Cervical Cancer Cell Lines are Sensitive to Sub-Erymethal UV Exposure.

Running Title: Cervical Cell Lines are sensitive to Low Dose UV

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ABSTRACT: High risk human papillomavirus (HPV) infections are the causative agent in virtually every cervical cancer as well as a host of other anogenital and oropharyngeal malignancies. These viruses must activate DNA repair pathways to facilitate their replication, while avoiding the cell cycle arrest and apoptosis that can accompany DNA damage. HPV oncoproteins facilitate each of these goals, but also reduce genome stability. Our data dissect the cytotoxic and cytoprotective characteristics of HPV oncogenes in cervical cancer cells. These data show that while the transformation of keratinocytes by HPV oncogene leaves these cells more sensitive to UV, the oncocenes also protect against UV-induced apoptosis. Cisplatin and UV resistant cervical cancer cell lines were generated and probed for their sensitivity to genotoxic agents. Cervical cancer cells can acquire resistance to one DNA crosslinking agent (UV or cisplatin) without gaining broad tolerance of crosslinked DNA. Further, cisplatin resistance may or may not result in sensitivity to PARP1 inhibition.

INTRODUCTION:

Cervical cancers are the third most common and second deadliest cancer in women worldwide. Over half a million new cases of cervical cancer are diagnosed annually killing nearly 300,000 people (Parkin and Bray, 2006). Access to healthcare is a major factor in the development of cervical cancer with as many as 80% of cases occurring in developing countries (Sherris et al., 2001; Siegel et al., 2012; Tota et al., 2011). In addition to differences in screening and care, environmental factors (smoking and UV exposure for instance) increase the risk of cervical cancer (Fonseca-Moutinho, 2011; Godar et al., 2014). Nearly every cervical cancer is the result of a human papillomavirus infection (zur Hausen, 2002). This very large family of double stranded circular DNA viruses is divided into 5 genera based on sequence differences in their L1 major capsid gene (Bernard et al., 2010). While members of both the genus alpha and beta of human papillomaviruses are associated with cancer (Godar et al., 2014; Howley and Pfister, 2015; Wendel and Wallace, 2017), members of the genus alpha human papillomaviruses are further divided into high risk and low risk papillomaviruses based on the relative ability to cause cancer. Among

the high risk alpha papillomaviruses, human papillomaviruses 16 and 18 cause 70% of cervical cancers (Winer et al., 2006). In this manuscript, we will refer to these two viruses as simply as HPV.

Much is known about the molecular basis of how HPV manipulates the host cell both to promote its life cycle and to cause malignant transformation. Although the E5 protein from these viruses has oncogenic potential, the two canonical HPV oncogenes are HPV E6 and HPV E7 (Roman and Munger, 2013; Wallace and Galloway, 2015). HPV E6 binds a cellular ubiquitin ligase (E6AP) and uses it to promote p53 degradation (Huibregtse et al., 1991; Martin Scheffner et al., 1993, p. 53; M. Scheffner et al., 1993). HPV E6 also activates the catalytic subunit of telomerase (Klingelutz et al., 1996). HPV E7 disengages cell cycle checkpoints by degrading RB and RB family proteins (Dyson et al., 1989; Roman and Munger, 2013; Zhang et al., 2006). The lack of evolutionary pressure to mutate or otherwise inactivate these tumor suppressors results in tumors that are dependent on continued HPV E6 and E7 expression (Chang et al., 2010).

In addition to their well-characterized ability to inactivate p53 and RB, HPV oncogenes have a complicated relationship with cellular response to UV (Wallace and Galloway, 2014). HPV E7 prevents HPV-transformed cells from undergoing the G1 arrest after UV necessary to avoid replication fork collapse (Flynn and Zou, 2011; Gujuluva et al., 1994; Morandell et al., 2012; Wieringa et al., 2016). HPV E6's degradation of p53 prevents the induction of p53 target genes (Gu et al., 1994). HPV E6 blocks the induction of NER proteins (McKay et al., 2001a) correlating with limited NER and more mutations after UV exposure (El-Mahdy et al., 2000; Havre et al., 1995; Rey et al., 1999). Both HPV oncogenes hinder the homologous recombination pathways that responds to double strand DNA breaks that can result from UV-induced replication fork collapse (Wallace et al., 2017). The repair mechanisms that respond to UV are also critical for repairing lesions caused a number by genotoxic drugs that cause DNA crosslinks (Alt et al., 2007; Furuta et al., 2002; Ho et al., 2006). It is not surprising then that HPV E6 and E7 sensitize cells to this class of chemotherapeutics (Koivusalo et al., 2002; Liu et al., 2000; Wallace et al., 2012). The

dependence of cervical cancers on these viral oncogenes suggests that these drugs would be particularly lethal to cervical malignancies.

