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# Vital role of HERV-K in malignant disease progression provides a novel target for cancer therapeutics

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SCHOOL OF MEDICINE

Thesis

**VITAL ROLE OF HERV-K IN MALIGNANT DISEASE PROGRESSION  
PROVIDES A NOVEL TARGET FOR CANCER THERAPEUTICS**

by

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B.A., University of California Berkeley, 2015

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requirements for the degree of  
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**ABSTRACT**

Human Endogenous Retroviruses (HERV) are segments of the human genome that are viral in origin and occupy approximately 8% of the human genome, which is nearly 3 times as much as functional protein coding genes (3%). Although most are defective due to accumulation of post insertional mutations, Human Endogenous Retrovirus Type K (HERV-K) retains the ability to produce functional particles and is activated during progression of malignant disease. The resulting proviral products have been associated with tumorigenesis through their presumed role in malignant cell production. While therapeutics that focus on HERV-K inhibition have not been manufactured, current Federal Drug Administration (FDA)-approved antiretroviral therapies are capable of decreasing expression of HERV-K in cancer cells. In summary, antiretroviral drugs may serve as a promising class of new anticancer drug by targeting and decreasing expression of HERV-K proteins.

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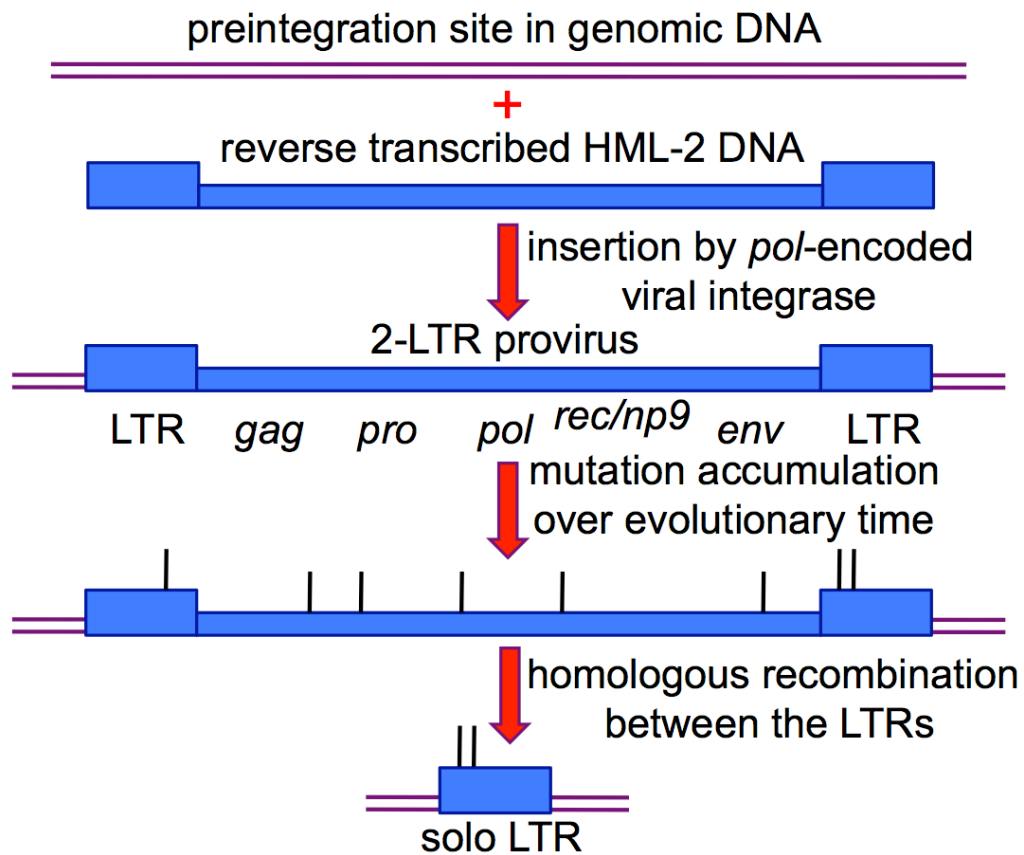
AR.....	Androgen Receptor
ART .....	Antiretroviral Therapy
BC.....	Breast Cancer
CSC .....	Cancer Stem Cells
EGFR.....	Epidermal Growth Factor Receptor
ERV .....	Endogenous Retrovirus
HAART .....	Highly Active Antiretroviral Therapy
HERV.....	Human Endogenous Retrovirus
HML.....	Human MMTV-like
hSGT .....	Human Small Glutamine-rich Tetratricopeptide
IN.....	Integrase
JSRV .....	Jaagsiekte Sheep Retrovirus
LTR.....	Long Terminal Repeat
MS .....	Multiple Sclerosis
NNRTI.....	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI .....	Nucleoside Reverse Transcriptase Inhibitor
ORF .....	Open Reading Frame
PLZF.....	Promyelocytic Leukemia Zinc-Finger
PFV.....	Prototype Foamy Virus
RA.....	Rheumatoid Arthritis
RT.....	Reverse Transcriptase

VSV ..... Vesicular stomatitis Virus

## INTRODUCTION

### HERVs Background

Human Endogenous Retroviruses (HERV) are segments of the human genome that are viral in origin. The genetic material derives from retroviruses, a broad group of RNA viruses that are able to integrate a DNA copy of the viral genome into a host's cell. If integrated into a germ line cell, the proviral element has the potential to become an integral and inherited part of the host genome, which can have significant consequences on the carrier (Hohn, Hanke, and Bannert 2013). Once germ lineage cells acquire the provirus, the HERV is a permanent part of the genome unless acted on by selective processes and genetic drift (Broecker et al. 2016). HERVs that lead to decreased fitness of the host are removed via negative selection. Those that provide a net neutral or positive effect will inevitably accumulate changes that result in eventual functional decay. Many HERVs are rendered inactive due to extensive post-insertional recombinations, deletions, and mutations that occurred over the course of human evolution. A common event that leads to HERV inactivation is homologous recombination between two long terminal repeats (LTRs) that removes the majority of functional viral information, resulting in a solo LTR (Lenz 2016)



**Figure 1: Formation of Solo LTRs.** A typical HERV sequence contains 4 genes (*gag*, *pro*, *pol*, and *env*) flanked by two LTR sequences. These LTRs that can undergo recombination events that lead to a solo LTR. In the figure, the small, black vertical lines represent the mutations that inevitably accumulated over time (Lenz 2016).

Due to incorporation of exogenous retroviruses that were present approximately 2-40 million years ago, the human population, regardless of genetic background, shares an extensive number of HERV loci in their genome. Consequently, HERVs occupy approximately 8% of the human genome, which is nearly 3 times as much as functional protein coding genes (3%). Despite their

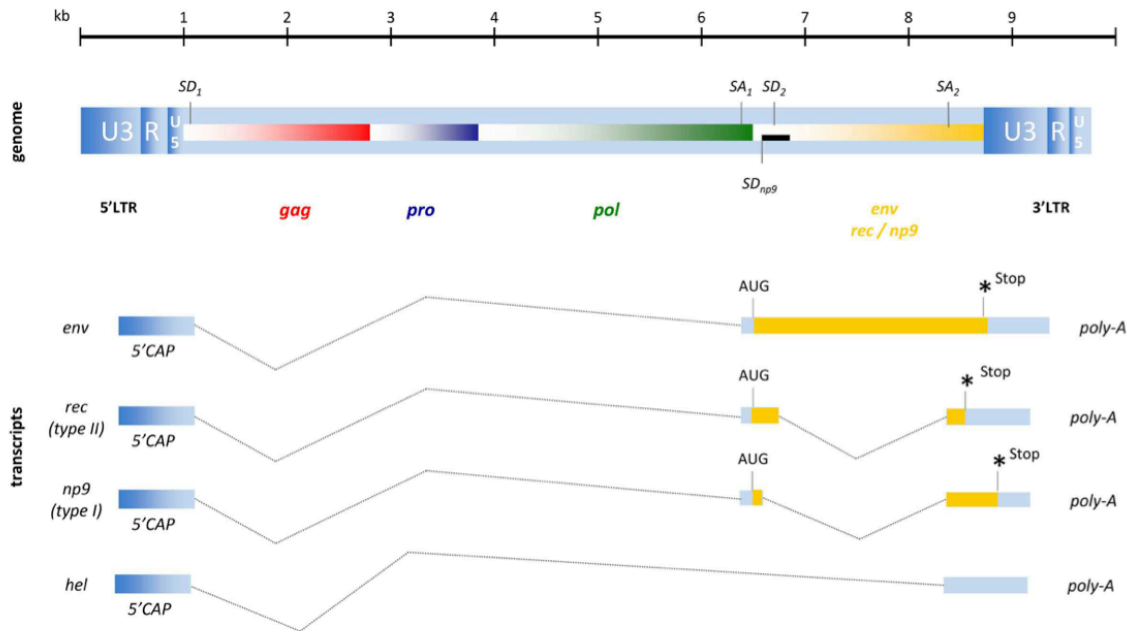
relatively large presence in the human genome, accumulation of post insertional modifications over the course of human evolution has diminished the activity of the majority of acquired HERVs (Mullins and Linnebacher 2012). Additionally, there are also cell mechanisms, such as chromatin remodeling, RNA silencing, and antiretroviral protein inhibition, that are initiated to actively counter HERV-K activity and that result in an apparent lack of function.

