**Mechanisms of the cytoprotective response to tobacco and its metabolites in esophageal epithelia**

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## ABSTRACT

Esophageal cancer, the 6th most common cause of cancer death worldwide, is strongly associated with cigarette smoking. Yet, malignant transformation of a single epithelial cell in response to cigarette smoke is a rare event, and the cytoprotective mechanisms within the epithelial cells that line the esophagus in response to exposure to tobacco and its metabolites are not well understood. In robust preliminary data, we demonstrate a novel mechanism that governs cellular responses to environmental insults in the esophageal epithelium. Specifically, we demonstrate that two transcriptional regulators, *Krüppel*-like factor 5 (KLF5) and p53, coordinately regulate whether esophageal epithelial cells undergo apoptosis or survival in response to an exogenous stress, UV radiation or $H_2O_2$. Here we seek to extend these findings to mechanistic studies of tobacco and its metabolites, including studies of both cigarette smoke and e-cigarettes. Our overarching hypothesis is that *KLF5 and p53 act as a molecular rheostat following exposure to tobacco and its metabolites, determining whether cells live or die in response to stress, and that disruption of this regulation underlies ESCC*. To test this hypothesis, we will pursue the following **Specific Aims**: (1) To define the cellular outcomes of exposure to tobacco and its metabolites in cellular and murine models with disruption of *KLF5 +/- p53*; (2) Using both candidate-gene and genome-wide approaches, to delineate the pathways downstream of KLF5 and p53 that determine specific esophageal epithelial cellular responses to tobacco and its metabolites. In this way, we will identify key cytoprotective mechanisms within the esophageal epithelium following tobacco exposure, mechanisms that may also have broad relevance to other tissues and the origins of other squamous cell cancers. Moreover, the research proposed in this project will provide important preliminary date for a NIH grant submission relevant to "**Health Effects** - Understanding the short and long term health effects of tobacco products" (RFA-OD-18-002), permitting the PI to conduct further studies in this field.

**PLAN TOWARD EXTRAMURAL FUNDING VIA THE PROPOSED PROJECT**

The proposed research will provide important preliminary data in “research priorities related to the regulatory authority of the Food and Drug Administration (FDA) Center for Tobacco Products (CTP).” Here, we seek to define key cytoprotective mechanisms within the epithelial cells that line the esophagus in response to exposure to tobacco and its metabolites. We anticipate that this research will define potential targets for early intervention along the carcinogenic pathway in the esophagus following exposure to tobacco and its metabolites and that the key cytoprotective mechanisms identified through this work will be relevant in other tissues, such as for squamous cell cancers of the lung or oropharynx. With a CEET Pilot Award, we will gain experience in studies of environmental carcinogens in neoplastic transformation, including through the use of the Vitrocell Smoking Machine and draw upon the expertise of the Penn CEET, including through the CEET Core and through collaborations with CEET. Ultimately, we anticipate that the research proposed here will lead to the submission of a R01 grant application related to “**Health Effects** - Understanding the short and long term health effects of tobacco products” (RFA-OD-18-002).

## SPECIFIC AIMS

Esophageal cancer is the 6th most common cause of cancer death worldwide, and more than 80% of these cases are esophageal squamous cell cancer (ESCC)[1-3]. While the risk factors for ESCC can vary by region, smoking is clearly and strongly associated with ESCC, and in Western countries, approximately 90% of ESCC can be directly attributed to tobacco use as well as use of alcohol[3-5]. The esophagus is normally lined by a layer of stratified squamous epithelial cells, and for each of these epithelial cells, malignant transformation is a rare event[6, 7]. Yet the molecular mechanisms by which esophageal epithelial are protected against malignant transformation in response to environmental insults such as exposure to tobacco and its metabolites are not well understood. This proposal is motivated to answer the following questions: (1) How do esophageal squamous epithelial cells typically respond to tobacco and its metabolites to maintain homeostatic control; (2) What molecular events disrupt this control, resulting in malignant transformation and neoplastic progression?

