Degradation of pRb by Retrovirally Infected Cell Lines

by

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Submitted in partial fulfillment of the requirements for graduation as an Honors Scholar at Point Loma Nazarene University, San Diego, California, June 12, 2021

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Abstract

The human papillomavirus (HPV) contains hundreds of serotypes that have been divided into low-risk versus high-risk variants. Whereas low-risk serotypes have been shown to cause benign skin lesions, the high risk serotypes are associated with various types of cancer, including cervical cancer. Research has demonstrated that high-risk HPV encodes an oncoprotein E7 that binds to and degrades the tumor suppressor retinoblastoma protein pRb. This project investigated the use of retroviral infection to establish a stable cell line to be characterized via western blot protein analysis. Both BJ and HT-1080 cell lines infected with HPV retroviral particles showed significant decreases in pRb expression in the western blot assay. In addition, the hydrophobicity of E7 protein sequences of low risk and high risk HPV serotypes was analyzed using the Grand Average of Hydropathy (GRAVY) calculator. Analysis of 24 well-classified serotypes demonstrated a negative correlation between risk level and hydrophobicity. Future experiments are needed to verify the presence of the viral protein E7 in HPV infected cells in order to confirm the linking of pRb degradation to E7 function.

Introduction

Human Papillomavirus (HPV) is the most common sexually transmitted disease in the United States today. Around 79 million people are currently affected with the virus, with about 14 million new infections occurring each year. Nearly 80% of sexually active people will be infected with the virus at some point in their lives.[1] However, this is not only an American problem. In developing countries, the spread of HPV continues to increase as a result of low socioeconomic status, lack of population awareness, and insufficient screening and vaccination problems.[2] Unfortunately, without proper attention developing countries will maintain a trend of worsening HPV rates.

Typically, chemical carcinogens and radiation are thought to be the main causes of cancer; however, around 15-20% of cancers worldwide are caused by viral infection.[2] Viruses require a host cell in order to survive and replicate. They use a cell's existing machinery to express viral proteins and to ensure viral replication and propagation.[3] The better the virus is at causing cells to divide rapidly and in turn generate more host cells, the better chance the virus has to survive and pass on its genetic information. Thus, viral proteins have become more and more tumorigenic over time because this provides the virus an evolutionary advantage. In many cases, viral proteins function so well to create uncontrolled cell growth that infection can lead to cancer.

HPV is an example of a cancer-causing virus. Currently, two classifications of HPV exist, low-risk and high-risk, based on the symptoms induced following infection. Infection with low-risk HPV serotypes leads to benign skin lesions such as genital warts. High-risk infection can lead to a multitude of cancers, including cervical, oropharyngeal and penile cancers. Shockingly, research shows that 99.7% of cervical cancer is associated with high-risk HPV.[4] Cervical cancer is currently the leading genital cancer in women worldwide, with nearly 500,000 new cases per year. In 2015, 526,000 women developed cervical cancer and 239,000 of these women died.[2]

Unfortunately, if the rate of HPV infection continues to rise worldwide, the amount of deaths from cervical cancer will follow suit.

One key protein attributed to HPV based cancer is the viral E7 protein expressed following infection. Both high-risk and low-risk serotype E7 contain an LXCXE pRb binding motif through which E7 can bind the tumor suppressing pRb protein. Binding of E7 to pRb renders pRb inactive and leads to the release of the E2F transcription factor.[5] Once released from its inhibitor pRb, E2F enters the nucleus where it promotes the transcription of genes involved in cell cycle progression. In addition, a distinct motif of the E7 protein recruits cyclic-AMP response element binding protein (CBP) and its paralog p300, which form a complex that acetylates the pRb and initiates its degradation via the ubiquitin proteasome pathway.[6] Thus, the viral E7 protein binds to tumor suppressor protein pRb, prematurely releasing the pRb-bound cell division factor E2F and marking pRb for degradation (figure 1). Currently, high-risk HPV E7 proteins have been shown to degrade pRb at higher rates than low-risk forms of E7.[7] Degradation of a tumor suppressor in cells disrupts the regulation of the cell cycle and can lead to uncontrolled cell growth. Therefore, viral E7 plays a significant role in causing HPV-linked cancers.

