Cooperating Oncogenic Alterations in Aggressive Pten-Deficient Breast Cancer

by

Sharon Wang

A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
Department of Laboratory Medicine and Pathobiology
University of Toronto

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2017

Abstract

PTEN is one of the most frequently inactivated tumor suppressors in human malignancies including breast cancer, and its mutation or loss is often implicated in advanced cancers and therapy resistance. Yet, germline mutations in PTEN induce non-malignant hamartomas in humans and mouse tumors with a wide spectrum of aggressiveness, indicating context-dependent effects. To develop effective anti-cancer therapies, a better understanding of how mutational events cooperate with PTEN-loss is required.

In this thesis, I show that most tumors derived from Pten-deleted mammary epithelium were highly differentiated and lacked transplantable tumor initiating cells following FACS isolation or transient propagation under non-adherent conditions. Surprisingly, a rare group of Pten-deficient tumors with distinct histology did contain transplantable TICs. In comparing these two tumor groups, I identified low miR-143/145 expression as a critical factor that determined transplantability. Knockdown of miR-143/145 in Pten-deficient tumor cells promoted secondary tumor engraftment, RAS pathway activation and sensitivity to MEK inhibitors. In basal-like breast cancer, 7% showed combined loss of PTEN and miR-145 as well as poor clinical outcomes. Thus, Pten loss can initiate benign or aggressive mammary tumors depending on miR-143/145 status. Patients with basal-like tumors and low-Pten/miR-143/145 expression should be identified and prioritized for aggressive therapy.
*Pten*-deletion also cooperated with *p53<sup>R270H</sup> mutation, resulting in diverse tumors including basal-like and claudin-low. Compared to mammary tumors arising from Pten<sup>Δf</sup>:p53<sup>Δf</sup> mice, Pten<sup>Δf</sup>:p53<sup>R270H</sup> mammary tumors were more capable of undergoing mesenchymal-epithelial transition (MET) and possessed greater metastatic potential. Together, these findings identified important oncogenic events that cooperate with PTEN-loss to induce aggressive breast cancer, enhance metastatic progression and influence response to therapy, and have significant implications for personalized breast cancer treatment.
Acknowledgments

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# Table of Contents

Acknowledgments ........................................................................................................... iv

Table of Contents ............................................................................................................. v

List of Tables ...................................................................................................................... ix

List of Figures .................................................................................................................... x

List of Acronyms ................................................................................................................ xii

Chapter 1 Introduction ....................................................................................................... 1
  1.1 Breast Cancer (BC) ..................................................................................................... 1
    1.1.1 Disease burden .................................................................................................. 1
    1.1.2 BC heterogeneity .............................................................................................. 1
  1.2 Tumor Suppressors in BC .......................................................................................... 5
    1.2.1 PTEN ................................................................................................................. 5
    1.2.2 MicroRNAs (miRNAs) .................................................................................... 17
    1.2.3 Tumor protein TP53 ........................................................................................ 20
    1.2.4 Other tumor suppressors in BC ...................................................................... 22
  1.3 Thesis rationale and outline ...................................................................................... 23

Chapter 2 ............................................................................................................................ 25

2 Materials and Methods ................................................................................................. 25
  2.1 Animals ...................................................................................................................... 25
  2.2 PCR genotyping ....................................................................................................... 25
  2.3 Primary cell isolation and enrichment of lineage-negative (Lin-) cell population ....................................................................................................................... 25
  2.4 Tumorsphere culturing in vitro, primary cell line creation and long-term cell labelling ....................................................................................................................... 26
  2.5 Lentivirus generation and generation of stably expressed and knockdown cell lines ......................................................................................................................... 26
  2.6 Fluorescence-activated cell sorting .......................................................................... 27
2.7 Orthotopic transplantation and tail vein injection ........................................... 27
2.8 Histology, immuno-histochemistry and immuno-fluorescence staining ............ 27
2.9 Western Analysis ............................................................................................ 28
2.10 RNA isolation, real-time PCR and microarray .............................................. 28
2.11 Quantitative RT-PCR .................................................................................. 29
2.12 DNA copy number and exome sequencing .................................................. 29
2.13 Bioinformatics ............................................................................................. 29
2.14 Gene Set Enrichment Analysis ................................................................. 29
2.15 Preswick Drug Screening .......................................................................... 30
2.16 MTT viability assay ..................................................................................... 30
2.17 Additional Statistical Analysis ..................................................................... 30

Chapter 3 ............................................................................................................ 31

