein as well as in the NRS element (Polony et al. 2003). Testing by mutagenesis generated a virus, LR-9 G919A, which contain a single silent point mutation in the NRS, that was able recapitulate the effects of ΔLR-9 with an even higher incidence (Polony et al. 2003). This result suggested that disruption of the NRS sequence allows increased viral readthrough, and increases the efficiency of splicing to downstream genes like MYB, which ultimately contributes to rapid tumor formation (Smith et al. 1997; O'Sullivan et al. 2002; Polony et al. 2003; Wilusz and Beemon 2006).

Prior to my thesis work, the tumors generated from these experiments with LR-9, ΔLR-9, and LR-9 G919A represent the majority of the original pool of samples used for recent integration site analysis from our lab (Yang et al. 2007; Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). In these studies, one of the most notable discoveries is the identification of clonal ALV integrations into the telomerase reverse transcriptase (TERT) promoter. This discovery implicates the maintenance or early upregulation of TERT expression as a selection factor for ALV-induced B-cell lymphomas. This discovery serves as the core source of inspiration for the majority of my thesis work covered in the following chapters.

Telomerase and Tumorigenesis

In humans, TERT encodes the reverse transcriptase component of telomerase and together with its RNA partner TR, which provides a template for TERT, constitute the telomerase reverse transcriptase enzyme. The canonical function of telomerase is to extend and maintain telomeres, preventing replicative senescence. Replicative senescence is the state in which cells stop dividing when their telomeres are critically short (Harley, Futcher, and Greider 1990). Senescence occurs in most cells because of telomere shortening with successive cell divisions (Blackburn 2000). With a few exceptions,

such as germ cells and stem cells, TERT is not actively transcribed and, consequently, telomerase activity in these cells is low (Wright et al. 1996). However, in cancer cells, TERT is often transcriptionally reactivated by various mechanisms, accounting for 90% of all human cancers (Shay and Bacchetti 1997). The reactivation of TERT enables replicative immortality, a hallmark of cancer characterized by the ability to grow endlessly (Hanahan and Weinberg 2011).

Mice have been extensively used as a model for human telomerase function. One direct way of accessing the function of telomerase is to knockout its expression *in vivo*. Knockout mice for the telomerase RNA component, *mTERC*, were initially generated by Blasco and colleagues in 1997 (Blasco et al. 1997). These knockout mice were viable, fertile, and phenotypically normal until the fifth generation. Cells grown past the fourth generation exhibit evidence of telomere shortening, lack of detectable telomere repeats at chromosome ends, aneuploidy, and chromosomal abnormalities that include end-to-end fusions, which is similar to observations in the propagations of normal somatic human cells (Blasco et al. 1997; Counter et al. 1992). In contrast, late generation *mTERC*, or *mTR*, knockout mice cells show the ability to overcome senescence/crisis like most mouse cell lines, which is not commonly observed in normal human somatic cells (Blasco et al. 1997; Counter et al. 1992). Furthermore, oncogenically transformed telomerase null mouse cells can form tumors, suggesting that telomerase is not required for tumor formation in mice (Blasco et al. 1997). *mTERT* knockout mice were first described by Yuan and colleagues in 1999, sharing similar observations to the *mTERC* knockout mice (Yuan et al. 1999).

While telomerase may not be required for tumorigenesis in mice, studies with targeted overexpression of *mTERT* suggest a role in promoting tumorigenesis (Gonzalez-Suarez et al. 2001; Canela et al. 2004; Cayuela, Flores, and Blasco 2005). In one study, a conditional mouse line, K5-Tert, is generated with targeted expression of *mTERT* to basal keratinocytes. While mice are viable and show histologically normal epithelia with high levels of telomerase activity and normal telomeres, they are

more susceptible to chemically induced carcinogenesis than their wild-type littermates (Gonzalez-Suarez et al. 2001). This susceptibility is determined to be dependent on the expression of *mTR*, implicating telomerase as the contributing factor (Cayuela, Flores, and Blasco 2005). Similarly, targeted overexpression of *mTERT* to T cells in two independent mouse lines shows higher incidences of spontaneous T-cell lymphoma than their corresponding age-matched wild-type controls (Canela et al. 2004).

Although studies in mice indicate an association between telomerase and tumorigenesis, these studies reveal that the association is likely telomere-independent. Overexpression of *mTERT* did not impact telomere length in mice (Canela et al. 2004; Cayuela, Flores, and Blasco 2005; Gonzalez-Suarez et al. 2001). Investigation into telomere-independent functions of telomerase is an area of active investigation (for a detailed review, see Low and Tergaonkar 2013)). Presently, noncanonical functions appear to be associated with telomerase and transcription of genes involved in various aspects of tumorigenesis. In mice, TERT expression is associated with the activation of Wnt-related pathways (Choi et al. 2008). This is in part achieved through direct interaction between TERT and a SWI/SNF-related chromatin remodeling protein, BRG1, which in turn activates Wnt-dependent genes (Park et al. 2009). In humans, TERT can also regulate NF-κB-dependent transcription, which can be mediated through the direct interaction between TERT and p65 subunit of NF-κB. This interaction recruits TERT to NF-κB regulated promoters and activates genes that promote cancer progression (Ghosh et al. 2012).

Using mice as a model of human telomerase function and telomere biology does present some limitations. Although telomerase appears to promote tumorigenesis in mice, mice possess significant differences in telomere biology when compared to humans. Constitutive telomerase expression is found throughout the lifespan of a mouse in both somatic and renewable tissues; furthermore, mice show a significantly higher susceptibility to spontaneous oncogenesis (Blasco et al., 1997; Forsyth et al., 2002; Sherr and DePinho, 2000; Wright and Shay, 2000). In addition, laboratory mice and other rodents

generally do not exhibit age-related or division-dependent telomere shortening (Forsyth, Wright, and Shay 2002; M A Blasco et al. 1997; Samper, Flores, and Blasco 2001; Wright and Shay 2000) and maintain long telomeres even at senescence (Parrinello et al. 2003).

In contrast, the chicken may present a optimal model for the study of telomeres and telomerase. Like humans, telomerase activity is observed early in development, maintained in renewable tissues and stem cells, and diminishes in most somatic tissues following embryogenesis in chickens (Forsyth, Wright, and Shay 2002; S.E. Swanberg and Delany 2003; Taylor and Delany 2000). Telomerase expression and activity is upregulated in most transformed cells as well (Swanberg and Delany 2003). In proliferating cells, division-dependent telomere shortening is observed in chicken somatic tissues *in vivo,* which also correlates with age (Taylor and Delany 2000; M. Delany et al. 2003).

Much less research has been published utilizing chicken as a model for the study of telomeres and telomerase. However, what is available suggest that telomerase functions in a similar manner to humans. Upregulated levels of chTERT and chTR, the chicken telomerase RNA component, is observed in a chicken lymphoma cell line, DT40, which also correlates with telomerase activity when compared to non-transformed chicken embryonic fibroblasts (CEF) (Swanberg et al. 2004). Furthermore, telomerase activation is associated with increase c-myc expression which has been shown to be involved with the reactivation of telomerase in many human cancers (K.-J. Wu et al. 1999).

In Marek's disease herpesvirus (MDV) induced T-cell lymphoma, the expression of a viral *TR* (*vTR),* which shares 88% sequence identity to chicken *TR,* driven by MDV is associated with increased incidence of T-cell lymphoma in chickens (Trapp et al. 2006). Constitutive expression of vTR in the chicken fibroblast cell line DF-1 is associated with phenotype consistent with oncogenic transformation (Trapp et al. 2006). More recently, overexpression of chTERT alone or in combination with chTR is sufficient to immortalize chicken preadipocytes *in vitro* (Wang et al. 2017). Two independent cell lines generated from this process display greater than 100 population doublings, high telomerase activity,

and lack any signs of replicative senescence (Wang et al. 2017). Interestingly, neither of the cell lines show any morphological features of malignant transformation, such as the development of cell cloning foci or loss of contact inhibition in culture (Wang et al. 2017). These observations suggest that TERT alone is not sufficient to drive malignant transformation in cultured chicken cells.

In chapter 2, I take advantage of our established model using ALV and chickens to test the effects of overexpression of chicken TERT in tumorigenesis. Overexpression expression of TERT can be achieved through the use of recombinant avian virus. As many lines of evidence suggest that early TERT activation is likely not sufficient to drive tumorigenesis per se, we can combine TERT overexpression with ALV infection to screen for potential cooperating genes through proviral integration site analysis.

Proviral and TERT Promoter DNA Methylation

Epigenetic events play a significant role in the development and progression of disease states. DNA methylation is one of these mechanisms. DNA methylation occurs by the covalent addition of a methyl group to the cytosine ring, resulting in 5-methylcytosine (5mC) (Bird 2002). This process is catalyzed by enzymes called DNA methyltransferases (DNMTs). This modification is most commonly associated with transcriptional silencing and found in nonexpressed or noncoding regions of the genome. In mammalian DNA, 5mC is found in approximately 4% of the genomic DNA and is located almost exclusively at cytosine-guanosine dinucleotides (CpGs). CpG sites occur relatively randomly throughout the genome with the exception of small stretches of DNA with high concentrations (>50% CpGs) of CpGs sites, called CpG islands (Bird 2002). These CpG islands are commonly found near promoter regions of genes, where transcription starts (Herman and Baylin 2003). While most CpG sites are often methylated, CpG islands are more dynamic, and changing methylation states depends on the cellular environment (Bird 2002).

Accessible methylation data for chicken are relatively limited compared to their mammalian counterparts. In 2011, Li and colleagues performed one of the first genome-wide methylome studies of

liver and muscle tissues from red jungle fowl and avian broilers. Their results suggested that chickens show analogous patterns of DNA methylation compared to mammals (Li et al. 2011; Eckhardt et al. 2006). More specifically, repetitive sequences are hypermethylated, most CpG islands remain hypomethylated, and gene body regions show relatively higher levels of DNA methylation than the flanking regions (Li et al. 2011). Furthermore, gene expression level is negatively correlated with DNA methylation in the proximal promoter regions (Li et al. 2011). This would suggest that DNA methylation functions in the traditional sense of suppressing transcription in chickens like in mammals.

In cancer, there is significant change in DNA methylation patterns that supports tumorigenesis. In normal cells, most CpG sites in intergenic regions are methylated, whereas CpG-island sites of active gene promoters are unmethylated. In contrast, cancer cells are often characterized with loss of methylation in normally silent regions of the genome, which leads to inappropriate expression of proto-oncogenes. In addition, selective hypermethylation of promoter regions is associated with aberrant silencing of many known tumor suppressor genes (Jones and Baylin 2002; Herman and Baylin 2003). Epigenetic silencing by DNA methylation has been shown to occur just as frequently as somatic mutations in the aberrant silencing of tumor-suppressor function (Jones and Baylin 2002; Herman and Baylin 2003).

The human TERT gene is characterized by a large (2 kb) CpG island, which starts 846 bp upstream from its transcriptional start site (TSS) and extends into the first intron, 1178 bp downstream of the TSS. Similarly, the chicken also has a large (1.1 kb) CpG island, extending from -337 to +746 bps relative to its TSS. The relationship between TERT promoter methylation and expression has proven to be more complex in human cancers. On the one hand, initial reports suggest that TERT promoter DNA methylation is associated with gene silencing (Liu et al. 2004; Lopatina et al. 2003; Shin et al. 2003). A decrease of TERT promoter activity is observed when human teratocarcinoma (HT) and human acute myeloid leukemia (HL60) cells undergo induced differentiation by retinoic acid treatment (Liu et al.

2004; Lopatina et al. 2003). This reduction in TERT promoter activity during differentiation correlates with a gradual accumulation of DNA methylation in the TERT promoter (Liu et al. 2004; Lopatina et al. 2003). Treatment with a common demethylating agent, 5-azacytidine, reduces TERT promoter methylation and is associated with a reactivation of TERT expression (Lopatina et al. 2003). Similar results are observed using normal cells. Actively replicating normal human oral keratinocytes (NHOK) express TERT and have telomerase activity, which gradually decreases and is inactivated during senescence (Kang, Guo, and Park 1998). The inactivation of TERT is associated with the gradual hypermethylation of the TERT promoter during senescence in NHOK (Kang, Guo, and Park 1998). Treatment of senescent NHOK with 5-azacytidine restores expression of TERT (Shin et al. 2003).

In contrast, other reports suggest a direct, positive correlation between TERT promoter methylation and expression (Devereux et al. 1999; Dessain et al. 2000; Nomoto et al. 2002; Guilleret and Benhattar 2004; Guilleret et al. 2002). The independent methylation status of different normal and tumor tissues and cell lines reveals that telomerase-negative samples are characterized by hypomethylation at the TERT promoter, and telomerase-positive samples by hypermethylation. Treatment with 5-azacytidine can induce expression in some cell lines (Devereux et al. 1999; Dessain et al. 2000). In addition, hypermethylation of the TERT promoter appears to be one of the most prominent biomarkers of cancer and may also correlate with cancer progression and relapse (Barthel et al. 2017; Castelo-Branco et al. 2013, 2016).

Further investigation suggests that these two seemingly contradictory observations may likely involve separate functional domains of the promoter. Baylin and colleagues later reported that breast, lung, and colon cancer cells retain alleles with little or no detectable methylation near the TSS (-150 to +150 around the TSS) while being hypermethylated in regions further upstream of the TSS (>600 bp) (Zinn et al. 2007). Hypomethylated regions are associated with active chromatin marks, while hypermethylated regions are associated with inactive chromatin marks, suggesting that DNA

methylation at the TERT promotor displays the usual dynamics of gene expression (Zinn et al. 2007). The mechanism behind TERT expression upregulation and hypermethylation of the TERT promoter is still under investigation.

In addition to the maintenance of cellular gene expression, DNA methylation is a known mechanism in suppressing any potentially harmful viral sequences, both endogenous and exogenous. One of the earliest evidence of proviral DNA methylation was observed using an interspecies model. In these studies, DNA methylation of RSV LTRs is investigated using a rat restriction cell line (XC). This cell line was established from rare rat sarcomas induced by inoculating rats with Rous sarcoma tissue from chickens (Svoboda 1960). At the time, RSV was known to be non-permissive in rats, or unable to replicate after infecting the host cells (Svoboda 1960). However, low frequency rat sarcomas may form in a large cohort of infected rats. Upon further investigation of the XC cells, the block on RSV replication is associated with methylation of stably integrated RSV proviral DNA which is later shown to suppress proviral transcription (Guntaka et al. 1980; J Hejnar et al. 1999; Svoboda et al. 2000). Transcriptional silencing can be achieved through different mechanisms that include proviral *de novo* DNA methylation. Daxx, a cytoplasmic Fas death domain-associated protein, was later discovered to be involved in the maintenance of DNA methylation of avian proviruses in mammalian cells (Shalginskikh et al. 2013). DNA methylation of LTRs is demonstrated to be associated with the silencing of related mammalian retroviruses including MLV (Stewart et al. 1982), RSV (J Hejnar et al. 1999), HIV-1 (Bednarik, Cook, and Pitha 1990), HTLV-1 (Koiwa et al. 2002), as well as endogenous retroviruses (Lavie et al. 2005).

The impact of integrating viruses on the methylation state at the site of integration is an area of active investigation. Elucidating contributing factors may offer insights into practical applications like optimizing viral vectors for gene therapy, as well as understanding proviral selection in virus-induced tumorigenesis. Presently, the resulting DNA methylation at the site of integration is dependent on a growing list of factors. In the case of the XC cell line, rat cells appear to suppress RSV transcription

through DNA methylation of the proviral LTRs; in contrast, RSV provirus is not significantly targeted by DNA methylation in chickens (Guntaka et al. 1980; J Hejnar et al. 1999; Svoboda et al. 2000). These observations support the importance of the properties of the host cell on the transcriptional activity of proviruses. In comparison, MLV integration into mouse cells is associated with *de novo* methylation of MLV LTRs (Jähner and Jaenisch 1985), suggesting that the properties of the integrating virus can impact its susceptibility to DNA methylation.

Further investigations reveal that proviral integrations can alter the methylation state of the host genome at the site of integration. RSV integration has been associated with transient hypomethylation of flanking genomic DNA in hamster cells (Hejnar et al. 2003). In contrast, MLV integration is associated with *de novo* methylation in mice (Jähner and Jaenisch 1985). The DNA methylation state of the host genome at the site of integration may also determine the resulting methylation state of the provirus. Proviruses integrated close to transcriptional start sites of active genes may have long-term transcriptional activity and be resistant to transcriptional silencing by DNA methylation; in contrast, proviruses in intergenic regions, which are characterized by high methylation, tend to become transcriptionally silenced (Senigl, Auxt, and Hejnar 2012). In human tumors, early reports investigating integrated Hepatitis B virus (HBV) and human papilloma virus (HPV) 16 suggest a direct correlation between the methylation state at the site of integration prior to proviral integration and the methylation states of the provirus after integration (Hatano et al. 2017; Watanabe et al. 2015). Consequently, human proviruses located near highly methylated regions, which are likely transcriptionally inactive, become methylated and, subsequently, transcriptionally inactive, while proviruses located near hypomethylated regions remain active.

Previously, our lab has reported that the TERT promoter is a hot spot for ALV integration – also called a common integration site – in ALV-induced B-cell lymphomas. Additionally, these TERT promoter integrations are one of the most clonally expanded – or most abundant unique – integrations in tumors

tested (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). Clonally expanded TERT integrations would suggest that selection for these integrations likely occurred early during tumorigenesis, implicating the importance of early TERT activation in chicken B-cell lymphoma. TERT activation may be achieved through promoter insertion or enhancer activation from the ALV provirus.

Interestingly, the majority of the TERT promoter integrations are found to be in the opposite orientation of the TERT gene, which suggest that the TERT promoter may be bidirectional or two promoters (Nehyba et al. 2016). This observation later led to the identification of a novel antisense long non-coding RNA (lncRNA), which we referred to as TERT antisense promoter associated RNA (TAPAS) (Nehyba et al. 2016). The viral promoter drives the expression of fusion transcripts containing viral sequences spliced to exons 4 through 7 of TAPAS, which suggest that its overexpression may be contributing to tumorigenesis (Nehyba et al. 2016). TAPAS expression appears to correlate directly with TERT expression in various normal chicken tissues, which suggest that TAPAS may be an active factor in normal chicken tissues in addition to tumorigenesis (Nehyba et al. 2016). The functional and downstream consequences of TAPAS overexpression is still under investigation.

In the case of TERT, placing the ALV LTR in the opposite orientation next to the endogenous TERT promoter has been shown to be sufficient to drive expression of downstream coding genes using an *in vitro* reporter assay in chicken cells (Yang et al. 2007). Alternatively, DNA methylation is likely to play a role in the activation of TERT in ALV-induced lymphomas. As previously mentioned, TERT promoter methylation is a ubiquitous mechanism of regulation of TERT expression during development (Liu et al. 2004; Lopatina et al. 2003; Shin et al. 2003) and, especially, cancer in humans and other animals (Jones and Baylin 2002; Herman and Baylin 2003). These observations likely extend to the chicken genome as it has been shown to share analogous patterns of DNA methylation to those of mammals (Li et al. 2011; Eckhardt et al. 2006). In chapter 3, we investigate the influence of DNA methylation at the TERT promoter following ALV integration of chicken tumors.

Understanding the interplay between the epigenome of the host DNA and provirus in chickens may reveal significant insights into TERT regulation that can further apply to human TERT regulation. In human cancers, there are similar observations that involve somatic changes at the TERT promoter and in expression. Interestingly, a group has recently reported that the human TERT promoter region is a common integration site in hepatitis B virus-associated hepatocellular carcinomas (Buendia and Neuveut 2015). Presently, the methylation status at these integration sites have not been tested. More recently, investigations into known common point mutations in the human TERT promoter in different cancers reveal that even a single point mutation is capable of activating TERT expression in an allele-specific manner – discussed in more detail in the following section (Stern et al. 2017). These tumors with TERT promoter mutations are associated with an allele-specific perturbation of normal maintenance methylation of the TERT promoter (Stern et al. 2017). More specifically, alleles with TERT promoter mutations show decreased methylation at the TERT promoter, which likely contributes to the activation of TERT expression (Stern et al. 2017).

TERT Promoter Mutations in Cancer

In 2013, a high penetrant disease-segregating causal germline mutation in the TERT promoter of a melanoma family was discovered (Horn et al. 2013). This mutation lead to the discovery of specific and recurrent somatic mutations in tumors from unrelated patients in the TERT promoter (Horn et al. 2013; Huang et al. 2013). This discovery provided an example of a bona fide direct mechanism for cancer-specific activation of TERT expression. In the original study, genetic linkage analysis reveals the disease segregating mutation as a cytosine to thymine transition 57 bps upstream of the ATG start site (Horn et al. 2013). The mutation carriers within the family developed rapid onset and aggressive melanoma, as well as, multiple malignancies (Horn et al. 2013). Consequently, this prompted rapid screening of tumors of unrelated patients with metastatic melanomas. Sequencing of the TERT promoter in melanoma cell lines detected two recurrent and mutually exclusive somatic mutations, a

cytosine to thymine transition at 124 and 146 bases upstream from the ATG start site in the TERT promoter (Horn et al. 2013; Huang et al. 2013).