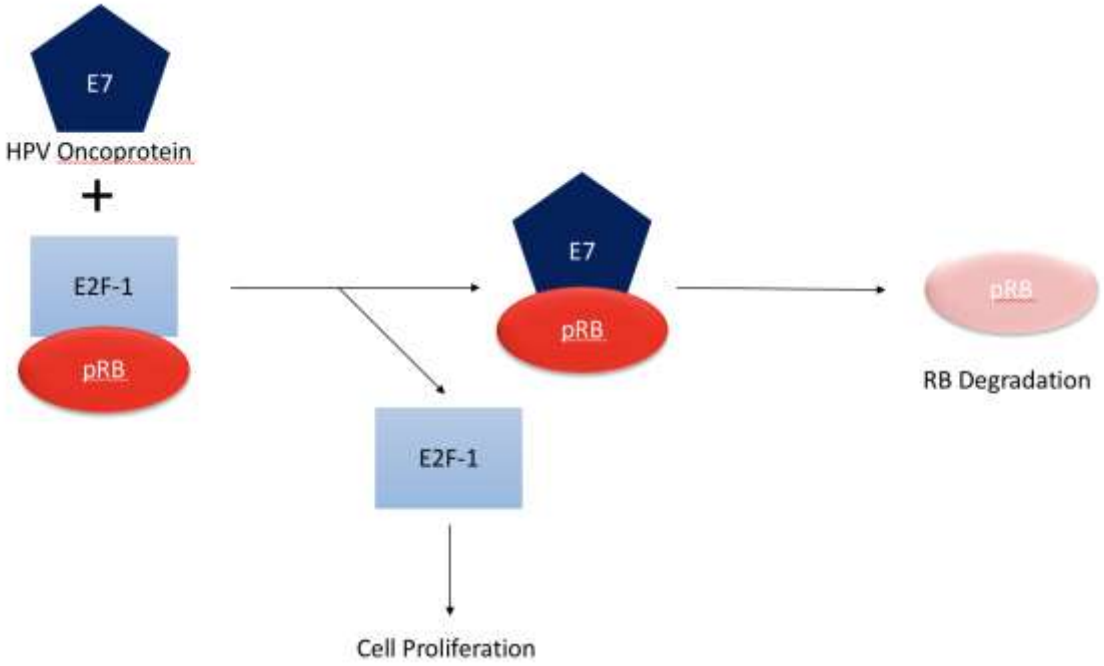

Figure 1. HPV protein E7 binds to pRb, displacing E2F-1 to enter the nucleus and promote cell proliferation. E7 binding initiates a cascade of events leading to degradation of pRb protein, resulting in a decrease of cell cycle inhibition.

Clearly, the viral proteins in high-risk HPV serotypes are equipped to dysregulate the cell cycle and cause cancer when compared to low-risk HPV, but the mechanism of action is not fully understood. It is also unclear why different HPV serotypes result in such different phenotypic outcomes. Research is currently studying differences between the high-risk and low-risk E7 proteins to explain why high-risk HPV is more cancerous. One finding suggests the important role of the amino acid directly preceding the LXCXE pRb binding motif. In the well-

known high-risk serotype HPV-16, the E7 LXCXE binding motif is preceded by an aspartic acid while the low-risk HPV-6 E7 motif is preceded by a glycine. A single mutation (G22D) in the low-risk HPV E7 to replace this glycine with an aspartic acid to mimic the high risk-sequence is enough to significantly increase the low-risk E7's affinity for pRb.[8] Other research points out the possible significance of phosphorylation of serine residues slightly downstream of the LXCXE binding motif.[7] The LXCXE binding motif is found in the second conserved region (CR2) of the E7 protein's intrinsically disordered regions.[5] Intrinsically disordered regions are highly exposed to the aqueous environment of the cell, so it would be safe to assume that a more polar protein sequence would be more stable in the cell. Phosphorylation makes a protein more polar and could stabilize the high-risk form of the E7 protein, allowing it to bind pRb more efficiently. Therefore, the degree of hydrophobicity of HPV E7 protein sequences must be studied to determine if a correlation exists between hydrophobicity and risk-level for cancer following HPV infection.

In the past, this lab has attempted to create a stable HPV cell line through transfection of an HPV plasmid but has had difficulty in visualizing Rb degradation in transfection models due to poor transfection efficiency. This study used retroviral infection of two cell lines (BJ and HT-1080) with either E7 from high-risk (HPV-16), E7 from low-risk (HPV-6), or vector control pLXSN viral particles to establish stable HPV-infected cell lines. Next, the cell lines were characterized and degradation of pRb was visualized using western blot analysis. Furthermore, high and low-risk HPV E7 protein sequences were analyzed using the Grand Average Hydropathy (GRAVY) calculator to ascertain a correlation between hydrophobicity and tumorigenicity.

Methods and Materials

Antibiotic Selection Kill Curve

100,000 cells were plated in each well of a 24 well plate with increasing concentrations of Geneticin antibiotic. Cell death was analyzed for 5 days to determine the optimal concentration of antibiotic to kill cells without the HPV plasmid which co-expresses the geneticin resistance gene . Each concentration was tested in duplicate for both BJ and HT-1080 cells. Cell survival (% confluency) was plotted for each concentration at day 5 to determine optimal concentration of Geneticin to induce cell death.