Despite epigenetic silencing factors, the HERV-K family, one of the most recently acquired families of HERVs, contains highly conserved proviruses from the HML-2 subgroup; nearly 90 proviruses have maintained open reading frames (ORFs) that encode functional viral proteins (Subramanian et al. 2011). In most cells, HERV-K transcription and translation is muted by epigenetic control, but the placenta and the testes basally produce HERV particles (Pérot et al. 2012). However, intact ORFs and subsequent amplification have turned HERV-K into the largest contributor of retroviral-derived proteins in the human genome (Hanke, Hohn, and Bannert 2016). The transcriptional influence over an unknown number of genes imposed by proviral sequences provides additional incentive to research these viral insertions.

A typical HERV K sequence, seen in Figure 1, contains transcribable gag, pro, pol, and Env genes flanked on each side by a 5' and 3' LTR. While the LTR regions do not transcribe functional proteins, they are capable of regulating transcription of HERV and host genes due to a transcriptional promoter and enhancer core (Chuong, Elde, and Feschotte 2016). Normally, the full HERV-K

encodes for structural proteins (George et al. 2011), but slippery sites and a resulting ribosomal frameshift leads to production of a protease (Pro) and a polymerase (Pol), where the latter exhibited reverse transcriptase (RT) and integrase (IN) activity (Hohn, Hanke, and Bannert 2013). Due to the various and diverse HERV-K loci found throughout the genome, traditional products of viral structural proteins can also be formed. The Env gene encodes different proteins depending on the manner its transcript is spliced. If exons are not removed, then the transcript should produce a standard retroviral envelope protein. However, functional Env proteins are rarely described, likely due to the accumulation of mutations (Hurst and Magiorkinis 2017). Three other proteins, Rec, Np9, and *hel*, are produced upon removal of several exons within the Env transcript.

Depending on the product of the Env transcript, most HERVs are either referred to as type 1 provirus (expressing Np9) or a Type II provirus (expressing Rec). Np9 differs from Rec via a 292 base pair frameshift deletion. The differences in their splicing details are indicated in Figure 2. While *hel* is not translated into a protein, Rec and Np9, on the other hand, are translated into products that are often associated with malignancies.



**Figure 2: Alternative Splicing of HERV-K Env Produces Different Products.** HERV transcripts can produce various products from the Env gene. Type II proviruses express Rec while type I proviruses express Np9, which has a 292 base pair deletion in the gene sequence indicated by the black bar. Hel does not have a protein coding capabilities and is poorly described in literature (Hohn, Hanke, and Bannert 2013).

## Physiological effects of HERVs

As mentioned previously, HERVs that are integrated into the human genome can elicit significant consequences, both positive and negative, on the host. Hypothetically, conservation of these HERVs thus far suggests that their proteins also provide beneficial effects to mask the detrimental effects to the host, and several studies have indicated possible evolutionary advantages of HERVs. One of the most notable examples is the expression of HERVs during placentogenesis (Kurth and Bannert 2010). From the perspective of the mother,

the placenta is identified as an allogeneic tissue. However, in the syncytiotrophoblast, which acts as an interface between the maternal tissues and the trophoblast, there are significantly high levels of HERV Env proteins that are not seen in the trophoblast. A common function of the viral envelope is to facilitate fusion of the cell membranes of the virus and its target cell while escaping detection of the immune system. In the context of placentogenesis, expression of HERV Env may therefore prevent rejection of the fetus by providing local immunological tolerance. The expression of HERV-K during placentogenesis is presumed to hold a similar role (Kurth and Bannert 2010). Another study also noted the HERV-K Rec proteins play a role in fetal development and differentiation (Andersson et al. 2002). Other hypothesized functions include, but are not limited to, promotion of genetic diversity (Brandt et al. 2005), repair of chromosomes (Teng, Kim, and Gabriel 1996), and role in telomerase production (Eickbush 1997).

Recently, HERV-K expression has also been indicated in brain development and function. In 2014, Karki et al demonstrated that transfecting human neuronal cell lines with a vector carrying HERV-K (HML-2) Env leads to increased transcription of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF). This would result in increased neuronal viability and protection from neurotoxicity (Bhat et al. 2014). Increased expression of  $\beta$ III-tubulin, a microtubule that is found almost exclusively in neurons that contribute to their neoplastic abilities, was also observed in cells with increased expression

of HERV-K env (Karki et al. 2013). This, however, may be a double edged sword; in the context of malignant diseases, this element of the tubulin family has also been associated with tumor aggressiveness, resistance to chemotherapy and poor patient survival (Karki et al. 2013). Contrary to its proposed role in brain development and function, HERV-K has demonstrated an association with several CNS related diseases. These conflicting roles suggest that HERV-K's neuroprotective actions may be secondary to its pathogenic influence as a means to ensure survival of the host and conservation of its proviral sequence in the human genome.

Despite its possible influence on reproduction and development, HERV K evolution is not meant to primarily benefit the host and have therefore been linked with pathologies. For several decades, HERVs have been associated with human chronic diseases such as autoimmune conditions and cancer (Hohn, Hanke, and Bannert 2013). For example, there are several studies investigating its role in the propagation of autoimmune disease such as rheumatoid arthritis (RA) and multiple sclerosis (MS). In RA, an autoimmune disorder where chronic inflammation of synovial joints culminates in cartilage and bones damage, molecular assays have showed a significant increase in the HERV-K gag, when compared to healthy controls (Freimanis et al. 2010). Expression is further enhanced by the presence of exogenous viral particles and antibodies generated for the HERV-K antigen may exhibit cross reactivity through similar properties of host proteins (Freimanis et al. 2010). MS is a disease where the insulating

sheaths of neurons in the central nervous systems are damaged by an inflammatory process, resulting in impaired neural communication. Compared to RA, disease etiology of MS is not as well understood and the likely multifactorial origins of pathogenesis make HERV-K contributions controversial. While data shows an elevation in the expression for HERV-K in these pathologies, their exact role in pathogenesis is not clearly understood and more research is necessary before confirming an association. However, HERV-'K's role in malignant diseases has been extensively researched, and its expression is presumed to play a crucial role in the development or maintenance of several cancers. Table 1 contains a list of the malignant diseases that are often associated with increase in HERV-K HML-2 activity.

**Table 1: List of cancers associated with activity of HERV-K particles (Hohn, Hanke, and Bannert 2013).**

Tissue	Cancer	HERV-K(HML-2) activity	Reference
Skin	Melanoma	Retroviral particles Enhanced transcription RT activity Expression of Env, Rec, Np9	Buscher et al. (6), Muster et al. (86), Hirschl et al. (124)
Testes	Germ cell tumors, gonadoblastoma, seminoma	Anti-Gag/Env-Ab Expression of Rec, Np9	Boller et al. (87), Kleiman et al. (92), Boller et al. (125)
Ovary	Ovarian clear cell carcinoma; ovarian epithelial tumors	Expression of Gag and Env	Gotzinger et al. (15), Wang-Johanning et al. (72), Iramaneerat et al. (126)
Breast	Breast cancer	Free viral RNA RT activity Virus particles Specific CTLs	Wang-Johanning et al. (112), Contreras-Galindo et al. (127), Wang-Johanning et al. (128)
Prostate	Prostate cancer	Enhanced Gag-production due to fusion to androgen-dependent ETV1 and ETS genes	Tomlins et al. (12), Lamprecht et al. (13), Ishida et al. (111)
Blood	Lymphoma	Free RNA RT activity Virus-like particles	Contreras-Galindo et al. (127)

## **NECESSITY OF RESEARCH AND SPECIFIC GOALS**

Currently, many cancer treatments focus on inhibiting the progression of the cell cycle and exploiting biological differences between healthy cells and cancerous cells in order to target malignant cells. However, these “unique” targets used to identify malignant cells are also found in normal healthy stem cells, particularly in the hair follicle, intestine, and hematopoietic system. While effective, cancer chemotherapeutics can have dangerous side effects if improperly used; In order to prevent permanent tissue damage or patient death, it is critical that the dosing regimens of these traditional anticancer agents must be carefully designed and administered. Targeting HERV-K transcription and translation would provide a novel target for pharmacological cancer therapeutics. Additionally, the epigenetic suppression of HERV- K in most cell types implies that it is unlikely that normal cells rely on the transcripts of HERV-K on a day to day basis (Hanke, Hohn, and Bannert 2016). Because of this, the repercussions of overdose are unlikely to be as severe as those seen with anticancer drugs. Therefore, targeting and inhibiting expression of these proviral sequences should not introduce additional harm to a patient. Yet, considering its basal levels of expression in a subset of healthy tissues and hypothesized contribution to reproductive processes, removal of the HERV-K sequences through new technologies such as CRISPR in hopes of healthier progeny should be avoided. However, temporary inhibition of HERV-K transcription and expression in cancerous diseases via administration of antiretroviral therapies may be a

potential way to treat and manage pathological states associated with raised HERV-K expression.

