Cervical cancer: An unanticipated consequence of high-risk human papillomavirus infection

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Abstract

Cancer is not a single story, but rather numerous often interwoven tales, each with its own characters and progression. In the case of human papillomavirus (HPV) induced cervical cancer (CaCx), the narrative is about the relationship between virus and host, with the consequences of evolution's shortsightedness driving the plot. Along with the increased proliferative state characteristic of cancer, cells experience frequent, inaccurate replication and replication stresses (ex. DNA damage and nucleotide starvation). To prevent replication fork stall and collapse generated by these stresses, the cell employs translesion synthesis (TLS). Notably, most of the genes in this pathway are upregulated in CaCx; however, the key protein polymerase eta is not. We have observed that upregulation in this pathway is complicated. It occurs at numerous levels, increasing both mRNA and protein abundance. This research further dissects how TLS upregulation occurs. Data shows that in CaCx-derived cell lines, the stability of some TLS proteins is increased, while the stability of other TLS proteins is unchanged. The increased proliferation, typical of these cell lines, cannot account for the enhanced stability. Despite increased TLS protein stability, these cells fail to adequately activate TLS increasing the risk of DNA damage. Genomic instability is a driving factor in HPV genome integration that prevents viral propagation and leads to cell transformation. It also raises mutagenesis rates, likely creating a selective pressure for tolerating failed TLS. The elevated mutation rate known to be associated with failed TLS could also provide a mechanism for acquired resistance to the drugs commonly used to treat CaCx. Changes in protein abundance are routinely used as biomarkers that can lead to the improved outcomes associated with early cancer detection. Elevated TLS protein could be leveraged to ensure cervical cancers are detected during Stage 1, when the 5-year survival rate is 80-90%, rather than at Stage IV, when the rate dips to around 15%

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Chapter 1 - Defining Cervical Cancer in an Evolutionary Context

In biology, as in many other fields, the dilemma of efficient communication of complex ideas has widespread consequences, the repercussions of which cancer is not immune to. Many conventional definitions of cancer exist throughout the biological community. Many of those definitions, to cover the broad spectrum of diseases classified as cancer, fail to encapsulate the stories of those cancers in their entirety by inadequately delving into their intricacies. The story of cancer is not a story, but rather numerous stories, each with its own characters and progression. In the case of human papillomavirus (HPV)-induced cervical cancers, the story is about the relationship between viruses and hosts, with the consequences of evolution's shortsightedness driving the narrative.

Although at first, it might seem counter-intuitive that any cancer might be caused by evolutionary processes, considering that cancer seems to defy the key aspects of evolution. One, evolution occurs at the level of a population and; two, carcinogenic traits should be selected against given their detrimental burden upon host survival. However, when waxing existential about the meaning of life, or at least reproduction, a frequently endorsed purpose is to pass on one's genetic information. A more accurate summary is that the purpose of existence is to pass on one's genetic information as accurately as possible, or inaccurately if it provides a benefit to the organism. This is where cancer subverts those seemingly essential characteristics of evolution. Cancer, despite occurring at the organismal level, does affect the population level within the context of cells. Cancer's characteristic increased, abnormal proliferation of cells is being selected for, providing a benefit to the cell, despite the cost to the host. Furthermore, in selecting for this phenotype, the long-term consequences of accurate genomic replication are not considered. These ramifications are often too downstream to be considered for selection until the

population eventually crashes due to the death of the organism. The inability to foresee the implications phenotypes have down the road is a staple of evolution. This “shortsightedness” is what frequently leads to “unintended”, or unanticipated consequences of evolution.

HPV- Epithelial Cell Interaction

To understand how the selection for increased fitness, in the form of propagation viability, drives the narrative of HPV-driven transformation, it is important to understand propagation as it occurs under the relevant contexts of HPV and cell interactions. The first context is the proliferation of healthy cells prior to HPV infection. The second context is the propagation of viral progeny and cells after HPV infection occurs. The final context is the transformation of cells and cessation of viral progeny production by HPV integration. All three of these contexts have selective pressures that need to be examined to understand how they contribute to the unanticipated consequence of cancer.

Uninfected epithelial cells

Understanding the context of HPV and cell interactions requires first understanding how HPV-infected cells typically proliferate. The target cells of HPV infection are solely the basal epithelial cells composing the inner layers of mucosal membranes lining the urogenital, anal, and oral tracts[1–4]. In the epithelium of the human body, division and differentiation are distinct processes that result in the allocation of cells to different sections of the epithelium. Basal epithelial cells either propagate to maintain the basal epithelial cell population or differentiate to contribute to the cells belonging to the cellular populations within the different layers of the stratified epithelium[1,5]. In stratified epithelium, there is a gradient in which eventual cessation of division and reliance on differentiation have a negative correlation. These cells shift towards focusing solely on differentiating to contribute to the cellular populations of the more apical

layers of epithelium, of which the most apical layer consists of degraded cells that are eventually shed[5,6]. Cells within the suprabasal population are regenerated when some of the basal layer cells, differentiate, allowing the suprabasal layer to continue the regeneration of the other layers[1,5,6] (Fig. 1). In short, this allocation of functions to different layers of epithelium means that HPV, although it solely infects basal epithelial cells, resides in cells within the layers of stratified epithelium as basal epithelial cells divide vertically and it allocates replicated genomes to the nascent cells. As a consequence of these differentiating cells foregoing division, they also consequentially, for HPV, reduce the percentage of S phase competent cells within those populations[1,2]. Alternatively, cells within these layers eventually die and are shed, thus warranting less monitoring by the immune system. This reduction in immune surveillance and response creates an environment in which viruses can replicate with reduced risk[7].

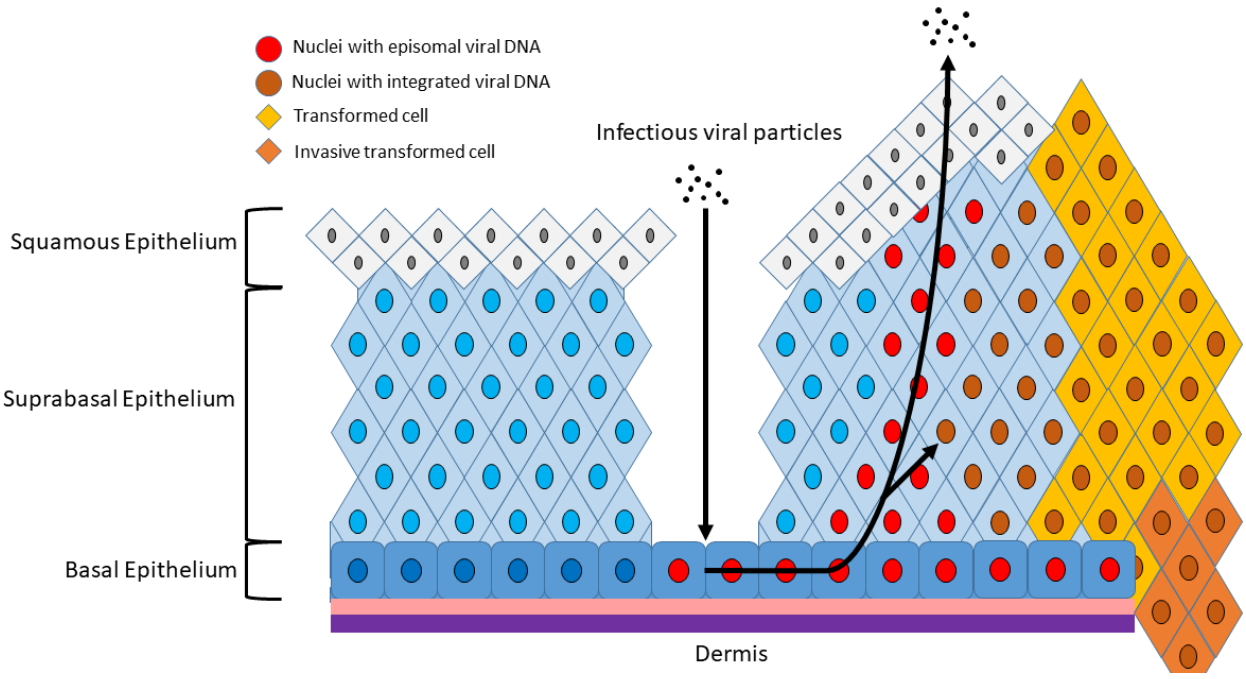


Figure 1 Epithelial cell proliferation in contexts of HPV interaction. Schematic of the proliferation and differentiation of epithelial cells in uninfected, infected, integrated, transformed, and invasive transformed HPV contexts. The branched arrow points to either viral release or oncogenic integration of the viral genome.

HPV-infected epithelial cells

The second context of HPV-epithelial cell interactions is initiated after the infection of an exposed basal epithelial cell by HPV via an abrasion in the mucosal membrane[4]. The normal viral propagation cycle of HPV is divided into five stages, the first of which was the previously described process of infection[1,2]. The other four stages are genome maintenance, genome amplification, viral packaging, and viral release[1,2]. These stages of the viral propagation cycle proceed as the cell in which the virus resides transitions to the apical layers of the epithelium[1,2,5,6]. Genome maintenance occurs in the basal epithelial layer as the infected basal epithelial cell proliferates horizontally to maintain the population of cells within that layer[2,6]. During this stage, the virus replicates its genome at 2n (two times the original amount) to ensure that it can allocate replicated viral genome to newly propagated cells within the layer of basal epithelium and maintain approximately fifty to one-hundred genome copies per cell[1,2,5,6]. During this time, the virus also expresses the E6, E7 (Early 6 and Early 7, named so as they are expressed early during the propagation cycle) oncogenes at relatively low levels[8–12]. Low levels of viral gene expression, in combination with the low levels of viral genomes and replication, aid in avoidance of immune detection and a subsequent immune response[7]. When an infected basal epithelial cell divides vertically, the viral propagation cycle transitions to the next stage.

The genome amplification stage of the HPV viral propagation cycle is initiated upon vertical proliferation of basal epithelial cells and persists in cells occupying the lower layers of stratified epithelium as cells differentiate[1,2]. During this phase of the cycle, HPV steers the cells away from their normal context of proliferation, wherein cells experience a gradient of shifting away from proliferation and towards differentiation[1,2,5,6] (Fig. 1,2). This graded alteration occurs when moving towards the apical layers of tissue, towards a more proliferation focused context.

Increased expression of the viral oncogenes, as well as other viral genes, no longer risk the same threat of immune detection and response as it would typically in the basal layers[7]. Thus, HPV experiences a shift in what pressures are acting upon its propagation, transitioning from the pressure to avoid host defenses to one in which it needs to change the normal conditions of the cell to facilitate increased genome replication, avoid cell cycle arrest, and prevent induced cell death[8,9,11,12]. HPV handles these pressures via the action of the E6, E7 oncogenes, which will be discussed in greater detail later on. During this time, HPV proteins participate in the activation of DNA damage repair (DDR) pathways and recruitment of proteins involved in those pathways for the purposes of viral replication[13–15]. At this phase of propagation, the HPV genome is drastically increased in comparison to the genome maintenance stage[1,2]. As a result, the number of HPV genome copies per cell increases while in this stage. Both the genome copy number and amount of replication continue to rise as the cells progress to the upper layers of stratified epithelium as part of this phase[1,2] (Fig. 2). However, as the cells progress to the uppermost layers of epithelium HPV enters the next stage of the propagation cycle.