However, HPV oncogenes also activate a plethora of DNA repair proteins including those in the pathways that respond to UV damage; nucleotide excision repair (NER), Fanconi anemia repair (FA) and translesion synthesis (TLS) (Alan and D'Andrea, 2010; Knobel and Marti, 2011; Schärer, 2013). HPV depends on DNA repair pathways to replicate its genome (Anacker et al., 2014; Gillespie et al., 2012a; Hong et al., 2015; Moody and Laimins, 2009; Spriggs and Laimins, 2017a). In addition to creating an environment that is seemingly primed to respond to crosslinked DNA, HPV oncogenes inhibit the apoptosis induced by these lesions (Garnett et al., 2006; Garnett and Duerksen-Hughes, 2006; Jackson et al., 2000; Leverrier et al., 2007). Thus in at least certain scenarios, HPV E6 can protect against DNA crosslinking agents (Koivusalo et al., 2002).

The balance between chemo-sensitization and chemo-resistance is critical in the management of any cancer. DNA repair and damage tolerance pathways are particularly important with regard to cervical cancer as a DNA crosslinking agent, cisplatin, is currently the standard of care (Lorusso et al., 2014). Resistance to cisplatin in advanced and recurrent cervical cancers results in only a 10-20% chance of living through the year (Diaz-Padilla et al., 2013). As a result, substantial effort has been made to identify potential mechanisms of cisplatin resistance as well as ways to re-sensitize cervical cancer cells (Kilic et al., 2015; Roy and Mukherjee, 2014; Zhu et al., 2016). The efficacy of cisplatin is at least partially determined by gene expression patterns in cervical cancer cells, with multiple DNA repair genes being identified as important predictors of cisplatin sensitivity (Garzetti et al., 1996; Hasegawa et al., 2011; Henríquez-Hernández et al., 2011; Kitahara et al., 2002; Saito et al., 2004; Zhu et al., 2016). Because cisplatin induces DNA damage, increased repair capability likely accounts for the resistance (Woods and Turchi, 2013).

Conversely, if HPV oncogenes block repair in a predictable manner, these defects could be leveraged therapeutically. The sensitivity of BRCA1 or BRCA2 deficient breast and ovarian cancers is the most publicized link between known repair deficiencies and improved cancer treatments (D'Amours et al., 1999; Jasin, 2002). A similar relationship exists for cisplatin sensitivity. Specifically, the response of TLS and NER to UV predicts the lethality of cisplatin exposure (Gueranger et al., 2008; Marteijn et al., 2014; Rosell et al., 2003; Srivastava et al., 2015). In this manuscript, we characterize the interplay between HPV oncogene-induced sensitivity to crosslinking agents and their protection from these genotoxins in the context of cervical cancer cell lines. We show that, compared to control cells, cervical cancer cells are more sensitive to sub-erythral UV exposure and low doses of cisplatin. We demonstrate that these cells have a greater tendency to undergo apoptosis after UV. Interestingly, HPV E6 and E7 also block apoptosis after UV. We generate UV- and cisplatin-resistance cervical cancer cell lines and define their cross sensitivity. Finally, we determine the toxicity of a chemotherapeutic agent (olaparib) with a different mechanism of action (PARP1 inhibition) in cervical cancer cells that are resistant to cisplatin.

METHODS AND MATERIALS:

MTT Assays: LD50s were determined by MTT assay. HeLa cells were seeded in a 96 well plates and treated with UV, cisplatin, or olaparib at specified doses. 48 hours after treatment, MTT solution was added to the cells and solubilized 24 hours later. Plates were then read for optical density by spectrophotometer with a dual reading at frequencies 550 and 655 nm.

