HPV-Positive Cervical Cancer Cells Show Generally Reduced Levels of IFN-Alpha

Sarah C. Bonaparte and Jennifer T. Thomas, Ph.D.

Cervical cancer is typically induced by the presence of Human papillomavirus, or HPV, the most common viral sexually transmitted infection in the United States. The presence of HPV has been shown to reduce the body's antiviral response by decreasing the levels of IRF-3, which is a transcription factor that controls the Interferon (IFN) antiviral proteins, within infected human cells. As an extension of these studies, we compared IFN-α levels in HPV-positive and HPV-negative cervical cancer cell lines. ELISA analysis was used to determine IFN-α protein levels in supernatants from cultured cell lines. Consistent trends suggest that HPV-positive cervical cancer cells show a reduction in IFN-α secreted when compared to HPV-negative cervical cancer cells, though significance was not found between the two cell lines. A reduction in IFN-α by HPV suggests HPV's ability to evade the body's immune response to viral infections. Understanding this mechanism may provide further insight into the development of cervical cancer.

INTRODUCTION

Cancer

Cancer is a disease characterized by abundant and unregulated cell proliferation that often results in the invasion of tissues. Over 100 different types of cancers have been identified and are named for the part of the body where the cancer originates (CDC, 2014, Cervical Cancer). Compared to healthy cells which follow the cell cycle in a methodical sequence, the consequences of unregulated cell growth in cancer can result in severe illness or death. A prominent feature of cancer is its ability to metastasize, or travel to other parts of the body through the blood or lymph systems, which can quickly result in organ obstruction or failure (National Institute of Health, n.d.).

Cervical cancer begins as an unregulated cell growth in the cervix, which is located at the lower end of the uterus, joining the uterus to the vagina in females. Cervical cancer, in contrast with other common cancers, often develops slowly and many successful treatments have been identified for premature stages of the disease. The transformation of benign to malignant cervical cancer cells is usually, but not always, induced by Human papillomavirus infections (Durst, 1987). There are multiple risk factors for cervical cancer that include having multiple sex partners, young or multiple pregnancies, extended use of oral contraceptives, smoking, and undergoing hormone replacement therapy. In addition, cervical cancer is a leading cause of female deaths in many developing countries. There is a crucial need for awareness and education among sexually active women in these developing countries, as well as in the United States, on the risks associated with cervical cancer (Faridi *et al.*, 2011).

According to Combes, *et al.*, 9.4% of cancers in women and 0.6% of cancers in men are related to Human papillomavirus infection, though the high percentage of HPV related cancers in

women is attributable to the prevalence of cervical cancer in developing countries. Additionally, HPV is linked to oropharyngeal cancers, or OPCs, cancers of the mouth and neck (National Institute of Health, n.d.). HPV-related OPCs occur in both men and women, though men may additionally or alternatively develop HPV-related penile and anal cancers. Though HPV-attributable OPC incidences are roughly equal among men and women, epidemiological evidence suggests that non-HPV related OPCs occur more frequently in men due to their generally more aggressive smoking and drinking behaviors (Combes *et al.*, 2014).

Human Papillomavirus

Human papillomavirus (HPV) is a small, double-stranded DNA virus that infects epithelial cells. There are over 100 recognized types of HPV, of which 40 types specifically infect genital epithelium. The different types of HPV are characterized by differences in genetic sequencing of the outer capsid protein, L1 (CDC, 2012, Human Papillomavirus). HPV is the most common sexually transmitted infection among sexually active men and women in the United States, and nearly all sexually active men and women are predicted to be infected with HPV at some point in their lifetime (Dunne et al., 2007). In fact, more than 10,000 women in the United States contract HPV each year. The virus can be spread through direct contact via oral, vaginal, or anal sex with an infected individual, even if the individual shows no visible symptoms of viral infection. The sites of infectivity of HPV include the genital areas of the vagina, cervix, rectum, anus, penis, and vulva as well as oropharyngeal regions (CDC, 2014, Genital HPV Infection). These sites are consistent with HPV's mechanism of infectivity in that HPV invades epithelial tissues via areas that are susceptible to trauma that could result in micro-lesions (Schiller, *et al.*, 2010).