Each point mutation generates new Ets binding motifs (Horn et al. 2013). Of the four well-characterized Ets transcription factors, the GA-binding protein (GABP) has been demonstrated to bind specifically to the new Ets binding site generated by either mutation (Bell et al. 2015). Following GABP binding, enrichment of active chromatin mark H3K4me2/3 as well as pol II binding occurs, leading to monoallelic expression of TERT (Stern et al. 2017, 2015; Huang et al. 2013). As previously mentioned, allele-specific hypomethylation is observed with mutated alleles. Although TERT is invariably upregulated in most tumors, tumors with the promoter mutations have been reproducibly shown to have significantly increased expression compared with the same type of tumors without mutations (Heidenreich, Nagore, et al. 2014; Heidenreich et al. 2015).

Following the initial discovery, screenings of other cancers reveal that the TERT promoter mutations are not specific to melanomas and occur in varying frequencies. An early exhaustive study looking at 60 different cancers types report that the frequencies of the mutations can generally be grouped into low (<15%) or high (>15%) frequency mutation types, suggesting that frequency may correlate with self-renewal capacity of the tissues (Heidenreich, Nagore, et al. 2014; Killela et al. 2013). Tumors originating from canonical telomerase-negative compartments are likely initially challenged with a telomere-dependent proliferative barrier which is alleviated with the acquisition of TERT promoter mutations. Mutations lead to an immediately advantageous proliferative effect, which could explain the high frequencies of TERT promotor mutations originating from tissues with relatively low rates of self-renewal that includes melanomas, liposarcomas, hepatocellular carcinomas, urothelial carcinomas, medulloblastomas and subtypes (Heidenreich, Nagore, et al. 2014; Killela et al. 2013).

In contrast, the effects of TERT promoter mutations in telomerase-positive compartments would likely be neutral, reducing selection for such alterations, in cancers that include the hematopoietic

system, gastrointestinal stromal tumors, lung, ovarian and uterine cervix cancer, and prostate carcinomas (Killela et al. 2013; Campanella et al. 2015; Wu et al. 2014; Stoehr et al. 2015; Panero et al. 2016; Huang et al. 2013). Nevertheless, more screening identified exceptions to this generalization that include prostate cancer and mantle cell carcinomas (Stoehr et al. 2015; Panero et al. 2016). Furthermore, frequencies of TERT promoter mutations also vary depending on histological sub-classes. For example, desmoplastic melanomas, or melanocytic lesions in chronically exposed skin, carry TERT promoter mutations at a frequency exceeding 80% (Shain et al. 2015). In contrast, acral and mucosal melanoma are nearly free of TERT promoter mutations (Liau et al. 2014). For adult gliomas, high frequencies are observed in primary glioblastoma (70-80%), followed by oligodendrogliomas (60-70%), oligoastrocytomas (35-55%), and astrocytomas (30-40%) (Killela et al. 2013).

From the literature, initial screens of hematological malignancies were limited to a few types that included leukemia and diffused large B-cell lymphomas or restricted to small sample sizes (Mosrati et al. 2015; Yan et al. 2013; Vinagre et al. 2013; Killela et al. 2013). These studies suggested that hematological malignancies may not be subjects for somatic promoter mutations in the TERT gene. While the observation regarding the relationship between self-renewal potential and frequency of TERT promoter mutations may fit the current data, exceptions were identified which included the presence of TERT promoter mutations in 33% of mantle cell lymphomas tested (Panero et al. 2016).

Although cells of hematological origin are characterized with persistent telomerase activity, evidence of early activation, as well as, late activation of TERT expression and telomerase activity is observed in hematological malignancies. In adult T-cell leukemia/lymphoma (ATLL), telomerase activity appears as a key event in the development and progression of the disease, and ATLL cells are characterized with long telomeres that suggest an early activation of TERT that may contribute to a delay in replicative senescence and prolonged time to acquire genetic alterations for the induction of a fully transformed phenotype (Dolcetti and De Rossi 2012; Kubuki et al. 2005). In contrast, in acute

myeloid leukemia (AML), chronic myeloid leukemia (CML), and B-cell disease, telomerase activity is not required for the initiation of disease but is required for its maintenance at later stages (Röth et al. 2003; Vicente-Dueñas et al. 2012; Bruedigam and Lane 2016; Deville, Hillion, and Ségal-Bendirdjian 2009). TERT promoter mutations may simply offer another method to achieve TERT activation and, subsequently, increase telomerase activity.

Taking into account the aforementioned factors, the prevalence of promoter mutations in hematological malignancies remains unclear. To improve upon current data, we screened a more robust collection of different hematological malignancies for TERT promoter mutations which is covered in chapter 4. If there are hematological malignancies with TERT promoter mutations, we can draw and test potential associations between our studies using ALV insertional mutagenesis in the chicken TERT promoter and TERT promoter mutations in humans.

Chapter 2. Investigation of TERT overexpression in ALV-infected chickens

Abstract

Avian leukosis virus (ALV) induces B-cell lymphoma and other neoplasms in chickens by integrating within or near cancer genes and perturbing their expression. The TERT promoter region have been previously identified as a common integration site in these lymphomas, suggesting a causal role in tumorigenesis. In addition, TERT promoter integrations are clonal, implicating selection for these integrations early in tumorigenesis. Tumors with TERT promoter integrations are associated with increased TERT expression. In this study, we test the effects of early TERT expression in chicken embryos on ALV-induced tumorigenesis. Overall, we were unable to conclude if TERT overexpression had any impact on tumor progression. Nevertheless, two tumors with TERT overexpressing provirus were detected. ALV integration site analysis implicates ARIB4B as a potential TERT cooperating gene. In addition, we observed an unexpected high frequency of ALV-A induced hemangiomas. Integration analysis of these hemangiomas reveal potential novel players in hemangioma development.

Introduction

Avian leukosis virus (ALV) is a simple retrovirus that primarily causes B-cell lymphomas in infected chickens (Beemon and Rosenberg 2012; Hayward, Neel, and Astrin 1981; Justice and Beemon 2013). ALV-induced lymphomas develop in a multi-stage process, originating initially as neoplastic follicles in the bursa, which can develop into primary bursal tumors. Primary tumors can then metastasize and form secondary tumors in other tissues, which commonly include the liver, kidney, and spleen (Neiman et al. 2003). This study uses a specific ALV strain that can induce rapid-onset lymphomas, which develop in less than 3 months after infection of 10-day chicken embryos (Polony et al. 2003; Smith et al. 1997).

Cellular transformation is caused by multiple genetic changes that result in the dysregulation of genes that promote tumorigenesis. ALV-induced lymphomas commonly occur through insertional mutagenesis by means of proviral integration. Generally, proviral integration induces the aberrant

expression of oncogenic factors via promoter insertion or enhancer activation that is mediated by its long terminal repeats (LTRs) (Beemon and Rosenberg 2012; Hayward, Neel, and Astrin 1981; Justice and Beemon 2013).

From the investigation of ALV integration sites in chicken tumors, sites that are often targets of integrations – or common integration sites – can be observed. These common integration sites implicate neighboring genes as potential oncogenic factors in tumorigenesis (Hayward, Neel, and Astrin 1981; Baba and Humphries 1986; Clurman and Hayward 1989; Yang et al. 2007; Justice, Morgan, and Beemon 2015; Nehyba et al. 2016; Jiang et al. 1997). Additionally, measuring the relative abundance of proviruses – or clonality – can provide insight into tumor development (Neiman et al. 2003; Yang et al. 2007; Malhotra, Winans, et al. 2017). Highly clonal integrations may implicate early activation of the associated neighboring genes during tumorigenesis. Previously, the TERT promoter region was identified as a common integration site (Yang et al. 2007). In addition, unique integrations in the TERT promoter were found to be one of the most clonally expanded integration sites. This implicates TERT promoter integration and, subsequently, TERT activation as an early event in tumorigenesis (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). Twenty-six unique integration sites were identified in the region in multiple independent tumors (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017).

Telomerase is a ribonucleoprotein complex that adds repeat sequences to chromosome ends, called telomeres. It is comprised of a catalytic protein component, TERT, as well as a noncoding telomerase RNA template component, TERC. Upregulation of telomerase activity has been associated with more than 90% of all human cancers, including lymphomas (Shay and Bacchetti 1997). Elevated telomerase activity diminishes replication-associated telomere shortening by maintaining telomere lengths, allowing continual proliferation and survival of cancer cells (Blasco 2005).

Elevated TERT expression is observed in chicken tumors with clonal TERT promoter integrations, implicating the activation of TERT expression by ALV proviral integration as an early selection factor for tumorigenesis. The effects of constitutive overexpression of TERT in whole organisms has been studied using transgenic mice. While overexpression of TERT has been shown to be associated with increased tumorigenesis in mice, the majority of studies point to mechanisms that are independent of telomere length (Gonzalez-Suarez et al. 2001; Canela et al. 2004; Cayuela, Flores, and Blasco 2005; Flores, Cayuela, and Blasco 2005). Currently, proposed telomerase-independent functions include increased wound healing (Cayuela, Flores, and Blasco 2005), mobilization of cells (Canela et al. 2004; Flores, Cayuela, and Blasco 2005), and cell survival (Lee et al. 2008).

Telomere independence of mice may be partly due to the contrasting properties of murine telomere biology, compared to humans. Constitutive telomerase expression is found throughout the lifespan of a mouse in both somatic and renewable tissues. Subsequently, most cells in mice are characterized with long telomeres throughout their lifespan. Furthermore, mice show a significantly higher susceptibility to spontaneous oncogenesis (Blasco et al. 1997; Forsyth, Wright, and Shay 2002; Sherr and DePinho 2000; Wright and Shay 2000). In contrast, the chicken may present a optimal model for the study of telomeres and telomerase. Like humans, telomerase activity is observed early in development, maintained in renewable tissues and stem cells, and diminishes in most somatic tissues following embryogenesis in chickens (Forsyth, Wright, and Shay 2002; S.E. Swanberg and Delany 2003; Taylor and Delany 2000). Consequently, division-dependent telomere shortening is observed in chicken somatic tissues *in vivo*, which also correlates with age (Taylor and Delany 2000; M. Delany et al. 2003).

Various converging lines of evidence suggest that TERT is likely not sufficient to drive tumorigenesis alone. Chicken tumors with clonal TERT promoter integrations show coinciding selection for integrations near other genes like MYB in multiple tumors, suggesting that tumors are the result of multiple cooperating genes (Malhotra, Winans, et al. 2017). Furthermore, while overexpression of TERT

is sufficient to immortalize chicken cells like adipocytes, cells overexpressing TERT do not show properties of malignant transformation such as loss of contact inhibition, foci formation, or anchorage-independent growth (Wang et al. 2017). In humans, melanoma patients with familial TERT promoter point mutations, which have been shown to promote TERT expression, are healthy until adulthood which is when they encounter higher incidences of melanoma compared to patients without mutations. This observation is consistent with the idea that TERT contributes to tumorigenesis, but additional factors are required during the patients' lifetime for malignant transformation (Horn et al. 2013).

In this study, we attempted to investigate the effects of early TERT overexpression in ALV-infected chicken embryos on tumorigenesis. TERT overexpression is achieved through the use of replication-deficient recombinant ALV-derived virions. Chickens are coinfected with recombinant virions and ALV. Coinfection with ALV is expected to contribute additional genetic hits to TERT overexpression cells and, subsequently, enables cells to undergo malignant transformation. Integration analysis of tumors with recombinant proviruses and ALV may provide potential insight into additional TERT-cooperating genes.

Materials and Methods

Cloning of chTERT Overexpressing Recombinant Virus

Chicken embryo fibroblast (CEF) RNA was extracted using Amsbio RNA-Bee. First strand cDNA synthesis was performed using Thermo Fisher SuperScript® III First-Strand Synthesis System. Full length chicken TERT cDNA was cloned flanked with ClaI sites by PCR using primer 1 and 2 and New England Biolabs (NEB) Phusion® High-Fidelity DNA polymerase (Figure 2.1 and Table 2.1). Cloned TERT was then cut with NEB ClaI and ligated with NEB T4 ligase into a vector called DAS, generating DAS TERT (Figure 2.1). DAS is a vector derived from ALV that lacks the env genes, allowing the encoded recombinant virus to accommodate large cDNA like TERT. Lacking the env gene, DAS clones are replication deficient and require a helper virus for packaging. Ligation products were used to transform XL-1 Blue competent

cells. Single clones from ampicillin selection were screened by colony PCR using primer 1 and 2 with Phusion polymerase. The 4041 bp full length chicken TERT cDNA was confirmed by conventional Sanger sequencing (Eurofins Genomics, Louisville, Kentucky), using staggered PCR primers: primer 3-7 (Figure 2.1 and Table 2.1).

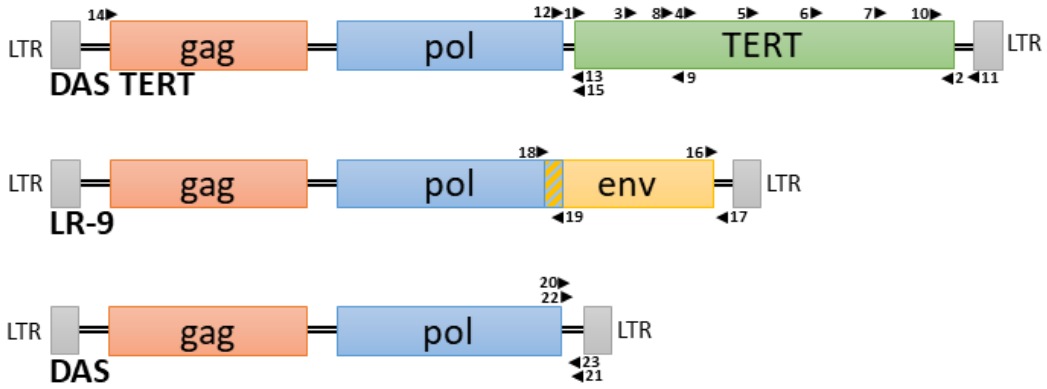


Figure 2.1. Schematic illustrations of viral vectors. At the top, a schematic of cloned TERT virus; middle, the specific strain of ALV, LR-9; bottom, the replication deficient viral vector used for cloning the TERT virus. Black arrows indicate the location of the different primers used throughout the study.

Primer	Sequence (5'-3')	Purpose
1	GCAT-ClaI-ATGGAGCGCGGGGCTCAG	Cloning chicken TERT into DAS
2	GCAT-ClaI-TTAGTCCAGTATAGTTTTGAAA	Cloning chicken TERT into DAS
3	CGGGCAACCAATTTATGAAC	Sequencing of TERT clone in DAS
4	CCTGTGGCAGACAAAAGCTC	Sequencing of TERT clone in DAS
5	TGGGAAAGAGTCCACTGAGG	Sequencing of TERT clone in DAS
6	ACTAAGCCGTGTTGTTGAAGG	Sequencing of TERT clone in DAS
7	CTCTGTGGGATCCAGAAGGA	Sequencing of TERT clone in DAS
8	CGTGATGGAGAACAGGTTGA	qPCR of total TERT cDNA (exon 2)
9	GTGCTACATGCCAGGAGGTT	qPCR of total TERT cDNA (exon 2)
10	CCCTTAAAGTCTATAAGATGCATCTGTTTGGG	Detection of DAS TERT viral cDNA and DAS TERT provirus in tumors
11	ATGTTGCTAACTCATCGTTACCATGTTG	Detection of DAS TERT viral cDNA and DAS TERT provirus in tumors
12	AGGAGAAACCGCTAGCAACA	Quantification of DAS TERT viral titer
13	GCTCCATATCGATGCCACAG	Quantification of DAS TERT viral titer
14	TCAAGCATGGAAGCCGTC	Detection of spliced proviral TERT cDNA
15	CTGAGCCCCGCGCTCCAT	Detection of spliced proviral TERT cDNA
16	ATTCACTCGGCTATCGCGAG	Detection of LR-9 viral cDNA in viral supernatant
17	CAAGCCTTGCCCCGTTACAG	Detection of LR-9 viral cDNA in viral supernatant
18	AACCGGACATCACCCAAA	Quantification of LR-9 viral titer
19	CCTCACACAAGACCAGGACA	Quantification of LR-9 viral titer
20	GAGCTGAGCTGACTCTGCTG	Detection of DAS cDNA in viral supernatant
21	CCCTCCCTATGCAAAAGCGAAAC	Detection of DAS cDNA in viral supernatant
22	TGAGCTGACTCTGCTGGTG	Quantification of DAS viral titer
23	GGACTCCTAACCGCGTACAA	Quantification of DAS viral titer

Table 2.1. Summary of primer sequences and purposes

Transfection, Infection, and Virion Production

CEFs were cultured in medium 199 (Thermo Fischer Scientific, Waltham, MA, USA) supplemented with 2% tryptose phosphate, 1% fetal calf serum, 1% chick serum, and 1% antibiotic/antimycotic (199 2-1-1) at 39°C and 5% CO_2. A specific mutant of ALV, called LR-9 G919A, was used as a helper virus for the packaging of DAS TERT and DAS virions. Henceforth, referred to as either LR-9 or ALV, LR-9 G919A contains a silent mutation in a regulatory element that results in rapid onset of B-cell lymphomas in chickens (Polony et al. 2003). LR-9 DNA was SacI digested and ligated to form recombinant competent products prior to electroporation. A 2:5 weight ratio of LR-9 to DAS TERT or DAS was used for electroporation. CEFs were transfected in Opti-MEM™ (Thermo Fischer Scientific, Waltham, MA, USA) with 1 x 220 mV 40 ms pulse. Cells were allowed to recover for 2 mins in 199 2-1-1 at room temperature. Transfected CEFs were transferred to a 60 mm plate and propagated until near confluency. All cells were then transferred to 100 mm plates and once again into 150 mm plates. Supernatant was collected daily for three consecutive days once cells were confluent in 150 mm plates, which took an average of nine days. Cells and supernatants were collected for further experiments.

Small aliquots of supernatant were collected throughout the propagation and to test for reverse transcriptase (RT) activity described in the following section. For infection, CEFs were trypsinized, resuspended in in a small volume (800 µl per 60 mm plate), and plated with 100 µl of viral supernatant that was positive for RT activity. Cell were incubated for 1 hour with occasional shaking and then allow to propagate until confluency. Supernatants were then collected to test for RT activity, and cells were collected for further experiments.

Reverse Transcriptase (RT) Assay

22 µl of viral supernatant was added to a 28.5 µl RT reaction mix – RT Assay buffer (20 mM $MgCl_2$, 0.2% Triton X-100, 2 mM EDTA, and 100 mM Tris pH 8.0), 2.5 µl of poly(rC)-p(dG) 12-18 RT substrate, 1 mM dGTP, α-^{32}P, and 0.1 M DTT. Reaction was incubated at 37°C for 1 hour. Reaction was

then subjected to speed vac to reduce the volume to approximately 10 µl. The entire reaction was then spotted on to DE81 DEAE cellulose paper and allowed to dry for 10 mins at room temperature. Spotted paper was washed 3 times for 5 mins each with 2x SSC buffer (300 mM NaCl, 30 mM sodium citrate) and followed by one wash for 5 mins in 100% EtOH. Paper was air dried for 5 mins and exposed to a phosphor screen. Phosphor screen was then imaged using a Typhoon Imager (General Electronic Healthcare, Piscataway, NJ, USA).

Telomerase Repeated Amplification Protocol (TRAP) Assay

Whole cell extracts were prepared with CHAPS buffer (10 mM Tris-HCl pH7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.5% CHAPS, 10% glycerol). Protein concentrations were determined by the Bradford method with Bio-Rad Protein Assay Reagent (Hercules, CA, USA). Protein extracts (20 µg of total protein) were first incubated with 0.1 µg of unlabeled TS primer and all four dNTPs (50 µM each) in 1x TRAP buffer (25 mM Tris-HCl pH 8.4, 63 mM KCl, 1.5 mM $MgCl_2$, 0.05% Tween 20, 1 mM EGTA), 0.5 mg/ml purified BSA (NEB), 1 mM spermidine, and 5 mM beta-mercaptoethanol in a total reaction volume of 50 µl for 45 mins at 37°C. Total volume of the extract was adjusted with CHAPS buffer. The reaction was stopped by incubation at 94°C for 2 min. CHAPS buffer was included as a negative control, and commercially available telomerase positive cell extract was used as a positive control. Heat inactivated extract was used as an additional control.

Aliquots of synthesis (1.25 µl) were then PCR amplified in 1x Herculase buffer with all four dNTPs (50 µM each). 0.5 mg/ml purified BSA, 0.05 µg Cy5-labeled-TS primer, 0.05 µg ACX primer, TSNT primer mix (0.1 µg of NT primer and 0.01 mol TSNT per 1 µl), and 0.25 µl of Herculase Hotstart (Thermo Fischer Scientific, Waltham, MA, USA) in 25 µl of total reaction volume. All oligonucleotides – TS, ACX, NT, TSNT were made as described by Kirn and Wu (1997). TS primer used in PCR amplification was labeled at 5' with Cy5, which was commercially available from Integrated DNA Technologies (Coralville, IA, USA).

PCR amplification started with 94°C for 2 min followed with 27-36 cycles (30 secs at 95°C, 30 secs at 55°C, and 1 min at 72°C). STOP/loading buffer (BF blue, glycerol, EDTA) was added to the TRAP PCR products and separated on 7.5% acrylamide gels (ratio of acrylamide to bis-acrylamide 19:1) in 0.5x TBE. Gel images were captured using Typhoon Imager in fluorescence mode (General Electronic Healthcare, Piscataway, NJ, USA).