3 microRNA-143/145 loss cooperates with Pten-deficiency to promote an aggressive subgroup of basal-like breast cancer ................................................................. 31

3.1.1 Abstract ....................................................................................................... 31
3.2 Introduction .................................................................................................... 31
3.3 Results ............................................................................................................ 34

3.3.1 Most Pten-deficient mammary tumors are not transplantable following cell sorting or short-term propagation as tumorspheres ........................................... 34
3.3.2 A fraction of Pten-deficient mammary tumors with distinct histology contains resilient TICs ................................................................. 38

3.3.3 Transplantable PDA but not non-transplantable AME cells continue to proliferate following orthotopic transplantation ........................................ 41

3.3.4 Distinct genetic alterations including reduced expression of microRNA145 in Pten-deficient PDAs versus AMEs revealed through analysis of mRNA and miRNA profiling, copy number variation and mutation .......................... 41

3.3.5 miR-145-low/PTEN-low tumors represent a distinct subgroup of human breast cancer ......................................................................................... 50
3.3.6 Functional cooperation between miR-143/145 and Pten loss promotes engraftment of non-transplantable Pten-deficient mammary tumors .......... 62
3.3.7 MiR-143/145 loss cooperates with Pten-deficiency at least in part by inducing RAS signalling .................................................. 66
3.4 Discussion .............................................................................. 71

Chapter 4 ..................................................................................... 84

4 Targeted Pten deletion plus p53-R270H mutation in mouse mammary epithelium induces aggressive claudin-low and basal-like breast cancer ................................................................. 84

4.1 Abstract ................................................................................ 84
4.1.1 Background ....................................................................... 84
4.1.2 Methods ............................................................................ 84
4.1.3 Results ............................................................................... 85
4.1.4 Conclusions ...................................................................... 85
4.2 Introduction ........................................................................... 85
4.3 Results .................................................................................. 87
4.3.1 Targeted Pten deletion plus expression of a p53-R270H mutant in mammary epithelium accelerate mammary tumorigenesis ..................... 87
4.3.2 Frequent tumor initiating cells (TICs) in poorly differentiated adenocarcinomas and spindle-like PtenAf:p53R270H tumors .................. 93
4.3.3 Poorly differentiated and spindle-like PtenAf:p53R270H tumors cluster with known mouse models of basal-like and claudin-low breast cancer ............... 93
4.3.4 Poorly differentiated and spindle-like PtenAf:p53R270H tumors cluster with human basal-like and claudin-low breast cancer ......................... 96
4.3.5 Spindle-like PtenAf:p53R270H mutant and PtenAf:p53Af deletion mammary tumors cluster closely together ........................................ 96
4.3.6 FDA-approved drug screens reveal similar sensitivity of PtenAf:p53R270H and PtenAf:p53Af claudin-low-like tumors to 8-azaguanine but no differential sensitivity to other drugs .................................................. 99
4.3.7 Claudin-low-like and basal-like PtenAf:p53R270H tumor cells have higher metastatic potential than PtenAf:p53Af spindle mammary tumor cells .......... 102
4.4 Discussion ............................................................................ 105
4.4.1 Limitations ................................................................................................................ 111
4.5 Conclusions .................................................................................................................. 111
Chapter 5 .............................................................................................................................. 112
5 Future Directions .............................................................................................................. 112
  5.1 Cooperation between PTEN-deletion and miR-143/145-loss .................................. 112
  5.2 Cooperation between PTEN-deletion and p53 mutation .......................................... 114
References ............................................................................................................................ 117
List of Tables

Table 3-1 Most $Pten$-deficient mammary tumors are not transplantable following cell sorting or short propagation as tumorspheres. .................................................................................................................. 36
List of Figures

Figure 1-1 Structure of PTEN tumor suppressor and major post-translational modifications. ..... 8

Figure 1-2 Schematic diagram of PTEN signaling and function. ........................................... 10

Figure 3-1 A small fraction of Pten-deficient mammary tumors is transplantable ............... 39

Figure 3-2 IHC and proliferation analysis of Pten-deficient mammary tumors .................... 44

Figure 3-3 AME tumor cells undergo attrition and fail to proliferate following orthotropic transplantation ........................................................................................................ 46

Figure 3-4 Proliferation and apoptosis analysis of AME and PDA cells following orthotopic transplantation into NOD/SCID mice ........................................................................ 48

Figure 3-5 Pten-deficient mammary PDAs and AMEs are molecularly distinct ................ 52

