Elucidating the role of Keratin 17 in Cervical Carcinogenesis

By:
Adriana Batazzi

Thesis submitted to the Johns Hopkins University in conformity with the requirements for the degree of Masters of Science.

Baltimore, Maryland
June 2014

© Adriana Batazzi
All rights reserved
Abstract

Cervical malignancies develop when viral genes from high-risk strains of the Human Papilloma Virus (HPV) such as HPV type 16 and 18 are expressed in the cervical epithelium. Expression of Keratin 17 (K17), an intermediate filament cytoskeletal protein that is robustly upregulated in a variety of epithelial-derived tumors, is positively correlated with the progression of cervical lesions and is considered a reliable prognostic biomarker for cervical cancer. We previously reported that the genetic loss of Krt17 attenuates skin tumorigenesis in a mouse model for spontaneous basaloid tumor formation, correlating with a modulation of T helper 1/T helper 17 (Th1/Th17) and T helper 2 (Th2) inflammatory cytokine expression, and resulting in dampened recruitment of immune cells to the site of tumor formation. The present study aims to understand the role of K17 in cervical carcinogenesis using the HPV16tg mouse model, which spontaneously forms squamous cell carcinoma-like lesions in cervical epithelia when females are treated with estrogen. We hypothesized that onset and progression of HPV16tg-induced cervical lesions would be delayed in the absence of K17, and that K17’s immunomodulatory role extends to the cervical epithelium. Compared to wildtype controls, HPV16tg/+ mice exhibit robust hyperplasia of cervical epithelia, as well as increased cervical epithelial proliferation and vasculature 3 months after receiving an estrogen implant. All of these trademarks of cancer were found to be attenuated with the genetic loss of Krt17. Whereas several Th1 pro-inflammatory cytokines are upregulated in HPV16tg/+ cervical tissue relative to wildtype control, these are further upregulated in the Krt17 null background, a finding that is consistent with
the literature on cervical carcinogenesis, but opposite to what prevails in the ear skin tumors occurring in the same mice. Collectively, these findings support the notion that K17 expression is positively correlated with the progression of tumors and extends the idea that K17 functions as an immune response modulator in both cervical and skin carcinogenesis. The observed differences in inflammatory readouts between ear and cervical epithelia suggest that K17’s impact on signaling pathways and gene expression occurs in a tissue and/or context-dependent manner.

Advisor: Dr. Pierre Coulombe

Readers: Dr. Pierre Coulombe and Dr. Fengyi Wan
Preface

This thesis is based on research conducted at the Johns Hopkins Bloomberg School of Public Health from June 2013 until June 2014. This twelve-month project was conducted in the Coulombe laboratory under the supervision of Dr. Pierre Coulombe, principal investigator, and Dr. Ryan Hobbs. All relevant literature was accessed through the Welch Library of the Bloomberg School of Public Health portal.

I would like to acknowledge Dr. Pierre Coulombe for his mentorship and guidance, and for allowing me to work on this project. I would also like to extend my gratitude to Dr. Ryan Hobbs for his invaluable help in conducting the experiments relevant to the project and for his unlimited patience. I would like to thank Minerva C. Han for all of the preliminary work that she accomplished prior to my taking on the project, and the members of the Coulombe laboratory for all of their help. Lastly, I would like to thank all the members of department of Biochemistry and Molecular Biology of the Bloomberg School of Public Health for the help and support provided to see this project pushed forward.
Table of Content

Title Page .......................................................................................................................... i
Abstract .................................................................................................................................. ii
Preface ....................................................................................................................................... iv
Table of Content ....................................................................................................................... v
List of Tables ............................................................................................................................ vi
List of Figures ........................................................................................................................... vii
Introduction ..............................................................................................................................
  1. The human papilloma virus: causative agent of cervical cancer ......................................... 1
  2. Keratin 17 in tumor growth and cervical carcinogenesis ......................................................... 8
  3. The immune response in cervical carcinogenesis .................................................................... 14
  4. Introducing the mouse model for cervical carcinogenesis ....................................................... 17
Materials and Methods .......................................................................................................... 19
Results ....................................................................................................................................... 25
  1. Keratin 17 expression increases with lesion progression in the cervical epithelium of the K14-HPV16tg mice ............................................................................................. 25
  2. Genetic loss of Krt17 delays onset and progression of cervical carcinogenesis ................. 27
  3. Keratin 17 modulates the immune response and affects immune cell recruitment .......... 34
Discussion .................................................................................................................................... 40
Conclusion .................................................................................................................................... 45
References .................................................................................................................................... 46
Curriculum Vitae ....................................................................................................................... 57
List of Tables

Materials and Methods

Table 1: Table of forward and reverse sequences of mouse primers used in qRT-PCR analysis of cervical and ear skin tissue

Results

Table 2: Summary of morphological features characteristic of cervical lesions observed in WT, HPV16\textsuperscript{tg/\textsuperscript{+}/K17\textsuperscript{+/+}} and HPV16\textsuperscript{tg/\textsuperscript{+}/K17\textsuperscript{--/--}} mice at one, three and six months post-implantation
List of Figures

Introduction

Figure 1: The HPV genome and the oncogenic actions of E6 and E7
Figure 2: The human reproductive tract.
Figure 3: The mouse reproductive tract
Figure 4: Two distinct but homologous families of Keratins
Figure 5: The structure of Keratins
Figure 6: Keratin 17’s expression pattern with CIN progression.
Figure 7: The K14-HPV16 Mouse model for cervical carcinogenesis

Results

Figure 8: K17 expression increases with lesion progression
Figure 9: HPV16tg/+K17/- mice have delayed lesion onset
Figure 10: HPV16tg/+K17+/+ mice have a hyperproliferative epithelium
Figure 11: HPV16tg/+K17/- mice have decreased evidence of vessel formation
Figure 12: HPV16tg/+K17/- mice have an enhanced pro inflammatory response in the cervix.
Figure 13: HPV16tg/+K17/- mice have a dampened pro inflammatory response in ear skin
Figure 14: HPV16tg/+K17/- mice have a higher number of intra-epithelial immune cells
Introduction

1. The Human Papilloma Virus: causative agent of cervical cancer

Cervical cancer is the second leading cause of cancer deaths among women worldwide. With over 500,000 new cases and 200,000 deaths yearly, it is to this day a major public health burden (Parkin et al., 2005). Prevention and treatment of this disease was largely aided when, in the 1990s, a causal relationship between certain strains of the human papilloma virus (HPV) and the development of cervical cancer was established (Walboomers et al., 1999). Among the hundreds of strains of the virus, a select few are termed “high-risk” and drive the process of tumorigenesis, not only in the case of cervical cancers but also for anal, vaginal, penile and oropharyngeal cancers (Chaturvedi, 2010). Of those high-risk strains, HPV16 is the most commonly found strain in cervical malignancies. The field of cervical carcinogenesis has therefore, in the last two decades, largely focused on investigating how the expression of HPV16’s viral genes leads to neoplastic transformation and ultimately cervical cancer.

HPV16 is a DNA virus with a circular, double stranded genome composed of early gene regions encoding proteins E1, E2, E4, E5, E6 and E7, and late gene regions encoding the L1 and L2 proteins (Figure 1a). Viral DNA replication occurs primarily in basal keratinocytes of stratified squamous epithelium (zur Hausen et al., 1981), where the virus replicates to produce episomes that have the potential to integrate the genome of the host's proliferative basal cells and further express viral proteins.
In the early stages of the HPV16 infection, the E1 and E2 proteins regulate both the number and maintenance of episomes and the transcription of the two viral oncogenes, E6 and E7, through mechanisms that are still under investigation (Kadaja et al., 2009). Upon viral integration, E1 and E2 gene expression are disrupted and the expression of E6 and E7 is no longer “properly controlled”. The epithelium subsequently becomes hyper-proliferative (McBride et al., 1991). An HPV infection is termed productive when a sufficient number of episomes have integrated the host’s genome to lead to the deregulated expression of E6 and E7.

Findings suggest that one of the ways whereby both the E6 and E7 oncoproteins cause abnormal cell growth is through a stimulation of proteasome-mediated degradation of important tumor suppressor gene products: p53 and pRb. The ubiquitin ligase E6-AP is altered by E6, leading to ubiquitination and subsequent proteasomal degradation of p53, thus compromising cell cycle checkpoints and rendering cells unable to undergo apoptosis (Thomas et al., 1999). Studies have also shown that E7 can direct proteasomal degradation of pRb by directly interacting with subunits of the proteasome (Gonzales et al., 2001). In addition to directing proteasomal degradation, E7 affects the association of pRb with the E2F family of transcription factors, which play a crucial role in cell cycle control. As a result, cells are driven into the G1-S phase, stimulating them to proliferate and hence promoting viral DNA replication (Hiebert et al., 1992; Nevins et al., 1991). However, a deregulated cell cycle represents only one aspect of HPV16’s role in malignant transformation. Some findings suggest that viral genome
integration also causes genomic instability in the host, leading to the accumulation of mutations (Duensing and Munger, 2002). The intricacies of the mechanisms whereby E6 and E7 cause the keratinocytes of the cervical epithelium to undergo neoplastic transformation have yet to be completely elucidated (Figure 1b).

