Visualizing the Degradation of pRb by the High-Risk HPV Protein E7

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Abstract

The oncoprotein E7 coded for by the human papillomavirus (HPV) is known to degrade the tumor suppressor retinoblastoma protein (pRb). Transient transfections of a plasmid coding for the E7 protein were not effective at visualizing degradation of pRb. Although transient transfection would be efficient in visualizing pRb degradation, the lack of success makes more reliable methods, such as the use of retroviruses, necessary.

Background

HPV, or Human Papillomavirus, affects 79 million Americans, with approximately 14 million new people being infected each year. HPV is a DNA virus from the papillomavirus family with more than 170 discovered strains, 40 of which spread through sexual contact. Viral infections like these are causal contributors of 15-20% of all human cancers, and “According to the World Health Organization’s (WHO) statistics, common cancers are one of the most prevalent causes of mortality worldwide with 8.2 million deaths in 2012” [1]. High-risk HPV DNA is found to be present in 99.7% of cervical cancer specimens, as high-risk HPV behaves differently than most HPV infections. 90% of HPV infections become inactive between and 12 and 24 months, however, high-risk types persist and increase the risk of cervical cancer [1]. HPV has been linked to vulvoginal and penile cancers, and “accumulating evidence suggests that HPV is associated with a subset of head and neck cancers, and in particular those of the tonsils” [2].

The papillomavirus genome is approximately 8000 base pairs in length and consists of three regions - early proteins, regulatory proteins, and oncoproteins. The capsid of the virus is coded by a 3000 base pair region that encodes L1 and L2, two structural proteins. E1, E2, and E4 are the regulatory proteins, and E5, E6, and E7 are the oncoproteins. “Sequence variations such as single-nucleotide polymorphisms or genetic mutations within L1, LCR, E6, and E7 regions of HPV can determine families, relatedness, and phylogeny of the HPV types” [1]. 60 out of 160 HPV types are associated with mucosal epithelial, and are categorized as Alphapapillomavirus genus, or alpha-PV. Alpha-PV can be classified into nine groups, and these groups include the oncogenic high-risk types.

Viruses, which infect a host cell to take over and reproduce, can cause cancer by promoting proliferation in order to reproduce rapidly, thus benefiting the virus. HPV, through the oncogenic E6 and E7 proteins, can infiltrate mucous membranes and cause uncontrolled proliferation. Thus, the virus can cause cancer and use rapid cell division to expand in its host. HPV 16, a high-risk type of the virus, has been classified as having a different phosphorylation rate in the E7 protein than other high-risk types [3]. The expression of the E7 protein can be observed in-vivo using human cell lines, specifically HT1080 cells (homo sapien connective tissue). In-vivo models can be used to transfect, express, and monitor the activity of the E7 protein, as well as the correlation between E7 expression, phosphorylation rates of E7, and high-risk types of HPV.

There are several different ways that the HPV virus can cause cancer. The two main proteins involved are the oncoproteins E6 and E7 and their ability to sabotage cellular

machinery to induce carcinogenesis [4],[5]. E7 has a wide variety of intracellular targets, including the Rb family member protein p130, but more importantly pRb [6]. This interaction between E7 and pRb is one of the main mechanisms known to cause cancer. It is known that E7 induces carcinogenesis through high-affinity binding to pRb [3]. When E7 binds to pRb, it binds to its pocket domain, usually involved in binding to E2F family transcription factors through its LxCxE motif, allowing a complexed CBP/p300 complex to acetylate pRb [3]. By binding and displacing these transcription factors, E7 effectively puts the cell in a constant state of division. Furthermore, the acetylation of pRb also leads to degradation of pRb through a ubiquitin proteasome pathway, so all tumor suppressive activity by pRb will be eliminated [7]. Phosphorylation of E7 by CKII has been implicated in the ability of the protein to bind pRb and subsequently degrade it, but there are significant differences in the phosphorylation between low risk and high-risk strains, with high risk being more phosphorylated [3]. While it is currently unknown what this difference is caused by, it has been predicted that the amount of serine residues at the active site is responsible. To test this hypothesis in vivo, lysates of cells infected with HPV can be analyzed using western blots to visualize the degradation of pRb. To determine the differences between high and low risk, several mutants of the HPV-16 E7 protein have to be made. To avoid the lengthy development of retroviral particles to express E7 and mutant versions in cells, transfection of DNA coding for E7 would be a preferred method, as it is much faster, allowing for more mutant versions of E7 and their ability to degrade pRb.

