Molecular Mechanisms of Esophageal Squamous Cell Carcinoma

Apple Long
University of Pennsylvania, applong@mail.med.upenn.edu

Follow this and additional works at: http://repository.upenn.edu/edissertations
Part of the Molecular Biology Commons

Recommended Citation
http://repository.upenn.edu/edissertations/1861

This paper is posted at ScholarlyCommons. http://repository.upenn.edu/edissertations/1861
For more information, please contact libraryrepository@pobox.upenn.edu.
Molecular Mechanisms of Esophageal Squamous Cell Carcinoma

Abstract

MOLECULAR MECHANISMS OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Apple Long

Anil K. Rustgi

Esophageal squamous cell cancer (ESCC) is the 6th leading cause of cancer related deaths amongst American men. Although rare, this disease has a high mortality rate with a 5-year survival of 17%. Incidentally TP53 mutation is the most common genetic alteration in ESCC, along with over expression of EGFR and CYCLIN D1. We have previously modeled the invasive features of ESCC, through an in vivo-like 3D organotypic culture system, utilizing primary epithelial cells that have been transformed by overexpression of mutant TP53 and EGFR. From this model, a RNA microarray was performed to determine critical genes that are upregulated at the front of invasion. WNT10A was found to be over 4-fold upregulated, along with a WNT-signaling gene signature in the invasive cells. We additionally found that increased WNT10A expression was associated with ESCC, compared to normal esophageal tissue and that increased WNT10A staining was associated with poor prognosis. Functionally, WNT10A was determined to induce increased proliferation, migration, invasion and stemness properties in transformed esophageal cancer cells. In addition to examining the oncogenic properties of WNT10A, we also focused on generating an in vivo model of mutant TP53 driven ESCC. We utilized a carcinogen, 4-nitroquinoline 1-oxide (4-NQO), as a challenge to induce genetic changes in the background of Tp53 mutation, specific to the oral squamous, esophagus and forestomach tissues. We found preliminarily that TP53 mutation, specifically the R172H mutant, can accelerate tumorigenesis as compared to loss of TP53 alone. Additionally, when RNA-seq was performed on the tumor derived TP53R172H/- and TP53/- cells, we found an increase of genes associated with an epithelial to mesenchymal transition (EMT), in the TP53R172H/- cells. Mesenchymal genes such as Cdh2, Zeb1, Zeb2, Twist, and Snail were increased, while epithelial genes such as Cdh1, Epcam, and Krt4 were decreased. Altogether, we surmise that the TP53 mutant, R172H, can induce gain-of-function (GOF), oncogenic properties in order to promote tumorigenesis, potentially by inducing an EMT phenotype.

Degree Type
Dissertation

Degree Name
Doctor of Philosophy (PhD)

Graduate Group
Cell & Molecular Biology

First Advisor
Anil K. Rustgi

Keywords
Cancer biology, Esophageal Squamous Cell Carcinoma, Mutant p53, Wnt signaling

This dissertation is available at ScholarlyCommons: http://repository.upenn.edu/edissertations/1861
Subject Categories
Molecular Biology
MOLECULAR MECHANISMS OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Apple Long

A DISSERTATION
in
Cell and Molecular Biology
Presented to the Faculties of the University of Pennsylvania
in
Partial Fulfillment of the Requirements for the
Degree of Doctor of Philosophy
2015

Supervisor of Dissertation

Anil K. Rustgi, M.D.

T. Grier Miller Professor of Medicine; Chief of Division of Gastroenterology, Perelman School of Medicine

Graduate Group Chairperson

Daniel S. Kessler, Ph.D.

Associate Professor of Cell and Developmental Biology

Dissertation Committee
Gerd Blobel, Ph.D. Frank E. Weise III Professor of Pediatrics, Perelman School of Medicine

Makoto Senoo, Ph.D. Assistant Professor of Developmental Biology, University of Pennsylvania School of Veterinary Medicine

Ellen Puré, Ph.D. Grace Lansing Lambert Professor of Biomedical Science, University of Pennsylvania School of Veterinary Medicine

Chair: Andy Minn, M.D., Ph. D. Assistant Professor of Radiation Oncology, Perelman School of Medicine
ACKNOWLEDGMENTS

I would like to thank all of the members of the Rustgi and Nakagawa lab, past and present for ideas, inspiration, experimental design help, and actual help with experiments and great advice. I would also like to thank our collaborators, Drs. Andres Klein-Szanto, Ju-Seog Lee, Shelley Berger, Morgan Sammons, and Jiajun Zhu for their instrumental help with several of our experiments and experimental design.
ABSTRACT

MOLECULAR MECHANISMS OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA

Apple Long

Anil K. Rustgi

Esophageal squamous cell cancer (ESCC) is the 6th leading cause of cancer related deaths amongst American men. Although rare, this disease has a high mortality rate with a 5-year survival of 17%. Incidentally TP53 mutation is the most common genetic alteration in ESCC, along with over expression of EGFR and CYCLIN D1. We have previously modeled the invasive features of ESCC, through an in vivo-like 3D organotypic culture system, utilizing primary epithelial cells that have been transformed by overexpression of mutant TP53 and EGFR. From this model, a RNA microarray was performed to determine critical genes that are upregulated at the front of invasion. WNT10A was found to be over 4-fold upregulated, along with a WNT-signaling gene signature in the invasive cells. We additionally found that increased WNT10A expression was associated with ESCC, compared to normal esophageal tissue and that increased WNT10A staining was associated with poor prognosis. Functionally, WNT10A was determined to induce increased proliferation, migration, invasion and stemness properties in transformed esophageal cancer cells. In addition to examining the oncogenic properties of WNT10A, we also focused on generating an in vivo model of mutant TP53 driven ESCC. We utilized a carcinogen, 4-nitroquinoline 1-oxide (4-NQO), as a challenge to induce genetic changes in the background of Tp53 mutation, specific to the oral
squamous, esophagus and forestomach tissues. We found preliminarily that TP53 mutation, specifically the R172H mutant, can accelerate tumorigenesis as compared to loss of TP53 alone. Additionally, when RNA-seq was performed on the tumor derived TP53^{R172H/-} and TP53^{-/-} cells, we found an increase of genes associated with an epithelial to mesenchymal transition (EMT), in the TP53^{R172H/-} cells. Mesenchymal genes such as Cdh2, Zeb1, Zeb2, Twist, and Snail were increased, while epithelial genes such as Cdh1, Epcam, and Krt4 were decreased. Altogether, we surmise that the TP53 mutant, R172H, can induce gain-of-function (GOF), oncogenic properties in order to promote tumorigenesis, potentially by inducing an EMT phenotype.
TABLE OF CONTENTS

ACKNOWLEDGMENTS .................................................................................................................. II

ABSTRACT ....................................................................................................................................... III

LIST OF TABLES............................................................................................................................... VII

LIST OF FIGURES.............................................................................................................................. VIII

CHAPTER 1: INTRODUCTION ........................................................................................................ 1

Esophageal cancer ............................................................................................................................. 2

Embryogenesis of the esophagus ...................................................................................................... 5

TP53: A potent tumor suppressor ...................................................................................................... 7

Gain of function roles of mutant TP53 ............................................................................................. 9

Role of mutant TP53 in 3D organotypic culture of transformed esophageal cells ......................... 12

WNTs and the canonical WNT pathway .......................................................................................... 15

WNTs in development ...................................................................................................................... 19

WNT pathway in cancer .................................................................................................................. 22

Figures and figure legends .............................................................................................................. 27

CHAPTER 2: WNT10A PROMOTES AN INVASIVE AND SELF-RENEWING
PHENOTYPE IN ESOPHAGEAL SQUAMOUS CELL CARCINOMA .......................................... 29

Abstract ........................................................................................................................................... 30

Introduction ..................................................................................................................................... 31

Results........................................................................................................................................... 34

Discussion....................................................................................................................................... 39

Materials and methods .................................................................................................................. 43
Figures and figure legends ................................................................. 48
Tables and table legends .................................................................. 56

CHAPTER 3: THE ROLE OF MUTANT TP53 IN THE DEVELOPMENT OF A MOUSE MODEL OF ESOPHAGEAL SQUAMOUS CELL CARCINOMA ................................................................. 59
Abstract .......................................................................................... 60
Introduction ...................................................................................... 61
Results ............................................................................................. 65
Discussion ........................................................................................ 71
Materials and methods ..................................................................... 76
Figures and figure legends ................................................................. 79
Tables and table legends ................................................................. 85

CHAPTER 4: THESIS SUMMARY AND DISCUSSION ............................................. 88

BIBLIOGRAPHY ...................................................................................... 96
LIST OF TABLES

Chapter 2- Supplementary Tables

Supplementary Table 1. Significantly upregulated pathways associated with invasive mutant TP53 gene signature

Chapter 3- Tables

Table 1. Genes associated with a mesenchymal gene signature

Table 2. Genes associated with an esophageal epithelial gene signature
LIST OF FIGURES

Chapter 1- Figures
Figure 1. Molecular mechanisms of esophageal cancer........................................... 28

Chapter 2-Figures
Figure 1. WNT pathway is significantly upregulated in invasive mutant TP53 cells…. 49
Figure 2. WNT10A is upregulated in invasive esophageal cells................................. 50
Figure 3. WNT10A promotes migration and invasion............................................... 51
Figure 4. WNT10A increases CD44High population and promotes self-renewal....... 52
Figure 5. WNT10A is upregulated during esophageal development and in cancer…… 53

Chapter 3-Figures
Figure 1. Mutations in TP53 with 4-NQO can induce dysplasia and invasive squamous carcinoma of the esophagus................................................................. 80
Figure 2. Mutations in TP53 with 4-NQO can lead to invasive squamous carcinoma of the esophagus and metastasis to the lymph node and lung............................. 82
Figure 3. Isolation of cells from mouse tumors....................................................... 84
CHAPTER 1: Introduction
Esophageal cancer

Esophageal cancer is the sixth leading cause of cancer related deaths worldwide and the eighth most common cancer type, with an average 5-year survival of approximately 15-20% (Altekruse SF et al., 2009). Esophageal cancer can be separated into two major subtypes, with esophageal squamous cell carcinoma (ESCC) being more common than esophageal adenocarcinoma (EAC), worldwide. ESCC is most prevalent amongst the “Asian esophageal cancer belt”, which spans from the Middle East and across China, suggesting multifactorial components of genetics and environment as contributors to disease. In the United States, however, EAC is more common and on the rise, and currently contributes to 80% of all esophageal cancer cases (Taylor et al., 2013; Mao et al., 2011).

Major risk factors of esophageal cancer include increased age, alcohol use, and smoking (Mao et al., 2011). Ethanol primarily inhibits DNA methylation and interacts with retinoid metabolism, in order to induce mutations along the aerodigestive tract (Toh et al., 2010). Moreover, metabolism of ethanol can lead to further mutagenic properties: specifically, in certain Asian populations, that may be deficient for expression of aldehyde dehydrogenase 2 (ALDH2), acetaldehyde build-up may occur, leading to bulky adduct formation that may escape DNA-repair pathways, and predisposition for ESCC (Yokoyama et al., 2002). Chemicals in cigarettes that are particularly carcinogenic include the polycyclic aromatic hydramines and N-nitrosamines (Toh et al., 2010). These chemicals can induce DNA methylation, bulky DNA-adduct formation along with genomic instability. Regional risk factors additionally play a strong role in ESCC,
especially in areas throughout the esophageal cancer belt, where alcohol and tobacco use is limited. Nitrosamines found in preserved fish and vegetable diets in northeast China are major contributors of disease (Napier et al., 2014). In northern Iran, frequent drinking of extremely hot tea has been shown to induce caustic injury and inflammation of the esophagus, which has been associated with high risk of ESCC (Mao et al., 2011).

The only known precursors of ESCC include esophageal squamous dysplasia (ESD), with severity of ESD highly associated with ESCC incidence. Primarily, ESD is characterized by hyperproliferation, nuclear atypia, loss of cellular polarity, and improper differentiation of the tissue, without invasion into the basement membrane. Dysplasia grading is stratified into low, middle, and high, depending on the level of infiltration of the epithelium. When all layers are affected, the pathological grade is considered to be esophageal intraepithelial neoplasia (EIN). Once the atypical epithelial cells penetrate the underlying lamina propria, it is considered to be invasive squamous cell carcinoma, with lymph node dissemination. Once ESCC has occurred, tumors may be of varying differentiation and rather heterogenous, with features of keratin pearls, cytokeratin bridges, and desmoplasia within the adventitia; ESCC may also be further stratified based on differentiation of the tumor, with poorly differentiated tumors correlating with worst outcome and shorter survival time (Taylor et al., 2013).

Prognosis of esophageal cancer highly correlates with staging at diagnosis. Commonly, patients present during later stages, when lymphatic and hematogenous spread has already occurred. Furthermore, since the esophagus wall lacks a serosa, tumors can expand into adjacent structures in the mediastinum and neck. Staging of
esophageal cancer is highly dependent on the TNM system. T staging represents the depth into which the tumor has invaded. T1/T2 tumors, which are usually treated with surgical resection, indicate tumor invasion into the lamina propria and muscularis, respectively. T3/T4 tumors require neoadjuvant therapy, of either chemotherapeutics or radiation, prior to surgical resection, if possible. These later stages usually indicates invasion into adventitia and adjacent structure. N staging indicates the absence (N0) or presence (N1) of periesophageal lymph node involvement, which is best assessed by endoscopic ultrasound (EUS). N1 automatically indicates requirement of neoadjuvant therapy prior to surgical resection. M staging indicates the level of metastatic spread. M0 indicates no tumor spread, as determined by computer tomography (CT) at the most common sites of metastasis: lung, liver and bone. Metastatic spread is stratified further into M1a versus M1b, with M1a an indicator of improved survival. M1a indicates spread to the celiac and cervical lymph node, whereas M1b, which is present in 15 to 20% of ESCC diagnosed, indicates distant spread. All together staging is extremely important in determining therapeutics and must be assessed appropriately with a variety of modalities, such as CT, EUS, and ¹⁸F-fluorodeoxyglucos positron emission tomography (FDG-PET) (Rice et al., 2010; Napier et al., 2014).

A vast majority of genes and dysregulated proteins have been associated with ESCC occurrence and prognosis. The most common genetic alterations of ESCC include overexpression of Epidermal Growth Factor Receptor (EGFR), CYCLIND1, mutation in TP53, and inactivation of p15⁵INK⁴B/p16⁴INK⁴A, and loss or mislocalization of p120 Catenin and CADHERIN-1 (E-CADHERIN) (Mandard et al., 2000; Stairs et al.,
Recent sequencing efforts have additionally shown that NOTCH1, NOTCH3, and FBXW7 were quite frequently mutated in ESCC, along with members of the WNT/B-Catenin signaling pathway (Song et al., 2014). Our lab has additionally previously shown the importance of PERIOSTIN in mediating migration, invasion, and as a diagnostic marker of ESCC (Michaylira et al., 2010; Wong et al., 2013b; a). One of the most drastic genetic alterations in ESCC is associated with TP53 loss or mutation. Overall, TP53 is estimated to be mutated in 40-50% of all cases of ESCC. Yet, recent sequencing efforts of ESCC in the Chinese population report exceedingly high rates of TP53 mutation, from 83 to 93%, with a predominance of missense mutations (Song et al., 2014; Gao et al., 2014). Most common missense mutations within TP53 include R175H and R248H, which account for 10% of all TP53 alterations, combined (Petitjean et al., 2007). As TP53 mutation is the most often mutated gene in ESCC, it is not surprising that it is suspected to be an early event during pathogenesis. Based on immunohistochemistry (IHC) staining along with sequencing of microdissected tissue, TP53 mutations are seen even during early dysplasia, suggesting TP53 mutation may be an initial driver of ESCC progression (Wang et al., 1993).

**Embryogenesis of the esophagus**

The esophagus originates as a joint structure with the trachea in a developmental intermediate known as the anterior foregut. This foregut is derived by ventral folding of the endodermal epithelial sheet during gastrulation (Wells and Melton, 1999). The separation of these two structures requires well-coordinated cues from
epithelial/mesenchymal interactions as well as specific cell intrinsic cues to proliferate and differentiate. Failure of these processes can lead to malformation and birth defects such as esophageal atresia and tracheoesophageal fistula.