In robust preliminary data, we demonstrate that two key transcriptional regulators, *Krüppel*-like factor 5 (KLF5) and p53, coordinately regulate the response of esophageal epithelial cells to environmental insults. Typically, cells make a decision to induce cell cycle arrest (and survival) or apoptosis in response to exogenous stress, an outcome mediated in large part by the tumor suppressor p53[8, 9]. KLF5 promotes proliferation and migration and controls homeostasis in normal esophageal squamous epithelial cells[10-12], and we have demonstrated that mutant *p53*, which is the most commonly mutated gene in human cancers[13], in cancers arising in association with tobacco smoke, and in ESCC[14, 15], acts as a "molecular switch" for KLF5 function[16, 17]. In non-transformed esophageal epithelial cells with wild-type *p53*, KLF5 and p53 dictate cellular responses to exogenous stress from UV radiation or $H_2O_2$ (**Figure 1**). Here we seek to extend these findings to mechanistic studies of tobacco and its metabolites, including studies of both cigarette smoke and e-cigarettes. Our overarching hypothesis is that *KLF5 and p53 act as a molecular rheostat following exposure to tobacco and its metabolites, determining whether cells live or die in response to stress, and that disruption of this regulation underlies ESCC*. To test this hyposthesis, we will pursue the following **Specific Aims**:

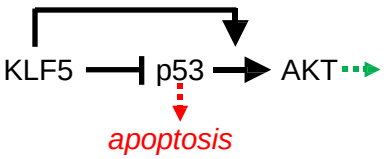


**Figure 1.** KLF5 and p53 mediate cell fate decisions in response to UV and $H_2O_2$ in esophageal epithelial cells.

**Aim 1. To define the cellular outcomes of exposure to tobacco and its metabolites in cellular and murine models with disruption of *KLF5 +/- p53***

Here, we will employ primary human esophageal epithelial cells with disruption of KLF5 and p53 in organotypic culture as well as murine models with esophageal specific *Klf5* deletion and *p53* mutation. We will examine the effects of both tobacco and its metabolites and will study the effects of e-cigarettes using the Vitrocell Smoking Machine belonging to the CEET and Abramson Cancer Center's Tobacco and Environmental Carcinogenesis Program.

**Aim 2. Using both candidate-gene and genome-wide approaches, to delineate the pathways downstream of KLF5 and p53 that determine specific esophageal epithelial cellular responses to tobacco and its metabolites**

In preliminary data, we demonstrate that AKT1 and AKT3 are important downstream mediators of KLF5 and p53 in cell survival following exogenous stress with UV or $H_2O_2$. Initially, we will determine whether AKT1/3 play similar roles in mediating cell fate decisions (apoptosis vs. survival) in response to tobacco and/or its metabolites and will define other mediators of esophageal epithelial cell survival or apoptosis downstream of KLF5 and p53 following exposure to tobacco using RNA-seq.

Through these approaches, we will define key cytoprotective mechanisms within the esophageal epithelium following tobacco exposure, and we anticipate that this research will delineate potential targets for early intervention along the carcinogenic pathway in the esophagus following exposure to tobacco and its metabolites. Moreover, as squamous cell cancers (SCCs) from different tissues have similar features, both molecular and otherwise[18], these findings should have relevance to the mechanisms underlying SCCs, such as lung or oropharyngeal cancers. Finally, we expect that the research proposed here will provide essential preliminary data for a R01 grant application related to "**Health Effects** - Understanding the short and long term health effects of tobacco products" (RFA-OD-18-002).

## RESEARCH PLAN

### Significance

Esophageal cancers, of which at least 80% are esophageal squamous cell carcinoma (ESCC), are the 8th most common cancer and 6th leading cause of cancer deaths worldwide[1, 2]. Moreover, because most of these cancers are detected at advanced stages, ESCC has a median survival of only 15 months[19, 20]. Tobacco and alcohol use together significantly increase the likelihood of developing ESCC, and in Western countries, approximately 90% of ESCC can be directly attributed to the use of tobacco use and alcohol[3-5]. The esophagus is normally lined by a layer of stratified squamous epithelial cells[6], and these types of stratified squamous epithelia form a remarkable protective barrier against environmental insults[21]. In fact, despite frequent exposure of esophageal epithelial cells to environmental stresses including cigarette smoke, malignant transformation of a single cell is a rare event[6, 7].

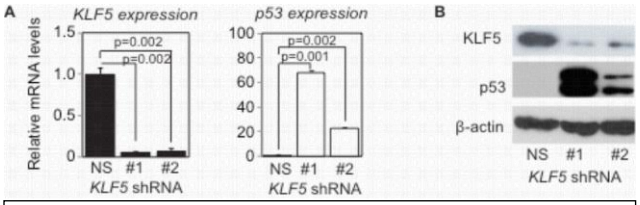


**Figure 1.** KLF5 suppresses p53 in unstressed human keratinocytes. (**A**) As assessed by quantitative real-time PCR, either of two *KLF5* shRNAs increased *p53* mRNA expression, compared to non-silencing control (NS), after 7 days of doxycycline induction in primary human esophageal keratinocytes. (**B**) Similarly, p53 protein levels increased markedly with *KLF5* silencing with shRNA for 7 days. β-actin served as a loading control.