Figure 3-6 GSEA reveals distinct pathways in PDAs versus AMEs ..................................... 54

Figure 3-7 g:Profiler analysis of AMEs versus PDAs. p<0.05 ............................................... 56

Figure 3-8 aCGH analysis showing distinct genetic alterations in PDAs versus AMEs ......... 58

Figure 3-9 Schematic representation of Cul9 (a) and Cbl (b), and relative location (asterisks) of mutations identified in PDAs ......................................................................................... 60

Figure 3-10 Low miR-145 expression in PTEN-deficient BC leads to poor clinical outcome .... 64

Figure 3-11 Disruption of miR-143/145 in AMEs promotes proliferation and tumor engraftment ......................................................................................................................................... 68

Figure 3-12 miR-143/145 knockdown cooperates with Pten loss by inducing phospho-Erk, leading to increased sensitivity to MEK inhibitors .............................................................................. 72

Figure 3-13 miR-143/145 knockdown cooperates with Pten loss by activating the Ras pathway 74

Figure 3-14 Distinct levels of activation of 18 signalling pathways in AMEs vs PDAs.......... 76
Figure 3-15 miR-143/145 overexpression targets the Ras pathway .................................................. 78

Figure 3-16 Models of oncogenic cooperation between PTEN deficiency and miR-143/145 loss .................................................................................................................................................. 80

Figure 4-1 Pten deletion plus p53-R270H point mutation cooperate to accelerate mammary tumor formation. .................................................................................................................................................. 89

Figure 4-2 Analysis of mutant p53 accumulation in tumor cells. ......................................................... 91

Figure 4-3 High frequency of TICs in PDA and spindle tumors. .......................................................... 94

Figure 4-4 Molecular classification of PD and spindle WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} mammary tumors. .................................................................................................................................................. 97

Figure 4-5 Molecular clustering of spindle-like Pten^{Δf}:p53^{R270H} and Pten^{Δf}:p53^{Δf} mammary tumors. .................................................................................................................................................. 100

Figure 4-6 HTP screens and GSEA reveal similarities and differences between Pten^{Δf}:p53^{R270H} and Pten^{Δf}:p53^{Δf} mammary tumors. .................................................................................................................................................. 103

Figure 4-7 WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} spindle and PDA tumor cells exhibit higher metastatic potential than WAP-Cre:Pten^{fl/fl}:p53^{fl/fl} spindle tumor cells. .................................................................................. 107
<table>
<thead>
<tr>
<th>Acronyms</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACL</td>
<td>ATP-citrate lyase</td>
</tr>
<tr>
<td>AGO</td>
<td>Argonaute</td>
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<td>AKT</td>
<td>Proprotein kinase B</td>
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<td>Adenomyoepithelioma</td>
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<td>APC/C</td>
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<td>Ataxia Telangiectasia Mutated</td>
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<td>HTP</td>
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<td>IFNγ</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>MEC</td>
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<td>Mesenchymal to epithelial transition</td>
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<td>MicroRNA</td>
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<td>MMTV</td>
<td>Mouse Mammary Tumor Virus</td>
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<td>mTOR</td>
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<td>RISC</td>
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<td>RNA</td>
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<td>Rhotekin</td>
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<td>RTKs</td>
<td>Receptor tyrosine kinase</td>
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<td>S6K1</td>
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<tr>
<td>shRNA</td>
<td>Short hairpin RNA</td>
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<td>VHL</td>
<td>Von Hippel-Lindau</td>
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<td>WAP</td>
<td>Whey Acidic Protein</td>
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Chapter 1
Introduction

1.1 Breast Cancer (BC)

1.1.1 Disease burden

Globally, over 1.8 million cases of BC are diagnosed every year (Fitzmaurice, Dicker et al. 2015). In 161 countries, BC is the most prevalent cancer among women, with an incidence of 123.1 cases per 100,000 population in the United States and 130.1 cases per 100,000 in Canada (Fitzmaurice, Dicker et al. 2015; Canadian Cancer Society 2016; Siegel, Miller et al. 2016). In developed countries, BC is most frequently diagnosed in women at the prime of their lives – between ages of 50-69 (51%), followed by women aged 70 and over (32%) and women aged 50 and under (17%). Over the last few decades much progress has been made in early BC diagnosis and treatment, leading to continuous improvements in 5-year survival rates (Canadian Cancer Society 2016; Siegel, Miller et al. 2016). However, the extensive heterogeneity of BC has posed significant challenges to understanding how myriad oncogenic changes cooperate to influence cancer initiation, progression and response to therapy (see 1.1.2), causing BC to remain as the leading cause of cancer death among women aged 20 to 59 (Siegel, Miller et al. 2016).