Before the final stage of the HPV propagation cycle, when viral progeny release, the viruses must first be packaged. When infected cells reach the upper layers of epithelium HPV begins expressing the L1 and L2 proteins (Late 1 and Late 2, named so as they are expressed late within the propagation cycle)[1,2,6]. These two proteins comprise the viral capsid. After the production of the late genes, the capsid is formed around copies of the HPV genome. This stage of the propagation cycle is termed viral packaging[1,2]. This phase, similarly to the genome amplification stage, is initiated by changes in gene expression characteristic of cellular differentiation signaling to the virus to transition to the appropriate stage[1,2,5] (Fig. 2). After the virus is packaged it is eventually released as cells of the apex layers of the mucosal membrane

are naturally sloughed off and the membranes are degraded allowing the virus access to the environment and potential infection of other cells[1,2,5] (Fig. 1). Utilizing this approach to viral release is another mechanism by which the virus tolerates the pressure of immune detection and response as it avoids the necessity of apoptosis or cell lysis to release progeny[7].

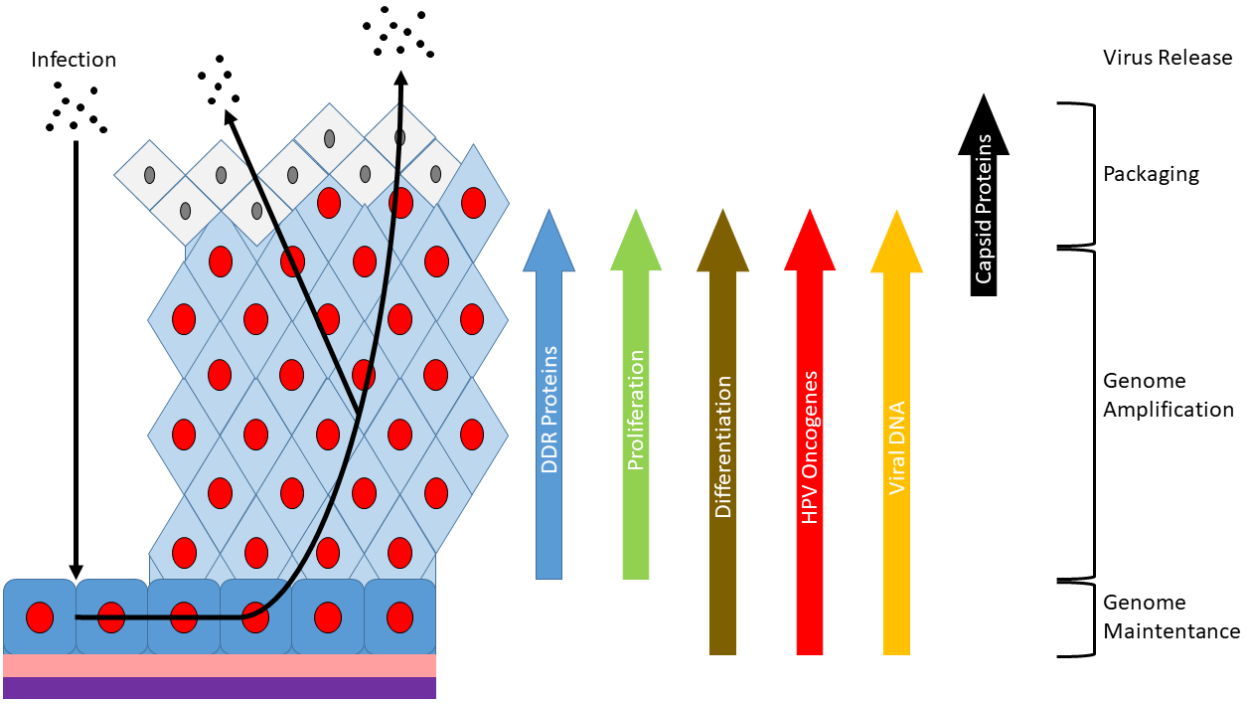


Figure 2 HPV gene expression and epithelial cell interaction. Schematic showing expression of HPV genes in epithelial tissue and alterations to epithelial cell dynamics. Colors of the arrows are unrelated to colors on the diagram of tissue.

HPV-induced epithelial cell transformation

The previously detailed context of epithelial cell HPV interaction is an important consideration when reflecting on how to define a complex concept like cancer. More specifically, it is a prime example of how broad stroke definitions of cancer such as, "the abnormal proliferation" are not universally valid. Although the HPV-driven transition for these cells to a state of abnormal proliferation is not the norm for those cells, it is the typical, or normal, consequence of HPV infection. This "atypical" state is remedied upon HPV's potential,

eventual removal; thus, it is not permanent. Most HPV infections are either asymptomatic or result in a benign lesion, especially those of the low-risk (LR) HPVs-HPVs commonly associated with lesions such as warts[16]. Even infection by one of the high-risk (HR) HPVs, those associated with malignant lesions, infrequently results in the transformation of infected cells[17,18]. Despite being essentially the sole cause of cervical cancers (~99%) and a major contributor to anal and head/neck cancers, a minute percentage of HR HPV infections lead to cancer[19]. In the US alone, which experiences an approximate occurrence of fourteen million new HPV infections yearly, around fourteen thousand new cases of HPV related cancers are experienced[16–19]. Thus, HPV is not the story of cancer being defined as "abnormal cellular proliferation". Instead, it is the story of abnormal cellular proliferation, within the context of the HPV replication cycle, as will be detailed in the following paragraph.

During HPV's infection and residence inside epithelial cells, integration of the viral genome into the host genome can occur. These events initiated by breakage of both the host and viral genomes are not a natural event within the HPV propagation cycle[20–22]. The unintended, infrequent occurrence of HPV genome integration is not enough to lead to the transformation of cells by itself. There are four components of the HPV genome essential to an integration event leading to the transformation of epithelial cells which are as follows: E2, E6, and E7 genes, as well as the long-non-coding region (LCR)[23–25]. Arguably, the most important gene is E2, as breakage of the viral genome must occur in the sequence for E2 during an integration event to lead to transformation. The E2 gene has many functions, although the most relevant is the ability to bind to a promoter within the LCR that regulates expression of the E6, E7 oncogenes[23]. When a breakage occurs within the E2 gene sequence, it disrupts the protein's regulatory function. Upon linearization and subsequent integration, rampant expression of E6 and E7 occurs[20–23,26]

(Fig. 1). Understanding the role of these oncogenes helps elucidate how genomic instability is generated to increase the likelihood of integration events, how HPV shifts the role of epithelial cells away from differentiation and towards proliferation, and how cells with the properly integrated HPV genome (where E2 is disrupted) are transformed.

The main characteristics contributing to E6 and E7s' oncogenic potential are the differences in their targets between the E7, E6 genes of LR versus HR HPVs. There are hundreds of HPVs, with the LR HPVs only causing benign lesions such as genital warts[16]. The E7, E6 genes of HR HPVs, unlike the LR HPVs', target retinoblastoma protein (pRB) and tumor suppressor p53, respectively[16,24,25,27]. pRB serves as a G1/S checkpoint regulator, among other things, binding to E2F. E2F is a family of transcription factors involved in the activation of a number of genes involved in the transition to the S phase of the cell cycle[24,25]. When the cell has accumulated the appropriate resources, S phase disassociation of E2F from pRB occurs, allowing activation of their targets and transitioning the cell into replication. Upon HPV infection, and subsequent E7 expression, E7 interacts with members of the cullin family of proteins (with E7s from different HR HPVs interacting with different cullins)[28]. HR HPV type 16 E7, with assistance from cullin-2 (CUL2) of the cullin-2 complex, chaperones pRB to the complex[28]. The CUL2 complex, a ubiquitin ligase, causes disassociation of E2F from pRB, ubiquitination of pRB, and subsequent pRB proteasomal degradation[24,25,28]. Thus, whether the cell is unprepared to enter S phase or, like the stratified epithelium cells, would normally shift away from proliferation towards differentiation it will still progress into S phase. This unprepared entry into a cellular phase would normally result in apoptosis.

Although the S phase progression driven by E7's interaction with pRB would normally result in apoptosis the expression of HR HPV E6 prevents this from occurring. Normally in a

cell, p53 interacts with and is ubiquitinated by mouse double minute 2 homolog (MDM2). MDM2, the transcription which can be activated by p53, thus induces the proteasomal degradation of p53[29,30]. However, upon DNA damage or unwarranted S phase, the interaction between p53 and MDM2 ceases, allowing p53 to accumulate and act as a transcription factor. As a transcription factor, p53 activates genes involved in cell cycle arrest and apoptosis[29,30]. When HPV infects a cell and expresses E6, E6 interacts with E6 associated protein (E6AP), an E3 ubiquitin-protein ligase which ubiquitinates and causes proteasomal degradation of p53[27]. This degradation occurs even when there is DNA damage regardless of the cessation of p53-MDM2 interaction[27,29,30]. E6's interaction with p53 prevents the cell from adequately responding to irregular cell cycle progression and the resulting consequences, such as mutations and transformation.

These aforementioned effects of E6 and E7 not only shift cells towards a proliferative state but also increase genetic instability, a prerequisite for HPV-induced transformation[20,21]. By forcing the cells preemptively and uncharacteristically into S phase, they are ill prepared to replicate their genomes effectively and accurately. The prevention of cell cycle arrest and apoptosis avoidance means those same cells can persist and proliferate. This combined with the recruitment of DDR proteins for viral propagation result in depletion of resources for cellular genome maintenance and increase the occurrence of breakages within the host genome[13,14,31]. In other words, HPV via the mechanisms it employs for mediating pressures faced when propagating has created the potential for a circumstance where viral propagation ceases. For the cell, on the other hand, increased, dysregulated expression of E6 and E7 creates a cellular environment that tolerates genome alterations potentially leading to cellular transformation.

Indeed, E6 and E7 expression in human foreskin keratinocytes (HFKs), keratinocytes being the cells HPVs infect, is enough to lead to immortalization[23,26].

Ramifications of HPV-Induced Epithelial Cell Transformation

The unanticipated consequence of HPV oncogene expression is detrimental to the virus by preventing viral propagation through genome integration and poses an overwhelmingly fatal threat to the host. However, this consequence is one that is selected for at the level of the cellular population. The increased proliferative ability of cells with an E2 disrupted, integrated HPV genome provides those cells with an increased fitness[20,21]. Even with the consequences of irregular proliferation potentially resulting in unviable cells, the cancerous cells are still the predominantly observed phenotype as cells that are not viable are not present within the population. This selection for increased fitness of cells in the form of dysregulated, increased propagation is shortsighted since it will result in the death of the individual and subsequently all cellular populations comprising that individual and housing the virus. Although, integration and rampant HPV oncogene expression is not the only consequence of "shortsighted" selection.

An important aspect of genome replication and consequentially proliferation is accuracy. Factors such as ensuring the cell has an adequate abundance of cellular growth factors and proper resolution of DNA damage are important components of accurate genome replication. Both inadequate levels of key components of replication, such as nucleotides, and lesions such as thymine-thymine dimers serve as means of inhibiting progression of the replicative machinery[31,32]. Inhibition of the replicative machinery can lead sequentially to stalling of the replication fork, uncoupling of the helicase, generation of single-stranded DNA (ssDNA) stretches, replication fork collapse, chromosomal breakage, and cell death[31–34]. To avoid this sequence of events requires utilization of specialized replicative machinery.

Typically, the cell employs one of the replicative polymerases, such as polymerase delta, to add nucleotides to nascent strands of DNA. These polymerases have high fidelity since their specific active sites primarily interact efficiently with nucleotides typically found in the genome, as opposed to analogues generated through some sort of stress[35,36]. However, with their high specificity, they are unable to bypass obstacles such as DNA crosslinks and continue replication. As previously detailed, this results in uncoupling of the helicase, that can still proceed past the lesion and prevent the sequence of events following failed bypass[32,35,36]. In order to prevent deleterious, possibly lethal damage from this blockage, the cell employs translesion synthesis.