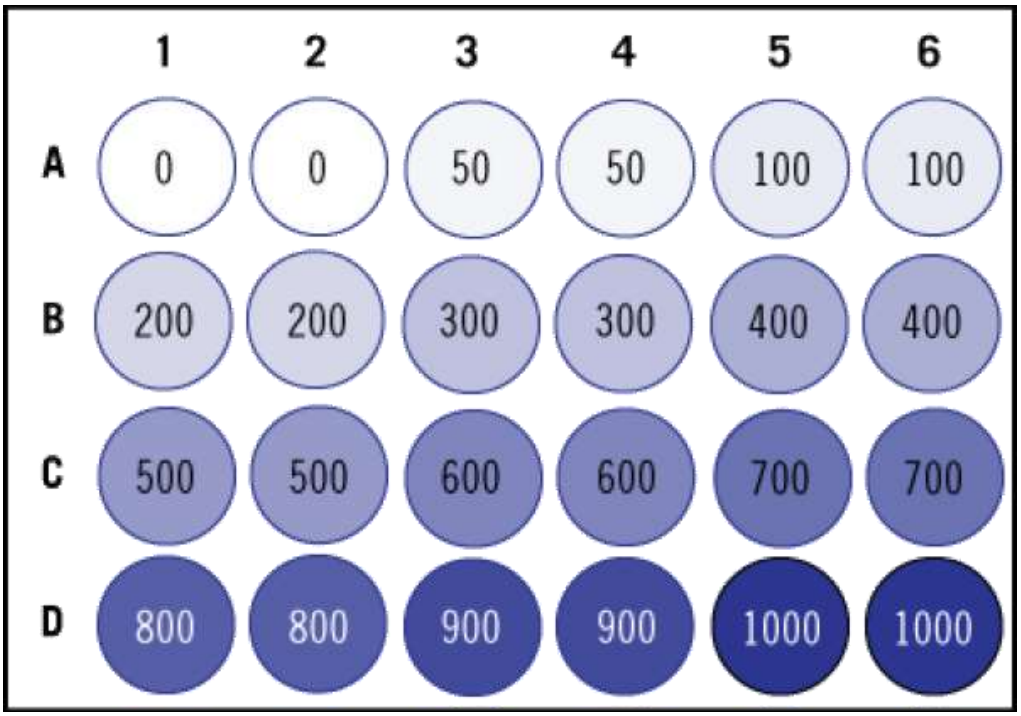

Figure 2. Plate setup for geneticin kill curve test. Each well contained 100,000 cells and concentration increased from 0ug/mL to 1 mg/mL.

Determining the Toxicity of Polybrene

Polybrene is a cationic polymer that can greatly enhance infection efficiency in cell lines. To determine the appropriate concentration to aid in HPV infection of BJ and HT-1080 cells and test for toxicity to cells, 25,000 BJ cells and 50,000 HT-1080 cells were plated with increasing concentrations of polybrene. No toxicity was observed to the cells so 5 ug/ul of PB were used in infection protocol.

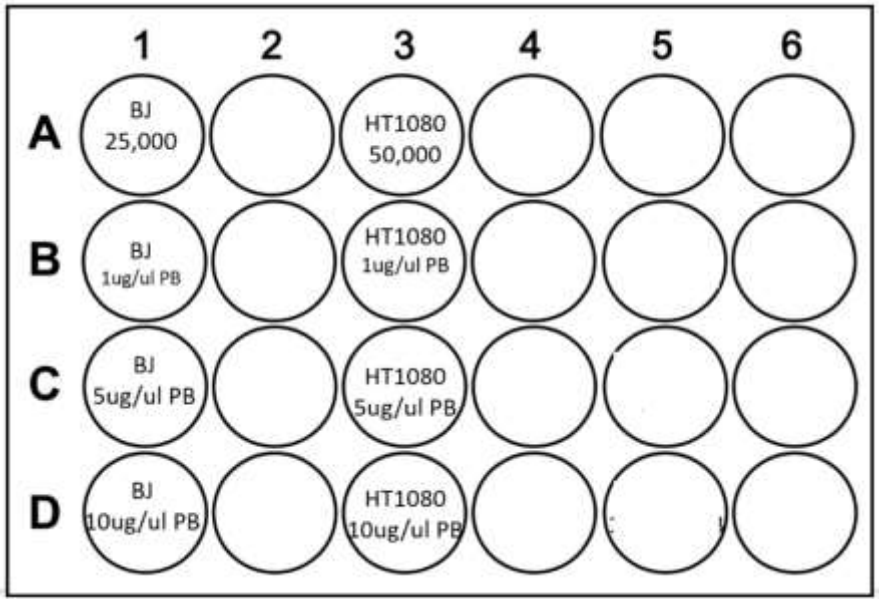

Figure 3. Plate setup for polybrene toxicity test.