1.1.2 BC heterogeneity

BC encompasses a collection of heterogeneous diseases characterized by a range of histological, molecular and clinical features, which form the basis for subtype classification.

1.1.2.1 Histological subtype

Histologically, the World Health Organization classifies breast tumors into 3 precancerous types (such as precursor, intraductal proliferative and papillary) and 22 carcinoma types (such as apocrine, cribriform, lobular, metaplastic, mucinous, papillary and tubular) (Sinn and Kreipe 2013). However, the clinical significance of histological classifications can be limited. For example, although the majority of breast tumors (~75%) are diagnosed as invasive carcinomas of no special type (previously known as invasive ductal carcinoma, not otherwise specified, IDC...
NOS), they still display wide variations in both clinical and molecular profiles (Li, Anderson et al. 2003; Li, Uribe et al. 2005; Weigelt, Horlings et al. 2008; Sinn and Kreipe 2013).

1.1.2.2 Subtypes Defined by Immunohistochemical Markers

BC can also be classified by the expression of immunohistochemistry (IHC) using antibodies against estrogen receptor alpha (ERα), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) (Hammond, Hayes et al. 2010; Senkus, Kyriakides et al. 2015). Information on IHC marker profiles plays an important role in diagnosis and treatment decision making (see 1.1.2.3). However, intratumor spatial heterogeneity both within the same primary tumor and between matched primary and metastatic lesions have complicated treatment assessment (Nassar, Radhakrishnan et al. 2010; Aurilio, Disalvatore et al. 2014).

1.1.2.3 Intrinsic Molecular Subtypes

Advances in gene expression microarray studies have revealed greater insights into BC heterogeneity. Seminal studies on gene expression profiling using the PAM50 predefined gene set have identified five main molecular BC subgroups: Luminal A and B, HER2 positive, Basal-like and Claudin-low (Perou, Sorlie et al. 2000; Sørlie, Tibshirani et al. 2003; Prat, Parker et al. 2012). Although molecular subtyping has been widely embraced by the research community, its translation into the clinic has faced significant challenges (Weigelt, Pusztai et al. 2011; Dai, Li et al. 2015).

1.1.2.3.1 Luminal subtypes

Luminal subtype A and luminal subtype B are characterized by high expression of genes normally expressed by luminal cells (Perou, Sorlie et al. 2000). Both are also ERα positive cancers that can be treated with therapies that inhibit ER activity (e.g., Tamoxifen) or inhibit estrogen-producing aromatase activity (e.g., Letrozole, Exemestane) (Buzdar, Douma et al. 2001; Sørlie, Tibshirani et al. 2003; Davies, Godwin et al. 2011; Bernhard, Luo et al. 2015). Patients with luminal A present with the most favourable clinical outcome of all BC molecular subtypes as measured by both relapse-free survival and overall survival (Sorlie, Perou et al. 2001; Sørlie, Tibshirani et al. 2003). In contrast, luminal B subtype tumors express lower levels of ERα, a stronger proliferative signature, more frequent mutations in tumor suppressors TP53 (32%) and
PTEN (24%) as well as worse prognosis (Sorlie, Perou et al. 2001; Sørlie, Tibshirani et al. 2003; Hu, Fan et al. 2006; The Cancer Genome Atlas Network 2012).

1.1.2.3.2 HER2 positive subtype

The HER2 positive subtype is defined by overexpression or amplification of the HER2/ERBB2/neu oncogene (Perou, Sorlie et al. 2000; Sørlie, Tibshirani et al. 2003). Histopathologically, approximately half of HER2 positive cancers display ERα and PR expression (Romond, Perez et al. 2005). HER2 positive cancers also show frequent mutations in tumor-associated genes including TP53 (75%), PIK3CA (42%) and PTEN (19%) (Sorlie, Perou et al. 2001; Sørlie, Tibshirani et al. 2003; The Cancer Genome Atlas Network 2012). Clinically, HER2 positive BCs are treated with therapies that bind to and/or inhibit signaling from the HER2 receptor such as Herceptin® (trastuzumab), Perjeta® (pertuzumab), Kadcyla® (ado-trastuzumab emtansine) and Tykerb® (lapatinib) (Geyer, Forster et al. 2006; Verma, Miles et al. 2012; Swain, Kim et al. 2013). However, despite these advances, HER2 positive cancers still exhibit an aggressive clinical course and patients with HER2+ tumors have poor survival (Sorlie, Perou et al. 2001; Sørlie, Tibshirani et al. 2003). A major mechanism by which resistance to HER2-targeted therapy develops is through activation of the PI3K signaling pathway (Nagata, Lan et al. 2004). This pathway is activated by HER2 receptors, and constitutive activation of the former will bypass anti-HER2 therapy.