Damage to the cell caused by environmental stresses induces a number of cellular responses including growth arrest, to prevent the replication of damaged DNA, and apoptosis, which eliminates aberrant cells[22, 23]. The tumor suppressor p53, the "guardian of the genome," is an important mediator of the damage response and protects against malignant transformation in normal human epithelia[8, 24]. p53 is the most commonly mutated gene in human cancers and in ESCC, and heavy smokers with ESCC have an increased risk for p53 mutation compared to non-smokers[14, 15, 18, 25, 26]. Yet, while p53 function and dysfunction have been studied extensively in cancer cells[24], much less is known about how normal epithelial cells respond to environmental insults. In particular, how does a single cell determine a specific output (growth arrest and repair or apoptosis) in response to environmental stressors?[27] Clearly, tight regulation of the DNA damage response is essential to ensure, for example, that damaged cells that cannot be repaired are not allowed to survive and proliferate.

Potential clues to the mechanisms of critical cell-transcriptional regulators of the DNA damage response that interact with p53. One of these transcription factors, the zinc-finger transcription factor KLF5 is an important regulator of cell cycle progression and apoptosis and interacts with p53 in multiple contexts[16, 28-30]; in particular, p53 acts as a "molecular switch" for KLF5 function in normal esophageal keratinocytes[17, 31]. These interactions between KLF5 and p53 are critical in both normal and cancer cells, yet significant questions remain about the "network architecture" of p53 in normal epithelial cells in response to stress[32], and the roles of KLF5 and p53 in the outputs of normal esophageal cells following environmental insults, in particular tobacco and its metabolites. To this end, this proposal is designed to test the hypothesis that KLF5 and p53 act as a molecular rheostat following exposure to tobacco and its metabolites, determining whether cells live or die in response to stress, and that disruption of this regulation underlies ESCC, and, as such is motivated to answer the following questions: (1) How do esophageal squamous epithelial cells typically respond to tobacco and its metabolites

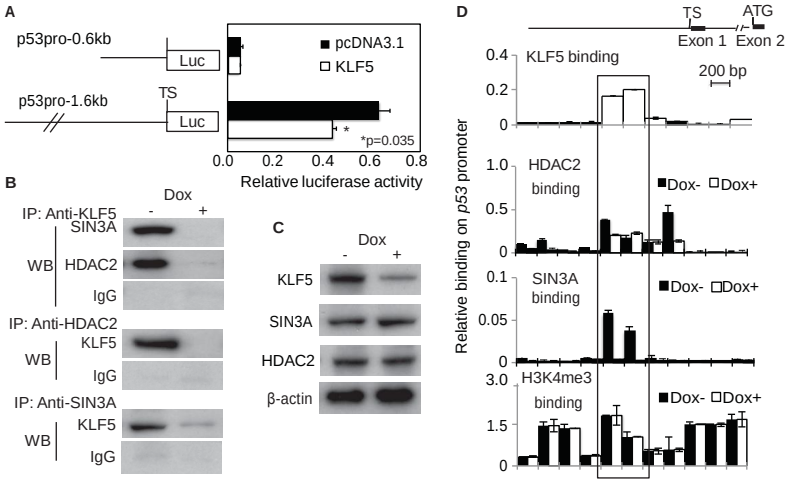


**Figure 2**. KLF5 recruits SIN3A and HDAC2 to form a repressive complex on the *p53* promoter. (**A**) KLF5 inhibited activity of a reporter containing a 1.6 kb region upstream of the *p53* translation start site. No effect of KLF5 was seen on a 0.6 kb reporter, indicating that the region from -0.6 kb to -1.6 kb was critical for KLF5 repressive functions on *p53*. (TS: transcriptional start site) (**B**). KLF5 bound to SIN3A and HDAC2 in primary human esophageal keratinocytes, and this binding was inhibited by *KLF5* knockdown. (**C**) In contrast, *KLF5* knockdown did not significantly alter SIN3A or HDAC2 levels on Western blot. (**D**) Quantitative ChIP using overlapping primers covering 4 kb of the *p53* promoter revealed KLF5, SIN3A, and HDAC2 binding within the region from -1.4 kb to -1.0 kb. Of note, binding of SIN3A in this region was nearly abolished with *KLF5* knockdown.

to maintain homeostatic control; (2) What molecular events disrupt this control, resulting in malignant transformation and neoplastic progression?