Constructs

The HPV16 E7 and HPV6 E7 plasmids used in retroviral infection of BJ and HT-1080 cells were obtained from the plasmid repository Addgene. The HPV16 E7 plasmid can be found at this link: https://www.addgene.org/52396/. The HPV6 E7 can be found at this link: https://www.addgene.org/52398/. The Moloney murine leukemia virus (MMLV) retroviral particles used in this research were synthesized by VectorBuilder. Packaging, storage information, and protocols can be found here: https://en.vectorbuilder.com/products-services/service/mmlv-retrovirus-packaging.html.

Determining the Multiplicity of Infection (MOI) and Retroviral Infection

A multiplicity of infection (MOI) is the number of infectious particles per cell in an infection media. It is important to determine the MOI before infection in order to maximize the amount of infected cells while minimizing the amount of viral particles used. An MOI was determined by plating cells with increasing MOIs of 0, 1, 5, and 10 and visualizing plasmid induced GFP expression using a fluorescent microscope at 48 hours and 72 hours. The protocol for MOI determination can be found here: https://docs.google.com/document/d/1BwYwdFUa9eutktCAR-PYDq-rimPzFT77/edit. The protocol for HPV E7 infection including the time course, dosing, and additional protocols can be found here: https://docs.google.com/document/d/1BwYwdFUa9eutktCAR-PYDq-rimPzFT77/edit.

Western Blotting

Expression of the pRb protein was analyzed via western blot analysis. Cell lysate was collected using RIPA buffer (supplemented with protease inhibitors). Cell lysate was centrifuged at 14,000xg. Final whole cell lysate protein concentrations were determined using the standard BCA protocol. 20 ug of protein lysate, 8.75 uL LDS, and 3.5 uL of reducing agent were added to each sample and water was added to a final volume of 35 uL. For the Rb control sample 10 ng of purified Rb protein was added to the well with 2.5 uL of LDS sample buffer (Invitrogen), 1 µL

of Bolt Reducing Agent (Invitrogen), and 5.5 ul of Milli-Q water. Sample mixtures were incubated for 10 min at 70 degrees C. Following incubation, experimental protein samples, control samples, and 3uL of iBright protein ladder were added to a Bolt 4-12% Bis-Tris Plus gel. The gel was run at 200 V and 40 mA for 1 hour. The gel was transferred to a methanol activated PVDF membrane using a blot sandwich. Order of assembly was cathode core, sponge pad, filter paper, gel, membrane, filter paper, two sponge pads, anode core. The transfer ran at 20V and 390mA for 1 hour. Following transfer, the membrane was washed with milli-Q water and blocked with a milk buffer in the fridge for 2 hours. Then the membrane was transferred to a primary antibody milk buffer solution containing a 1:500 Rb antibody (catalog number: ab181616) solution and 1:2500 GAPDH antibody (catalog number: ab8245). GAPDH was used as the loading control. The membrane was in the primary antibody wash on a rocking platform in the fridge overnight. The following day, the membrane was washed 6 times with TBST to remove the primary buffer solution and placed into a secondary antibody solution for 1 hour. This secondary antibody solution consisted of 1:10000 goat anti-rabbit secondary (HRP catalog number 31460) to bind Rb primary antibody and 1:2000 goat anti-mouse secondary (HRP catalog number 62-6520) to bind GAPDH primary antibody. Following the hour, the membrane was washed and the membrane was placed in a peroxide Luminol/Enhancer working solution for 5 min in preparation for visualization. Lastly, the membrane was placed in the imaging machine and ran on chemiluminescence setting to visualize the protein. The western blot protocol can be found here: https://docs.google.com/document/d/1q0Q7U_uEAAuFhEtC4Hnl_BFGnPnH2yAtfs9e5_ZCpJg/edit

Grand Average of Hydropathy Determination

HPV serotype sequences were obtained from NCBI protein database (https://www.ncbi.nlm.nih.gov/protein/). Sequences obtained in FASTA format were copied and pasted into the GRAVY calculator website (http://www.gravy-calculator.de) which calculates a grand average of hydropathy of the sequence. This value is calculated by summing the assigned hydropathy values of each individual amino acid in the sequence and then dividing by the protein sequence length. The higher the value, the more hydrophobic that sequence is. Well-classified sequences, based on literature support, were grouped into high risk (red) and low risk (green) then graphed together in Excel. The compiled sequence information, as well as the final data table and graph can be found here: https://docs.google.com/document/d/1ZElkkuNlbWWzulVHbc7WCpUqXUwizY-UBY6mnClMXY8/edit.