**Figure 1: The HPV genome and the oncogenic actions of E6 and E7.** (a) Schematic of the early and late gene regions of the HPV16 genome. (Munger, 2002) (b) Schematic of the actions of E6 and E7. Upon loss of E2, E6 and E7 get overexpressed and lead to the degradation of p53 and unbound E2F leads to deregulated transcription of genes that control the cell cycle. (Janiceck & Averette, 2001)

The HPV infection initially causes a cervical intraepithelial lesion (CIN), which can then progress through increasing stages of dysplasia upon viral genome integration because of the uncontrolled cell growth, and ultimately develop into malignant cervical carcinomas. In clinical terms, CINs at stage 1 are low-grade squamous lesions while CINs at stages 2 and 3 have become high-grade squamous
lesions that can then cause a carcinoma *in situ* before developing into squamous cell carcinoma (Mitchell et al., 1994). It is important to note that cervical intraepithelial lesions caused by HPV infections do not all have malignant potential and do not typically develop into cervical carcinomas; for instance, in women aged 25 years and under, CINs at stage 1 spontaneously regress over time in over 90% of the cases as opposed to progressing to the stage of high grade lesions, and in 70-80% of the cases in women 25 years or older (Cox et al., 2003; Moscicki et al., 2004). In addition, cervical cancer only develops decades after exposure to the virus (Watson, 2005). This suggests that cervical carcinogenesis is a multifactorial process, involving factors other than viral protein overexpression.

The human cervical epithelium is metaplastic. The portion of epithelium that is exposed to the vagina is termed the ectocervix and is stratified and squamous, while the endocervix, lining the upper portion of the cervix, is composed of columnar epithelial cells. The two types of cells meet in a region termed the squamocolumnar junction, and studies have shown that viral protein overexpression in this “hot spot” of the cervical epithelium is more likely to cause a cancerous infection (Herfs et al., 2012 and Herfs et al., 2013) (Figure 2).
Metaplastic epithelium can be found in other parts of the body, such as the esophagus, respiratory tract or gut, and there is increasing evidence that these sites of junction of different cell types are more susceptible to abnormal cell growth when exposed to cancer-causing agents (Birchmeier et al., 1995; Hirota et al., 1999; Kuipers, 1998; Kim et al., 1997). Whether or not an HPV infection is productive therefore also depends on the location and type of cells in which the oncoproteins are expressed.

In addition to this histology-related factor, estrogen levels are also thought to affect the productivity of the HPV infection, as increased estrogen levels were
reported to promote cervical carcinogenesis. The most well documented mechanism by which estrogen acts as a carcinogen is through its binding to the nuclear Estrogen Receptor alpha (ERα) and stimulating cell proliferation (Chung et al., 2010). In the normal cervix, basal cells of the squamous epithelium, metaplastic cells and endocervical glandular cells are all ERα positive, so increased and prolonged estrogen exposure is correlated to disease progression, and lower estrogen levels could be correlated to lesion regression.

Mouse models have been invaluable in the investigation of all of the factors and mechanisms of cervical carcinogenesis. Although there are anatomical differences between the human and murine reproductive tract, the histological similarities have allowed for translation of findings from murine studies to humans. In mice, the cervical canal splits to form the cervico-uterine transformation zone, and the two canals lead to the uterine horns. The outer cervix, or cervico-vaginal region, the cervical canal and the cervico-uterine transformation zone are all composed of stratified squamous epithelium, while in the uterine horns the canals are composed of columnar epithelium (Manocha and Graham, 1970). Mice therefore also have a squamocolumnar junction, and mouse models used to study cervical carcinogenesis typically develop lesions in the cervico-uterine transformation zone (Elson et al., 2007) (Figure 3).
In patients with malignant lesions, immature basal-type squamous cells progressively replace suprabasal squamous cells in the entire cervical epithelium, but this was shown to not be the case in murine models of cervical cancer. To establish the grade of neoplastic progression in these models, parameters such as cell proliferation, dysplasia, localization of anaplastic cells in suprabasal epithelial layers, angiogenesis and mast cell recruitment are used (Riley et al., 2003).

Figure 3: The mouse reproductive tract. Composite H&E image of the reproductive tract of a K14-HPV16 transgenic mouse. Stratified squamous epithelium is found in the outer cervix, the cervical canal and the cervico-uterine transformation zone, and columnar epithelium is found in the canals of the uterine horns. (Riley et al., 2003)
The current method of detection for cervical cancer is the Pap smear, a histological and cytological analysis of cells scraped from the opening of the cervix of patients, with HPV DNA testing as an adjunct method (Franco et al., 2003). While these methods allow for early detection of abnormal cells, they have poor specificity for whether or not the identified lesions have malignant potential, requiring repeated testing in subjects with abnormal histological and cytological results (Mitchell et al., 1994). This lead to the current thought that the use of novel biomarkers that would monitor molecular events in histological and cytological samples would help in identifying lesions that are at higher risk of developing into cervical carcinomas. A number of potential biomarkers are currently under investigation for cervical cancer screening, but of particular interest to this study is Keratin 17 (K17).

2. Keratin 17 in tumor growth and cervical carcinogenesis

Keratins are divided into 28 type I or 26 type II intermediate filament proteins (Schweizer et al., 2006) that co-assemble in an obligatory and pairwise fashion to give rise to 10 nm wide intermediate filaments (Fuchs and Cleveland, 1998; Kim and Coulombe, 2007) (Figure 4). All keratins and intermediate filament proteins share a tripartite structure consisting of the central $\alpha$-helical rod domain, a head and a tail (Figure 5). The expression of many of the specific pairings of type I and type II keratins is regulated in a tissue-differentiation, and context-specific fashion (Kim and Coulombe, 2007).
Figure 4: Two distinct but homologous families of Keratins. Keratins are sub grouped into Type I and Type II keratins and share sequence homology. (Adapted from Coulombe, 2001)

Figure 5: The structure of Keratins. (a) Schematic of Keratin secondary structure. All keratins share a tripartite structure consisting of a head, a tail, and a central α-helical rod domain that is the major determinant of self-assembly. (b) Electron microscopy image of in vitro reconstructed filament from purified Keratin 5 and Keratin 14 proteins. Scale bar = 500um. (Adapted from Gu and Coulombe, 2005)
In surface epithelia, in particular, keratin filaments make a crucial contribution to mechanical support and cytoarchitecture (Coulombe and Wong, 2004; Fuchs and Cleveland, 1998; McGowan and Coulombe, 1998). In such epithelia, keratin filaments are very abundant and are organized into a cytoplasmic network that is anchored at the surface of the nucleus and typically extends to the cell periphery, with attachment at sites of cell-cell and cell-matrix interactions (Coulombe and Lee, 2012). Interference with the structural support role of keratin filaments, e.g., through genetically inherited mutations in individual keratin proteins, engenders cell fragility and susceptibility to mechanical trauma-induced cell lysis (Omary et al., 2004). For example, missense mutations in either the Keratin 5 or Keratin 14 genes, which are respectively type II and type I keratins that partner together and are constitutively expressed in the basal layer of the epidermis, cause epidermolysis bullosa simplex, a disease characterized by skin blistering upon trivial mechanical trauma (Bonifas et al., 1991; Gu and Coulombe, 2005; Ma et al., 2001).

Additional functions have been defined for keratin proteins, as could be anticipated from their diversity and dynamic properties, and the context-dependent regulation of keratin proteins (Hobbs et al., 2012). For instance, keratins have been implicated in cell signaling pathways that regulate key cellular processes such as protein synthesis, maintenance of homeostasis, cell growth and apoptosis in a context-specific manner (Kim et al, 2006; Paladini and Coulombe, 1998; Tong and Coulombe 2006).
A number of studies have shown that the pattern of keratin expression changes with neoplastic progression in the cervical epithelium (Smedts et al., 1990; Maddox et al. 1999). This is consistent with the notion that most tumorigenic processes are, early on, accompanied by aberrations in differentiation. A study published by Frank Smedts et al in 1992 shows a screen of the keratins expressed in normal cervical epithelium and cervical epithelium of patients with low grade and high-grade neoplastic lesions. K17, although present at very low levels in normal cervical epithelium, is present in relatively low amounts at the CIN1 stage and shows increased expression with increasing severity of lesions. Furthermore, the expression of K17 seems restricted to the basal cells of the cervical epithelium at the CIN1 stage, but becomes apparent in suprabasal layers as the lesions progress (Figure 6).