Translesion synthesis (TLS) is the process by which the cell switches out the normal replicative polymerase for a TLS polymerase, such as polymerase eta. These polymerases have lower fidelities than the replicative polymerases resulting in less accurately replicated genomes[35,36]. Having a lower fidelity also allows for efficient bypass of a lesion[32,35]. Then, after the lesion has been bypassed the replicative polymerase can be switched back with the TLS polymerase and normal replication can proceed. Efficient employment of TLS is thus a critical aspect of ensuring a replicating cell's survival.

Cancerous cells with their increased proliferation would theoretically be required to employ more TLS, as with increased synthesis of DNA cells would encounter damage and resource limitation during S phase with higher frequency. In short, cancerous cells should require increased, efficient employment of TLS to accommodate increased uncoupling of the helicase. Failure to do so would result in cellular unviability. This is where the second consequence of shortsighted selection becomes relevant to the story of HPV induced cervical cancer.

As will be discussed in the later chapters of this thesis, it has been observed that HPV increases the abundance of TLS proteins. This increase in abundance is accounted for in a

number of different ways. Firstly, gene expression changes occur at various levels such as mRNA abundance, protein levels, and protein stability[13]. Secondly, TLS protein abundance changes are not accounted for in a ubiquitous manner. Different proteins within the TLS pathway have their increased abundance accounted for by either multiple or singular levels of gene expression[13]. However, one notable protein whose abundance is not altered is polymerase eta. This key component of the TLS pathway and the only TLS polymerase with a link to oncogenesis does not showcase increased mRNA or protein levels. The typical response to stresses such as increased levels of DNA replication is the induction of polymerase eta. Failed induction of pol-eta, failure of observed cervical cell lines to form polymerase eta foci at stalled replicative machinery, and increased cellular sensitivity to genotoxic agents is indicative of an inability of these cells to reliably account for DNA damage and stress (Fig. 3).

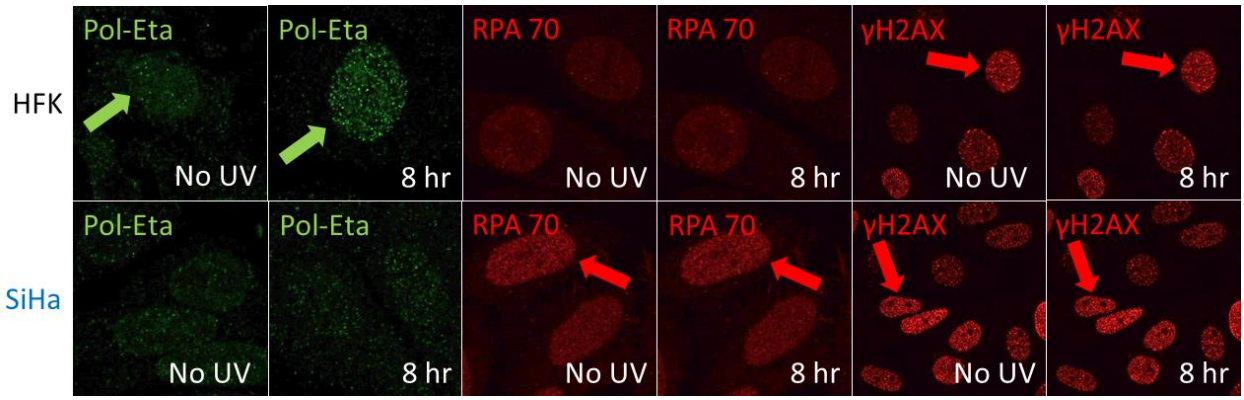


Figure 3 Failed translesion synthesis activation in cervical cancer cell lines. Results generated by Dr. Sebastian Wendel (Dr. Nicholas Wallace's Lab-Kansas State University). Immunofluorescence microscopy showcasing foci formation of TLS and TLS-related proteins in HFK and SiHa cella 4 and 8 hours post UV treatment. RPA70 foci are a biomarker of an uncoupled helicase, γH2AX is a biomarker of DSBs. Arrows point to representative cells with foci of the indicated protein. Cell line names are color coded to match color coding for other experiments.

Chapter 2 - Methods for Studying Cancer

In association with the behemoth task of adequately defining the diverse spectrum of diseases that fall under the term cancer is the equally foreboding task of studying those diseases. To define cancer requires an understanding of what cancer, within the context of each disease, is and entails a scientific examination for a proper dissection. This dissection requires the employment of individuals whose expertise draw from various fields including biology, chemistry, biochemistry, medicine, bioinformatics, and biostatistics. Each one of these disciplines provides an integral component of information which contributes to advancing our knowledge of the enigma that is cancer. Thus, the story of cancer entails understanding processes that are polymerization of multiple fields.

Cell Models

One of the fundamental aspects to understand cancer and the processes involved are the model systems used. The systems for modeling and studying cancer are just as varied as the techniques utilized, ranging from the non-cancerous cellular level, 3d cellular models of cancers, live tumor samples, and many other systems that fall within the spectrum of cellular transformation. In the case of cervical cancer, and other HPV associated cancers, a unique system exists to study the stages in the transition from healthy cells to cancerous ones.

HPV negative models

HPV-associated transformation can be broadly categorized into four stages, which were analyzed in the previous chapter, that will be discussed here in the context of cellular models. The uninfected stage is best modeled using primary tissue (tissue samples taken directly from an organism) culture techniques, including isolation of keratinocytes. A frequently used tissue sample for keratinocyte isolation is human foreskins as they are a readily available and often

discarded source. Human foreskin keratinocytes (HFKs) serve as a control cell line to compare results in cell lines at other stages of HPV-associated transformation. Although foreskins are often readily available, isolating HFKs requires proper tissue technique to keep them alive, ensure they remain sterile, and since they are non-cancerous and do not survive indefinitely stocks must be consistently replenished.

Acute oncogene expression models

The next stage of transformation, acute HPV gene expression, is modeled by HFKs either expressing the HPV genome or the HPV oncogenes at relatively low levels. This models early HPV infection, prior to the buildup of genomic instability. Expressing the entire HPV genome models an environment where integration events have not occurred and thus E2's regulation of E6/E7 has not been disturbed. Expressing only the HPV oncogenes models a system where an integration event has occurred, with levels of oncogene expression controlled by the promoter from the vector. Utilizing a weak promoter, like a long-terminal repeat (LTR) promoter, ensures representation of an acute expression cellular environment. By modeling acute expression of HPV oncogenes within the cell events that might lead to alteration of the cellular environment, in the form of increased genomic stability, can be studied to understand the occurrence of integration events. The problem with modeling this particular stage via this system is that it can potentially prove problematic when trying to generate stable cell lines expressing either the HPV genome or oncogenes, as the efficiency of HFK transduction is frequently low. This obstacle is exasperated by the fact that this system, unless expressing both oncogenes in some form, is not sufficient to transform cells, thus this system is subject to the same necessity as uninfected HFKs of frequent regeneration.

Long-term acute oncogene expression models

When expressing both of the HPV oncogenes in HFKs, whether just E6/E7 or the entire HPV genome in a disrupted E2 integration context, over long periods of time the next stage of transformation can be modeled. Conjoint expression of E6 and E7 in HFKs has been verified as a sufficient mechanism to immortalize keratinocytes; however, this process takes time. Employment of high-passage oncogene-positive HFKs allows for an analysis of the long (on the scale of decades) transitionary process where the cellular environment is altered between HPV infection, HPV integration, and cellular transformation. One limitation for this system is the proper establishment of the stable cell lines and the time allotment required for maintaining the cells long enough for the transitionary phases to be observed. Other limitations are the acquisition of samples. Patient variation must be taken into account for different samples, with some samples potentially being unusable due to high melanocyte concentrations (melanocytes have a high tendency to contaminate samples). There is also a disparity in the frequency of circumcision, with the practice seeing a diminishment and illegalization in various countries. This shift in cultural practice has reduced availability of foreskin samples for HFK isolation. Luckily, as these cells are transformed by the activity of the oncogenes, they can be used to self-regenerate stocks.

Chronic oncogene expression models

The final stage of HPV transformation is the post-transformation stage, represented by cancer cell lines. In cervical cancer cell lines, SiHa and HeLa are the most prominent representatives of HPV-associated transformation. Other cell lines include C33A (HPV-), HT3 (HPV30), and CaSKI(HPV16). SiHa and HeLa contain E2 disrupted α HR HPV types 16/18 genomes, respectively. These two HPV types account for approximately 70% of HPV-associated

cervical cancers, with HPV-associated cervical cancers comprising approximately 99% of cervical cancer cases. Hence, SiHa and HeLa are two of the most frequently used cancer cell lines in the field. C33A are also commonly used and primarily serve as HPV negative control, due to being HPV negative. All these cervical cancer cell lines are easy to maintain, immortal, and allow for analysis of changes to the cellular environment as a byproduct of chronic E6/E7 expression over decades of time. In other words, these cells lines showcase the effects of transformation and high genomic instability, albeit in a context that does not account for three-dimensional cellular interactions.

Models of post-infection microenvironments

Understanding the model systems used to study a topic is an essential first step in understanding the research itself. The four aforementioned *in vitro* models of HPV-associated transformation are an integral component of the HPV field of study, although they are not the only systems used. These cells are so invaluable not only because they represent the transitions between normal to cancerous cells but are also the cells that HPV infects. Although not the cells HPV typically infects human foreskin fibroblasts (HFFs) serve as a control cell line without many of the limitations imposed on experiments when working with HFKs. HFFs grow in Dulbecco's modified eagle medium (DMEM), a media typically utilized for maintaining HeLa and SiHa. HFKs do not grow in DMEM and thus potentially introduce a variable in experiments concerned with altering the growth medium (ex. growth or starvation experiments). HFFs are also more resilient to different stresses, they have a longer lifespan and are more tolerant of enzymatic manipulation. HFFs are just as readily available as HFKs and more easily obtainable from human foreskin samples. HFFs are also often used as feeder cells in raft cultures, that assist in modeling post-infection and tumor microenvironments that are only recently being

understood. All these cell lines whether used for convenience or due to biological relevance have established the current state of the HPV field of research.

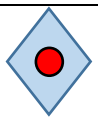
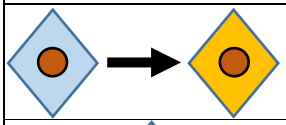
Cell Type Modeled	Advantages	Disadvantages	Examples
	• Low mutation load • Represent HPV infection microenvironment	• Donor variation • Not immortal	• Human foreskin fibroblasts (HFFs)
	• Low mutation load • Cells that HPV infects	• Donor variation • Not immortal	• Human foreskin keratinocytes (HFKs)
	• Dissect effects of acute oncogene expression	• Reductionist approach • Donor variation	• Oncogene+ HFKs: ○ E6/E7/E6E7
	• Shows effects of whole genome	• Donor variation	• HR HPV genome+ HFKs
	• Ethical, stepwise transformation model	• Big time investment • No control	• HFK E6E7 ○ Passage: 50-200
	• Immortal • Result of actual HPV infection	• High mutation load	• SiHa • HeLa • HT3

Table 1 Models for studying cervical cancer. Table highlighting various cellular models for studying cervical cancer. The "cell type modeled" column contains images of cells represented in Figure 1. The portion of this column with two cells indicates that those models illustrate both cell types and their transition.

Databases

Another essential aspect of understanding an area of research is the analyses employed to make conclusions from data sets. Although some of the analytical techniques used to summarize data sets relating to cancer research will be discussed later with the relevant molecular approaches, it is important to understand one characteristic of the statistical mechanisms behind conclusions. This aspect is the fact that much of the data related to cancer research that is being analyzed/has the potential to be analyzed is present within publicly available databases. The

availability of information has allowed for substantial progress in cancer research, in part due to its intrinsic ability for a collaborative effort. Huge data sets resulting from techniques like RNA-sequencing, that allows for the confirmation of the presence and quantity of RNA in large sample groups, can undergo largescale analyses looking at alterations to entire pathways by outside laboratories, as a result of this data being shared by the various institutes they originated from. Analytical processes utilizing database datasets can often also allow an analysis of real-life tumor samples, bridging the gap between the scientific and medical fields, as well as the tissue systems previously described and the most biologically relevant *in vitro* system. The final advantage databases provide is an ability for researchers to have a shotgun approach in determining pathways of interest that are altered in cancerous cellular environments, determining which pathways are upregulated or downregulated with techniques like RNA-seq. All these advantages that database analyses provide drastically increases the efficiency of cancer research.