Cell Culture: HeLa cells are HPV 18 positive *Homo sapiens* adenocarcinomas grown in Dulbecco's Modified Eagle Medium (ThermoFisher Scientific, catalog number:12100046), supplemented with 10% fetal bovine serum (VWR, catalog number:89510-194) and Pen-strep (Calsson Labs, catalog number:PSL02-6X100ML). Human foreskin keratinocytes or HFKs were derived from neonatal human foreskins and were grown in EpiLife medium supplemented with calcium chloride (60 μ M), human keratinocyte growth supplement (Cascade Biologics, Portland, OR), and penicillin-streptomycin. Multiple

cell lines were derived from several donors for this work. The cells were incubated at 37°C in a 5% CO₂ environment. Cells were washed with 0.1% EDTA (Invitrogen catalog #15-576-028) and raised from plate with 0.05% Trypsin (Sigma-Aldrich catalog # T4049-500ML)

Immunoblotting: HeLa Cells transfected by different plasmids (PLL3.7, 18E6-1, 16E7-1) were cultured for different times (0 h, 3 h, 6 h, 24 h) in 6 cell-well plate after using UV light dose (8 mJ/cm²) treatment, then both adherent and floating cells were collected and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM ethylene diamine tetraacetic acid (EDTA), 1 mM ethylene glycol tetraacetic acid (EGTA), 20 mM NaF, 1% Nonidet P-40 (NP-40), 1 mM phenylmethylsulfonyl fluoride (PMSF), 100 mM Na3VO₄, 1% Triton X-100, 10 mg/mL Aprotinin and 10 mg/mL Leupeptin) for 30 min. The lysates were centrifuged at 12,000 rpm for 10 min and the supernatant was collected. Equal amount of protein lysates were electrophoresed on a 12% SDS polyacrylamide gel and transferred onto nitrocellulose filter (NC) membrane. The membrane was washed 3 times for 10 min in TBST (Tris-Buffered Saline and Tween-20), then after blocking with 2.5% skim milk suspended in TBST for 1 hour, membranes were individually incubated respectively with the appropriate primary antibodies Tubulin, P53, Bax, HPV E7 (1:1000) overnight followed by secondary antibodies (1:1000) for 1 h at room temperature. Protein bands were detected using ECL assay kit (Invitrogen, Australia) and exposed using a Kodak medical Xray processor (Kodak, USA). Anti-human p53 and β -tubulin monoclonal antibodies were purchased from Sigma (Sydney, Australia). Anti-HPV 18E7 polyclonal antibody was purchased from Santa Cruz Biotechnology. Anti-human Bax polyclonal antibody was from Cell Signaling Technology.

Resistant Colony Generation and Isolation: Colony isolation was performed using sterile cloning disks (SP Scienceware catalog # F37847-0001). Cells were grown at low confluence in a 10cm plate and treated 4 times every 2 weeks for a 2-month period. Cells were observed from single cells, with their growth monitored microscopically. They were washed with 0.1% EDTA, before colonies were isolated by placing a sterile cloning disks soaked in trypsin on them. Individual disks were then put in a well of a 24-

well plate containing growth media, allowing cells to detach from the disk. Cells were then expanded, and acquisition of resistance was assessed.

Colony Formation Assays: Colony formation assays were performed by seeding low concentrations of cells on six well plates. They were then treated with UV 48 hrs after seeding and allowed to grow until colonies appeared. Twenty-four hours after the first colonies with 15 or more cells were seen, colonies were stained with a crystal violet solution (0.2% crystal violet, 5% ascertic acid, and 2.5% 2-propanol) and hand counted.

shRNA Transfections: Cell transduction was basically according to the method described previously (Gu et al., 2006). Briefly, HeLa cells were maintained in complete DMEM medium (Gibco-Invitrogen) and were plated in T75 flasks (4×10^5 /flask) overnight culture. LV-shRNAs was diluted in 2.0 ml cultural medium containing polybrene (8 μ g/mL) and added to the cells for culture for 1 hour at 37 °C. After this, 8 mL of fresh polybrene medium was added to the cells and incubation continued for 24 hours. Polybrene medium was then replaced with fresh DMEM culture medium, and the cells were further cultured for assays.

UV Irradiation: To prepare cells for UV irradiation the media was aspirated, and the cells were washed with 1X PBS (Bio Basic catalog # PD8117). A thin layer of PBS was added to the wells or plate and then cells were irradiated in a UV Stratalinker 2400 (Stratagene Catalog # 400075-03) at specified doses. Media was then added, and the cells were incubated at 37°C.

Flow Cytometry: Flow cytometry analysis was carried out with the FACSCalibur™ (Becton Dickinson). The acquisition and analysis of data was done with the program Cell Quest Pro.