1.1.2.3.3 Basal-like and Claudin-low subtypes

Molecularly, the Basal-Like subtype is defined by a gene expression profile resembling basal and/or myoepithelial cells (Perou, Sorlie et al. 2000). Basal-like cancers exhibit frequent mutations in PTEN (35%), TP53 (84%) and RB1 (20%), a strong association with germline BRCA1 mutations (30%), and preferential metastasis to the lung and brain, instead of to the bone (Sørlie, Tibshirani et al. 2003; Kennecke, Yerushalmi et al. 2010; Stefansson, Jonasson et al. 2011). The Claudin-Low subtype is molecularly characterized by low expression of claudins (tight junction protein), enrichment of epithelial-to-mesenchymal transition (EMT) markers, immune response genes and cancer stem cell-like features (Herschkowitz, Simin et al. 2007; Prat, Parker et al. 2010; Taube, Herschkowitz et al. 2010; The Cancer Genome Atlas Network 2012).
Both basal-like and claudin-low subtypes are grouped under the pathology term Triple Negative Breast Cancers (TNBCs) due to the absence or low level expression of ERα, PR and HER2. A clustering analysis of aggregated gene expression profiles involving 587 TNBC cases identified 6 molecular TNBC subtypes including Basal-like 1, Basal-like 2, Immunomodulatory, Mesenchymal-like, Mesenchymal Stem-like and Luminal AR, that also displayed distinct sensitivity profiles to pharmacological inhibitors (Lehmann, Bauer et al. 2011). TNBCs are treated with systemic chemotherapies such as anthracyclines or taxanes (Wahba and El-Hadaad 2015). As such, patients with TNBC exhibit poor clinical outcome and effective therapies are urgently needed (Sorlie, Perou et al. 2001; Sørlie, Tibshirani et al. 2003; Prat, Parker et al. 2010). Notably, TNBC patients harboring BRCA1/BRCA2 mutations respond well to PARP inhibitors (such as olaparib), but effective treatments for other TNBCs are clearly lacking (Brown, Kaye et al. 2016).

1.1.2.4 Subtype by integrated copy-number and gene-expression profiles

BCs also demonstrate remarkable heterogeneity in genomic architecture. One analyses of nearly 2000 BC patients showed that as much as 39% of genes are impacted by germline and somatic copy number aberrations (Curtis, Shah et al. 2012). Integration of copy-number data with gene-expression profiling identified 10 “integrative clusters” or subgroups that correlated with distinct clinical outcome (Curtis, Shah et al. 2012).

1.1.2.5 Subtype by pathway activity

Classification strategies based on signaling pathway activity have also been developed. The Nevins group generated gene expression signatures for 18 different pathways including ER, PR, p53, β-catenin, E2F1, PI3K, Myc, Ras, IFNα, IFNγ, Akt, p63, Src, Her2, EGFR, TGFβ, STAT3 and TNFα. Using patterns of pathway deregulation, they identified 17 BC subgroups that correlated with distinct copy number and clinical profiles (Gatza, Lucas et al. 2010).

1.1.2.6 Lessons from molecular heterogeneity

In-depth molecular analyses have furthered our understanding of BC complexity. An evaluation of copy number variations in 100 BC cases identified driver mutations in 40 cancer genes (Stephens, Tarpey et al. 2012), and a whole-genome sequencing analysis identified 93 probable
driver mutations, including TP53, PIK3CA, MYC, CCND1, PTEN, ERBB2, FGFR1, GATA3, RB1 and MAP3K1 (Nik-Zainal, Davies et al. 2016). These and other studies show that although molecular alterations are frequently observed in BC, driver mutations typically occur at low frequencies (Stephens, Tarpey et al. 2012; The Cancer Genome Atlas Network 2012; Nik-Zainal, Davies et al. 2016). Such complexity and heterogeneity with multiple alterations occurring at low frequencies (<1%) poses a serious impediment to anti-cancer therapy. To develop effective treatments, we need to understand the oncogenic contributions of cancer-related alterations (such as PTEN loss) and how different events cooperate to influence cancer initiation, progression and response to therapy.