The anterior foregut separates in a ventral/dorsal manner, giving rise to the trachea and esophagus, respectively. Specific transcription factor expression cues the distinction between these two tissues; SOX-2 is expressed in the epithelial cells of the dorsal region, whereas NKX2.1 marks the ventral epithelium (Que et al., 2007; Harris-Johnson et al., 2009). These transcription factors are critical for these cell fate decisions, as hypomorph mutations in SOX-2 and deletion of NKX2.1 can lead to esophageal atresia and fistulation (Que et al., 2007; Minoo et al., 1999). Extrinsic signaling factors, such as WNT signaling (which will be covered more in depth in a later chapter) and bone morphogenic protein (BMP) signaling have also shown to play a role in this process, as defects in their signaling also produce esophageal atresia phenotypes in mice (Rodriguez et al., 2010).

Following separation of the dorsal foregut, a ciliated simple columnar lumen is formed as a precursor to the esophageal stratified epithelium (Yu et al., 2005). SOX-2 here plays a critical role in inducing the proliferation and stratification of the epithelial cells, as SOX-2 hypomorph mutant mice maintain columnar structure and develop goblet-like mucin producing cells (Que et al., 2007). Similarly, TP63 null mice also exhibit a similar phenotype in the proximal forestomach, which recapitulates the distal gastroesophageal junction in humans (Daniely et al., 2004; Wang et al., 2011). TP63 is another critical factor in the induction of stratification of epithelium in both skin and
esophagus, through its role in maintaining the self-renewal capacity of stratified epithelial. TP63 null mice notably are born with little or eroded epithelium (Parsa et al., 1999; Yang et al., 1999). Interestingly, BMP signaling is silenced during the stratification of the esophageal epithelium, as it may negatively regulate SOX-2 expression, but it is re-expressed shortly after stratification in the suprabasal layers of the esophagus in order to allow for proper differentiation of the tissue (Domyan et al., 2011; Rodriguez et al., 2010).

**TP53: A potent tumor suppressor**

TP53 was first described as a protein associated with the simian virus 40 large T antigen in virally transformed cells (Lane and Crawford, 1980; Linzer and Levine, 1979). Initial descriptions of TP53 suggested that it was acting as an oncogene, as it became upregulated when cell lines were transformed by viruses, irradiation or chemical carcinogens. Furthermore, its overexpression was able to immortalize primary cells and transform them in cooperation with the expression of Ras (Eliyahu et al., 1984). In actuality, the original clone of TP53 studied was actually a mutant version (DeLeo et al., 1979; Rotter, 1983). Thus, the wildtype version of the protein was indeed a tumor suppressor that would become upregulated in response to cellular insults.

*TP53* encodes a tetrameric multidomain protein that is important in responding to a variety of stress signals, such as genotoxic damage, oncogene activation, and loss of normal cell contact, amongst others (Lane, 1992; Vousden and Prives, 2009). While primarily a nuclear transcription factor, capable of activating and repressing downstream
target genes, TP53 has additionally been described to have non-transcriptional functions. TP53 is composed of three domains: an N-terminal trans-activating (TA) domain, a central DNA-binding domain (DBD) and a C-terminal oligomerization domain (OD) (Bullock and Fersht, 2001). Almost every amino acid of TP53 can be modified by posttranslational modifications, such as phosphorylation, acetylation, ubiquitination, amongst others, in order to initiate downstream tumor suppressing functions (Meek and Anderson, 2009). TP53 has been shown to participate in a myriad of functions, including induction of apoptosis, senescence, cell cycle arrest, DNA damage response, autophagy, metabolism, miRNA transcription, and inhibition of embryonic cell reprogramming, thus making TP53 such a potent tumor suppressor (Vousden and Prives, 2009).

Not unexpectedly, TP53 is the most mutated gene in cancer, and is estimated to be mutated in over 50% of all cancers. Additionally, individuals with germ line mutations in TP53 have a cancer predisposition syndrome, known as Li-Fraumeni syndrome. Over 70% of TP53 mutations in cancers are missense mutations and 30% of them fall within one of six hotspot codons in the DBD (Petitjean et al., 2007). The mutations of TP53 generally fall into three categories. (1) Nonsense mutations, which account for the minority of mutations in esophageal cancer, induce loss of production of full-length protein. These mutations, when homozygote, lead to TP53 null cells, which do not have the ability to induce a TP53 tumor-suppression response. (2) Single amino acid alterations that lead to missense mutations can also cause loss of wildtype TP53 functions. Additionally though, they can also act in a dominant negative fashion if there is maintenance of the second wildtype allele. Since TP53 binds to DNA in a tetrameric
fashion, one mutated allele can induce hetero-oligermerization to inhibit proper DNA binding. Of note, mutations in TP53 confer increased stability compared to their wildtype counterparts due to a combination of mechanisms, which are not completely understood. Nevertheless, stabilization of the mutant copy over the wildtype protein of TP53 induces a majority of defunct tetramers. Lastly, a newly accepted notion of mutant TP53 is its (3) gain-of-function (GOF) oncogenic properties. Given the high propensity of TP53 missense mutations across many cancers, it has been suggested that certain mutations, specifically at the hotspot codons, can provide greater survival advantage, through GOF oncogenic properties. GOF is especially apparent when there is loss of heterozygosity of the second TP53 allele, in order to ensure that no dominant negative effects can occur. Therefore, in order to most rigorously study GOF properties of mutant TP53, investigators must perform all studies under TP53 null conditions (Brosh and Rotter, 2009; Oren and Rotter, 2010; Freed-Pastor and Prives, 2012; Oren and Rotter, 2015).

**Gain of function roles of mutant TP53**

GOF mutations in the DBD region of TP53 are most common, as they cause TP53 to lose its specificity to DNA sequences, while maintaining transactivation functions (Petitjean et al., 2007). These mutations can either cause global conformation changes or interfere with promoter-specific contact. The R175H mutation, a hotspot mutation, for instance, causes destabilization at a zinc-binding site, which structurally distorts the conformation of TP53 (Olivier et al., 2010). This mutant may even expose hydrophobic epitopes that can aggregate into large B-sheaths that may act as a sponge for other
proteins. R273H, on the other hand is a contact defective mutant, which cannot bind the
canic TP53 DNA-binding sites in a sequence specific fashion (Xu et al., 2011). Other
hotspot mutations include G245S, R248W, R249S and R282S (Petitjean et al., 2007).
The prevalence of hotspot mutation sites suggests a specific survival advantage for these
mutations, and thus potentially oncogenic potential of these specific mutants.
Additionally, in esophageal cancer, up to 75% of all tumors with TP53 mutation have
coinciding additional alterations of the second allele (either mutated or promoter
methylated), suggesting that GOF properties may be highly prevalent (Hu et al., 2001).

Some of the first studies to implicate GOF roles of mutant TP53 occurred over 20
years ago. When TP53 null fibroblasts were transfected with mutant TP53, it was shown
that mutant TP53 overexpressing cells had a greater propensity for forming tumors in null
mice, compared to the TP53 null fibroblasts. Transfection of mutant TP53 in a null
human osteosarcoma line also induce greater soft agar plating efficiency, as compared to
the control (Dittmer et al., 1993). This seminal study implicated the potential for
additional oncogenic functions of mutant TP53 and led to decades more of continuing
researching into the mutant TP53 GOF field. In 2004, two groups showed through
independent mouse models the in vivo GOF effects of mutant TP53. Each group
expressed a knock-in mutation of TP53 at the endogenous promoter, which can be
induced by a tissue specific Cre-Lox system. In a 129S4/SVJae background, mice, with
Protamine-Cre induced knock-in mutations of R172H and R270H of TP53 (equivalent to
R175H and R273H respectively in the human), mice developed carcinomas at higher
frequencies, when compared to TP53 null mice, although lifespan of mice were
equivalent. While most TP53 null mice succumbed to hematological illness, almost none developed carcinomas, suggesting the necessity of mutations in TP53 in order to induce transformation in epithelial cells (Olive et al., 2004). In the C57BL/6 background of knock-in p53R172H mice, there were no differences in tumor site spectra between mutant TP53 mice and TP53 null mice. Instead, there was a greater predilection for the mutant TP53 knock-in mice to develop metastases, as compared to the null controls (Lang et al., 2004). This phenomenon is reinforced in recent metastasis models utilizing cells derived from murine pancreatic cancer driven by KRASG12D and TP53R172H mutations. When these cells were deprived of mutant TP53, there was a decreased propensity of these cells to colonize into the lungs and liver when injected orthotopically into pancreata. The mechanism by which mutant TP53 induced increased metastases was shown to be through repression of TP73/NF-Y complex leading to induction of PDGFB (Weissmueller et al., 2014).

Studies on GOF mutant TP53 indicate that the oncogenic properties of mutant TP53 can arise from a variety of mechanisms. Specifically, mutant TP53 has been shown to participate in novel protein-protein interactions, trans-activate novel gene targets, or a combination of the two. Additionally, since mutant TP53 is normally expressed at rather high protein concentrations due to its inability to be properly degraded, normally weak protein-protein interactions can be exacerbated. When mutant TP53 participates in novel protein-protein interactions, it can either inhibit or potentiate the functions of the binding partner. Proteins that mutant TP53 have been shown to inhibit include MRE11 and the TP53 family members, TP63 and TP73 (Freed-Pastor and Prives, 2012). Inhibition of
MRE11, for instance inhibits its proper binding to the MRE11-NBS-RAD50 complex, which is required for proper ATM-mediated DNA-repair. Thus mutant TP53 can potentiate genomic instability in its GOF role (Song et al., 2007). TP53 interaction with various isoforms of TP63, via the DNA-binding domain can initiate a pro-invasive phenotype, either through activation of downstream TP63 targets or through induction of an integrin recycling pathway (Adorno et al., 2009; Muller et al., 2009). Additionally, mutant TP53 can interact with a variety of transcription factors in order to be recruited to their discrete binding sites. In turn, mutant TP53 can either trans-activate or repress these downstream gene targets as its trans-activation domain is not commonly mutated (Freed-Pastor and Prives, 2012; Muller and Vousden, 2013). These trans-activation domains are capable of recruiting transcriptional activators such as p300 and TATA binding protein, in order to induce transcription of novel targets (Di Agostino et al., 2006; Liu et al., 2011; Dell’Orso et al., 2011). Transcription factors that have been shown to co-opt mutant TP53’s transactivation functions include NF-Y, SP1, ETS-2, VDR, and SREBP2 (Liu et al., 2011; Chicas et al., 2000; Do et al., 2012; Stambolsky et al., 2010; Freed-Pastor et al., 2012). Downstream functions of mutant TP53 can lead to a variety of transforming roles including: migration, invasion, proliferation, drug resistance, and induction of genomic instability (Freed-Pastor and Prives, 2012).

**Role of mutant TP53 in 3D organotypic culture of transformed esophageal cells**

Considering the high prevalence of mutations in TP53 in ESCC, our lab generated cells lines that overexpressed the different TP53 mutations in a transformed setting.
Primary normal epithelial esophageal cells (EPC2) were obtained from a human biopsy, and were immortalized by the overexpression of hTERT (Harada et al., 2003). These immortalized, EPC2-hTERT cells could be further transformed by the expression of a combination of oncogenes, such as overexpression of EGFR and mutations in TP53 (Okawa et al., 2007). Of note, the wildtype allele of TP53 was maintained in these cells, while the mutant allele was transgenically overexpressed. These cells were shown to be transformed by a variety of mechanisms such as through migration, invasion, colony formation, soft agar growth, and xenograft implant assays (Okawa et al., 2007).

Furthermore, these cells were grown in an in vivo-like 3D organotypic culture system (OTC), which recapitulated the microenvironment of epithelial cells of the esophagus (Michaylira et al., 2010). This 3D OTC system consists of a bottom matrigel/collagen acellular layer, which is overlaid with low passage fetal esophageal fibroblasts in the middle layer. After seven days, these layers contract into a feeder matrix, and epithelial cells can be grown on top. These cells can divide in an asymmetrical fashion in order to induce the stratified layer of the esophageal epithelium. Furthermore, upon introducing an air-liquid interface, the stratified epithelium can induce keratinization, which is seen in human skin and mouse esophagus. These 3D culture systems can be harvested and embedded into paraffin blocks in order to allow for Hematoxylin and Eosin (H&E) and/or immunohistochemistry (IHC) staining (Kalabis et al., 2012).

Although the transformed EPC2-hTERT derivatives that have overexpression of mutant TP53 can induce stratification in this 3D OTC system, they additionally show
features of invasive downward growth into the fibroblast-enriched matrix. Primarily these invasive regions are not differentiated and maintain basal cell features, such as strong TP63 staining, a marker of the undifferentiated epithelial cell (Okawa et al., 2007). Interestingly, there are different degrees of invasion, with the R175H mutation being the most invasive. This process was further explored, in order to better understand some of the mechanisms of mutant TP53-mediated invasion, as a potential GOF property. In order to do so, our lab utilized laser-capture microdissection of the invasive and non-invasive regions of the 3D OTC samples that grew different TP53 mutations (R175H, R248W, R273H and V143A). We proceeded to harvest RNA from each these sections in order to perform comparative microarray analysis on the invasive and non-invasive region. One of the top genes that was upregulated in each microarray set was PERIOSTIN, a matricellular protein that not only was induced by mutant TP53, but also worked in concert with mutant TP53 in order to induce a permissive microenvironment to induce invasion (Ohashi et al., 2010a; Michaylira et al., 2010; Wong et al., 2013b).

Additional pathway analysis was performed on the invasive gene signature, and from the combination of all the mutants, it was shown that there was a significant enrichment of the WNT signaling gene signature. Specifically, genes such as \textit{WNT10A} and \textit{WNT5B} were upregulated, while the WNT signaling negative regulator \textit{DKK1} was downregulated across multiple mutants (Long et al., 2015). As the WNT signaling pathway is frequently dysregulated in over 40% of all ESCC (Song et al., 2014), we decided to investigate this pathway further, specifically via \textit{WNT10A}. \textit{WNT10A} upregulation was not only associated in the WNT signaling pathway, but also
contributed to other significantly enriched pathways, associated with the invasive gene signature, such as regulation of the epithelial to mesenchymal transition pathway and induction of human embryonic stem cell pluripotency.

**WNTs and the canonical WNT pathway**

The WNT family of ligands is critical in both development and disease across all metazoan species, with conservation from the *Hydra vulgaris* to the higher vertebrates (Kusserow et al., 2005). *Wnt1* was first cloned in 1982, under the name *int1*. Notably, *int1* was a common insertion site for the retrovirus, Mouse Mammary Tumor Virus; this specific insertion could induce the upregulation of the proto-oncogene *Wnt1*, in order to induce transformation of mouse mammary tissue (Nusse and Varmus, 1982). Concurrently, developmental biologists, studying *Drosophila melanogaster*, were able to generate mutants that disrupted segment polarity in embryonic development. *Wingless* was (*Wg*), was one of the genes discovered in this mutant screen, along with *Armadillo*, *Dishevelled*, *Porcupine*, and *Arrow* (Sharma and Chopra, 1976; Nüsslein-Volhard and Wieschaus, 1980). Indeed, the *Drosophila* homologue of *int1* mapped closely to *Wingless*, with a similar localization pattern of a striped larvae. By the 1990s, the naming of the gene combined, to form ‘WNT’ (Wingless-related integration site), with *Wnt1* (*int1*) becoming the first member of a family of related genes (Nusse et al., 1991).

Mice and humans have over 19 members in the WNT family, which fall into twelve subgroups. Between mouse and human, WNTs are conserved 99% at the amino acid level. While at least eleven WNTs existed in the last common ancestor between
deuterostomes and arthropods, duplication events occurred over the last 500 million years, in order to generate many of the paralogs of WNT 3, 5, 7 and 10 (Sidow, 1992). Interestingly, *Drosophila* and *Caenorhabditis* lost some of their WNT homologs, and only have seven and five WNTs, respectively. *WNT10* was duplicated along with the *WNT1-WNT6-WNT10* cluster. Over time, *WNT6* was lost from the *WNT1-WNT10B* cluster, where as *WNT1* was lost from in the *WNT6-WNT10A* cluster (Nusse, 2001). Maintenance of two different *WNT10* genes may suggest diverse and specific functions of these two genes in higher organisms, although redundant roles may still exist. Additionally, this unique grouping of WNT genes suggests coordinated regulation of each cluster, similar to another developmental grouping, the Hox cluster.