Apoptosis Assay: The Dead Cell Apoptosis Kit (ThermoFisher Scientific catalog #V13242) and the Countess™ II FL Automated Cell Counter (ThermoFisher Scientific catalog #AMQAF1000) were used to measure apoptosis. Cells were seeded at 50,000 cells/well in a 6-well plate and incubated at 37°C overnight. Cells were then UV irradiated at specified intervals and incubated at 37°C for 48 hrs. Cells

were then washed with 0.1% EDTA and incubated with Trypsin for 3 minutes at 37°C. DMEM was then added to cells and they were incubated at 37°C for 30 min. Cells were then pelleted by centrifuging at 188 x g for 5 min. After aspiration of DMEM, they were resuspended in $\sim 1 \times 10^6$ cells/ml in 1X annexin binding buffer and incubated with propidium iodide for 15 min at RT. Cells then were loaded onto slides and imaged using appropriate fluorescent filters on Countess™ II FL Automated Cell Counter.

Reagents:

Cisplatin: cisplatin (Sigma-Aldrich catalog # 479306-1G) is a platinum based antineoplastic agent that forms cytotoxic adducts with the DNA dinucleotide d(pGpG) that induces intrastrand cross-links².

Olaparib: olaparib (LC laboratories catalog # O-9201) is a selective inhibitor of PARP1/2 with IC₅₀ of 5 nM/1 nM in cell-free assays¹.

RESULTS:

HPV+ cervical cancer cell lines are sensitive to sub-erythral doses of UV.

Cells mitigate the toxicity of low dose UV by pausing the cell cycle and inducing a highly coordinated signaling response. HPV oncogenes transform keratinocyte cells in part by disrupting both processes. HPV E7 degrades the master cell cycle regulator, RB. While both HPV E6 and E7 induce aberrant activation of DNA damage proteins. We hypothesize that the continued expression of HPV E6 and E7 in cervical cancer cell lines will make them less able to respond to UV and as a result more sensitive to UV exposure. This hypothesis was tested by defining the sensitivity of a HPV+ cervical cancer cell line (HeLa) to UV. As a control, untransformed primary keratinocytes derived from neonatal foreskins (HFKs) were also observed because HPV infects and transforms keratinocytes. HeLa cells were significantly more sensitive to a gradient of low dose UV (0 – 10 mJ/cm²) than HFKs when viability was measured by MTT assay (Figure 1A).

As a complimentary measure of UV sensitivity, a colony formation assay was used to measure the toxicity of the same range of UV doses. These results confirm the data obtained by the MTT assay. Indeed, HeLa cells were significantly more sensitive to all exposures of UV in the colony formation assay, with virtually no colonies visible after 1 mJ/cm² of exposure (Figure 1B and Supplemental Figure 1). Conversely, HFKs formed readily detectable colonies at all observed doses of UV.

The sensitivity resulting from an inability to properly respond to UV damage is likely manifest in elevated apoptosis. Unfortunately, neither a colony formation assay or a MTT detect apoptosis directly and thus they cannot distinguish apoptosis from other types of cell death. To determine if HPV+ cervical cancers have an increased propensity to undergo apoptosis after UV exposure, we measured propidium iodide (PI) staining as a standard indicator of apoptosis (Figure 1C). PI is a membrane impermeable dye that intercalates between DNA base pairs. We used a fluorescence-based assay to detect PI. Cell membranes become permeable during apoptosis allowing PI uptake measurable at 617nm. Taking the ratio of cells that are stained with PI to the total cell count allows us to determine the percentage of apoptotic cells after UV exposure. We found HeLa cells had significantly higher levels of PI staining compared to HFKs when both were exposed to either 2 or 4 mJ/cm² of UV (Figure 1C).

HPV oncogenes decrease sub-erythematous UV-induced apoptosis.

This increased sensitivity is likely due to HPV E6 and E7 disrupting cell cycle regulation and causing abnormal activation of DNA damage response genes. However, HPV oncogenes also mitigate the deleterious consequences of DNA damage by inhibiting apoptosis. To define the ability of HPV oncogenes to block the apoptosis in the context of a cervical cancer cell line exposed to UV, a shRNA system (PLL E61) was used to partially deplete HPV oncogenes in HeLa cells. HeLa cells with PLL E61 cells had decreased HPV E7 levels 2 and 4 days after treatment with shRNA (Figure 2 A). Because these genes share a promoter, their expression is known to be linked. Indeed, a recent study was unable to achieve knockdown of HPV E6 or HPV E7 individually (Wechsler et al., 2018). Thus, decreased HPV E7