1.2 Tumor Suppressors in BC
In this thesis, I investigated the role of tumor suppressor PTEN and its cooperations with miR-143/145 and TP53 in aggressive BC.

1.2.1 PTEN
The tumor suppressor PTEN (phosphatase and tensin homolog deleted on chromosome ten) was identified in 1997 by deletion mapping of breast, prostate and brain cancers with frequent deletions at chromosome 10q23 (Li and Sun 1997; Li, Yen et al. 1997; Steck, Pershouse et al. 1997; Steck, Pershouse et al. 1997). Subsequent cloning and characterization revealed PTEN to be an evolutionary conserved gene involved in many cellular processes. Research over the last few decades firmly established PTEN as one of the most frequently inactivated tumor suppressors in human cancers (e.g., mutated in 29% of glioblastoma, 35% of endometrial carcinoma) and its mutation or loss is often implicated in advanced cancers and therapy resistance (Catalog of Somatic Mutations in Cancer; Ali, Schriml et al. 1999; Nagata, Lan et al. 2004; Kokubo, Gemma et al. 2005; Yin and Shen 2008; Ferraldeschi, Nava Rodrigues et al. 2015; Forbes, Beare et al. 2015; Peng, Chen et al. 2016). Interestingly, tissue specific deletion of Pten in mouse models lead to the development of tumors with a wide spectrum of aggressiveness (see 1.2.1.3). Further, tumorigenicity of these Pten-null tumors can be enhanced by other oncogenic mutations (see 1.2.1.3). To develop effective therapies, a better appreciation of how oncogenic events cooperate with mammary-specific PTEN loss is required.
1.2.1.1 PTEN functions

PTEN is a lipid and protein phosphatase (Li and Sun 1997; Li, Yen et al. 1997; Song, Salmena et al. 2012). The PTEN protein consists of five functional domains: a PIP2 binding domain (PBD), a phosphatase domain, a C2 domain, a carboxy-terminal tail and a PDZ-binding domain (Figure 1-1). 1) The N-terminal PBD domain is highly conserved and is important for PTEN membrane targeting and orientation (Walker, Leslie et al. 2004). Mutations in this domain (such as K13E) can compromise phosphatase and tumor suppressive activities. 2) The phosphatase domain consists of amino acids 7-180 and contains a catalytic signature motif HCXXGXXR (where “X” is any amino acid) present in other protein tyrosine phosphatases (PTPs) and dual specificity protein phosphatases (DUSPs) (Li, Yen et al. 1997; Steck, Pershouse et al. 1997). Approximately 40% of all PTEN germline mutations occur in this phosphatase core motif (Eng 2003). Beyond homology of the catalytic motif, the first 190 amino acids in PTEN resembles tensin and auxilin 2 (Li, Yen et al. 1997; Steck, Pershouse et al. 1997). Structurally, the phosphatase domain contains an active site pocket that is deep and wide, positively charged and capable of accommodating PIP3 substrates (Lee, Yang et al. 1999). 3) The C2 domain is important for mediating PTEN interactions with the plasma membrane (Lee, Yang et al. 1999). Mutations in the C2 domain (such as M-CBR3 and M-α2) can lead to reduced affinity for membranes and diminished ability to suppress cell proliferation (Lee, Yang et al. 1999). The PTEN C2 domain bears structural homology to other C2 domains that regulate calcium-dependent membrane recruitment of signaling proteins (Lee, Yang et al. 1999). However, the PTEN C2 domain is calcium independent. 4) Finally, the carboxy-terminal tail maintains protein stability and the PDZ binding site mediates protein-protein interactions (Vazquez, Ramaswamy et al. 2000; Valiente, Andres-Pons et al. 2005). Tumor-associated mutations in PTEN are found throughout the protein, indicating that all domains are important for tumor suppressive functions of PTEN (Song, Salmena et al. 2012).

1.2.1.1.1 Cytoplasmic functions of PTEN

On the plasma membrane and endosomal membranes, PTEN acts as the central negative regulator of the proto-oncogenic Phosphatidylinositol 3-kinase (PI3K) signaling by dephosphorylating phosphatidylinositol 3,4,5-trisphosphate (PIP3) at position D3, thereby generating phosphatidylinositol 4,5-bisphosphate (PIP2) (Figure 1-2) (Salmena, Carracedo et al.
Thus, PTEN, as a lipid phosphatase, antagonizes the PI3K pathway (Maehama and Dixon 1998; Stambolic, MacPherson et al. 2001).