**Figure 6: Keratin 17's expression pattern with CIN progression.** Although restricted to the basal layer of the cervical epithelium at CIN1, Krt17 expression increases with lesion progression into high-grade squamous lesions and locates to suprabasal layers. (Adapted from Smedts et al., 1992)
In addition, a very recent study by Escobar-Hoyos et al (April 2014) reports the results of a large proteomic analysis of human cervical tissues in an attempt to identify prognostic and diagnostic biomarkers. High K17 expression was seen in patients with high-grade squamous lesions, and was associated with poor survival of squamous cell carcinoma patients. Interestingly, upregulated K17 expression has been associated with lesion progression and poor prognosis in a number of epithelial cancers other than cervical cancer, including those occurring in the skin, breast, oral cavity, bile duct, esophagus and larynx, lung, ovary and gut (Chen et al., 2005; Che et al., 2005; Cohen-Kerem et al. 2004; DePianto et al., 2010; Ide et al., 2012; Kitamura et al., 2012; Toyoshima et al., 2012; van de Rijn et al., 2002; Wang et al., 2012). This suggests that K17, in addition to being a potentially very faithful biomarker of malignant potential, plays a role in epithelial tumorigenesis.

K17 is constitutively expressed in ectoderm-derived epithelial appendages such as the hair follicle, nail beds or sebaceous glands. Furthermore, it is induced upon wounding for tissue repair, which along with carcinomas is a setting of enhanced cell proliferation and altered differentiation (Mansbridge and Knapp, 1987; McGowan and Coulombe, 1998). The functional relevance of this increase in K17 expression is not yet known, but contributes to the idea that this keratin is implicated in signaling pathways that regulate cell proliferation and differentiation. Indeed, it is reported that K17 modulates hair follicle cycling in a TNFα dependent manner, and that it is required for the persistence of the anagen state, a state of rapid cell growth (Tong and Coulombe, 2006). Interestingly, K17 is also involved in pathways that are potentially oncogenic. It is shown to bind to the adaptor protein
14-3-3σ and stimulate the mTOR pathway, thus regulating protein synthesis and epithelial cell growth at the wound edge (Kim et al., 2006). Deregulation of multiple elements of the mTOR pathway has been reported in many types of cancers such as colon, gastric and hepatocellular carcinomas, and has a significant effect of tumor progression (Kim and Eng, 2011; Populo et al., 2011; Villanueva et al., 2008). In addition, K17 expression is correlated with the epidermal growth factor receptor expression, with EGFR activation stimulating Krt17 promoter activity and increasing the fraction of soluble K17 protein (Chung et al., 2012). EGFR overexpression is associated with a poor prognosis in head and neck, ovarian, cervical and esophageal cancers (Bauknecht et al., 1989; Ozawa et al., 1980; Pfeiffer et al., 1989; Sainsbury et al., 1987). There is therefore a body of evidence that suggests that K17 is implicated in signaling pathways that have been formally implicated in tumor growth, and that it could be a driver of tumorigenesis.

The Krt17 gene is highly conserved between human and mouse, and as a result mouse models for epithelial cancers are used to investigate the role of K17 in tumorigenesis. A study by DePianto et al. published in 2010 provided evidence that tumor development may be affected by K17 expression. In the Gli2 transgenic (Gli2tg) mouse model, in which basal cell carcinoma-like lesions spontaneously develop with great reproducibility in ear skin, the genetic loss of Krt17 was found to delay the onset of the lesions and attenuate their progression. Loss of Krt17 in this setting correlated with reduced tumor keratinocyte proliferation and a profound attenuation of inflammation and modulation of cytokine expression. Specifically, the Th1 “pro inflammatory” immune response is decreased, and the Th2 “anti-
inflammatory” response is upregulated (see next section for further information). This was manifested through the decreased expression of pro inflammatory cytokines and presence of inflammatory immune cells in the Krt17 null background.

In considering whether or not K17 plays a similar role in the context of cervical carcinogenesis, it is important to review the current understanding of the immune system’s response to the HPV infection and its response to malignant progression in cervical epithelium.

3. The immune response in cervical carcinogenesis

Naïve CD4+ T lymphocytes, also called Th0 cells, differentiate into subsets that are distinguished by the cytokines they produce. The Th1 response occurs when T cells differentiate into cells that specifically produce pro inflammatory cytokines, the canonical one being IFNγ, and represents a key component of cell-mediated immunity through the production of cytotoxic T lymphocytes, or CD8+ T cells. The Th2 response occurs when T cells differentiate into cells that produce anti-inflammatory cytokines such as IL4, IL5 and IL13, and through these cytokines mediate the activation and maintenance of the humoral immune response. The Th2 response counteracts the Th1 response, and ideally, when facing an immune challenge, a well-balanced Th1 and Th2 response suited to that challenge is generated (Szabo et al., 2010; Zhu et al., 2010).

In the context of cancer, clinical outcome is strongly dependent on the ability of host cells to generate an immune response that would ultimately result in tumor
regression (Shurin et al., 1999). There is evidence that a strong $T_\text{h}1$ response leads to the infiltration of $T_\text{h}1$ cells within the tumor microenvironment, thus promoting the killing of tumor cells, either through direct cell-cell contact or through cytokine-mediated mechanisms, and hence inhibit tumor growth (Coussens and Werb, 2002). Evidence shows that in several types of tumors, the occurrence of a shift from a $T_\text{h}1$ to the $T_\text{h}2$ phenotype correlates with the transition between a precancerous to an invasive tumor stage (Demaria et al., 2010). Such a decrease in so-called pro-inflammatory cytokines allows tumor cells to promote angiogenesis and infiltrate the surrounding tissue (Grivennikov et al., 2010). The $T_\text{h}1$ response is therefore associated with a good prognosis when the $T_\text{h}1$ cells control tumor invasion. On the other hand, chronic inflammation has been defined as a key contributor to tumor growth and a number of inflammatory diseases are associated with a greater risk for cancer (Balkwill and Mantovani, 2001). In this context, inflammatory cells play a pro-tumorigenic role, and this was found to occur in part through the inhibition of apoptosis and the destruction of host cell-mediated immune responses. The reduction of inflammation is characterized by a reduction of wound healing-like processes like angiogenesis or tissue remodeling, and leads to better clinical outcomes (Mantoveni et al., 2008). This was further confirmed when a correlation between the use of anti-inflammatory drugs and better patient outcomes in a number of cancer types was established (Rayburn et al., 2009).

The role of inflammatory cells in the tumor microenvironment is therefore ambiguous and likely context dependent. Tumor progression is largely dependent on the immune response to malignant cells, and how inflammation can either
protect or destroy tissues is an emerging issue in the field of tumor immunology. A better understanding of the interplay between immune cells, inflammation and cancer could help in promoting the immune response against cancer in affected patients.

In the case of cervical cancer, the upregulation of pro-inflammatory cytokines upon HPV infection is known to promote cell-mediated immunity and thus has a potent anti-tumor effect. Findings suggest that the regression of HPV lesions is related to the upregulation of the interferon pathway, and clinically, a shift away from the T_h1 response is associated with poorer clinical outcomes in cervical cancer patients (de Jong et al., 2004; Scott et al., 1999). In addition, IFNγ has been shown to directly inhibit tumor growth in cervical cancer cell lines (Sharma et al., 2002).

HPV genes are expressed in keratinocytes of the cervical epithelium, and these cells are therefore crucial to the development of an effective immune response to viral proteins. Infected keratinocytes are important initiators of the innate immune response, and promote a successful adaptive immune response along with the dendritic cells of the cervical lining (Black et al., 2009; Nestle et al., 2009). As such, the HPV virus causes a robust inflammatory response early in the stages of infection in human cervical epithelium. However, the virus eventually evades the immune system of the host (Stanley, 2012). This occurs in part through the actions of the E6 and E7 oncoproteins, which have been reported to down regulate interferon pathways and modulate cytokine expression in cervical keratinocytes, allowing for lesion progression and the development of cervical cancer (Kanodia et al., 2007; Karim et al., 2011; Um et al., 2002).
The expression of both IFNγ and IFNγ-induced cytokines, such as Cxcl9, Cxcl10 and Cxcl11, and pro-inflammatory cytokines like Cxcl5 or IL1β, have been shown to be Krt17-dependent in the basaloide tumors spontaneously arising in the ear skin of Gli2tg mice (DePianto et al., 2010). In such ear skin lesions, expression of these cytokines is robustly upregulated compared to wildtype controls, and is associated with a state of chronic inflammation and tumor growth. Genetic loss of Krt17 leads to a reduction of inflammation and slower progression of ear skin tumors in these Gli2tg mice, correlating with markedly reduced expression of several powerful cytokines including CxCl5, CxCl9, CxCl10, CxCl11, and several others (DePianto et al., 2010). Current research in the Coulombe laboratory focuses on understanding if K17’s influence on the immune response is through direct control of gene expression, which other cofactors are involved, and how these pathways could be targeted for therapeutic applications.