abundance is a reliable marker of HPV E6 for which a dependable antibody does exist. To determine if this knockdown was persistent, oncogene levels were measured at 1 and 2 weeks after transfection with shRNA. Transfection with PLL E61 was able to detectably decrease the abundance of HPV E7 at these late time points (Figure 2B). As a final measure of the PLL E61's ability to diminish HPV oncogene levels, the abundance of HPV oncogenes was determined after transfection with increasing amounts of PLL E61. Immunoblot analysis shows a dose dependent decrease in HPV E7 levels (Figure 2C). Since there is no viable commercially available antibody to HPV E6, the degradation of its target protein, p53, is used as a surrogate for HPV E6 expression. P53 levels were determined by immunoblot after treatment with PLL E6. Notably, the reduction in oncogene abundance remains below the levels that result in apoptosis (data not shown).

Having characterized PLL E61's ability to decrease the amount of HPV oncogenes in HeLa cells, the approach was used to define the role of HPV oncogenes in the apoptosis induced by low doses of UV. Supporting a role for HPV oncogenes in repressing UV-induced apoptosis, both MTT and colony formation assays demonstrated that UV was more toxic to HeLa cells when HPV E6 and E7 were targeted by shRNA (Figure 3A-B). Specifically, the knockdown of HPV oncogenes combined with exposure to a low dose of UV resulted in lower cellular viability and fewer colonies than either knockdown or UV exposure alone.

As noted, these assays do not directly measure apoptosis, so the abundance of BAX, a pro-apoptotic protein, was defined by immunoblot. After exposure to a sub-erythral amount of UV, HeLa cells demonstrated a mild induction of both BAX and p53 (Figure 4A). In contrast, UV-induced increases in BAX and p53 abundance were enhanced by HPV E6 and E7 knockdown. Notably, PLL E6.1 also increased the abundance of p53 in untreated HeLa cells further confirming the knockdown of HPV E6. To more directly detect the role of HPV oncogenes in protecting cervical cancer cells against apoptosis, flow cytometry was used to define the population of sub-G0/G1 cells after UV. The results from this assay are consistent with HPV oncogene-mediated inhibition of apoptosis as the percentage of sub-G0/G1 cells

after UV was significantly increased by oncogene knockdown (Figure 4B). As a control and to further confirm that the increase in the sub-G0/G1 population was due to BAX-induced apoptosis, BCL2, an anti-apoptotic BAX antagonist was transfected into HeLa cells. BCL2 expression prevented the accumulation of sub-G0/G1 cells after UV (Figure 4B).

Generation of UV- and cisplatin-resistant HPV+ Cervical Cancer Cells:

The ability of HPV oncogenes to both sensitize cells to UV and protect them from UV-induced apoptosis portrays the complexity of their manipulation of the cellular damage response. To further probe this relationship, UV-resistant HeLa cell lines were generated by repeated exposure to UV followed by a recovery period (Figure 5A). This process was repeated at least 4 times and until individual resistant colonies could be isolated using trypsin soaked cloning rings. These colonies were then expanded and their sensitivity to UV was compared to parental HeLa cells via MTT assay. The LD50 was calculated for each resistant cell line and used to select highly resistant cells for further analysis (Figure 5B and Table 1).

Cisplatin is a genotoxic agent that is quite effective at killing HPV+ cervical cancers. Cisplatin works by inducing DNA crosslinking that activate many of the same cellular responses induced by UV exposure (McKay et al., 2001b). For comparison with the UV-resistant HeLa cells, cisplatin-resistant HeLa cells were generated using the previously described treatment-recovery cycle (Figure 5A). Resistance was again established using MTT assays to determine LD50 values (Figure 5C and Table 1). This approach allowed resistant cells to be identified and selected for further analysis. UV resistant HeLa A and B as well as cisplatin resistant HeLa A were chosen because they had the most robust resistance.

Sensitivity of UV- and cisplatin-resistant HPV+ cervical cancer cells to other genotoxic agents:

We postulated that resistance to one type of crosslinking agent may not necessary result in resistance to agents that similar damage DNA. To begin testing this hypothesis and to characterize the mechanisms of resistance in these cell lines, MTT assays were conducted to define the LD50 for UV for

the most cisplatin-resistant HeLa lines (Figure 6A and Table 1). Although individual matched data points were not significantly between resistant and parental cell lines, a statistical analysis comparing the full data sets to each other revealed that a significant increase in UV resistance accompanied acquired cisplatin resistance in HeLa cells (Wilcoxon Matched Pairs Test, $p < 0.05$). The average LD50 increased from 65.47 in parental HeLa to 108.6 in the cisplatin resistant colony demonstrating an almost 2-fold difference in sensitivity. Next, MTT assays defined the LD50 for cisplatin in the most UV-resistant HeLa lines (Figure 6B and Table 1). This analysis demonstrated that cells that acquired UV resistance could become more sensitive to other crosslinking agents. Finally, a recent report indicated that acquired cisplatin resistance could sensitize cells to PARP inhibition (Michels et al., 2013). To determine if this was true for the cisplatin-resistant HeLa cells described in this report, MTT assays were used to measure sensitivity to olaparib, a commercially available inhibitor of PARP1 (Figure 6C and Table 1). This analysis demonstrated that the sensitivity to PARP1 inhibition in HeLa cells that acquired cisplatin resistance depended on the concentration of inhibitor. At lower concentrations, cisplatin resistance was associated with increased resistance to PARP1 inhibition, while at very high concentrations of inhibitor the cells were notably more sensitive. Since this result differed from published reports, we generated a pool cisplatin-resistant cervical cancer cell line using SiHa cells (Supplemental Figure 2). Unlike clonal populations of resistant cells, this cell line is likely to have gained resistance through multiple mechanisms providing a broader representation of resistance mechanisms. SiHa cisplatin resistant pooled cells were more sensitive to PARP1 inhibition via olaparib (Supplemental Figure 3 and Table 1), suggesting that the increased dependence on PARP1 activity varies depending on the individual tumor or more likely the mode of resistance.

DISCUSSION:

We investigated the response of cervical cancer cells to low dose UV exposure. Particularly, our efforts illuminate the increased likelihood of UV-induced apoptosis in these cells. HPV oncogenes seem to counter the inclination towards programmed cell death as their shRNA mediated knockdown caused

increased p53- and BAX-associated apoptosis, suggesting dying cervical cancer cells sustain a greater abundance of DNA damage. This is somewhat suspected given the ability of HPV oncogenes to impair cellular DNA repair. We also generated clonal populations of cervical cancer cells that were resistant to chemical- or radiation-induced DNA crosslinks and measured their sensitivity to other crosslinking agents and a PARP1-inhibitor.

HPV oncogenes have both protective and detrimental effects on a cell's response to UV.

One realization from this study is that HPV oncogenes sensitize cells to UV while also protecting them from UV-induced apoptosis. HPV oncogenes cause constitutive activation of both ATM and ATR, two keystone repair kinases (Gillespie et al., 2012b; Johnson et al., 2017; Moody and Laimins, 2009). While this indicates repair initiation, successful repair also requires resolution of these signaling events as well. HPV E6 also mislocalizes repair proteins from sites of damage allowing the existence of active repair complexes without resolution of the damage they were activated in response to (Mehta and Laimins, 2018; Wallace et al., 2017). Such a disruption of repair signaling is consistent with the increased sensitivity to crosslinked DNA. HPV oncogenes protect the cell from BAK-mediated apoptosis, consistent with the virus's extensive efforts to avoid immune detection by minimizing its cytotoxic effects (Jackson et al., 2000).

While the benefits of evading the immune system are obvious, the evolutionary pressures that drove HPV to sensitize cells to UV are more cryptic. Unlike cutaneous members of the papillomavirus family, infections in the genital tract would not encounter sunlight often, making it unlikely they evolved a mechanism to change the cellular response to UV. The increased toxicity is more likely an unintended consequence of the role repair factors play in replicating the HPV genome. Perhaps being recruited to sites of viral replication prevents ATM and ATR from coordinating the robust response required to avoid the deleterious effect of UV-damage.

Resistance to crosslinking agents does not guarantee resistance to other genotoxic agents.

We generated cervical cancer cell lines that were resistant to two different sources of DNA crosslinks. The clonal populations of resistant cells insured that the acquisition of resistance occurred separately in each cell line. Although we did not determine the mechanism of resistance in these cells, the clonality suggests that cells gained the ability to survive either UV or cisplatin through diverse means. A significant amount is known about the ways that cells become tolerant of genotoxic drugs. Potential resistance strategies include reducing the functional concentration of the drug in the cell by pumping it out of the cell or obtaining a mutation that restores expression of a repair factor (Michels et al., 2013; Rosell et al., 2003; Srivastava et al., 2015; Zhu et al., 2016). Less is known about the ways cells come to be more tolerant of UV, but our observations are in line with previous reports indicating UV-resistance does not confer resistance to other DNA crosslinking agents (Petersen et al., 1995). Instead, UV-resistance appears to have fitness costs as cells grow slower and can be more sensitive to cisplatin (Data not shown, Figure 6 and (Petersen et al., 1995)). Similarly, sensitivity to small molecule PARP1 inhibitor may or may not accompany resistance to cisplatin (Figure 6 and Table 1). Moreover, cisplatin resistance was not accompanied with proportionate resistance to UV (Figure 6 and Table 1). Together, this adds to the evidence that understanding underlying mechanisms of resistance is critical for predicting the response of tumors to other genotoxic drugs.