Quantitative PCR and End Point PCR of TERT and Viral RNA

All samples used were extracted using Amsbio RNA-Bee. First strand cDNA synthesis was performed using Thermo Fisher SuperScript® III First-Strand Synthesis System. All quantification of cDNA used standard qPCR conditions with SYBR® Green Master mix (Bio-Rad, Hercules, CA, USA) in a CFX96 real-time PCR detection system (Bio-Rad, Hercules, CA, USA). All primer locations with respect to their target sequences are illustrated in Figure 2.1, and sequences and purposes are listed in Table 2.1. Quantification of total TERT cDNA in transfected and infected CEFs and tumor samples used primer 8 and 9, located in exon 2 of chicken TERT. For the detection of viral cDNA in supernatants from transfected and infected CEFs and tumors, primers targeting the region between the 3' LTR and the nearest genes were used. Primer 10 and 11 were used for DAS TERT, primer 16 and 17 for LR-9, and primer 20 and 21 for DAS. Tumors were screened for DAS TERT provirus by PCR using primer 10 and 11.

Quantification of viral titers in supernatants used primers targeting the junction between pol and the sequence downstream of each viral cDNA (Figure 2.1). Primer 12 and 13 were used for DAS TERT, primer 18 and 19 used for LR-9, and primer 22 and 23 for DAS. Viral titers were calculated by back calculating from qPCR measurements against a standard curve generated from known concentrations of corresponding vectors. Spliced proviral TERT transcripts were quantified by qPCR using primer 14 and 15, located just upstream of the gag splice donor site and downstream of the env splice acceptor sites, respectively.

Tumor Induction and Tissue Isolation

12-day old chicken embryos were inoculated intravenously with viral supernatants prepared from transfected CEFs. Three different conditions were used: ALV only (LR-9), vector control and ALV (DAS + LR-9), and DAS TERT and ALV (TERT + LR-9) (Table 2.2-2.4). An additional mock group that was sham-inoculated with medium was used as a negative control. Viral titers for inoculation were normalized to equivalent concentrations of ALV (1.49×10^5 virions/ml). In addition to ALV, the DAS group was inoculated with 3.31×10^5 DAS virions/ml and the TERT group was inoculated with 1.56×10^5 TERT virions/ml, as estimated by qRT-PCR. In total, 9 birds infected with ALV only, 26 birds with DAS and ALV, and 27 birds with DAS TERT and ALV were followed and scored (Table 2.2-2.4).

Birds were kept in isolators for 14 weeks. Isolator units were cleaned weekly, and containment was maintained throughout the 14 weeks. Birds were removed as they died or became moribund. A bird was considered moribund if it was unable to move. Tumors tissues were excised at the time of death or sacrifice, snap frozen in liquid nitrogen, and stored at -80°C. Tumor tissues included primary bursal (B) tissue, secondary tumors in the liver (L), kidney (K), spleen (S), muscle (M), and leg (LE). All birds were sacrificed at the final take-down on the 15th week. All birds were necropsied, and cause of death was determined according to ALV lesions as listed in Diseases of Poultry. Summary tables of the bird data can be found in Table 2.2-2.4.

LR-9	Bird ID	Hatch Date	Death Date	Weeks after hatching	Lymphoid	Hemangioma	Other	Multiple lesions
	882	01-28-2015	03-08-2015	5.6	-	+	+	Y
	883	01-28-2015	05-14-2015	15.1	-	+	-	N
	884	01-28-2015	03-29-2015	8.6	+	-	-	N
	885	01-28-2015	05-07-2015	14.1	-	+	-	N
	886	01-28-2015	05-14-2015	15.1	+	-	+	Y
	887	01-28-2015	02-23-2015	3.7	-	-	-	N
	888	01-28-2015	05-14-2015	15.1	-	+	-	N
	889	01-28-2015	05-14-2015	15.1	+	-	-	N
	890	01-28-2015	04-18-2015	14.4	-	-	+	N
Total Birds	9				3	4	3	2

Table 2.2: Birds infected with LR-9 IDs, time of death, and neoplasms observed

DAS + LR-9	Bird ID	Hatch Date	Death Date	Weeks after hatching	Lymphoid	Hemangioma	Other	Multiple lesions
	81	01-29-2015	04-18-2015	11.3	+	+	-	Y
	82	01-29-2015	02-26-2015	4.0	+	-	-	Y
	83	01-29-2015	03-04-2015	4.9	-	+	-	N
	201	02-18-2015	05-04-2015	10.7	+	-	-	N
	202	02-18-2015	04-23-2015	9.1	-	+	-	N
	203	02-18-2015	03-29-2015	5.6	-	-	+	N
	204	02-18-2015	05-05-2015	10.9	+	+	-	Y
	205	02-18-2015	06-04-2015	15.1	-	+	-	N
	206	02-18-2015	04-15-2015	8.0	+	-	-	N
	207	02-18-2015	06-04-2015	15.1	-	-	-	N
	208	02-18-2015	04-16-2015	8.1	+	-	-	N
	209	02-18-2015	05-29-2015	14.3	+	+	-	Y
	210	02-18-2015	06-04-2015	15.1	-	-	-	N
	211	02-18-2015	04-05-2015	6.6	-	+	-	N
	212	02-18-2015	04-26-2015	9.6	+	-	-	N
	213	02-18-2015	05-01-2015	10.3	-	+	+	Y
	214	02-18-2015	04-09-2015	7.1	-	+	-	N
	215	02-18-2015	06-04-2015	15.1	+	-	+	Y
	216	02-18-2015	03-17-2015	3.9	-	-	-	N
	217	02-18-2015	04-17-2015	8.3	-	+	-	N
	218	02-18-2015	04-15-2015	8.0	-	+	-	N
	219	02-18-2015	03-26-2015	5.1	-	+	-	N
	220	02-18-2015	04-12-2015	7.6	+	+	-	Y
	221	02-18-2015	06-04-2015	15.1	-	+	-	N
	222	02-18-2015	06-04-2015	15.1	-	-	-	N
	223	02-18-2015	06-04-2015	15.1	-	-	+	N
Total Birds	26				10	14	4	7

Table 2.3: Birds infected with DAS and LR-9 IDs, time of death, and neoplasms observed

TERT + LR-9	Bird ID	Hatch Date	Death Date	Weeks after hatching	Lymphoid	Hemangioma	Other	Multiple lesions
	781	01-30-2015	05-04-2015	13.6	+	-	-	N
	782	01-30-2015	02-28-2015	4.1	-	+	-	N
	783	01-30-2015	05-14-2015	15.0	+	+	+	Y
	784	01-30-2015	02-22-2015	3.3	-	-	-	N
	785	01-30-2015	03-03-2015	4.6	-	+	-	N
	787	01-30-2015	05-14-2015	15.0	-	-	-	N
	788	01-30-2015	02-21-2015	3.1	-	-	-	N
	789	01-30-2015	05-14-2015	15.0	+	-	+	Y
	790	01-30-2015	05-14-2015	15.0	-	+	-	N
	791	01-30-2015	04-01-2015	8.9	+	-	-	N
	792	01-30-2015	05-14-2015	15.0	-	+	-	N
	793	01-30-2015	04-07-2015	9.6	-	-	+	N
	794	01-30-2015	03-13-2015	6.1	+	+	-	Y
	795	01-30-2015	05-14-2015	15.0	+	-	-	Y
	796	01-30-2015	02-23-2015	3.6	+	-	-	Y
	797	01-30-2015	05-14-2015	15.0	+	-	+	Y
	798	01-30-2015	03-27-2015	8.1	-	+	-	N
	799	01-30-2015	03-26-2015	8.0	+	+	-	Y
	892	01-30-2015	04-01-2015	8.7	-	-	-	N
	91	02-18-2015	04-16-2015	6.9	-	+	-	N
	92	02-18-2015	04-07-2015	6.9	-	-	+	N
	93	02-18-2015	05-18-2015	12.9	+	+	-	Y
	94	02-18-2015	04-01-2015	6.1	-	-	+	N
	95	02-18-2015	04-16-2015	8.3	+	+	-	Y
	96	02-18-2015	06-04-2015	15.3	-	-	+	N
	97	02-18-2015	06-04-2015	15.3	+	+	+	Y
	98	02-18-2015	05-25-2015	13.9	-	+	-	N
Total Birds	27				12	13	8	10

Table 2.4: **Birds infected with TERT + LR-9 IDs, time of death, and neoplasms observed**

Integration Site Mapping and Quantification

DNA from tumors was isolated. The sequencing libraries were prepared as described previously (Justice et al., 2015). Ten µg of purified genomic DNA was sonicated with a Bioruptor UCD-200 (Diagenode Inc., Denville, NJ, USA). End repair, A-tailing, and adapter ligations were performed as described previously (Gillet et al. 2011) (P-GATCGGAAGAGCAAAAAAAAAAAAAAA, and adapter long arm, CAAGCAGAAGACGGCATACGAGATXXXXXXGTGACTGGAGTTCAGACGTGTGCTCTTCCGATC*T, where "X's" denote the barcode sequence, "P" denotes phosphorylation, and "*" denotes a phosphorothioate bond). Nested PCR was performed to enrich the library for proviral junctions. In the first round of PCR, 23 cycles were used with an ALV-specific primer (CGCGAGGAGCGTAAGAAATTTCAGG) between the 3' LTR and *env* and a primer (CAAGCAGAAGACGGCATACGAGAT) within the adapter that was attached by ligation. In the second round of PCR, a primer (AATGATACGGCGACCACCGAGATCTACACXXXXXXXXGACG-ACTACGAGCACATGCATGAAG) near the 3' end of the LTR was used. This primer has an additional barcode for increased multiplexing and ended 12 nucleotides short of the junction between viral and genomic DNA. This primer was paired with an adapter-specific primer on the opposite side of the fragment, which overlapped the adaptor's bar code sequence (CAAGCAGAAGACGGCATACGAGATXXXXX-X). Libraries were quantified by quantitative PCR (qPCR) and then under-went single-end 100-bp multiplexed sequencing on the Illumina Hi-Seq 2000. A custom sequencing primer (ACGACTACGAGCACATGCATGAAGCAGAAGG) was used, which hybridized near the end of the viral 3' LTR, 5 nucleotides short of the proviral/genomic DNA junction. The resulting reads could be validated as genuine integrations by verifying that they began with the last 5 nucleotides of the proviral DNA, CTTCA. The last two nucleotides of the unintegrated proviral DNA, TT, were cleaved by ALV integrase upon integration, so the lack of these 2 nucleotides in the read acted as further validation of a true viral integration.

Sequence Analysis

Reads were curated with a custom python script to remove sequences that did not start with the last five nucleotides of viral DNA, "CTTCA" (J. F. Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). The files were uploaded to Galaxy (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010). In Galaxy, quality scores were converted to Sanger format with FastQ Groomer v1.0.4 (Blankenberg et al. 2010). CTTCA and adapter sequences were removed using Galaxy Clip tool v1.0.1. This tool also removed reads containing ambiguous nucleotides and reads less than 20 nucleotides after adapter removal. Reads were mapped with Bowtie (Goecks et al. 2010) against the *Gallus gallus* 4.0 genome (Nov. 2011). Sequences were aligned using a seed length of 28 nucleotides, with a maximum of 2 mismatches permitted in the seed. All alignments for a read were suppressed if more than one alignment existed to avoid multiple mapping and ensure reads correspond to only unique integration sites. 50,000 random reads were selected from each sample to be used for further analysis. If less than 50,000 reads were obtained, all available reads were used. A custom Perl pipeline developed in the lab was used to analyze the aligned reads from bowtie (Justice, Morgan, and Beemon 2015). The custom pipeline identified unique integration sites, hotspots of integration, and common integration sites among multiple samples. Integrations from two unrelated barcodes on the same sequencing lanes were omitted.

Statistical Analysis

For statistical analysis of data Microsoft Excel 2016 was used. Cumulative mortality rates were estimated using linear regression. Statistical significance between mortality rates were calculated by t-test comparing the slope coefficients of any two linear regressions (Soper 2018). Statistical significance between two population proportions, or percentages, were tested using Fisher's exact test for a 2 x 2 contingency table for each category of interest (Soper 2018).

Results

Cloning of Chicken TERT into a Recombinant ALV-derived Vector

Full length TERT cDNA was cloned into DAS, a vector encoding a replication-deficient recombinant virus derived from RSV. The resulting clone is named DAS TERT (Figure 1.1). The 4041 bp long TERT insert was confirmed by Sanger sequencing using staggered primers (Figure 2.1). Sequence was identical to reference full length TERT transcript reported on Ensembl (ENSGALT000000021539.2). Transfection of CEF with DAS TERT or DAS TERT and ALV increased total TERT expression by 36-fold or 568-fold, respectively (Figure 2.2A). CEF transfected with DAS TERT and ALV had increased telomerase activity (Figure 2.2B). In contrast, transfection by DAS and ALV did not significantly increase telomerase activity (Figure 2.2B).

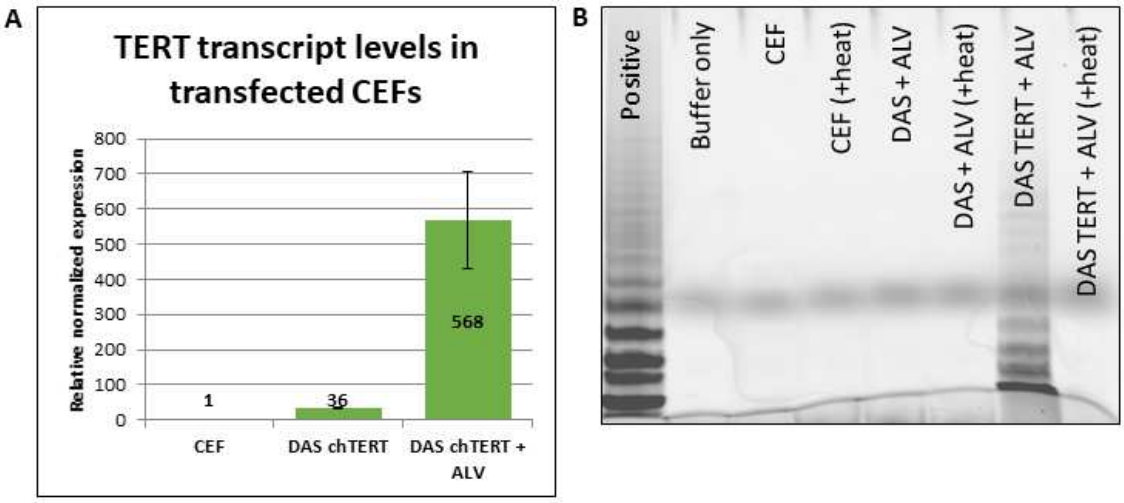


Figure 2.2. TERT overexpression in DAS TERT transfected CEFs. A) qPCR of total TERT transcripts in CEFs 8 days post-transfection with DAS TERT vector or cotransfection with DAS TERT and ALV. B) Typhoon image of TRAP assays with the indicated samples. Commercially available telomerase positive cells were used as a positive control.

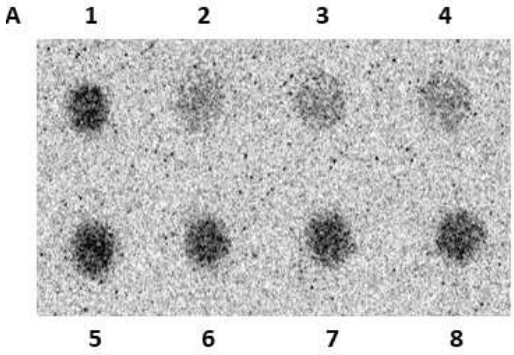
Virion Production and Infection with TERT Virus

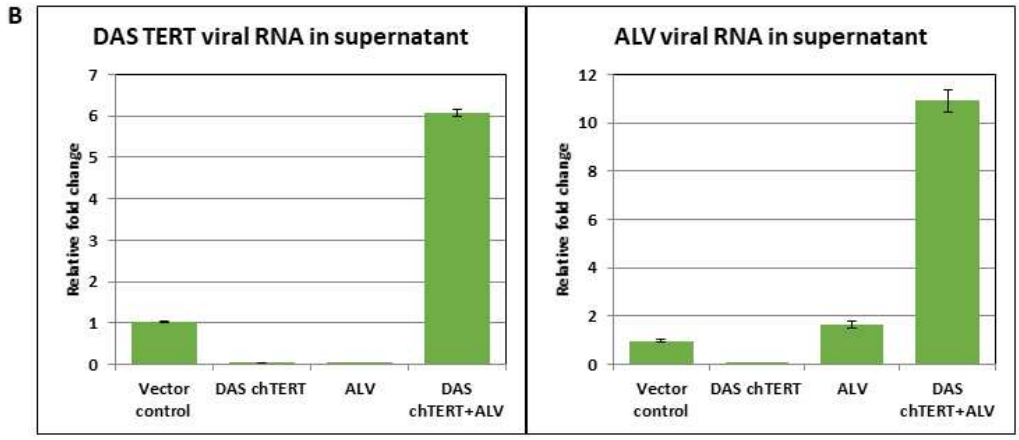
Supernatant from CEFs cotransfected with DAS TERT and ALV tested positive for RT activity, indicating successful virion production post-transfection (Figure 2.3A). Virions produced from coinfection were able to produce supernatant positive for RT activity, indicating successful virion production post-infection (Figure 2.3A). Supernatant from CEFs cotransfected with DAS TERT and ALV

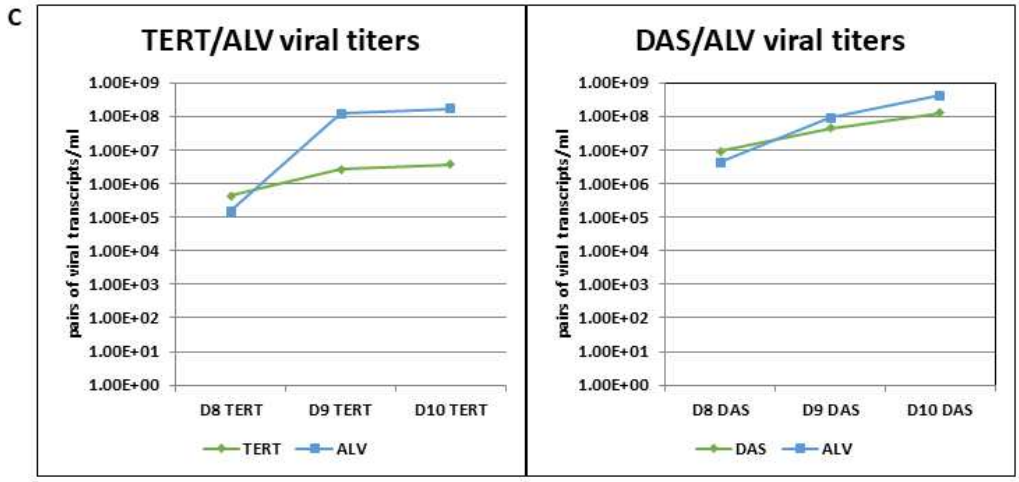
have detectable viral TERT and ALV cDNA (Figure 2.3B). Quantification of TERT, DAS, and ALV viral titers was possible using qRT-PCR, targeting unique sequencing to each viral RNA (Figure 2.3C).

Higher proportions of TERT virions were produced at earlier time points – eight days post transfection being the earliest with appreciable titers (Figure 2.3C). An increase in the relative titer of ALV compared to TERT virions was observed over time in viral samples with TERT and ALV (Figure 2.3C). In contrast, viral samples containing DAS and ALV did not show any significant difference in virion production (Figure 2.3C). Comparing the titers of TERT and DAS, packaging may be more efficient with smaller viral genomes. Viral titers prepared from chicken experiments eight days after transfection were selected in order to maximize yield of TERT virions.

CEFs infected with viral supernatant from DAS TERT and ALV transfected cells show increased telomerase activity (Figure 2.3D). In contrast, CEFs infected with DAS and ALV did not show significant increase in telomerase activity (Figure 2.3D).







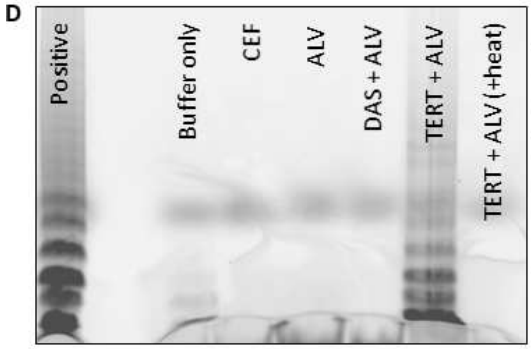


Figure 2.3. TERT, DAS, and ALV virion production. A) On the left, Typhoon image of a phosphor scrren of RT assays with samples indicated on the right. B) qRT-PCR of DAS TERT viral RNA on the left and ALV viral RNA on the right in supernatant samples listed. Corresponding vectors for each viral cDNA was used as a positive control. Expressions were relatively to a known amount of vector control C) qPCR quantification of the number of viral transcripts in supernatant containing TERT and ALV virions on the left and DAS and ALV virions on the right from three consecutive days (day 8-10). D) Typhoon image of TRAP assays of CEFs 6 days post-infection with viral supernatants. Commercially available cells positive for telomerase activity were used as a positive control.

Significant Changes in Mortality/Morbidity Rates Between Chicken Groups

High morbidity in birds was observed as early as 4 weeks after hatching (Figure 1.3). The first birds were sacrificed at the end of 4 weeks in both the DAS group and the TERT group. Cumulative mortality appeared to increase in a linear manner with respect to time for all infected groups (Figure 2.3). Mortality rates were statistically significant between each group where DAS show the highest rate, followed by TERT, and LR-9. At the end of 14 weeks, LR-9 had 44% (4/9) chickens sacrificed or died, the DAS group had 69% (18/26), and the TERT group had 56% (15/27). No significant difference in morbidity was observed between the chicken groups at the end of 14 weeks, which is the week before the final takedown at week 15.