4. Introducing the HPV16tg mouse model for cervical carcinogenesis

The present study uses a well-established mouse model by Arbeit et al., 1996 for cervical cancer, namely the K14-HPV16 transgenic mice (HPV16tg) chronically administered 17-β estradiol by pellet implantation. These mice have been serially backcrossed in the FVB/n background, a strain known to be permissive for epidermal carcinogenesis, another end point of interest to our laboratory. Viral gene expression is driven by human K14 promoter, which is active in the basal progenitor layer of all stratified epithelia including the cervix. When combined, viral protein
expression and elevated estrogen levels lead to the development of cervical lesions one to three months post implantation, and invasive carcinomas six months post implantation, in treated HPV16tg mice. The model and timeline of the protocol being used (treatment, harvest) are shown in Figure 7.

We hypothesized that the genetic loss of Krt17 would lead to a delayed lesion onset and attenuated tumor progression in this model, since K17 expression is correlated with cervical lesion prognosis and its overexpression is associated with a poor clinical outcome. We also hypothesized that the effect would be related to K17’s immunomodulatory function. The mouse model allowed for a comparative analysis of tumor development between wild type, HPV16tg/+K17+/+ and HPV16tg/+K17−/− mice, to elucidate the role of K17 in cervical carcinogenesis.

![Diagram of HPV16 Early region genes (E1-E7) and timeline of treatment and harvest]

**Figure 7: The K14-HPV16 Mouse model for cervical carcinogenesis.** Mice express the early region genes of the HPV virus in the cervical epithelium. All mice are implanted at 1 month of age (p30). Mice were harvested at 1 month (p30), 3 months (p60) and 6 months (p210) post implantation, and a second implant was done for the mice harvested at 6 months post implantation. (Adapted from Arbeit et
Materials and Methods

Transgenic Mice and Hormone Treatment

The K14-HPV16 mouse (HPV16tg) has been previously described (Arbeit et al., 1996). Wildtype (WT), HPV16tg and Krt17/- female mice, maintained on the FVB/n genetic background (obtained from the National Cancer Institute), were used for generating the desired genotypes (WTK17+/+, HPV16tgK17+/+ and HPV16tgK17/-) through selective crossings. Genotyping protocols have been previously described for the Krt17, and HPV16tg genes (National Cancer Institute Mouse Repository, strain code #01XT3). At one month of age, virgin female mice of the appropriate phenotypes were anesthetized using short exposure to 100% ether and implanted with 90-day release, 0.05mg 17β-estradiol pellets (Innovative Research of America, Sarasota, FL) in the dorsal region of the neck. Implanted mice were harvested for tissue, RNA and protein at three time points: one, three and six months post-implantation. Groups of 7 to 10 mice were harvested per time point and per genotype. Mice harvested six months post-implantation were re-implanted after the first 90-day release period was completed (Figure 7). All mice were housed in the Broadway Research Building animal housing facility of Johns Hopkins University, individually or with littermate controls, and were fed rodent chow and water ad libitum upon weaning. All procedures involving mice were performed using protocols reviewed and approved by the Johns Hopkins University Animal Care and Use Committee.
Tissue Procurement

The cervix was collected by excision of both uterine horns anteriorly, and the upper vagina area posteriorly. Excised tissue was embedded in Sakura Tissue-Tec OCT compound (Torrance). 5-7uM thick sections were stained with hematoxylin-eosin (Thermo Scientific) or toluidine blue, or processed for indirect immunofluorescence with relevant antibodies or a TUNEL assay.

RNA/Protein isolation

Cervix samples used for RNA/Protein isolation were prepared using Trizol (Invitrogen). Tissue was homogenized and directly underwent phase separation. Protein and RNA were isolated post-phase separation as directed by the standard protocol, and RNA samples underwent the RNAeasy Minikit cleanup protocol (Qiagen). All RNA samples were checked for purity via spectrophotometry.

Antibodies and Western Blotting

Primary antibodies used for indirect immunofluorescence include rabbit polyclonal antisera directed against K17 (McGowan, JCB, 1998), K14 (Covance), PECAM-1 (Santa Cruz Biotechnology, Inc.), phospho-histone H3 (Cell Signaling), F4/80 (AbD Serotec) and CD207 (eBioscience); and rat monoclonal directed against CD11b (Novus Biologicals), and CD4 (BD Pharmigen). The secondary antibody used was Alexa 488 (Invitrogen).

Primary antibodies used for Western Blot analysis in this study include rabbit polyclonal antisera directed against K17 (McGowan, JCB, 1998), a rabbit polyclonal
against HPV E7 (Invitrogen) and a mouse monoclonal against actin (Sigma).
Secondary Antibodies include goat anti-rabbit HRP and goat anti-mouse HRP (Sigma). Blots were developed using ECL Select developing solution according to the manufacturer’s instructions (GE Healthcare).

**Immunofluorescence**

All antibodies were diluted in a block solution consisting of 5% Natural Goat Serum in 1× PBS. All cryosections were left unfixed before block solution application. All antibodies yield a staining pattern consistent with previously published studies. Rabbit IgG (Santa Cruz biotechnology) was used as a negative control for all rabbit immune antibodies, and rat IgG (Santa Cruz biotechnology) for all rat immune antibodies. Sections were imaged using a Zeiss fluorescence microscope with Apotome attachment at the Johns Hopkins University Bloomberg School of Public Health, department of Biochemistry and Molecular Biology. Images were acquired at equal exposures for each type of preparation (i.e any given protocol) and equally brightened, contrasted and cropped with the Axiovision software.

**Determination of Apoptosis**

For analysis of DNA degradation representative of apoptosis in the cervical epithelium, the TUNEL assay (Roche) was used. Sections were digested with Proteinase K (Invitrogen) for one hour at 37° C. The positive control sample was exposed to DNase I (Roche) for 30 minutes at room temperature. The negative control was incubated in a solution lacking the enzyme. All slides were incubated at
37°C for two hours with a reaction mixture consisting of TUNEL enzyme and TUNEL label in dilution buffer, as directed by the standard protocol. DAPI, made up in 1X PBS solution, was used to stain the nuclei, thus revealing tissue architecture. Images were acquired in the same way as with all other immunofluorescence stains (see Immunofluorescence section).

Signal quantification/Cell counts/Measures of epithelial thickness

Signal quantification of Western Blots and K17 and PECAM-1 immunofluorescence images was done using the Image J software.

A manual cell count was performed for the quantification of phosphohistone H3-positive cells, mast cells and TUNEL positive cells. Three biological replicates per genotype were tested, and 10-15 images of different regions of the cervico-uterine transformation zone and the cervico-vaginal region were acquired per mouse. The number of cells stained positive relative to negative controls was manually counted and averaged first per mouse, and then per genotype.

Epithelial thickness was measured on H&E images acquired with a Zeiss microscope at the Johns Hopkins University Bloomberg School of Public Health, department of Biochemistry and Molecular Biology, using the Image J software.

qRT-PCR

Following RNA extraction, 1 µg of RNA was reverse-transcribed with the RT² iScript kit (BioRad). qRT-PCR was performed on the first strand cDNA using SSO-Advanced SYBR green (BioRad) as described by the manufacturer. PCR parameters for custom
qRT-PCR screen = 95°C for 5 minutes, followed by 40 cycles of 95°C for 10 sec + 55°C for 30 sec. No template controls, no reverse-transcriptase controls, standard curves, and a melt curve were included with every PCR plate. Data analysis for commercial qRT-PCR arrays was performed using the template provided online by SABiosciences. Actin and 18S were used as reference genes for normalization of the data. Fold enrichments and error bars for custom qRT-PCR data were determined using the traditional ΔΔC_q method as calculated in Microsoft Excel. A list of all custom qRT-PCR primers used is provided in Table 1.
**Table 1:** Table of forward and reverse sequences of mouse primers used in qRT-PCR analysis of cervical and ear skin tissue