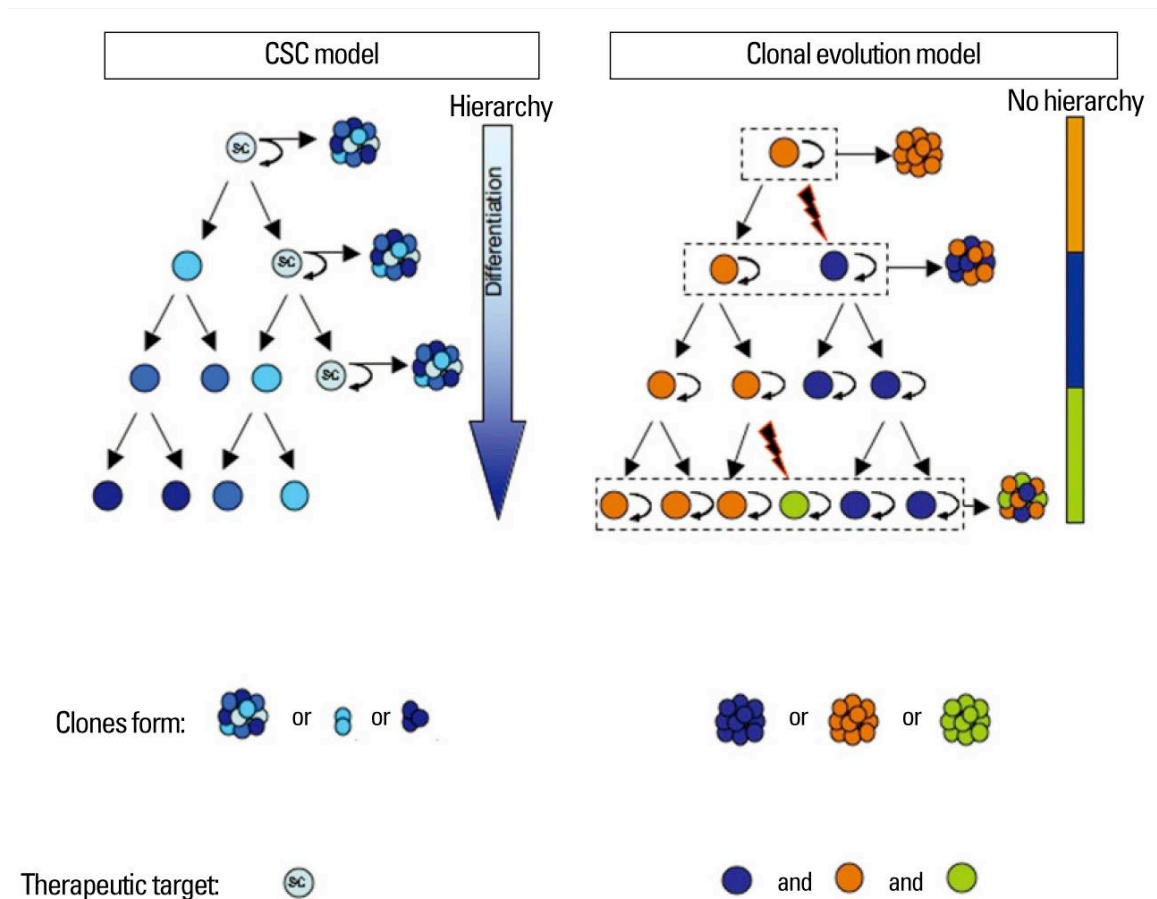
The specific aim of this thesis is to cross reference studies to identify how HERV-K plays a vital role in cancer development and speculate on applications of HERV-K targeted antiretroviral therapies. This paper also intends to suggest plausible directions of future therapeutic research.

## **HERV-K PROTEINS IN CANCER DEVELOPMENT**

### **Cancer Development Theories**

Cancer develops when cell signaling pathways become impaired. These aberrant cells are unresponsive to apoptotic signals and tend to aggressively multiply. There are a couple theories to the development of cells with a deviant phenotype. The stem cell theory of cancer proposes that cancerous cells arise from a subset of cancer cells that have characteristics of normal stem cells (Laks, Visnyei, and Kornblum 2010). These cancer stem cells (CSC) undergo differentiation and self-renewal and act as a generator of the cancerous cells that will result in a tumor, which contrasts with the clonal evolution cancer theory where any cell that escape regulation of the cell cycle can become a cancerous cell. These differences are visualized in Figure 3. The stem cell theory has several implications for cancer therapeutics. Malignant cells that have a phenotype characteristic of a CSC often persevere after chemotherapy. Because

of their ability to generate new cancer cells, it is hypothesized CSCs are also responsible for the propagation and relapse of cancer (Argaw-Denboba et al. 2017). Regardless of the development theory, several studies have suggested that HERV-K is associated with cancer, especially in deviant cells displaying aggressive and metastatic tendencies. In other words, targeting cells with extensive HERV-K expression covers the therapeutic targets indicated in both cancer models.



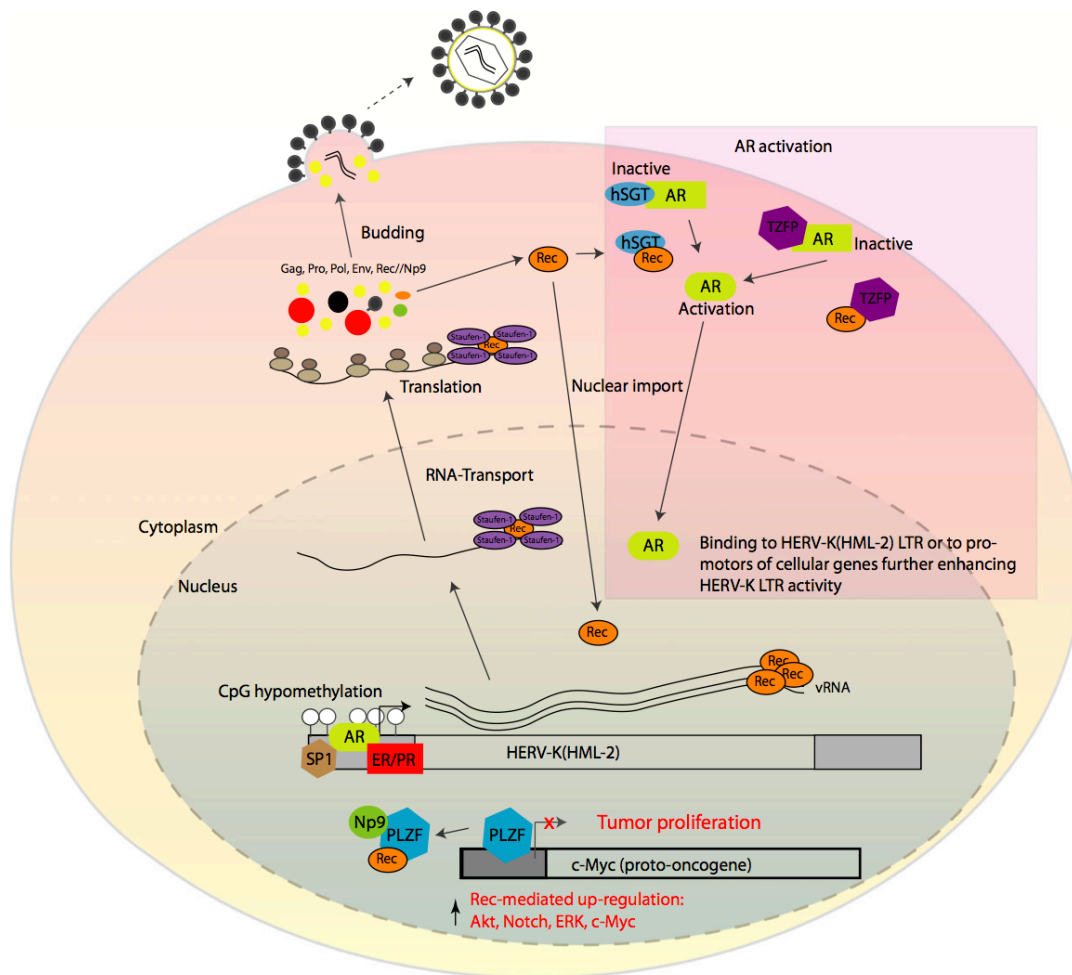
**Figure 3: CSC and Clonal Evolution Models of Cancer Development.** The CSC model suggests that cancerous cells arise from differentiation of a small population of cancer stem cells, and therapeutics should target these CSCs. On the other hand, the clonal evolution model implies that any cell has the potential

to switch a cancerous phenotype, providing a larger cell population for therapeutic agents (Laks, Visnyei, and Kornblum 2010).

### **Mechanisms of increased HERV-K expression**

Expression of HERV-K transcripts are considerably elevated in germ cell tumors (GCT), ovarian cancers and melanoma, to name a few, while the majority of healthy tissues and benign tissues show little if any signs of transcription (Hohn, Hanke, and Bannert 2013). Protective mechanisms against HERV-K particles are also enacted by the cell, and research has shown that HERV-K transcription activity often correlates with level of CpG methylation in cancer cell lines (Stengel et al. 2010). Cells that showed an increase in HERV-K transcripts also produced antibodies, which appear to have anti-tumorigenic properties (Zhou et al. 2015). Furthermore, a strong association between the presence of HERV-K antibodies and the clinical manifestations of GCT have been characterized (Hanke, Hohn, and Bannert 2016).

The debate whether cancer enhances HERV-K expression or whether proviruses induce malignant pathogenesis is ongoing, but it is still important to identify mechanisms leading to increased HERV-K transcriptional states. Within a cell, possible intracellular mechanisms for HERV-K activation include chromatin remodeling via hypomethylation and transcription factor binding.