Implications for therapeutic intervention.

Virtually every cervical cancer is the result of a human papillomavirus infection. HPV transforms cells in part by blocking tumor suppressors (p53 and RB) and by activating the catalytic subunit of telomerase. HPV E6 and E7 also have a well-documented ability to disrupt DNA damage repair (Bristol et al., 2017; Moody, 2018; Spriggs and Laimins, 2017b). HPV transformed cells require continued expression of HPV oncogenes. Since HPV E6 and E7 hinder a cell's ability to protect itself from DNA crosslinks, every cell in HPV-associated tumors are predicted to share this defect. This could explain the efficacy of crosslinking agents like cisplatin in the treatment of malignancies caused by HPV.

Therapeutics could be better designed to target HPV-associated cancers if a greater understanding of the

mechanisms by which HPV oncogenes induce sensitivity to DNA crosslinks is gained. We show that it is possible to acquire resistance to one source of DNA crosslinks while remaining sensitive to crosslinks from other origins, suggesting that tumors that have acquired resistance to cisplatin could remain sensitive to other crosslinking drugs such as chlorambucil or mitomycin c.

Finally, the importance of measuring the effects of sub-erythral doses of UV should be noted for studies addressing most clinically relevant questions, particularly those relevant to sensitivity or tumorigenicity. While exposure to high doses of UV or other crosslinking agents may provide interesting molecular insights, they likely induce different repair responses than the levels seen in common exposure to UV or after chemotherapy (Quinet et al., 2016). Further, excessive damage is more likely to induce an apoptotic response rather than a full-fledged effort to repair lesions.

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Figure Legends:

Figure 1: Sensitivity of cervical cancer to UV. A. This graph depicts the sensitivity of cervical cancer cell lines to ultraviolet radiation when compared to primary human foreskin keratinocytes (HFK) as measured by MTT. The black square points and solid line represent HFKs. The black circle points and dotted line represent HeLa cells, a cervical cancer cell line. B. This graph depicts the combined results of the colony formation assays. The solid black line and solid black squares represent HeLa and the dotted line and dot points are non-cancer HFK. C. This graph depicts the percentage of apoptotic (propidium iodide positive) cells 48 hours after increasing doses of UV. For all, n=3, *p<0.05 by unpaired t-test and error bars represent mean $\pm$ SD.

Figure 2: Confirmation of lentiviral transduction. A. Representative immunoblot showing E7 levels in HeLa cells transduced with the empty vector PLL, shRNA E6-1, or shRNA E6-2. Lysates were collected 2 days post lentiviral infection (PI) or 4 days PI. Tubulin is the loading control. B. Representative immunoblot showing E7 levels in HPV18 HeLa cells transduced with empty vector PLL or a gradient of shRNA 18E6-1. C. Representative immunoblot showing E7 levels in HeLa cells transduced with the empty vector PLL or shRNA E6-1. Lysates were collected 1 and 2 weeks PI. Tubulin is the loading control. D. Graphs showing relative E7 protein determined by densitometry.

Figure 3: Increased UV sensitivity in E6/E7 knockdown HeLa cells. A. This graph depicts UV sensitivity in HeLa cells transduced with the empty vector PLL or shRNA E6-1 as measured by MTT assay. B. This image depicts UV sensitivity in HeLa cells transduced with empty vector, a shRNA Control, or a shRNA 18E6E7 by colony formation assay (CFA). C. This graph depicts UV sensitivity in HeLa cells transduced with the empty vector PLL or shRNA 18e6-1 by cell counting. For all, n=3, *p<0.05 by unpaired t-test and error bars represent mean $\pm$ SD.

Figure 4: Increased cell cycle arrest and apoptosis due to UV sensitivity in E6/E7 knockdown HeLa cells.