Techniques to Study Cancer

Now that the basics of model systems and database analysis have been detailed, some of the techniques used in the field of cancer research can be discussed. It is important when discussing techniques in this discipline to understand their application in determining alterations to the cellular environments. For many techniques, this can be summarized as ways to dissect alterations to cellular pathways. This dissection entails figuring out at what levels within the pathway alterations to regulation occur and whether the result is upregulation or downregulation. The levels at which these alterations can be broadly categorized are the mRNA level, protein level, and localization. These can be further expanded to mRNA abundance, mRNA stability, translation efficiency, protein abundance, modified protein abundance, protein stability, complex formation, and localization. Researchers often have numerous techniques at their disposal to

assess changes at each of these levels. As such, not all of the techniques for each of these levels will be discussed, nor will any of the techniques for some levels be discussed. The main techniques that will be focused on pertain to categories that are often easier to study and are relatively widespread in the field of cancer research.

RT-qPCR

Techniques that analyze mRNA levels are essentially the first glimpse into pathway upregulation or downregulation as mRNA abundance is one of the first byproducts of gene expression. These analyses provide an answer on whether the mRNA is either increased, decreased, or unaltered. While, analyzing other categories, like mRNA stability, provides a mechanism for adjusted abundances. The first technique to be discussed, reverse transcription quantitative polymerase chain reaction (RT-qPCR), pertains to this first level of pathway alteration, mRNA abundance. The technique itself is conducted by generating templates associated with the genes of interest. Primers, one example, are constructed segments of DNA designed to target specific sequences and initiate the replication of a DNA product. Designing these primers is a component of RT-qPCR that is a process in and of itself. These templates are required to span introns of DNA, be specific to the gene of interest, have little to no affinity to binding itself, have an annealing temperature within a specific temperature range, an annealing temperature close to the annealing temperature of the complementary template, and a reasonable product size (approximately 200 base pairs [bp]). Luckily, as many criteria as there are for generating these templates, there are widely available programs which can generate templates based on a particular given sequence with set parameters for these conditions, as well as online services that allow for premade templates to be ordered. RNA is harvested from the appropriate source of interest, then reverse transcribed into DNA (referred to as complementary DNA

[cDNA]). cDNA, as opposed to regular DNA, does not contain introns. These stages although not components of RT-qPCR itself are essential prerequisites.

The newly generated cDNA is then combined with the primers, nucleotides, dyes, and enzymes in a heat block to create RNA products from the cDNA. This itself is the process of RT-qPCR. The concentration of the template used is important. If using a pre-generated template from an online service, they will often provide the user with information regarding the appropriate concentration of each template to use for the reaction. However, if the templates were generated by the user using a program, they will need to perform a dilution curve. This involves following the same protocol as described below but with a diluted gradient of template present to determine, based on later analyses, the appropriate concentration. The heat block changes to different temperatures for various lengths of times based on the programming of the researcher. Although the temperature, time of each step, and the number of times the steps are repeated can be changed, there is a basic formula to the process. Often times the first stage in the process is to adjust the environment so the enzyme can function, this is dependent on the enzyme being used but involves the heat block changing to a specific temperature for a set amount of time. Activating the enzyme allows DNA to be replicated. The next stage is repeated, usually around forty times, and involves heating up the cDNA to denature it, heating up the mixture to allow for annealing of the templates to the cDNA, and then holding at a determined temperature to allow for elongation of the DNA product. The final stage, the melt stage, is a process in which the temperature of the heat block is incrementally increased with the block being held at each temperature for a set amount of time. This process continues until the block reaches a higher temperature which is guaranteed to have melted all the products generated during previous stages. Determining the appropriate conditions for these components of RT-qPCR can often be a

long process involving conducting the protocol to fine-tune the settings but is an essential precursor to analyzing the resulting data.

Two sets of data can be analyzed from RT-qPCR results, generated during the repeated steps in the protocol by setting the heat block to measure the signal of dyes at the end of each repetition of those stages. Although it happens second in the protocol, the melting step will be discussed first. This step generates what is known as a melt curve, the melt curve is a curve showing the loss of dye signal from generated products and can be analyzed to assess the specificity of the templates used and verifying contamination. A highly specific template will generate one closely organized set of curves with repeated runs of the same mixture. This is a result of these primers making one product with one melt temperature, determined by the nucleotide content and amount within the product. If a template is not specific, multiple sets of curves will be generated, due to numerous products with varying nucleotide content, and if curve sets contain multiple curves within the same repetition templates, it might be dimerizing. Primer dimers are hybridized primers and due to their low base pair length typically appear in melt curves as peaks at low temperatures. By including a negative control, usually the reaction mixture containing water as opposed to cDNA, contamination can be detected. The negative control should produce no melt curves. Analysis of the melt curve is thus an important quality control assessment of the templates and mixture components being used.

The other repeated stage of RT-qPCR, in which DNA products are formed, provides the means to determine changes in mRNA levels. By measuring changes in dye signal after each completed cycle, the machine is effectively, indirectly measuring the RNA concentration for a gene of interest. Analyzing where curves indicating a change in dye signal occur within the number of cycles is an analysis of how easy it was to generate a large volume of DNA product.

This process is conducted by analyzing three values which can be generated by software often included with heat blocks requiring little more than defining contents of each of the wells of a run. These three values are the R^2, efficiency (E), and quantitation cycle (Cq). The R^2 value is a determination of how well the variability can be explained by reasonable, natural variations in the process. A low value means that a large amount of the variability cannot be explained, and the process must be redone. Efficiency is an analysis of how much product is generated relative to the amount of cDNA present and is the value analyzed during a dilution curve to determine the appropriate concentration of template. A value as close to 100% is ideal as it means you are generating one product per respective unit. Determination of the E comes from analyzing the slope of the line resulting from analysis of the Cq values in your dilution gradient. Changes to the Cq value should mirror the dilution factor to get an E value of 100%. Finally, the Cq value is the number of cycles required for the dye signal fluorescence to be detected or quantified. The crossing point (Cp) and the cycle threshold (Ct) are also valid and similar ways of measuring this. A lower Cq value means fewer cycles were required to generate product and thus there was a larger amount of cDNA, considering this cDNA was generated from RNA there is consequentially higher levels of mRNA.

Western blots

The second technique of interest is utilized to assess changes to the cellular environment at the protein level. Western blots allow protein abundance to be compared between different cell lines or treatments similar to how RT-qPCR allows a comparison of mRNA abundance. However, unlike RT-qPCR western blots have several alterations available that expand the utility of the technique. The basis for western blots is similar to many other blot techniques. Cellular lysates are centrifuged to allow proteins to be isolated. The concentrated proteins in these lysates

are then measured via bicinchoninic acid assay (BCA), a colorimetric assay quantifying the interaction between polypeptides and copper. Conducting a BCA allows proteins within lysates to be equally loaded into gels and electrophoresis helps separate the proteins by molecular weight. These gels are then used to adhere the proteins to a membrane through interactions, such as hydrophobic interactions binding proteins to nitrocellulose. Afterwards, different proteins can be probed for by utilizing antibodies targeted to proteins of interest. A primary antibody is used to probe for the protein of interest, then a secondary antibody targets the antibody of the organism that the primary antibody originated from. This secondary antibody is conjugated with horseradish peroxidase (HRP), these membranes when exposed to HRP substrate emit light and when imaged in a machine capable of detecting that wavelength can be used to semi-quantitatively detect protein abundance. As a semi-quantitative technique, western blots can determine relative protein abundance. However, as previously mentioned, westerns can be expanded and altered to further the possible generation of results.

Densitometry

The first addition that can be applied to westerns is an analytical one. Densitometry is a further quantitative analysis of western blots. Densitometry is an approach to measuring the quantity of a particular protein, analyzing the pixilation occupying a defined area on a western blot, representative of the band for the protein of interest. When normalized to levels of a loading control protein, usually the product of a housekeeping gene that will typically not have the abundance altered, variations in loading can be accounted for. This provides a more reliable, representative quantification of protein abundances under the condition of interest. Although densitometry, like western blots in general, is a semi-quantitative technique, it does allow for a narrower semi-quantitative conclusion and opens up the possibility of further analyses.

Protein stability assay

The second addition to western blots allows for an analysis of at what level protein abundance might be altered, specifically looking at protein degradation. Cycloheximide, a drug that inhibits protein biosynthesis by preventing translocation, helps arrest protein production in treated cells. As a result, changes in protein levels from untreated cells should be a result of protein degradation. This provides insight into the stability of proteins and when combined with densitometry can be used to quantify half-lives for proteins under specific conditions. This addition to westerns can also be combined with the utilization of proteasome inhibitors, such as MG132, to determine if proteasome inhibition is the cause of changes to stability.

Growth starvation

The last addition of interest to western blots is the utilization of growth starvation. It is important to note that the term here is used to refer to using media deprived of growth factors and does not deprive cells of other nutrients. This application is especially useful when studying cancer as it can help detect whether changes to the cellular environment are a result of changes to cellular proliferation, as is characteristic of cancerous cells, or due to some other alteration to the cell. This technique is relatively simple, requiring the cell line/treatment of interest to be switched over to media with different levels of growth factors in tandem with a control cell line and then running lysates on the same blot for visual analysis and densitometry.

Immunofluorescence microscopy

Studying cancer is not only a study of alterations to the normal environment of the cell but also of how these changes to the cellular environment can be taken advantage of to treat cancer. Finding a technique to reliably assess alterations, such as complex formation, is essential. Immunofluorescence (IF) microscopy is an invaluable means to analyze changes inside the cell.

IF allows for the detection of proteins using antibodies, similar to western blots. However, unlike western blots, IF allows for a visualization of protein content and localization within the context of the cellular environment, via the formation of fluorophore foci. Another advantage of IF is the ability to use multiple antibodies to assess protein localization in relation to other proteins. Thus, IF allows for an analysis of how particular pathways might be functioning as a measure of correct protein content and localization/colocalization within both spatial and temporal contexts.

The applicability of IF is not that simple, and manipulation of the general procedure can produce techniques refined towards answering more specific questions relating to a particular environment. One of the first components of an IF protocol is determining what cells will be fixed and imaged on. Utilizing a 96-well plate provides a broader image of numerous cells/cell populations and treatments. On the other hand, coverslips provide a more detailed, higher magnified image to gain a more intricate image of what's happening in individual cells. Combined, these two platforms for imaging allows for a diverse analysis.

Characterization of DSB repair complex formation and resolution

The particular aspects of the cellular environment of interest, that correlate with the story of cervical cancer as previously detailed, are those pertaining to the repair of double-stranded breaks (DSBs) in DNA within the host genome. IF helps serve to measure if protein abundance and localization allows for the adequate formation of repair complexes and thus resolution within the context of the cells of interest. However, as an essential step in this process is the generation of DSBs within cells to provide an opportunity to assess the cellular response[37].

Assessment of DSB repair complex formation and resolution begins with using one of various genotoxic agents or an endonuclease to help induce DSB[37]s. Genotoxic agents such as hydrogen peroxide provide the means of generating the aforementioned breaks and subsequent

analysis of repair complex formation and resolution[37]. Analysis of repair complex formation allows for a determination of whether the correct proteins are being made, are in the proper abundance, and are localizing with the other appropriate components of the complex. Analysis of repair complex resolution clarifies whether complexes and their respective component proteins are localizing correctly in both a spatial and temporal sense to a sufficient degree[37–39]. The analysis that repair complex formation and resolution can, like other aspects of IF microscopy, be further expanded upon by altering the procedure.