## Preliminary Studies

p53 loss or mutation is typically an early event in ESCC, and inactivation of p53 is not sufficient for the development of ESCC[33, 34]. In contrast, KLF5 expression increases in esophageal squamous cell dysplasia and is lost in esophageal cancer[16]. To determine the function of KLF5 and p53 in the normal esophageal epithelium in response to stress, we utilized non-transformed primary human esophageal keratinocytes[35]. In normal, unstressed epithelial cells, p53 was maintained at low levels within the cell, and knockdown of *KLF5* knockdown with shRNA resulted in a surprising and dramatic increase in p53, both at the RNA and protein levels (**Figure 1**). The mechanism of *p53* repression by KLF5 was transcriptional repression, as KLF5 formed a repressive complex with the co-repressor SIN3A and HDAC2 (**Figure 2**); interestingly, this complex was disrupted in response to UV stress (**Figure 3**), providing a mechanism for *p53* induction in esophageal epithelial cells exposed to an environmental stressor.

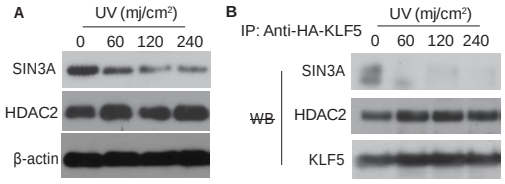


**Figure 3.** The KLF5 repressive complex on *p53* is disrupted by stress, providing a mechanism for *p53* induction during stress. (**A**) On Western blot, SIN3A levels decreased slightly with UV stress, while HDAC2 levels were unchanged. (**B**) By co-immunoprecipitation, SIN3A binding to KLF5 was abolished by UV stress, even as HDAC2 still bound to KLF5.

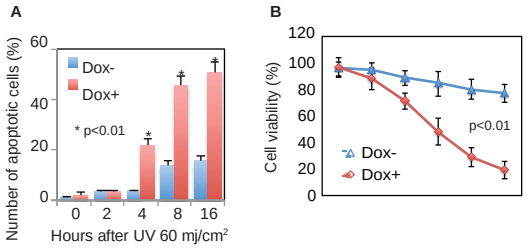


**Figure 4.** KLF5 knockdown increases apoptosis in response to UV stress. (**A**) Apoptosis, assessed by flow cytometry at different time points after 60 mj/cm$^2$ of UV, was increased following *KLF5* knockdown by doxycycline induction. (**B**) *KLF5* knockdown also decreased cell viability, as assessed by trypan blue exclusion.

When primary esophageal epithelial cells with KLF5 knockdown were exposed to UV irradiation, cells had an increase in apoptosis and a decrease in cell viability, compared to cells without p53 knockdown (**Figure 4**). Since *p53* was induced in both contexts (data not shown), these findings suggested that KLF5 must have an additional role in normal esophageal epithelial cells and the stress response besides *p53* repression and derepression. Though RNA-seq and ChIP-seq (data not shown), we identified *AKT1* and *AKT3* as KLF5 targets during UV stress. In fact, both KLF5 and p53 were

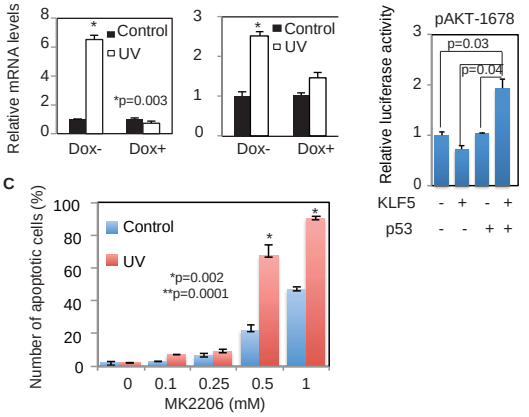


**Figure 5.** KLF5 and p53 cooperatively activate *AKT* in response to UV stress. (**A**) By qPCR, *AKT1* and *AKT3* were induced by UV stress, and this induction was blocked by *KLF5* knockdown. (**B**) Luciferase reporter assays using 1678 bp upstream of the translational start site of the AKT1 promoter demonstrated that KLF5 and p53 cooperate to upregulate *AKT*. (**C**) Apoptosis increased in primary epithelial cells when AKT was inhibited with the AKT inhibitor MK-2206 for 8 hours after 60 mj/cm$^2$ UV irradiation.