Results

The first step in establishing a stable cell line is determining an antibiotic selection concentration. The geneticin kill curves for BJ and HT-1080 cells (fig. 4a and 4b) were completed in order to find the optimal concentration of antibiotic to kill uninfected cells without the plasmid induced geneticin resistance. After 5 days in geneticin, it was determined that a concentration of 300 ug/mL was sufficient to kill BJ cells and a concentration of 400ug/mL was

sufficient to kill HT-1080 cells. These concentrations were chosen based on the sharp drops in cell survival after 5 days of exposure to each concentration of geneticin (fig. 4a and 4b). Moving forward, these concentrations of geneticin were used for selection. Polybrene is a polymer that can greatly enhance retroviral infection efficiency, so a test for toxicity of polybrene at various concentrations was conducted to determine a beneficial dose to add to our infection protocol. Using the plate setup shown in figure 3, the test demonstrated no toxicity to BJ or HT 1080 cells. As a result, a concentration of 5ug/uL was used in the infection protocol.

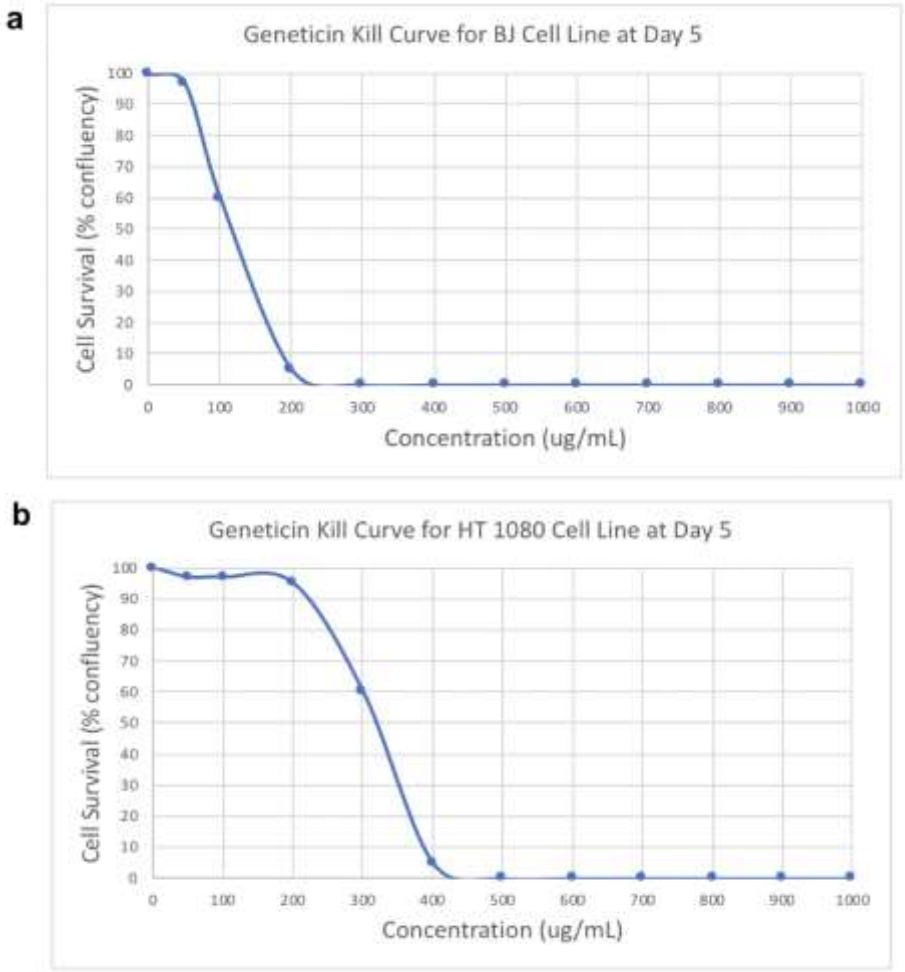

Figure 4. Antibiotic kill curves to determine optimal concentration for selection of HPV plasmid induced antibiotic resistance in BJ and HT-1080 cells. Cells were treated with increasing concentrations of G418 (Geneticin) antibiotic and analyzed for cell death on day 5. a.) A concentration of 300 ug/mL was sufficient to kill off all BJ cells by day 5 and thus the optimal concentration of geneticin to select for HPV-infected BJ cells. b.) A Geneticin concentration of 400 ug/mL was used to select for antibiotic resistance in HT-1080 cells.

The fluorescent microscopy images in figure 5 are a representative group of images taken of HT-1080 cells 48 hours post-infection. Cells were infected with increasing MOIs of 0, 1, 5, and 10 viral particles per cell. The blue in the top row of images is DAPI staining of cell nuclei

used to visualize all cells. The green in the bottom row highlights plasmid induced GFP expression which indicates a successfully infected cell. An optimal MOI would achieve maximum infection while using as few viral particles as possible. Analysis of the many images represented by figure 5 demonstrated an increase in infected cells from 1 to 5; however, this increase was not 5-fold. Therefore, an MOI of 2.5 was used moving forward to avoid diminishing returns associated with high MOIs.