The 40 different genital types of HPV are further classified into low risk (non-oncogenic and wart inducing) or high risk (oncogenic) types. Of the high risk types of HPV, 13 are known to be associated with cervical cancer. Typically, a healthy immune system can clear the body of virus upon HPV infection within two years. Additionally, infections may occur without symptoms and be cleared without the knowledge of the infected individual based on their relative health and the invading HPV type. However, when the immune system is unable to eliminate high risk types, the virus may induce the development cancer in any given amount of time (Dunne *et al.,* 2007). Oncogenic viral proteins may, then, interfere with normal the functions of the human cell that allow for apoptosis of virally infected cells, creating a persistent infection. The majority of previous studies investigate the relationship between high risk HPV and cervical cancer specifically. The development of other cancers induced by the presence of HPV has more recently become a topic of public health. For example, high risk HPV types 16 and 18 are also associated with penile and oropharyngeal cancers in men, with diagnoses of the later increasing significantly over the past 20 years (National Institute of Health, n.d.).

HPV infections may be identified by testing the DNA or RNA of a sample of cells (National Institute of Health, n.d.). Although there is no medical cure for HPV infections (National Institute of Health, n.d.), medications are available to reduce the common symptom of genital warts (CDC, 2015, Human Papillomavirus). There are currently three methods of vaccination against HPV that are both safe and effective for prevention. Gardasil®, approved by the FDA in 2006, works as a tetravalent HPV vaccine, providing protection against HPV types 6, 11, 16 and 18. Cervarix®, approved by the FDA in 2009, works as a bivalent HPV vaccine, providing

protection against types 16 and 18, though it cannot be distributed to males (CDC, 2012, Human Papillomavirus). The Food and Drug Association recently approved the administration of Gardasil 9® as a 9-valent vaccine, providing protection against oncogenic HPV types 16, 18, 31, 33, 45, 52, and 58 as well as non-oncogenic types 6 and 11. The inclusion of 5 other HPV types ultimately protects against an additional 20% of cervical cancer incidences (Printz, 2015). All three vaccines require three shots administered over, at minimum, a six month period of time. Doctors recommend that initial vaccines are received around age 11-12 and that a booster is received in the recipient's mid-twenties (CDC, 2014, Genital HPV Infection). The antigen used for vaccines is the L1 protein, the dominant capsid protein of HPV, which forms into noninfectious and non-oncogenic units called "virus like particles." Impressively, 99% of HPV vaccine recipients internally develop an antibody buildup just one month after finishing the sequence of shots (CDC, 2012, Human Papillomavirus).

Antiviral Immune Response

The immune system is composed of two distinct but interrelated parts - innate immunity and adaptive immunity. The innate immune system generally defends the body from foreign microbes, while the adaptive immune system protects the body by specifically responding to the particular invading microbe. All people are born with an innate immune system which guards from harmful microbes at birth. Conversely, adaptive immunity can only be built upon exposure to microbes foreign to the body (Sompayrac, 2008).

Papillomaviruses are the only known class of viruses that commence their infection extracellularly. Human papillomavirus is unable to bind to keratinocytes and can only induce infection in basal cells of stratified epithelium. The virus can only bind to human cells via L1 protein, the dominant protein constructing the virus capsid. The L1 capsid protein binds to heparin sulfate proteoglycans (HSPGs) on segments of exposed basement membrane. Since HPV can only bind to exposed areas of basement membranes, infection commonly occurs in epithelial cites susceptible to trauma that results in micro-lesions or abrasions (Schiller *et al.,* 2010).

The recognition of an invading pathogen begins when dendritic cells or macrophages, which are antigen-presenting immune cells, encounter and recognize pathogen via pattern recognition receptors (PRRs). Toll like receptors (TLRs), which are a particular class of PRRs, are hosted on the surface of these cell types to recognize specific pathogenic invaders. Once the binding of TLR on immune cell and pathogen associated molecular pattern (PAMP) on invading pathogen occurs, cellular signaling is initiated, leading to the activation of transcription factors (Abbas, 2014). Antiviral transcription factors widely include Interferon Regulatory Factors that monitor the transcription of antiviral interferon genes. Specifically, the expression of Interferon Regulatory Factor-3 (IRF-3) is a known part of an innate response to foreign viral microbes. IRF-3 usually remains in the cytoplasm of cells and is only activated upon viral invasion. When viral invasion occurs, IRF-3 reacts by promoting the expression of antiviral Type I Interferons IFN-α and IFN-β. The secretion of IFN-α and IFN-β promotes protein binding to receptors on virally uninfected cells to warn of the presence of a virus (Ronco *et al.*, 1998). IFN-α and IFN-β are also able to bind to receptors of virally infected cells and induce apoptosis to discontinue viral replication (COPE, n.d.). Additionally, IFN-α was identified as a regulator of the vascular endothelial growth factor C (VEGF-C), functioning to limit tumor angiogenesis and to prevent