When using various DSB inducing genotoxic agents to assess cellular response to DNA damage discerning between the processes of repair complex assembly, localization, and resolution can prove to be problematic[37–39]. This analysis when limited to few instances of time after induction of DNA damage makes it hard to differentiate when repair is inhibited or just delayed (ex., mislocalized versus delayed localization), as well as whether seemingly normal or excessive repair response activation is instead an inability to inactivate or resolve a repair response[37–39]. To subvert this limitation of DNA damage IF analysis utilization of a mechanism for long-term DSBs can be utilized. Enzymatic cleavage of DNA, by a rare-cutting endonuclease such as I-SceI, is one method of generating these prolonged DSBs[37,40]. Lesions generated by these endonucleases are renewed upon repair, utilizing long-term lesions allows for the formation of foci independently of a temporal restriction due to delayed localization[37]. This continuous lesion also helps refine immunodetection of the various components of the repair complex machinery because of the large repair foci[37]. Although this method does have limitations, such as the complications involved with introducing endonuclease activity recognition sites, the possibility of discerning between mislocalization or delayed localization as components of the changed cellular environment of a cancer is an invaluable tool[37].

Cell sensitivity alteration analysis

The last component of cancer research that will be discussed is what can be done to take advantage of the cellular environment in cancerous cells. These techniques are the precursor to the implementation of different treatment studies. These components of cancer research do not require the other components of cancer research to be implemented to progress to this stage but do require those components to help explain why certain treatment options are effective at selectively targeting cancerous cells. The foundation of techniques that fall under this category is utilizing some treatment that damages cells then assessing cell survival, with the treatment ideally targeting cancerous cells more efficiently due to the altered cellular environment. Utilizing DNA cross-linkers, such as cisplatin or UV, to generate lesions in DNA can target cancerous cells deficient in responding to that damage. Generating these lesions can lead to replication fork stall, collapse, DSBs, and cell death. Assessment of cell survival can then be conducted through microscopy or through a metabolic assay. Using metabolic activity as an assay for cell survival can be performed using MTT. MTT is a colorimetric assay in which NAD(P)H-dependent cellular oxidoreductase enzymes, which are generated by metabolically active cells, reduce tetrazolium dye MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to its insoluble formazan. This reduction produces insoluble purple crystals from a yellow liquid. The crystals are then solubilized with the addition of solubilization buffer and the absorbance is read on a plate reader. Increased absorbance represents increased metabolic activity and thus cell survival. Ideally, when utilizing a treatment, the cancerous cells showcase low absorbance values, while normal cells have high absorbance indicating selective targeting and sensitivity of cancerous cells.

Chapter 3 - Alterations to TLS Protein Stability in CaCx Cell Lines

Introduction

DNA damage involves a myriad of DDR pathways to properly repair different types of DNA damage. In terms of severity, nucleotide excision repair (NER) lies at the lower end of the DNA damage severity spectrum. NER is involved in excising mutated nucleotides, such as thymine-thymine dimers generated by UV-induced DNA damage[41]. Repair of more severe damage such as DSBs requires pathways such as homologous recombination (HR) and non-homologous end joining (NHEJ). These DSBs represent a greater risk to the cell than mutated nucleotides, with greater deleterious damage potentially occurring when the damage is unrepaired[41–44]. αHPV types 16 and 18 have been shown to inhibit the activation of numerous DDR pathways partially due to their recruitment of factors in those pathways for viral propagation.

To optimize DDR, said damage must be minimized, further progression of DNA damage increases the risk for potentially lethal consequences such as chromosomal breakage. Minimizing DNA damage means bypassing crosslinks during replication[31,34,45]. When a lesion is encountered during replication, the polymerase stalls unable to synthesize past the damaged DNA. Despite the stalling of the replication fork, the helicase progresses producing long stretches of breakage-prone single-stranded DNA. Allowing these stretches to persist predisposes the replication fork for collapse into a DSB[31,46–49]. The functional alternation between the normal replicative polymerase and a translesion polymerase is essential to prevent this scenario. We hypothesized, based on observed stifling of DDR pathways in HR HPV positive cells, that DNA damage tolerance (DDT) pathways, such as TLS, would also be affected.

TLS inhibition would result in the generation of more deleterious damage, combined with HR and NHEJ obstruction would likely result in cell death. Cisplatin, which is the standard of care for cervical cancer, induces crosslinks in strands of DNA. It is possible that the effectiveness of cisplatin treatment stems from failed DDT and subsequent failure of DDR. Whether this is the case and why HPV leads to changes in how the TLS pathway is regulated are both currently unresolved questions in the field of cervical cancer research. The focus of research in this chapter was to further dissect changes to TLS protein abundance that were observed, as well as to provide insight into increased protein stability as a possible mechanism initiating those changes. Increased levels of DDR proteins have been observed as a sign that repair potential is inhibited and the same might be true of TLS and tolerance potential[13].

Methods

Tissue culture

All cell lines were maintained in 10 cm plates with 10 mL of the appropriate media. HFKs were cultured in low-calcium Epilife® (500 mL calcium-free Epilife®, 5 mL 100x HKGS, 5 mL penicillin-streptomycin, 250 µL 0.06M CaCl) while HFFs, HeLa, and SiHa were cultured in DMEM containing FBS and penicillin-streptomycin. During all experiments containing HFKs the HFKs were centrifuged to form a pellet whenever they were passaged, media was aspirated, and cells were suspended in new media as part of normal HFK maintenance. All other cell lines were taken through the same procedures of spinning down the cells and resuspending the pellet in media to reduce variability in tissue culture techniques.

During the experiments where varying concentrations of FBS were used, all cells were grown in 10% FBS DMEM (the standard FBS concentration for the cell lines used) then lifted and spun down in 10% FBS DMEM to assist in neutralizing trypsin. The media was then

aspirated off the pellet and the cells were suspended in DMEM with the appropriate FBS concentration. After passaging and seeding cells, they were maintained in the appropriate FBS concentration for 24 hours.

Protein stability assay

Cycloheximide (CHX) was dissolved in DMSO at 1mg/mL, 100 µL of this stock was added to plates for a final concentration of 100 µg/mL of CHX and a final concentration of 1% DMSO. Prior to adding either DMSO or CHX to plates, the media was aspirated and replenished. DMSO was added to the mock treatment time point at 24 hours prior to collection. Each CHX time point had DMSO added at 24 hours, then at the appropriate time prior to collection, the media was aspirated, replaced, and XHX was added. Ex., for the 2-hour time point, DMSO was added at the same time as the mock sample, then 2 hours prior to collection (22 hours after DMSO was added), CHX was added.

Immunoblotting

Cell lysates were collected by treating cells for 5 minutes with RIPA lysis buffer (with protease and phosphatase inhibitors). Lysates were loaded into 10% Tris-Glycine SDS-PAGE gels. All western blots were conducted in triplicate with protein levels of the cell lysates normalized by BCA. Primary antibodies used were as follows: TOPBP1 (Santa Cruz Biotechnology®), RPA70 (Cell Signaling Technology®), Rad18 (Cell Signaling Technology®), Rad6 (Abcam®), nucleolin (Santa Cruz Biotechnology®), KI67 (Abcam®), Pol-Eta (Santa Cruz Biotechnology®), PCNA (Cell Signaling Technology®), Ub-PCNA (Cell Signaling Technology®), and GAPDH (Santa Cruz Biotechnology®).

For the experiments analyzing the impact of FBS concentration on protein stability, HFKs were included to ensure CHX was inhibiting protein synthesis. Densitometry was

performed on all proteins, and levels were normalized for each cell line and treatment to the appropriate control treatment, nucleolin (TLS stability experiments) or GAPDH (FBS concentration TLS stability experiments). For instances where bands appeared faint, multiple exposures were obtained to get a darker exposure and ensure that the faintness was not skewing densitometry.

Software

Image J and Graph Pad were used for densitometry and statistical analysis, respectively. One-phase decays were used for analysis of half-lives and two-tailed t-tests were used to determine significant differences in protein stability for each time point in the time series.

Results

The half-lives of translesion synthesis proteins in keratinocytes were less than 24 hours

We have previously found that the abundance of essentially all the proteins involved in TLS is increased in cervical cancer cell lines (Dr. Sebastian Wendel, personal communication). This, at least in part, can be explained by our RNA-seq analysis that showed that replication fork and TLS gene expression are upregulated in cervical cancer samples in comparison to healthy cervical tissue samples (Dr. Sebastian Wendel, personal communication). However, whether or not this upregulation at the gene expression level accounts for the increased protein abundance in its entirety has yet to be determined. Notably, the HR HPVs have been previously shown to upregulate gene expression at a number of different levels. Work by Johnson, Aloor, and Moody has shown HR HPVs have upregulated various genes involved in DDR pathways, with the explanation for this upregulation being a complicated story[13]. Different proteins were upregulated solely at one level of gene expression, while others were upregulated at various

levels of gene expression[13]. One particular way that these DDR proteins were upregulated was by increasing protein stability.

We hypothesized the stabilities of some TLS proteins was increased in cervical cancer cell lines. In order to determine the "normal" half-lives for these proteins, we analyzed the degradation of TLS proteins in HFKs. Conducting a time series with HFKs was essential to provide insight on the proper concentration of CHX to use, as the concentration used in the literature for the cell lines of interest were varied. This time series would also indicate the proper time points to use. To properly determine the half-lives of TLS proteins sufficient representation of the degradation is essential. This requires time points prior to and either at or after proteins reach half their original abundance.

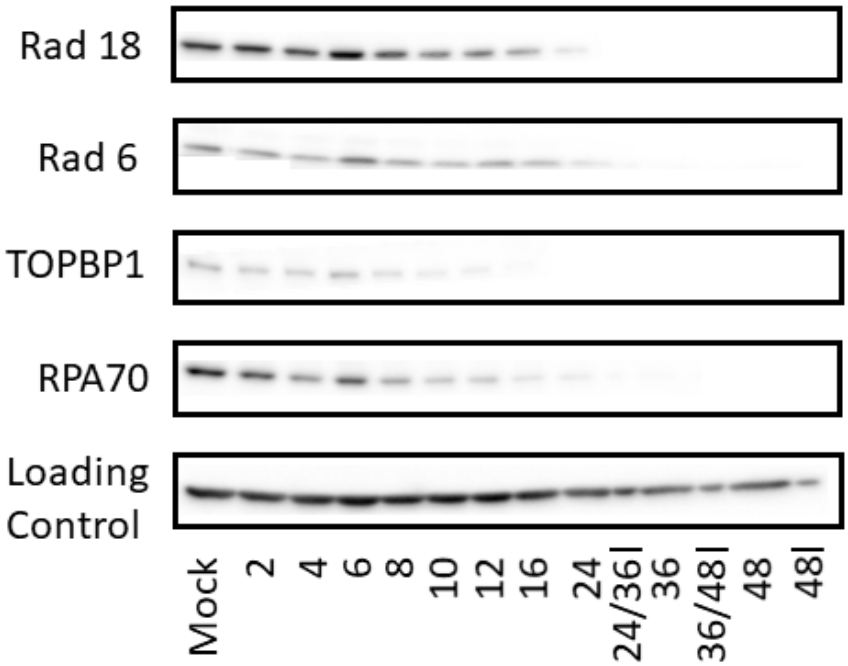


Figure 4 Translesion synthesis protein half-lives in keratinocytes. Representative western of TLS protein stability across 48 hours post CHX treatment. Mock treatment was treated with DMSO for 48 hours. Lanes with two time points below a bar (ex., 24/36) were left empty in order to accommodate potential overflow from adjacent lanes due to the high volume of lysate loaded.