**Figure 4: Proposed Mechanisms of HERV-K Activation and Tumor Proliferation.** Major intracellular methods of activation of HERV-K transcription include hypomethylation of HERV-K promoters, binding of transcription factors and hormone receptors, and expression of Rec and Np9 accessory proteins. More specifically, Rec and Np9 bind to regulators of AR and c-Myc, leading to an increase in AR and C-Myc activity, known to induce pro-proliferative cell states (Hanke, Hohn, and Bannert 2016).

HERV-K LTRs are often hypomethylated in tumorigenic states, leading to activation of proviral transcription in various cancers (Stengel et al. 2010).

Although global hypomethylation is a common epigenetic event seen in malignant disease, HERV-K hypomethylation is also correlated with age, and is

therefore capable of inducing cancer development as a patient ages (Wallace et al. 2014).

Additionally, numerous transcription factors also contribute to upregulation of HERV-K expression. Sp1 and Sp3 are common zinc finger proteins that bind to GC boxes of many promoters (Fuchs et al. 2011). They are responsible for regulating genes that control cell growth, differentiation, apoptosis, DNA damage, and chromatin remodeling. In the context of HERV-K, the 5'LTR regions contains several binding sites for transcription factors. Of the binding sites that are conserved in the majority of the 5' LTR region, binding sites for both Sp1 and Sp3 are included (Manghera and Douville 2013). Interestingly, the TATA box promoter is absent from HERV-K LTRs and an alternative initiator sequence is not present. Instead, many of these LTR regions contain 4 G-rich regions, providing nucleosome-free binding regions for Sp1 and Sp3. HERV-K transcripts undergo a distinct initiation process that is mediated through binding of these transcription factors to HERV-K LTRs (Fuchs et al. 2011). Although proviral promoter activity depends on the degree of accumulated mutations and insertions, Sp1 and Sp3 binding can free some of the many HERV-K transcription start sites, which allow for various degrees of TATA-independent transcription of HERV-K genes (Hurst and Magiorkinis 2017). The presence of alternative transcription start sites also provides more than one location at which transcription can be initiated, and represents an additional mechanism for heightened HERV-K expression (Persson et al. 2016).

Common hormones also play a role in stimulation of HERV-K expression through their ability to act as transcription factors. HERV-K expression is often associated with malignancies in hormone-regulated tissues and analysis of HERV-K promoters indicate the presence of several response elements (RE) that react to estrogen, progesterone, androgens, and glucocorticoids (Manghera and Douville 2013). Studies have demonstrated that a significant increase in HERV-K transcription follows estradiol and progesterone treatment in breast cancer cell lines (Golan et al. 2008), and that androgen-regulated cell lines increase proviral protein expression in the presence of androgens (Hanke et al. 2013). Furthermore, accessory proteins, Rec and Np9, originating from the alternative splicing of HERV-K sequence can contribute to increased transcription of proviral sequences. The proposed route of increased HERV-K expression and resulting tumor proliferation due to these proteins, will be discussed further on in this paper.

In addition to the molecular mechanisms recruited to increase HERV-K expression, it is possible that horizontal infection of HERV-K particles can stimulate transcriptional events in neighboring cells. While research has yet to identify HERVs in the human genome that code for fully infectious virus, contributions of functional HERV-K genes from the diverse number of HERV-K loci have retained the coding capacity for every protein necessary for viral infection (Belshaw et al. 2004). Currently, the predominant view is to doubt the infectious potential of HERVs, but recent studies have identified that HERV-K

particles are transmissible and their packaged genetic information can undergo reverse transcription once fusion with a neighboring cell is complete (Contreras-Galindo et al. 2015). After fusion, most complementary DNA sequences formed 1LTR and 2LTR episomes, but chromosomal integration was not identified. However, the nature of the experiment may have favored autointegration rather than chromosomal integration, and this ambiguity prompts the necessity of further studies. Nevertheless, it is likely that these episomes are able to transcribe and translate HERV-K material (Contreras-Galindo et al. 2015). This could contribute to the elevated levels and potentiate the horizontal infection into neighboring cells. Additionally, acquisition of viral envelopes such as vesicular stomatitis virus (VSV) would increase the success of the HERV-K infection by several fold (Tyagi et al. 2017), as indicated in figure 8a, but mutations necessary to develop a similar envelope are unlikely to occur.

It has also been theorized that many retroviruses can act synergistically and research, although conflicting, has indicated that HIV particles have also been associated with heightened levels of HERV-K (van der Kuyl 2012). Efficiency of HERV-K horizontal infection is also positively influenced in presence of HIV-1 Tat and Vif (Contreras-Galindo et al. 2015), as well as the HERV-K Rec accessory protein. Due to HIV's ability to increase the expression of HERV-K, it is not surprising that HIV-infected persons show a higher risk for cancer development; the enhanced risk can range from a 2-3 fold risk for cancers such as melanoma, lung cancer, and hepatocellular cancer, to a staggering ten to

thirty fold risk for anal cancer and non-Hodgkin's lymphoma (Sigel et al. 2011). However, it is uncertain which viral particle instigates propagation so further research is required.

### **Associations and Mechanisms of HERV-K Mediated Oncogenesis**

There are three likely mechanisms through which HERV-k proteins can contribute to oncogenesis: immunosuppression, insertional mutagenesis, and direct oncogenic activity. Due to its immunosuppressive domain, escape from immune system surveillance is a typical role of most retroviral Env proteins, and would be a plausible contributor to cancer development. This function is demonstrated in a viral particle's ability to fuse with a cell without an immune response during the viral reproductive cycle and in processes such as placentogenesis.

Insertional mutagenesis refers to activation of proto-oncogenes via insertion of proviral sequences. HERVs are known to pose an influence over neighboring genes by acting as transcription factors through provision of alternative promoters (Dunn, Medstrand, and Mager 2003). These promoters, which are often solo LTRs generated from recombination events that remove internal gag, pro, pol, and Env genes, are often hypomethylated and allow for recruitment and binding necessary transcription factors and proteins, ultimately leading to increased expression of downstream genes and, potentially, cell proliferation. This mechanism of HERV induced pathogenesis is seen in

Hodgkin's Lymphoma, resulting in impaired regulation of the proto-oncogene colony-stimulating factor 1 receptor (CSF1R) (Lamprecht, Bonifer, and Mathas 2010). However, translocation of HERV sequences can also disrupt the stability or lead to the inactivation of tumor suppressor genes (Kurth and Bannert 2010).

Furthermore, HERV-K inserts lead to expression of transcription factors that lead to oncogenesis. After transfecting embryonic kidney 293T cells line with a vector expressing the HERV-K Env, Tsang et al found that HERV-K was able to modify expression of 86 genes (17 upregulated and 69 downregulated). Of the upregulated genes, those that showed greatest increase in expression were transcription factors that include EGR1, ETV4, ETV5 and FosB. These transcription factors have been associated with promotion of epithelial to mesenchymal transition (EMT) and tumor aggression in cancers such as prostate and endometrial cancer (Currie et al. 2017). While the conclusion drawn from this study are indeed interesting, more studies should be done *in vitro* so the sample subject is more representative of the environment, eliminating potential threats to the study's external validity. Nevertheless, this correlation can serve as a segue into HERV-K's direct oncogenic role.

The third and most direct mechanism of malignant disease progression is the possibility that HERVs encode for proteins that are capable of switching a healthy cell's phenotype to that of a cancerous cell. Previously, studies have indicated that Env protein from retroviral sequences had the ability to induce tumor formation on its own, as well as demonstrated phenotypic switching,

leading to tumor formation in vivo (Hofacre and Fan 2004). A recent study demonstrated that expression of HERV-K induces epithelial-mesenchymal transition (EMT), where a loss of cell polarity induces a loss of function. The change in phenotype is seen in the cell's increased mobile capability and, therefore, metastatic potential (Tsang et al. 2017). The study used a non-transformed breast epithelial MCF10A cell line, showing HERV-K Env increased the levels of fibronectin and N-cadherin while decreasing E-cadherin, a result characteristic of EMT.

Furthermore accessory proteins Rec and Np9 are other potential oncogenes due to their interaction with cancer-related structures. Rec is one of the notable products from alternative splicing of the HERV-K Env transcript and facilitates nuclear export of HERV-K mRNA (Contreras-Galindo et al. 2015). Additionally, it may contribute to development of a precancerous state; mice with increased Rec expression lesions similar to those seen in humans with classical seminoma. In cells with co-expression of Rec and promyelocytic leukemia zinc-finger protein (PLZF) show an increase in cell proliferation and reduced apoptosis. PLZF functions as a negative regulator of the c-myc protooncogene, but its proficiency is impaired when Rec binds (Denne et al. 2007). In contrast to Np9, Rec also has the ability to increase HERV-K replication. In the testes, Rec can also form complexes with testicular zinc-finger protein (TZFP) and human small glutamine-rich tetratricopeptide (hSGT) and prevent binding to the androgen receptor (AR). Because TZFP and hSGT usually depressed AR's

actions, Rec therefore tampers with hSGT and TZFP's ability to hold AR in the inactive state. The presence of androgens has been linked to further expression of HERV-K via recruitment of transcription factors to HERV-K LTRs (Manghera and Douville 2013). This propagates the expression of HERV-K particles, and amplifies other effects of proviral expression seen in Figure 4.