apopotosis increased in a dose-dependent manner when AKT was blocked pharmacologically in primary human esophageal epithelial cells exposed to UV, suggesting that AKT was important for pro-survival responses of these cells to environmental stresses. Effects were similar in cells treated with $H_2O_2$ (data not shown). Taken together, these data demonstrate that KLF5 and p53 together determine the cellular output in non-transformed human esophageal epithelial cells following environmental stress, in this case with UV irradiation and $H_2O_2$. The stress responses and the mechanisms of the mechanisms of these responses may vary based on the type of stressor, the dose, and the chronicity. Through the approaches outlined below, we seek to define key cytoprotective mechanisms within the esophageal epithelium following tobacco exposure.

## Methods

### Aim 1. To define the cellular outcomes of exposure to tobacco and its metabolites in cellular and murine models with disruption of *KLF5 +/- p53*

*Hypothesis: Disruption of KLF5 and/or p53 within the esophageal epithelium predisposes to the development of dysplasia and malignant transformation*

To define the functions of KLF5 and p53 in esophageal epithelia exposed to tobacco and its metabolites, we will employ primary

human esophageal epithelial cells in three-dimensional organotypic culture and murine models with *Klf5* disruption, alone or in combination with *p53* mutation, specifically in esophageal epithelia. We have already established primary human esophageal keratinocytes with *KLF5* knockdown and *p53* mutation ($p53^{R175H}$, a hotspot mutation in human cancer)[16]. For organotypic culture, $5 \times 10^5$ keratinocytes will be seeded onto matrix in 6-well plates and maintained for a total of 7 days, then raised to the air-liquid interface. The experimental design is indicated in **Table 1**. Cells in organotypic culture will be exposed to cigarette smoke or e-cigarettes using the Vitrocell Smoking Machine belonging to the CEET and Abramson Cancer Center's Tobacco and Environmental Carcinogenesis Program. Initially, we will confirm that there is no massive viability loss associated with the humidity, starting with cells in the clean air controls. We will then expose cells to 1 cigarette for 8 minutes, followed by 1 day recovery; if necessary, we can adjust the flow rate, the humidity, and the dilution factor. Similar experiments will be performed using e-cigarettes. Following the recovery, we will isolate cells and examine markers of apoptosis (TUNEL), as well as proliferation (Ki-67 and keratin 14, a marker of proliferating keratinocytes[36]) and differentiation using the differentiation marker keratin 4[37, 38] to evaluate for disruptions in normal homeostasis. We will also assess levels of adducts of oxidative stress (we will draw upon the expertise of the CEET for these studies).

| **Table 1.** Experimental design for organotypic culture experiments | Non-silencing control | *KLF5* knockdown |
|---|---|---|
| *p53* wild-type | | |
| $p53^{R175H}$ | | |

To determine the functions of KLF5 and p53 in the stress response to tobacco metabolites *in vivo*, 6 week-old mice will be treated with 4-Nitroquinoline 1-oxide (4-NQO)[39] daily in drinking water at 50 µg/ml for 8 weeks and sacrificed 12 weeks after completion of treatment. We will examine the esophagus, grossly and histologically, for changes in morphology and for dysplasia and neoplasia. To investigate proliferation, we will perform IHC with anti-BrdU and keratin 14 and for apoptosis, we perform TUNEL staining. We will quantify proliferating and apoptotic cells by counting, in a blinded manner, cells staining positive for BrdU or TUNEL in epithelia from at least 10 high-powered fields (hpf) of well-oriented esophageal cross-sections from at least 2 control and 2 mutant mice. These results will be expressed as mean number of positive cells/hpf ± SEM. We will also examine p53 expression in *ED-L2/Cre;Klf5*$^{loxP/loxP}$ mice. We will examine differentiation using IHC for keratin 4 and markers of keratinocyte differentiation[37, 38].

| **Table 2.** Experimental design for murine models | Control | *Klf5* deletion |
|---|---|---|
| *p53* wild-type | | |
| $p53^{R172H}$ | | |

*Anticipated Results, Potential Pitfalls, and Alternative Approaches*
We anticipate that, in the presence of *KLF5* knockdown and/or *p53* mutation, primary human esophageal keratinocytes exposed to cigarette smoke will have increased survival and decreased apoptosis, compared to controls, and we anticipate that mice with *Klf5* deletion and/or *p53* mutation will have increased esophageal squamous cell dysplasia and/or cancer in response 4-NQO treatment. We expect that some dose modulation and other modifications will be necessary for the use of the smoking machine and we will draw on the expertise within CEET for these studies. In addition, it is possible that the consequences of KLF5 and p53 loss or mutation may be different in response to cigarette smoke, e-cigarettes, and 4-NQO than in response to UV irradiation and $H_2O_2$. Nonetheless, these functional analyses will be important. In future directions, we will examine KLF5 expression in human esophageal tissues from smokers, compared to non-smokers, and examine changes in cellular senescence in our cell culture models.