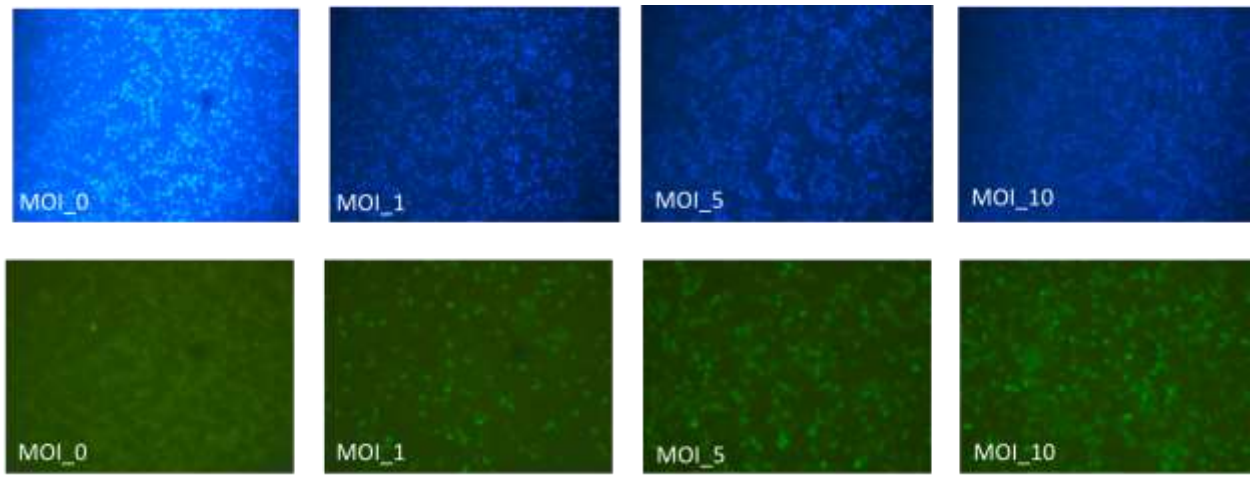

Figure 5. To determine the optimal multiplicity of infection (MOI) of BJ and HT-1080 cells, increasing MOI's were tested on cells. The top row shows DAPI staining of the cell nuclei while the bottom row shows green fluorescence associated with a GFP control plasmid. Above is a representative image of HT-1080 cells 48 hours after viral infection with an MOI of 0, 1, 5, and 10. BJ and HT 1080 cells were analyzed at 48 and 72 hours post infection to determine an MOI of 2.5 maximizes infection while using minimal viral particles.

Having determined no toxicity to cells by polybrene (figure 3) and having established an effective MOI using GFP control plasmids (figure 5), retroviral infection of BJ and HT-1080 cells was completed. Cells were incubated in media containing 5 ug/uL of polybrene and an MOI of 2.5 overnight to achieve infection. 72 hours post-infection, cells were exposed to geneticin at the concentrations determined in figure 4 to ensure HPV E7 and pLXSN plasmids had successfully been transduced. Viral plasmids contained geneticin resistance genes, therefore only successfully infected cells would survive the selection period. After 5 days of selection, only infected cells remained. Cells were continuously cultured in media containing geneticin in order to maintain newly established infected cell lines.

Once two HPV infected cell lines were established (HPV16 and HPV6), as well as the control plasmid pLXSN, western blotting was used to characterize the cell lines. The high risk HPV-16 E7 protein has been shown to increase degradation of pRb more than low risk HPV-6 E7. Thus, it was hypothesized that cells infected with high risk HPV-16 would have less pRb protein than low risk HPV-6 infected cells. In a western blot, this would be observed by a thicker and darker band being presented in wells from low risk infected cells. Nevertheless, some degradation would be expected in the low-risk well compared to control cells without any viral E7. Accordingly, the pRb band was expected to be brightest in control wells, followed by low risk

wells, with high risk wells having the least pRb. In addition to wells containing HPV infected cell lysate, a control pLXSN plasmid cell line lysate was used to ensure that plasmid infection did not disrupt pRb levels. Rb levels in this cell line were expected to be the same as control cells without the vector control plasmid. GAPDH was used as a loading control to ensure equal loading of cell lysates into each well. Equal sized GAPDH bands validates comparisons made on pRb expression. Figure 6 demonstrates increased pRb degradation in high-risk HPV-16 infected cells compared to low risk HPV-6 infected cells and control cells for both BJ and HT-1080 cells. For an unknown reason, BJ cells infected with pLXSN plasmid did not show pRb. Surprisingly, control HT-1080 cells exhibit higher amounts of pRb compared to pLXSN HT-1080 cells, but this is likely due to a slight overloading of cell lysate in the well, evidenced by higher amounts of GAPDH in control HT-1080 cells. Differences can be accounted for by the differential loading, so it was concluded that HPV-16 E7 significantly degrades pRb and HPV-6 E7 moderately degrades pRb compared to control cells.