The abundance of all TLS proteins fell below 50% by 24 hours and later time points such as 36 and 48 hours had almost no protein left for those proteins. For some TLS proteins, the abundance fell below 50% before 24 hours (Fig. 4). From these time points, we concluded that 24 hours was sufficient to compare the half-lives of these proteins.

Lastly, considering time points prior to 24 hours showcased a gradual degradation of these TLS proteins indicated that 100 µg/mL was as sufficient concentration of CHX to accommodate cell sensitivity and protein synthesis inhibition. This time series also helped determine cell sensitivity to the DMSO concentration used. A 1% final concentration was determined to be appropriate.

The stability of Rad18 and RPA70 were increased in cervical cancer cell lines

With the approximate half-lives for TLS proteins determined in HFKs, the next step was to assay how transformation affected the stability of those proteins. In order to measure whether HPV-associated transformation altered TLS protein stability, SiHa and HeLa were used as models of HR HPV types 16- and 18-associated transformations. Although variations might exist between how these HPV types interact with cells during transformation and other HPV types, they are a relevant *in vitro* system, as these two cell lines contain HPV types that account for approximately seventy percent of cervical cancers[17–19].

As can be seen by immunoblots and densitometry (Fig. 5) and half-life summaries (Table 2), both RPA70 (24+ vs. 10.87) and Rad18 (24+ vs. 8.43) have increased stability in cervical cancer cell lines in comparison to HFKs. On the other hand, Rad 6 and TOPBP1, did not have significantly different stabilities. Although not statistically different, the half-lives of TOPBP1 (24+ vs. 11.8) and Rad6 (19.71 vs. 5.78) in SiHa do approach a significant difference. Also of note is the trends seen between SiHa and HeLa. All TLS proteins in SiHa have a less steep curve than HeLa. The steepness of curves is also important as the cancerous cell lines, with their increased proliferation, have typically higher protein concentrations, but this does not account for the levels seen in later time points. This can be observed when analyzing the steepness of the curves, if the initially increased abundance was a factor the degradation, curves would parallel the degradation curve of HFKs.

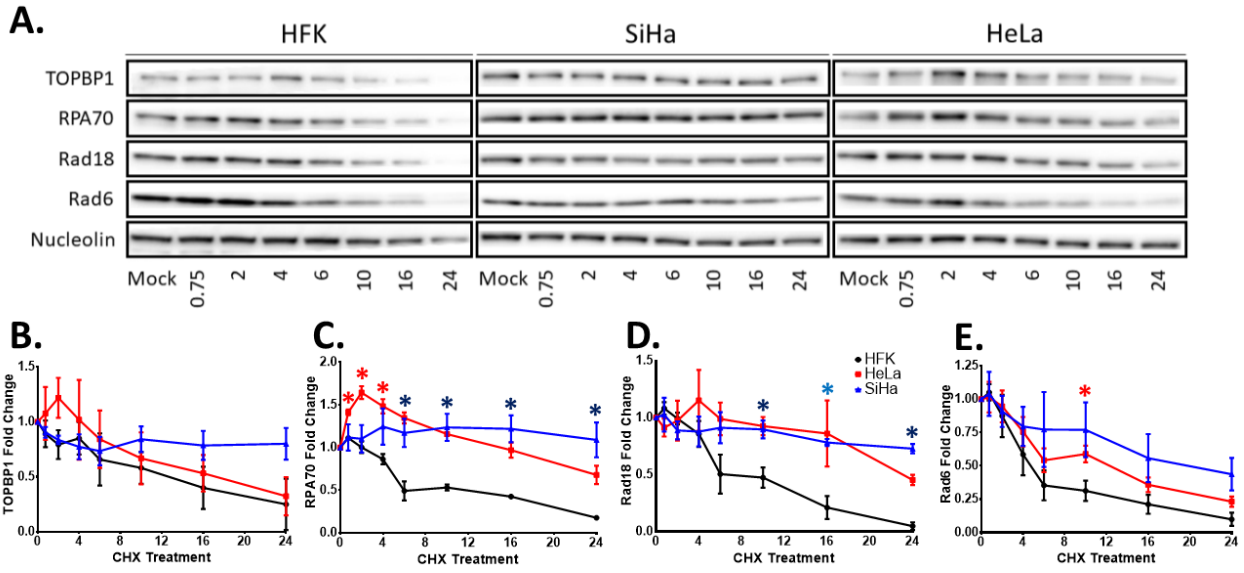


Figure 2 Translesion synthesis protein stability in cervical cancer cell lines. A, representative westerns blots of TLS protein stability up to 24 hours in HFKs and cervical cancer cell lines. Mock treatment was treated with DMSO for 24 hours. B-E, densitometry summary for the stability of TLS proteins normalized to nucleolin. Asterisks represent significant differences from the values for HFKs for each time point with the color of the asterisk corresponding to which cell line had significantly different stability for that protein during that time point (Red=HeLa, Blue=SiHa, Purple=HeLa and SiHa).

From analyses of densitometry (Table 2) the half-lives of the four TLS proteins of interest were determined. The half-lives for these proteins was increased in both cervical cancer cell lines, although this increase, in either cell line, was only significant for RPA70 and Rad18.

Cell Line	Post CHX Treatment (hrs)	TOPBP1	RPA70	Rad18	Rad6
HeLa	0.75	0.925158	0.001983	0.151863	0.795067
	2	0.768064	0.003166	0.927822	0.735512
	4	0.697798	0.003866	0.367947	0.422771
	6	0.792157	0.002755	0.095468	0.261344
	10	0.232804	0.000118	0.019544	0.049406
	16	0.174459	0.003812	0.101607	0.17727
	24	0.114742	0.009709	0.001981	0.101558
SiHa	0.75	0.925158	0.972109	0.74185	0.952516
	2	0.768064	0.597557	0.335058	0.86997
	4	0.697798	0.157914	0.907923	0.381858
	6	0.792157	0.027671	0.137477	0.23931
	10	0.232804	0.012394	0.023256	0.105562
	16	0.174459	0.007623	0.005737	0.148602
	24	0.114742	0.011154	0.000204	0.061987

Table 2 Densitometry of translesion synthesis protein stability in cervical cancer cell lines. Different colored cells represent differences in significance in protein abundance normalized to mock treatment in comparison to respective HFK time points (Green=Nearly significant <0.065, red=significant <0.05, orange= <0.01, light orange= <0.005, yellow= <0.001).

However, similar to the analysis of the degradation curves the half-lives for Rad6 and TOPBP1, especially for SiHa, approached significance. It is important to consider that although densitometry analysis does convey information about alterations to stability up to 24 hours that increasing the timespan for the time series might showcase that Rad6 and TOPBP1 have increased stability at a later time point compared to HFKs.

Cell Line	Protein	Half-Life (hrs)	95% CI (hrs)
HFK	TOPBP1	11.8	7.71-18.66
	RPA70	10.87	8.68-13.71
	Rad18	8.43	6.38-10.95
	Rad6	5.78	4.31-7.72
HeLa	TOPBP1	18.97	10.34-24+
	RPA70	24+	N/A*
	Rad18	24+	20.91-24+
	Rad6	10.75	8.48-13.71
SiHa	TOPBP1	24+	N/A*
	RPA70	24+	N/A*
	Rad18	24+	N/A*
	Rad6	19.71	12.31-24+

Table 3 **Half-lives of translesion synthesis protein in cervical cancer cell lines.** Proteins whose half-lives are labeled as "24+" did not reach half of their original abundance within 24 hours. Confidence intervals where one of the intervals is labeled as "24+" had the abundance for that protein not reach half of the original abundance for at least one of the repeats, while confidence intervals labeled as "N/A*" had none of the repeats reach half their original abundance at 24 hours (Red=significant <0.05, light orange= <0.005, yellow= <0.001).[Half-life in column – remove cap L]

Increased proliferation did not drive increased TLS protein stability

One important consideration when dissecting alterations to gene expression in cancerous cell lines is determining if the increased protein stability is the result of elevated proliferation. We have previously shown that proliferation has an impact on TLS, increasing protein levels in

HFFs. We hypothesized based on this information that increased proliferation accounts for the increased TLS protein stability observed in cervical cancer cell lines.

To test this, we assayed stabilities of TLS proteins in HFFs and cervical cancer cell lines in media containing either 0 or 10% FBS. When comparing the normalized densitometry of 0 and 10% FBS concentration, HFFs have three groups in which proteins can be divided. The first group, composed solely of KI67, is not expressed in 0% FBS HFFs for either DMSO- or CHX treated samples; therefore, the changes in protein stability are incalculable. However, KI67 is expressed in 10% FBS HFFs. Although changes in stability are not calculable, changes in abundance are. When observing KI67 abundance between the two HFF FBS treatments, there is a significant difference. The second group, composed of PCNA, RPA70, and TOPBP1, do not have their stability increased with proliferation. The amount of these proteins degraded in HFFs in 10% FBS is more than the amount degraded in HFFs in 0% FBS. The normalized abundances for PCNA and TOPBP1 in 0% FBS HFFs are significantly different from the normalized abundances for HFFs in 10% FBS, while RPA70 approaches significance (Table 5). The final group, composed of both polymerase eta (pol-eta) and ubiquitinated PCNA (Ub-PCNA), have protein stabilities in which in 0 and 10% FBS HFFs they are approximately at one when normalized after 24 hours. The stability of pol-eta does not significantly change when in 10% FBS HFFs and although the stability of Ub-PCNA does significantly change in 10% FBS HFFs, it does not fall drastically below one. In the latter two cases, it is evident that there is no alteration to protein stability in HFFs, that proliferation does not normally positively correlate with protein stability. In the first group, an analysis of alterations to protein stability cannot be

made as there is no expression of KI67 in either DMSO- or CHX-treated HFFs in 0% FBS (Fig. 2).