**Aim 2. Using both candidate-gene and genome-wide approaches, to delineate the pathways downstream of KLF5 and p53 that determine specific esophageal epithelial cellular responses to tobacco and its metabolites**
*Hypothesis: AKT1 and AKT3, as well as other as yet undefined targets and pathways, mediate KLF5 and p53 effects on esophageal epithelial cell-fate decisions in response to exposure to tobacco and its metabolites*

In preliminary data, we demonstrate that AKT1 and AKT3 are important downstream mediators of KLF5 and p53 in cell survival following exogenous stress with UV or $H_2O_2$. Here, we will determine whether AKT1/3 play similar roles in mediating cell fate decisions (apoptosis vs. survival) in response to tobacco and/or its metabolites and will define other mediators of esophageal epithelial cell survival or apoptosis downstream of KLF5 and p53 following exposure to tobacco using RNA-seq. To determine the role of whether AKT1 and AKT3, we will block AKT signaling, both using shRNA and the chemical inhibitor MK-2206. Primary human esophageal epithelial cells will be grown organotypic culture using the experimental design detailed in **Table 1**,

and we will assess proliferation, differentiation, and apoptosis as per **Aim 1**. In addition, to define additional mediators and pathways downstream of KLF5 in the stress response to tobacco and its metabolites, we will isolate DNA and RNA from primary esophageal epithelial cells exposed to cigarette smoke in the Vitrocell Smoking Machine and perform ChIP-seq, using an antibody that we have validated for ChIP[16], and RNA-seq. Prior to sequencing, sample quality will be evaluated on an Agilent 2100 Bioanalyzer, and samples will be prepared for sequencing using standard protocols from the Next-Generation Sequencing Core. Sequencing will be performed on an Illumina HiSeq-2500. Of note, analytic pipelines are constantly evolving and will be selected as appropriate in consultation with the Penn Bioinformatics Core. The goal of the ChIP-seq experiments is to define promoters bound by KLF5 in response to cigarette smoke, compared to untreated cells; thus for these experiments, we will utilize control human esophageal epithelial cells without any genetic modifications. The goal of the RNA-seq experiments is define the effects of KLF5 loss and/or p53 mutation on the transcriptional profiles of normal human esophageal keratinocytes in response to cigarette smoke; thus the experimental design will be as per **Table 1**.

| **Table 3.** Experimental design for ChIP-Seq | Control human esophageal epithelial cells |
|---|---|
| Unexposed | |
| Exposed to cigarette smoke | |

*Anticipated Results, Potential Pitfalls, and Alternative Approaches*

We anticipate that AKT1 and AKT3 will play important roles in mediating cell survival downstream of KLF5 and p53 in esophageal epithelial cells exposed to cigarette smoke and that the ChIP-seq and RNA-seq approaches will identify additional relevant mediators and pathways. As noted for Aim 1, the mechanisms of the stress response may differ depending on the environmental stressor, and this will be important to delineate. In Future Directions, we will validate and functionally characterize any potential target genes identified from the ChIP-seq and RNA-seq analyses. Taken together, we anticipate that these experiments will define key cytoprotective mechanisms within the esophageal epithelium following tobacco exposure, and we anticipate that this research will delineate potential targets for early intervention along the carcinogenic pathway in the esophagus following exposure to tobacco and its metabolites.