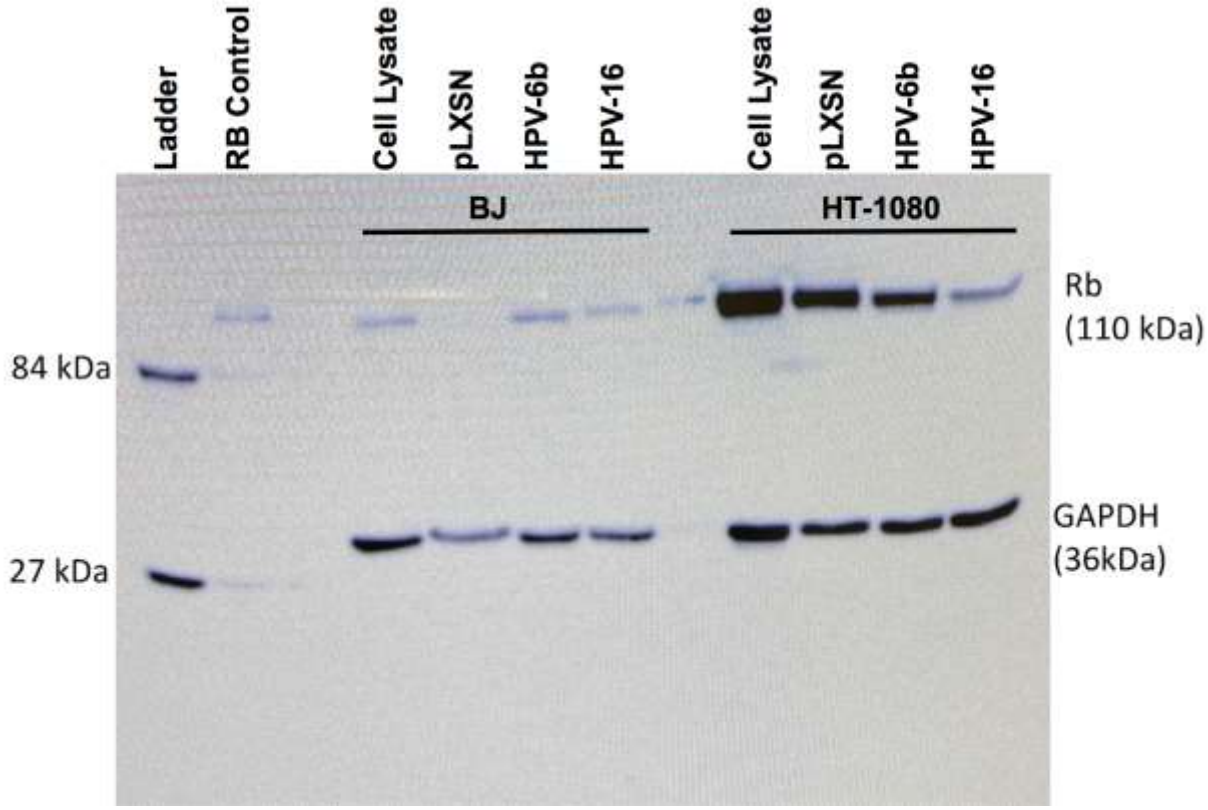

Figure 6. Western blot of BJ and HT-1080 protein lysates. A protein ladder was used in lane one to determine protein sizes and identify pRb and GAPDH. An Rb protein sample was loaded into lane two to test the effectiveness of our Rb antibody. Both cell lines showed a decrease in retinoblastoma protein (pRb) in high-risk (16) HPV infected cells compared to low-risk (6b) HPV infected cells. This provides evidence that the viral E7 protein of high-risk HPV degrades pRb better when compared to low risk HPV E7 protein. GAPDH was used as a loading control to ensure equal loading of protein in the wells.

Figure 7 shows the Grand Average Hydropathy Values for various well-classified high risk and low risk HPV serotype E7 amino acid sequences. The green low risk bars demonstrated higher hydropathy values overall compared to the red high risk serotypes. The higher the hydropathy value, the more hydrophobic the sequence was overall. Therefore, it was concluded that low risk E7 sequences were more hydrophobic than the high risk E7 sequences. Furthermore, this data demonstrates an inverse correlation between hydrophobicity and risk level in HPV E7 protein sequences.

Figure 7. Grand Average of Hydropathy (GRAVY) values for various HPV E7 protein sequences showed an inverse correlation between hydrophobicity and HPV risk level. Low risk serotypes are colored green, with the well known HPV 6 sequence checkered. High risk serotypes are colored in red, with well known HPV 16 and 18 sequences checkered. The higher the hydropathy value, the more hydrophobic.

Discussion

Using the geneticin selection concentrations and MOI determined in figures 4 and 5, retrovirally infected BJ and HT-1080 cells were successfully created. Standard cell culture practices were used to grow up these cell lines, and infected cells were frozen down for future experiments. The next step was to characterize these retrovirally infected cell lines using a western blot to visualize pRb degradation through the well studied activity of HPV E7 proteins.