Members of the WNT family are small, secreted proteins, which are cysteine-rich. Many of these cysteine sites are predicted to form disulfide bonds in order to maintain the globular structure of WNT. In addition, WNTs are also highly post-translationally modified with various glycosylation and acylation sites; these modifications contribute to multiple band formation of overexpressed WNTs, when they are run on an immunoblot. Glycosylation events are primarily at asparagine residues, and are suggested to preceded palmitoylation events (Komekado et al., 2007). Perhaps the best-described modification of WNTs is the palmitoylation of various cysteine and serine residue by the ER protein PORCUPINE (PORCN) (Willert et al., 2003; Takada et al., 2006). These modifications are crucial for the recognition of WNTs by WNTLESS (WLS), a sorting receptor that sits at the golgi membrane and transports WNTs to the plasma membrane (Bänziger et al., 2006). Mutations in Porcupine in mice lead to embryonic lethality (Barrott et al., 2011;
Biechele et al., 2011), whereas mutations in human \textit{PORCN} (an X-linked gene), can lead to lethality in males and focal dermal hypoplasia in females (Grzeschik et al., 2007; Wang et al., 2007). These findings suggest the necessity of palmitoylation of WNTs, in order for proper secretion and signaling functions.

Considering the high levels of lipidation amongst WNTs, it was proposed that these proteins were highly hydrophobic. Indeed the hydrophobic nature of WNTs eluded researchers in the ability to purify the protein, for over 30 years. The first purification was of the murine WNT3A in 2003 (Willert et al., 2003), while the first structure of WNT (Wnt8 in \textit{Xenopus}) was not solved until 2012 (Janda et al., 2012). The hydrophobic nature of WNTs would suggest its poor ability to diffuse across long distances extracellularly. Primarily, WNTs act as morphogens that generate a gradient, and act as paracrine cellular signals in a concentration dependent manner. Often times, WNTs act across very short distances, such as across the neuromuscular junction or within a given stem cell niche (Korkut et al., 2009; Sato et al., 2004). Long-range functions of WNTs have recently been proposed to involve either binding with other interacting proteins or through transport via exosomes (Mulligan et al., 2011; Gross et al., 2012).

Classically, WNT signaling is separated into a canonical and non-canonical pathway.Canonical WNTs usually include WNT1, WNT3A, and WNT8, and the classic non-canonical WNT is WNT5A. Yet, depending on cellular context and available receptors, many WNTs can feed into either pathway (Grumolato et al., 2010). Canonical WNT signaling is best described by its ability to induce nuclear localization of B-
CATENIN and activation of its downstream target genes. Non-canonical WNT signaling, on the other hand, is more diverse and can act antagonistically to canonical WNT signaling. It is divided primarily into three categories: the planar cell polarity (PCP), WNT/Ca$^{2+}$ and JNK pathways (Gómez-Orte et al., 2013). The PCP pathway regulates cellular polarity during morphogenesis, and is involved in such processes as gastrulation and neural tube closure. It utilizes mainly the FRIZZLED (FZ) receptors in order to activate downstream RH0A and JNK effectors (Schambony and Wedlich, 2007; Minami et al., 2010). In addition to developmental roles, the WNT/Ca$^{2+}$ pathway can also play a role in cancer, inflammation, and neurodegeneration (De, 2011). Its main effector is the activation of phospholipase C, which triggers intracellular calcium release, in order to activate downstream kinases and phosphatases ultimately toward an N-FAT driven transcriptional response (Gómez-Orte et al., 2013). The JNK pathway can utilizes ROR2 receptors to induce JUN and ATF2 target genes (Schambony and Wedlich, 2007). Depending on the WNT, the cell type, and/or the receptor complement, different WNT signaling pathways can be elicited, suggesting high diversity and specificity of each context.

The canonical WNT pathway is perhaps the better studied of the two pathways. Many of the proteins were elucidated through genetic screens and epistasis experiments in Drosophila, in order to determine the order of the pathway components (Sharma and Chopra, 1976; Nüsslein-Volhard and Wieschaus, 1980). Canonical WNT signaling is initiated with the binding of WNT to the FZ family of receptors and the homologue of Drosophila Arrow, low-density lipoprotein (LDL) receptor-related protein (LRP). In
addition to WNTs, the FZ/LRP5/6 receptors can also bind to WNT antagonists, such as DICKKOPF (DKK), in order to disrupt WNT binding (Semënov et al., 2008). Other inhibitors of WNTs include the secreted Frizzled-related proteins (sFRPs) and WNT inhibitory protein (WIF), which can sequester away functional WNTs (Bovolenta et al., 2008). Nevertheless, binding of WNT to its heteromeric receptor complex leads to the recruitment of DISHEVELED (DVL) to the FZ receptor, and phosphorylation of the LRP tail by CK1α and GSK3 (Tamai et al., 2004). These coordinated events lead to the binding of AXIN to the cytoplasmic tail of LRP5/6 (Mao et al., 2001). AXIN is large scaffolding complex that houses the destruction complex of B-CATENIN, which include adenomatous polyposis coli (APC) and the kinases CK1α and GSK3. Relocalization of AXIN to the LRP tail leads to the inhibition of B-CATENIN phosphorylation by CK1alpha and GSK3, preventing its degradation (Kishida et al., 1998). The build-up of B-CATENIN induces its nuclear translocation, where it can act with Lymphoid Enhancer-Binding Factor 1/T cell-specific Transcription Factor (TCF/LEF) binding partners to induce transcription of target genes (Aberle et al., 1997).

**WNTs in development**

The diversity in number of WNTs and their high conservation suggest their criticalness in multicellular organisms, especially in embryogenesis. One of the first studies to demonstrate the importance of WNTs in development utilized ectopic overexpression of a murine Wnt1 mRNA in *Xenopus laevis* blastomeres at the ventral end, in order to induce a duplicated dorsal axis (McMahon and Moon, 1999). This
experiment demonstrates the role of WNT signaling in development patterning and additionally highlights the importance of the temporal and spatial restriction of WNTs. Over the years, WNTs have been described in a variety of processes in development and homeostasis including: proliferation, cell fate specification and differentiation, cellular migration, cell adhesion regulation as well as stem cell maintenance, amongst others. Wingless, the WNT1 orthologue in *Drosophila*, in addition to mediating proper segmental patterning in embryonic and larvae stages, is critical for the proliferation of the wing discs; as its namesake suggests, mutants are wingless (Giraldez and Cohen, 2003). Additionally, canonical WNT signaling target genes include *MYC* and *CCND1* in colon cancer cells (He et al., 1998; Shtutman et al., 1999), implicating a role of WNT signaling in proliferation in specific cellular contexts.

Knockout models in mice validated additional roles of WNTs and their signaling pathways in embryogenesis. Almost every murine WNT molecule has been knocked out either alone or as a double knockout with other WNTs. Many of these knockout mice are embryonic or perinatal lethal due to the developmental defects. Herein, I provide a few examples to demonstrate the varied tissue types affected by WNTs. *WNT1* knockout mice do not form midbrains and suffer from ataxia when viable, but succumb shortly after birth (Thomas and Capecchi, 1990; McMahon and Bradley, 1990). Similarly, the *WNT1/WNT3* compound knockout mice are embryonic lethal by E.18.5 with reduced numbers of neural crest precursors (Ikeya et al., 1997), suggesting that WNT1 and WNT3A may have redundant roles in induction of proliferation. WNT2 is expressed embryonically at the mesenchymal component of the ventral foregut, and when knocked
out in mice, leads to embryonic lethality due to lung hypoplasia and reduction of NKX2.1 expression (Goss et al., 2009). This phenotype is exacerbated with concomitant knockout of *WNT2B* and is also observed in *WNT7B* null mice (Goss et al., 2009; Shu et al., 2002). *WNT10B* knockout mice, interestingly, develop normally, potentially due to compensation by the expression of *WNT7B*. With aging, *WNT10B* null mice do exhibit a decrease in bone density (Stevens et al., 2010). Additionally, *WNT10B* null myoblasts displayed increased capability to form adipocytes, consistent with previous described work, demonstrating WNT10B’s role in inhibiting adipogenic differentiation (Ross et al., 2000; Cawthorn et al., 2012) (Ross 2000). *WNT10B* mutation concurrently has been shown to be associated with a propensity for obesity in humans (Christodoulides et al., 2006).

The *WNT10A* knockout mouse has not been fully described, but functional aspects of WNT10A in development can be surmised by its mutation in human disease. *WNT10A* is one of the primary genes mutated in odontyl-onychol dermal dysplasia (OODD) and Schopf-Schulz-Passarge syndrome (SSPS), which are both characterized by a variety of ectodermal dysplasias, affecting the hair, teeth, nail, skin, and tongue (Cluzeau et al., 2011). Prominent phenotypes of individuals with *WNT10A* missense and nonsense mutations include smooth tongues, barrel shaped teeth, and palmar plantar keratoderma (Tziotzios et al., 2014). These phenotypes suggest a role of WNT10A in proliferation and differentiation of the ectodermal layer of these tissue types. Localization studies of *Wnt10a* expression in the embryonic mouse show that *Wnt10a* predominates at the surface ectoderm, and more specifically in epithelial cells of the anterior apical
ectodermal ridge (AER) (Summerhurst et al., 2008). Wnt10a expression has additionally been implicated during hair follicle morphogenesis. The proliferation and downward growth of the hair follicle placode is a well-orchestrated process involving careful communication between the epithelial and mesenchymal layers. During embryonic day (E) 14.5 of mouse, Wnt10a is expressed throughout the epidermis of the skin, but during the start of the hair follicle growth cycle, at E18.5, its expression condenses in the highly proliferative hair follicle placode. As hair follicle morphogenesis progresses, Wnt10a expression relocates to the inner root sheath. During adult homeostasis, Wnt10a’s expression pattern is similar to its embryonic counterpart. Its expression condenses at the dermal papilla during the start of anagen (growth phase in hair follicle) and then inhabits the inner root sheet during anagen before it is shut off during telogen (rest phase) (Reddy et al., 2001). Our own work demonstrates that in addition to ectodermal surfaces of mouse embryo and the growth stages of adult hair follicles, WNT10A is highly localized to the E14.5 esophagus, during stratification induction by SOX2 and TP63 activity. Interestingly, no esophageal phenotypes have been reported in patients with WNT10A mutation, suggesting potential redundant roles of other WNTs during esophageal development and homeostasis.

**WNT pathway in cancer**

Considering the varied functions of WNTs in development, it is unsurprising that WNT signaling can also contribute to disease. Many diseases and developmental defects have been alluded to previously. The primary focus of this next section will be to
describe the varied roles of WNTs and members of its pathway in cancer. Of note, while in many cases, upregulation of canonical WNT signaling can lead to increased proliferation, there are exceptions, in which active WNT signaling may be associated with improved survival in human cancers (Anastas and Moon, 2013). APC was the first described member of the WNT pathway to demonstrate an association with human cancer. Based on its namesake, germline APC mutations were frequently seen in patients with a hereditary cancer predisposition syndrome, familial adenomatous polyposis (FAP), which largely affects the large intestines (Kinzler et al., 1991; Nishisho et al., 1991). These mutations would lead to truncation of APC, and its inability to mediate binding of the B-CATENIN destruction complex (Groden et al., 1991). APC is also incidentally mutated in 84% of all sporadic colorectal cancer cases (Ashton-Rickardt et al., 1989).

In addition to APC, many other components of the B-CATENIN destruction complex are frequently mutated in human cancer. Truncation of AXIN or missense mutations in the X-chromosome linked Wilms tumor (WTX) gene, both lead to destabilization of the B-CATENIN destruction complex (Salahshor and Woodgett, 2005; Major et al., 2007). AXIN mutations are frequently seen in hepatocellular carcinoma, where as WTX is oftentimes mutated in Wilms tumor, a pediatric kidney cancer (Satoh et al., 2000; Rivera et al., 2007). Mutations in CTNNB1 itself, specifically at phosphorylation sites in the N-terminal, inhibit its ability to be ubiquitinated and destroyed. Although these mutations occur in only 1% of colorectal cancer cases, they are more frequently seen in other cancers such as medulloblastoma, hepatocellular carcinoma, as well as Wilms tumors (Koesters et al., 1999; Polakis, 2007).
WNT receptors and inhibitors can also be dysregulated in human cancers. Receptor upregulation can lead to activation of canonical WNT signaling. FZ7 is frequently upregulated in triple-negative breast cancer; coincidentally in vitro assays indicate that its knockdown in both triple-negative breast cancer and hepatocellular cancer cells can lead to reduction of xenograft tumor growth (Yang et al., 2011; Wei et al., 2011). Truncation mutations in LRP5 are prevalent in parathyroid cancer and associate with reduced proliferation and increased B-CATENIN activity in xenograft models and cell culture (Björklund et al., 2007). LRP5 is additionally a candidate gene of the frequently amplified site 11p13.2 in esophageal cancer (Shi et al., 2014). As previously mentioned, sFRPs can bind to FZ receptors to block the transmission of WNT signaling. In cancers such as colorectal, medulloblastoma and pancreatic adenocarcinoma sFRP1 is hypermethylated, and down-regulated (Suzuki et al., 2004; Vincent et al., 2011; Kongkham et al., 2010).

Finally, overexpression of WNTs themselves is commonly seen in many human cancers. Similar to murine mammary tumors, human breast cancer is also associated with increase expression of WNT1 (Wong et al., 2002). Interestingly, WNT2 was shown to be highly expressed in esophageal cancer, specifically within the tumor-associated fibroblasts. Secreted WNT2 was shown to promote proliferation and migratory ability of the epithelial cells in cell culture (Fu et al., 2011). WNT5A, on the other hand, can play a more complicated role in cancer. In some cases, WNT5A can act as a tumor suppressor, and its expression can inhibit proliferation, migration and invasiveness in thyroid tumor and colorectal cancer cell lines (Kremenevskaja et al., 2005). In gastric cancer though,
WNT5A was shown to promote cell migration by induction of the focal adhesion kinase (FAK); its upregulation is additionally associated with poorer prognosis (Kurayoshi et al., 2006).

The role of WNT10A in cancer has also been briefly touched up in the recent literature. While it has not be classified as a canonical or non-canonical WNT, based on homology to WNT10B, which has been confirmed to be a canonical WNT, and based on its expression pattern in hair follicle morphogenesis, in relation to induction of nuclear B-CATENIN, it is highly suggested to act as canonical WNT. The first suggestion of WNT10A playing a role in cancer, occurred in 2001, when it was published that WNT10A mRNA expression levels were upregulated in a variety of gastrointestinal cancer cell lines, including several esophageal cancer cell lines (Kirikoshi et al., 2001a). Upregulation of WNT10A is also seen in chronic lymphocytic leukemia, as compared to normal B-cells (Lu et al., 2004). Conflictingly, WNT10A has been shown to be downregulated in a mesenchymal population of oral squamous cell carcinoma cells, via DNA hypermethylation at a nearby CpG island (Kurasawa et al., 2012). In these previous reports, WNT10A has only been shown to be correlative with certain cancer types, without specificity to mechanism. In renal cell carcinoma, however, WNT10A was determined to play a definitive role in enhancing proliferation, migration, invasion and chemoresistance, through its induction of active B-CATENIN. WNT10A expression is also associated with poorer survival, especially in concert with high nuclear B-CATENIN and CYCLIND1 staining (Hsu, 2012). We will present data demonstrating the
relationship between WNT10A and ESCC, as well as its functions in tumorigenic properties (Figure 1).
Figures and figure legends
Figure 1. Molecular mechanisms of esophageal cancer. Model demonstrating that mutant TP53 (R175/2H) can drive tumorigenesis and invasion both in vitro and in vivo. Through a mutant TP53-driven mouse model of ESCC, we demonstrate that mutant TP53 can promote an epithelial to mesenchymal gene signature. Additionally, in both models, we demonstrate the upregulation of WNT10A, which can promote proliferation, migration, invasion and stemness properties in vitro.
CHAPTER 2: WNT10A promotes an invasive and self-renewing phenotype in esophageal squamous cell carcinoma

The data in chapter 2 have been published in *Carcinogenesis*.