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer</th>
<th>Sequence (5'--&gt;3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>18S</td>
<td>Forward</td>
<td>CCTGTGCGCTTTCTTTGGGA</td>
</tr>
<tr>
<td>18S</td>
<td>Reverse</td>
<td>CATTCGAAGCTCTGGCCCTTATC</td>
</tr>
<tr>
<td>Actb</td>
<td>Forward</td>
<td>GGCTGTATTCCCCCTCCATCG</td>
</tr>
<tr>
<td>Actb</td>
<td>Reverse</td>
<td>CCAGTTGGAACAATGGCCATGT</td>
</tr>
<tr>
<td>Aire</td>
<td>Forward</td>
<td>AGGTCAGCTTCAGAGAAAAACCA</td>
</tr>
<tr>
<td>Aire</td>
<td>Reverse</td>
<td>TCACTCCAGACACTCTGAGA</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Forward</td>
<td>TTAAAAACCTGGATCGGAACCAA</td>
</tr>
<tr>
<td>Ccl2</td>
<td>Reverse</td>
<td>GCATTAGCTCTAGATTTACGGGT</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>Forward</td>
<td>CCAAGTGTGCACGTCATTCTTT</td>
</tr>
<tr>
<td>Cxcl10</td>
<td>Reverse</td>
<td>GGCTCGACCCGGATGATTCCA</td>
</tr>
<tr>
<td>Cxcl11</td>
<td>Forward</td>
<td>GGCTTCCTTATGTTCACAAACG</td>
</tr>
<tr>
<td>Cxcl11</td>
<td>Reverse</td>
<td>GCCTGATCTGCATGCAATCAA</td>
</tr>
<tr>
<td>Cxcl15</td>
<td>Forward</td>
<td>TCCAGCTCCATTCTAGC</td>
</tr>
<tr>
<td>Cxcl15</td>
<td>Reverse</td>
<td>TTGGCGCTATAGCTGAGGAG</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>Forward</td>
<td>TCTTTTGGGATCGCATATCTC</td>
</tr>
<tr>
<td>Cxcl9</td>
<td>Reverse</td>
<td>TTGTAGTGGAACGCTGACCATG</td>
</tr>
<tr>
<td>Eif4e</td>
<td>Forward</td>
<td>AACGGAAAAACCAACCCCTAC</td>
</tr>
<tr>
<td>Eif4e</td>
<td>Reverse</td>
<td>CTCTGGGTTAGGCAACCTC</td>
</tr>
<tr>
<td>Eif4ebp1</td>
<td>Forward</td>
<td>GGGGACTACAGCACACCAC</td>
</tr>
<tr>
<td>Eif4ebp1</td>
<td>Reverse</td>
<td>GTTCGACACTCCATGAGAAAT</td>
</tr>
<tr>
<td>Hnrnpk</td>
<td>Forward</td>
<td>ACTGATGAGATGGTGAATTGCG</td>
</tr>
<tr>
<td>Hnrnpk</td>
<td>Reverse</td>
<td>CTGCAGTCTGAGTCTGACGG</td>
</tr>
<tr>
<td>Ido1</td>
<td>Forward</td>
<td>GCTTTGCTCTACACATCCAC</td>
</tr>
<tr>
<td>Ido1</td>
<td>Reverse</td>
<td>CAGCGCTTGAACCTCTGCT</td>
</tr>
<tr>
<td>Ifng</td>
<td>Forward</td>
<td>ATGAAGCCTACAGCCATG</td>
</tr>
<tr>
<td>Ifng</td>
<td>Reverse</td>
<td>CCATCCCTTGGAGCTTCC</td>
</tr>
<tr>
<td>Il1b</td>
<td>Forward</td>
<td>GAAATGCCTACCTGGTACATG</td>
</tr>
<tr>
<td>Il1b</td>
<td>Reverse</td>
<td>CTTAGCTCTAGCTGAGACAC</td>
</tr>
<tr>
<td>Il10</td>
<td>Forward</td>
<td>GCTCTGATGAGACATG</td>
</tr>
<tr>
<td>Il10</td>
<td>Reverse</td>
<td>CCGAGCTTAGGAGCATG</td>
</tr>
<tr>
<td>Il17a</td>
<td>Forward</td>
<td>TTTAATCTCCCTTGCCGCAAAAA</td>
</tr>
<tr>
<td>Il17a</td>
<td>Reverse</td>
<td>CTCTCCCTCTCGCAATTTGAC</td>
</tr>
<tr>
<td>Il18</td>
<td>Forward</td>
<td>GACTCTTGCAACTTCAAGG</td>
</tr>
<tr>
<td>Il18</td>
<td>Reverse</td>
<td>CAGGCTGCTCTTTGTCATAG</td>
</tr>
<tr>
<td>Il22</td>
<td>Forward</td>
<td>ATGAGTTTCTTTCTTATGGG</td>
</tr>
<tr>
<td>Il22</td>
<td>Reverse</td>
<td>GCCTGAGATGCTGACACTC</td>
</tr>
<tr>
<td>Krt17</td>
<td>Forward</td>
<td>ACCATCGGCGAGTATCACTC</td>
</tr>
<tr>
<td>Krt17</td>
<td>Reverse</td>
<td>CTAAGGCAGGCACACTG</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Forward</td>
<td>CTGGGACAGCCGACACACTAAAG</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Reverse</td>
<td>CTGCGGCAAGTCTCATGAG</td>
</tr>
<tr>
<td>Tgfb</td>
<td>Forward</td>
<td>CTCCCCCTGGCGCTTACG</td>
</tr>
<tr>
<td>Tgfb</td>
<td>Reverse</td>
<td>GCCTAGTTGGACAGGATCTG</td>
</tr>
<tr>
<td>Tnfa</td>
<td>Forward</td>
<td>CTTGCGGCGAGTACGAAG</td>
</tr>
<tr>
<td>Tnfa</td>
<td>Reverse</td>
<td>GCCTTGTACGAGTATTTTACG</td>
</tr>
</tbody>
</table>


Results

1. Keratin 17 expression increases with lesion progression in the cervical epithelium of the K14-HPV16tg mice

The site of tumor onset in this mouse model was previously reported to be the cervico-uterine transformation zone (TZ) (Elson et al., 2000). Both the cervico-uterine transformation zone and the cervico-vaginal region are shown in Figure 8a. K17 expression in the cervical epithelium of the transgenic mice was assessed via immunofluorescence and western blotting. Immunofluorescence images show that K17 is expressed at low levels in the basal layer of the TZ of the cervical epithelium in mice harvested one month post implantation. At three months post implantation, expression of K17 increases in the TZ, along with evidence of epithelial thickening and papillation. At six months post implantation, K17 expression is seen throughout the thickened, hyperplastic cervical epithelium, not only in the basal compartment but also in all suprabasal layers (Figure 8b). This was further confirmed via western blotting, showing the amount of K17 antigens increasing over time (Figure 8c). Thus, expression of K17 in the cervical epithelium is consistent with previous reports on the HPV16tg mouse model and findings in human cervical epithelium, thereby setting the stage for an analysis of the impact of Krt17 genetic loss on cervical carcinogenesis.
Figure 8: K17 expression increases with lesion progression. (a) Reconstructed H&E image of mouse cervical epithelium. The site of tumor onset in the HPV16tg mice is the cervico-uterine transformation zone. Signs of neoplastic progression could also be observed in the cervico-vaginal region. (b) K17 Immunofluorescence staining in the cervico-uterine transformation zone of the HPV16tg/+K17+/+ mice at one month, three months and six months post-implantation. Note the increase in expression and the localization of K17 in suprabasal cells over time. (c) K17, E7 and Actin protein expression over time in total protein extracts of the cervix of the HPV16tg/+K17+/+ mice. E7 expression is maintained and K17 expression increases over time, as confirmed by signal quantification. (Scale = 100uM)
2. Genetic loss of Krt17 delays onset and progression of cervical carcinogenesis

Hallmarks of onset of cervical intraepithelial lesions include the first evidence of dysplasia, papillomatosis and epithelial thickening. The degrees to which the TZ of WT, HPV16\textsuperscript{tg/+K17+/+} and HPV16\textsuperscript{tg/+K17−/−} mice harvested at all three time points showed disorganized, dysplastic cells and invagination of the basal layer was visually assessed on H&E stained cervical tissue sections. At one-month post implantation, no evidence of dysplasia, papillation or epithelial thickening is observed for any genotype (Figure 9a-c). At three months post implantation, HPV16\textsuperscript{tg/+K17+/+} mice show evidence of moderate dysplasia with some degree of papillation, characteristic of lesion onset. In contrast, WT and HPV16\textsuperscript{tg/+K17−/−} mice have similar histology and show a smoother, more continuous epithelium in the TZ than the HPV16\textsuperscript{tg/+K17+/+} mice (Figure 9d-f). The onset of the lesions is therefore delayed in the HPV16\textsuperscript{tg/+K17−/−} mice assessed at three months post implantation. At six months post implantation, HPV16\textsuperscript{tg/+K17+/+} and HPV16\textsuperscript{tg/+K17−/−} mice both show severe dysplasia, papillomatosis, and some evidence of microinvasive or fully invasive lesions. The WT mice also start displaying some mild hyperplasia (Figure 9g-i). The prolonged chronic estrogen administration is likely to be the culprit for the hyperplastic epithelium in wild type mice and may account for the lack of apparent differences between HPV16\textsuperscript{tg/+K17+/+} and HPV16\textsuperscript{tg/+K17−/−} mice at six months post implantation, since elevated levels of estrogens are known to accelerate the progression of tumors.
Quantitative measurements of inner epithelial thickness of the TZ show a significant difference at three months post implantation between the \textit{HPV16}^{tg/*K17^{+/}} mice and the \textit{HPV16}^{tg/*K17^{-/-}} mice (p=0.004) and between the \textit{HPV16}^{tg/*K17^{+/}} mice and \textit{WT} controls (p=0.007). There was no statistical difference between the wildtype controls and the \textit{Krt17^{-/-}} knockout mice (Figure 9j). The features used to assess the onset of cervical intraepithelial lesions in the mice used in this study and the degree to which they were present are summarized in Table 2.