Np9, another alternatively spliced product of the HERV-K Env transcript, has similar abilities to Rec but does not exhibit any influence over the enhanced replication of HERV-K sequence. Considering Np9 shares the same starting sequence of amino acids as Rec, it is not surprising that it shares Rec's ability to bind to PLZF protein, leading to increased c-myc transcription and malignant cell properties (Denne et al. 2007). Additionally, misregulation of the MAPK Pathway is also a demonstrated result of Np9 expression. Numb and Notch are both regulative protein of the MAPK pathway, the former acting as an antagonist to the latter, which is a transcription factor that promotes the Ras signaling cascade. Interaction of Np9 with a ligand of Numb ubiquinates the negative regulator of the Ras cascade, and leading to proteolytic degradation of Numb (Armbruester et al. 2004). However, it is important to note that the mechanism through which these accessory proteins act is still elusive and that both are expressed in various types of human tissue cells, indicating that their expression is not restricted to disease states (Schmitt et al. 2015).

## **Melanoma**

HERV-K expression has long been associated with melanoma, which has the highest mortality among skin cancers. Studies demonstrated that UV radiation, the most established environmental risk associated with melanoma occurrence, leads to specific HERV-K activation (Schanab et al. 2011), and increased levels of HERV-K particles and associated antibodies and T-lymphocytes could be detected in melanoma patients (Stengel et al. 2010). Despite efforts to characterize HERV-K's role in melanoma progression, it was uncertain if the provirus actively participated in tumorigenesis and concealment from immune surveillance or it was activated as a byproduct of malignant transformation (Schmitt et al. 2013).

However, recent studies have implicated that malignant melanoma cells with CD133+ stemness features require HERV-K to induce the phenotype switch (Argaw-Denboba et al. 2017), adding to the evidence that HERV-K has direct oncogenic activity. In melanoma, tumors are comprised of cells that show distinct morphological and phenotypic profiles, and are therefore referred to as heterogeneous tumors. Different intra- and extra-cellular markers can be used to target and characterize cells that show high self-renewal capacity and tumorigenic potential within the population of cancerous cells. Of the markers used to identify CSCs, studies have suggested that those supporting CD133 positive (CD133+) features had particularly troublesome capabilities. Due to a CD133+ cell's association with enhancement of tumor initiation and metastasis,

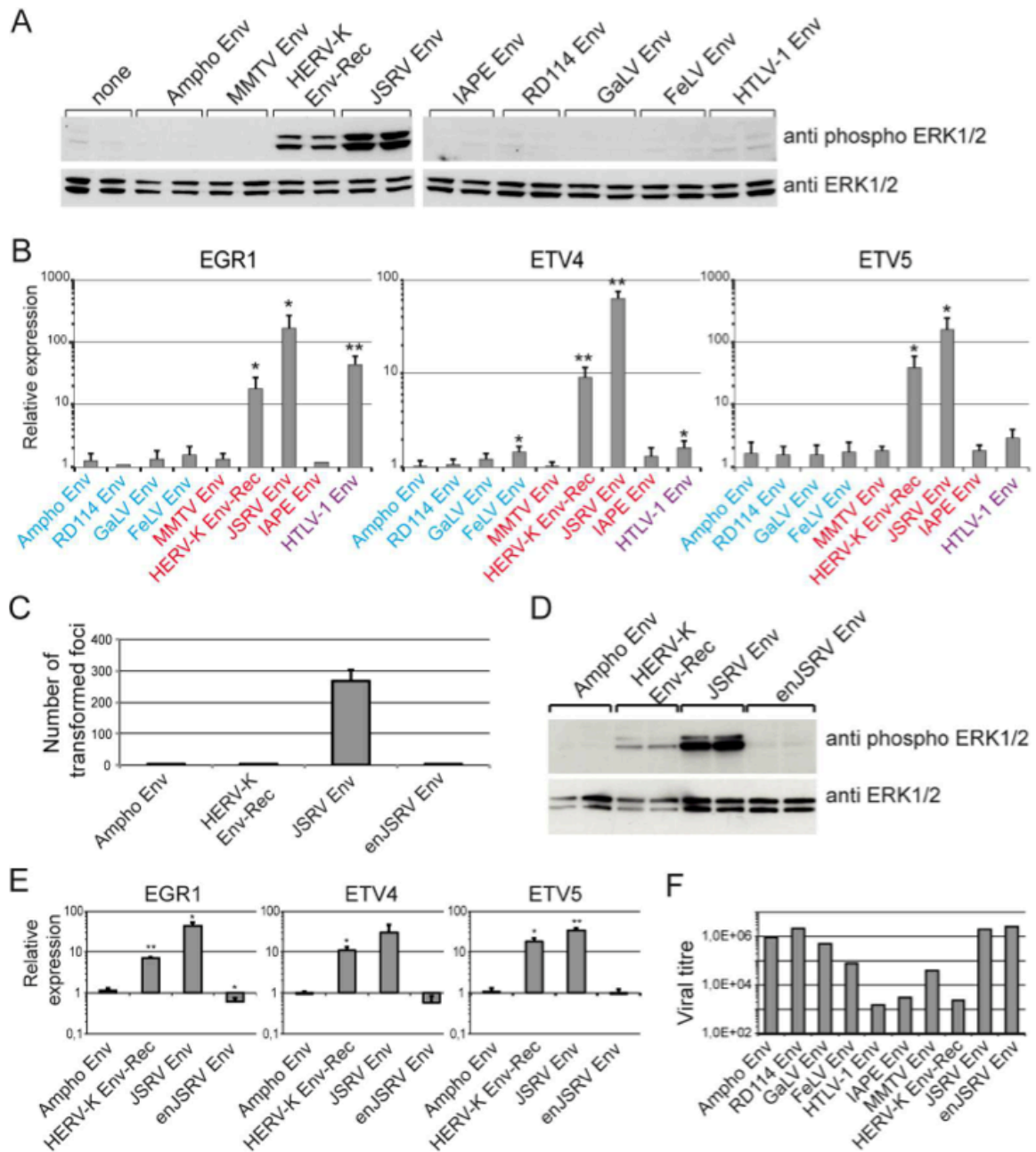
inhibition of apoptosis, and resistance to chemotherapy, the CD133 cell marker is widely used to characterize and isolate melanoma CSCs in studies.

When comparing the ability for different melanoma cell lines to undergo phenotype switching that lead to the expression of CD133, only those with a significant increase in relative expression of HERV-K became CD133+ (Argaw-Denboba et al. 2017). Furthermore, analysis of relative composition of CD133+ cell lines where HERV-K expression was inhibited indicated a significant decrease in CD133+. Cell cultures demonstrated a visible reduction in cell growth in these HERV-K inhibited lines (Argaw-Denboba et al. 2017).

A possible mechanism through which HERV-K fosters CD133+ expression and emerging CSCs is via activation of the Ras-Raf-Mek (MAPK) Pathway. Notably, RAS mutations are the most common type of abnormality in protooncogenes in human tumors and protooncogenes were first discovered in retroviruses. Several studies have indicated that 80% of all cutaneous melanoma arise from irregular activation of this pathway (Wang and Qi 2013), and although cutaneous melanoma is not the most common result of the mutation, it is the most aggressive. A recent study had indicated that HERV-K Env activates the MAPK Pathway, via ERK1/2 phosphorylation and transcription factor expression (Tyagi et al. 2017). Phosphorylation leads to activation of kinase activity, which plays a major role in regulating cell division. Similar activation of kinases is seen in Jaagsiekte Sheep Retrovirus (JSRV), which has a strong oncogenic effect (De

las Heras et al. 2006). However, this ability not observed in any other retroviral Env, as indicated in Figure 5.

Considering JSRV's oncogenic effect has been described previously, the parallels seen in between HERV-K and JSRV suggests that HERV-K modification of the MAPK pathway is a plausible means to oncogenesis (Tsang et al. 2017). Results from studies exploring HERV-K role in pancreatic cancer also demonstrated that downregulation of the HERV-K Env protein leads to decreased expression of MAPK intermediates (Li et al. 2017), strengthening the proposed mechanism through which HERV-K initiates cancer.



**Figure 5: Oncogenic Properties of Different Retroviral Envs.** (A) Shows ability for various retroviral Envs to phosphorylate ERK1/2. (B) Expression of transcription factors EGR1, ETV4, and ETV5 following transcription of Env particles. (C) Transforming activity of Ampho, HERV-K, JSRV, and enJSRV. (D) Phosphorylation of ERK 1/2 in cells expressing Ampho, HERV-K, JSRV, and enJSRV. (E) Expression of transcription factors EGR1, ETV4, and ETV5 following transcription of Env particles. (F) Levels of viral titre of Env particles (Tsang et al. 2017).

## **Breast Cancer**

Of all of the cancers relevant to HERV-K, breast cancer is the most common and the leading cause of cancer death in woman worldwide. In breast cancer there are four intrinsic subtypes determined by The Cancer Genome Atlas Network: basal, Her2E, Lum A, and Lum B (Johanning et al. 2017). Like most cancers, the HERV-K is overexpressed in breast cancer in several studies despite epigenetic silencing of HERV-K in most healthy tissues, but the subtypes that demonstrated this elevation were not identified (Johanning et al. 2017). To resolve if one or more subtypes showed increase in HERV-K expression, Johanning et al measured the levels of expression of 4 different HERV-K loci in each of the breast cancer subtypes. Interestingly, their research demonstrated that the greatest levels of HERV-K expression is seen in the basal subtype, which is notably the most aggressive of the breast cancers. This postulates the aggressive behavior of the basal breast cancers cells may be driven by the difference in expression of HERV-K, as serum HERV-K levels are strongly indicative of metastatic potential at the time of breast cancer diagnosis (Wang-Johanning et al. 2014).