Previous research done in this lab has shown that HT1080 cells can be successfully transfected, and pRb can be visualized through western blotting. The plasmid used to study E7 and its effect on pRb used a MMLV promoter, which proved to be ineffective at expressing E7. Therefore, research going forward will compare the old plasmid to a new plasmid with a CMV promoter, which has been shown to highly express the target gene [8]. The new plasmid was used to study whether the plasmid with a CMV promoter was effective at inducing expression of E7 in transfected cells so that the degradation of pRb could be studied via western blot. If the results of the wild type E7 showed successful degradation, mutants could be made using the same plasmid and promoter with a mutated E7 gene in order to determine the distinctions between high and low-risk HPV.

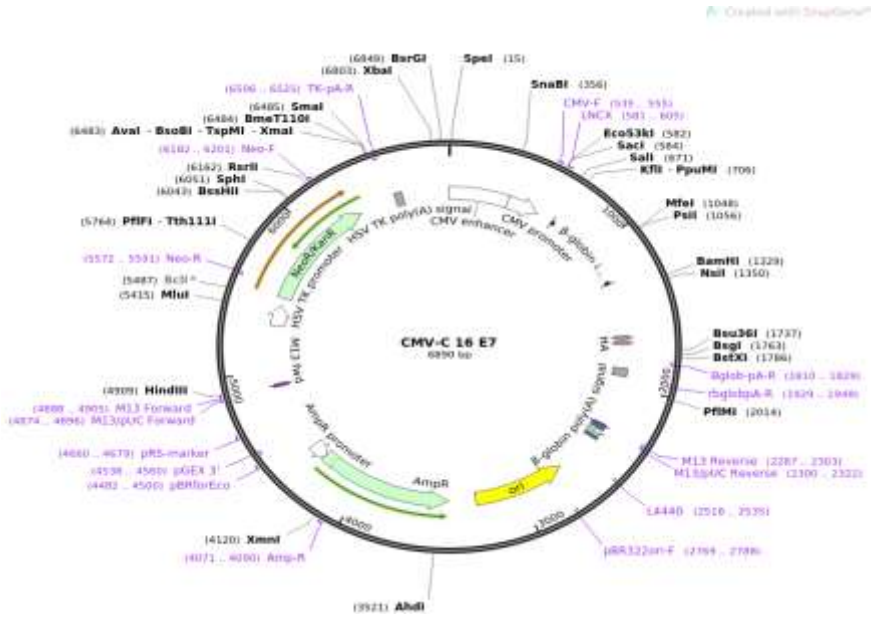

Figure 1: Diagram showing the HPV-16 E7 plasmid containing the CMV promoter. Different genes are labeled, and the cut sites for restriction enzymes are also labeled.

Results:

Fragment 1 (1576 bp)

Fragment 2 (5314 bp)

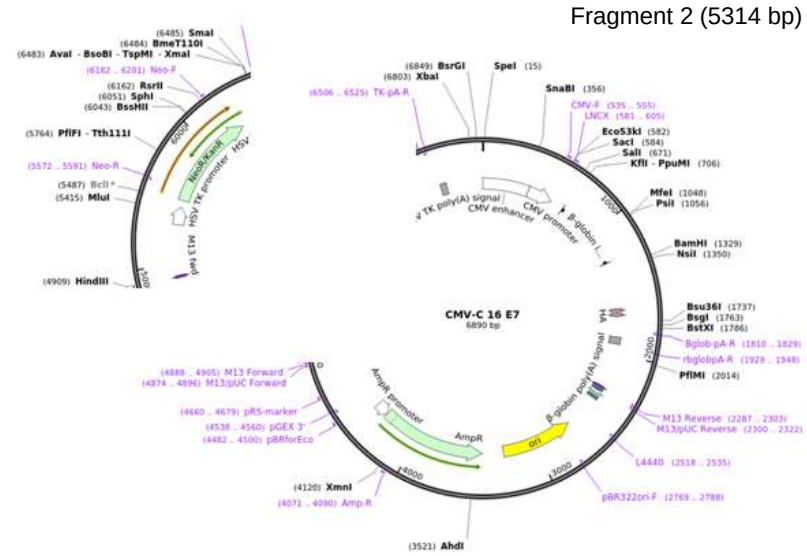

Figure 2: Visualization of the double digestion with HindIII and SmaI restriction enzymes. The result is two fragments, one of which is 1576 bp, while the other is 5314 bp.