Abstract

Esophageal cells overexpressing Epidermal Growth Factor Receptor (EGFR) and TP53 mutation can invade into the extracellular matrix when grown in 3D organotypic cultures (OTC) and mimic early invasion in esophageal squamous cell carcinoma (ESCC). We have performed laser capture microdissection with RNA microarray analysis on the invasive and non-invasive tumor cells of TP53<sup>R175H</sup> overexpressing OTC samples to determine candidate genes facilitating tumor invasion. *WNT10A* was found to be >4-fold upregulated in the invasive front. Since *WNT10A* is also prominently upregulated during placode promotion in hair follicle development, a process that requires epithelial cells to thicken and elongate, in order to allow downward growth, we hypothesized that WNT10A may be important in mediating a similar mechanism of tumor cell invasion in ESCC. We have found that WNT10A expression is significantly upregulated in human ESCC, when compared with normal adjacent tissue. Furthermore, high WNT10A expression levels correlate with poor survival. Interestingly, we observe that WNT10A is expressed early in embryogenesis, but is reduced dramatically postnatally. We demonstrate that overexpression of WNT10A promotes migration and invasion, and proliferation of transformed esophageal cells. Lastly, we show that WNT10A overexpression induces a greater CD44<sup>High</sup>/CD24<sup>Low</sup> population, which are putative markers of cancer stem cells, and increases self-renewal capability. Taken together, we propose that WNT10A acts as an oncofetal factor that is highly expressed and may promote proper development of the esophagus. During tumorigenesis, it is aberrantly overexpressed in order to promote ESCC migration and invasion, and may be linked to self-renewal of a subset of ESCC cells.
Introduction

Esophageal squamous cell carcinoma (ESCC) is the sixth leading cause of cancer-related death amongst American men, and sixth overall cause worldwide (Altekruse SF, et al., 2009). Common genetic alterations of ESCC include overexpression of Epidermal Growth Factor Receptor (EGFR) and CYCLIND1, as well as mutation in TP53 and either loss or mislocalization of p120 CATENIN (Enzinger and Mayer, 2003; Lu et al., 2003; Song et al., 2014; Stairs et al., 2011). A recent genome sequencing effort from a cohort of 158 Chinese ESCC samples revealed that WNT and NOTCH signaling pathways are also highly deregulated (Song et al., 2014).

We have shown previously that overexpression of mutant TP53 (R175H) along with EGFR in primary immortalized esophageal epithelial cells induces transformation (Okawa et al., 2007). Furthermore, when these engineered cells were grown in 3D organotypic culture (OTC), which mimics the stratified epithelium and its crosstalk with the underlying stroma, they invade into surrounding stroma, similar to early invasion observed in ESCC (Okawa et al., 2007). To understand what molecular mechanisms may be responsible for invasion, we dissected out the invasive and non-invasive regions from these 3D cultures and performed comparative microarray analysis (Michaylira et al., 2010). PERIOSTIN, was found to be the highest upregulated gene, and has been described previously as a potential biomarker for ESCC (Wong et al., 2013b; a). Strikingly, we observed additional dysregulation of a variety of Wnt pathway genes, such as upregulation of WNT ligands, WNT10A, WNT5B, and downregulation of the WNT pathway inhibitor DKK1 (Michaylira et al., 2010). WNT signaling is critical in the
embryonic development of different invertebrate and vertebrate organisms. In particular, WNT signaling is critical in the regulation of axis patterning, cell fate specification, cell proliferation and cell migration during development (Clevers, 2006; Macdonald et al., 2009). WNT ligands are secreted glycoproteins that are cysteine-rich and comprise a short N-terminal signal sequence with a mature segment that has variable length (Willert and Nusse, 2012). There are nearly 20 different WNT proteins, the expression of which is spatially and temporally regulated during development, and maintain homeostasis and drive cancers in a context dependent manner (Polakis, 2000; Staal et al., 2008). Mouse WNT10A is synthesized as a 417 amino acid precursor that contains a 382 amino acid mature region, the latter of which contains two potential glycosylation sites. Mouse, rat and human WNT10A are highly conserved and WNT10A’s amino acid sequence is 64% identical to WNT10B (Wang and Shackleford, 1996; Kirikoshi et al., 2001b). Developmentally, WNT10A is best studied in the context of ectodermal lineages. It is studied primarily in the deregulation of ectodermal tissues resulting in a variety of disorders classified as: odonto-onychal dermal dysplasia (Adaimy et al., 2007; Bohring et al., 2009). Manifestation of WNT10A mutations in humans can result in defects in dentinogenesis, tooth morphogenesis, odontoblast differentiation, hair follicle development, nail formation, papillae of the tongue and sweat gland, and regeneration of the epidermis (Bohring et al., 2009; Nawaz et al., 2009; Cluzeau et al., 2011). Contributing to this developmental phenotype, WNT10A messenger RNA has been shown to strongly localize to the dermal condensates during the earliest stages of embryonic hair follicle formation and postnatal anagen (Reddy et al., 2001). This process requires coordinated cross-talk between epithelial cells and underlying dermal cells in
order to facilitate elongated epithelial cell down growths (Reddy et al., 2001; Itin, 2014). Moreover, publically available in situ hybridization data indicates that in addition to localization in ectodermal tissues, \textit{WNT10A} messenger RNA also strongly localizes to the embryonic esophagus at embryonic day 14.5, suggesting a role in esophageal development (Visel et al., 2004). \textit{WNT10A} has been previously implicated in a variety of cancers and has been shown to promote proliferation, migration and chemoresistance in renal cell carcinoma cell lines by regulation of \textit{B-CATENIN} (Hsu et al., 2012; Li et al., 2001; Kirikoshi et al., 2001a; c). Other reports suggest that \textit{WNT10A} is also upregulated in esophageal cancer, gastric and colon cancer cells and tumors (Kirikoshi et al., 2001a). By contrast, the \textit{WNT10A} promoter has been suggested to be hypermethylated in head/neck squamous cell carcinoma and oligodendroglioma cell lines (Kurasawa et al., 2012; Ordway et al., 2006). Herein, we show that \textit{WNT10A} is upregulated both in early development, as well as in early and late stages of ESCC, due its ability to promote proliferation, migration, invasion and self-renewal. Taken together, we suggest that \textit{WNT10A} may act as an oncofetal factor in the context of both esophageal development and tumorigenesis.
Results

WNT10A is preferentially expressed in the invasive compartment of transformed epithelial cells in 3D OTC

We have identified WNT10A as a prominent upregulated gene in invasive versus non-invasive EPC2-hTERT-EGFR-TP53^{R175H} cells grown in 3D OTC, an in vivo like model of ESCC (Michaylira et al., 2010). WNT10A was a strong candidate for further study, as it was upregulated significantly in the invasive region of another TP53 mutant (R248W) (unpublished data). Furthermore, based on pathway analysis of all TP53 mutants analyzed (R175H, V143A, R248W and R273H), there was a significant enrichment for WNT/B-catenin signaling genes (Figure 1). Interestingly, WNT10A was included in other significantly upregulated pathways, such as regulation of epithelial to mesenchymal transition and human embryonic stem cell pluripotency (Supplementary Table 1).

We sought to confirm this expression of WNT10A through immunofluorescent staining of 3D OTCs. WNT10A stained predominately in the cytoplasm of epithelial cells. Strikingly, we saw stronger staining within the invasive regions (I), as compared with the basal non-invasive (NI) regions (Figure 2A). We quantified the mean intensity of the staining, and saw that invasive regions had an ~4-fold increase in fluorescent level as compared with the basal layer (Figure 2B). We next confirmed that WNT10A expression was increased through quantitative PCR. Using a non-invasive cell line, EPC2-hTERT-EGFR-empty vector cells, we confirmed that the more invasive EPC2-hTERT-EGFR-TP53^{R175H} cells expressed significantly higher levels of WNT10A messenger RNA.
These results were replicated with several primer sets for WNT10A as compared with GAPDH and HPRT (data not shown).

**WNT10A overexpression promotes migration and invasion in transformed esophageal keratinocytes**

Since WNT10A was upregulated in the invasive front of transformed esophageal cells grown in 3D OTC, we next wanted to determine the role of WNT10A in mediating migration and invasion. To test this hypothesis, we first generated EPC2- hTERT-EGFR-R175H cells that either overexpressed WNT10A or an empty vector control (herein known as WNT10A and empty vector, respectively). We confirmed WNT10A expression by western blot (Figure 3A). Several bands were visualized for WNT10A, indicative of posttranslational modifications, such as glycosylation and lipidation, mainly at the 40 and the 50 kD molecular masses (Willert and Nusse, 2012). Interestingly, overexpression of WNT10A resulted in a slight increase in β-catenin protein (Figure 3A), suggesting increased stabilization of total β-catenin. WNT10A overexpressing cells exhibited increased migratory and invasive capability, as compared with empty vector controls in Boyden chamber assays (Figure 3B and C). WNT10A cells exhibited also a significant increase in proliferative ability (Figure 3D).

**WNT10A promotes a self-renewal phenotype**

Based upon our microarray results, WNT10A was suggested to be important in a category of genes that promote stem cell pluripotency, along with genes such as WNT5B and SOX2. We therefore wanted to investigate the role of WNT10A in stemness marker expression, as well as its ability to promote self-renewal through single sphere assays.
CD44, a cell surface marker for hyaluronic acid, has been previously implicated as a putative stem cell marker in ESCC, and has been suggested to be regulated by the WNT/B-catenin signaling pathway (Wielenga et al., 1999; Zhao et al., 2011). Additionally, CD44^{High} and CD24^{Low} expressing cells have also been described to represent a tumor-initiating population with mesenchymal-like properties (Al-Hajj et al., 2003; Mani et al., 2008). By fluorescence-activated cell sorting-analysis, WNT10A overexpressing cells exhibited a marked increase in CD44^{High} and decrease in CD24^{Low} cells (Figure 4A), resulting in a nearly 2-fold increase in CD44^{High}/CD24^{Low} cells with WNT10A overexpression (Figure 4B). We next performed single cell sphere formation assays in ultra-low attachment plates in the presence of sphere-promoting media. WNT10A overexpressing and empty vector control cells were plated in a single cell suspension of 100 cells per well. After 7 days, there was a significant increase in the average number of spheres formed per well in the WNT10A overexpressing cells as compared with control cells (Figure 4C), although there was no difference in the size of the spheres that formed. Together, these data demonstrate that WNT10A increases migratory and invasive capabilities and promotes a shift toward CD44^{High} cells as well as self-renewal.

**WNT10A expression in esophageal development and ESCC**

WNT10A has been implicated previously to be upregulated during early follicle morphogenesis as well as in a variety of other ectodermal tissue types (Reddy et al., 2001; Narita et al., 2005). WNT10A is rarely expressed in the normal adult mouse esophagus. Interestingly though, RNA in situ hybridization data of whole mount embryos...
suggest that in addition to ectodermal lineages, WNT10A is also expressed in the developing esophagus (Visel et al., 2004). To confirm this finding, we conducted WNT10A IHC in the esophagus at embryonic days (E) 12.5, 18.5, postnatal day (P) 1 and adult tissue. We found that WNT10A is expressed as early as E12.5, but exhibits the strongest staining at E18.5 (Figure 5A). Interestingly, by P1, WNT10A staining is mostly abrogated, although there may be some residual low levels. In normal adult stages, WNT10A is expressed minimally. This expression pattern suggests that WNT10A may play a role in the development of the early mouse esophagus.

In order to examine WNT10A expression in pre-malignant and malignant stages of ESCC, we utilized a mouse model of ESCC. The 4-NQO is a quinolone derivative that causes DNA-adduct formation similar to carcinogens in tobacco, leading to oral squamous and esophageal dysplastic lesions, similar to that of human head neck squamous cell and esophageal carcinomas (Tang et al., 2004). In mice treated with 4-NQO ad libitum in drinking water, we saw increased hyperplastic and dysplastic lesions in the esophagi, with downward growths into the submucosa, consistent with invasive cancer, as compared with mice on water (control) (Figure 5B). Interestingly, 4-NQO-treated mice exhibited greater cytoplasmic WNT10A staining in the esophagi, especially in the invasive regions, as compared with control mice (Figure 5B). We additionally screened a panel of ESCC cell lines via western blot analysis for expression of WNT10A. As compared with immortalized primary epithelial cells (EPC2-hTERT), we saw an increase in WNT10A expression in three out of the eight ESCC cell lines screened (Figure 5C), namely TE6, TE10 and TE11.
In order to determine if WNT10A expression could also serve as a biomarker for ESCC prognosis, we utilized a tumor microarray (TMA) of 92 normal-matched ESCC tumors and 40 additional tumors, along with 5-year survival data on the patients. IHC indicated a faint nuclear pattern in samples from normal esophagus samples, while high-grade dysplasia and invasive tumors presented with overall stronger cytoplasmic staining (Figure 5D). An independent pathologist blinded to patient prognosis status scored the TMA for cytoplasmic signal (Cy0, Cy1, Cy2 and Cy3). Interestingly, most normal tissues expressed either very low (Cy1) or no (Cy0) staining, whereas tumor tissues were mostly scored as Cy2 or Cy3 (Figure 5E). When the tumor samples (n = 132) were stratified for 5-year survival, there was a decrease in median survival time (MST) between the high expressers (Cy2, Cy3) MST = 17.5 months versus the low expressers (Cy0, Cy1) MST = 33.5 (Figure 5F). Additionally, there was a significant (P = 0.0322) decrease in overall survival in the high expressers, based upon the Gehan–Breslow–Wilcoxon test, and was trending (P = 0.0916), based on Log-rank. Taken together, these findings suggest that WNT10A is over-expressed in ESCC tumors and cell lines, as well as in early esophageal development in the mouse, and that it may be an important biomarker for poor survival in ESCC.
Discussion

WNTs are critical signaling factors, required for the development of numerous tissue types, as well as maintenance of homeostasis in regenerative tissues, such as of the gastrointestinal tract and the skin. Hence, dysregulation of the WNT signaling cascade leads to a variety of cancers and diseases. Herein, we have demonstrated WNT10A to be expressed in mouse esophageal development, peaking at embryonic day 18.5 but diminished substantially postnatally. It is further re-expressed during esophageal carcinogenesis as observed in both a mouse model of esophageal epithelial dysplasia as well as in human ESCC. These findings suggest that WNT10A may serve as an oncofetal factor in the esophagus.

Interestingly, WNT10A embryonic expression has been described in another context of esophageal development. Notably, FOXA3Cre;Cdx2 conditional knockout mice, which exhibit esophagealization of the intestine, express increased levels of WNT10A in both the mutant intestines, as well as the embryonic esophagus, as these mice are embryonic lethal (Gao et al., 2009). In conjunction with our data, these findings would lend credence to the premise that differentiation of the esophagus during late development may involve WNT10A. SOX2 is a critical transcription factor, also highly expressed in embryonic development of the esophagus. It is vital in the division of the trachea and the esophagus, in order to promote a single esophageal tube, as well as fostering the stratified squamous epithelium within the esophagus (Que et al., 2007). In adult life, SOX2 is limited to the basal compartment of esophageal epithelial cells, where it promotes proliferation and differentiation (Que et al., 2007). In other squamous
epithelial types, both SOX2 and WNT10A have been shown to be important in normal development. Interestingly, Sox2 hypomorphic mice and humans carrying WNT10A mutation both present with malformation of the fungiform papillae, and ultimately smooth tongues, as WNT signaling has been shown to drive SOX2 expression (Adaimy et al., 2007; Okubo et al., 2006). These observations suggest a potential connection between WNT10A driving SOX2 expression in esophageal development. Indeed, SOX2 is amplified in ESCC (Bass et al., 2009), and it would be interesting to determine to what extent WNT10A and SOX2 are overexpressed concurrently in ESCC.

Employing a 3D OTC model of ESCC, we found that WNT10A is upregulated in the invasive front compared with the non-invasive epithelium. We further demonstrate that overexpression of WNT10A enhances esophageal cells ability to proliferate, migrate and invade. Loss of function or germline mutation of WNT10A specifically leads to malformation of ectodermal appendages during development. These developmental processes require careful communication between epithelial and mesenchymal lineages, for proper migration of epithelial cells to form placodes and eventually ectodermal tissues (Itin, 2014). Specifically, during the hair follicle development, and then later in anagen, a period of growth during the hair follicle cycle, WNT10A is upregulated near the dermal condensate and later dermal root sheet, and may be required for the downward growth of the hair follicle (Reddy et al., 2001). Therefore, we propose that WNT10A may be normally required for epithelial migration and proliferation during development, and that the oncogenic state is co-opting a developmental process to facilitate carcinogenesis. This pattern is frequently seen in the context of other oncofetal factors.
We also observe that WNT10A expression is also increased in an ESCC TMA when compared with adjacent normal esophagus, and correlates with decreased MST. Patients with ESCC that express higher WNT10A levels had an overall significant decrease in 5-year survival time, as well. These data suggest that WNT10A can annotate the invasive compartment of ESCC and could potentially be used as a biomarker for poorer prognosis in ESCC. Similarly, WNT10A has been shown to be upregulated in renal cell carcinoma biopsies and drives proliferation, migration and chemoresistance of renal carcinoma cell lines (Hsu et al., 2012). Taken together, these data suggest that WNT10A can act as an oncogene in a variety of tissue types.