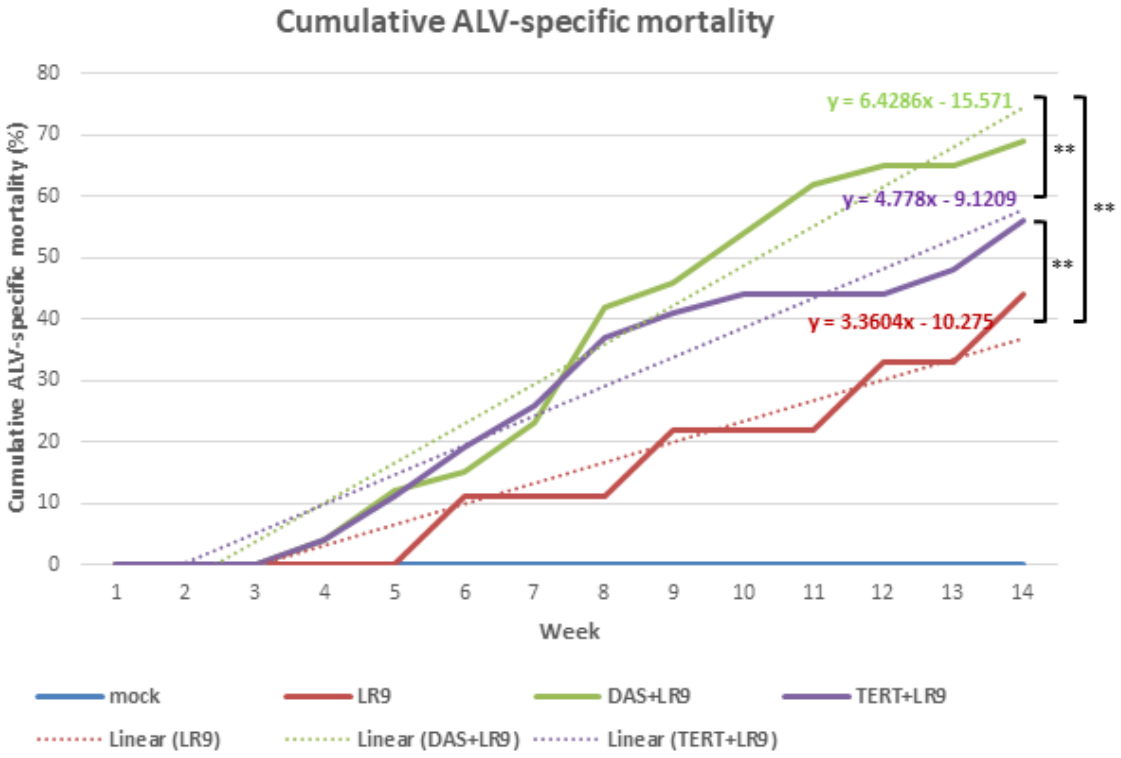


Figure 2.4. Mortality and morbidity in infected chickens. Percent cumulative mortality is plotted at the end of each week for each chicken group. Linear regression lines and equation are depicted as an estimate of the rate of mortality from ALV-specific lesions. Test of significance are performed using t-test between the slope coefficients of the linear regression lines (n = 14, ** $P < 0.01$) Remaining chickens were sacrificed at end of 15 weeks.

No Significant Difference in Types of Lesions or Tumor Burden Between Chicken Groups

Types of lesions were scored and categorized into lymphoid, hemangiomas, or other – lesions that did not classically fit into lymphoid or hemangiomas – and tumor burden was reflected in the occurrence of multiple lesions in a single bird. In all 3 chicken groups, hemangiomas accounted for the majority of tumors ranging from 44% to 54%, followed by lymphoid lesions ranging from 33% to 44%, and, lastly, lesions that were ambiguous ranging from 15% to 30% (Table 2.5). Percentage of multiple lesions ranged from 22% to 37% (Table 2.5). No statistical significance was found between the chicken groups across all four variables.

Group	Lymphoid	Hemangioma	Other	Multiple lesions	Any lesions
LR-9	3/9 (33%)	4/9 (44%)	2/9 (22%)	2/9 (22%)	8/9 (89%)
DAS + LR-9	10/26 (38%)	14/26 (54%)	4/26 (15%)	7/26 (27%)	22/26 (85%)
TERT + LR-9	12/27 (44%)	13/27 (48%)	8/27 (30%)	10/27 (37%)	23/27 (85%)

Table 2.5: Number and percentage of birds with specific types of lesions and tumor burden

Chicken Tumors with TERT Recombinant Provirus

Using primers specific for the TERT recombinant provirus, a PCR screen of tumors from chickens coinfected with the TERT recombinant virus and ALV revealed two tumors out of 32 tumors (1 out of 27 birds) that had an integrated TERT recombinant provirus (Figure 2.5A and 2.5B). The two liver tumors, 791L1 and 791L2, show the predicted amplification product of the unique TERT-ALV sequence in the TERT recombinant provirus (Figure 2.5B).

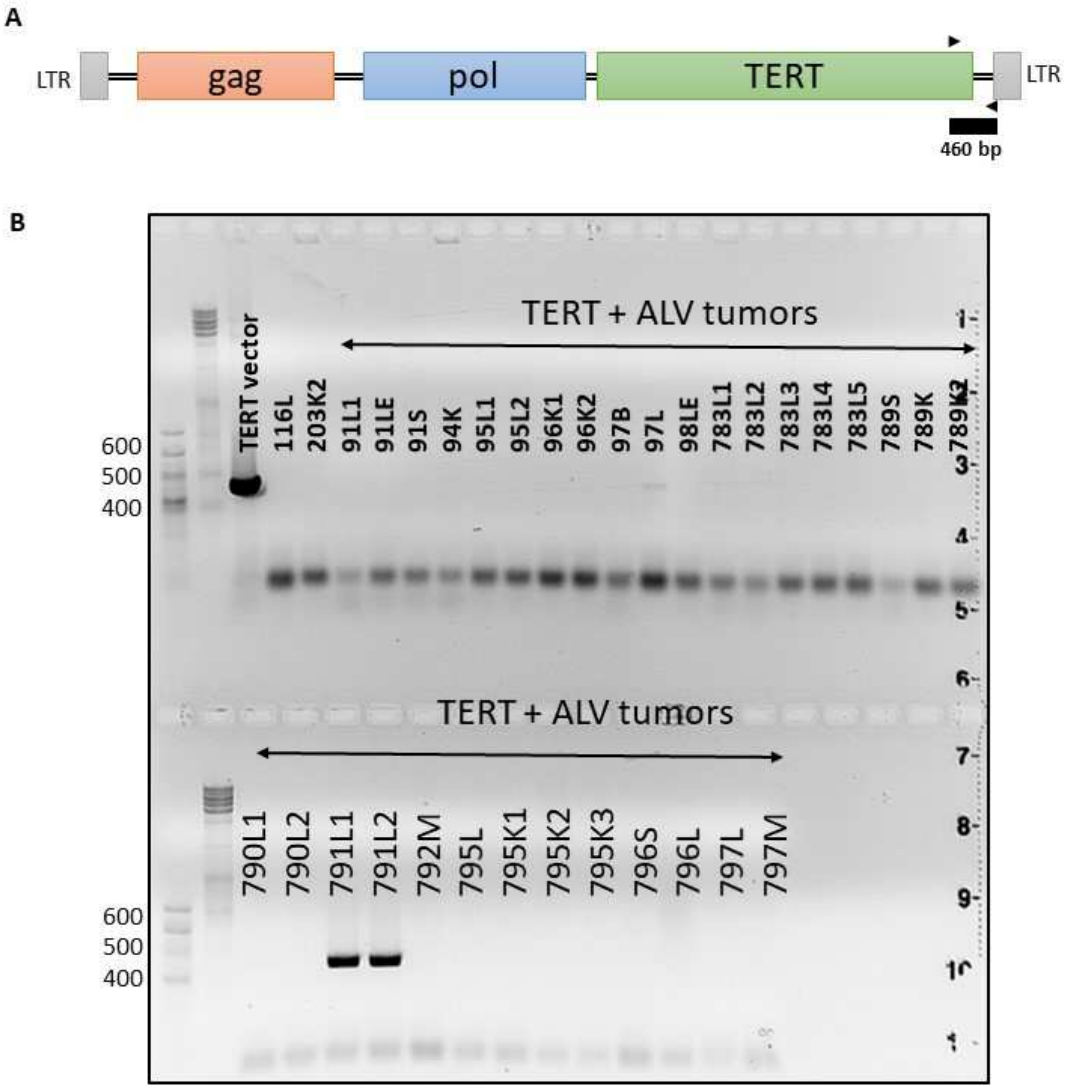


Figure 2.5. Identification of tumors with TERT provirus. A) Schematic depicting the TERT provirus, primers used to identify TERT provirus, and the predicted amplication product B) Agarose gel image of the PCR reactions using tumors from chickens infected with TERT virus and ALV. 100 bp ladder is on the left hand side of each row of lanes and markers 400, 500, and 600 bp markers are labeled for reference. DAS TERT vector was used as a positive control. Normal liver (116L) and a tumor from the DAS group were used as negative controls.

Two different spliced proviral TERT transcripts were detected from PCR amplification of cDNA from both tumors (Figure 2.6A and 2.6B). qRT-PCR for total TERT expression (Figure 2.6C) of both tumors shows greater than 40-fold expression compared to normal bursal control (Figure 2.5C). Both tumors show greater than 20-fold expression compared to D2L, a previously identified tumor that was shown to have increased endogenous TERT expression (Figure 2.5C) (Yang et al. 2007; J. F. Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). Sequencing analysis of ALV proviral

integrations of 791L1 and 791L2 suggest that the tumors were likely derived from two independent clonal expansions (Figure 2.gD). ARID5B was the most abundant unique integration in 791L1. A unique integration near MYB and a pair of integrations near mir-155 were the most abundant in 791L2.

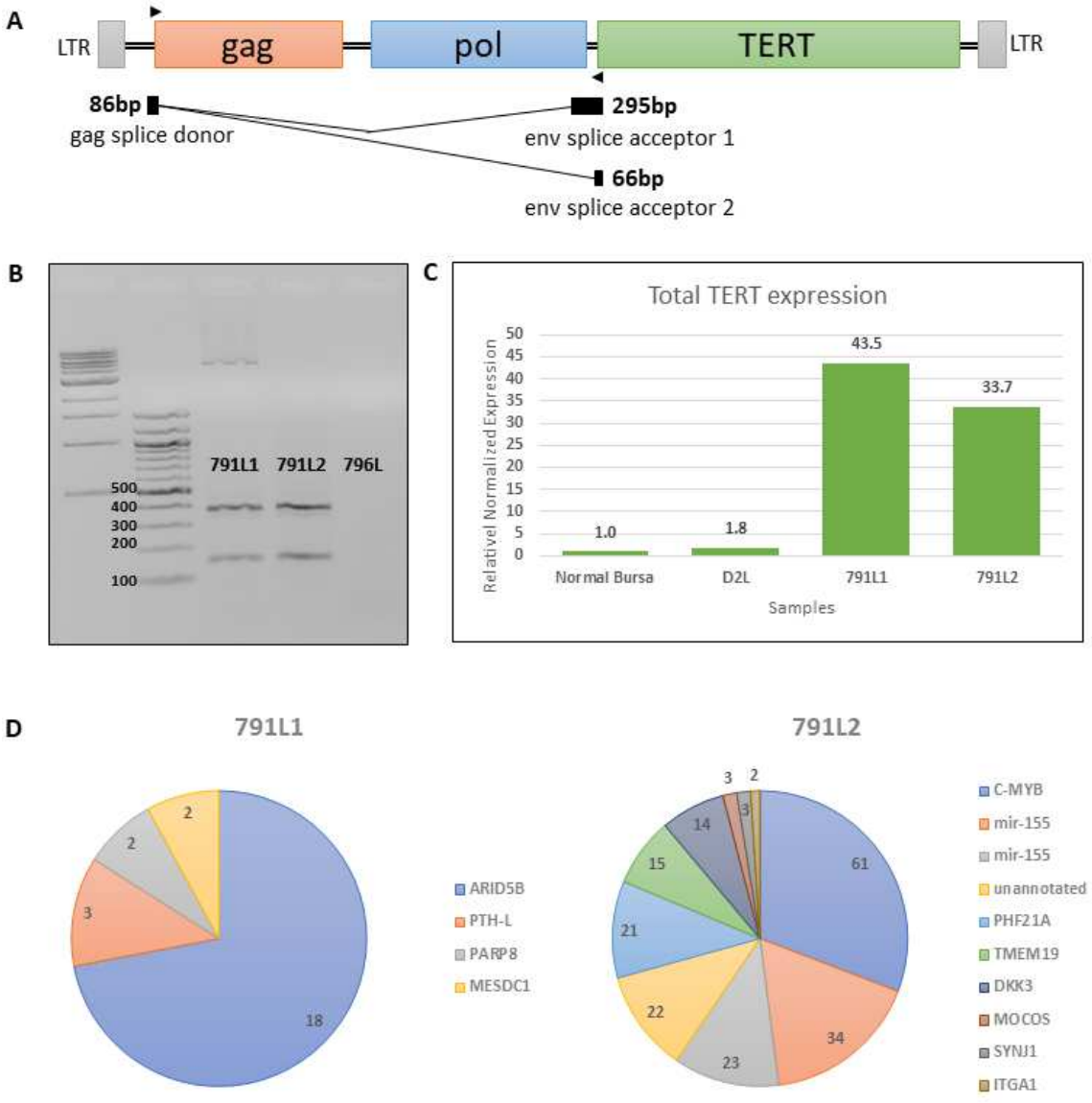


Figure 2.6. Characterization of tumors 791L1 and 791L2. A) Schematic depicting the TERT provirus, primers (black arrows) used to detect spliced proviral TERT transcripts, the two spliced proviral TERT transcripts validated by Sanger sequencing, and primers (orange arrows) used for qPCR for total TERT expression B) Agarose gel image of PCR reactions for spliced proviral TERT transcripts using cDNA extracted from tumor 791L1 and 791L2. 796L was used as a negative control. C) qPCR of total TERT expression in normal bursal tissue, D2L, 791L1, and 791L2. Expression was normalized to GAPDH. D) Pie charts depicting the distribution of integrations sites in tumor 791L1 and 791L2. Within each pie chart, each unique integration is represented by a slice with the corresponding number of sonication breakpoints. The list of the most proximal host genes is denoted next to the pie chart.

Multiple Tumors with Common Expanded Integration Sites

High-throughput sequencing analysis of integration sites of ALV-induced tumors show different group of genes associated with lesion type. Consistent with previous reports, multiple tumors harbored expanded integrations sites near MYB, mir-155, MYC, and TERT (Figure 2.7). Tumors 81L, 206L, 215B, and 791L2 had unique clonal integration sites near MYB. Tumors 81L, 206L, 783L5, and 791L2 had integrations near mir-155. Tumors 886L1, 215B, 783L5 had integration near MYC. Tumors 206L and 208L2 had integrations near TERT. For each gene, there was at least one tumor that had coinciding integrations near another of the four genes, and a tumor that was exclusive to one of these 4 genes. With the exception of 81 liver hemangioma and a mixed liver tumor in 783, tumors with expanded unique integrations near MYB, mir-155, MYC, or TERT were enriched in lymphoid tumors and liver tissue.

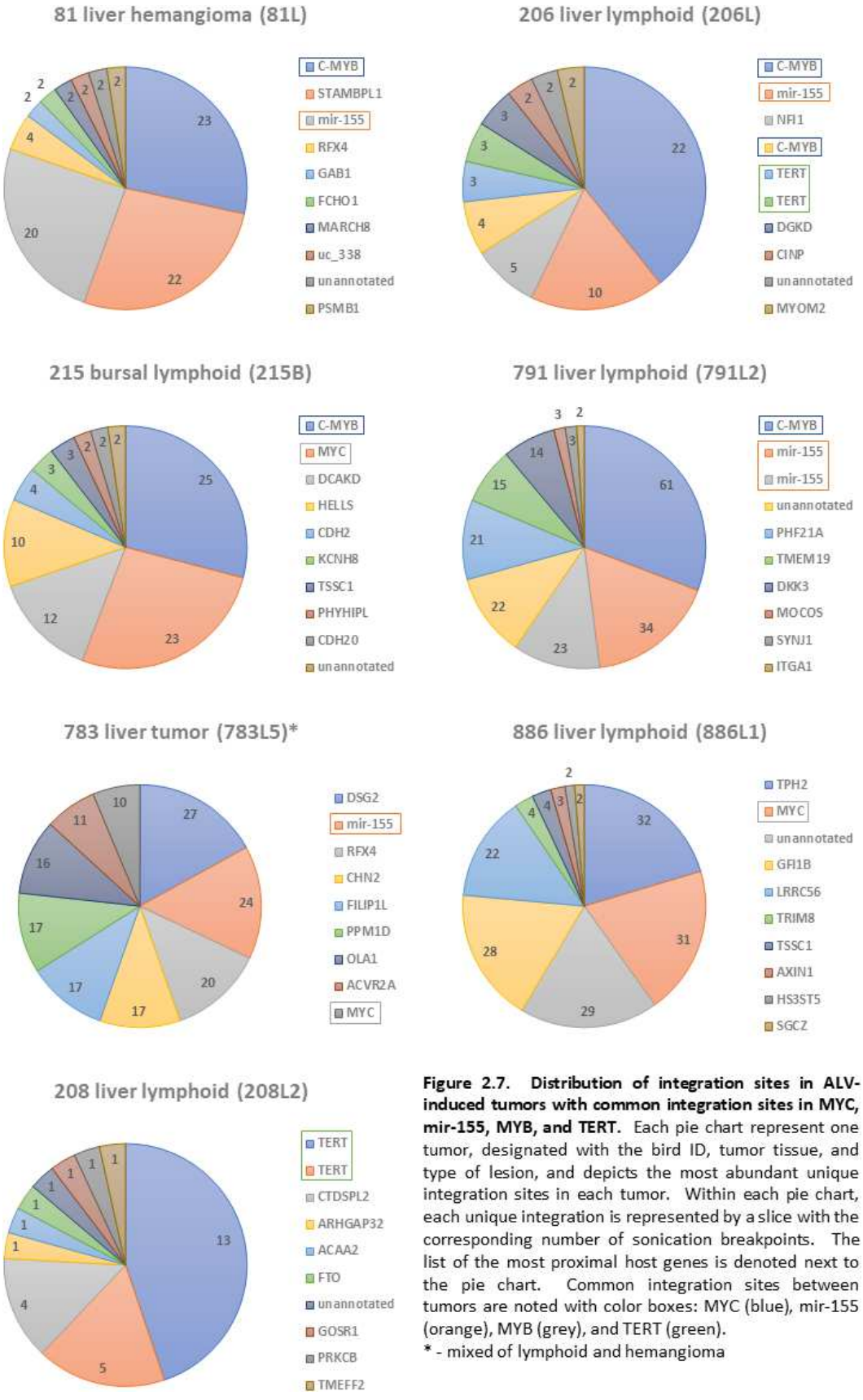


Figure 2.7. Distribution of integration sites in ALV-induced tumors with common integration sites in MYC, mir-155, MYB, and TERT. Each pie chart represent one tumor, designated with the bird ID, tumor tissue, and type of lesion, and depicts the most abundant unique integration sites in each tumor. Within each pie chart, each unique integration is represented by a slice with the corresponding number of sonication breakpoints. The list of the most proximal host genes is denoted next to the pie chart. Common integration sites between tumors are noted with color boxes: MYC (blue), mir-155 (orange), MYB (grey), and TERT (green).
* - mixed of lymphoid and hemangioma

In addition to previously reported genes, multiple tumors harbored expanded integrations sites near FRK, PLAG1, and GLIS3 (Figure 2.8). Tumors 83LE and 792M had integrations near FRK. Tumor 223K and 795K3 had integrations near PLAG1. Tumor 94K and 792M have integrations near GLIS3. Tumors with FRK and PLAG1 integrations did not coincide with any previously reported sites. In addition, a GLIS3 integration coincided with a FRK integration in tumor 792M, and a GLIS3 integration coincided with a mir-155 integration in tumor 94K. In contrast to MYB, mir-155, MYC, and TERT, tumors with expanded unique integrations near FRK, PLAG1, and GLIS3 were enriched in hemangiomas and tumors that did not have classical lymphoid phenotypes.