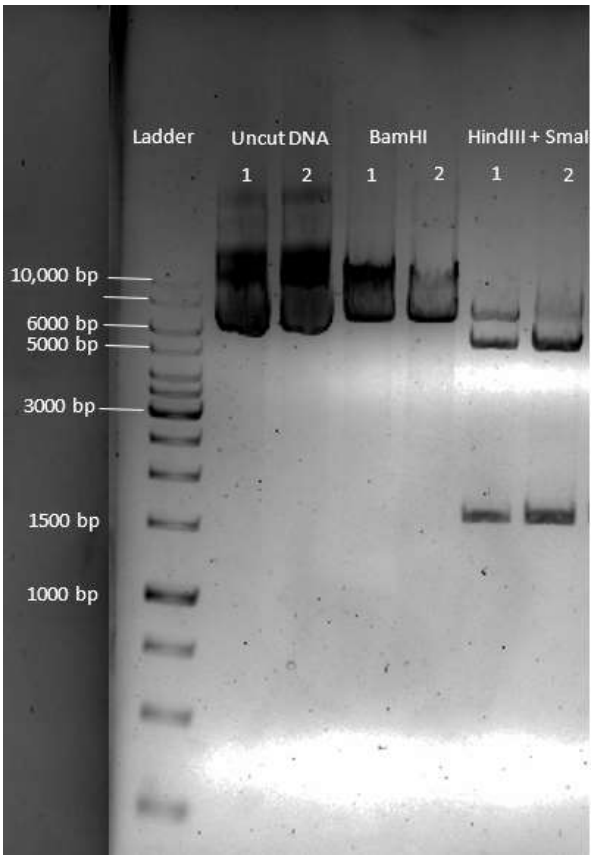

Figure 3: Gel electrophoresis of DNA digestion products. The DNA ladder shows various sizes of DNA and their location on the gel. Uncut DNA 1 and 2 are the two HPV-16 E7 plasmid with the CMV promoter without addition of restriction enzymes. The two plasmids are from two different minipreps, done previously, and are both tested to ensure that the E7 gene is correctly inserted. BamHI 1 and 2 are the two plasmids with a single cut at the BamHI cut site. Lastly, HindIII and SmaI were used in a double cut on both plasmids (refer to Figure 2 for restriction enzyme cut sites the resulting fragments). The results show that the plasmids isolated from the minipreps are the plasmids expected, and they do have the E7 insert.

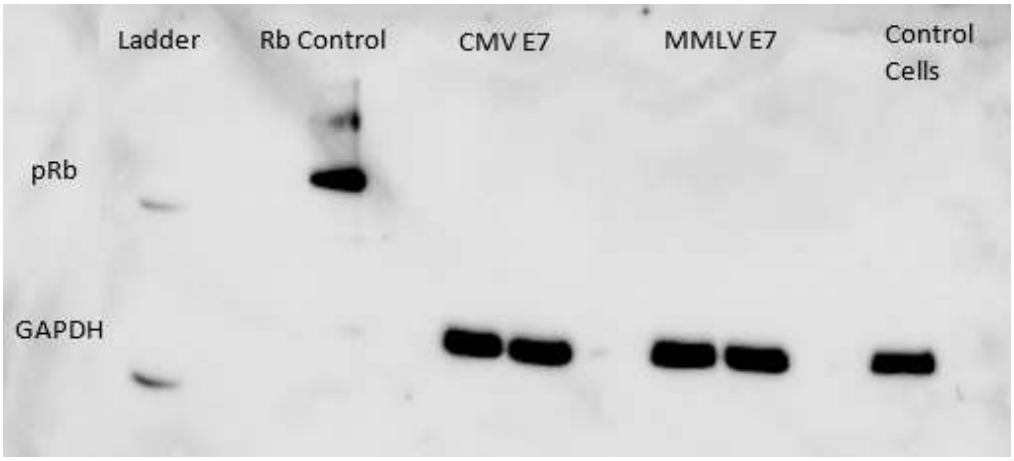

Figure 4: Western blot of lysates obtained from transfected HT1080 cells. A protein ladder was included to determine the size of the proteins. A control of isolated pRb was run to show pRb protein location for comparison to experimental lysates. CMV E7 refers to cells transfected with HPV-16 E7 plasmids containing the CMV promoter. MMLV E7 refers to plasmids used in previous research that contain the MMLV promoter. Control cells are cell lysates from cells not transfected with any plasmid. GAPDH was visualized as a loading control. Results from this blot are inconclusive due to an error in the negative control cells.