We additionally demonstrated that WNT10A could induce stem cell-like properties by promoting self-renewal. These data are supported by our pathway analysis of invasive mutant TP53 cells, which suggests that in addition to epithelial to mesenchymal transition, WNT10A may also play a role in maintaining embryonic stem cells. Indeed, WNT10A is secreted by mammary stem cells and it may be required for maintaining this population (Ji et al., 2011). Wnt signaling is also required for the maintenance of a variety of stem cell niches, such as in intestinal crypts and hematopoietic systems (Korinek et al., 1998; Reya et al., 2003). Interestingly, we have demonstrated previously that PERIOSTIN, a type of matricellular protein involved in remodelling the tumor stroma during tumor cell invasion, is upregulated in ESCC, mediates ESCC invasion and is critical in tumorigenesis in vivo (Michaylira et al., 2010; Wong et al., 2013b; a; Wong and Rustgi, 2013). Indeed, metastatic tumor cells induce stromal PERIOSTIN expression in the secondary target organ (e.g. lung) to initiate
colonization (Malanchi et al., 2012; Holland et al., 2013). PERIOSTIN is needed for cancer stem cell maintenance and recruits WNT ligands for increased WNT signaling in cancer stem cells (Malanchi et al., 2012). Whether PERIOSTIN and WNT10A interact in this context is an area for future investigation.

Our study is the first report to demonstrate a role for WNT10A in both esophageal development and in tumorigenesis. Our data suggest that in a TP53 mutant background, WNT10A mediates tumor cell migration and invasion, while increasing a population of self-renewing CD44\textsuperscript{High} cells. Interestingly, up to 83% of ESCC cases exhibit mutant in TP53, while altered genes in the WNT pathway represent almost 85% cases of ESCC (Song et al., 2014). These mutations may work synergistically to promote more aggressive and invasive tumors in ESCC.
Materials and methods

Network analysis

Ingenuity Pathway Analysis was used to identify potential upstream regulators that control gene expression patterns enriched in invasive TP53 mutant cell lines. The analysis is based on prior knowledge of expected effects between transcriptional factors and their target genes stored in the Ingenuity Knowledge Base (Krämer et al., 2014). Briefly, the analysis examines the known targets of each transcription factor in the signature and compares their direction of change (expression in invasive relative to the non-invasive gene signature) to what is expected from the literature. If the direction of change is consistent with the literature across the majority of targets, then the regulator is predicted to be active in invasive cells, whereas if the direction of change is mostly inconsistent (anti-correlated) with the literature, then the regulator is predicted to be inactive in invasive cells. Regulation z-score was used to estimate the activation state of the regulators. The overlap P values generated by Fisher’s exact test were used to estimate the statistical significance of overlap between the dataset genes and the genes regulated by a regulator.

Cell culture

EPC2-hTERT cells and derivatives, established and extensively characterized by us, were grown in keratinocyte serum-free media (Invitrogen, Carlsbad, CA), supplemented with 40 µg/ml of bovine pituitary extract, 1ng/ml epidermal growth factor and 1% penicillin/streptomycin, as described previously (6,7,32). The TE series human ESCC cells were a gift from Dr. Nishihira who established the cell lines and were grown in Dulbecco's modified Eagle's medium supplemented by 10% fetal calf serum (Sigma–
Aldrich, St. Louis, MO) and 1% penicillin/streptomycin (Invitrogen) as described previously (Nishihira et al., 1993; Nakagawa et al., 1995). The earliest frozen stocks of all cell lines have been stored at the Cell Culture Core of the University of Pennsylvania. We have propagated cells from frozen stocks of the original vials that were authenticated by short tandem repeat analysis for highly polymorphic microsatellites FES/FPS, vWA31, D22S417, D10S526 and D5S592 as performed by the Cell Culture Core to validate the identity of cells by comparing cells at the earliest stocks we have and those grown >8–12 passages. All cell lines have been tested for mycoplasma contamination on a regular basis. WNT10A was a gift from Marian Waterman (Addgene plasmid # 35920), and was subcloned into pBabe-Blast empty vector (Najdi et al., 2012). Retroviral spinfection was performed as described previously (Michaylira et al., 2010). 3D OTCs Epithelial cells were grown as described (Kalabis et al., 2012), in order to recreate the microenvironment of ESCC, by supplying extracellular membrane components, including collagen and fetal esophageal fibroblasts.

**Mouse treatments**

4-nitroquinoline 1-oxide (4-NQO) experiments were performed as described previously and under Institutional Animal Care and Use Committee (IACUC) approval (Tang et al., 2004). In brief, 6-week-old C57Bl/6 and mixed background mice were given 10mg/ml of 4-NQO diluted in 10% propylene glycol in their drinking water ad libitum. Mice were randomized amongst littersmates into control versus treatment groups. Mice were euthanized either directly after treatment or after a 12-week wash out period. Three to five mice, per condition, were euthanized and tissues were processed for histology and IHC.
Tumor microarrays and IHC/immunofluorescence

ESCC tissue along with adjacent non-cancerous mucosa were obtained as surgical biopsies from Kagoshima University Hospital, as described previously (37). The clinical materials were obtained from informed consent patients according to the Institutional Review Board standards and guidelines. IHC and immunofluorescence were performed as described previously (37). About 5 µM paraffin embedded sections were incubated with WNT10A (Ab-106522, Abcam, Cambridge, MA 1:200) or WNT10A (SC-376028, Santa Cruz Biotechnologies, Santa Cruz, CA 1:100). Immunofluorescence signal was quantified in at least four independent high-powered fields per slide for mean intensity using ImageJ (Schneider et al., 2012).

RNA isolation and quantitative PCR

Total RNA was isolated using GeneJet RNA Purification Kit (Thermo Fisher Scientific, Waltham, MA) and cDNA was synthesized utilizing the Taqman Reverse Transcription Reagents kit (Applied Biosystems, Foster City, CA) according to the manufacturer’s instructions. Quantitative PCR was performed with primer sequences for WNT10A Forward: 5’-ATCCACGAATGCCAACACCA-3’, Reverse: 5’-CTCTCTCGGAAAACCTCTGCT-3’ and ACTB Forward: 5’-CCTGGCACCCAGGACAAT-3’, Reverse: 5’-GCCGAGCCACCGGATACT-3’ using Power SYBR Green PCR Master Mix (PE, Applied Biosystems), according to manufacturer’s instructions.

Western blotting

Proteins were separated through 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis Bis-Tris gel (Invitrogen) and then transferred to a poly membrane
Membranes were then blocked with 5% non-fat milk in TBS-T (1× TBS and 0.01% Tween-20), probed with primary antibody (1:500 WNT10A SC-376028, Santa Cruz, 1:1000 β-CATENIN D10A8, Cell Signaling, and 1:1000 GAPDH, Millipore) diluted in blocking buffer overnight at 4°C, washed with TBST-T and incubated with horseradish peroxidase-coupled secondary antibodies (GE Healthcare, Pittsburgh, PA). Signal was visualized with Amersham ECL-Prime (GE Healthcare) as per manufacturer’s instructions.

**Migration and invasion assays**

Boyden chambers (8-μm pore size, FluoroBlok-HTS inserts; BD Biosciences, San Jose, CA) were used for migration and invasion assays, as described previously (6). In brief, Boyden chambers were coated with 200 μg/ml of Matrigel Matrix (BD Biosciences) 2h before cell plating. Inserts were placed in a 24-well plate containing full keratinocyte serum-free medium to stimulate cell migration and invasion. Cells (5×10⁴) in keratinocyte basal media (Lonza, Basel, Switzerland) were placed in each insert. Twenty-four hours later, migrating or invading cells were labeled with 4 μg/ml Calcein AM dye (Invitrogen) in Hanks’ buffered salt solution buffer (Invitrogen) for 1h. Labeled cells were then read on a Synergy HT multidetection microplate reader (BioTek, Winooski, VT) at 485-nm excitation and 528-nm detection. Data represents the average of three independent experiments performed in triplicate for each genotype.

**Cell proliferation assay**

A total of 3×10³ cells were cultured in 96-well plates. Cell proliferation was quantified using the WST-1 colorimetric assay (Roche, Penzberg, Germany) according to manufacture’s instructions. Absorbance was measured on a microplate reader (Tecan
Sunrise, Männedorf, Switzerland) at a wavelength of 450nm. Data represents the average of five independent experiments performed in quintuplicate.

**Flow cytometry and fluorescence-activated cell sorting**

Flow cytometry was performed using LSRII (BD Biosciences) and FlowJo (Tree Star, Ashland, OR). Cells were suspended in phosphate-buffered saline (Invitrogen) containing 1% BSA (Sigma–Aldrich) and stained with PE/Cy7-anti-CD24 (1:40; BioLegend, San Diego, CA) and APC-anti-CD44 (1:20; BD Biosciences) on ice for 30min. Flow cytometry data represent at least three independent experiments.

**Self-renewal assays**

About 100 cells were plated in single suspension in an ultra-low attachment 96 well-plate in 100 µl of mammary epithelial cell growth medium (Invitrogen). After 7 days, spheres were visualized and counted. All spheres consisted of greater than three cells. Data represents three independent experiments performed in 96 replicates for each cell type.

**Statistics**

Each experiment is presented as mean ± standard error (at least n=3) and was analyzed by paired or non-paried two-tailed Student’s t-test. $P < 0.05$ was considered significant. Overall survival curves were plotted by the Kaplan-Meier method and subjected to the Log-rank and Gehan-Wilcoxon test. Chi-square test was used to determine differences in proportion of WNT10A scoring in normal versus tumor samples.
Figures and figure legends
Figure 1. WNT pathway is significantly upregulated in invasive mutant TP53 cells. Ingenuity Pathway analysis shows networks of genes, known to be regulated by WNT signaling in gene expression data, are significantly associated with TP53 mutations. Upregulated and downregulated genes are indicated by red and green, respectively. The lines and arrows represent functional and physical interactions and the directions of regulation, as indicated in the literature.
Figure 2

Figure 2. WNT10A is upregulated in invasive esophageal cells. (A) Representative Hematoxylin and Eosin (H&E), WNT10A and DAPI staining of EPC2-hTERT-EGFR-TP53R175H cells grown in 3-D organotypic culture (OTC). Non-invasive (NI) and invasive (I) regions are delineated by white dots (B) Quantification utilizing ImageJ for mean fluorescent intensity of WNT10A staining in the non-invasive versus invasive front (n=4 independent experiments). Fluorescent intensity of invasive front is relative to non-invasive regions. Bar graphs represent relative mean ± SEM. **p<.005 (paired student’s t-test) (C) qRT-PCR of WNT10A expression relative to ACTB comparing non-invasive EPC2-hTERT-EGFR cells versus invasive EPC2-hTERT-EGFR-TP53R175H cells (n=3 experiments). Bar graph represents fold change ± SEM. **p<.005 (unpaired student’s t-test).
Figure 3. WNT10A promotes migration and invasion. (A) Western blot (cropped image) of EPC2-hTERT-EGFR-p53<sup>R175H</sup> either overexpressing empty vector or WNT10A. Multiple bands indicate post-translational modifications of WNT10A. B-catenin densitometry indicates a modest 1.3-fold increase in total β-catenin relative to GAPDH, with WNT10A overexpression (n=3 experiments). (B) Transwell Boyden Chamber migration assay of Empty Vector compared to WNT10A cells along with representative images of migratory cells stained with Calcein AM. (C) Transwell Boyden Chamber invasion assay of Empty Vector compared to WNT10A cells with representative images of invasive cells stained with Calcein AM. Scale bars =100 µM. n=3 in triplicate. Bar graphs represent fold changes ± SEM. *p<.05, **p<.005 (paired student’s t-test). (D) Proliferation assay utilizing a WST-1 colorimetric assay. (n= 5 experiments in quintuplicate). *p<.05 (paired student’s t-test).
Fig. 4. WNT10A increases CD44\textsuperscript{High} population and promotes self-renewal. (A) Representative histograms of CD44 and CD24 levels between Empty Vector and WNT10A cells, as expressed in a logarithmic scale. (B) Quantification of the CD44\textsuperscript{High}/CD24\textsuperscript{Low} population between Empty Vector and WNT10A cells (n=3 experiments). Bar graphs represent fold change ± SEM within 3 different passages of cells within one representative experiment. **p<.005 (unpaired student’s t-test). (C) Quantification of the average number of spheres formed per well between Empty Vector and WNT10A cells (n=3 experiments in 96 replicates). Bar graph represents fold change ± SEM. *p<.05 (paired student’s t-test). Representative images of the spheres from each cell type indicate no difference in the size of the spheres formed. Each image was taken at a magnification of x100.
Figure 5. WNT10A is upregulated during esophageal development and in cancer.

(A) WNT10A IHC of embryonic (E12.5, E18.5), postnatal day 1 (P1), and adult esophagus (E). Epithelial (e) and mesenchyme (m) are as indicated. (B) Representative WNT10A staining in esophagus of control mice versus mice treated with 10mg/ml of 4-NQO for 16 weeks. Downward growths and hyperplasia/dysplasia are indicative of early ESCC. Bar graph represents average scoring of esophagi ± SEM. *P < 0.05 (unpaired Student’s t-test) of three control and five experimental mice. (C) Western blot (cropped image) for WNT10A on a panel of ESCC human cell lines, as compared to normal, immortalized epithelial cells (EPC2-hTERT).
**Figure 5.** WNT10A is upregulated during esophageal development and in cancer.