metastasis of cancerous cells (He *et al.*, 2010). Evidence from the lab of Ronco *et al.* (1998) suggests that IRF-3 is suppressed in HPV-positive cells, preventing IFN-α secretion as well as likely keeping the innate immune system from functioning normally and from preventing the development and spread of cancerous tissues or viral infections.

The progression from Human papillomavirus infection to the development of cervical cancer is specifically due to the E6 and E7 genes in the viral genome. E6 viral proteins block cell cycle monitors by inhibiting the tumor suppressor gene p53, a transcription factor important for inducing apoptosis in infected cells. E7 viral proteins block the tumor suppressor gene Rb (retinoblastoma), an important cell cycle regulator. Collectively, these two genes alter somatic cells to allow virally infected cells to continue to survive and replicate, ultimately leading to tumor development (Faridi *et al.*, 2011).

Previous studies from Everhart and Thomas (2010) suggest that faulty tumor suppressor protein response resulted in a decrease in IRF-3 expression upon HPV infection. Furthermore, LeBlanc and Thomas (2014) discovered faint levels of IFN-α and IFN-β secreted by HPV-positive and HPV-negative cervical cancer cell lines through Western Blot analysis, though they observed no difference between the two cell lines. These studies led us to predict that a decrease in secreted IFN-α levels, a downstream product of IRF-3, would result in HPV-positive cervical cancer cells when compared to HPV-negative.

MATERIALS AND METHODS

Cell Culture

Cell lines derived from cervical cancer tissue were purchased from American Type Culture Collection (Manasas, VA) and grown and maintained in the tissue culture lab at Belmont University. The cell lines include:

C33A: HPV-negative cervical cancer cells
HeLa: HPV type 18 positive cervical cancer cells

These cell lines were chosen because they both are immortal cervical cancer cells but differ only in Human papillomavirus infectivity.

The frozen cell lines were thawed and aseptically transferred to a growth plate of 15 mL of EMEM (Eagle's Minimal Essential Media) (Life Technologies Corporation, Grand Island, NY) with 10% fetal bovine serum (Life Technologies Corporation, Grand Island, NY) and 5% gentamycin antibiotic (Life Technologies Corporation, Grand Island, NY) on plastic tissue culture plates. EMEM allows cell growth and proliferation in culture with essential nutrients while fetal bovine serum provides supplementary nutrients and gentamycin prevents the growth of additional microorganisms that could alter the study. The cell lines were incubated at 37 ºC and 5% CO_2 for cell proliferation to occur until the cells were 70-80% confluent. The cells were then harvested to new plates for further growth or freezing by aspirating them off of the EMEM, washing with 10 mL phosphate buffered saline (PBS) (Life Technologies Corporation, Grand Island, NY), and breaking adhesion to the growth plate using 2 mL 0.05% trypsin EDTA (Life Technologies Corporation, Grand Island, NY). Typically a 1:6 or 1:8 split was followed based on the cells confluence (Rush and Thomas, 2013). Cells were frozen for long-term storage using EMEM freezing media (Life Technologies Corporation, Grand Island, NY).

Supernatant Preparation
Since IFN-α proteins are secreted, the media was harvested for analysis. Four to five milliliters of the HPV-positive HeLa or HPV-negative C33A cell culture media was harvested and centrifuged at 1,500 rpm for 10 minutes at 4 ºC. Directly following centrifugation, 100 microliters of the supernatant of each cell type was directly aliquoted to the corresponding wells of the IFN-α ELISA kit (Abcam, n.d.).