The PI3K pathway controls diverse cellular processes important to cancer development including cell survival, cell growth, proliferation, angiogenesis and metabolism (Fruman and Rommel 2014). There are three classes of PI3K enzymes, with Class 1A being the most widely implicated in cancer (Samuels, Wang et al. 2004). PI3K is a heterodimer composed of p110 catalytic and p85 regulatory subunit (Yu, Zhang et al. 1998). In the absence of signal, p85 inhibits p110 catalytic activity (Yu, Zhang et al. 1998). p110 activation is initiated by ligand binding to receptor tyrosine kinases (RTKs) or G protein coupled-receptors (GPCR). RTK activation induced by growth factor binding results in receptor dimerization and autophosphorylation (Thorpe, Yuzugullu et al. 2015). p110 is then recruited to the plasma membrane through direct interaction between the p85 SH2 domain and phosphorylated RTK motifs, leading to removal of p110 basal inhibition by p85 (Yu, Zhang et al. 1998). Similarly, ligand-bound GPCRs stimulate p110 through direct binding with G protein subunits (Dbouk, Vadas et al. 2012). Other mechanisms of activation involve the adaptor protein growth factor receptor-bound protein 2 (GRB2). GRB2 interacts with GRB2-associated binding protein (GAB), leading to p85 binding and p110 activation. GRB2 can also activate son of sevenless (SOS), leading to RAS activation and stimulation of p110 independent of p85 (Rozakis-Adcock, McGlade et al. 1992; Buday and Downward 1993; Chardin, Camonis et al. 1993; Egan, Giddings et al. 1993; Li, Batzer et al. 1993; Ong, Hadari et al. 2001; Pawson 2004; Cully, You et al. 2006). Once activated, p110 phosphorylates PIP2, producing PIP3 (Fruman and Rommel 2014). Accumulation of PIP3 at the membrane leads to recruitment of proteins containing plekstrin homology (PH) domains, including phosphoinositide-dependent protein kinase 1 (PDK1) and AKT (Fruman and Rommel 2014). PDK1 then phosphorylates AKT at Thr308, thereby activating this major downstream effector (Alessi, Deak et al. 1997). Full AKT activation is subsequently achieved via another phosphorylation step at Ser473, in this case by mammalian target of rapamycin complex 2 (mTORC2) or DNA-dependent protein kinase (DNA-PK) (Feng, Park et al. 2004; Sarbassov, Guertin et al. 2005).

Activated AKT dissociates from the membrane and translocates to the cytoplasm and nucleus, where it phosphorylates a plethora of downstream signaling proteins, modulating many cellular
processes (Calleja, Alcor et al. 2007). AKT promotes protein synthesis and cell growth through activating the mammalian target of rapamycin complex 1 (mTORC1). This is achieved through inhibitory phosphorylation of two mTORC1 negative regulators: 1) proline-rich Akt substrate of 40 kDa (PRAS40), which inhibits mTORC1 activity through direct binding, and 2) tuberous sclerosis protein 2 (TSC2) (Gao and Pan 2001; Inoki, Li et al. 2002; Manning, Tee et al. 2002; Potter, Pedraza et al. 2002; Sancak, Thoreen et al. 2007). TSC2 forms a complex with TSC1 to inhibit Ras homolog enriched in brain (RHEB). Phosphorylated TSC2 relieves its inhibition on

Figure 1-1 Structure of PTEN tumor suppressor and major post-translational modifications.

PTEN has 5 functional domains: PBD (PIP2 binding domain), Phosphatase, C2 (C2 domain), C-tail (C-terminal tail) and PDZ-BD (PDZ-binding domain). Select sites of post-translational modifications are noted.

PTEN-long can exit the donor cell and enter neighbouring cells, where it functions as a canonical PTEN tumor suppressor to antagonize PI3K signaling
Figure 1-2 Schematic diagram of PTEN signaling and function.