Not only did we visualize degradation of pRb in HPV infected cells compared to cells infected with the control plasmid pLXSN, we also observed a difference in degradation levels between high and low risk forms of the virus. The high risk HPV16 infected cells degraded pRb substantially more than low risk HPV6 infected cells in both BJ and HT-1080 cell lines (see figure 6). Thus, we successfully established two HPV infected cell lines and characterized these cells by visualizing tumor suppressor degradation.

Lastly, our research found an interesting trend in E7 protein sequences of high risk and low risk HPV serotypes. Using only well classified strains, figure 7 shows the GRAVY hydropathy values of low risk serotype's E7 sequence were more hydrophobic than high risk sequences. In the aqueous environment of the cell, this trend indicates a relationship between E7 hydrophobicity and its ability to bind to pRb and mark it for degradation. Further studies are needed to narrow in hydropathy analysis on the intrinsically disordered regions of the E7 protein. These regions are loosely structured and more exposed to the aqueous environment of the cell, yet incredibly important to E7 function. The more hydrophobic an amino acid sequence is, the less exposed it will be in the cell's aqueous environment. Thus, if these essential regions of E7's sequence exhibit the same trend of low risk forms being more hydrophobic than high risk forms, we will have provided a valid explanation for the difference in E7 degradation capabilities between cancerous high risk and benign low risk forms of HPV. Further single point mutation studies will be done to discover key amino acid differences accounting for the varying high risk and low risk severities. For example, sequence alignment tools can be used to discover hydrophobicity differences of key amino acids throughout high risk and low risk E7 sequences. This will provide clues on which amino acids might play a crucial role in optimal E7 protein fold and function. If altering the hydrophobicity of a single amino acid of the E7 protein changes pRb levels in a western blot from mutant E7 infected cells, the hydrophobicity of this amino acid is important to E7 function. Therefore, the trend in hydrophobicity found in this research provides insight into future E7 single point mutation experiments which can be analyzed using western blot visualization of pRb.

Additional research can be used to further characterize our established HPV infected cells. First, using immunocytochemistry, we can visualize E7 proteins in HPV infected cells to confirm E7 as the causative agent for pRb for degradation as seen in the western blot shown in figure 6. In addition, to analyze the relative tumorigenicity of high risk vs low risk cells, a cell viability assay could be used. E7 proteins release E2F to enter the nucleus and promote continuation of the cell cycle and cell division, leading to rapid cell proliferation. If E7 functions better in high risk serotypes, our HPV16 cells should show faster rates of division and more cells in a viability assay. Accomplishing these experiments would significantly increase our confidence in the validity of our HPV infected cell lines.

References

1. Human Papilloma Virus (HPV): Causes, Testing, Treatment & Prevention. Accessed April 3, 2021. https://my.clevelandclinic.org/health/diseases/11901-hpv-human-papilloma-virus

2. Chan CK, Aimagambetova G, Ukybassova T, Kongrtay K, Azizan A. Human Papillomavirus Infection and Cervical Cancer: Epidemiology, Screening, and Vaccination - Review of Current Perspectives. *J Oncol*. 2019;2019. doi:10.1155/2019/3257939
3. Ryu W-S. Virus Life Cycle. In: *Molecular Virology of Human Pathogenic Viruses*. Elsevier; 2017:31-45. doi:10.1016/b978-0-12-800838-6.00003-5
4. Lehoux M, D'Abramo CM, Archambault J. Molecular mechanisms of human papillomavirus-induced carcinogenesis. *Public Health Genomics*. 2009;12(5-6):268-280. doi:10.1159/000214918
5. Lee C, Kim DH, Lee SH, Su J, Han KH. Structural investigation on the intrinsically disordered N-terminal region of HPV16 E7 protein. *BMB Rep*. 2016;49(8):431-436. doi:10.5483/BMBRep.2016.49.8.021
6. Jansma AL, Martinez-Yamout MA, Liao R, Sun P, Dyson HJ, Wright PE. The high-risk HPV16 E7 oncoprotein mediates interaction between the transcriptional coactivator CBP and the retinoblastoma protein pRb. *J Mol Biol*. 2014;426(24):4030-4048. doi:10.1016/j.jmb.2014.10.021
7. Heck D V., Yee CL, Howley PM, Munger K. Efficiency of binding the retinoblastoma protein correlates with the transforming capacity of the E7 oncoproteins of the human papillomaviruses.*Proc Natl Acad Sci U S A*. 1992;89(10):4442-4446. doi:10.1073/pnas.89.10.4442
8. Zhang B, Chen W, Roman A. The E7 proteins of low- and high-risk human papillomaviruses share the ability to target the pRB family member p130 for degradation. *Proc Natl Acad Sci U S A*. 2006;103(2):437-442. doi:10.1073/pnas.0510012103