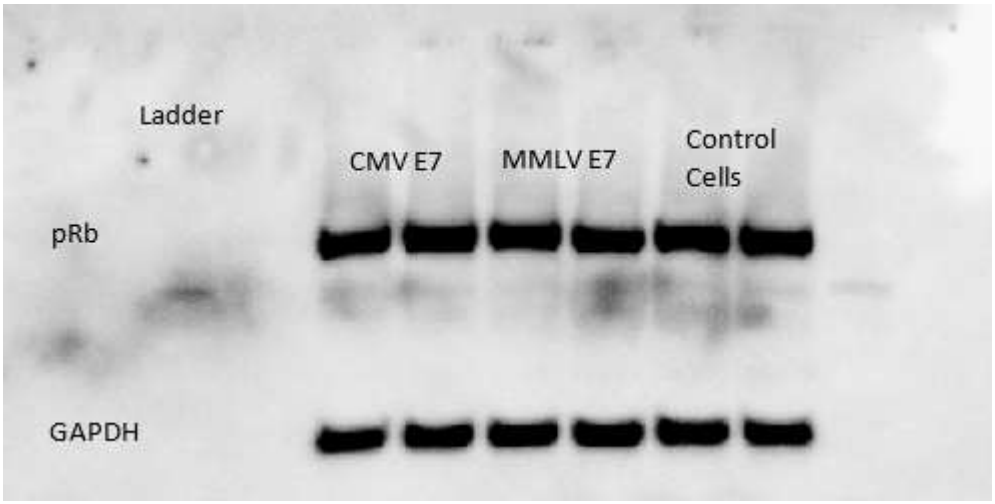

Figure 5: Western blot of cell lysates using the same procedure as the experiment in Figure 3. GAPDH was again used as a loading control. Unlike Figure 3, pRb was able to be visualized in the experimental samples.

Table 1: Results of Beta-galactosidase Assay for Each Day in the Time Course Experiment

Sample Absorbance at 405 nm				
	CMV E7	MMLV E7	pLXSN	PBS+Reagent
Day 1	0.061	0.061	0.065	0.055
Day 3	0.441	1.196	1.342	0.061
Day 5	1.287	1.099	0.075	0.056

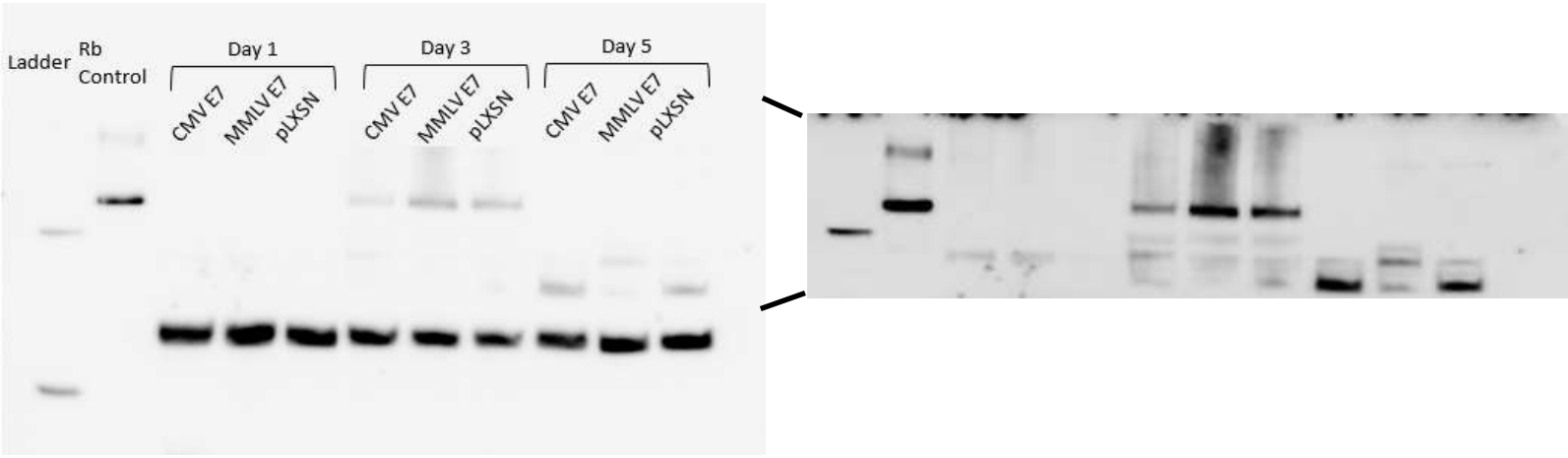

Figure 6: Western blot of the time course experiment. Cells transfected with CMV E7, MMLV E7, and pLXSN were lysed at 1, 3, and 5-day intervals. The ladder was used to confirm protein size, and a Rb control was used to identify Rb in the experimental samples as mentioned previously. GAPDH was used as a loading control. The section to the right is a zoom in of the Rb section and was overexposed in order to visualize faint bands that might have not been visualized due to the strong bands coming from GAPDH drowning out any fluorescence. The data is consistent in what happened in the experiment shown in Figure 3, and no real conclusion could be made.