In their experiment, HERV-K108, HERV-K109, HERV-K113, and HERV-K115 were selected because these loci were capable of creating infectious viral particles capable of integrating additional HERV-K sequences in neighboring cells and can be found on different chromosomes throughout the genome. Because it is unlikely for HERV-K transcription to be limited to one chromosome,

the variation in chromosome location provides a more representative response of transcriptional events in the human genome. Interestingly, regardless of the locus, the basal subtype consistently expressed higher levels of HERV-K transcripts when compared to the other three (Figure 6) (Johanning et al. 2017).

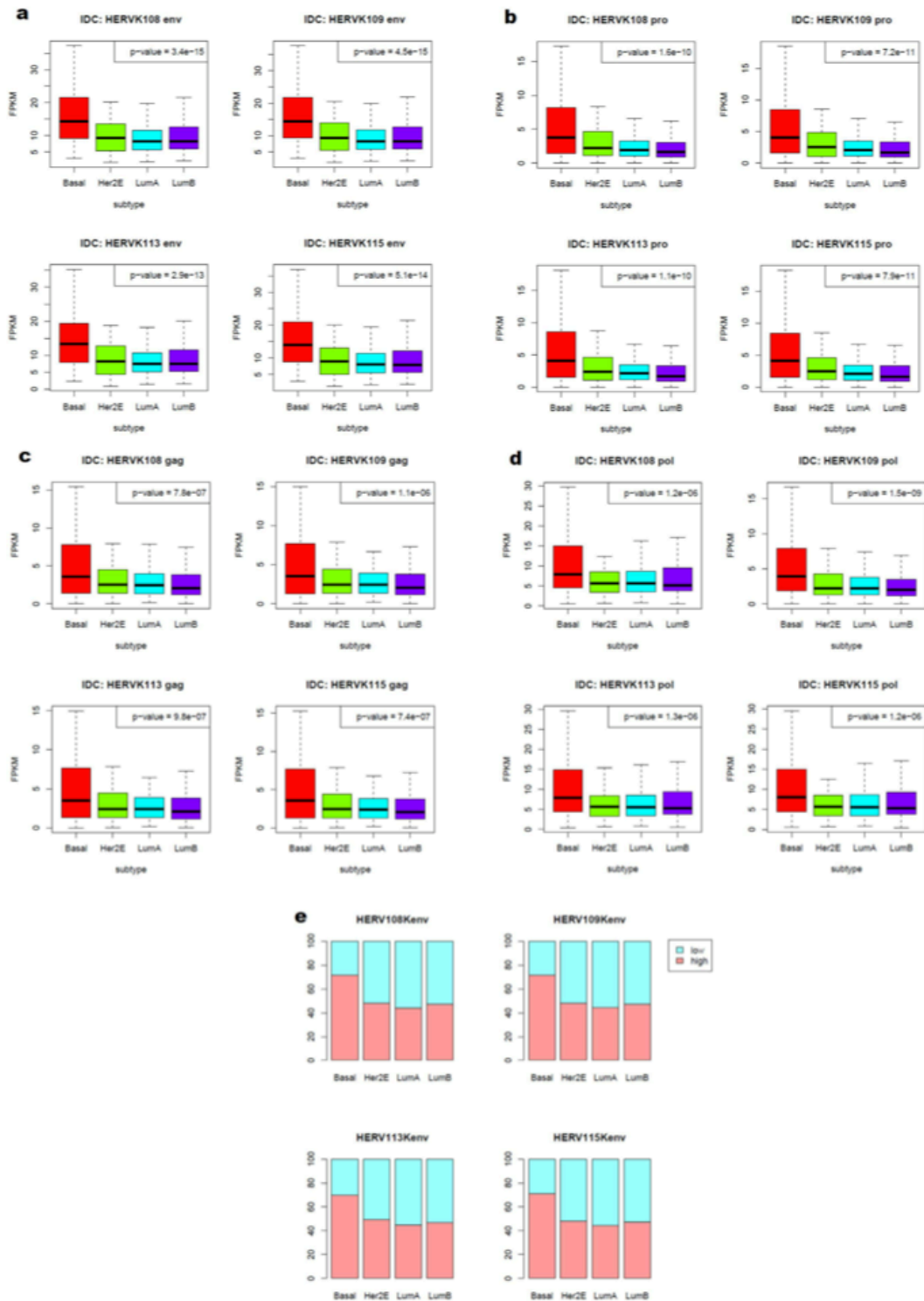


Figure 6: Levels of HERV-K Expression in BC Patient Tumors. Expression of

HERV-K **(a)** env, **(b)** pro, **(c)** gag, and **(d)** pol transcripts were from different loci (HERV-K108, K109, K113, and K115) in BC tumors was evaluated. **(e)** Percentage of cells expressing higher or lower than average levels of Env proteins depending on BC subtype (Johanning et al. 2017).

This finding, consistent through each combination of subtype and HERV-transcript, implies that the basal subtype has additional mechanisms that lead to upregulation and increased integration of HERV-K sequences (Johanning et al. 2017). Additionally, although the increase was significant throughout each HERV-K gene, the Env transcript had smaller p-values by several orders of magnitude, and further studies clarifying the different roles of HERV-K transcripts and products should be initiated.

Contributions of HERV-K Env protein to breast cancer development have also been explored. In vivo studies have witnessed a decrease in breast tumor size and weight in mouse xenografts of 3 different breast cancer (BC) cell lines transduced with a HERV-K inhibiting or control vector (Zhou et al. 2015). Significantly greater expression of HERV-K was observed in mice infected with the control vector, conserving the plausibility that tumor progression is attributable to heightened levels of HERV-K env. Long term cultures of the same cells yielded similar results; there was a significant reduction in the size and number of colonies in the BC cell lines that were not able to effectively produce HERV-K products (Zhou et al. 2016). Using the Ingenuity Pathway Analysis program, a comprehensive analysis tool that identifies relevant signaling and metabolic pathways associated with certain levels of expressed genes, HERV-K

knockdowns show a decrease in epidermal growth factor receptor (EGFR) expression. EGFR contains downstream pathways that regulate EMT, resulting in enhanced migration and metastatic abilities, and is usually overexpressed in BC cells. Reduction in vimentin and CK-19, characteristic mesenchymal and epithelial markers, respectively, upon an increase in HERV-K expression confirmed that the observed phenotype switch followed enhanced transcription.

Similar to the research seen with CD133+ melanoma cells, signaling changes in the MAPK pathway was observed after knockdown of HERV-K (Argaw-Denboba et al. 2017). Due to depression of Ras, p-ERK  $\frac{1}{2}$ , and p-RSK, all of which are players in the MAPK pathway, which have been proposed to play an important role in activation of HERV-K expression (Huang et al. 2013), knockdown cells showed less proliferative and migratory abilities.

Many of the studies investigating HERV-K's correlation and role in malignant disease have reached similar conclusions: increased production of retroviral particles leads to malignant disease via interruption of the MAPK pathway and induction of EMT. The shared result makes HERV-K products a promising target for cancer pharmaceuticals and is certainly worth investigating.

The next part of the paper will explore the possible applications of antiretroviral drugs that may depress expression of HERV-K transcripts.

## **ANTIRETROVIRAL DRUGS**

Antiretroviral Therapy (ART) is the use of any of several classes of antiretroviral agents that act to inhibit different stages of the viral life cycle. While these therapeutics will not directly kill viruses, inhibition of vital process can slow down rate of infection. Currently, there are 26 FDA-approved antiretroviral drugs, which function via inhibition of proteins crucial to the retroviral replication cycle. Depending on which protein it targets, these drugs can be classified as entry (fusion) inhibitors, protease inhibitors, integrase (IN) inhibitors, or reverse transcriptase (RT) inhibitors, which is further broken down into nucleotide RT inhibitors (NRTIs) and non-nucleotide RT inhibitors (NNRTIs). These drugs are often used in combination to delay the progression of HIV infection, a practice more commonly known as highly active antiretroviral therapy (HAART).

### **Efficacy of ART in lowering HERV-K Expression**

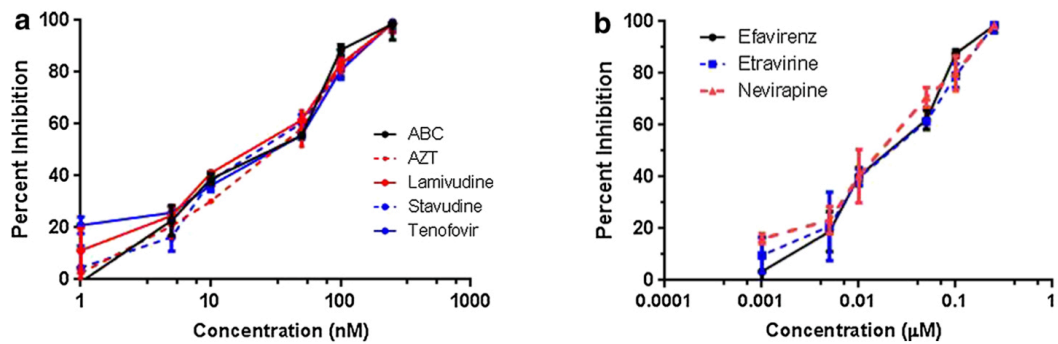
Entry inhibitors block targets relating to viral binding, fusion and entry into a host cell. However, because HERV's are endogenous elements and their infectious potential is not widely supported, this class of therapeutics is unlikely to exert a significant effect on the HERV-K lifecycle and research regarding this class's effect on HERV-K expression has not been conducted.