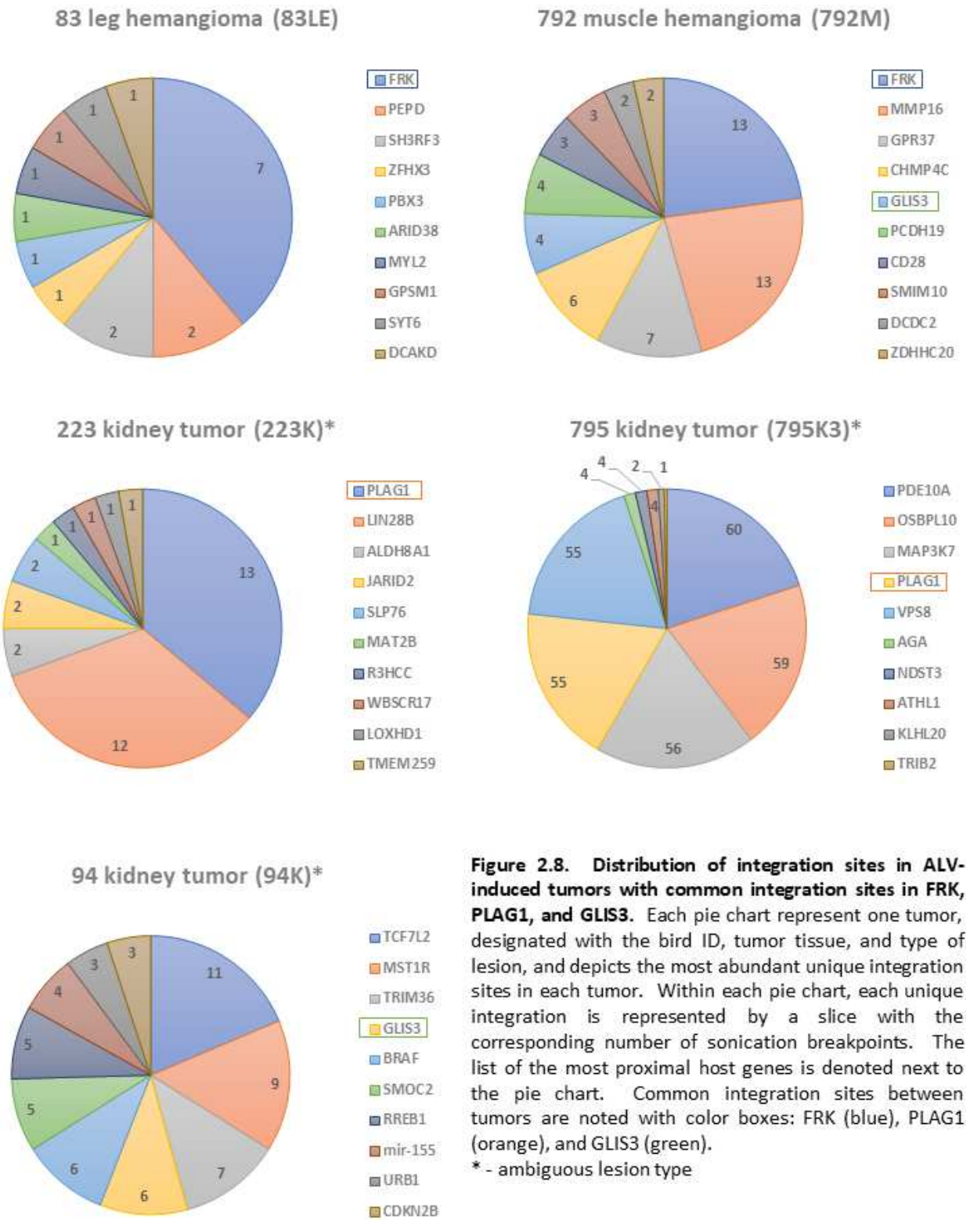


Figure 2.8. Distribution of integration sites in ALV-induced tumors with common integration sites in FRK, PLAG1, and GLIS3. Each pie chart represent one tumor, designated with the bird ID, tumor tissue, and type of lesion, and depicts the most abundant unique integration sites in each tumor. Within each pie chart, each unique integration is represented by a slice with the corresponding number of sonication breakpoints. The list of the most proximal host genes is denoted next to the pie chart. Common integration sites between tumors are noted with color boxes: FRK (blue), PLAG1 (orange), and GLIS3 (green).
* - ambiguous lesion type

Discussion

In this study, we tested the effects of TERT overexpression on ALV-induced tumorigenesis by coinfecting chicken embryos with a TERT recombinant virus and ALV. Overall, we cannot conclude if TERT overexpression contributes to tumor progression in ALV-induced tumorigenesis. This conclusion was largely attributed to converging evidence that cells were not saturated with TERT virus during the course of the chicken experiment.

There was a notable difference in mortality rates, where the DAS group showed a significantly higher rate, followed by TERT, and then ALV. This difference in mortality rates is associated with the difference in initial total titers used for infection. Both DAS and TERT virus may contribute to tumorigenesis by proviral integration. If the chicken embryo was saturated, we would likely observe either a difference attributed to TERT overexpression or no difference in mortality rate. In our case, the association between mortality rate and total initial infection titers suggests that the embryos were not saturated with DAS and TERT virus.

To achieve TERT overexpression, a replication-deficient ALV-derived vector was used to accommodate the size of full length chicken TERT cDNA (4 kb). Consequently, unlike ALV, the TERT virus is not able to replicate on its own. Thus, ALV is needed as a helper virus for the packaging of new recombinant virions in cells initially coinfected with both viruses. From the process of generating virus for the initial infection, ALV appears to prefer to package itself and DAS more efficiently than TERT recombinant virus over time. This may be attributed to the greater genomic size of the TERT virus. Consequently, DAS and ALV probably replicated better than the TERT virus, which may also contribute to the increased mortality rate observed with DAS. In the future, a scrambled sequence or an equally sized gene that is known to be unrelated to tumorigenesis could be used to mitigate this difference.

No significant differences were found in overall mortality at the end of 14 weeks or in tumor incidence at the end of the experiments at 15 weeks. As the cells were likely unsaturated by TERT and

DAS virus, the actual events necessary for transformation likely became more dependent on the replication competent ALV, over time. Being replication competent, ALV would be expected to saturate the available target cells eventually during chicken development.

Furthermore, we did not rule out the potential lethality related to the overexpression of TERT in B cells. Loss of B cells would reduce the pool of potential ALV target cells, which may lead to a reduction of malignant transformation in B cells. This reduction in B cell transformation may then lead to a reduction in mortality rate and lymphoid lesions in our chicken experiments. With the reduction of the characteristic rapid-onset B-cell lymphomas, ALV infection of endothelial cells may occur and lead to the prevalence of hemangiomas that we observed. However, we did not observe a noticeable difference in chick viability post-infection and CEFs post-transfection.

Consistent with these ideas, we observe a low frequency of tumors coinfected with the TERT virus and ALV (2 out of 11 lymphoid tumors in a total of 32 tumors tested). In addition, no DAS provirus was detected in the DAS group. Both tumors were found in the liver of chicken 791. While tumor 791L2 has clonal integration near MYB and mir-155, which is consistent with previous chicken tumor data, 791L1 had a clonal integration in gene ARID5B, suggesting that this gene may potentially cooperate with TERT. AT-rich interactive domain-containing protein 5B, ARID5B, is a transcription coactivator that acts as a binding domain for the histone demethylase PHD finger protein 2 (PHF2) and histone deacetylases (Hata et al. 2013; Leong et al. 2017). Interestingly, tumor 791L2 also had a clonal integration near PHF2, which further supports the involvement of ARID5B and PHF2 in ALV-induced lymphomagenesis.

Genome-wide association (GWAS) studies have revealed several single nucleotide polymorphisms in ARID5B that are significantly associated with risk for hematological malignancies in humans (Papaemmanuil et al. 2009; Treviño et al. 2009). Interestingly, ARID5B has been shown to positively regulate the expression of MYC in t-cell acute lymphoblastic leukemia (Leong et al. 2017). MYC has been previously shown to be a major player in ALV-induced B-cell lymphomas (Hayward, Neel,

and Astrin 1981). The clonal integration near ARID5B is located in the promoter region of ARID5B in the opposite orientation, which suggests enhancer activation of this gene in the chicken tumors.

Alternatively, our data does not rule out the possibility that malignant transformation may be the result of proviral integrations from the TERT recombinant provirus activating nearby genes. In order to test this, we can identify the location of the provirus by using inverse PCR and Sanger sequencing. As previously noted, the DAS genome is ALV-derived; however, the LTR is varied enough to provide unique priming sites for amplification of the TERT specific provirus in tumor samples with LR-9 proviruses. The data from tumors 791L1 and 791L2 suggest that the approach was sound in the identification of potential TERT cooperating genes, despite the many technical caveats mentioned.

In order to address the primary issue of infection saturation, we could seek a method to generate TERT virions without the need of a helper virus or any other agent that may contribute to tumorigenesis and infect chickens with just the TERT recombinant virus. We could then use a titer that may guarantee saturation of the cells in the chicken embryo to ensure that most of the cells are infected.

An attractive approach to achieving TERT overexpression in the developing chicken embryo is to utilize CRISPR/Cas9. Recently, this method of genome editing is shown to be feasible in the developing chicken embryo (Williams et al. 2018). Rapid somatic gene knockout, as well as activation of endogenous target promoter, was shown to be possible in chicken embryos (Williams et al. 2018). We could utilize a similar approach to activate TERT expression and observe any changes in cancer phenotype. This approach also has the added benefit of mitigating confounding effects of proviral integration in approaches using recombinant retroviruses.

In contrast to previous reports, hemangiomas were the predominant type of lesions observed in this study. One possible explanation may be attributed to the potential variance in the genetic background of the chickens. Genetic variance even in highly inbred chicken lines have been shown to

impact various aspects of chicken biology. Interestingly, an area of active investigation involving genetic variance among inbred chickens concerns the susceptibility of chickens to Marek's disease, a disease caused by an alphaherpesvirus (Luo et al. 2013). Copy number variation is shown to be associated with Marek's disease resistance that can be transmitted vertically (Luo et al. 2013). Considering this idea, one can reason that the sudden occurrence of hemangiomas induced by chickens infected with a previously characterized ALV may be partly attributed to genetic variance of the chickens.

Nevertheless, this occurrence presented an opportunity to characterize the tumors for any patterns that may delineate among the different types of lesions observed in our chicken experiment. Integration site analysis suggests that there is selection for different genes between lymphoid tumors and hemangiomas. Consistent with previous reports, integration sites near MYB, mir-155, MYC, and TERT were found to be associated with lymphoid tumors of liver tissue. In comparison, multiple hemangiomas had integrations sites near FRK, PLAG1, and GLIS3.

Retroviral induced hemangiomas in chickens is not entirely new. Currently, ALV-J is a major economic problem in Asia. ALV-J is known to cause hemangiomas and myeloid tumors in chickens. Recently, our lab had identified the MET gene as a common integration target in ALV-J-induced chicken hemangiomas (Justice et al. 2015). In this study, ALV-A appears to also have the capacity to induce hemangiomas in chickens, and our integration data implicates FRK, PLAG1, and GLIS3 as potential genes involved in the development of hemangiomas.

There is not a known association among the three genes identified in our ALV-A study and the MET gene implicated by our ALV-J study in hemangiomas. All three genes have been previously associated with some type of cancer. FRK and PLAG1 appears to be the most extensively studied, and much less is known about GLIS3. The non-receptor tyrosine kinase Fyn-related kinase (FRK) is a member of the family of kinases that are distinctly related to the Src family kinases (Goel and Lukong 2016). Interestingly, Src is one of the first viral oncogenic gene products characterized through the investigation

of RSV (Beemon and Hunter 1978). Not surprising, FRK has been shown to play a role in various human cancers albeit there is some controversy. Originally discovered to potentially have tumor suppressive function, FRK has been recently shown to have oncogenic roles as well (Goel and Lukong 2016). The functional paradox appears to be dependent on the tissue-specific context (Goel and Lukong 2016).

Interestingly, translocation activation of FRK has been observed in a specific hematological malignancy. More specifically, patients with acute myelogenous leukemia are shown to carry a translocation that results in the fusion of exon 4 of an ETS transcription factor, ETV6, to exon 3 of FRK (Hosoya et al. 2005). The resulting fusion transcript is expressed as a chimeric protein consisting of the entire oligomerization domain of ETV6 and the kinase domain of FRK, which is shown to have kinase activity and be sufficient in driving transformation in Ba/F3 cells and NIH3T3 cells (Hosoya et al. 2005). Our data shows that the two expanded unique integrations in tumor 83LE and 792M are located in exon 1 of FRK and are in the sense orientation, suggesting that FRK is likely activated by ALV. This would likely implicate FRK as a potential oncogenic factor in driving ALV-induced tumorigenesis.

Pleomorphic adenoma gene 1 (PLAG1) was first discovered in pleomorphic adenomas of the salivary gland (Kas et al. 1997). Unlike FRK, the majority of the research suggests that PLAG1 is an oncogenic factor, which was later shown to be associated with other human cancers such as lipoblastoma, hepatoblastoma, and, interestingly, hematological malignancies like acute myeloid leukemia (Astrom et al. 2000; Zatkova et al. 2004; Landrette et al. 2005). Interestingly, one of the main mechanisms of PLAG1-induced cancers is a reciprocal chromosomal translocation event. Translocation events involve the swapping of the PLAG1 promoter with that of an ubiquitously expressed gene (Juma et al. 2016). The chromosomal breakpoints are located between the upstream regulatory region and the coding region of both translocation partners; thus, preserving the coding sequence and functionality of the translated proteins downstream (Juma et al. 2016). In the case of PLAG1, expression becomes driven by strong promoters, leading to overexpression in human cancers. This is reminiscent of

activation by retroviral promoter insertion, which is consistent with the two clonal integrations observed in our study in exon 1 of PLAG1.

Lastly, Gli-similar 3 (GLIS3) is part of a subfamily of Krüpple-like zinc-finger proteins, also termed Krüpple-like factors (KLBFs), which have the capability to bind to GC-rich DNA sequences. Originally named from the study of segmentation defects in Drosophila, KLFs are involved in many aspects of biological processes, including differentiation, development, proliferation, and apoptosis (Pearson et al. 2008). Not surprisingly, the activation of KLFs like GLIS3 are associated with many human cancers (Chou et al. 2017). Once again, GLIS3 has been shown to also be activated by means of an abnormal chromosomal translocation event resulting in the fusion of GLIS3 and another oncogenic factor, cleft lip and palate transmembrane protein 1-like (CLPTM1L), in cases of fibrolamellar hepatocellular carcinoma (Xu et al. 2015). This translocation event between chromosome 5 and 9 produces a fusion protein that contains the first 7 exons of CLPTM1L and exons 5-11 of GLIS3 that promotes cancer phenotypes in hepatocellular carcinoma cell lines (Xu et al. 2015).

What makes this particularly interesting are the sites of translocation. In humans, CLPTM1L is upstream of TERT and the TERT-CLPTM1L locus is a known target for recurrent somatic chromosomal translocations to immunoglobulin sites in human B-cell neoplasms (Nagel et al. 2010). In these cases, TERT is transcriptionally activated by promoter insertion of immunoglobulin promoters (Nagel et al. 2010). Interestingly, the human TERT-CLPTM1L locus contains the coding region for a human TERT Antisense Promoter Associated (TAPAS) lncRNA (Malhotra, Freeberg, et al. 2017). The TERT-CLPTM1L locus is conserved in chickens and is the site of the clonally expanded TERT promoter integrations we previously reported, as well as the coding region of chicken TAPAS (Yang et al. 2007; Justice, Morgan, and Beemon 2015; Nehyba et al. 2016). Furthermore, our integration data shows that the unique integration site in GLIS3 in tumor 792M and 94K are located upstream of exon 3 in the sense direction,

which may implicate the upregulation of a truncated GLIS3 product similar to what is observed in fibrolamellar hepatocellular carcinoma.

In conclusion, this study expands the number of genes associated with ALV-induced tumorigenesis. In addition to previously reported genes, new potential genes of interest are observed. Tumor 791L1 implicates ARID5B as a potential associated gene for TERT overexpressing tumors while 791L2 reaffirmed our previous reports of potential cooperation between MYB and TERT in ALV B-cell lymphomas (Malhotra, Winans, et al. 2017). In addition, the analysis of ALV-A-induced hemangiomas offers new potential players, FRK, PLAG1, and GLIS3, in hemangioma development. All three genes appear to be associated with activating translocation events in various human cancers. This may implicate a relationship between selection for sites of translocation and common sites of proviral integration in tumors.

Chapter 3. ALV Integration and DNA Methylation at the Site of Integration

Adapted from Lam G and Beemon K. (2018) **ALV Integration-Associated Hypomethylation at the TERT Promoter Locus.** Viruses. 10(2), 74; doi:10.3390/v10020074

Abstract

Avian leukosis virus (ALV) is a simple retrovirus that can induce B-cell lymphoma in chicken(s) and other birds by insertional mutagenesis. The promoter region of telomerase reverse transcriptase (TERT) has been identified as an important integration site for tumorigenesis. Tumors with TERT promoter integrations are associated with increased TERT expression. The mechanism of this activation is still under investigation. We asked whether insertion of proviral DNA perturbs the epigenome of the integration site and, subsequently, impacts the regulation of neighboring genes. DNA cytosine methylation, which generally acts to suppress transcription, is one major form of epigenetic regulation. In this study, we examine allele-specific methylation patterns of genomic DNA from chicken tumors by bisulfite sequencing. We observed that alleles with TERT promoter integrations are associated with decreased methylation in the host genome near the site of integration. Our observations suggest that insertion of ALV in the TERT promoter region may induce expression of TERT through inhibition of maintenance methylation in the TERT promoter region.

Introduction

Avian leukosis virus (ALV) is a simple retrovirus that can induce B-cell lymphoma in chickens and other fowl by means of insertional mutagenesis. Proviral integration can upregulate the expression of proximal genes through enhancer and promoter elements in the viral long terminal repeat (LTR) sequences (Beemon and Rosenberg 2012; J. Justice and Beemon 2013). Previous studies have shown common integration sites in ALV-induced lymphomas near MYC, MYB, BIC and, more recently, the telomerase reverse transcriptase (TERT) genes (Justice, Morgan, and Beemon 2015; Hayward, Neel, and Astrin 1981; Baba and Humphries 1986; Clurman and Hayward 1989; Yang et al. 2007; Nehyba et al. 2016; Malhotra, Winans, et al. 2017). Previously, we found that integrations in the TERT promoter region were one of the most clonally expanded—or most abundant unique—integrations in tumors tested from ALV infected chickens (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017).

This suggests that TERT promoter integrations occurred early during ALV-infection in tumors with abundant copies of a unique TERT integration, implicating them as important early events in tumorigenesis (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017).

TERT encodes the catalytic subunit of telomerase, which has been shown to be upregulated in 90% of different types of human cancers surveyed, including lymphomas (Shay and Bacchetti 1997). Elevated TERT expression contributes to telomerase-dependent maintenance of telomeres that is often required for long-term proliferation and survival of cancer cells (Blasco 2005). Similar phenotypes can be achieved through a telomerase-independent process, known as alternative lengthening of telomeres (ALT), which has been observed in both humans (Bryan et al. 1997, 1995) and chickens (O'Hare and Delany 2011). Expression of TERT is tightly regulated through many mechanisms, including epigenetic modification of the promoter region to regulate telomerase activity in most somatic cells (Delany and Daniels 2004; Zhu, Zhao, and Wang 2010). Systematic analysis of the Cancer Genome Atlas database revealed that methylation of the TERT promoter region is one of the most prevalent markers associated with TERT expression in human cancers, in addition to the discovery of common somatic point mutations in the TERT promoter (Barthel et al. 2017; Horn et al. 2013; Huang et al. 2013).

DNA methylation is generally associated with repression of gene expression and occurs almost exclusively at regions of DNA where a cytosine nucleotide is followed by a guanine nucleotide (CpGs) in vertebrates (Li et al. 2011; Bird 2002; Weber and Schübeler 2007). The vast majority of DNA is highly methylated at CpGs; however, a small fraction of DNA comprising CpG islands, areas containing a high concentration of CpGs (at least 200 bp long with >60% GC), show differential methylation during development and disease states (Li et al. 2011; Bird 2002; Weber and Schübeler 2007). These CpG islands are frequently associated with gene promoters (Li et al. 2011; Bird 2002; Weber and Schübeler 2007). In the case of TERT, the relationship between TERT promoter methylation and expression has proven to be complex and is still under active investigation. Surprisingly, early studies suggest a direct

relationship between TERT promoter methylation and expression, and, subsequently, telomerase activity (Devereux et al. 1999; Dessain et al. 2000; Nomoto et al. 2002; Guilleret and Benhattar 2004; Guilleret et al. 2002). In multiple studies, normal human somatic cells that do not express TERT are associated with unmethylated or hypomethylated promoters, while some cancer lines with completely hypermethylated TERT promoter regions express TERT (Devereux et al. 1999; Dessain et al. 2000; Nomoto et al. 2002; Guilleret and Benhattar 2004; Guilleret et al. 2002). In contrast, other reports of TERT promoter DNA methylation suggest that methylation is associated with gene silencing (Liu et al. 2004; Lopatina et al. 2003; Shin et al. 2003). Further investigations reveal that the activation of TERT expression can be allele-specific in cancer cells, which are under pressure to maintain active alleles protected from DNA methylation (Zinn et al. 2007). Most recently, common TERT promoter mutations are shown to be associated with allele-specific hypomethylation of the TERT promoter in cancer cells with TERT expression (Stern et al. 2017).

DNA methylation also plays an important role in the regulation of retroviral proviruses. First introduced by Katz and co-workers, evidence of proviral DNA methylation was observed in a rat restriction cell line (XC) that was established from rat sarcoma tumors induced through heterotransplantation by inoculating newborn rats with suspensions of Rous sarcoma tissue (Svoboda 1960; Guntaka et al. 1980). Using this model, Svoboda and co-workers demonstrated that DNA methylation was involved in transcriptional silencing of avian proviruses (Svoboda et al. 2000; Hejnar et al. 1999). Daxx, a cytoplasmic Fas death domain-associated protein, was later discovered to be required for long-term maintenance of silencing and full viral DNA methylation of avian proviruses in human cells (Shalginskikh et al. 2013).