IFN-α ELISA Protocol
A Verikine™ Human IFN-α Multi-Subtype Enzyme-Linked Immunosorbent Assay kit from PBL Assay Science (Pestka Biomedical Laboratories, Piscataway, NJ) was utilized for testing the IFN-α levels of HPV-positive HeLa and HPV-negative C33A cervical cancer cell lines. The ELISA kit was chosen based on its specificity in detecting minute levels of a specific protein. The test functions by a process of antibody binding to create signal upon reaction between substrate and antibody-bound enzyme. Ultimately, sample interferon concentration is calculated based on that of a known, or control, spectrum of interferon concentration.

The ELISA test was carried out according to manufacturer's protocol. HeLa or C33A supernatant of EMEM and standard samples provided by the kit were pipetted to corresponding wells of the 96-well plate. The plate was incubated at room temperature for one hour, during which the interferon present in the media bound to the antibodies present in the bottom of each well. The wells were then washed with diluted wash buffer. Diluted IFN-α antibody solution was pipetted into each well and the plate was incubated at room temperature for one hour, during which the secondary antibody bound to the interferon already bound to the antibody on the bottom on the wells. After washing with diluted wash buffer, HRP (horseradish peroxidase) solution was pipetted into each well. The plate was incubated a room temperature for one hour, during which time the HRP bound to the secondary antibody at the bottom of each well to create signal. After washing with diluted wash buffer, TMB (tetramethylbenzidine) substrate was pipetted into each well, and the plate was incubated in the dark for 15 minutes at room temperature. Stop solution was added to each well and a color change was visibly observed (PBL Science Assay, 2013).

Quantitation of Data
Within five minutes of adding stop solution, the absorbance of the supernatant in each well was read individually with a spectrophotometer at 450 nm. The number of cells per plate of supernatant media was calculated using a hemocytometer to normalize the amount of protein secreted among the different trials conducted. The concentration of IFN-α secreted by each cell was calculated based on the known IFN-α concentrations of the standard curve.

Statistical Analysis
Collectively, six positive readings from each cell line were used for statistical analysis following normalization based on cell count for each run. Statistical analysis was conducted by running a standard T-test on only these positive results from the ELISA tests to calculate a P value.

RESULTS

IFN-α Levels Generally Reduced in HPV-Positive Cervical Cancer Cells

We sought to determine the difference in IFN-α levels between HPV-positive HeLa and HPV-negative C33A cervical cancer cell lines with an ELISA test. Positive data from four separate experiments, of the six runs conducted, was used in statistical analysis. Evaluation of IFN-α levels in harvested supernatants of HPV-positive and HPV-negative cervical cancer cell lines by an ELISA kit and following T-test reveal no statistical difference between the two cell lines. As shown in Figure 1, a reduction of IFN-α levels in the HeLa HPV-positive cervical cancer cell line was observed in three out of the four individual experiments. Though clear trends were observed indicating that the C33A HPV-negative cells secreted more IFN-α than the HeLa HPV-positive cells, no statistical difference was found between the cell lines (p=0.104).

Picograms of IFN-α were determined based on IFN-α standards provided by the ELISA kit. Picogram levels were normalized based on cell count. A general reduction of IFN-α levels in the HeLa HPV-positive cervical cancer cell line was observed in three out of the four individual experiments.

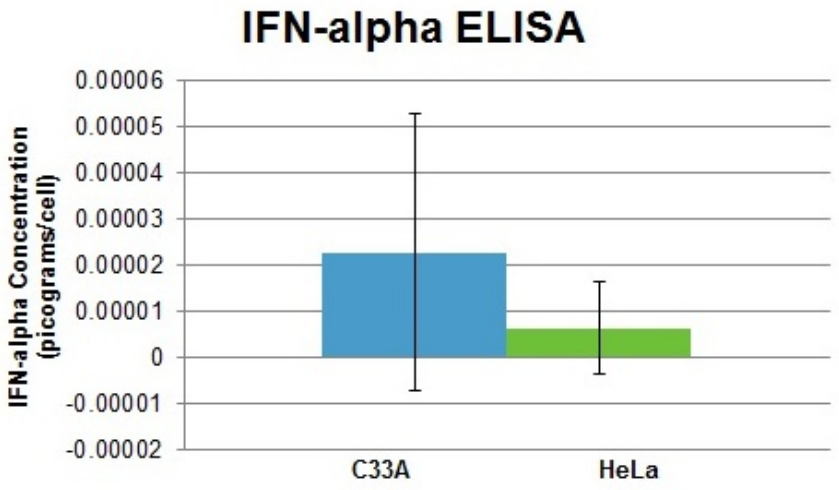

Figure 1. ELISA analysis of IFN-α levels in supernatants of HPV-positive and HPV-negative cervical cancer cell lines (p=0.104).