**Summary and a brief outline of how the results from the pilot study will enable the submission of a subsequent NIH grant and a detailed plan for this subsequent grant submission:** The proposed research will provide important preliminary data in "research priorities related to the regulatory authority of the Food and Drug Administration (FDA) Center for Tobacco Products (CTP)." With a CEET Pilot Award, we will gain experience in studies of environmental carcinogens in neoplastic transformation, including through the use of the Vitrocell Smoking Machine and draw upon the expertise of the Penn CEET (we have already preliminary discussions regarding use of the smoking machine). While we have expertise in the molecular mechanisms of cellular proliferation, differentiation, and carcinogenesis in the esophagus, studies of the stress response are a new direction in the lab. Moreover, our initial experiments were performed using UV irradiation and $H_2O_2$, and we seek to extend our exciting preliminary results into a physiologically relevant system such as studies of the impact of tobacco smoke on the esophageal epithelium. We anticipate that this research will define potential targets for early intervention along the carcinogenic pathway in the esophagus following exposure to tobacco and its metabolites and that the key cytoprotective mechanisms identified through this work will also be relevant in other tissues, such as for squamous cell cancers of the lung or oropharynx. Ultimately, we anticipate that the research proposed here will lead to the submission of a R01 grant application related to "**Health Effects** - Understanding the short and long term health effects of tobacco products" (RFA-OD-18-002).

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**BUDGET WITH PERSONNEL AND BUDGET JUSTIFICATION**

| **Personnel** | |
|---|---|
| Yizeng Yang, M.D., Ph.D (3.6 calendar months) | |
| *Salary* | $20,012 |
| *Benefits* | $6,803 |
| **Supplies** | |
| *Reagents* | $8,185 |
| *Disposables* | $4,000 |
| *Animal Work* | $6,000 |
| *Next generation sequencing* | $5,000 |
| **Total** | $50,000 |

**Personnel**:
Yizeng Yang, M.D., Ph.D. (3.6 calendar months), Senior Research Investigator. Dr. Yang is extremely well-qualified in biochemistry, molecular biology, cell culture, and the generation and analysis of animal models. Dr. Yang received his Ph.D. degree from the internationally-renowned Karolinska Institute in Stockholm, Sweden and has been a valuable member of Dr. Katz's laboratory since 2003, first as a postdoctoral researcher and since 2009 as a Senior Research Investigator. Dr. Yang has published in the *Journal of Biological Chemistry*, *Biochimica et Biophysica Acta*, the *Journal of Lipid Research*, the *FASEB Journal*, *Cancer Biology and Therapy*, the *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *Biochemical and Biophysical Research Communications*, *Cell Cycle*, and *Cancer Research*, many of these as 1st author from the PIs lab. He will participate in studies proposed in both aims of this proposal, and the work embodied in this proposal will comprise 3.6 calendar months of his total effort.

**Supplies**:
*Reagents*: This category includes the following: common enzymes needed for subcloning and analyses; reagents for cell culture and organotypic culture; antibodies; reagents and kits for RNA isolation; oligonucleotides for sequencing, PCR, and other analyses

*Disposable*s: This category includes glassware, plastic ware, slides, gloves and similar items.

*Animal Work*: Included are the costs for housing all of the mice. We also include the costs for embedding, sectioning, and routine staining of tissues.

*Next generation sequencing:* This category includes the costs for library prep for ChIP-seq and RNA-seq, the costs for performing next generation sequencing through the Next Generation Sequencing Core at the University of Pennsylvania, and the costs for data analyses through the Penn Bioinformatics Core.

**VERTEBRATE ANIMALS**

A protocol regarding the proposed use of animals (**Protocol # 804001**) was approved by the University of Pennsylvania Institutional Animal Care and Use Committee (IACUC) on October 2, 2017. All mice necessary for the proposed experiments have already been generated by us or obtained from others (collaborators or commercially) and are housed within our colony in the basement of BRBII/III. The information below addresses the required 4 points of the Vertebrate Animal Section:

**Description of Procedures**

We anticipate that the experiments in this proposal will require, in total, the use of 184 mice. Both male and female mice will be used for these analyses and for perpetuating the mouse lines. A table outlining the proposed use of these animals is below:

| **Mouse Line** | **Time points** | **# of each genotype** (control, *Klf5* loss, *p53* mutant, *Klf5* loss + *p53* mutant) | **Total # per time point** | **# required for generation of relevant genotypes and maintenance of the lines** | **Total # of animals required** |
|---|---|---|---|---|---|
| *ED-L2/Cre;Klf5*$^{loxP/loxP}$ X *p53LSLR172H* **Treated with 4-NQO** | 1 | 58 | 174 | 10 | 184 |