Table 2: Results of qPCR for Each Day in the Time Course Experiment

	E7	NORM
MMLV1	13.76	16.76
MMLV3	13.89	17.58
MMLV5	14.72	16.82
CMV1	15.33	16.63
CMV5	19.13	17.06
PLXSN1	25.94	16.94
PLXSN5	27.64	17.09

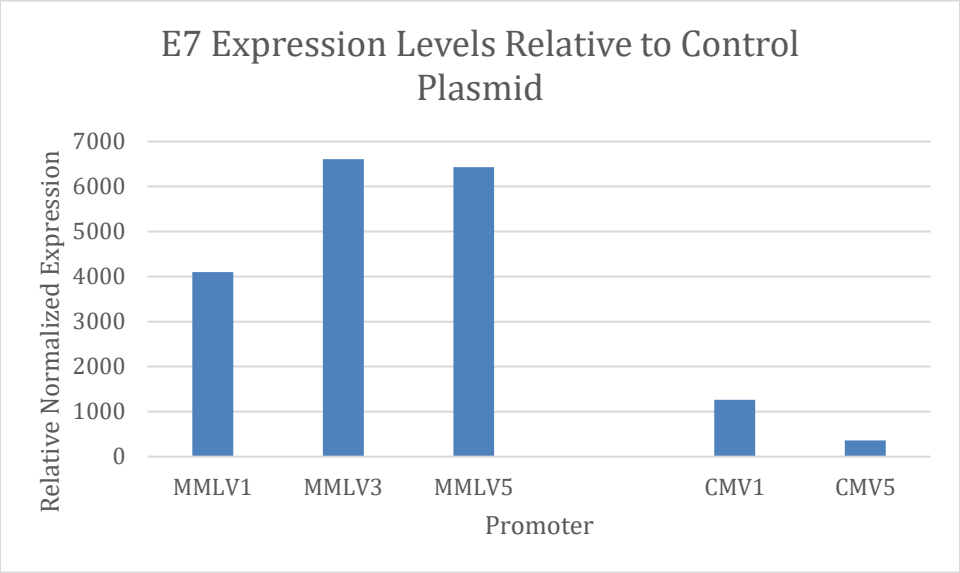

Figure 7: qPCR results depicting E7 expression levels relative to control plasmid. The MMLV promoter had greater expression levels across the three days than the CMV promoter (only days 1 and 5 shown in graph).

The DNA digest in Figure 3 was completed to determine if the gene coding for HPV-16 E7 was present in the new plasmid. The plasmid containing the E7 gene was expected to be 6890 bp. Therefore, we expected a single cut in the DNA from BamHI to yield a single band in the gel that was 6890 bp. It is also expected that a double cut from HindIII and SmaI, resulting in

two DNA fragments, would yield two bands that were 1576 bp (shown as Fragment 1 in Figure 3) and 5314 (shown as Fragment 2 in Figure 3). Two different minipreps of the plasmid were loaded in wells labeled Uncut DNA 1 and 2. As seen in the figure, the single cuts in lanes labeled BamHI 1 and 2 matched the uncut DNA exactly, and the bands traveled between the 6000 and 8000 bp bands given by the ladder. In addition to this, the double cuts labeled HindIII and SmaI had three distinct bands. The first being DNA that had not been cut, as it matched the uncut DNA. Another band in between 5000 and 6000 bp is seen, which would correlate to the expected 5314 bp (Fragment 2) band resulting from the double digestion with BamHI and SmaI. The third band appearing at around 1500 bp would match the expected band resulting from the second half of the BamHI and SmaI cut at 1576 bp (Fragment 1). Since all of the band sizes matched the expected band sizes based off of the plasmid correctly having the E7 insert, it was concluded that the plasmid used in the following experiments had the E7 gene.