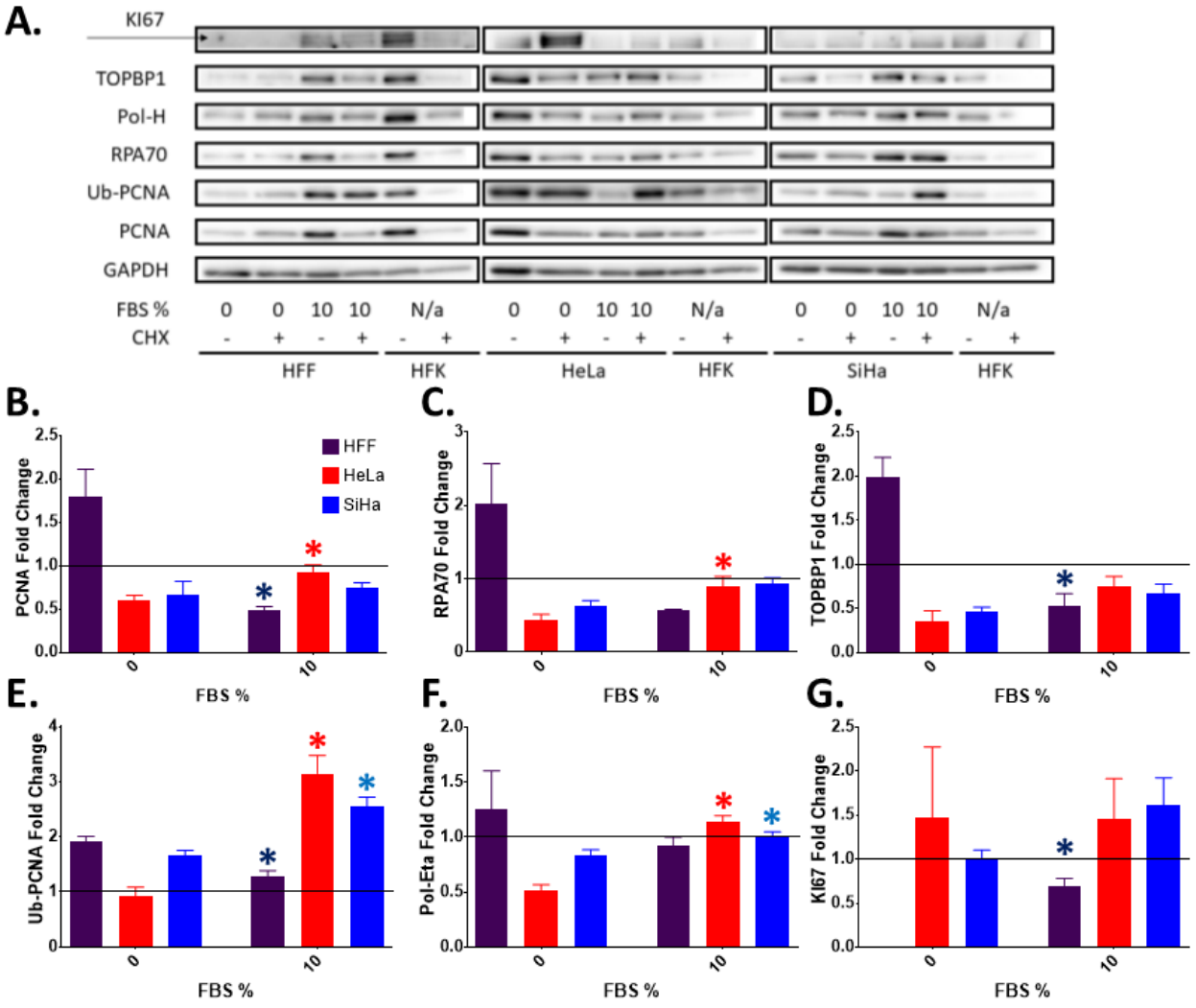


Figure 5 Translesion synthesis protein stability based on proliferation. A, representative western blot of TLS protein stability at 24 hours in HFFs and cervical cancer cell lines. CHX negative treated cells were treated with DMSO for 24 hours. HFKs were not grown in media containing FBS for any treatment. KI67 was measured as the topmost band. B-G, densitometry analysis of translesion synthesis protein abundance. The line at one indicates the values for all normalized DMSO-treated xxx abundances, with each cell line being normalized to the appropriate GAPDH levels for 0 or 10% FBS-treated cells from the same cell line. Asterisks represent significant differences between the stability of the protein in 0 versus 10% FBS, with the color corresponding to the cell line (Purple=HFFs, red=HeLa, blue=SiHa).

When comparing the stabilities of TLS proteins in cervical cancer cell lines the trends cannot be grouped under the same constraints as in HFFs. PCNA, RPA70, and TOPBP1 have their abundance fall to approximately 50% (CHX vs. DMSO) at 24 hours in 0% FBS in cervical cancer cell lines. Of these proteins, PCNA and RPA70 have their stabilities significantly altered in 10% FBS HeLas. Although, the stability of RPA70 does approach significant differences in SiHa. Pol-eta's protein abundance reaches or approaches 50% (CHX vs. DMSO) in HeLa and SiHa in 0% FBS, respectively, and has its stability significantly increased for both cell lines when in 10% FBS. Finally, Ub-PCNA and KI67 (which is notably expressed in 0% FBS cervical cancer cell lines) are at approximately one of their original abundances in 0% FBS. Both HeLa and SiHa follow the same trend for Ub-PCNA and KI67 in 10% FBS, where Ub-PCNA has a significant increase in abundance comparing 0 and 10% FBS treated cells, while KI67 has no significant change (Fig. 3B-G, Table5).

Cell Line (0% FBS)	Protein	Half-Life (hrs)	95% CI (hrs)
HFF	PCNA	24+	N/A*
	RPA70	24+	N/A*
	TOPBP1	24+	N/A*
	Pol-Eta	24+	N/A*
	Ub-PCNA	24+	N/A*
	KI67	Not expressed	Not expressed
HeLa	PCNA	24+	23.38-24+
	RPA70	19.88	14.47-24+
	TOPBP1	15.81	10.18-24+
	Pol-Eta	24+	20.73-24+
	Ub-PCNA	24+	N/A*
	KI67	24+	N/A*
SiHa	PCNA	24+	21.28-24+
	RPA70	24+	24-24+
	TOPBP1	21.67	17.72-24+
	Pol-Eta	24+	N/A*
	Ub-PCNA	24+	N/A*
	KI67	24+	N/A*

Table 4 Half-lives of translesion synthesis proteins in cervical cancer cell lines without growth factors. Proteins whose half-lives are labeled as "24+" did not reach half of their original abundance within 24 hours. Confidence intervals where one of the intervals is labeled as

"24+" had the abundance for that protein not reach half of the original abundance for at least one of the repeats, while confidence intervals labeled as "N/A*" had none of the repeats reach half their original abundance at 24 hours.

Cell Line (10% FBS)	Protein	Half-Life (hrs)	95% CI (hrs)
HFF	PCNA	23.06	19.23-24+
	RPA70	24+	N/A*
	TOPBP1	24+	15.5-24+
	Pol-Eta	24+	N/A*
	Ub-PCNA	24+	N/A*
	KI67	24+	N/A*
HeLa	PCNA	24+	N/A*
	RPA70	24+	N/A*
	TOPBP1	24+	N/A*
	Pol-Eta	24+	N/A*
	Ub-PCNA	24+	N/A*
	KI67	24+	N/A*
SiHa	PCNA	24+	N/A*
	RPA70	24+	N/A*
	TOPBP1	24+	N/A*
	Pol-Eta	24+	N/A*
	Ub-PCNA	24+	N/A*
	KI67	24+	N/A*

Table 5 Half-lives of translesion synthesis protein in cervical cancer cell lines with growth factors. Proteins whose half-lives are labeled as "24+" did not reach half of their original abundance within 24 hours. Confidence intervals where one of the intervals is labeled as "24+" had the abundance for that protein not reach half of the original abundance for at least one of the repeats, while confidence intervals labeled as "N/A*" had none of the repeats reach half their original abundance at 24 hours.

Protein	HFF	HeLa	SiHa
PCNA	0.016334	0.0321	0.654464
RPA70	0.053572	0.044951	0.051663
TOPBP1	0.004875	0.075633	0.133262
Pol-Eta	0.398672	0.001311	0.039997
Ub-PCNA	0.01174	0.003796	0.011067
KI67	0.001136	0.98634	0.124656

Table 6 Densitometry comparing translesion synthesis protein stability in cells without and with growth factors. Different colored cells represent differences in significance in protein abundance normalized to mock treatment in comparison to respective HFK time points (Green=Nearly significant <0.065, red=significant <0.05, orange= <0.01, light orange= <0.005).

Discussion

When trying to deduce the causation for upregulations to gene expression in the TLS pathway, it is important to remember that the story of any cancer is predisposed to be a complicated story. Drastic variation, even within the cell lines used to study those cancers, is common. Alterations to a particular gene pathway might occur to different degrees for different genes. These genes might also have individual stories when it comes to what levels of gene expression they are altered at. Cervical cancer is not entirely removed from this frequent characteristic of cancer with this concept previously being showcased when looking at DDR pathways[13]. As we have shown, the DDT pathway, TLS, also seems to be subject to this characteristic.

Many proteins involved in the TLS pathway seem to have their gene expression upregulated at the protein level, with some of this accounted for at the protein stability level. Although this does not explain the increase in abundance for all TLS proteins and is not the sole contributor to observed changes. For essentially all TLS proteins, notably excluding pol-eta, their gene expression is up at the level of mRNA expression. However, this increase to gene expression does not necessarily complete the story explaining raised protein abundances for either proteins whose stabilities are or are not altered. The significance of determining alterations to protein stability, or changes at any other level of gene expression, is not to promote them as the complete, succinct story. Rather, the significance is highlighting up and downregulations are occurring and are at least partially explained by changes at a particular level.

It is worth mentioning that there have been experiments that have shown that treatment with CHX can increase activation of the TLS pathway[49]. Inhibited protein synthesis leads to reduced levels of replication fork proteins resulting in replication fork stall and TLS activation.

This, however, is only a factor if it disproportionately alters the activation of TLS in cervical cancer cell lines versus HFKs. As it is this does serve as a possible explanation for the levels of Ub-PCNA in the experiments with variable FBS concentrations experiments. CHX only inhibits protein synthesis and does not prevent protein modifications, like ubiquitinations, from occurring. Increased TLS due to CHX treatment potentially provides an explanation for the jump in protein abundance in cervical cancer cell lines in comparison to the mock treatments as well. Even if this is the case, and assuming it affects cervical cancer cell lines disproportionately, this does not negate the fact that the decay curves for the TLS proteins are steeper in HFKs than in cervical cancer cell lines.

The other consideration with upregulation of gene expression, the mechanism, is an important factor in discerning its causation. In the case of cancer, questions arise whether increases might be a byproduct of mutations commonly associated with cancer, characteristics commonly associated with cancerous cells, or a mechanism driven by the carcinogenic factor. For cervical cancer, this means discerning whether the causative factor is due to characteristics associated with the transformation of cells or activity of the HPV oncogenes.

Similarly, to the story of whether TLS protein stability was amplified in cervical cancer, the story of whether the increase in proliferation, that is characteristic of cancer, drives increased stability is complicated. The most important conclusion that can be made is that magnifications to protein stability are not driven by cervical cancer cell lines proliferating faster. In HFFs, it appears that increased proliferation, at a minimum, does not increase the stability of TLS proteins. Based on the data, it appears that increased proliferation might lead to a decrease in TLS protein stability in HFFs.

A possible explanation for the previous claim might be attributed to a set level of protein being maintained within the cellular environment. In other words, HFFs in 0% FBS might appear to have increased stability because TLS protein levels might be maintained at low levels due to inability to proliferate. If this is the case, then the protein levels could potentially persist at the same abundance for 24 hours as a safety net and the stability could be misrepresented. The potential remedy to this circumstance is to continue a CHX treatment time series beyond 24 hours to the point where the abundances for TLS proteins falls to zero. However, a limitation to implementing this experiment is the sensitivity of HFFs. The stressful, possibly compounding, issues of inhibiting proliferation and protein synthesis causes HFFs to die at a higher percentage than cervical cancer cell lines. This limits the viability of continuing a time series beyond 24 hours as the likelihood of having a high enough quantity of cells to lyse for a manageable protein concentration is rather low.

As previously discussed, TLS in normal cells functions to bypass DNA damage during replication[32,36]. The normal function of this pathway involves the expression of the TLS genes as well as proper localization and alternation between normal, replicative and TLS machinery. Our insights into TLS protein stability elevations as a byproduct of increased proliferation have provided further insight into how TLS changes in cervical cancer cell lines. Stability appears not to be a limiting factor in the proper activation of TLS. It is apparent from our results that TLS proteins do not have reduced stabilities and thus might be degraded before functional bypass of damage can occur. Instead, it appears that the stabilities for some of these proteins is increased, contributing to a larger abundance of TLS proteins. The TLS pathway is being altered in cervical cancer cell lines, but these results do not provide evidence that the pathway is being changed in a detrimental way.

Considering the increased proliferation typical of cancerous cells, it logically follows that increased TLS would be necessary to accommodate the increased replicative stresses[31]. It appears that these cells accommodate this increased need by not only altering transcription but also by altering protein stability. However, despite altered proliferation being a logical driving force for increased TLS, it does not appear to be the mechanism for reduced degradation of TLS proteins. Instead, it appears that the underlying mechanism is one that might pertain to some other characteristic of HPV associated transformation, potentially some interaction of the oncogenes with the HR E6/E7 targets. As will be discussed in the next chapter this interaction could be symptomatic of either overlap in function between TLS proteins in other pathways, such as DDR. This would be justified due to HPV's utilization of DDR machinery for its own genome maintenance and amplification. On the other hand, upregulation of the TLS pathway could be a pre-emptive step to efficiently conduct TLS when it is properly activated/an attempt by the cell to troubleshoot improper TLS activation. As briefly mentioned in the introductory chapter, TLS is hindered in cervical cancer cell lines by inadequate expression and localization of pol-eta, as well as the formation of TLS machinery foci. The cell might try to circumvent this hindrance thinking inadequate levels of other TLS proteins are the causative factor or might increase expression of other TLS genes to ensure it is sufficiently prepared when pol-eta is expressed/TLS foci are formed.