Further investigation revealed a dynamic relationship between the methylation state of the proviruses and the context of the integration site. The integrations of ALV-related retroviruses like Rous sarcoma virus (RSV) and Moloney murine leukemia virus (MLV) can perturb the methylation state of

flanking host DNA in different ways. RSV integration has been associated with transient hypomethylation of flanking genomic DNA in hamster cells (Hejnar et al. 2003). In contrast, MLV integration is associated with de novo methylation in mice (Jähner and Jaenisch 1985). Proviruses integrated close to transcriptional start sites of active genes may have long-term transcriptional activity and be resistant to transcriptional silencing by DNA methylation; in contrast, proviruses in intergenic regions tend to become transcriptionally silenced (Senigl, Auxt, and Hejnar 2012). Studies with other human viruses suggest that there is a direct correlation between the methylation state at the site of integration prior to proviral integration and the methylation status of the provirus (Watanabe et al. 2015; Hatano et al. 2017). Ultimately, local changes in DNA methylation are dependent on a growing list of factors that include the host cell properties, integrating virus, and the site of integration.

Chicken and human telomerase activity and telomere biology share some key similarities. High levels of telomerase activity are observed in early stage chicken embryos and human prenatal organs, and telomerase activity is downregulated in a temporal and tissue-specific manner for most somatic tissues (Forsyth, Wright, and Shay 2002; Taylor and Delany 2000; Swanberg et al. 2010). With some exceptions, constitutive activity is observed in renewable tissue types and diminished telomerase activity in most differentiated somatic tissues (Forsyth, Wright, and Shay 2002; Taylor and Delany 2000; Swanberg et al. 2010). Division-dependent telomere shortening occurs in chicken somatic tissues, and telomerase activity upregulation is present in transformed chicken cells (Swanberg and Delany 2003). Furthermore, chicken TERT also has CpG islands spanning from the TERT promoter region and into the coding region. More specifically, there is a smaller CpG island from −337 to −471 relative to the TERT transcriptional start site (TSS), and a larger one from −200 to +746 spanning across the first TERT exon and into the first intron (Figure 3.1). In comparison, human TERT has CpG islands spanning from −846 to +1178 relative to the TERT TSS.

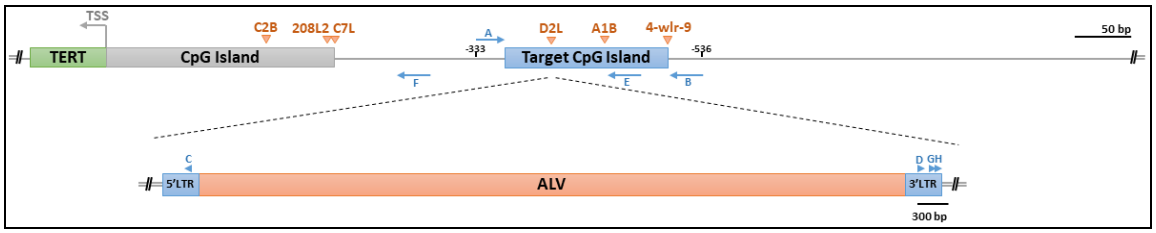


Figure 3.1. Schematic illustration of PCR primers, the cytosine-guanine dinucleotide (CpG) islands, and avian leukosis virus (ALV) telomerase reverse transcriptase (TERT) integrations in the chicken TERT promoter region. Primers are labeled A–H and depicted as letters and blue arrows. The targeted CpG island of interest is depicted within 333–536 bp upstream of the TERT transcriptional start site (TSS). The six ALV integrations tested are labeled and indicated with orange arrows at the site of integration. At the bottom, a schematic of ALV provirus in tumor D2L is depicted with primers labeled in the long terminal repeats (LTRs).

The influence of ALV integration on the methylation of the chicken TERT promoter has not been characterized previously in ALV-induced lymphomas. Our lab has observed that the clonally expanded integrations in the TERT promoter remain transcriptionally active in chicken tumors. Expression analysis revealed the upregulation of a novel TERT antisense promoter-associated (TAPAS) long non-coding RNA (lncRNA), in chicken tumors with TERT promoter integrations. TAPAS transcripts corresponded to a region downstream of the common ALV integration site in the TERT promoter region. Expression of TAPAS was shown to be partly driven by the ALV proviral promoter, which was supported by the detection of spliced proviral transcripts fused with TAPAS (Nehyba et al. 2016). Furthermore, the same subset of tumors was associated with increased TERT expression (Yang et al. 2007; Nehyba et al. 2016). Thus, we studied the epigenetic state of the ALV provirus and the TERT promoter in chicken lymphomas with clonally expanded TERT promoter integrations. To better understand the interplay between the epigenome of the host DNA and provirus, we compared the DNA methylation status of the unoccupied allele (without a TERT promoter integration) with the occupied allele (with a TERT promoter integration) in individual tumors with known clonally expanded TERT integrations. In this study, we found that ALV integrations in the TERT promoter are associated with hypomethylation of the flanking host genomic DNA and of the ALV LTRs.

Materials and Methods

Tumor Induction and Samples

Groups of 5- and 10-day-old chicken embryos were infected with ALV by injection with either ALV-LR9, ALV-ΔLR9, ALV-G919A, or ALV-U916A as described previously (Nomoto et al. 2002; Justice, Morgan, and Beemon 2015; Polony et al. 2003). Chickens injected include inbred single comb (SC) White Leghorn line embryos from Hy-Line International (Dallas Center, IA, USA) and specific pathogen free embryos from Charles River (North Franklin, CT, USA). ALV-LR-9 mutants have a deletion (ALV-ΔLR9) or silent mutation (ALV-G919A and ALV-U916A) in a 5′ regulatory element that results in rapid onset of B-cell lymphomas in chickens. Chickens were observed daily after hatching and euthanized when moribund or at the end of 10–12 weeks. Once sacrificed, tumor tissue and normal tissue were harvested from infected birds and frozen at −80 °C. Normal tissues were collected from uninfected chickens that were sham injected with media as controls. In total, five normal tissues (bursa, kidney, liver, spleen and non-tumor tissue from an infected bird, C2K), nine tumors without any known TERT promoter integrations, nine tumors with known TERT promoter integrations, and two chicken cell cultures were used for bisulfite sequencing (Table 3.1) (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017).

Sample group	Tissue type
Normal control tissues	normal bursa (NB), normal kidney (NK), normal liver (NL), normal spleen (NS), C2K
Chicken cell culture	primary chicken embryo fibroblast (CEF), bursal lymphoma cell line (DT-40)
Tumors without telomerase reverse transcriptase (TERT) promoter integration	205L1, 209L, 214L4, 215K1, 218K, 796L, 223K, 791L1, B8L
Tumors with TERT promoter integration	206L1, 208L2, A1B, C2B, C6L, C7B, C7L, D2L, 4-w-lr-9

Table 3.1. Chicken samples tested for DNA methylation

Chicken experiments were performed at the University of Delaware. These experiments were approved by the Institutional Animal Care and Use Committee (AUP Number 1271-2016-2), which was renewed on 1 August 2016.

DNA Extraction and Bisulfite Treatment

50 mg of tissue was homogenized with a Kimble-Chase Kontes pellet pestle and digested with proteinase K at 50 °C for 15 h. DNA was extracted with two rounds of phenol-chloroform extraction, with a 2 µg RNase A treatment for 1 h at 37 °C in between the rounds, followed by ethanol precipitation. DNA concentration was measured with a Thermo Scientific Nanodrop 2000c instrument (Waltham, MA, USA). For bisulfite treatment, an optimized protocol was adapted from Pappas et al. (Pappas, Toulouse, and Bradley 2013). In brief, 5 µg of genomic DNA was sonicated briefly with a Bioruptor UCD-200 instrument (Diagenode Inc., Denville, NJ, USA). DNA samples were then denatured using 3 N NaOH at 37 °C for 15 min. Sulfonation of unmethylated cytosines was performed by the addition of sodium bisulfite-hydroquinone solution in a thermal cycler at 95 °C for 4 min followed by 55 °C for 2 h. DNA was then desalted using a Thermal Scientific GeneJet PCR purification Kit (Waltham, MA, USA) and deaminated with the addition of 3 N NaOH at 37 °C for 15 min. Lastly, desulfonation and precipitation were performed by adding glycogen, 10 M ammonium acetate, chilled in 95% ethanol and incubated overnight at −20 °C. Samples were centrifuged at 13,000× *g* for 30 min, washed with 70% ethanol, air-dried, and stored at −20 °C for PCR amplification and sequencing.

PCR Amplification and Sequencing Analysis

Target sequences for tumor samples 4-wlr-9, A1B, and D2L were amplified by one round of PCR using bisulfite treated DNA. A schematic representation of the positions of corresponding primers, targeted region, and ALV integration sites are shown in Figure 3.1. Henceforth, alleles without a TERT promoter integration will be referred to as unoccupied alleles and alleles with an integration as occupied alleles. For amplification of the unoccupied alleles, a target CpG island within 333–536 bp

upstream of the chicken TERT TSS was amplified using primer A (GTTTTTGTTTTGTTTTTGAGGAGAT) and primer B (AACTAAAAATTTTCTCTATCAAATTATATT). For the amplification of occupied alleles, primers flanking the proviral-host junction at the site of integration were used. For 4-wlr-9 and A1B, primer A and primer C (CAACCCAAATACACACCAATATAATAA) were used to amplify the 5'LTR-host junction. For D2L, primer B and primer D were used to amplify the 3'LTR-host junction. Amplification of occupied alleles in C7L, C2B, and 208L2 required a semi-nested PCR approach. The first round of PCR was performed using primer E (TCACAAAAATAAATAAAAAACATTACTT) and primer G (TATATTGGTGTGTATTT-GGGTTGAT). In the second round, 3'LTR was amplified using primer F (AAAAAAACCTCTAAAATCACTTAA-TCC) and primer G, while the target CpG island was amplified using primer H (ATGTAGAAGTAGAAGGTT-TTATTT) and primer F. PCR amplifications were performed using Thermo Scientific Phusion U Hot Start DNA polymerase (Waltham, MA, USA) with optimized conditions adapted from manufacturer's instructions.

PCR amplicons were analyzed by conventional sequencing provided by Eurofins Genomics services (Louisville, KY, USA). Quantification of methylation was performed manually by digitally measuring the peak height of chromatograms. For each CpG site, percent methylation was calculated by dividing the cytosine peak height by the total peak height of cytosine and thymidine and multiplying by 100. Percent methylation means were calculated by averaging percent methylation of all CpG sites sequenced region of one representative sample. All unmethylated CpG sites had undetectable cytosine, or showed only thymidine peaks. All non-CpG cytosine showed complete conversion to thymidine. At least two technical repeats were performed from extracted genomic DNA.

PCR amplification and sequencing analysis of the larger CpG that was closer to the TSS proved to be beyond the limitations of the method described and, subsequently, was not analyzed in this study.

Results

Chicken TERT Promoters Were Significantly Methylated in Unoccupied Alleles

Limited data were available about the methylome of chicken tumors. In order to assess the effects of ALV integration on the methylation status of the TERT promoter, we required data on the methylation status of a CpG rich region in the TERT promoter of unoccupied alleles. We performed conventional bisulfite sequencing of genomic DNA samples that include five normal tissues, two chicken cell culture samples, nine tumors that do not have clonal TERT promoter integrations, and nine tumors that do have clonal TERT promoter integrations (Table 3.1). Sequencing was performed on PCR amplicons 333–536 bp upstream of the chicken TERT TSS of the unoccupied allele (Figure 3.1). This specific region was selected because multiple chicken tumors have neighboring TERT promoter integrations (Figure 3.1). CpG site distribution is depicted in this region in Figure 3.2. All tissues tested show the same pattern of DNA methylation across the target CpG island (Figure 3.2).

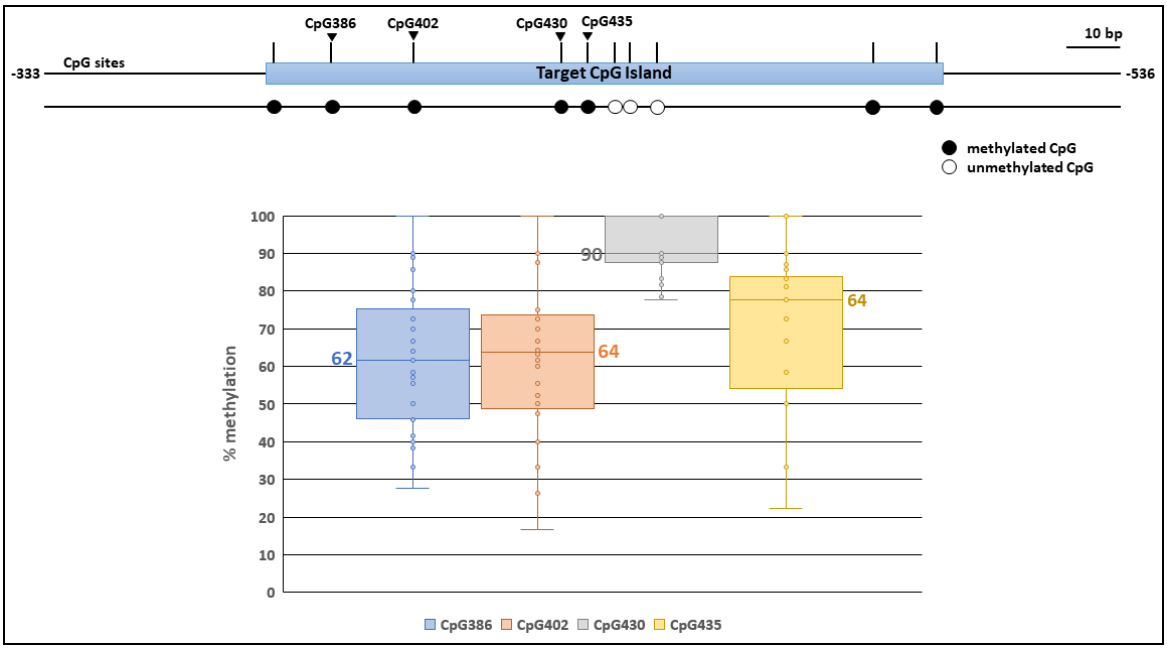


Figure 3.2. CpG methylation profiles of the targeted TERT promoter region in unoccupied alleles. Schematic representation of the CpG dinucleotide distribution is shown at the top. Four CpG sites of interest (CpG386, CpG402, CpG430, and CpG435) are indicated. Bisulfite sequencing analysis of the target region is depicted as a linear array of open circles representing non-modified CpG residues and closed circles representing methylated CpG residues. At the bottom, a box and whisker plot shows percent methylation distribution between each sample tested at four CpG sites that varied in the targeted CpG island of the TERT promoter region. Each dot may represent more than one measure of percent methylation in one sample at the indicated CpG site when percent methylation is the same. A total 25 samples are tested across the four CpG sites. Median percent methylation for each site is indicated next to each box in the corresponding color.

Variation in methylation between samples was observed at 4 of 10 CpG sites measured: CpG386, CpG402, CpG430, and CpG435 (Figure 3.2). Percent methylation at CpG386, CpG402, CpG430, and CpG435 ranged from 28–100, 17–100, 78–100, and 22–100% with a median of 62, 64, 90, and 64%, respectively (Figure 3.2). The remaining CpG sites were either unmethylated or completely methylated as indicated, across all samples tested (Figure 3.2). Variation in methylation between the samples did not separate into any obvious groups, like normal and tumor tissues. Taken together, significant methylation of unoccupied alleles was observed in both normal and tumor tissues with no obvious trends between sample groups.

ALV LTRs of Proviruses in the TERT Promoter Were Not Methylated

To examine the methylation state of ALV LTRs, we performed conventional bisulfite sequencing of six different tumors (4-wlr-9, A1B, D2L, C7L, C2B, and 208L2) with known clonally expanded TERT promoter integration sites. Sequencing was performed on PCR amplicons generated using primers flanking the proviral-host junction (Figure 3.1). With ALV integrations downstream of the target CpG island, tumors 4-wlr-9 and A1B were used to analyze the U3 region of the 5'LTR. In both cases, there were no detectable evidence of modified CpG dinucleotides. With integrations upstream of the target CpG island, tumors D2L, C7L, C2B, and 208L2 were used to analyze the R and U5 region of the 3'LTR. No evidence of methylation was detected in the 6 samples. Taken together, there was no evidence of methylated CpG dinucleotides throughout the LTR (Figure 3.3). Thus, ALV integration into the chicken TERT promoter region is not associated with de novo methylation of LTRs in B-cell lymphomas.

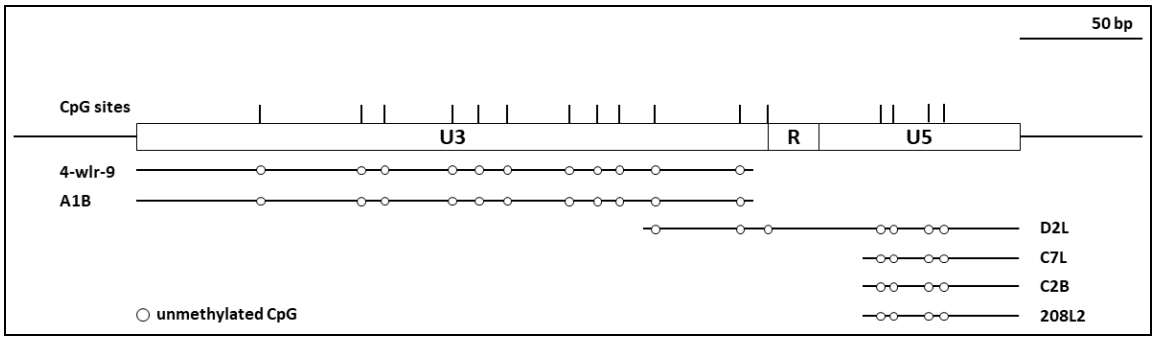


Figure 3.3. CpG methylation profiles of TERT promoter proviral LTRs in selected tumor samples. Schematic representation of the CpG dinucleotide distribution in the ALV LTR is shown at the top. Bisulfite sequencing analysis of the LTR sequences are depicted as a linear array of open circles representing non-modified CpG residues and closed circles representing methylated CpG residues. Each line represents a representative sequenced result of the LTR region from host-proviral PCR amplicons. No methylation was detected in proviruses of tumor tissues tested.

Occupied Alleles in the TERT Promoter Had Decreased Methylation in Flanking Host DNA

To examine the effects of ALV integration on the methylation of the flanking host DNA in the TERT promoter, bisulfite sequencing was performed on six different tumors (4-wlr-9, A1B, D2L, C7L, C2B, and 208L2). They all had known clonally expanded TERT promoter integrations near the target CpG island. For each tumor, two parallel sequencing experiments were performed. For the

unoccupied alleles, PCR amplicons were generated from the region that is 333–536 bp upstream of the chicken TERT TSS containing the target CpG island, using primers for the host genome. For the occupied alleles, PCR amplicons were generated using an LTR specific primer for the LTR closest to the target CpG island of the TERT promoter and a shared primer in the host genome (Figure 3.1).

In all six cases, a decrease in methylation was detected in the occupied allele compared to the paired unoccupied allele (Figure 3.4). Mean percent methylation decrease ranged from a complete loss of methylation in 4-wlr-9 and A1B to an approximately two-fold decrease in D2L, C7L, C2B, and 208L2 (Figure 3.4). Decreases in methylation at individual CpG sites appeared to be more dramatic closer to the site of integration (Figure 3.4). For example, occupied alleles of D2L show a complete loss of methylation at CpG sites immediately downstream of the integration but show retention of methylation at CpG sites farther downstream. Being farther upstream, occupied alleles from C2B, 208L2, and C7L show methylation at some sites that are unmethylated in occupied alleles of D2L, A1B, and 4-wlr-9. Taken together, ALV integrations near methylated sites in the host genome are associated with a decrease in methylation of the local host genome.

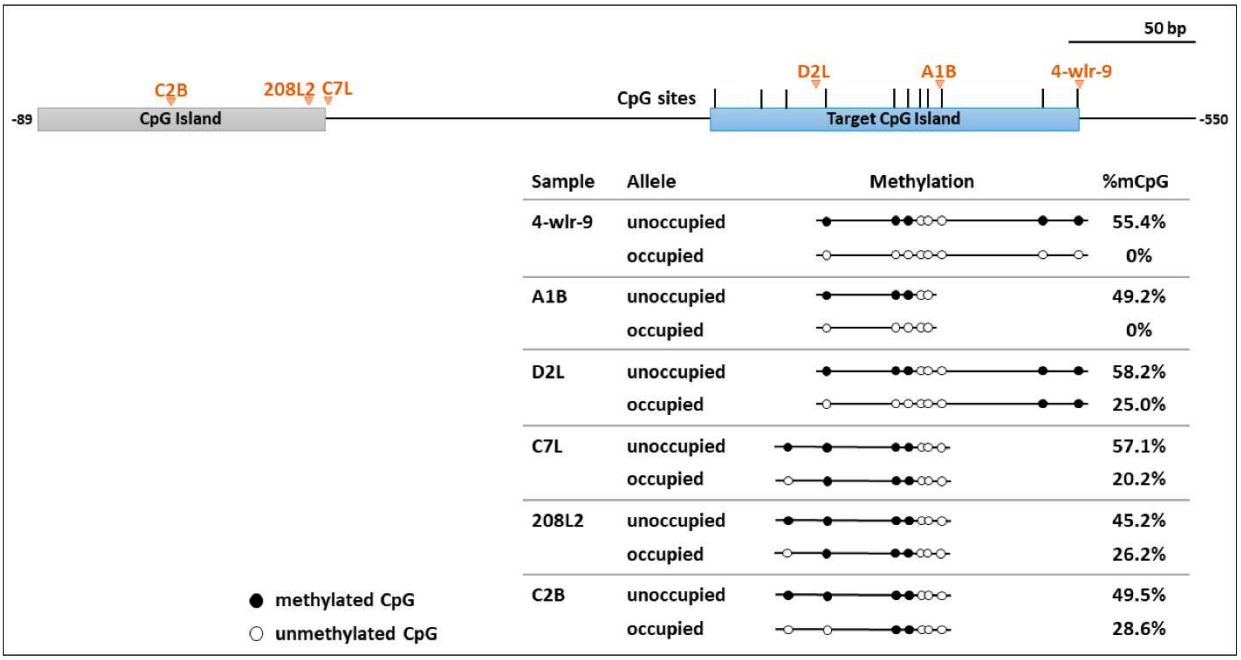


Figure 3.4. CpG methylation profiles of host genomic DNA in selected tumor samples. Schematic representation of the CpG dinucleotide distribution in the target CpG island in the TERT promoter region is shown at the top. The six TERT promoter integrations tested are labeled and indicated with orange arrows at the site of integration. For each tumor sample listed, bisulfite sequencing analyses are depicted as linear arrays of open circles representing non-modified CpG residues and closed circles representing methylated CpG residues. For each tumor sample, CpG methylation data in the target CpG island is shown for the unoccupied allele and the occupied allele. Corresponding mean percent CpG are listed, which were calculated by averaging percent methylation of all CpG sites in the displayed array.