Of the 3 proteins relevant to the HERV-K lifecycle, RT plays the most central role, making it the biggest target for pharmaceutical inhibition. RT

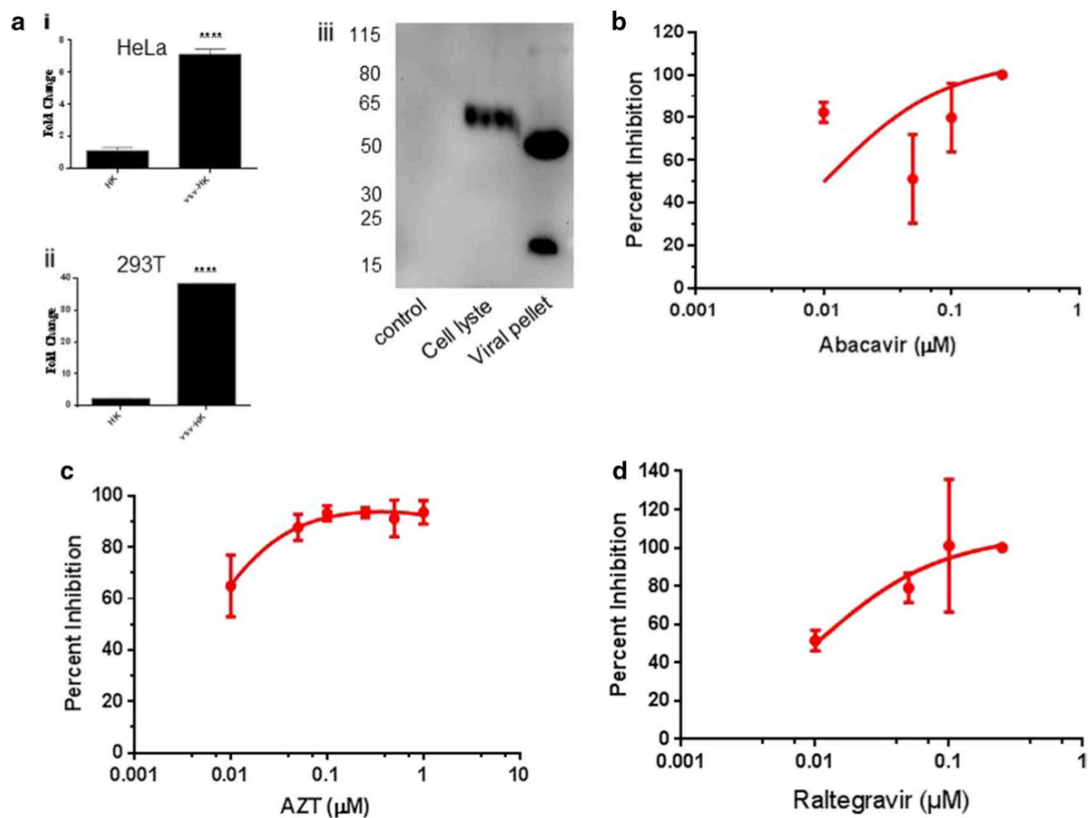
Inhibitors can be divided into 2 subgroups. NRTIs are structural analogues of the bases that make up genetic information. However, they lack a 3'OH, which is necessary to continue elongation of a DNA strand. Incorporation by RT into a growing DNA strand results in premature termination (Tyagi et al. 2017).

On the other hand, NNRTIs, as indicated in their name, are not analogous in structure to nucleotides, and will, therefore, not act to terminate a growing DNA sequence. Instead, NNRTIs bind to the NNTRI binding pocket, found near the polymerase active site of the RT, effectively preventing the alignment of the 3' end with the polymerase responsible for extension. In other words, the conformational shift caused by NNTRI interaction diminished the efficiency and ability of the RT itself (Argaw-Denboba et al. 2017).

When attempting to characterize the effect of FDA-approved antiretroviral drugs on HERV-K expression and products, Tyagi et al. found that nucleotide RT inhibitors (NRTI) and non-nucleotide RT inhibitors (NNRTI) lead to a significant dose-dependent inhibition of HERV-K RT activity (Figure 7; Tyagi et al. 2017)). The efficacy of these FDA-approved HIV medications also was reflected in similar IC90 values, indicating that the necessary dose to show the desired effect would be safe in the human population. Concomitantly, a decrease in overall HERV-K expression was observed, as measured by relative expression of HERV-K gag (Figure 8).



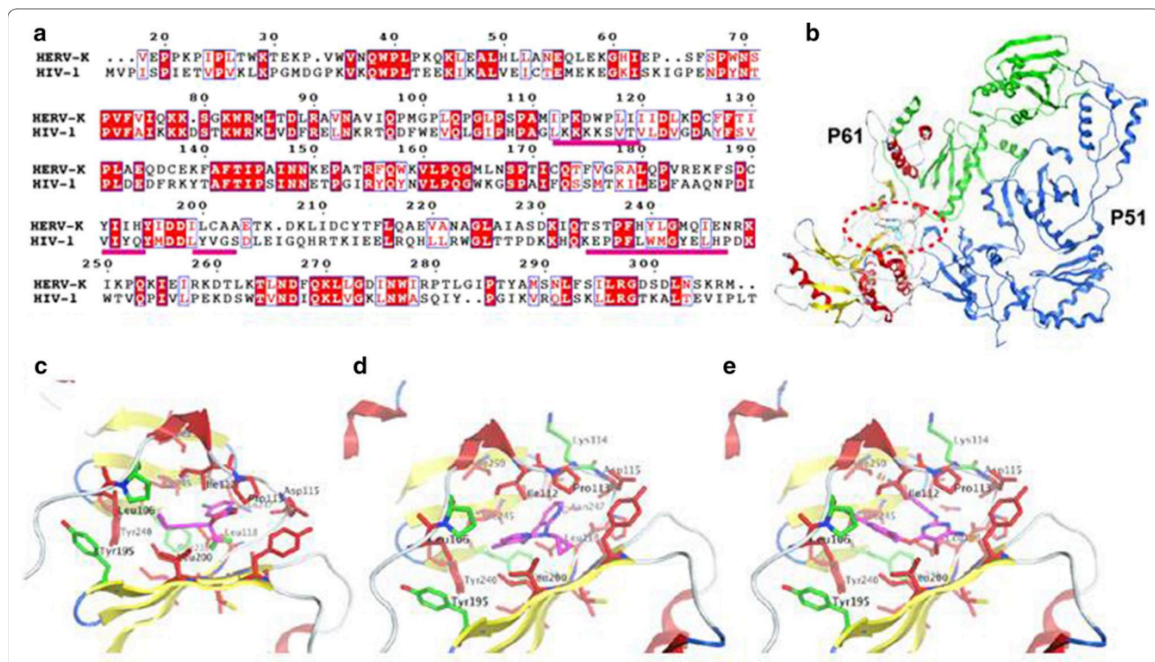
**Figure 7: Reverse Transcriptase Inhibitors reduce activity of HERV-K RT.** The percent inhibition of HERV-K RT compared to no treatment of (a) Nucleoside RT Inhibitors and (b) Non-nucleotide RT Inhibitors was measured. The control did not receive any treatment and is assumed to have 0% inhibition at all concentrations (Tyagi et al. 2017).



**Figure 8: HERV-K Expression is Effectively Inhibited by Different Classes of Antiretroviral Drugs.** (a) VSV-G pseudotyped particles facilitated more

successful HERV-K infection and increased expression by several fold. **(b-d)** HERV-K infected cells showed inhibition of proviral expression upon administration of **(b)** Abacavir, a NRTI, **(c)** AZT, a NNRTI, and **(d)** Raltegravir, an IN inhibitor (Tyagi et al. 2017)

The ability of RT inhibition by HIV-1 RT inhibitors is likely attributed to the fact that RT derived from a consensus HERV-K sequence has a 25% sequence homology with HIV-1 RT, as demonstrated through a sequence alignment. Comparative modeling using HIV-1 RT complexing with Efavirenz, Nevirapine, and Etravirine as a template suggests that NNRTIs bind to HERV-K RT in a hydrophobic cavity at an allosteric site (Tyagi et al. 2017). The model, shown in Figure 9, shows an inconsistent composition of amino acids in the NNRTI-binding pocket, but despite the fact that not all the components of the binding are conserved, small NNRTIs (Efavirenz and Nevirapine) were still able to bind. Etravirine, which is much larger molecular compound, showed signs of steric hindrance. Although small adjustments to amino acid composition eventually allowed docking of Etravirine, it would seem that future therapeutic attempts at creating NNRTIs for HERV-K RT inhibition should avoid bulkier molecules.



**Figure 9 Comparative modeling of HERV-K RT and HIV RT. (a)** Sequencing alignment of HERV-K and HIV RT. **(b)** Final Model of HERV-K RT showing P51 and P66 subunits. The *red oval* indicates the NNRTI-binding region. **(c–e)** Efavirenz, Nevirapine, and Etravirine, respectively bound to the NNRTI-binding pocket. Non conserved residues are colored *red*, *green* the conserved between HERV-K and HIV-1 RT. The NNRTIs are *colored* with their carbon atom in *magenta*. The *magenta underlined* sequence are residues lining the NNRTI-binding pocket (Tyagi et al.).