\textbf{Table 2:} Summary of morphological features characteristic of cervical lesions observed in \textit{WT}, \textit{HPV16}^{tg/*K17^{+/}} and \textit{HPV16}^{tg/*K17^{-/-}} mice at one, three and six months post-implantation.\(^1\)

<table>
<thead>
<tr>
<th>Features</th>
<th>\textit{WT}</th>
<th>\textit{HPV16}^{tg/*K17^{+/}}</th>
<th>\textit{HPV16}^{tg/*K17^{-/-}}</th>
<th>\textit{WT}</th>
<th>\textit{HPV16}^{tg/*K17^{+/}}</th>
<th>\textit{HPV16}^{tg/*K17^{-/-}}</th>
<th>\textit{WT}</th>
<th>\textit{HPV16}^{tg/*K17^{+/}}</th>
<th>\textit{HPV16}^{tg/*K17^{-/-}}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dysplastic Epithelium</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Papillation</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>Invasive Lesion</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Epithelial Thickening</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

\(^1\) Features are scored on a scale from 0 (none observed) to + (some evidence) to ++ (high evidence) based on observations of 10-15 images per mouse used in this study.
**Figure 9: HPV16+/+K17−/− mice have delayed lesion onset.** (a-i) H&E images of the TZ of the mice (a-c) At one month post implantation, WT and transgenic mice show no signs of lesions. (d-f) At three months post implantation, the wild type and knockout mice show no signs of dysplasia, but the HPV16+/+K17+/+ mice show evidence of invagination of the cervical epithelium (papillation) and a dysplastic, thickened epithelium in the TZ. (g-i) At six months post implantation, all transgenic mice have severe papillomatosis and dysplasia. WT controls show some evidence of hyperplasia (Scale = 100uM). (j) Quantitative measurements of the inner epithelial thickness at three months post implantation based on H&E images of the TZ. The cervical epithelium is thickest in the HPV16+/+K17+/+ mice (Scale = 50uM).
The grade of neoplastic progression in murine models of cervical cancer is established using hallmarks of tumor progression such as the number of mitotically active cells in the basal layer of the epithelium, evidence of recruitment of cells able to induce angiogenesis, and number of stromal mast cells, known to be important orchestrators of angiogenesis in squamous cell carcinomas (Coussens et al., 1999). Phosphohistone H3 (PH3) immunostaining, a known marker of mitotically active cells, indicates an increased number of PH3 positive cells in the basal layer of the cervical epithelium of HPV16tg/+K17+/+ mice compared to WT controls (p=0.02) and HPV16tg/+K17−/− mice (p=0.003). The WT controls and the HPV16tg/+K17−/− mice show no statistical difference (Figure 10a). In contrast, TUNEL positive cells, which are apoptotic and found in the outermost suprabasal layers of the cervical epithelium, show no difference in count across genotypes of transgenic mice (Figure 10b).
Figure 10: HPV16tg/+K17+/+ mice have a hyperproliferative epithelium. (a) Immunofluorescence of PH3 at three months post implantation. Note that the HPV16tg/+K17+/+ mice have a higher number of PH3 positive cells in the basal layer of the epithelium of the TZ than the knockouts or the WT controls. (b) TUNEL positive cell quantification as a marker of apoptosis. The transgenic mice have no differences in the number of apoptotic cells in the outermost suprabasal layer of the epithelium of the cervico-uterine transformation zone. (Scale = 100uM)
Immunofluorescence staining of PECAM-1, a cell surface marker for blood vessels, and quantification of the signal seen up to 10 µm away from the cervical epithelium suggests that there is no statistical difference in the number of PECAM-1 positive cells between WT controls and \textit{HPV16\textsuperscript{tg/+K17/-}} mice. However, there is a net increase in the number of PECAM-1 positive cells around the cervical epithelium of \textit{HPV16\textsuperscript{tg/+K17/+}} mice compared to both the \textit{WT} controls (\(p=0.01\)) and the \textit{HPV16\textsuperscript{tg/+K17/-}} mice (\(p=0.02\)), suggesting that the genetic loss of \textit{Krt17} leads to a reduction in the recruitment of cells that contribute to angiogenesis (Figure 11a).

Although the positive correlation between angiogenesis and progression of neoplasms in the female genital tract has been documented, it is still unclear whether noninvasive lesions such as cervical intraepithelial lesions are also directly contributing to angiogenesis (Obermair et al., 1996). Studies have shown that there was a positive correlation between mast cell density and vessel density in CIN lesions (Utrera-Barillas et al., 2010). A mast cell count following a Toluidine blue stain indicates that at three months post implantation, the number of mast cells in the stromal component of the cervix is highest in the \textit{HPV16\textsuperscript{tg/+K17/+}} mice compared to the other genotypes (Figure 11b). Increased stromal mast cells could contribute to the differences observed in vasculature across genotypes.
Figure 11: *HPV16tg/+*K17/* mice have decreased evidence of vessel formation.* (a) Immunofluorescence of platelet derived endothelial cell adhesion molecule 1 (PECAM-1), marker of cells involved in angiogenesis, in the TZ at three months post implantation. The *HPV16tg/+*K17/* mice have higher staining signal up around the basal layer of the epithelial lining compared to the knockout mice and the wild type controls. (b) Toluidine blue stained tissue sections to assess the number of stromal mast cells in the TZ three months post implantation. The *HPV16tg/+*K17/* mice have a higher number of stromal mast cells compared to knockout mice and wild type controls. (Scale = 100uM)
3. Keratin 17 modulates the immune response and affects immune cell recruitment

The balance between the T_h1 and the T_h2 immune response is crucial in the anti-tumor immune response, and K17’s role as an immunomodulator in the BCC-like skin lesions of the Gli2tg mouse model indicates that the genetic loss of Krt17 leads to cytokine profiles that are favorable to the deceleration of neoplastic progression. We hypothesized that this would also be the case in the cervical epithelium of this mouse model.