(D) Representative images of WNT10A scoring classifications of adjacent normal (upper left panel) versus carcinoma *in situ* (CIS), and tumor tissue. Scoring was based on cytoplasmic intensity (Cy0, Cy1, Cy2, and Cy3). All scale bars are 100µM. (E) Scoring stratified between low staining intensity (Cy0 and Cy1) and high staining intensity (Cy2 and Cy3) WNT10A staining between normal adjacent tissue and tumor sections, respectively (red square box). $p<.0001$ (Chi-square test).
Figure 5. WNT10A is upregulated during esophageal development and in cancer. (F) Kaplan-Meyer survival curve of tumor staining stratified between low WNT10A staining intensity versus high WNT10A staining intensity, with median survival time (MST).
Tables and table legends
## Supplementary Table 1:

<table>
<thead>
<tr>
<th>Ingenuity Canonical Pathways</th>
<th>P-values</th>
<th>Molecules</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Cycle Control of Chromosomal Replication</td>
<td>5.88844E-07</td>
<td>CDC7,MCM2,MCM6,MCM4,MCM5,CDC6</td>
</tr>
<tr>
<td>Mitotic Roles of Polo-Like Kinase</td>
<td>1.23027E-05</td>
<td>CDC7,PRC1,PLK4,CDC20,HSP90AB1,CCNB1,CDC27</td>
</tr>
<tr>
<td>Regulation of Cellular Mechanics by Calpain Protease</td>
<td>0.000549541</td>
<td>CAPN3,CNGB1,TLN1,CCNA2,ACTN1</td>
</tr>
<tr>
<td>VDR/RXR Activation</td>
<td>0.002238721</td>
<td>KLK6,IGFBP3,CALB1,CSNK2A1,IGFBP6</td>
</tr>
<tr>
<td>Cyclins and Cell Cycle Regulation</td>
<td>0.002238721</td>
<td>CCND2,CCNA2,HDAC4,HDAC9,CCNB1</td>
</tr>
<tr>
<td>Role of IL-17A in Arthritis</td>
<td>0.003715352</td>
<td>CCL7,CCL2,PIK3C2B,CXCL5</td>
</tr>
<tr>
<td>IGF-1 Signaling</td>
<td>0.005754399</td>
<td>SOCS3,PIK3C2B,IGFBP3,CSNK2A1,IGFBP6</td>
</tr>
<tr>
<td>p53 Signaling</td>
<td>0.00602596</td>
<td>CCND2,BIRC5,PIK3C2B,ADCK3,HDAC9</td>
</tr>
<tr>
<td>Regulation of the Epithelial-Mesenchymal Transition Pathway</td>
<td>0.00616595</td>
<td>WNT10A,PARD6B,WNT5B,PIK3C2B,EGR1,JAG, HGF</td>
</tr>
<tr>
<td>Eicosanoid Signaling</td>
<td>0.006456542</td>
<td>ALOX15B,RARRES3,PLA2G4F,PTGES</td>
</tr>
<tr>
<td>Paxillin Signaling</td>
<td>0.007943282</td>
<td>TLN1,PIK3C2B,ITGB8,PARVA,ACTN1</td>
</tr>
<tr>
<td>IL-17A Signaling in Fibroblasts</td>
<td>0.00817638</td>
<td>FEN1,LIG1</td>
</tr>
<tr>
<td>BER pathway</td>
<td>0.009772372</td>
<td>S100A7,CXCL5</td>
</tr>
<tr>
<td>Role of IL-17A in Psoriasis</td>
<td>0.01</td>
<td>CAPN3,TLN1,PIK3C2B,ITGB8,PARVA,ACTN1,ARPC1B</td>
</tr>
<tr>
<td>IL-17 Signaling</td>
<td>0.01023293</td>
<td>MMP3,CCL2,PIK3C2B,CXCL5</td>
</tr>
<tr>
<td>Role of IL-17F in Allergic Inflammatory Airway Diseases</td>
<td>0.014791084</td>
<td>CCL7,CCL2,CXCL5</td>
</tr>
<tr>
<td>Atherosclerosis Signaling</td>
<td>0.015135612</td>
<td>MMP3,CCL2,ALOX15B,RARRES3,PLA2G4F</td>
</tr>
<tr>
<td>GADD45 Signaling</td>
<td>0.020417379</td>
<td>CCND2,CCNB1</td>
</tr>
<tr>
<td>Human Embryonic Stem Cell Pluripotency</td>
<td>0.020892961</td>
<td>WNT10A,WNT5B,PIK3C2B,SOX2,BDNF</td>
</tr>
<tr>
<td>Insulin Receptor Signaling</td>
<td>0.020892961</td>
<td>PPP1R3D,PPP1R14A,SOCS3,PIK3C2B,GYS2</td>
</tr>
<tr>
<td>L-glutamine Biosynthesis II (tRNA-dependent)</td>
<td>0.023442288</td>
<td>GATB</td>
</tr>
<tr>
<td>Chronic Myeloid Leukemia Signaling</td>
<td>0.023988329</td>
<td>MECOM,PIK3C2B,HDAC4,HDAC9</td>
</tr>
<tr>
<td>CDK5 Signaling</td>
<td>0.029512092</td>
<td>PPP1R3D,PPP1R14A,EGR1,BDNF</td>
</tr>
<tr>
<td>Telomerase Signaling</td>
<td>0.029512092</td>
<td>PIK3C2B,HDAC4,HSP90AB1,HDAC9</td>
</tr>
<tr>
<td>Ascorbate Recycling (Cytosolic)</td>
<td>0.034673685</td>
<td>GLRX</td>
</tr>
<tr>
<td>Glutathione Redox Reactions II</td>
<td>0.034673685</td>
<td>GLRX</td>
</tr>
<tr>
<td>4-aminobutyrate Degradation I</td>
<td>0.034673685</td>
<td>ALDH5A1</td>
</tr>
<tr>
<td>Cell Cycle: G1/S Checkpoint Regulation</td>
<td>0.039810717</td>
<td>CCND2,HDAC4,HDAC9</td>
</tr>
<tr>
<td>Role of NANOG in Mammalian Embryonic Stem Cell Pluripotency</td>
<td>0.041686938</td>
<td>WNT10A,WNT5B,PIK3C2B,SOX2</td>
</tr>
<tr>
<td>Role of MAPK Signaling in the Pathogenesis of Influenza</td>
<td>0.045708819</td>
<td>CCL2,RARRES3,PLA2G4F</td>
</tr>
<tr>
<td>GDNF Family Ligand-Receptor Interactions</td>
<td>0.045708819</td>
<td>NRTN,PDLM7,PIK3C2B</td>
</tr>
<tr>
<td>Remodeling of Epithelial Adherens Junctions</td>
<td>0.045708819</td>
<td>ACTN1,HGF,ARP1C1</td>
</tr>
<tr>
<td>Macropinocytosis Signaling</td>
<td>0.045708819</td>
<td>PIK3C2B,ITGB8,HDAC4</td>
</tr>
<tr>
<td>Hereditary Breast Cancer Signaling</td>
<td>0.046773514</td>
<td>PIK3C2B,HDAC4,HDAC9,CCNB1</td>
</tr>
<tr>
<td>Growth Hormone Signaling</td>
<td>0.047863009</td>
<td>SOCS3,PIK3C2B,IGFBP3</td>
</tr>
<tr>
<td>Wnt/β-catenin Signaling</td>
<td>0.048977882</td>
<td>WNT10A,WNT5B,DKK1,SOX2,CSNK2A1</td>
</tr>
</tbody>
</table>

57
Supplementary Table 1. Significantly upregulated pathways associated with invasive mutant TP53 gene signature. Upregulated pathways associated with invasive TP53 mutant (R175H, R248W, R273H, V143A) gene signature P-values (Fisher’s exact test) estimate the statistical significance of the overlap between the dataset genes and genes regulated by the regulator. Bolded boxes indicate upregulated pathways associated with WNT10A
CHAPTER 3: The role of mutant TP53 in the development of a mouse model of esophageal squamous cell carcinoma
Abstract

Wild-type *TP53*, a potent tumor suppressor gene, is responsible for initiating appropriate responses to a variety of cellular stresses and is the most commonly mutated gene in human cancers. Strikingly, most *TP53* mutations are missense mutations within one of six hotspot codons in the DNA-binding domain (DBD). Recent evidence suggests that the DBD mutants can confer neomorphic activity or act as gain-of-function (GOF) mutants, in order to promote tumor progression. Over 80% of esophageal squamous cell carcinomas (ESCC) contain *TP53* mutation, with the majority within the DBD. Major risk factors of ESCC include smoking, alcohol use, and factors that induce tissue stress, such as irradiation and caustic injury of the esophagus. In order to determine how *TP53* mutation affects the progression of ESCC, we developed a mouse model of ESCC, which is driven by an esophageal specific Cre-recombinase, in order to induce *Tp53* mutation, and a chemical mutagen, 4-Nitroquinoline 1-oxide (4NQO). Mice are additionally lineage labeled with an *YFP*+ allele, to allow for detection of recombined cancer cells in metastatic sites, such as lymph nodes and lung. We found that mutation in *Tp53* (R172H) significantly accelerates initiation and progression of ESCC by 4 to 6 weeks, as compared to total *Tp53* loss. This finding suggests that mutant TP53 can promote tumorigenesis in a GOF mechanism. Furthermore, in order to further elucidate the role of *Tp53* mutation in ESCC, we performed RNA-seq on established cancer cell lines from mouse tumors, with or without TP53 mutation or loss. Our preliminary findings suggest mutant TP53 may promote an epithelial to mesenchymal transition (EMT) gene signature, leading to a potential GOF role of mutant TP53 in ESCC metastases.
Introduction

Esophageal squamous cell carcinoma (ESCC) is one of the deadliest tumor types due to its highly invasive and early infiltrative nature (Altekruse et al., 2009). It is characterized by high-grade dysplasia of the epithelial layers with invasion into the lamina propria and potential extension into the muscle layers and surrounding tissue, depending on tumor grade (Taylor et al., 2013). Major risk factors of certain etiologies of ESCC include smoking and alcohol use (Toh et al., 2010). Additionally, it is one of the cancers with the highest rate of TP53 mutation, suggesting that TP53 mutation may be a driver of carcinogenesis.

Our lab has previously described an in vitro role of mutant TP53 in ESCC. When primary epithelial immortalized cells overexpress mutant TP53 in conjunction with Epidermal Growth Factor Receptor (EGFR) overexpression, they develop enhanced proliferative, migratory, invasive and colony formation capabilities (Okawa et al., 2007). More specifically, these cells can induce invasion in an in vivo-like system of 3D organotypic culture (OTC), with cellular protrusions into the underlying fibroblast/matrigel/collagen support layer (Michaylira et al., 2010; Kalabis et al., 2012). Incidentally, different hotspot DNA binding domain (DBD) mutations of TP53 exhibited different tumorigenic properties in the cell culture model of esophageal cancer, with the conformation-altering mutant R175H being the most invasive (Michaylira et al., 2010). These hotspot mutants have previously been suggested to confer gain-of-function (GOF) or neomorphic properties to induce additional oncogenic capabilities in cancer cells.
In order to better elucidate the mechanisms by which mutant TP53 may be promoting ESCC, we chose to generate an in vivo mouse model of ESCC driven by the R172H mutation (mouse orthologue of human R175H). Indeed, previous mouse models of ESCC have been derived, through other mechanisms, which will be briefly discussed below.

(1) Namely, the fastest and most highly penetrant model of mouse ESCC utilizes 4-nitroquinoline 1-oxide (4-NQO) in the drinking water. 4-NQO is a carcinogen that can induce bulky adducts in DNA, as well as DNA breaks, similar to cigarette smoke. Mice are administered 4NQO in their drinking water for an initial 16-week period with an additional 12-week wash out period. After the end of the 28-week experimental period, mice on 4NQO develop 100% penetrance of hyperplasia, papilloma, and invasive carcinoma. A caveat of this model is its stochastic nature, which is purely carcinogen driven (Tang et al., 2004).

(2) Concurrent loss of E-CADHERIN and Transforming Growth Factor Beta-receptor II (TGFBR2) in vitro has also proven to induce invasion of esophageal cancer cells in vitro (Andl et al., 2006). When these genes were deleted in mice with the L2 promoter driven Cre recombinase (L2-Cre), which is specific for the oral squamous, ventral tongue, esophageal, and forestomach, there was development of oral squamous cell carcinoma over a period of 18 months. Notably though, these mice also demonstrated metastasis in lymph node and lung tissue (Nakagawa et al., 1997; Andl et al., 2014).
(3) SOX2 is a driver of proliferation and stratification of the esophageal lineage in development (Que et al., 2007). Furthermore, its re-expression has been shown to be prominent in various squamous cell cancer types, especially in ESCC. When SOX2 overexpression is induced by the *Keratin 5 (Krt5)* promoter, which is specific for the basal layer of the epidermis and aerodigestive tract, mice develop carcinomas in the forestomach over the period of 5 months. These carcinomas display increased nuclear STAT3 localization, which suggest an inflammatory component requirement in the development of carcinoma. Only when SOX2 is overexpressed concomitantly with nuclear STAT3, can esophageal carcinoma develop. Limitations of this model include the localization of the carcinoma, which is not esophageal specific and the dermatitis development in these mice, due to expression of *Krt5* in the basal layer of the epidermis, as well (Liu et al., 2013).

(4) Another inflammatory driven model of esophageal cancer utilizes deletion of *Ctnnb2* (p120 CATENIN). In human ESCC samples, p120 CATENIN has been demonstrated to be either lost or mislocalized away from the cell membrane, where it would normally tether E-CADHERIN. Interestingly, when p120 CATENIN was deleted with the L2-Cre recombinase, not only did E-CADHERIN appreciably decrease but there was additionally increased expression of nuclear NF-KAPPAB. Over the course of 8 to 12 months, these mice developed dysplasia and invasive carcinoma of the oral tissue, esophagus and forestomach. Treatment of these mice with dexamethasone, led to a decrease in the proliferation and tumor invasion, suggesting that inflammation can also drive esophageal carcinogenesis (Stairs et al., 2011).
Taken together, we decided to utilize genetic and environmental components, in order to generate a model of murine ESCC. We chose a common mutation of $TP53$ in humans, as the R175H mutant accounts for 6% of all $TP53$ mutations in ESCC and utilization of 4-NQO, as it can accelerate the progression of ESCC in mice. Additionally, we chose to utilize the L2-Cre recombinase, as it is most specific for esophageal tissue.
Results

Generation of a mouse model of ESCC.

We utilized mice that had a knock-in mutation at the R172H codon of Tp53 (Olive et al., 2004). By crossing these knock-in mutants to mice with a L2-Cre recombinase transgene and additional floxed alleles surrounding the Tp53 loci, we were able to generate compound Tp53 mutant mice, specifically in the oral squamous, esophageal, and forestomach tissues (Nakagawa et al., 1997; Marino et al., 2000). Ultimately, we generated five different combinations of genotypes at the Tp53 loci: +/+ , +/-, R172H/+, -/-, and R172H/-. Additionally, we crossed in at least one copy of the R26-stop-EYFP allele to each mouse. This allele allows expression of the EYFP gene at the Gt(Rosa)26Sor loci when a Cre-recombinase is expressed to delete the lox-p flanked STOP sequence, thus allowing lineage labeling of all recombined cells. At 8-12 weeks of age, mice were subjected to treatment of 10 mg/mL 4-NQO ad libitum in their drinking water for 16 weeks and then euthanized (Figure 1A).

Initial histology of the esophagus of these mice showed varying degrees of hyperplasia, dysplasia, downward growths, micro invasion, and frank invasive squamous cell carcinoma, depending on genotype. In TP53+/+ control mice, the predominant features of the esophagi were hyperproliferation of the basal layer and a mild inflammatory response (Figure 1B). When one allele of Tp53 was lost, TP53+/− mice exhibited increase in basal layer proliferation along with dysplastic features of suprabasal layer. Downward growths can also initially be seen (Figure 1C). In TP53R172H+/+ mice, increased dysplasia and inflammation is seen, with signs of microinvasion into the
underlying lamina propria (Figure 1D). TP53<sup>+/−</sup> mice, herein known as TP53 null mice, displayed variability in phenotype between microinvasion and invasive squamous cell carcinoma (Figure 1E). Lastly, TP53<sup>R<sub>172H</sub></sup> mice, herein known as compound mutants, displayed thickened esophagi with macroscopic tumors, causing food impaction (data not shown) and invasive squamous cell carcinoma to both the lamina propria and surrounding muscle layers (Figures F and G).

TP53 immunohistochemistry staining was confirmed in the null mice and the compound mutants (Figures 1H-J). No antibody control (Figure 1H) displayed no TP53 staining, and TP53 null mice exhibited either low levels or no nuclear staining. Positive nuclear TP53 staining in the TP53 null histological samples may signify immune infiltrates, which maintain two wildtype copies of the Tp53 gene (Figure 1I). Compound mutant mice exhibited high levels of TP53 nuclear staining both in the epithelial layer as well as underlying stroma and muscle layers, indicative of invasive epithelial cells. TP53 staining was performed on TP53<sup>+/+</sup>, TP53<sup>+/−</sup> and TP53<sup>R<sub>172H</sub>/+</sup> mice and showed intermediate levels of nuclear TP53 staining, indicative of either active wildtype or mutant TP53, as the two alleles are indistinguishable based on IHC staining (data not shown). YFP expression was also confirmed by a fluorescent microscope, as a marker of recombination (Figure 1K-L), indicating strongest fluorescence in tumor tissue.

**Mutant TP53 may decrease tumor latency**

Having established a mutant TP53 driven mouse model of ESCC, but with varying histological patterns, we next wanted to determine if there were differences
amongst the different genotypes of mice. We additionally wanted to determine if the TP53<sup>R172H</sup> mutation could confer GOF properties over the null allele. In order to examine tumor latency, we chose to treat all experimental mice with the same 16 weeks of 4-NQO in the drinking water ad libitum but additionally included a washout period. During the 16-week treatment period as well as the 12-weeks following, we carefully monitored mice for signs of distress and significant weight loss (Figure 2A).