Discussion

Our lab has previously shown that the TERT promoter region is a common integration site in ALV-induced B-cell lymphoma. These integrations are the most clonally expanded in a subset of tumors, suggesting that proviral integration into this region is an important early event in lymphoma development (Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). Tumors with TERT promoter integrations are associated with increased TERT expression (Yang et al. 2007; Nehyba et al. 2016). The mechanism of this activation is presently under investigation. The present study investigates the influence of ALV integration on the TERT promoter region methylation status at the site of integration in ALV-induced lymphoma. In this study, we observed that the chicken TERT promoter is significantly methylated in unoccupied alleles across normal and cancer tissues. Tested tumors with

TERT promoter integrations appear to have proviruses that are free of any detectable CpG methylation in the LTRs. Adjacent host sequences show a decrease in methylation in occupied alleles compared to unoccupied alleles.

DNA methylation of promoter regions is often associated with transcriptional silencing (Li et al. 2011; Bird 2002; Weber and Schübeler 2007). We observed significant methylation in the chicken TERT promoter region of unoccupied alleles, suggesting that in normal tissues and tumors, cellular factors were actively repressing transcription of TERT by means of DNA methylation. This is consistent with the downregulation of telomerase activity observed in chicken somatic tissues after the embryonic stage (Forsyth, Wright, and Shay 2002; Swanberg et al. 2010; Taylor and Delany 2000; S.E. Swanberg and Delany 2003). In contrast, there was a complete lack of detectable methylation in the ALV LTRs of proviruses in the TERT promoter region in lymphomas. As methylation in the LTR generally suppresses ALV proviral transcription (Svoboda et al. 2000; Hejnar et al. 2003; Senigl, Auxt, and Hejnar 2012), this result suggests that the ALV proviruses were likely transcriptionally active, which is consistent with the upregulation of spliced proviral transcripts containing TAPAS (Nehyba et al. 2016).

More importantly, we observe an allele-specific decrease in methylation in the host DNA near the site of ALV integration in the TERT promoter in the occupied alleles. This decrease in methylation may lead to derepression of the TERT gene and contribute to the observed increase in TERT expression in an allele-specific manner. Additional studies are required to determine if the methylation status directly impacts TERT expression. This is confounded by the presence of the LTR enhancer that has been demonstrated to activate transcription from the TERT promoter in cultured cells (Yang et al. 2007). To decouple the effects on TERT expression from the LTR enhancer and integration site methylation, future studies comparing expression from reporter constructs comprised of methylated and unmethylated TERT promoter region and ALV LTR may provide further insight. Taken together, the pattern of

methylation observed would suggest that methylation behaves in the conventional sense as a repressive marker of expression at the chicken TERT promoter.

Presently, the relationship between human TERT promoter methylation and TERT expression is still under investigation. On the one hand, increased TERT expression correlates with the hypermethylation of the TERT promoter region in many human cancers (Barthel et al. 2017). The mechanism of activation of TERT expression by increased TERT promoter methylation is presently unclear in humans. On the other hand, TERT promoter hypermethylation is associated with gene silencing (Liu et al. 2004; Lopatina et al. 2003; Shin et al. 2003); thus, in the tumor environment, one would expect selection for cells that acquired different mechanisms to protect the TERT promoter from DNA methylation. Our observations support the latter. Furthermore, the allele-specific hypomethylation of TERT promoter region that is associated with ALV integration is in agreement with recent work in humans. This idea of allele-specific activation of TERT was recently explored by multiple groups in different contexts. Common somatic TERT promoter mutations were recently discovered in many human cancers (Horn et al. 2013; Huang et al. 2013). Further investigation revealed that these somatic mutations were associated with an allele-specific switch to an active transcriptional state and monoallelic TERT expression of the allele containing the TERT promoter mutation across multiple human cancers (Huang et al. 2015; Stern et al. 2015). More recently, allele-specific hypomethylation was associated with TERT promoter mutations (Stern et al. 2017).

In regards to the seemingly contradictory observations about TERT promoter methylation, TERT hypermethylation may represent a broadly applicable prognostic marker for TERT expression and cancer progression (Castelo-Branco et al. 2013, 2016) that is distinct from what is observed in cancers with observed mechanisms of TERT activation like proviral- or mutation-associated hypomethylation. Presently, most of the published data looked at the average methylation across a population of alleles in tumors. Reanalyzing these samples and data, taking in account allele-specific methylation, may present

novel insight into the relationship between TERT promoter methylation and cancer. Perhaps in some human cancers, there is a decrease in TERT promoter methylation in the activated allele while on average there was an increase in methylation across the population of alleles when compared to corresponding normal tissue.

Concurrently, investigations into the effects of human integrating viruses on methylation in tumors were performed. Human herpesvirus 6B has been shown to integrate into subtelomeric regions and induce hypomethylation in these regions (Engdahl et al. 2017). Notably, the human TERT gene is located in the subtelomeric region of chromosome 5. Integrated human papillomavirus and hepatitis B virus show allele-specific methylation changes at the site of integration (Watanabe et al. 2015; Hatano et al. 2017). In these studies, methylation of the integrated viral genomes directly correlated with the methylation state of the integrated site prior to viral integration at specific sites tested (Watanabe et al. 2015; Hatano et al. 2017). Interestingly, the most frequent hepatitis B virus integrations were found in the TERT promoter region (Buendia and Neuveut 2015). To our knowledge, the methylation status at these integration sites have not been tested.

Future studies into the impact of human integrating viruses on DNA methylation and TERT expression in human cancers offers an exciting direction to test our observations observed in chicken tumors. Furthermore, chickens with TERT promoter integrations represent a subset of a larger collection of ALV-induced lymphomas. Ongoing work with other common integration sites may present novel trends that may provide further insight into the role DNA methylation plays in cancer. From these initial findings, a more comprehensive approach, such as high-throughput sequencing of integration sites, may be designed to address the specificity of this phenomenon.

We propose an additional selection factor in the growing model of ALV-induced tumorigenesis in chicken B-cell lymphoma. During early embryonic development, chicken embryos can become infected with ALV. Cells that acquire proviral integrations into the TERT promoter receive an

advantageous enhancement to proliferation and survival by means of TERT upregulation. This upregulation is in part associated with changes in the methylation state of the TERT promoter through the insertion of ALV. Additional studies are required to understand the mechanism and impact of the methylation changes on TERT expression and the specificity of this phenomenon.

Chapter 4. TERT promoter mutations in human B-cell malignancies

Adapted from Lam G., Xian R., Yingying L., Burns K., Beemon K. (2016) **Lack of TERT Promoter Mutations in Human B-Cell Non-Hodgkin Lymphoma**. Genes. 7(11), 93; doi:10.3390/genes 7110093

Abstract

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of immune cell neoplasms that comprise molecularly distinct lymphoma subtypes. Recent work has identified high frequency promoter point mutations in the telomerase reverse transcriptase (*TERT*) gene of different cancer types, including melanoma, glioma, liver and bladder cancer. *TERT* promoter mutations appear to correlate with increased TERT expression and telomerase activity in these cancers. In contrast, breast, pancreatic, and prostate cancer rarely demonstrate mutations in this region of the gene. *TERT* promoter mutation prevalence in NHL has not been thoroughly tested thus far. We screened 105 B-cell lymphoid malignancies encompassing nine NHL subtypes and acute lymphoblastic leukemia, for *TERT* promoter mutations. Our results suggest that *TERT* promoter mutations are rare or absent in most NHL. Thus, the classical *TERT* promoter mutations may not play a major oncogenic role in TERT expression and telomerase activation in NHL.

Introduction

Non-Hodgkin lymphomas (NHL) are a heterogeneous group of B, T, and natural killer cell neoplasms that arise primarily in lymph nodes. Most NHL in the western hemisphere are B-cell derived and comprise a variety of lymphomas, with diffuse large B-cell lymphoma (DLBCL), follicular lymphoma (FL), and chronic lymphocytic leukemia/small lymphocytic lymphoma (CLL/SLL) being the most common (Campo et al. 2011). Recent advances in molecular genetics have confirmed the molecular heterogeneity of NHL. Classically, NHL can be characterized by chromosomal translocation events that have been shown to occur frequently with different subtypes of NHL (Barrans et al. 2003; Bertoni et al. 2006; Biagi and Seymour 2002). Whole exome sequencing has further expanded molecular characterizations of NHL. Parallel sequencing experiments with DLBCL patients (Lohr et al. 2014) and FL patients (Okosun et al. 2014) have identified recurrent mutations in functionally relevant genes as well as novel genes that have not been previously implicated. Despite these

advances, NHL remains a heterogeneous group of malignancies, with many less characterized subtypes that remain difficult to diagnose and treat with current therapeutic strategies (Ansell 2015).

Recently, non-coding sequences have become an emerging field of active investigation in cancer research (Diederichs et al. 2016). In 2013, specific high frequency promoter mutations in the telomerase reverse transcriptase (*TERT*) gene in melanoma were reported, and were associated with a two- to four-fold increase in transcriptional activity (Horn et al. 2013; Huang et al. 2013). *TERT* encodes the catalytic subunit of telomerase, an enzyme that preserves chromosomal ends through telomere maintenance. The reported somatic transitions −124C>T and −146C>T in the *TERT* promoter region create a novel binding site for the ETS transcription factor GABP, which increases transcription of *TERT* (Bell et al. 2015). Increased TERT expression may confer increased proliferative potential and cell survival, which are essential factors in tumorigenesis (Cao et al. 2002). Strikingly, *TERT* promoter mutations are not unique to melanomas, but have been later found to be frequent in many other malignancies such as hepatocellular carcinoma, bladder cancer, and glioblastoma (Heidenreich, Rachakonda, et al. 2014; Killela et al. 2013; Weinhold et al. 2014; Vinagre et al. 2013; Liu et al. 2013; Rachakonda et al. 2013; Bell et al. 2016). However, *TERT* promoter mutations are not universal. Mutations have been shown to be absent, or rarely observed, in other cancer types like breast, pancreatic, and prostate cancer (Heidenreich, Rachakonda, et al. 2014; Killela et al. 2013; Bell et al. 2016).

Our lab has used the avian leukosis virus (ALV) as a tool to screen for common proviral integration sites in the host chicken genome to assess events involved in lymphoma development. By high-throughput sequencing and inverse PCR, we have previously shown that early chicken TERT (chTERT) expression through proviral integrations is associated with a similar two- to four-fold increase in transcriptional activity (Buendia and Neuveut 2015; Yang et al. 2007; Justice, Morgan, and Beemon 2015) and is likely important in lymphomagenesis. Although lymphocytes are known to be a

cell type characterized by high telomerase activity throughout their life cycle, lymphoid malignancies are associated with elevated TERT expression like the majority of cancers, suggesting a requirement for persistent TERT activity in transformed cells (Davison 2007; Lobetti-Bodoni et al. 2010).

We sought to investigate whether *TERT* promoter mutations play a role in TERT activation in human lymphomas. Presently, published work on the *TERT* promoter status of NHL is limited. Since the original reports in melanoma, we have found some published work that suggests *TERT* promoter mutations are absent in DLBCL and CLL (Weinhold et al. 2014; Vinagre et al. 2013). In contrast, *TERT* promoter mutations were detected in primary central nervous system lymphoma (Bruno et al. 2015). Here, we report a *TERT* promoter mutation screen of a collection of 105 human B-cell malignancies encompassing nine different subtypes of NHL. Our results indicate that *TERT* promoter mutations are absent across all tested NHL. These findings suggest that *TERT* promoter mutations are not major drivers for TERT up-regulation in lymphomas in contrast to the aforementioned cancers.

Materials and Methods

Patients and Samples

Representative cases of a variety of B-cell neoplasms were obtained from archived formalin-fixed paraffin-embedded (FFPE) tissues as well as frozen cells and tissues previously banked as de-identified research samples after obtaining institutional review board approval (Johns Hopkins Institution Review Board no. NA_00028682). The FFPE archives were searched from 2000 to 2014 for cases of Burkitt lymphoma, chronic lymphocytic leukemia/small lymphocytic lymphoma, diffuse large B-cell lymphoma, follicular lymphoma, lymphoplasmacytic lymphoma, mantle cell lymphoma, marginal zone lymphoma, myeloma/plasmacytoma, and plasmablastic lymphoma. Cases of glioblastoma and reactive lymph nodes were also queried as expected positive and negative control cases for formalin-fixed paraffin-embedded (FFPE) tissue. Representative cases with unambiguous pathologic diagnoses

and sufficient material were selected for histologic re-review by a board-certified Pathologist (Rena R. Xian). Both tumor neoplastic cell content and tissue adequacy was assessed, and only cases with at least 25% neoplastic cells and sufficient tissue were selected for the study. FFPE tissue sections were then acquired from the respective archival cases for subsequent analysis. B-cell acute lymphoblastic leukemia (B-ALL) bone marrow aspirate samples with at least 15% lymphoblasts, and normal bone marrow aspirate samples without phenotypic abnormalities were consecutively collected over a two-month period from remnant material from routine clinical flow cytometric testing. Fresh aspirate material collected in EDTA blood tubes was obtained and frozen until further analysis.

DNA Isolation and Mutational Analysis

DNA extraction from bone marrow was performed using Qiagen DNeasy Blood and Tissue Kit (Valencia, CA, USA) according to manufacturer's protocol. DNA extraction from FFPE tissue slides was performed using Pinpoint Slide DNA Isolation System™ (Irvine, CA, USA) according to manufacturer's protocol. Primers with the sequences 5'-M13F-CGGGCTCCCAGTGGATTCGC-3' and 5'-CGGGGCCGCGGAA-AGGAA-3' were used to PCR-amplify the proximal TERT promoter region containing −124C>T (chr5:1295228, NM_198253, GRCh37/hg19) and −146C>T (chr5:1295250, NM_198253, GRCh37/hg19). Amplified products were then sequenced using standard Sanger sequencing techniques (Louisville, KY, USA) with the universal sequencing priming site, M13F.

Results

Absence of TERT Promoter Mutations in NHLs

NHLs (Table 4.1) were screened for *TERT* promoter mutations. The PCR amplified region encompasses the two most commonly mutated nucleotides −124C>T (chr5:1295228G>A) and −146C>T (chr5:1295250G>A) upstream of the translational start site of *TERT* (Figure 4.1A). Altogether, 93 tumor samples were evaluated with at least 7 samples of each subtype in additional to a subset of gliomas as positive controls, and reactive lymph nodes and normal bone marrows as negative controls (Table 4.1).

We confirmed *TERT* promoter mutations in glioblastomas (n = 2), which is lower than expected (Bushman and Craigie 1990; Heidenreich, Nagore, et al. 2014; Weinhold et al. 2014; Vinagre et al. 2013) but demonstrates that we can detect the mutation. A glioma control trace that is heterozygous for the −124C>T mutation is shown (Figure 4.1B). Glioblastoma samples (n = 7) previously identified with promoter mutations were used as additional positive controls. All 7 control samples were confirmed to have the −124C>T mutation. No *TERT* promoter mutations were detected in any NHL samples in the amplified promoter region. A representative NHL trace showing the wildtype *TERT* promoter sequences at both positions are shown from a mantle cell lymphoma tumor sample (Table 4.1 and Figure 4.1B).

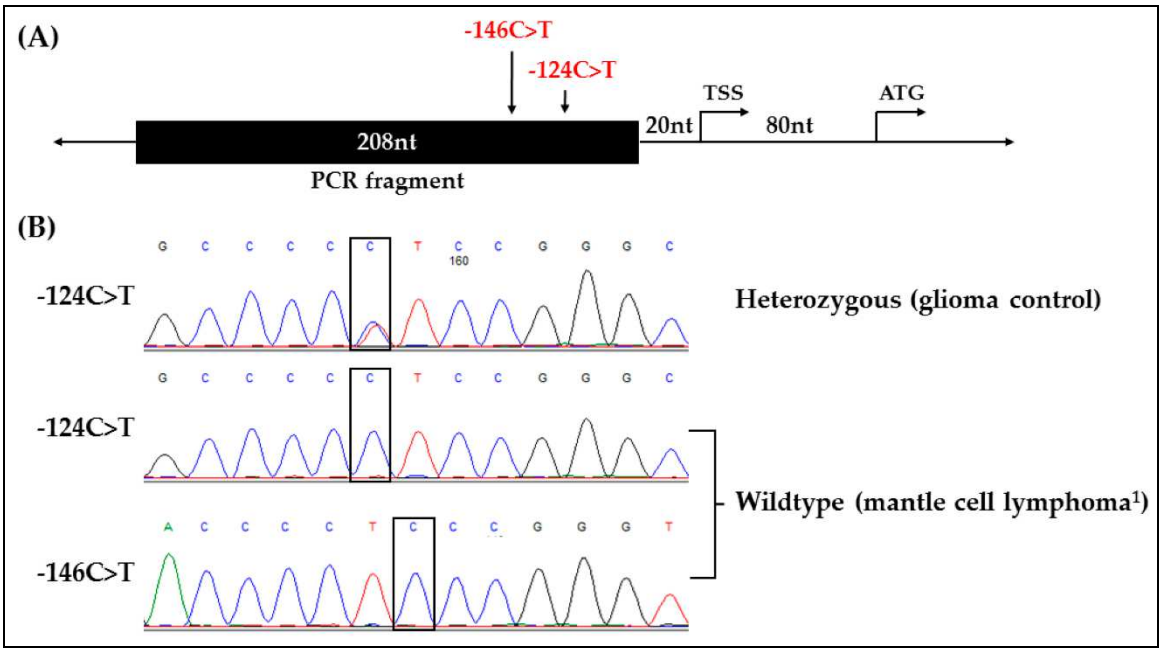


Figure 4.1. Screening of *TERT* promoter mutations in Non-Hodgkin lymphomas (NHLs). (**A**) Schematic of the amplified region and the location of −124C>T and −146C>T in the *TERT* promoter. (**B**) Sequencing chromatographs of the *TERT*promoter locus in a glioma control that is heterozygous for −124C>T (top) and a representative NHL tumor sample that is wildtype at both positions (middle and bottom). [1] A representative trace for wildtype at both positions.

Tumor Type	No. of Tumors	No. of Tumors Mutated
B-cell acute lymphoblastic leukemia	12	0
Burkitt lymphoma	9	0
Chronic lymphocytic leukemia	11	0
Diffuse large B-cell lymphoma	9	0
Follicular lymphoma	13	0
Lymphoplasmacytic lymphoma	7	0
Mantle cell lymphoma	12	0
Marginal zone lymphoma	16	0
Myeloma/plasmacytoma	9	0
Plasmablastic lymphoma	7	0

[1] Glioblastoma tissues were used as positive controls (n = 11); reactive lymph nodes (n = 13) were used as negative controls for formalin-fixed paraffin-embedded (FFPE) samples; normal bone marrow samples (n = 13) were used as negative controls for B-cell acute lymphoblastic leukemia (B-ALL) samples

Table 4.1 Samples tested for telomerase reverse transcriptase (TERT) promoter mutations[1]

Discussion

As a terminally differentiated cell type, normal human lymphocytes have atypical telomere and telomerase biology. In contrast to other cell types, lymphocytes have above average telomere length and telomerase activity (Davison 2007; Bruno et al. 2015). Despite the presence of longer telomeres, and enhanced telomerase activity, lymphocytes still experience division-dependent telomere shortening. Malignant transformation is associated with increased TERT expression and telomere length (Machiela et al. 2016). Furthermore, longer telomeres and higher telomerase activity are associated with more aggressive NHL than indolent ones, and have been suggested to be a prognostic risk factor for NHL (Ohyashiki et al. 2002). Our lab has repeatedly observed TERT activation in chicken lymphomas via ALV integration into the *chTERT* promoter region as an early event in avian B-cell lymphomagenesis (Yang et al. 2007; Justice, Morgan, and Beemon 2015).

Taken together, to overcome the restriction of telomere shortening, and support higher proliferative potential and survival, we hypothesized that lymphocytes may acquire *TERT* promoter mutations in the process of malignant transformation, which can directly up-regulate TERT expression and drive telomerase function. However, our data suggest that the NHLs tested were free

of the two most prevalent *TERT* promoter mutations (Horn et al. 2013; Huang et al. 2013). This result does not exclude the possibility of promoter mutations further upstream of the area we investigated, and the small sample size does not exclude the presence of low-frequency *TERT* promoter mutations, which would require much larger screens to resolve.