DISCUSSION

Though vaccines are currently available against four different types of HPV, the development of cervical cancer does still occur, especially with low-risk HPV types or in non-vaccinated individuals. Understanding how the virus functions provides understanding of the mechanism behind cervical cancer development inflicted by high risk HPV infections. Investigating this pathway could provide valuable information for further studies aimed at discovering different techniques of prevention or treatment of cervical cancer. The lab of Ronco *et al.* (1998) proposed that IRF-3 is suppressed in HPV-positive cervical cancer cells when compared to HPV-negative. These findings led us to question if IFN-α, a downstream product of IRF-3, would be

also suppressed. The secretion IFN-α in human cells generally follows this trend, suggesting that HPV maintains complex mechanism of human immune evasion (Everhart and Thomas, 2010).

The inability to obtain statistical significance could be attributed to a lack of consistently positive results. Only half of the overall data obtained was fit for statistical analysis. Though our conclusions cannot be supported by statistical evidence, consistent trends suggest that HPV indeed utilizes a mechanism of evading cellular antiviral responses. A better understanding of how to obtain fully positive readings from the spectrophotometer, in regards to the standard curve, would improve this experiment. A probable cause of difficulty in the ELISA protocol was variation in the preparation of media used for samples and standards. Despite the accuracy of the standards, some sample readings among different assays were negative. To test the variability in our methods, media incubation and centrifugation temperatures were minimally altered in some assays. Additionally, the ELISA kit recommends the use of a plate reader to read the absorbance of each well at once. The use of a spectrophotometer to read the absorbance of each well individually instead of the whole plate at once deviates from the ELISA protocol. However, this should not have affected resulting data. Stop solution was added to each well at the end of each assay to halt further reactions from occurring that could alter individual readings.

The cell lines used were optimal as subjects for the study because they are immortalized, cervical, cancerous, but differ in HPV type 18 infectivity. Both HPV-positive HeLa and HPV-negative cell lines are adherent to the bottom of growth plates. Confidence in this study and optimal results for statistical analysis could be confirmed in the future by testing multiple immortal cervical cancer cell lines beyond HeLa and C33A. HPV-positive cervical cancer cell lines that could be tested and compared with the results from the HeLa cell line include:

CaSki: HPV type 16 positive epidermoid carcinoma (ATCC, n.d., Ca Ski)
SiHa: HPV type 16 positive squamous cell carcinoma (ATCC, n.d., SiHa)
C4I: HPV type 18 positive carcinoma infected (ATCC, n.d., C-4 I)

HPV-negative cervical cancer cell lines that could be tested and compared with the results from the C33A cell line include:

DoTc2: uninfected carcinoma (ATCC, n.d., DoTc2 4510)

All of these cell lines are adherent and would be compatible with the ELISA protocol. In future studies, validation of results could be achieved by an in-depth analysis of the results of successful ELISA protocol carried out with the same and different high-risk types of human papillomavirus. Ultimately, cross examination between both HPV-positive and HPV-negative as well as between the two different types of human papillomavirus would provide a large amount of data and bring further confidence to the study.

In the future, analyzing IFN-α levels in cervical cancer cells infected with different bacteria and fungi would provide even more insight into cervical cancer development. The role of vaginal microbe presence in HPV infectivity, as well as cervical cancer development, has been uncertain. However, it has been suggested that HPV is required, but not sufficient, in cervical cancer development. Because less than 1% of HPV-positive individuals develop cervical cancer, there must be other factors at play to lead to the development of cancer (CDC, 2012, Human Papillomavirus). The work of Werner *et al*. (2010) suggests that the role of other non-viral infections could take part in cervical cancer development. This research was analyzed and expanded on at Belmont University by Rush and Thomas (2013), who showed that HPV-positive

cervical cell lines show a decrease in IRF-3 levels when further infected with bacteria and fungi when compared to un-infected HPV-positive cervical cancer cell lines. Additionally, the lab of Lee *et al*. (2013) established that women who test HPV-positive also possess a greater range in vaginal microbiota than those who test HPV-negative. Extending the work of Rush and Thomas (2013) by measuring and analyzing IFN-α levels, a downstream target of IRF-3 activity, upon infection of HPV-positive and HPV-negative cell lines with microbes potentially found in the vagina, would provide a great amount of information about cervical cancer development. Potential microbes include the Gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa*, Gram-positive bacteria *Staphylococcus aureus*, and a fungus *Candida albicans*.