When viral sequences are translated from mRNA, the resulting polypeptide sequence contains several individual proteins that are unable to function until they are separated from their genetic neighbors. This is the function of viral proteases: to cut the protein chain into individual enzymes, allowing gene products to act and exhibit their intended effect. HERV-K protease is encoded in the pol gene of the HERV-K sequence. Although it only shares a 28% amino acid consensus with HIV-protease, HERV-K protease contains many of the same active domains and a signature motif of Asp-Thr-Asp when compared to HIV

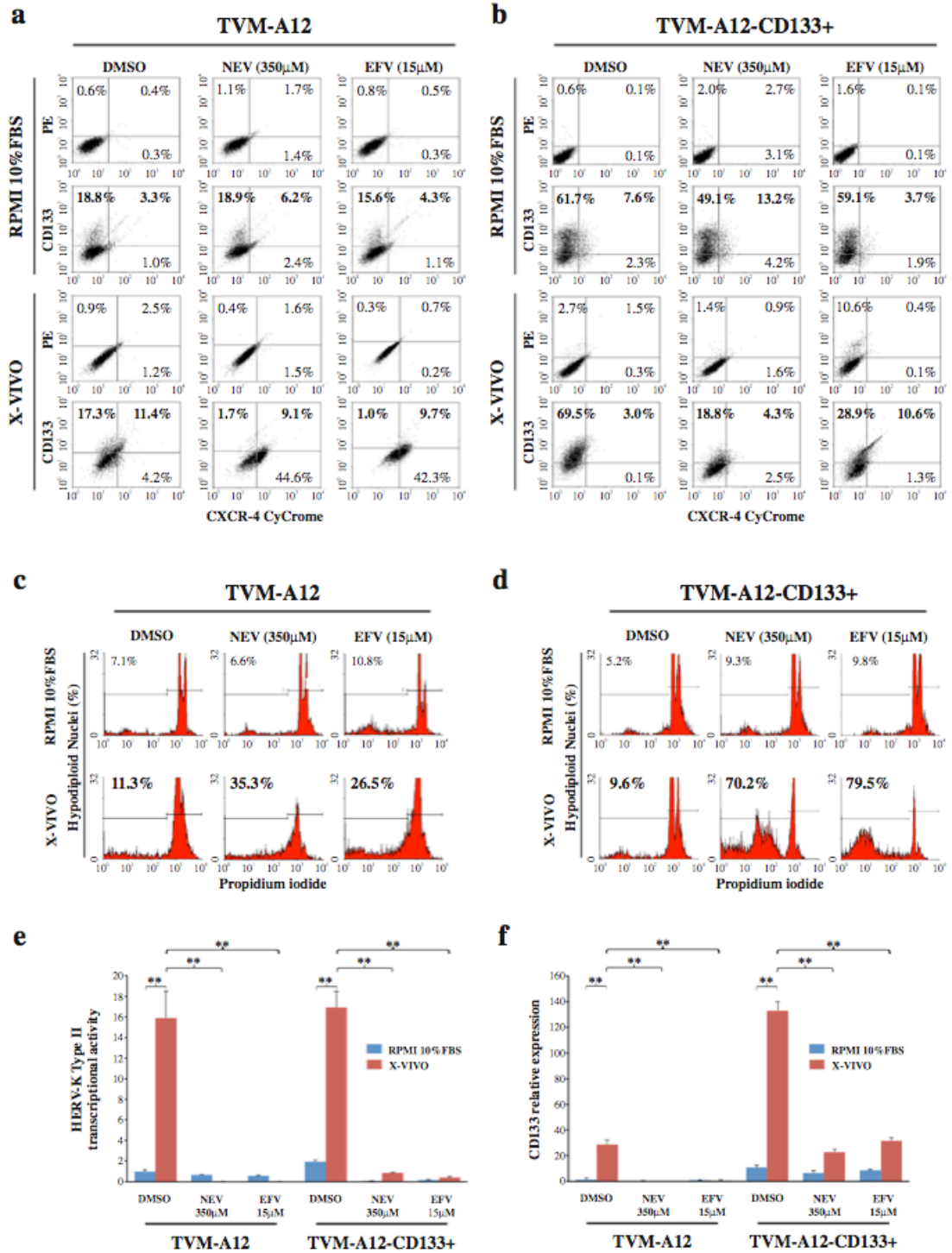
protease (Tyagi et al. 2017). These shared domains provide a plausible mechanism through which HIV protease inhibitors could depress HERV-K protease function. Tyagi et al demonstrated that all protease inhibitors were capable of significantly inhibiting the production of functional HERV-K proteins in a dose dependent manner, shown by the absence of mature HERV-K proteins in cell lysates and viral particles (Tyagi et al. 2017). Ultimately, HERV-K replication was unable to proceed in the presence of protease inhibitors. While HIV-protease inhibitors were able to significantly inhibit the functional abilities of HERV-K protease, the IC<sub>90</sub> of the most effective inhibitors still was 20-50 times higher than that needed for HIV protease inhibition. This staggering IC<sub>90</sub> poses a health concern for patients: the concentration of inhibitor needed to be effective may approach a lethal concentration. The results indicate that the currently available array of protease inhibitors is not appropriate for HERV-K protease inhibition, but antiretroviral therapy for HERV-K could still be effective if a more specific and potent inhibitor for HERV-K was developed.

The effects of integrase (IN) inhibitors on HERV-K function have also been explored. IN is another enzyme vital to the retroviral life cycle. Like protease, it is coded in the pol gene, but functions to integrate cDNA into the host genome (Hare et al. 2010). Comparative modeling demonstrated that the active sites of HERV-K IN and prototype foamy virus (PFV) IN are nearly identical; the only noted difference was substitution of a proline with a serine residue (Tyagi et al. 2017). Because PFV IN can be effectively inhibited by Raltegravir, one of the 3

IN inhibitors that are FDA approved, it is not surprising that the drug was also capable of inhibiting HERV-K replication (Hare et al. 2010).

### **Efficacy of lowering oncogenic properties in cancer cell lines**

While more studies are needed to analyze the effect of antiretroviral drugs on cancerous cells, the few studies that have been published have gathered evidence supporting the effectiveness of these drugs against malignant disease progression. Previously, two NNTRI's used to treat HIV, Nevirapine and Efavirenz, were shown to be effective at reducing proliferation and inducing differentiation of TVM-A12 melanoma cell lines (Sciamanna et al. 2005), but follow up studies have also identified that these NNTRIs were also effective in inducing apoptosis of CD133+ melanoma cells (Argaw-Denboba et al. 2017). In an *ex vivo* medium, where increased HERV-K expression is usually observed along with an increased percentage of CD133+ expressing cells, cell lines subject to Nevirapine and Efavirenz were unable to activate HERV-K expression and showed a parallel decrease in CD133+ expression (Argaw-Denboba et al. 2017, 13). The data show in Figure 10 provides evidence that the endogenous transition to cells with enhanced self-renewal, metastatic, and aggressive characteristics can potentially be halted using existing anti-retroviral drugs.



**Figure 10: NNRTIs are effective to restrain HERV-K activation and induce apoptosis in TVM-A12 CD133+ cells. Effects of NNRTIs on the expansion and**

maintenance of CD133+ cells in TVM-A12 **(a)** and TVM-A12-CD133+ **(b)** cell lines analyzed by flow cytometry analysis. Effects of NNRTIs on apoptosis levels in TVM-A12 **(c)** and TVM-A12-CD133+ **(d)** analyzed by flow cytometry analysis after nuclei staining with propidium iodide. Relative mRNA expression of HERV-K Env gene **(e)** and CD133 **(f)** analyzed by Real-time PCR. Data represent the results of three independent experiments (Argaw-Denboba et al. 2017, 13).

## **CONCLUSION AND FUTURE RESEARCH DIRECTIONS**

Although previously written off as “junk DNA,” some HERVs maintain the ability to produce functional products despite major inactivation by post-insertion mutations and silencing by epigenetic factors. Increased HERV-K expression is associated with age, but can also be mediated through several cell signaling pathways and affected by the presence of other viral particles. Consequently, increased levels of HERV-K proviral activity is hypothesized by several studies to lead to the development of cancer. The notion to use antiretroviral drugs to combat cancer progression is a fairly novel idea, and as a consequence much more research needs to be conducted before drug development becomes reasonable. As of now, research has identified that HERV-K proteins are highly sensitive to NRTIs, NNRTIs, and IN inhibitors, and these therapeutics can be administered to effectively decrease the activity of HERV-K products. While protease inhibitors were also effective, the doses required to observe the desired effect were too large to be safe and additional research would be necessary to develop a protease inhibitor more specific to HERV-K protease. In relation to antiretroviral drugs on malignant disease progression, NNRTIs were shown to be

extremely effective in inducing apoptosis in melanoma cells with CSC features by reducing their proviral expression. In addition to being much more affordable than classic chemotherapy treatments, antiretroviral drugs also provide alternative treatments for other pathologies associated with HERV-K expression outside the scope of cancer. The results and additional benefits suggest that HERV-K can serve as a promising new target in pathologies not limited to cancer. Developing more HERV-K specific therapies can increase potency and efficacy of antiretroviral compounds, but future research should continue focus on the exact mechanism of HERV-K mediated pathogenesis to clarify and confirm the proposed association.

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## CURRICULUM VITAE