A. Representative immunoblot showing BCL-2 related X protein (Bax) and p53 levels in shRNA 18E6-1

transduced HeLa cells with and without UV. Tubulin is the loading control. B. This graph depicts the percentage of HeLa cells in sub G1 after or without UV treatment. Cells were transduced with shRNA E6E7, lentiviral control, and BCL-2 exogenously expressed. For all, $n=3$, $*p<0.05$ by unpaired t-test and error bars represent mean $\pm$ SD.

Figure 5: Creating cross-linker resistant HeLa and SiHa cell lines. A. This image depicts the steps used to generate the cross linker resistant cell lines. Briefly, cells were treated 4 times with either 10 μ M cisplatin or 5mJ UV and given recovery periods between treatments. The resulting colonies were then isolated and transferred to individual wells of a 6-well plate for expansion. The resulting clonal populations were then tested for acquired resistance by MTT. B. This chart depicts the LC50s (amount required to kill 50% of cells) for each isolated cell line. The color gradient represents increasing resistance with black being the most resistant. C. This graph depicts UV sensitivity of the 2 most UV resistant HeLa cell lines as measured by MTT. The solid black line and circles represent the parental HeLa. The grey line, square, and triangle represent the 2 most resistance colonies. D. This chart depicts the LC50s for each isolated cell line with error bars representing the 95% confidence intervals. The color gradient represents increasing resistance with black being the most resistant. E. This graph depicts the cisplatin sensitivity of the isolated cell lines as measured by MTT. The solid black line and circle points represent Parental HeLa. The light grey line and square points represent the most resistant colony. For all, $n=3$, $*p<0.05$ by unpaired t-test and error bars represent mean $\pm$ SD.

Figure 6: Sensitivity to cross-linking agents in UV and cisplatin resistant cells. A. This graph depicts the sensitivity of HeLa cells as measured by MTT to ultraviolet radiation before and after acquiring resistance to cisplatin. The square points and solid line represent parental HeLa. The circle points and dotted line represent the Clone A of HeLa cells that acquired resistance to cisplatin. B. This graph depicts the sensitivity of HeLa cells to cisplatin measured by MTT before and after acquiring UV resistance. The square points and solid line represent the parental HeLa. The circle points and dotted line depict data from Clone A of UV resistant HeLa cells. The triangle points and the dashed line represent data from Clone B

of UV resistant HeLa cells. C. This graph depicts the PARP1 inhibitor (Olaparib) sensitivity of HeLa cells measured by MTT before and after cisplatin resistance was acquired. Black squares represent the parental HeLa line while open circles depict data from Clone A of cisplatin resistant HeLa cells. The untreated controls are set at 100. For all, $n=3$, $*p<0.05$ by unpaired t-test and error bars represent mean $\pm$ SD.

Table 1: Lethal Concentrations in UV and cisplatin-Resistant HeLa and SiHa. This table depicts the toxicities in cell lines before and after acquisition of resistance to cisplatin and UV. LC50 denotes the concentration or dose required to kill 50% of the cells calculated from MTT data (Figure 6). 95% CI denotes the 95% confidence intervals. * denotes significance difference compared to parental cell line determined by Student T-test ($p \text{ value} \leq 0.05$).

Declaration of Interest Statement:

The authors declare they have no conflicts of interest relevant to this manuscript.

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Abbreviations List:

HPV	Human Papillomavirus
UV	Ultraviolet Radiation
E6AP	E6 Associated Protein
RB	Retinoblastoma Protein
TLS	Translesion Synthesis
NER	Nucleotide Excision Repair
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide

Table 1

CELL TYPE	LC₅₀	LC₅₀ 95% CI
UV Toxicity (mJ/cm ²)		
HeLa Parental	6.547	5.46 to 7.8
HeLa Cisplatin Resistant A	10.8	8.49 to 13.73
Cisplatin Toxicity (μM)		
HeLa Parental	19.08	17.3 to 21.1
HeLa UV Resistant A	19.89	16.7 to 23.6
HeLa UV Resistant B	6.113*	5.9 to 6.3
Oliparib Toxicity (μM)		
HeLa Parental	845.3	674.8 to 1084
HeLa Cisplatin Resistant A	982	650 to 1656
SiHa Parental	636	556 to 731.1
SiHa Cisplatin Resistant Pooled	523.6	499.1 to 549.6

Figure 1

A. **B.** **C.** **D.**

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A.

Figure 7

Figure 8

Figure 9

Accepted Manuscript

Cervical cancer cell lines are sensitive to sub-erythral UV exposure

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