Additional future studies using similar assays could be aimed at investigating other molecular pathways known to contribute to cellular antiviral activity. Multiple interferon regulatory factors are known to contribute to a cellular antiviral response in conjunction with IRF-3. IRF-7 is another transcription factor known to induce IFN-α and IFN-β secretion. Sato *et al.* (2000) suggest that the co-expression of both antiviral transcription factors IRF-3 and IRF-7 is necessary for production of normal levels of type 1 interferons α and β. Investigating the result of HPV infection in cervical cancer cells in IRF-7 levels, and further IFN-α products, could provide further insight about the possibility of other mechanisms at play in cervical cancer development.

Human papillomavirus, and therefore cervical cancer, prevention is a tangible luxury in many parts of the world. However, an effective treatment for HPV is not available to prevent the development of HPV-related cancers otherwise. The most important steps we can currently take to combat the development of HPV-related cancers are to educate individuals in developing countries about the dangers of unprotected sex and HPV infections, make vaccinations affordable and available to individuals in all parts of the world, and develop a treatment of HPV infections before the development of cancer can ensue. Even with vaccinations available in a large part of the world, HPV infections are common to those in developing countries and potential in individuals who are sexually active but have not been vaccinated. Additionally, vaccination is available against a maximum of seven oncogenic HPV types, while there are thirteen known oncogenic types of HPV. Even with the best current vaccine, HPV infectivity is still a possibility to all that engage in risky behaviors. Understanding more about the mechanism of cervical cancer development from HPV infection, and other HPV-related cancers, could ultimately reveal a new target that could be effective in the development of therapeutic techniques aimed at clearing viral infection and preventing the development of HPV-related cancers.

LITERATURE CITED

Abbas, A. K., Lichtman, A.H. (2014). *Basic Immunology: Functions and Disorders of the Immune System*, 4th ed. Saunders Elsevier, PA.

Abcam. (n.d.) *ELISA sample preparation guide.* Retrieved April 18, 2014, from Abcam: http://www.abcam.com/?pageconfig=resource&rid=14666

American Type Culture Collection. (n.d.). *C-4 I (ATCC® CRL-1594).* Retrieved January 27, 2015, from ATCC: http://www.atcc.org/Products/All/CRL-1594.aspx

American Type Culture Collection. (n.d.). *Ca Ski (ATCC® CRL-1550).* Retrieved January 27, 2015, from ATCC: http://www.atcc.org/Products/All/CRL-1550.aspx

American Type Culture Collection. (n.d.). *DoTc2 4510 (ATCC® CRL-7920).* Retrieved January 27, 2015, from ATCC: http://www.atcc.org/Products/All/CRL-7920.aspx

American Type Culture Collection. (n.d.). *SiHa (ATCC® HTB-35).* Retrieved January 27, 2015, from ATCC: http://www.atcc.org/products/all/HTB-35.aspx

Centers for Disease Control and Prevention. (2014, January 28). *Cervical Cancer.* Retrieved April 3, 2014, from CDC: http://www.cdc.gov/cancer/cervical/

Centers for Disease Control and Prevention. (2014, March 20). *Genital HPV Infection - Fact Sheet.* Retrieved April 3, 2014, from CDC: www.cdc.gov/std/hpv/stdfact-hpv.htm

Centers for Disease Control and Prevention. (2012, May 7). *Human Papillomavirus.* Retrieved April 3, 2014, from CDC: http://www.cdc.gov/vaccines/pubs/pinkbook/hpv.html

Centers for Disease Control and Prevention. (2015, January 22). *Human papillomavirus - treatment.* Retrieved April 21, 2015, from CDC: http://www.cdc.gov/hpv/treatment.html