The degradation of pRb in HT1080 cells transfected with HPV16-E7 with a CMV promoter, HPV16-E7 with a MMLV promoter, and non-transfected cells was compared (Figure 4). GAPDH, a housekeeping protein that is stably expressed, was visualized with pRb as a control level of protein expression and is seen in all samples in the dark bands across the bottom of the image labeled GAPDH. Expression of Rb is seen in the dark band pRb but is not seen in any other columns (CMV E7, MMVL E7, Control Cells). If pRb was not seen in CMV E7 or MMVL E7 but it was seen in control cells, it could be concluded that pRb degradation occurred in both samples, since E7 is known to degrade pRb. However, as pRb was not seen in Control Cells, the results for this western blot are inconclusive. It is not entirely clear what resulted in this outcome. Since pRb was clearly visualized in the pRb protein control, it could not have been an issue with the antibodies used to tag the protein for fluorescence. It also could not have been an issue with not loading enough of the protein lysate, since there were strong GAPDH bands for each sample, showing that there was enough protein loaded to be visualized.

In the following experiment using the same protocol as the previous experiment, GAPDH is seen in all samples, as well as Rb (Figure 5). Dark bands are present in CMV E7, MMLV E7, and Control Cells for pRb, however, no degradation is seen (Figure 5). Since E7 is expected to degrade pRb, there should be no band in either of the samples transfected with the E7 plasmid but should be present in normal cells not transfected with the plasmid. Since no degradation was seen in any of the samples, which matches what is seen in the negative control, it can be concluded that the cells transfected with E7 did not have pRb degraded. The results of Figure 5 can be analyzed via direct comparison to Figure 4, as pRb is present in all samples. The presence of pRb in Control Cells is a positive result, however, presence of pRb without degradation in both CMV E7 and MMLV E7 provides inconclusive data for this western blot. The results of Figure 5 disprove that any disappearance in bands in Figure 4 could have been a result of degradation by E7, since no changes to the protocol were made. Again, it is unclear why the pRb bands were not visible in Figure 4, but in light of the results of Figure 5, it can be concluded that pRb is not being degraded by E7. This is likely a result of poor transfection efficiency. Based off of previous research done, the plasmid with the CMV promoter should be expressed in about 40-50% of cells, while the MMLV promoter had less than 10% expression. Unfortunately, this trend is not visualized in this data. No changes to the transfection protocol were made. This leads us to believe that E7 might not be expressed immediately, or might need more time to degrade Rb, and thus see a disappearance of the Rb bands on a western blot.

Along with this it is possible that E7 may be degrading pRb efficiently, however, the pRb from the non-transfected cells override any loss of pRb in those with successful E7 transfection. To determine this, a time course of 1, 3, and 5 days was performed.

Figure 6 depicts a time course experiment that was run to determine if allowing the cells to survive for longer amounts of time after transfection would result in degradation. GAPDH is seen in the row of dark bands in all samples of days 1, 3, and 5 of the time course experiment. This proves that a sufficient amount of protein was added to each well. pRb was loaded as a control as done in previous experiments, as seen in the column titled *Rb Control*. Day 1 and day 5 have no visible pRb bands, even for the negative control. Day 3, however, was the only day in the time course to see pRb expression in CMV E7, MMLV E7, and pLXSN. The pRb band in these samples were much fainter than the control band, showing possible degradation by E7. Since it was expected that the plasmid with the CMV promoter would result in more expression of E7, it was assumed that there would be more pRb degraded than what would be seen in cells transfected with the plasmid containing the MMLV promoter. This is shown in the western blot, as the Rb band for the CMV plasmid is fainter than the MMLV plasmid. This conclusion cannot be fully stated, however, as pRb was not seen in day 1 or 5 of the time course in any sample (CMV E7, MMLV E7, pLXSN), and therefore, it is unclear whether the faintness of the bands is due to actual degradation or the error that resulted in the disappearance of the bands seen in day 3 and 5. This can be seen further in the zoom in of the pRb section of Figure 6. It is unclear, again, why the bands would be disappearing, especially from cells with a control pLXSN plasmid which does not code for E7.

For this experiment, cells were also transfected with a Beta-galactosidase plasmid, so that a Beta-galactosidase assay could be run as a means to determine if there was successful transfection. In this assay, cells that were successfully transfected would produce the Beta-gal protein, which, when introduced to a reagent, causes a color change to yellow, which can be visualized by absorbance. Higher amounts of absorbance correspond to more Beta-gal production, and this production would indicate that the cells were transfected. Results are shown in Table 1. If it could be concluded that Beta-gal was successfully transfected, it could also be concluded that any other plasmid that was co-transfected with it, like HPV-16 E7, would have also successfully been transfected into the cells. Based off of the data for this assay, it is clear that there was successful transfection in both day 3 and day 5 cells, except for pLXSN day 5 which would not significantly impact results, since pLXSN serves as a control anyway, and would have the same effect as cells not transfected at all. Day 1 cells did not see much of a difference in absorption compared to control levels of absorption, so these cells were not successfully transfected. It is also possible that the beta-gal did not have time to turn on and cause fluorescence. Since they were not successfully transfected, it would be expected that there would be prominent pRb bands, due to E7 not being transfected, however, no Rb bands were visualized, even in the control. This further confirms that it was not likely that the possible degradation seen in day 3 was the result of E7. Similarly, with day 5, the previous conclusion would still stand even though the cells were successfully transfected, because the control pRb could not be visualized.