When mice displayed signs of illness, they were euthanized and examined grossly and via histology for invasive squamous cell carcinoma, and we plotted tumor incidence at specific time points for each genotype (Figures 2B and 2C). When sick mice were euthanized and found not to contain an esophageal tumor, these mice were marked as only censored at that particular time point. We firstly compared the tumor incidence curves between the TP53<sup>R172H/+</sup> and TP53<sup>+/−</sup> mice, which would allow us to compare the effects of a TP53<sup>R172H</sup> allele versus the null allele when one wildtype copy of Tp53 is still present (Figure 2B). In effect, we saw no difference between these two curves in terms of tumor incidence. Additionally, the TP53<sup>R172H/+</sup> and TP53<sup>+/−</sup> mice had median tumor incidence of 24.5 weeks and 23 weeks respectively. We next compared directly the compound mutant mice the null mice. There was a significant difference in terms of their tumor incidence curves (Gehan-Breslow-Wilcoxon test p=.0470) (Figure 2C). Additionally, the median survival time between both of these groups were shorter compared to the TP53<sup>R172H/+</sup> and TP53<sup>+/−</sup> group; the compound mice and null mice had a median tumor incidence time of 18 weeks and 22 weeks, respectively. Wildtype mice
treated with 4-NQO were also monitored, but these mice did not begin developing tumors until 14 to 16 weeks after the washout period begin (Data not shown).

Interestingly, during the 12-week washout period, mice of all genotypes began developing metastases to adjacent lymph nodes as well as micrometastases to the lungs. Lymph nodes were dissected out and examined under the fluorescent microscope and determined to be YFP+, indicating epithelial origin (Figure 2D and E). Additionally, histologically, dysplastic epithelial cells can be found in the lymph node. Lung tissue also showed signs of micrometastases by histology (Figure 2F). These findings suggest that TP53 mutation or loss in concert with 4-NQO treatment can induce a metastatic model of ESCC in mice.

**Mutant TP53 may confer an epithelial-mesenchymal transition (EMT) signature**

To further dissect out the GOF role of mutant TP53 in ESCC, we generated cell lines from the tumors of both the null and compound mutant mice in order to perform *ex vivo* experiments. In these initial experiments, we were able to generate two different TP53 compound mutant cell lines and one null cell line. Cell lines were further confirmed to express YFP (Figure 3A), to exclude contaminating, non-esophageal epithelial cells. Furthermore, TP53 was shown to be stabilized and highly upregulated in both compound mutant cell lines but not expressed in the null cell lines (Figure 3B).

In order to determine the transcriptional profile of these cells, we performed RNA-seq in duplicate for each of these cell lines, along with a wildtype mouse esophageal cell line control. Reads were adjusted to sequencing read depth and then
quantile normalized. Fold change was determined as compared to the wildtype cells. Classic wildtype TP53 targets such as Mdm2 and Cdkn1A were downregulated in the compound mutant and null cells (data not shown). Since the TP53 null cells were also tumor derived, many expression changes could be attributed to the transformation process. Differences between the compound mutants versus the null cells may be attributed to direct and indirect functions of mutant TP53, although stochastic carcinogen induced events cannot be entirely excluded.

Interestingly, preliminary analysis of the RNA-seq data show an increase in expression of mesenchymal genes in the compound mutant cells, as compared to both the null cells and wildtype cells, along with a decrease in epithelial genes. Pathway analysis additionally indicated epithelial to mesenchymal transition (EMT) as the most statistically significant pathway dysregulated in both compound mutant cells, as compared to the null cells (data not shown). EMT is critical in development and wound healing but can also become co-opted during tumorigenesis to induce invasion and metastasis. During EMT, epithelial cells lose junctions and change cellular shape; the classic change of EMT includes loss of Cdh1 (E-cadherin) and gain of Cdh2 (N-cadherin). Expression changes in Twist, Zeb1/2, Snail, Sparc, Vimentin, which have all previously been suggested of supporting mesenchymal phenotypes (Thiery et al., 2009; Lamouille et al., 2014; Vannier et al., 2013) are shown to be upregulated in the compound mutant cells. Correspondingly, the compound mutant cells exhibit a decrease in expression of genes associated with the epithelial phenotype, such as Cdh2 and Epcam, as compared to the TP53 null cells and wildtype control. Furthermore, Ctnnb2, the gene,
which encodes for the protein p120 CATENIN is highly down regulated in mutant compound cells. Keratins, which are associated with esophageal keratinocytes, such as *Krt4* and *Krt8* are additionally downregulated in the compound mutants, suggesting a loss of epithelial identity.

Although many of these gene targets need to be confirmed, there is indeed a trend toward EMT induction in the compound mutant cells. Since EMT may be critical for tumor cell dissemination and extravasation, future experiments will focus on the ability of compound mutant cells to induce metastases in a retro-orbital injection model of lung metastasis. In order to perform these experiments utilizing isogenic controls, we knocked down mutant *Tp53* in both compound mutant cell lines with two different short hairpins against mouse *Tp53* (Figure 3C). These cells will be utilized for future experiments to examine the role of mutant TP53 in EMT, mesenchymal to epithelial transition (MET) and metastasis.
Discussion

We have demonstrated that mutations in TP53, in conjunction with a 16-week regiment of 4-NQO in the drinking water, can lead to high-grade dysplasia and induction of invasive squamous cell carcinoma. Based on our tumor incidence curves, we observe (1) TP53 null and compound TP53 mutants demonstrate a decreased median tumor latency time, as compared to their heterozygote controls, and (2) TP53 compound mice display the fastest tumor latency as compared to all other genotypes. This first finding supports the notion that a wildtype copy of Tp53 can delay the onset of tumorigenesis, similar to patients who exhibit germ-line mutations of Tp53 at one allele (Malkin et al., 1990). Although we have not yet sequenced the tumors from the heterozygote mice, we expect that loss of heterozygosity at the Tp53 loci would be necessary for full transformation of the tissue, as previously reported (Olive et al., 2004). Furthermore, our data suggest that there is no statistical difference in tumor incidence curves between mutant TP53 and loss of TP5 when a wildtype allele is still present.

The second finding suggests that mutant TP53 may have oncogenic functions in order to induce faster progression of ESCC as compared to loss of TP53 alone. Previous mouse models of mutant TP53 driven carcinomas have demonstrated varied results on the role of mutant TP53 versus loss of TP53 alone during tumor progression. Global compound TP53 mutant mice, with the R172H allele, compared to TP53 null mice showed no difference in tumor latency, but rather, showed either a difference in tumor spectrum or increase in metastases potential in the TP53 compound mice (Olive et al., 2004; Lang et al., 2004). In a mammary tumor model, driven by overexpression of WNT-
1, addition of a humanized R175H knock-in mutation of TP53 did not potentiate faster tumorigenesis as compared to addition of loss of TP53. Yet the mutant compound mice exhibited increased number of mammary tumors as compared to the null controls (Lu et al., 2013). Interestingly though, in an APC^{fl/+} model of intestinal carcinoma, addition of a TP53^{R172H/+} allele in mice led to significant increase in invasive tumors, as compared with the addition of a TP53^{+/-} allele, with no difference in overall survival (Muller et al., 2009).

Although our results are currently not powered to make definitive conclusions on the role of mutant TP53 in tumor induction and progression, we find further evidence to suggest that mutant TP53 may play an oncogenic roles in inducing EMT, based on our ex vivo RNA-seq results. Incidentally, wildtype TP53 can repress EMT through multiple mechanisms. Loss of TP53 has been shown to downregulate miR-200c, leading to an induction of an EMT program, with increases in levels of N-CADHERIN and ZEB1 (Chang et al., 2011). TP53 can also repress the expression of SLUG, through its downstream target, MDM2. Slug, in turn, can promote EMT through its regulation of E-CADHERIN and MMP-2 (Wang et al., 2009).

Yet, in our dataset, many EMT associated genes are further deregulated in the TP53 compound mutant cells as compared to the TP53 null cells, suggesting additional roles of mutant TP53. Previous notions of GOF mutant TP53 in EMT and invasion have been reported. In prostate cancer cell lines, the R175H mutant has been shown to upregulate TWIST by alleviating repressive marks at the promoter (Kogan-Sakin et al., 2011). Several works have additionally highlighted the role of mutant TP53 in the inactivation of various isoforms of TP53 family members, TP63 and TP73, in order to
induce invasion. Inhibition of TAp63 through binding at the DBD by mutant TP53, leads to inhibition of integrin and EGFR recycling, and thus a more invasive phenotype (Muller et al., 2009). Moreover, TGF-β was shown to stabilize the interactions of mutant TP53 and TP63, leading to downregulation of the TP63 targets, *SHARP-1* and *CCNG2*, and cellular migration (Adorno et al., 2009). Finally, in pancreatic cancer, mutant TP53 can inhibit the TP73/NFY repression of *PDGFRB*, in order to promote invasion and metastasis (Weissmueller et al., 2014).

Incidentally, our dataset did not indicate any changes in expression of *Tp63* or *Tp73*, at least not in the RNA-level. However, we do observe marked increases in multiple mesenchymal transcription factors such as *Zeb1/2*, *Twist1* and *Snail*. In addition to regulation of one another, these transcription factors can work both alone and in concert in order to induce repression *CDH1* and activation of *CDH2* (Montserrat et al., 2011). Our RNA-seq data, in agreement, show that in the compound mutant cells, there is indeed a cadherin switch, with downregulation of *CDH1* to upregulation of *CDH2*. VIMENTIN, a major cytoskeletal feature of mesenchymal cells, is additionally upregulated. Correspondingly, the compound mutant cells lose epithelial identity through the downregulation of basal and suprabasal keratins, K14 and K4, respectively.

In ESCC, EMT can be promoted through various potential mechanisms. One of the most potent methods of EMT induction is through TGF-β signaling (Mani et al., 2008; Ohashi et al., 2010b). Within a 14-day TGF-β treatment, both transformed epithelial cells and esophageal cancer cells can induce an E-CADHERIN to N-CADHERIN switch, along with induction of ZEB1/2, and acquisition of a spindle shaped
morphology (Ohashi et al., 2010b). NOTCH3 can inhibit the TGF-β mediated changes, whereas reactive oxidative species may promote the EMT (Ohashi et al., 2011; Kinugasa et al., 2015). Of note, the cells that undergo EMT, additionally shift cell surface marker expression to a CD44\textsuperscript{High} - CD24\textsuperscript{Low} population (Ohashi et al., 2010a; Mani et al., 2008). These markers have previously been implicated as putative cancer stem cell markers. Our RNA-seq data set do not indicate a strong inclination towards increased \textit{Cd44} expression in the compound mutant cells, although we do see a marked decreased in \textit{Cd24} expression. Yet, \textit{Cd44} induction may be regulated at the protein level, and thus it would be of interest to investigate the protein expression of CD44 in the compound mutant cells as compared to null cells through fluorescence-activated cell sorting-analysis. In addition to promoting metastases and potentially cancer stem cell-like phenotypes, EMT can also induce increased tumor budding in ESCC. Tumor budding, which is a poor prognostic marker in ESCC, describes isolated cells out side the margin of a tumor and is associated with high levels of VIMENTIN and low levels of E-CADHERIN (Niwa et al., 2014).

Future directions will focus on the effect of mutant TP53 on EMT, invasion, and metastasis. In addition to generating more tumor-derived cell lines of both compound mutant TP53 cells and TP53 null cells, we will perform quantitative PCR to validate the EMT gene signature derived from our RNA-seq. Since EMT has also been associated with a more stem-like phenotype, we will also determine if changes in stemness markers are affected in the compound mutant TP53 cells at the protein level, as we did not detect changes in the RNA-level (Mani et al., 2008). These acquired stemness properties by EMT cells are also critical for survival during the extravasation process, and eventual
metastasis. We will functionally test if compound mutant TP53 cells can produce metastasis in a retroorbital injection model of lung metastasis. Knockdown of mutant TP53 has been previously reported to decrease metastatic potential to both the lung and liver in a pancreatic cancer model (Weissmueller et al., 2014). Our experiments will validate the potentially crucial role of mutant TP53 in mediating EMT, metastasis and overall poorer survival in ESCC.

Finally, we plan to mechanistically decipher the role of mutant TP53 in ESCC, and determine if mutant TP53 affects EMT-associated genes in a direct or indirect manner. We will perform a ChIP-seq for mutant TP53 in the compound mutant cell lines. Since, wildtype TP53 is deleted through recombination, we would specifically be immunonopercipitating for mutant TP53. This data set in combination with our RNA-seq results could indicate future direct targets of mutant TP53, and its potential GOF properties.
Materials and methods

Generation of mouse model

The Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania approved all animal studies. L2-Cre transgenic mice were generated by cloning Cre recombinase under the control of the EBV ED-L2 promoter (Nakagawa et al., 1997; Opitz et al., 2002) and were genotyped as previously described (Stairs et al., 2011). TP53^{R17H}, TP53^fl and Rosa\textsuperscript{YFP} mice have previously been described (Olive et al., 2004; Marino et al., 2000; Srinivas et al., 2001). 4-nitroquinoline 1-oxide (4-NQO) experiments were performed as described previously (Tang et al., 2004; Long et al., 2015). In brief, 8 to 12-week-old mixed background mice, bearing the L2-Cre, Tp53 mutant and/or wildtype alleles, and YFP transgene were given 10mg/ml of 4-NQO diluted in 10% propylene glycol in their drinking water ad libitum. Mice were euthanized either directly after 16-weeks of treatment or during the 12-week wash out period. Tissues were processed for histology and immunohistochemistry (IHC) or cell lines were generated from the tumor tissue.

Histology and immunohistochemistry (IHC)

Tumor tissue were embedded in paraffin blocks and cut in 5 μM sections. Hematoxylin and eosin (H&E) and IHC staining were performed as previously described (Long et al., 2015). All histology was read blinded by an independent pathologist. Paraffin embedded sections were incubated with mouse TP53 antibody (CM5, Vector Labs, Burlingame, CA 1:250).
Cell isolation and culture

Tumor derived cancer cells were generated as previously described (Stairs et al., 2011). In brief, freshly isolated esophageal tumors were digested in dispase (Thermo Fisher Scientific, Waltham, MA) for 10 minutes at 37° on a shaker. Esophagi tumors epithelial cells were then peeled from the submucosa and subjected to incubation in trypsin-EDTA (Thermo Fisher Scientific) for 10 minutes at 37° on a shaker. Detached cells were filtered through a 40 µM strainer into soybean trypsin inhibitor (Thermo Fisher Scientific). Cells were then centrifuged and resuspended and plated in calcium-free keratinocyte serum-free media (Invitrogen, Carlsbad, CA), supplemented with 40 µg/ml of bovine pituitary extract, 1ng/ml epidermal growth factor, 18 µM of CaCl₂, and 1% penicillin/streptomycin. Short hairpins against murine TP53, cloned into the pLKO.1 vector were a gift from Dr. Berger. Lentiviral spinfection was performed as described previously on cultured tumor cell lines (Michaylira et al., 2010). Lentiviral infected cells were selected in 2 µg/mL of puromycin. Knockdown was confirmed via western blot.

RNA isolation and sequencing

Cells were grown to 80% confluency in 10 cm dishes. Cells were harvested and polyA+ mRNA was isolated by double selection with mRNA Direct Dynabeads (Invitrogen). PolyA+ mRNA were fragmented to an average size of 200bp using Mg₂⁺-containing buffers at 94°C for 15 minutes. First-strand synthesis was performed using Protoscript II reverse transcriptase and random hexamer primers (New England Biolabs, Ipswich, MA). Directional second-strand synthesis was performed using the dUTP method (New England Biolabs). End-repair, adapter ligation, and 12 cycles of PCR were
performed as per the NEBnext Ultra protocol to produce barcoded, Illumina-compatible sequencing libraries. Insert size distribution was measured using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA) and sequencing library molarity was quantified by the Kapa method using absolute quantification standards. Reads were mapped as previously described (Sammons et al., 2015). Relative reads were normalized to the wildtype cell line.