Homozygous null mice for *Klf5* die by embryonic day 9, and the use of the Cre-loxP system allows us to bypass this early lethality. *ED-L2/Cre* mice, which we generated (Tetreault et al, *Gastroenterology*, 139:171-181, 2010) and have deposited in the NCI Mouse Repository, demonstrate *Cre* expression and the ability to recombine floxed alleles in esophageal epithelia. *Klf5*$^{loxP/loxP}$ mice were obtained from Dr. Ryozo Nagai of Jichi Medical University (Takeda et al, *Journal of Clinical Investigation,* 120:254–265, 2010). *p53LSLR172H* mice express the mutant *p53*$^{R172H}$ allele, the mouse homologue of the human *p53*$^{R175H}$ mutation, only in tissues expressing Cre recombinase. For chemical carcinogenesis experiments with 4-NQO, 6 week-old mice will be treated with the carcinogen 4-NQO daily in drinking water at 50 µg/ml for 8 weeks and sacrificed 12 weeks after completion of treatment. Esophagi will be examined grossly and used for histology, immunohistochemistry, RNA, and protein analyses. Control mice have a cancer incidence of ~33% (Tang et al, *Clinical Cancer Research*, 10:301-13, 2004) with 4-NQO treatment, and I have estimated a cancer incidence of 50% for *ED-L2/Cre;Klf5*$^{loxP/loxP}$ mice and *p53LSLR172H* mice treated with 4-NQO and of 75% for *ED-L2/Cre;Klf5*$^{loxP/loxP}$;*p53LSLR172H* mice treated with 4-NQO. When the sample size in each group is 58, a 0.05 level Chi-square test will have 80% power to detect the difference between groups with proportion of 0.50 and the group with proportion of 0.75. In total this experiment will require **184** mice.

| | Cancer |
|---|---|
| Test significance level, α | 0.05 |
| **Loss of *KLF5* alone group** proportion w/ outcome | 0.50 |
| ***p53LSLR172H* alone group** proportion w/ outcome | 0.50 |
| ***p53LSLR172H* and loss of KLF5 group** proportion w/ outcome | 0.75 |
| Power (%) | 80 |
| N per group | 58 |
| Total sample size needed (per time-point) | 174 |

**Justifications**

The use of mice is necessary to investigate the role of KLF5 in esophageal homeostasis and disease *in vivo*, and the proposed experiments are a natural and necessary extension of our *in vitro* experiments. Currently, mice are the only mammalian species amenable to genetic manipulation which closely resemble human physiology and allow for the modeling of human disease. The genetic mouse models used for these experiments will be engineered to lack *Klf5* and express mutant *p53* specifically in esophageal epithelia. These experiments will be complemented by studies using primary esophageal epithelial cells derived from

knockout and mutant mice, allowing us to investigate the mechanisms of KLF5 and p53 in non-transformed primary cell lines. These *in vitro* studies should decrease the number of animals needed for our experiments by allowing us to test some hypotheses but are in no way a substitute for *in vivo* analyses. Overall, these studies will provide important new insights into the mechanisms of tobacco-mediated esophageal squamous cell cancer and the functions of KLF5 in this process.

**Minimization of Pain and Distress**

Mice will be monitored daily for signs of distress by University Lab Animal Resources (ULAR) staff. If animals develop pain or distress, a staff veterinarian will be consulted and/or the animals will be sacrificed (euthanized) by $CO_2$ asphyxiation or CO2 anesthesia followed by cervical dislocation, consistent with the current Report of the AVMA Panel on Euthanasia. Since some of the mice used in this proposal are expected to develop tumors, animals will also be examined at least every other day by laboratory personnel for signs of pain or distress (eg. impaired mobility or hunched habitus/withdrawn behavior). Mice will be weighed weekly, and animals whose body weight is less than 80% of the mean of the control group or whose body weight is less than 80% of projected weight, based on growth curves for that species and strain, will be euthanized. However, a stable weight may be misleading in mice, as growing tumors can contribute to a stable weight even though the animal is in a debilitated condition. Thus, we will also determine the body condition score (Ullman-Culleré and Foltz, *Laboratory Animal Science*, 49:319-23, 1999), and mice will be euthanized if the body condition score is 1/5 or the body condition score is 2/5 and the mouse has decreased activity/responsiveness. At the conclusion of the experiments, all animals will be sacrificed by $CO_2$ asphyxiation or $CO_2$ anesthesia followed by cervical dislocation, consistent with the current Report of the AVMA Panel on Euthanasia, and tissues will be harvested for appropriate analyses. No unusual procedures will be performed for these experiments.

**Euthanasia**

All animals will be sacrificed by $CO_2$ asphyxiation or $CO_2$ anesthesia followed by cervical dislocation, which are consistent with the current Report of the AVMA Panel on Euthanasia.