Quantitative RT-PCR analysis of cervical samples shows a distinct cytokine profile across the HPV16tg/+K17+/+ and HPV16tg/+K17-/- genotypes. At one month post estrogen implantation, both the HPV16tg/+K17+/+ and the HPV16tg/+K17-/- mice exhibit an increase in the mRNA levels of the T_h1-related proinflammatory cytokines such as Cxcl5, Cxcl9, Cxcl10, Cxcl11, IFNγ, TNF α and IL1β relative to the WT controls, with the HPV16tg/+K17-/- mice exhibiting up to a 10 fold increase in the mRNA of these cytokines compared to the HPV16tg/+K17+/+ mice. Additionally, the T_h2-related cytokines such as IL10, IL18 and IL22, are up to 10 fold lower in the HPV16tg/+K17-/- mice compared to HPV16tg/+K17+/+ relative to the WT controls (Figure 12a). Therefore the levels of T_h1 and T_h2 cytokines are significantly different between the analyzed genotypes. The HPV16tg/+K17-/- mice exhibit a pro inflammatory cytokine profile that is consistent with the observation of delayed and mitigated cervical lesion onset and progression early in cervical tumorigenesis. By three months post implantation, however, genotypic differences in cytokine profiles are no longer as pronounced (Figure 12b).
Figure 12: HPV16tg/+K17−/− mice have an enhanced pro-inflammatory response in the cervix. (a) At one month post implantation, qRT-PCR analysis shows that pro-inflammatory targets such as Cxcl9, Cxcl10, Cxcl11, IFNγ, TNFα and IL1β have a higher fold change in the HPV16tg/+K17−/− mice than the HPV16tg/+K17+/+ mice compared to the WT controls. (b) At three months post implantation, the expression of these selected targets shows no difference across genotypes compared to WT controls.
Relative to cervical tissue, such differences across genotypes do not occur in the ear skin of this mouse model at one month post implantation, but are observed at three months post implantation, with $HPV16^{tg}/+K17^{-/-}$ mice exhibiting a dampened pro inflammatory immune response (Figure 13a). This is consistent with what has been observed in the $Gli2^{tg}$ mouse model (DePianto et al., 2010), although there does not seem to be a clearly defined polarization towards the $T_h2$ response in the ear lesions of these mice (Figure 13b).
Figure 13: *HPV16*α+/*K17*/- mice have a dampened pro-inflammatory response in ear skin. (a) At one month post implantation, qRT-PCR shows that there are no differences in the expression of targets across genotypes of transgenic mice compared to WT controls. (b) At three months post implantation, the expression of selected pro-inflammatory targets such as Cxcl9, Cxcl11, IFNg and Cxcl5 is higher in *HPV16*α+/*K17*/+ mice than *HPV16*α+/*K17*/- mice compared to WT controls. Note that the expression of pro-inflammatory targets is opposite in the ear skin at three months post implantation than it is in the cervix tissue at one month post implantation.
Intraepithelial lymphocytes such as macrophages and Langerhans/dendritic cells were previously described as resident immune cells of the cervical epithelium (Amador-Molina et al., 2013). In addition, the TZ was reported to be the major site of induction of the cell-mediated immune response since it contains a high density of intraepithelial T cells (Pudney et al., 2005). In response to the HPV infection, keratinocytes as well as T cells and dendritic cells contribute to the promotion of a pro-inflammatory response, through both cytokine signaling and additional immune cell recruitment. Immunofluorescence stainings for CD11b and F4/80, which are macrophage markers and CD4 as a T cell marker, were performed. We observed that there are considerably more intraepithelial immune cells at three months post estrogen implantation in HPV16tg/+K17−/− mice compared to WT controls or HPV16tg/+K17+/+ mice (Figure 14). Altogether, the data suggest that downstream of the robust pro-inflammatory immune response seen in cytokine profiles at one month post implantation, recruitment of both adaptive and innate immune cells known to be key in cell mediated immunity is affected at three months post implantation.
Figure 14: *HPV16*+/+*K17−/−* mice have a higher number of intra-epithelial immune cells. Immunofluorescence of CD4 T cells (top row), F4/80 macrophages (middle row) and CD11b macrophages and neutrophils (bottom row) in the TZ. All three immune cells are resident cells of the tissue and are present in the WT controls. Their presence is enhanced in the transgenic mice at three months post implantation. They are highly detectable within the cervical epithelium of the *HPV16*+/+*K17−/−* mice but not in the *HPV16*+/+*K17+/+* mice or the WT controls. (Scale = 100uM)
Discussion

The recent implication of K17 in signaling pathways that have been formally associated with tumor growth suggests that it could be a driver of tumorigenesis. K17 expression levels are correlated with cervical intraepithelial lesion progression and higher K17 levels are associated with poorer clinical outcomes in cervical carcinoma patients, contributing to the idea that K17 directly impacts tumorigenesis in the context of cervical cancer. In this study, we establish that K17 expression increases with lesion progression in the K14-HPV16 mouse model for cervical carcinogenesis, and that upon it genetic ablation, cervical intraepithelial lesion onset is delayed and progression is attenuated. We also observe that the loss of Krt17 leads to an enhanced Th1 immune response, a response that is protective in cervical carcinogenesis and which is not as pronounced when K17 is present. This is in striking contrast to the situation prevailing in ear skin, where the genetic loss of Krt17 also attenuates tumorigenesis but dampens the “pro-inflammatory” Th1 immune response, which is ultimately also protective to the tissue. Although the ear skin and the cervical epithelium exhibit opposite cytokine profiles upon Krt17 loss, the phenotype of the lesions remains consistent. Therefore, consistent with our hypothesis, K17 is a driver of tumorigenesis in cervical cancer, and has substantial immunomodulatory function in both the ear skin and the cervical epithelium.

Similarly to the fact that the “pro-inflammatory” Th1 response can be both protecting and damaging in different contexts, K17’s role in regulating the expression of Th1 related genes likely differs depending on the tissue where cancers
develop. The cervical epithelium and the epidermis of ear skin are likely to have different co-factors or transcription factors involved in the regulation of gene expression and possibly directly interacting with K17, contributing to the observed differences in cytokine profiles. Since the genetic ablation of Krt17 leads to an altered T cell differentiation pathway in both the cervical epithelium and the ear skin, we can speculate that K17 is involved in pathways relevant to T cell differentiation in a context and tissue dependent manner. One pathway of interest is the mTOR pathway: mTOR acts intrinsically through its mTORC1 complex to promote T_{h1} differentiation. T_{h1} cells fail to differentiate into a T_{h1} phenotype upon suppression the mTORC1 complex (Delgoffe et al., 2011, Hoshii et al., 2014), and the mTORC1 complex was recently found to be a key regulator of cell proliferation at the site of TPA-induced inflammation in mouse skin (Checkley et al., 2011). Interestingly, abnormal activation of the mTOR pathway has been reported in cervical cancer, and a gradual increase in activity was correlated with lesion progression (Wu et al., 2013). Although there is evidence of a K17 dependent stimulation of cell growth and Akt/mTOR activity in keratinocytes (Kim et al., 2006), its implication in the pathway is poorly understood in a cancer setting. Further investigation relating K17’s role in the Akt/mTOR pathway in ear skin tumors and cervical neoplasms could help in elucidating its role not only as a key contributor to tumor progression, but also as an immunomodulator.

Another pathway of interest is the IFN-JAK-STAT1 pathway. Interferon receptor chains, which are receptors expressed on keratinocytes, have tyrosine kinase activity. Upon binding of IFNγ to its receptor, the Jak1 and Jak2 proteins are
recruited and activate the STAT1 transcription factor after a series of phosphorylation events. STAT1 translocates to the nucleus and stimulates the transcription of specific genes. The pathway was reported to have an anti tumor effect in epithelial cancers such as cervical, prostate and breast cancer (Chan et al., 2012; Lee et al., 1999; Wee et al., 2014), in part through STAT1’s stimulation of the transcription of apoptotic genes such as p65 or Bcl2, or genes that mediate immune functions (Khodarev et al., 2012). Interestingly, STAT 1 has been implicated in Th1 cell differentiation and IFNγ regulation. The loss of IFNγ signaling in cells was reported to lead to an impaired Th1 phenotype. A proposed mechanism is the activation of T-bet, a Th1specific transcription factor, by STAT1 and IFNγ in T cells, stabilizing the Th1 phenotype (Szabo et al., 2000).

Findings have shown that in a setting of skin inflammation associated with high IFNγ levels, K17 expression is induced through the activation of STAT1 by IFNγ in epidermal keratinocytes (Jiang et al., 1994; Komine et al., 1996). Interestingly, HPV’s oncogenes E6 and E7 were found to selectively suppress STAT1 in skin keratinocytes. The increase in STAT1 levels upon keratinocyte differentiation is reported to be inhibited with the presence of the oncogenes (Hong et al., 2011). The possible interactions between STAT1 and E6 and E7 in cervical tissue have not been investigated. Since IFNγ expression was found to be K17 dependent in both mouse ear skin and cervical tissue in this report, we can speculate that a feedback loop between K17-STAT1-IFNγ could be disrupted by the E6 and E7 oncogenes in a context dependent manner and affect T cell differentiation.
Although the oncogenic principle (HPV16) is the same in both ear skin and cervical epithelium, disparities in cell types, baseline cytokine expression and resident immune cells could account for the differences in responses in the two tissues. In the female reproductive tract, normal physiological homeostasis requires the presence of pro-inflammatory cytokines, which are secreted by mucosal epithelial cells that cross talk with local leukocytes and protect the tissue (Fahey et al., 2005). The presence of these pro-inflammatory cytokines in normal epidermis has not been reported, so the pathways of cross talk between immune cells and keratinocytes are likely different in cervix and skin epithelia. In addition, it has been reported that cervical cancer development occurs when the HPV virus targets a discrete population of cervical epithelial cells, namely the squamocolumnar (SC) junction cells in the transformation zone. The cells in this junction are known to have a distinct immunophenotype, and a study reporting faithful markers of SC junction cells showed that these markers were not present in keratinocytes of human foreskin (Herfs et al., 2012). Interestingly, K17 is not expressed in normal epidermis, but has been reported to be present at very low levels in a population of cells called the reserve cells of human cervical epithelium, which are known to have stem-like properties (Martens et al., 2004). These disparities in the baseline epithelial environment could account for differences in viral-host cell interactions across the cervical epithelium and ear skin, affecting the crosstalk between immune cells and epithelial cells differently and ultimately causing discrepancies in gene expression upon Krt17 ablation. Also, since SC junctions are present in other epithelial layers (McNairn and Guasch, 2011), investigating the nature of the tumor
immune response in tissues that are reported to exhibit K17 overexpression in carcinogenesis and have such junctions could contribute to elucidating the mechanisms whereby K17 affects tumorigenesis.
Conclusion