Since it was unclear whether the data observed in the previous western blots was a result of random error, degradation, or a combination of the two, a qPCR was run on the samples to determine if there was any E7 expression in these cells. Low, or early C_q values

indicate a large amount of template available at the start of the reaction. Fewer cycles were needed to amplify these low sections, displayed in MMLV1, MMLV3, and MMLV5 in Table 2. MMLV1, MMLV3, and MMLV 5 had C_q values of 13.76, 13.89, and 14.72, respectively. CMV1 and CMV5 had C_q values of 15.33 and 19.13, respectively, which were greater than all MMLV C_q values. The qPCR results indicated that the MMLV plasmid had greater E7 expression than the CMV plasmid, however, both showed signs of expression. This is visualized in Figure 7, which shows the difference in relative normalized expression between the two promoters during the 5 day time course.

Discussion:

Although we do not know absolute levels, E7 expression was seen in the qPCR analysis. Unfortunately, there was inconsistency with pRb visualization and degradation, which impairs the testing of any mutant strains due to lack of consistency in the WT strain. There are multiple explanations as to why pRb is not being degraded, and a final conclusion from the experiments that provides a viable future direction for testing the HPV mutants.

The first explanation as to why pRb is not being degraded comes as a result of malfunctioning E7. It was found that E7 expression was adequate, however, pRb degradation was not seen. While possible, it is unlikely, since the sequence coding for the E7 protein is known to be the sequence for the WT version of HPV-16 E7. A second explanation addresses the levels of E7 expression, as adequate expression can disguise a low transfection efficiency. High levels of E7 in a minority of the cells will result in the degradation of pRb in those cells, however, a majority of those cells will not have had successful transfection. Without transfection of most cells, full pRb levels will be present and the cell lysate will reflect a lack of pRb degradation. A third explanation for the lack of pRb degradation is a lack of time for E7 to fully degrade pRb. E7 is not being kept in the cells long enough for proper degradation to occur, and this is reflecting a consistent lack of degradation. This might have been answered in the time course experiment, if there were not inconsistencies in the pRb bands.

Along with these explanations, a combination of possibilities may be working together to form these results. While another time course could be run to obtain clearer results, it is unlikely considering the inconsistencies with previous experiments, as well as failures to visualize pRb degradation. Overall, a transient transfection will not be adequate for future studies. A retroviral transfection will be more useful and successful in inducing expression of E7 in a much greater proportion of cells. Retroviral transfection poses restrictions with the cost and general safety concerns of repeated exposure and use of retroviruses. Retroviruses will be more tedious when multiple E7 mutants are made, since the creation of these retroviruses can be a lengthy process. This was part of the benefit of transient transfections, however, moving forward, retroviral transfections will provide the necessary means for studying the ability of E7 and its mutant forms, in degrading pRb, to determine what distinguishes high and low risk variant.

Methods:

<u>Cell Culture:</u>

HT1080 cells were maintained in EMEM (ATCC), 10% FBS (Gibco), and 1X Anti-Anti (Gibco) in a 5% CO2 incubator maintained at 37 degrees C.

Transfection, Beta-galactosidase assay, BCA assay:

Transfections were carried out using the Lipofectamine 3000 Transfection Kit (Invitrogen). For a 6-well plate, 7.5 µg of DNA, 125 µL of OptiMEM, 5 µL of P3000, and 3.75 µL Lipofectamine 3000 were used per well. A DNA mix consisting of the DNA, OptiMEM, and P3000 was added to the wells first, followed by a Lipofectamine mix. The amounts of each had been optimized. Cells were collected from the plate using trypsin and spun at 600 x g for 5 minutes. The pellet was then resuspended and washed with PBS and spun down again. The pellet was lysed using RIPA buffer and spun at 14,000 x g for 15 minutes or was lysed with Buffer RLT and spun for 3 minutes at 14,000 x g if the sample was to be analyzed by qPCR. After the spin, the supernatant was separated from the debris. The lysate was frozen until qPCR could be performed. In samples lysed with RIPA, transfection was confirmed by running a mammalian B-gal Assay (Thermo Scientific). Protein concentration was determined by using Pierce BCA Protein Assay Kit (Thermo Scientific). It was previously determined that of the 100 µL lysate, 20 µL could be used for the Beta-gal assay, and 10 µL could be used for the BCA. The remaining lysate was frozen until a western blot was performed. To complete the Beta-gal assay, 20 µL of Beta-gal reagent was added to the 20 µL of lysate and incubated at 37 C for 30 minutes. To get a base absorbance reading, 20 µL of Beta-gal reagent was added to 20 µl of PBS. The BCA assay followed the protocol outlined by the Thermo Scientific kit exactly.