Combes, J., Chen, A., & Franceschi, S. (2014). Prevalence of Human Papillomavirus in Cancer of the Oropharynx by Gender. *Cancer Epidemiology Biomarkers & Prevention, 23*(2954), 2954-2958. Retrieved April 22, 2015, from http://cebp.aacrjournals.org/content/23/12/2954.full

Cytokines and Cells Online Pathfinder Encyclopedia (COPE). (n.d.). *IFN-α.* Retrieved April 3, 2014, from COPE: http://www.copewithcytokines.de/cope.cgi?key=IFN%2dalpha

Dunne, E., Unger, E., Sternberg, M., McQuillan, G., Swan, D., Patel, S., Markowitz, L. (2007). Prevalence of HPV Infection Among Females in the United States. *The Journal of the American Medical Association.* 249(8): 813-819. Web. 08 Sep. 2014.

Durst, M., Croce, C.M., Gissman, L., Schwarz, E., Huebner, K. "Papillomavirus sequences integrate near cellular oncogenes in some cervical carcinomas." *PNAS*. 84.4 (1987): 1070-1074. Web. 28 Feb. 2014.

Everhart, B., & Thomas, J. (2010). Interferon Regulatory Factor-3 Levels are Decreased in Cell Lines Expressing Human Papillomavirus. *Journal of Proceedings Belmont University Undergraduate Research Symposium,* 21, 85-92.

Faridi, R., Zahra, A., Khan, K., Idrees, M. (2011). Oncogenic Potential of Human Papillomavirus (HPV) and Its Relation with Cervical Cancer. *Virology Journal,* 8 (269).

He, M., Cheng, Y., Li, W., Liu, Q., Liu, J., Huang, J., Fu, X. (2010). Vascular endothelial growth factor C promotes cervical cancer metastasis via up-regulation and activation of RhoA/ROCK-2/moesin cascade. *BMC Cancer,* 10: 170. Web. 08 Sep. 2014.

LeBlanc, C., & Thomas, J. (2014). The Evaluation of Type I Interferon Levels in HPV-Positive Cervical Cancer Cell Lines.

Lee, J.E., Lee, S., Lee, H., Song, Y., Lee, K., *et al.* 2013. Association of the Vaginal Microbiota with Human Papillomavirus Infection in a Korean Twin Cohort. PLoS ONE 8(5): e63514. doi: 10.1371/journal.pone.0063514

National Institute of Health. (n.d.). *HPV and Cancer.* Retrieved April 8, 2014, from the National Cancer Institute: http://www.cancer.gov/cancertopics/factsheet/Risk/HPV

PBL Assay Science (2013). *VeriKine™ Human IFN-α Multi-Subtype ELISA Kit.*

Printz, C. (2015). FDA approves Gardasil 9 for more types of HPV. *Cancer, 121*(8), 1156-1157. Retrieved from http://onlinelibrary.wiley.com/doi/10.1002/cncr.29374/full

Ronco, L., Karpova, A., Vidal, M., Howley, P. (1998). Human papillomavirus 16 E6 oncoprotein binds to interferon regulatory factor-3 and inhibits its transcriptional activity. *Genes and Development* 12: 2061-2072.

Rush, K., and Thomas, J. (2013). IRF-3 Levels Decreased in Human Papillomavirus Cervical Cell Lines Infected with Bacteria and Fungi.

Sato, M., Suemori, H., Hata, N., Asagiri, M., Ogasawara, K., Nakao, K., . . . Taniguchi, T. (2000). Distinct and Essential Roles of Transcription Factors IRF-3 and IRF-7 in Response to Viruses for IFN-α/β Gene Induction. *Immunity, 13*(4), 539-548. Retrieved April 22, 2015, from http://www.sciencedirect.com/science/article/pii/S1074761300000534

Schiller, J., Day, P., & Kines, R. (2010). Current Understanding Of The Mechanism Of HPV Infection. *Gynecologic Oncology, 118*. Retrieved April 22, 2015, from http://www.ncbi.nlm.nih.gov/pubmed/20494219

Sompayrac, L. (2008). *How the Immune System Works,* 3rd ed. Blackwell Publishing, MA.

Werner, J., DeCarlo, C.A., Escott, N., Zehbe, I., Ulanova, M. (2010). Expression of integrins and toll-like receptors in cervical cancer: effect of infections agents. *Innate Immunity* 0(0): 1-15.