Concurrent to this study, *TERT* promoter mutations were reported to be present in 33% of circulating mantle cell lymphoma (Panero et al. 2016). In this study, a limited subset of other lymphoid neoplasms was reported to be free of *TERT* promoter mutations. While we detected no *TERT* promoter mutations in our set of mantle cell lymphoma, there are a few possible reasons for this apparent discordance. First, the small sample size of mantle cell lymphoma (n = 12) evaluated in the current study may be too small to detect a change that may be present in a small fraction of cases. Second, the source of neoplastic B-cells in our study was different from the concurrent study. The peripheral blood source of mantle cell lymphoma in the concurrent study indicates that all patients had circulating leukemic-phase disease, which is associated with advanced stage disease, and worse prognosis when coupled with nodal involvement (Pittaluga et al. 1996). This is compared to the node-based disease selected in our study, irrespective of circulating cells, which may harbor different clonal abnormalities commensurate with the stage of the lymphoma. *TERT* promoter mutations may be more prevalent in a particular stage in mantle cell lymphomagenesis. This has been previously shown to be true in melanoma, in which *TERT* mutations are associated with different histology types of the disease, and are more commonly found in melanoma without regression as compared to melanoma with regression (de Unamuno Bustos et al. 2016).

Our findings suggest that activation of TERT expression by acquired *TERT* promoter mutations is not a major driver for TERT activation in NHL. As observed previously, the frequency of this phenomenon is perhaps associated with the intrinsic proliferative potential of the cell type, in which cells with higher proliferative potential like lymphocytes are less likely to have *TERT* promoter

mutations (Weinhold et al. 2014). In the case of lymphocytes, perhaps activation of TERT expression indirectly through the up-regulation of other genes like *MYC* is far more common and supplants the requirement of other mechanisms of TERT activation like *TERT* promoter mutations. Despite our results, the non-coding sequences of NHL tissues remain an uncharted territory, as novel mutated regulatory sites are being discovered across many different cancers (Melton et al. 2015), and future analysis by whole-genome sequencing may lead to the discovery of novel mechanisms in lymphomagenesis. As whole-genome patient sequencing and clinical data becomes available, we can begin to explain these observations and apply them to enhance our understanding of cancer biology and the treatment of cancer.

Chapter 5. Conclusion and future directions

Introduction

In this thesis, I describe our work characterizing the effects of overexpressing chicken TERT in chickens coinfected with ALV in chapter 2. I also analyze ALV integrations in the resulting tumors in chapter 2. Overall, no conclusions could be made about TERT overexpression and tumor progression in ALV-induced tumorigenesis. Nevertheless, we identified one chicken out of the 27 chickens that carried two tumors (791L1 and 791L2) that retained the recombinant TERT virus. Investigation into the ALV integration pattern of these two tumors revealed that the tumors had different clonal integrations. While tumor 791L1 was associated with genes previously observed like MYB, tumor 791L2 implicated ARID5B as a potential cooperating gene in transformation of TERT overexpressing cells. No DAS provirus was detected in tumors from the DAS group.

Unexpectedly, we also observed an increased incidence of hemangiomas across every chicken group, which accounted for the majority of the lesions observed. In contrast, ALV-A has been reported to commonly be associated with B-cell lymphomas. ALV integration analysis of these hemangiomas implicated a distinct subset of genes that may play a causal role in hemangioma development. Notable genes include FRK, PLAG1, and GLIS3, which were associated with multiple tumors that did not show a clear lymphoma phenotype. No TERT proviruses or TERT promoter integration sites were detected in hemangiomas.

In chapter 3, we further characterized the effects of ALV integration at the site of integration. Specifically looking at clonal integrations in the TERT promoter, we identified a decrease of DNA methylation in the flanking genomic DNA of ALV-occupied alleles compared to unoccupied alleles in every tumor tested. This allele specific ALV-associated hypomethylation at the TERT promoter presents another potential mechanism of gene activation in ALV-induced tumorigenesis.

Lastly, we investigated the frequency of TERT promoter mutations in human hematological malignancies. We did not observe any previously described TERT promoter mutations in any of the ten

types of malignancies tested. This result is consistent with earlier reports investigating smaller subsets of hematological malignancies (Killela et al. 2013). Our results suggest that TERT promoter mutation is not a primary driver of TERT activation in hematological malignancies.

Taken together, this body of work tackle the role of TERT in tumorigenesis from various angles. Previous data and data presented here provide converging evidence that TERT may provide essential oncogenic functions in cooperation with a specific network of genes in lymphoma development. ALV-induced tumors show that TERT may be activated directly by ALV proviral integrations in the TERT promoter in a subset of lymphomas. This activation may be mediated by proviral LTR enhancers. In addition, hypomethylation of the TERT promoter associated with ALV proviral integration may be another mechanism in achieving TERT activation. Other lymphomas implicate the activation of genes that have been shown to activate TERT, like MYC. Together, these genes and TERT are recurring factors in lymphomas. Tumors 791L1 and 791L2 further support this association between MYC and TERT by implicating additional players, ARID5B and PHF2.

The activation of TAPAS by TERT promoter integrations presents another layer to the potential interaction with TERT function and regulation and its associated genes. The relationship between TAPAS in chickens and humans are currently being explored. In chickens, TAPAS expression correlates with TERT expression in adult tissues and during chick development (Nehyba et al. 2016). Both show increased expression in ALV-induced tumors with TERT promoter integrations (Nehyba et al. 2016). In contrast, TAPAS expression is negatively correlated with TERT expression in human cancer patients (Malhotra, Freeberg, et al. 2017).

The discovery of ALV-A induced hemangiomas constitutes another dimension of tumorigenesis that requires more exploration. Our initial investigation suggest that hemangiomas may be driven by an exclusive set of genes. Many of the genes, including FRK, PLAG1, and GLIS3, are still under active investigation and do not have clearly defined functions in tumorigenesis. At the moment, no association

is known between the genes implicated in the hemangiomas and lymphomas in our study. How these genes in hemangiomas may be related to the oncogenic functions provided by TERT and associated genes in lymphomas presents an exciting new direction in understanding the role each set of genes play in their corresponding lesion types. The approach used in this work could be optimized to comprehensively expand the pathways associated with TERT, as well as, other genes of interest. While TERT promoter mutations may not be a primary mechanism of TERT activation in human hematological malignancies, our preliminary results from our chicken experiments may have identified alternative players involved in TERT function, like ARID5B and PHF2.

This work expands our understanding of retroviral induced tumorigenesis and continues to support the valuable application of using chicken as a model for cancer studies. Several avenues for future research are described below in this chapter.

How is the expression of the genes of interest affected by ALV integration?

The resulting impact on the expression of neighboring genes from ALV integration can be unpredictable. While promoter insertion and enhancer activation are common mechanisms of activation, epigenetic changes, like DNA methylation, may also play a role in regulation which is explored in chapter 3. Quantifications of endogenous transcripts, as well as any proviral fusion transcripts are required to determine the net change in expression of neighboring genes. A candidate approach using qPCR can be used to validate any changes in total expression of genes of interest.

The clonal integration near ARID5B is located in the opposite orientation in the promoter region of ARID5B, suggesting that ALV may induce expression by means of enhancer activation in a manner analogous to TERT. Induction of ARID5B would be consistent with the reported association of ARID5B expression and tumorigenesis in humans (Leong et al. 2017). Integration sites identified in FRK, PLAG1, and GLIS3 are all in the same orientation at the 5’ end of the gene. Both integrations for FRK and PLAG1 are located in exon 1, and GLIS3 integrations are located in intron 2. Consequently, the expression of

these three genes is likely to be increased by promoter insertion. Increased expression of these genes would be consistent with their associations with various human cancers (Goel and Lukong 2016; Astrom et al. 2000; Chou et al. 2017). In the case of the FRK, PLAG1, and GLIS3, proviral driven expression may lead to fusion transcripts that overexpress truncated proteins from their respective genes similar to what happens with MYB. All three genes have been previously shown to be activated by reciprocal translocation events in human cancers where the resulting fusion proteins are comprised of modified proteins of the two genes involved (Hosoya et al. 2005; Juma et al. 2016; Xu et al. 2015).

Does ARID5B cooperate with TERT in tumorigenesis in chickens?

From our integration site analysis, we observed a clonal integration near ARID5B in tumor 791L1, which harbored the TERT recombinant provirus. Consistent with our tissue culture experiments, the recombinant provirus was able to drive TERT overexpression in the tumor. The clonal integration near ARID5B suggest selection for the activation of ARID5B in the TERT overexpressing tumor. We could test the effects of ARID5B expression by establishing an analogous environment using CEFs. CEFs are primary cells that have been observed to stop dividing over time in tissue culture, and this observation coincides with a decrease in TERT expression (data not shown).

Knowing this, we can establish a stable TERT overexpressing CEF cell line using our TERT recombinant virus. This can be achieved by transfecting our TERT recombinant vector into CEFs and propagating primary CEFs for at least a few weeks, which is often when CEFs begin to stop dividing. Propagation will naturally select for cells that are immortalized by TERT overexpression. This method has previously been shown to work for chicken adipocytes (Wang et al. 2017). After establishing immortalized CEFs, we can overexpress ARID5B by similar means and look for any phenotypic indicators of malignant transformation. As ARID5B has been shown to be sufficient in driving malignant transformation in human cells (Leong et al. 2017), comparing ARID5B and TERT overexpression with ARID5B overexpression alone may present some interesting new findings for future investigation.

Furthermore, the establishment of immortalized CEFs from the overexpression of TERT may prove to be useful for any future experiments involving TERT. Immortalized cells may present an excellent tool for the production of reagents that may benefit the poultry industry, such as vaccine production.

Is there an association between the distinct subsets of genes that define lymphomas and hemangiomas in ALV-induced tumors?

Extending from the previous question about potential cooperation between ARID5B and TERT, we could also look to similar experiments altering expression of different genes in each subset of genes that are attributed to lymphomas and hemangiomas and observe if any of these genes are associated with changes in other genes in their corresponding group. With the data we have collected with ALV-induced tumors, we know that MYB, MYC, mir-155, TERT, and, possibly ARID5B appears to be associated primarily with lymphomas. On the other hand, our investigation of hemangiomas introduces FRK, PLAG1, and GLIS3 as potential factors in hemangioma development in addition to the previously described MET.

With the exception of TERT, all the noted genes have canonical functions in regulating many downstream targets. MYB, MYC, PLAG1, and GLIS3 are known transcription factors, ARID5B is a transcription coactivator, FRK is a Src-like tyrosine kinase, and mir-155 is a miRNA. The possibility that some of these may activate one another in the associated lesion types would not be surprising. To test this idea, we could initially take a candidate approach and look at expression of these genes in the corresponding tumor types and investigate if there is a significant association between the genes. If a correlation exists, we would then test if the associated genes are sufficient to drive the expression of related genes in tissue culture to account for other spurious effects that may be driven by ALV integrations. Together, this data may reveal novel associations that are important to lymphoma and hemangioma development in chickens.

Are abnormal translocation sites common integration sites for retroviral integration?

Understanding the determinants of proviral integration site preferences is an active area of investigation. Information regarding site preferences can be applicable to many areas of biology, especially, research involving the use of retroviral vectors. In our lab, we have rigorously profiled integration site selection and preferences of ALV-A (J. F. Justice, Morgan, and Beemon 2015; Malhotra, Winans, et al. 2017). Our analysis of the hemangiomas observed in our most recent chicken experiments showed that integrations into the 5' region of FRK, PLAG1, and GLIS3 were selected for in tumors tested. Interestingly, all three of these genes have been previously described to be the target of abnormal reciprocal translocation events in human cancers (Hosoya et al. 2005; Juma et al. 2016; Xu et al. 2015). The clonal integration sites identified in our study are near the reported breakpoints of the translocation events of each gene.

There may be an association between sites prone to translocation and common integration sites for retroviral integration in tumors. While translocation information is limited in chickens, there is more information regarding translocation events in humans. However, translocation events are largely restricted to disease states, as well as integration site studies of human integrating viruses. To better characterize this association, we can perform analogous integration site studies using primary human cells in tissue culture. Primary human cells can be infected with an integrating virus of interest and integration sites can be used to compare with the current reported translocation events. Furthermore, any transformed cells can be isolated by means of a traceable marker and propagated for comparison. A similar approach was recently applied to the investigation of genomic features that enable long-term proviral expression (Miklík, Šenigl, and Hejnar 2018). The findings could provide insight into another contributing factor in site selection of proviral integration.

Is the chicken TERT promoter an exceptional region for ALV-associated hypomethylation?

In chapter 3, we observed an ALV-associated hypomethylation of the flanking genomic DNA. The relationship between DNA methylation at the site of integration prior to proviral integration and the type of integrating virus is a complex one as explored in the introduction to my thesis. Our investigation into TERT promoter integrations suggest that the significantly methylated state of the chicken TERT promoter was not associated with methylation of the ALV provirus after integration. In contrast, analysis of human cancers associated with integrating viruses showed a positive correlation with the methylation status of the genomic DNA prior to integration and the methylation state of the provirus (Watanabe et al. 2015; Hatano et al. 2017).

In both studies, various loci were tested, and allele-specific methylation analysis suggested that the HBV and HPV16 integrants remained hypomethylated when the flanking host genome was hypomethyled (Watanabe et al. 2015; Hatano et al. 2017). In contrast, after integration into highly methylated human genome regions, integrates became methylated (Watanabe et al. 2015; Hatano et al. 2017). Interestingly, the most frequent hepatitis B virus integrations were found in the TERT promoter region (Buendia and Neuveut 2015). Coincidentally, the methylation status of the flanking genomic region and a HBV integrant in the human TERT promoter was tested (Watanabe et al. 2015; Hatano et al. 2017). Both the flanking TERT promoter region and provirus were shown to be hypomethylated (Watanabe et al. 2015; Hatano et al. 2017).

The relationship of the type of integrating virus and proviral silencing was explored recently. HIV, MLV, and ALV showed different extents of silencing upon infection of human cells (Miklík, Šenigl, and Hejnar 2018). In this study, the group concluded that the proximity to active regulatory chromatin segments correlated with stable provirus expression for all three retroviruses even though the extent of silencing globally varied (Miklík, Šenigl, and Hejnar 2018). Our observations in the TERT promoter are

consistent with this association between active epigenetic markers, in our case DNA hypomethylation of the flanking genomic DNA was associated with stable proviral activity, which is inferred by activation of TERT expression and proviral fusion transcripts of ALV and TAPAS.

However, in our specific case, analysis of the unoccupied allele suggests that the TERT promoter is repressed in the same tumor cells. Thus, this would suggest that the resulting DNA methylation status at the integration site may be more dependent on the integrating virus, ALV. A natural follow-up question would concern the specificity of this phenomenon. To test site specificity, other clonal ALV integrations can be tested using the same approach. Preliminary results suggest that clonal integrations at the MYB locus do not appear to change between the occupied and unoccupied alleles. However, the difference in the location of the integration sites with respect to the gene may contribute significantly to the results. Common integration sites are located in intragenic region of MYB (Kanter, Smith, and Hayward 1988), in contrast to integrations in the TERT promoter. The relationship between DNA methylation and expression in intragenic regions varies more than promoter regions (Bird 2002).

Specific targeting strategies like CRISPR can be utilized in chickens to attain more direct control over site selection (Véron et al. 2015). To test genomic site specificity, a minimal ALV LTR can be targeted to various gene promoters that have varied DNA methylation states, and comparison of allele specific methylation can be performed with the same approach used previously. The reverse relationship can be tested using the same CRISPR approach. In this case, LTRs from different viruses can be tested at a single genomic site.

Integrating high-throughput sequencing may offer a more comprehensive approach to analyzing the relationship between proviral integration and methylation status at the site of integration. Using our established method of mapping provirus integration, a parallel experiment with some modifications using bisulfite treated tumor DNA can be used to investigate the methylation status of flanking genomic DNA. With enough coverage and depth, comparison of the two datasets will theoretically screen the

methylation status of flanking sequences throughout the genome. Expression can then be correlated by RNA-seq experiments.

Alternatively, one can map proviral integration while tracking transcriptional activity by adapting a method originally designed for HIV proviruses called barcoded HIV ensembles (B-HIVE) described in detail by Chen et al. 2017. This method involves the generation of a barcoded library of minimal viral vectors of the virus of interest. The library will utilize barcodes with enough complexity to assure that each viral vector in the library contains a unique barcode in between the LTR and essential viral genes. Once packaged, virions can infect a host of interest. Integrations can be mapped from genomic DNA using inverse PCR coupled with high-throughput sequencing. Quantification of proviral expression can be achieved by measuring barcode abundance from the ratio of read counts after RT-PCR on the RNA pool and the DNA pool. Data from these experiments may provide valuable insight into the determinants of DNA methylation at the site of ALV integration, which can also be applied to other integrating viruses.

The cellular environment also plays a role in the silencing of proviruses. While ALV is largely permissive in chicken cells, ALV is efficiently silenced in mouse (Guntaka et al. 1980) and human cells (Miklík, Šenigl, and Hejnar 2018). However, the artificial insertions of a CpG island core element into the LTR of an RSV-derived vector has been shown to be able to provide efficient protection of the integrated vector from silencing and gradual CpG methylation in rodent and human cells (F. Senigl, Plachy, and Hejnar 2008). The TERT promoter may act in a similar manner. One can test this idea by performing an analogous experiment by artificially inserting TERT promoter next to an ALV LTR and observing the methylation status of the recombinant ALV provirus in non-permissive mammalian cells. Furthermore, any elements identified from the above experiments may also provide potential candidates that alter the susceptibility to proviral silencing. Such experiments may provide further insight into the

determinants that restrict permissiveness in heterologous infections, as well as useful information for the development of genetic tools.

What are other mechanisms of TERT activation in hematological malignancies?

In chapter 4, we attempted to investigate potential somatic changes in the TERT promoter that may be major drivers of TERT expression in human cancers. To this end, we investigated the prevalence of TERT promoter mutations in a large subset of hematological malignancies and concluded that TERT promoter mutations are not a prominent mechanism of TERT activation. Human integrating viruses may offer another direct way of activating TERT expression. Human herpesvirus 6B has been shown to integrate into subtelomeric regions and induce hypomethylation in these regions (Engdahl et al. 2017). Notably, the human TERT gene is located in the subtelomeric region of chromosome 5. As mentioned previously, the most frequent hepatitis B virus integrations were found in the TERT promoter region (Buendia and Neuveut 2015). These observations may offer potential sources to test if any of our observations with ALV in chickens translates to human cancers.

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Curriculum Vitae

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EDUCATION

2011-2018 **Ph.D. Cellular, Molecular, Developmental Biology and Biophysics**
Johns Hopkins University, Baltimore, MD
Supervisor: Dr. Karen Beemon

2005-2009 **Bachelor of Science, Biochemistry and Molecular Biology** *summa cum laude*
Lebanon Valley College, Annville, PA
Supervisor: Dr. Walter Patton

RESEARCH EXPERIENCE

2011-present **Ph.D. Thesis**, Johns Hopkins University
Exploring TERT Expression and Regulation in Tumorigenesis: Lessons from ALV

2009-2011 **Post-baccalaureate Research**, Cancer Research Training Fellowship, NCI
Anti-EGFR therapy in skin carcinogenesis

2006-2009 **Undergraduate Research**, Lebanon Valley College
Kinetic characterization of *E. coli* GMP synthetase

PRESENTATIONS

May 2015 **Cold Spring Harbor Laboratory Meeting: Telomeres & Telomerase**
Cold Spring Harbor, New York
Presented a poster entitled: *TERT promoter/enhancer mutations in chickens and humans*

PUBLICATIONS

1. **Lam G**, Beemon K. (2018) ALV Integration-Associated Hypomethylation at the TERT Promoter Locus. Viruses. 10(2)

2. Malhotra S, Winans S, **Lam G**, Justice J, Morgan R, Beemon K. (2017) Selection for avian leukosis virus integration sites determines the clonal progression of B-cell lymphomas. PLoS Pathog. 13(11)

3. **Lam G**, Xian RR, Li Y, Burns KH, Beemon KL. (2016) Lack of TERT Promoter Mutations in Human B-cell Non-Hodgkin Lymphoma. Genes. 7(11)

4. Mascia F, **Lam G**, Keith C, Garber C, Steinberg SM, Kohn E, Yuspa SH. (2013) Genetic ablation of epidermal EGFR reveals the dynamic origin of adverse effects of anti-EGFR therapy. Sci Transl Med. 5(199)

5. Abbott JL, Newell JM, Lightcap CM, Olanich ME, Loughlin DT, Weller MA, **Lam G**, Pollack S, Patton WA. The effects of removing the GAT domain from E. coli GMP synthetase. Protein J. 25(7-8)

RESEARCH INTERESTS

Retroviruses
Transposable elements
Epigenetics
Gene Therapy
Cancer
High-throughput technology

TEACHING EXPERIENCE

2018 Spring	Immunology Teaching Assistant	Johns Hopkins University
2017 Fall	Genetics Teaching Assistant	Johns Hopkins University
2017 Spring	Cell Biology Teaching Assistant	Johns Hopkins University
2016 Fall	Genetics Teaching Assistant	Johns Hopkins University
2015 Fall	Molecular Biology Teaching Assistant	Johns Hopkins University
2014 Fall	Biochemistry Laboratory Assistant	Johns Hopkins University
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