Chapter 4 - Insights into Implications and Future Directions

Consequences of TLS Upregulation and Inhibition by HR HPVs

With upregulation to the TLS pathway observed in cervical cancer cells, and increased proliferation not serving as the mechanism, it is plausible that the HR HPVs' genomes are the basis for the observed changes. Therefore, it is worth consideration to ponder what this might mean for cells prior to transformation. If cells pre-integration and transformation are susceptible to TLS upregulation. The consequences, both positive and negative, that potentially result would be an essential aspect of the story of HPV induced cervical cancer. When pondering these implications, it is also important to keep in mind the effects on not only the virus but the host as well.

Although the consequences of HPV infection for cells have been well studied, they are worth revisiting with the additional context of generated results detailed in the previous chapter. HPV-infected epithelial cells have their proliferation and differentiation adjusted to better suite HPV. Notably, in the stratified epithelium, cells are gradually shifted away from differentiation, driven to proliferate[5,6,9]. The cellular environment is skewed during this time, to one where HPV can take better advantage of cellular resources. DDR proteins are recruited for viral propagation, depleting their availability for the cell[13]. The resulting genomic instability is deleterious for both the cell and virus, creating a scenario promoting the viral genome integration[22,31]. Integration results in loss of propagation for the virus and can lead to malignant transformation. Upregulation of TLS might allow for recruitment of TLS proteins by the virus since there are proteins with functionality in both TLS and DDR pathways, for example, TOPBP1 and ATR, and could mean that activating TLS might further deplete their availability for the cell. Thus,

upregulation of TLS offers a chance for increased viral recruitment of proteins involved in propagation, but with an increased risk of genomic instability and subsequent integration.

As briefly discussed during the introduction, upregulation of TLS in cervical cancer cell lines does not increase TLS resolution, with TLS being impeded from proper activation. A possible explanation for inhibiting TLS could be to cause DNA damage as a means to increase recruitment of DDR proteins to viral genomes. Nucleotide starvation potentially generated by increased proliferation, as well as other exogenous or endogenous forms of damage, could generate replication fork stall[31]. Improperly dealing with the stress would result in fork collapse[34]. Since HPV positive cells are shifted towards a more proliferative state and HPV recruits DDR proteins, it serves as at least a basal justification for this rationale[6,13]. Of course, generating DNA damage via failed TLS invites the possibility that the cell dies. Obviously, this is not an ideal situation for either the virus or host. However, the potential for cell death and a halt to the viral propagation cycle might be outweighed by the increase in viral propagation efficiency.

Potential Mechanisms of TLS Upregulation

Although evidence shows that upregulation of the TLS pathway occurs in cervical cancer cell lines, the mechanism is currently not fully understood. We show that increased stability is not a byproduct of increased proliferation. We can make other inferences minimizing the scope of potential mechanisms based on the fact that altered TLS is evident in cervical cancer cell lines. Considering E2 must have been disrupted for transformation to occur in these cell lines, it cannot be a possible source of the mechanism[23]. To further narrow down likely mechanisms, two possible paths should be considered. First, that the mechanism is related to non-essential HPV gene function. These are functions associated with genes that are not present in all HPV types,

having distinct characteristic functions in the HR types and thus not required to be a successful HPV. E6 and E7 of the HR HPVs, with their targeting of p53 and pRB, respectively, comprise this path. Second, is that the mechanism is related to essential HPV gene functions. These are functions of genes essential for HPV propagation and thus do not differ amongst the LR and HR HPV types. Candidate genes for testing the hypothesis that the mechanism is related to essential HPV genes might be shared targets of LR and HR E6 and E7.

The first hypothesis, although narrowed down to only the HR HPV types, still is a daunting task to tackle. In an attempt to make discerning the mechanism more approachable, a more narrowed hypothesis is essential. E7's degradation of pRB would be the most logical place to start. E7 has been previously shown to have an impact on the stabilities of other proteins. Not only does this limit the scope to a single protein, but this enhanced stability was linked to a specific domain. E7's pRB binding domain is essential to elevate other proteins' stability[13]. This suggests a mechanism specific to HR HPV types. If E7 is involved in the process, this could also provide an explanation for the observed differences in SiHa and HeLa. Although both cervical cancer cell lines contain HPV types with E7s that interact with pRB (types 16 and 18), they utilize different cullin family proteins to initiate this interaction. Notably, these experiments showed that HR types other than 16 and 18 showcased this phenotype of altering protein stability[28]. Finally, these experiments found that cell lines expressing the entire genome of HR HPV types showcased comparable protein stability enhancement to cell lines expressing the E7 gene solely[13]. This suggests that it is possible that TLS protein stability is elevated prior to HPV integration and cellular transformation.

Consequences of TLS Upregulation and Inhibition for HPV Positive Cervical Cancer Cells

Although the causation of TLS alteration is not fully understood and the possible implications that the causative factor might have prior to integration and transformation are left unresolved, we know that this process is occurring in transformed cells. This raises the question of what implications this has for cancer cells. It has been shown that cervical cancer cell lines fail to establish pol-eta foci, while forming foci of failed TLS markers (Fig.3). Whether or not the mechanism which impedes these processes is linked to the mechanism with which other aspects of TLS is altered is not known. However, the fact that both these alterations are present in the same cell lines does paint a possible story of the consequences they incite.

By impeding proper resolution of TLS cervical cancer cell lines have created a cellular environment where replication fork stall and subsequent collapse are prevalent upon DNA damage or replication stress induction. This increased chance of replication fork collapse also means activation of DDR pathways, to repair the subsequent DSBs, by the cell and recruitment of the proteins in those pathways by HPV[42–44]. This increased rate of damage and possible failed resolution of that damage would normally increase the potential that cells undergo apoptosis. However, given the expression of E6 in these cells and its interaction with p53 inhibiting apoptosis this is not the case[27]. This scenario means that cells are undergoing numerous mutations from improper DDR. Even if some cells were undergoing proper apoptosis under these circumstances, lethal phenotypes are not observed. Only genotypes resulting in viable cells, those impeding apoptosis, would be the ones that are observable. The cells undergoing apoptosis would be removed from the population while the other cells prevail.

This story of TLS alteration and impedance concludes with cells whose cellular environment has been changed resulting in a higher mutagenesis rate. This increased rate of mutation might mean that some cells die due to the terminal nature of some mutations. Although similar to cells that might still undergo apoptosis, lethal phenotypes would quickly be removed from the cellular population. This increased mutation rate also means that cells that develop a resistance to these stresses would likely have their genotypes selected for. These favorable genotypes, by being able to sustain these stresses, would decrease their likelihood of being removed from the population. Unfortunately, considering as these stresses relate to DDT and DNA damage, tolerating them might lead to cells resistant to cancer treatments. Considering the basis for cisplatin, as an essential component of cervical cancer's standard of care, generates crosslinks which require TLS to bypass, these cells would likely be predisposed to tolerate cisplatin[32]. In the case cisplatin was used in a situation where these cells were present, it would further select for those cells and allow their genotypes to take over the population. This scenario would further decrease the survival rate of the patient, essentially negating any treatment options.

Implications for Other HPVs and HPV-Associated Diseases

The fact that the mechanism for TLS alteration might not be unique to cervical cancer or even HR HPV types has significance for other HPV associated diseases. LR HPV types which cause benign lesions, such as genital warts, also affect cellular proliferation and if TLS alteration was a shared characteristic between LR and HR types it would provide further information to approach treatment of diseases associated with those HPV types[16–18]. On the other hand, the HR types do not solely cause cervical cancer. They are also responsible for contributing to cases of urogenital, anal, head, and neck cancers[16,18]. If TLS alteration was shared amongst the HR types, independent of which portion of the body they infected, then it would mean all HPV induced

cancers have a specific, limited homogeneity. This common thread amongst these cancers, outside of HPV integration might allow advances in knowledge or treatment of one cancer to be applied to, or serve as a stepping stone, to another cancer. Despite the high prevalence and association of cervical cancer with HPV, the other types of diseases have a huge impact in their own right. In the end, the stories of these cancers might share common threads, might be intertwined.

Future Directions

Considering the unresolved mechanistic details of TLS alteration in cervical cancer cells, most of the future directions that should be pursued relate to dissecting those aspects. These details can be grouped into three parts. One, whether the mechanism occurs prior to integration and transformation. Two, discerning if the process relates to the unique, discussed targets of HR HPVs (p53 and pRB). Three, determining the specificity of the procedure amongst the HPV types.

The first category could be approached by analyzing stabilities of TLS proteins in HPV oncogene-positive HFKs and cell lines expressing the entire HPV genome. Analyzing HFKs expressing the oncogenes would provide insight on whether transformation is required and would be representative of post-integration cells. These cells could further be grouped into three categories. The first is the expression of either E6 or E7 alone to understand the effects each oncogene had on cells individually. The second is an expression of both oncogenes together which would help analyze the additive or multiplicative effects that they might have, as well as addressing the fact that the mechanism might require both functional oncogenes. The final category is high passage E6/E7 expressing HFKs. This would be HFKs expressing both oncogenes (as both are required for transformation) at several different passages. This would

allow analysis of the process of transformation in these cells and how TLS was altered in parallel.

The second category could be approached by limiting the unique functions of the E6 and E7 genes. E6 interaction with p53 could be inhibited by using RNAi against E6AP, the protein it interacts with to cause the proteasomal degradation of p53[27]. E7 has a mutant available whose pRB domain has been removed, thus restricting interaction with pRB to cause E2F's disassociation with it, as well as pRB's proteasomal degradation[13]. This domain should logically serve as the starting point due to experiments conducted in other labs. However, other E7 binding domains (PDZ and hTERT) should be studied if the pRB binding domain fails to resolve any questions. Both of these approaches would allow a deeper analysis of what aspects of those genes might be playing a role in TLS alteration as well as whether or not it was specific to the unique functions of the oncogenes found in HR HPVs. By doing comparisons amongst cell lines expressing the full genome, individual oncogenes, or both oncogenes, in combination with or lacking the proper inhibitor, the significance of p53 and pRB targeting functions could be analyzed.

The final category could be addressed both through the experiments detailed in the previous paragraph, as well as by studying the effect of full genome expression of other HPV types in cells. Elucidating protein stability in these cell lines would provide an understanding of whether or not the mechanism was specific to HR HPV types or was ubiquitous amongst the LR and HR types. These experiments would also provide insight into whether the results from experiments studying alterations to TLS could be applied to other HPV associated diseases.

Final Remarks

Elucidating the mechanistic details of TLS upregulation and inhibition has three main components: address when it occurs during HPV interaction with epithelial cells, determine the potential homogeneity of TLS alteration amongst HPV types and discern the mechanism by which it occurs. This undertaking is no simple task, with each component serving as a sufficient project for a research project. However, the knowledge to be gained offers a plethora of applicability. Treating HPV-associated diseases, as well as furthering our understanding of both cancer and the TLS pathway stand to benefit from such information. Discerning at what levels upregulation to the pathway is accounted for and the reason HPV causes this alteration is a conundrum. What purpose does this serve for either the cell or the virus? The same question could be asked of the failure to adequately activate TLS by failed polymerase eta foci formation. Answers to these questions are currently only hypothesized, but the implications of evolutionary consequences in relation to the story of HPV-induced cervical cancer, highlight the importance of adopting seemingly obscure mindsets when studying and defining cancer.

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