This study aimed at elucidating the role of K17 in cervical carcinogenesis by using a well-established mouse model for this cancer, the K14-HPV16 model, first described by Arbeit et al. We hypothesized that K17 was a driver of tumorigenesis and modulated the immune response in the context of cervical cancer, a finding that was previously confirmed in the context of basaloid tumors. Consistent with this previous set of findings, there was a positive correlation between lesion severity and K17 expression during cervical carcinogenesis. By comparing the states of cervical epithelium morphology, basal cell proliferation, tissue vasculature and cytokine expression profiles between $HPV16^{tg/+}K17^{+/+}$, $HPV16^{tg/+}K17^{-/-}$ and WT controls, we established that the genetic loss of $Krt17$ lead to a delay in lesion onset, attenuated tumor progression and an enhanced protective immune response, thereby confirming our hypothesis. However, the mechanisms whereby K17 modulates the immune response to tumors has yet to be well defined. In cervical carcinogenesis, pro-inflammatory $T_h1$ cytokines levels are up to 10-fold higher in the $Krt17$ null background, whereas this response is dampened in ear skin tissues of the same mice. Collectively, our findings contribute to the body of evidence that K17 is faithful biomarker of cervical lesion progression, and that there is therapeutic promise in understanding how K17 is involved in signaling pathways that relate to tumor growth and modulates gene expression in a context dependent manner.
References


positive cervical cancer is associated with impaired CD4+ T-cell immunity
against early antigens E2 and E6. Cancer Res, 64(15), 5449-5455. doi:
10.1158/0008-5472.can-04-0831

Delgoffe, G. M., Pollizzi, K. N., Waickman, A. T., Heikamp, E., Meyers, D. J., Horton,
M. R., . . . Powell, J. D. (2011). The kinase mTOR regulates the differentiation of
helper T cells through the selective activation of signaling by mTORC1 and

Immunother, 33(4), 335-351. doi: 10.1097/CJI.0b013e3181d32e74

promotes epithelial proliferation and tumor growth by polarizing the immune
response in skin. Nat Genet, 42(10), 910-914. doi: 10.1038/ng.665

duplication errors: modeling the origins of genomic instability. Oncogene, 21(40),
6241-6248. doi: 10.1038/sj.onc.1205709

(2000). Sensitivity of the cervical transformation zone to estrogen-induced

Escobar-Hoyos, L. F., Yang, J., Zhu, J., Cavallo, J. A., Zhai, H., Burke, S., . . . Shroyer,
K. R. (2014). Keratin 17 in premalignant and malignant squamous lesions of the
cervix: proteomic discovery and immunohistochemical validation as a diagnostic
and prognostic biomarker. Mod Pathol, 27(4), 621-630. doi:
10.1038/modpathol.2013.166

cytokines and chemokines by polarized human epithelial cells from the female
reproductive tract. Hum Reprod, 20(6), 1439-1446. doi: 10.1093/humrep/deh806

prevention and the role of human papillomavirus infection. CMAJ, 164(7), 1017-
1025.


the retinoblastoma tumor suppressor by the human papillomavirus type 16 E7


Adriana Batazzi, B.S., BSc.
2927 East Baltimore Street, Baltimore, MD 21224
352.328.5546
adriana.batazzi@gmail.com

Education

Masters of Science in Biochemistry and Molecular Biology
Bloomberg School of Public Health | Johns Hopkins University | Baltimore, Maryland.
September 2012 - June 2014
GPA: 3.49 - Degree conferral: August 2014

Bachelor of Science (B.S.) in Biology
University of Tampa | Tampa, Florida | August 2010-December 2011
GPA: 3.6 while working 20 hours a week.

Bachelor of Science (BSc.) in Biochemistry and Chemistry
University of Johannesburg | Johannesburg, South Africa | January 2007 to May 2010

Relevant Coursework:
- Biology: Molecular Biology, Physiology, Molecular Genetics, Microbiology, Immunology
- Chemistry: Intermediate and Advanced Organic, Physical, Analytical and Inorganic Chemistry, Biochemistry
- Physics

French Baccalaureat, Scientific Section with Biology concentration.
Lycée Français Jules Verne | Johannesburg, South Africa | June 2006
Graduated Magna Cum Laude

Academic Achievements and awards
- Student Representative of the Masters Class | Johns Hopkins Bloomberg School of Public Health | September 2012 – August 2014
- Outstanding Senior Award | University of Tampa | Spring 2012
- Phi Kappa Phi Honor Society Candidate | University of Tampa | Fall 2011
- Skull&Bones/Alpha Epsilon Delta honoree | Fundraiser chair for Spinal Muscular Atrophy | Spring 2011
- Student Leadership and Academic Achievement Award | University of Tampa | Spring 2011
- Dean’s list honoree | University of Tampa | Fall 2010
- Volunteer of the month for P.E.A.C.E office | University of Tampa | Fall 2011
Clinical Experience

Gastrointestinal Medicine Shadowing Experience
Supervisor: Dr. M.D. Duncan, MD.
Johns Hopkins University Bayview Medical Center | Baltimore, MD | January 2013 - August 2013

Neurology Internship
Supervisor: Dr. S. Subramony, MD.
University of Florida | Gainesville, FL | January 2012 – June 2012
Shadow Physicians and work in the Center for Movement Disorders and Neurorestoration Clinic.

Surgical Oncology Internship
Supervisor: Dr. Antonio Bolognese, MD.
Policlinico Umberto I | Rome, Italy | June 2011- August 2011
Assisted in all procedures performed by Dr. Bolognese.
Participated in rounds and reviewed patient files to learn about conditions.

Research Experience
- Cervical Carcinogenesis and the role of Keratin 17 in tumor growth: Mouse genetics, Microscopy, Protein and RNA biochemistry/molecular biology, Tissue Handling. 
  Pierre Coulombe, Ph.D., Johns Hopkins University School of Public Health

- Duchenne Muscular Dystrophy Research: MRI Imaging Analysis and Data Analysis
  S.H. Subramony, MD, Chief of Neuromuscular Division, University of Florida
  D. Lott, PT/Ph.D., University of Florida

- Presentation on experimental analysis of recombinant DNA technologies
  MJ Cronje, Ph.D., University of Johannesburg

Languages spoken
- Fluent in English, French and Italian, Spoken and Written.

Service To The Community
- People Exploring Active Community Experience - Volunteered 150+ hours | Tampa, Florida | November 2010 - December 2011

- Volunteered as dance teacher and High School tutor for The Yenzani Foundation – Volunteered 400+ hours | Johannesburg, South Africa | June 2005 - August 2009
Work Experience

- Physics/Organic Chemistry Tutor | Academic Center for Excellence | University of Tampa | November 2010 – May 2011

- Volunteer Coordinator | P.E.A.C.E volunteer center | University of Tampa | November 2010 – May 2011
   Event organizing and coordination with organizations such as Metropolitan Ministries, the Humane Society, Shriner’s children hospital, Glazers children’s museum, as well as large scale events such as Carnival For Kids or sporting events.

- Freelance Stylist | Urban Degree | South Africa | July 2008 – May 2010
   Retail competency, client handling and sales work.

- Travel Agent | Sandown Travel Agency | South Africa | August 2006 – December 2006
   Client handling and administrative work.

References

– Pierre Coulombe, Ph.D., E.V. McCollum Professor and Chair, Biochemistry and Molecular Biology Department, Johns Hopkins University School of Public Health.
  o coulombe@jhu.edu

– S. H. Subramony, MD. Chief of Neuromuscular Division, University of Florida.
  o s.subramony@neurology.ufl.edu

– Donovan Lott, PT/Ph.D, University of Florida.
  o djlottpt@phhp.ufl.edu

– Antonio Bolognese, MD. Director of Pietro Valdoni Oncological Division.
  o antonio.bolognese@uniroma1.it

– Megan Frisque, Advisor, P.E.A.C.E/Alternative Breaks Office, University of Tampa.
  o mfrisque@ut.edu

– Janice Law, Director of the Academic Center of Excellence, University of Tampa.
  o jlaw@ut.edu