**Western blot**

To confirm protein expression, western blotting was performed as previously described (Long et al., 2015). Proteins were separated through a 4–12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis Bis-Tris gel (Invitrogen) and transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Membranes were blocked with 5% non-fat milk in TBS-T (1× TBS and 0.01% Tween-20), probed with primary antibody (1:1000 TP53 CM5, Vector Labs, 1:1000 GAPDH, Millipore), diluted in blocking buffer overnight at 4°C, washed with TBST-T and incubated with horseradish peroxidase-coupled secondary antibodies (GE Healthcare, Pittsburgh, PA). Signal was visualized with Amersham ECL-Prime (GE Healthcare) as per manufacturer’s instructions.
Figures and figure legends
Figure 1. Mutations in TP53 with 4-NQO can induce dysplasia and invasive squamous carcinoma of the esophagus (A) Schematic of experimental treatment plan indicating genotypes of mice and time period of 4-NQO treatment. (B-G) Representative Hematoxylin and Eosin (H&E) images of the esophagus after 16-weeks of 4-NQO treatment in (B) TP53+/+ (C) TP53+/− (D) TP53R172H+/+ (E) TP53−/− (F-G) TP53R172H−/− mice. Scale bars represent 100 µM.
Figure 1

**Figure 1.** Mutations in TP53 with 4-NQO can induce dysplasia and invasive squamous carcinoma of the esophagus. (H-J) Representative TP53 staining of mouse tumors. (H) No antibody control (I) staining in a TP53<sup>−/−</sup> tumor and (J) staining in a TP53<sup>R172H/−</sup> tumor. Bars represent 100 µM (K-L) Representative images of esophageal tumors in (K) brightfield and with (L) YFP filter. Bars represent 5 mM.
Figure 2. Mutations in TP53 with 4-NQO can lead to invasive squamous carcinoma of the esophagus and metastasis to the lymph node and lung (A) Schematic of experimental treatment plan indicating genotypes of mice and time period of 4-NQO treatment along with washout period with normal drinking water. (B-C) Tumor incidence curves of (B) TP53\(^{-/-}\) and TP53\(^{R172H/+}\) mice and (C) TP53\(^{-/-}\) and TP53\(^{R172H/-}\) mice. No statistical difference is seen in tumor incidence between TP53\(^{-/-}\) and TP53\(^{R172H/-}\) mice. Difference in tumor incidence curve between TP53\(^{-/-}\) and TP53\(^{R172H/-}\) mice is statistically significant based on Gehan–Breslow–Wilcoxon test (P = 0.04).
Figure 2. Mutations in TP53 with 4-NQO can lead to invasive squamous carcinoma of the esophagus and metastasis to the lymph node and lung. (D-F) Representative images of lymph nodes from tumor-burdened mice in (D) bright field (E) YFP-filter and (F) H&E stained. (G) Representative H&E of micrometastasis of squamous cell carcinoma in lung parenchyma. Bars in (D-E) represent 5 mM and (F-G) represent 10 µM.
Figure 3

(A) Representative images of cells isolated from an TP53<sup>R172H/-</sup> tumor, which displays a mix of epithelial and spindle-shaped morphology. Brightfield and YFP filtered images are shown at x200. (B) Western blot for TP53 (1:1000, CM5) and GAPDH (1:1000) in two different TP53<sup>R172H/-</sup> cell lines and one TP53<sup>/-</sup> cell line. (C) Western blot for TP53 and GAPDH after lentiviral transduction of pLKO.1 shSCR or shp53 constructs were stably expressed in both TP53<sup>R172H/-</sup> cell lines.
Tables and table legends
<table>
<thead>
<tr>
<th>Gene</th>
<th>R172H/- (1)</th>
<th>R172H/- (2)</th>
<th>-/-</th>
<th>+/+</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cdh2</td>
<td>59.81751582</td>
<td>49.93173493</td>
<td>3.444777445</td>
<td>1</td>
</tr>
<tr>
<td>Itga5</td>
<td>1.595422603</td>
<td>0.807512798</td>
<td>0.695034127</td>
<td>1</td>
</tr>
<tr>
<td>Serpine1</td>
<td>1.444222711</td>
<td>4.071795564</td>
<td>4.117733044</td>
<td>1</td>
</tr>
<tr>
<td>Slug</td>
<td>1.049709519</td>
<td>0.845504489</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Snail</td>
<td>105.1897074</td>
<td>2.328456105</td>
<td>1.625126135</td>
<td>1</td>
</tr>
<tr>
<td>Sparc</td>
<td>19.49080083</td>
<td>28.36893606</td>
<td>7.4036831</td>
<td>1</td>
</tr>
<tr>
<td>Twist1</td>
<td>1.721886975</td>
<td>2.645723427</td>
<td>0.891134343</td>
<td>1</td>
</tr>
<tr>
<td>Twist2</td>
<td>2.753315833</td>
<td>12.92097264</td>
<td>8.604172423</td>
<td>1</td>
</tr>
<tr>
<td>Vimentin</td>
<td>1429.164941</td>
<td>3116.429364</td>
<td>281.908284</td>
<td>1</td>
</tr>
<tr>
<td>Zeb1</td>
<td>51.68922707</td>
<td>84.34181017</td>
<td>6.646976372</td>
<td>1</td>
</tr>
<tr>
<td>Zeb2</td>
<td>9.869476577</td>
<td>6.45025041</td>
<td>1.243385188</td>
<td>1</td>
</tr>
</tbody>
</table>

Table 1: Genes associated with a mesenchymal gene signature. Genes that are highly upregulated in the TP53<sup>R172H/-</sup> samples as compared to both the TP53<sup>-/-</sup> and TP53<sup>+/+</sup> cells are highlighted in green. All values are expressed as fold change over reads in TP53<sup>+/+</sup> cells.
Table 2: Genes associated with an esophageal epithelial gene signature. Genes that are highly downregulated in the TP53^{R172H/-} samples as compared to both the TP53^-/- and TP53^+/+ cells are highlighted in pink. All values are expressed as fold change over reads in TP53^+/+ cells.
CHAPTER 4: Thesis summary and discussion
The focus of this thesis was to describe the multiple mechanisms, in which esophageal squamous cell carcinoma (ESCC) pathogenesis can occur and the consequences of such genetic perturbations. Indeed the initial emphasis of this project was on the role of mutant $TP53$, as it is the most commonly mutated gene in cancer and in ESCC (Song et al., 2014; Gao et al., 2014). Previous $in$ $vitro$ work utilizing novel transformed esophageal cell systems and $in$ $vivo$ like 3D organotypic (OTC) cultures laid the groundwork for this project (Okawa et al., 2007; Michaylira et al., 2010). Mutant TP53, when over expressed, in cooperation with overexpression of other oncogenes such as EGFR and CYCLIN D1, can lead to enhancement of tumorigenic properties, such as increased proliferation, migration, invasion, anchorage-independent growth and even tumor formation in nude mice (Okawa et al., 2007; Michaylira et al., 2010; Ohashi et al., 2010b).

$In$ $vitro$ studies of mutant TP53-mediated invasion led to the investigation of the role of WNT signaling in ESCC and how specifically WNT10A plays a role during tumorigenesis. WNT10A was discovered to be upregulated in an invasive gene signature microarray in EPC2-hTERT-EGFR-TP53$^{R175H}$ cells. Although we did not find a direct relationship between mutant TP53 and WNT10A, we did observe that WNT10A could act as an oncogene in ESCC, in its own respect. Of note, our RNA-seq dataset, derived from mouse tumor-derived cancer cells, show that, although Wnt10a was not upregulated significantly between TP53$^{R172H/-}$ and TP53$^{-/-}$ cells, there was at least a 2-fold increase in Wnt10a expression levels in all three cancer cell lines as compared to wildtype esophageal cells (data not shown). Wnt6 and Wnt10b were incidentally also upregulated.
in the cancer cell lines, based on RNA-seq data. WNT10A and WNT10B may have redundant functions, as they are closely related paralogs, induced by a duplication event at the last common ancestor of jawed-vertebrates. WNT6, is also frequently co-transcribed with WNT10A, as they reside at the same genetic loci with no intervening genes (Nusse, 2001). Although we did not actively pursue the impact of these other closely related WNTs in our studies, we did note that in EPC2-hTERT-EGFR-TP53\textsuperscript{R175H} transformed cells, WNT10B was also prominently upregulated via quantitative PCR, as compared to control cells (data not shown).

Our emphasis on WNT10A demonstrated that its upregulation could be used as an indicator for poor prognosis in ESCC. High levels of WNT10A staining in a tumor microarray (TMA) indicated a significant reduction in overall survival, as well as a halved median survival time, when compared to low levels of WNT10A staining. In fact, WNT10A staining was rarely present in normal tissue. We showed through immunohistochemistry (IHC) staining for WNT10A that this protein was additionally upregulated in 4-Nitroquinoline 1-oxide (4-NQO) induced hyperplasia and dysplasia of the esophagus. Staining for WNT10A in the 4-NQO induced compound mutant TP53 mice also showed high WNT10A expression levels in the invasive squamous carcinoma regions (data not shown). WNT10A expression was additionally highly upregulated in a panel of ESCC cancer cell lines, when compared to normal wildtype cells. It would be interesting to determine if WNT10A knockout mice would have the same propensity for tumor induction and latency as wildtype mice, when treated with 4-NQO. This
experiment could impart an in vivo role for WNT10A in ESCC tumorigenesis, and complement the human data and in vitro functional studies.

Finally, we show in chapter 2 the definitive role of WNT10A as functionally as an oncogene in ESCC, by promoting proliferation, migration, invasion, and stemness properties. These findings recapitulate previous reports of WNT10A as a driver in tumorigenesis in renal cell carcinoma (Hsu et al., 2012). In these studies, WNT10A can induce canonical WNT signaling, in order to induce stabilization of nuclear B-CATENIN, in order to drive downstream targets such as CCND1. Our results indicate that over-expression of WNT10A can increase B-CATENIN levels, but we have yet to confirm the activation of this signaling pathway, as reporter assays are hindered by the difficulty of transient transfection in our primary keratinocyte cell line. Future studies would focus on the downstream effects of WNT10A, such as its ability to induce the canonical WNT signaling pathway, which receptors are utilized by WNT10A in ESCC, and how WNT10A is being regulated in ESCC.

Previous 3D OTC invasion assays performed in the transformed esophageal cells, demonstrated the variability of the different mutant TP53s in their ability to induce invasion and hinted at the potential gain of function (GOF) roles of mutant TP53 in ESCC. Nevertheless, all previous experiments utilized overexpression of mutant TP53 in the presence of a wildtype TP53 allele. This experimental setup did not exclude potential dominant negative functions of mutant TP53. In order to fully study the potential GOF roles of mutant TP53, we realized the necessity to perform all future experiments in a TP53 null background. We therefore chose to utilize Cre-Lox recombination mouse
model systems to knockout wildtype \textit{Tp53}, while introducing the R172H missense mutation. R172H, which is the mouse homologue of the human R175H hotspot mutation, was chosen because it had previously been described to promote carcinoma formation and increase metastasis (Olive et al., 2004; Lang et al., 2004). Additionally, the R175H mutation induces the highest degree of invasion in the 3D OTC model (Okawa et al., 2007). R175H is also one of the two most commonly mutated sites of \textit{TP53} in ESCC, and it had the unique property of being a conformational altering mutant (Petitjean et al., 2007). By altering the conformation of the folded TP53 protein, the R175H mutation can expose new epitopes for potential novel protein-protein or protein-DNA interactions (Bullock and Fersht, 2001).

Ultimately, our goal was to generate a mutant TP53-driven mouse model of ESCC, in order to study its role in tumorigenesis. Unfortunately, the murine esophagus has many protective barriers, which inhibit transformation. Previous genetic based mouse models of ESCC frequently require a 9-18 month tumor latency period, indicating the resistance of this tissue to transformation (Stairs et al., 2011; Andl et al., 2014). Additionally, the epithelium of the mouse esophagus is keratinized, unlike that in human esophagus, which provides additional protection from extracellular insults. We found that with L2-Cre driven \textit{Tp53} mutation alone, mice displayed relative normal morphology in the esophagus. Therefore, we found a necessity to challenge the TP53 mutant mice with additional carcinogenic insults. 4-NQO was utilized, as it had previously been shown to induce invasive squamous cell carcinoma of the tongue and esophagus (Tang et al., 2004). Our utilization of a genetic and environmental-based model of mouse ESCC may
in fact be more physiological to human disease, as multi-factorial components contribute to the pathogenesis of human ESCC.

Our overall findings indicate that compound mutations in TP53 indeed accelerate the pathogenesis of ESCC in our mouse model, as compared to wildtype mice treated with 4-NQO alone. The dynamics of tumor latency amongst the different alleles is still an area of ongoing investigation, as we do not see differences in tumor incidence curves in TP53\(^{R172H/+}\) mice versus TP53\(^{-/-}\) and the differences that we do see in tumor incidences between TP53\(^{R172H/-}\) and TP53\(^{-/-}\), although significant, requires increase number of mice to power the study. Of note, previous findings in different mouse models of cancer that are driven by mutant TP53, showed no difference in tumor latency or survival when comparing TP53\(^{R172H/-}\) mice versus TP53\(^{-/-}\) mice (Muller et al., 2009; Lu et al., 2013; Weissmueller et al., 2014; Ahronian et al., 2015).

Nevertheless, our mouse model generated additional tools for future studies on mutant TP53 GOF as well as pathogenesis of ESCC. As we aged the mice longer beyond the 16-week 4-NQO-treatment period, we found that our mice were able to develop lineage-labeled metastasis to the periaortie and cervical lymph node and lungs. Another area of active investigation is the role of mutant TP53 on induction of metastasis. Although we are able to generate metastasis amongst our TP53 mutant mice, our system is currently flawed to study this phenomenon. Firstly, since we are utilizing a carcinogen, mutations may be stochastic and the specific role of mutant TP53 in inducing invasion and metastasis may be unclear. Secondly, the dynamics of primary esophageal tumor growth hinder our ability to study metastasis as an isolated model, since depending on
primary tumor growth patterns (intramural versus extramural growth), mice may be required to be euthanized before metastasis can occur. Therefore, an accurate interpretation of the extent of metastasis would require extremely large number of mice, when comparing multiple genotypes. In order to simplify the system, we plan to utilize a retro-orbital injection model to study metastatic potential of various tumor-derived mutant TP53 cell lines. The dissemination pattern of retro-orbital injection leads to lung metastasis, which is one of the primary metastatic niches of ESCC. Compound mutant TP53 cells can further be genetically manipulated, such as through knockdown of the mutant allele to generate isogenic TP53 null control cells. Additionally, specific targets of mutant TP53 may also be perturbed genetically, in order to examine downstream mechanisms of mutant TP53 functions.

From the lineage-labeled tumor derived cell lines that we have generated from our mouse model, we have obtained RNA-seq data comparing wildtype, non-tumorigenic cells, to TP53−/− and TP53R172H−/− cancer cells. Since the TP53−/− and TP53R172H−/− have been exposed to additional mutagenic changes through 4-NQO treatment, and have undergone a selection process through the culturing system, we decided to prioritize our focus on genes that were specifically affected in the TP53R172H−/−, but remain unaffected in the TP53−/− cells. This experiment is a first pass examination of the transcriptome changes that TP53R172H mutation may induce in ESCC. From a cursory examination of the data, we have determined that a set of EMT-associated genes is highly dysregulated in the TP53R172H−/− cells. This finding supports our hypothesis that mutant TP53 may play a GOF role in mediating invasion and metastasis in ESCC. In order to fully determine direct
mechanisms of mutant TP53 GOF functions, we plan to perform a mutant TP53 chromatin immunoprecipitation sequencing (ChIP-seq) for novel mutant TP53 targets. Previous labs have performed either ChIP-seq or RNA-seq in different mutant TP53 expressing cell lines in order to discover components of the mutant TP53 transcriptome (Do et al., 2012; Freed-Pastor et al., 2012; Garritano et al., 2013; Xiong et al., 2014). These datasets have utilized different mutations of TP53 and in different cancer cell contexts. From these studies, it was shown that mutant TP53 may have a propensity to localize at ETS-binding sites. Yet these studies also demonstrate the unique role of mutant TP53 GOF properties, which may be both cell-type specific as well as mutant specific. Analysis of our RNA-seq data set may indicate specific transcription factor signatures that are being co-opted by mutant TP53 in ESCC, through protein-protein interactions. By interrupting these interactions either through knockdown of the partner transcription factor or re-activation of wildtype TP53 through a small molecule compound, we may curtail the GOF effects of mutant TP53.

Our in vivo model of murine ESCC is reproducible, fast, and highly specific to esophageal lesions. The lineage label in our ESCC model also allows for ex vivo cell sorting as well as tracing of primary and metastatic lesions. Potentially, this mouse model can be utilized in the future, not only to study different components of mutant TP53 induced ESCC, but also be used as mouse model for therapeutic studies.


101


Marino, S., M. Vooijs, H. Van Der Gulden, J. Jonkers, and A. Berns. 2000. Induction of medulloblastomas in p53-null mutant mice by somatic inactivation of Rb in the


Willert, K., J.D. Brown, E. Danenberg, and A.W. Duncan. 2003. Wnt proteins are lipid-modified and can act as stem cell growth factors. 423.