DNA Digestion and Electrophoresis:

The reaction for a single cut at the BamH1 cut site was made with 1uL of restriction enzyme, 1 ug of DNA, 5 µL of 10X Buffer K (Invitrogen), and nuclease free water up to a total reaction volume of 50 µL. The reaction was incubated in a PCR for 1 hour at 37 C. The reaction was stopped by incubating at 65 C for 15 minutes. For the double digestion cut at the HindIII and SmaI sites, 1 ug of plasmid was added, and 5 µL of 10X Buffer T (Invitrogen), 1 µL of SmaI, and water up to 50 µL. This was incubated at 25 C for 15 minutes. 1 µL of HindIII was then added and incubated at 37 C for 1 hour. The reaction was stopped by incubating at 65 C for 15 minutes. Once the digestion was complete, they were loaded on a 1% agarose gel with each well consisting of 500ng of the digestion product and 5 µL of loading dye. The gel was run at 150 V for 1 hour.

MaxiPrep:

Protocol called for by the Qiagen Plasmid Maxi Kit was followed exactly. DNA product concentration was determined using Nanodrop.

RNA Isolation, Reverse Transcription, and qPCR:

RNA was isolated using the RNeasy Plus Mini Kit (Qiagen) and then cDNA was made through reverse transcription using SuperScript IV (Invitrogen). Concentration of the cDNAs was determined through Nanodrop and diluted to a concentration of 5 ng/µL. Reactions were made by combining 2 µL of cDNA, 1 µL of forward and reverse E7 primers (IDT), 10 µL of 2X SYBR

Green, and 17 µL of RNase/DNase free water. Each cDNA was run in triplicate. Control expression was determined by GAPDH (IDT). *ENTER the qPCR protocol that it was ran on*

Western Blotting:

The protein ladder used was iBright Prestained Protein Ladder (Thermo Scientific), and 3 µL of it was added. 10 ng of Recombinant Rb (Sigma-Aldrich) was added as a control with 2.5 µL of LDS sample buffer (Invitrogen), 1 µL of Bolt Reducing Agent (Invitrogen), and 5.5 ul of Milli-Q water. 20 µg of lysate, 8.75 µL LDS, and 2.5 µL Reducing agent, and Milli-Q water up to a total volume of 35 µL was added for each experimental sample. Everything except the ladder was incubated at 70 C for 10 minutes. The ladder, recombinant Rb, and experimental lysates were added to a Bolt 4-12% Bis-Tris Plus gel (Invitrogen). The gel ran at 200V and 40mA for 1 hour. The gel was transferred to a PVDF membrane that was activated with methanol for 30 seconds. To transfer, the blot sandwich was assembled in the order of: cathode core, sponge pad, filter paper, gel, membrane, filter paper, two sponge pads, anode core. Everything was soaked in 1X transfer buffer and bubbles were removed. Transferred using 20V and 390mA for 1 hour. After the transfer was complete, the membrane was washed with Milli-Q twice for 5 minutes, then blocked with milk buffer for 2 hours. The membrane was then transferred to a primary antibody wash overnight. The primary antibody wash consisted of a 1:500 concentration of Rb antibody and 1:2500 concentration of GAPDH antibody. These antibodies were added together. The following day, the membrane was washed with 1X TBST six times for 5 minutes, then it sat in secondary antibody for 1 hour. The secondary antibody wash consisted of a 1:10000 Goat anti-Rabbit secondary antibody which bound to the Rb antibody, and a 1:2000 Goat anti-Mouse antibody used for GAPDH. The antibodies were mixed in the same solution since the antibodies would not bind to each other. Following the secondary, the membrane was washed with 1X TBST five times for 4 minutes, then washed with a stable peroxide Luminol working solution for 5 minutes. Excess working solution was taken off by pressing the edge of the membrane on a Kimwipe, and the membrane was visualized for chemiluminescence.

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