In the cytoplasm, phosphatase and tensin homologue (PTEN) negatively regulates the phosphoinositide 3-kinase (PI3K, composed of p85 and p110 subunits) pathway by dephosphorylating phosphatidylinositol-3,4,5-trisphosphate (PIP3) to Phosphatidylinositol 4,5-bisphosphate (PIP2). Following PTEN loss, PIP3 accumulation leads to recruitment of phosphoinositide-dependent kinase 1 (PDK1) and AKT. AKT is then phosphorylated at Thr308 by PDK1 and at Ser473 by mTOR complex 2 (mTORC2). Activated AKT phosphorylates downstream targets including mTOR complex 1 (mTORC1), p21, p27, glycogen synthase kinase 3 (GSK3), glucose transporter 1 (Glut1), glucose transporter 4 (Glut4), hexokinase II (HK2), ATP-citrate lyase (ACL), mouse double minute 2 homolog (MDM2), Bcl-2-associated death promoter (BAD) and forkhead box class O family member protein (FOXO), leading to increased cell survival, proliferation, protein synthesis and metabolic changes.

In the nucleus, PTEN controls chromosomal stability and cell cycle. An alternative version of PTEN, called PTEN-long contain an additional 173 N-terminal amino acids, is secreted from cells and enter other cells where it opposes the PI3K pathway.
RHEB, leading to mTORC1 stimulation. Stimulated mTORC1 promotes protein synthesis and cell growth by inactivating eukaryotic translation initiation factor 4E binding protein 1 (4EBP1) and activating ribosomal protein S6 kinase, 70 kDa, polypeptide 1 (S6K1) (Fingar, Salama et al. 2002). AKT promotes cell proliferation by phosphorylating the cyclin-dependent kinase inhibitors p21 and p27, thereby preventing their translocation to the nucleus (Zhou, Liao et al. 2001; Liang, Zubovitz et al. 2002). AKT also drives proliferation by suppressing glycogen synthase kinase 3 (GSK3)-mediated proteasomal degradation of G1 cyclins (cyclin D1), c-Jun and c-Myc (Cross, Alessi et al. 1995; Gregory, Qi et al. 2003; Wei, Jin et al. 2005). AKT promotes angiogenesis in endothelial cells through phosphorylation and activation of endothelial nitric oxide synthase (eNOS), leading to NO production and angiogenesis (Dimmeler, Fleming et al. 1999; Fulton, Gratton et al. 1999). AKT also blocks apoptosis by inhibiting pro-apoptotic Bcl-2 homology domain 3 (BH3)-only proteins such as BAD and MDM2, as well as FOXO transcription factors. For example, AKT phosphorylates BAD and triggers its release from target proteins. Phosphorylation of MDM2 by AKT promotes its translocation into the nucleus, where it negatively regulates p53. Phosphorylated FOXO transcription factors are displaced from pro-apoptotic target genes such as the BH3-only protein BIM (Datta, Dudek et al. 1997; del Peso, Gonzalez-Garcia et al. 1997; Mayo and Donner 2001; Zhou, Liao et al. 2001; Dijkers, Birkenkamp et al. 2002; Tran, Brunet et al. 2003). Thus, cytoplasmic loss of PTEN leads to excessive PIP3 accumulation and AKT activation, resulting in protection from apoptosis and increased proliferation.

Finally, the PI3K/AKT/mTOR pathway is important for metabolic reprogramming or the “Warburg effect”. The Warburg effect describes the shift by cancer cells from catabolic metabolism – utilizing oxidative phosphorylation to maximize ATP production, to anabolic metabolism – increasing nutrient uptake and diverting metabolism synthesis to anabolic synthesis of nucleotides, fatty acids and amino acids, thereby facilitating growth and proliferation (Ward and Thompson 2012). For example, the PI3K/AKT/mTOR pathway increases nutrient uptake through increased expression of the GLUT1 glucose transporter and promotes plasma membrane docking by translocation of GLUT1 and GLUT4 (Cong, Chen et al. 1997; Barthel, Okino et al. 1999; Rathmell, Fox et al. 2003). The PI3K/AKT/mTOR pathway also diverts nutrients to anabolic metabolism at multiple nodes. For example, AKT promotes glucose processing by enhancing the activity of hexokinase – an enzyme that catalyzes the first
rate-limiting step of glycolysis by phosphorylating glucose to create glucose 6-phosphate (G6P) (Roberts, Tan-Sah et al. 2013). Also, mTORC1-mediated upregulation of hypoxia-inducible factor 1-alpha (HIF-1α) induces pyruvate dehydrogenase kinase 1 (PDK1) expression, leading to inhibitory phosphorylation of pyruvate dehydrogenase (PDH) (Kim, Tchernyshyov et al. 2006; Duvel, Yecies et al. 2010). PDH is responsible for the conversion of pyruvate to acetyl-CoA, and acetyl-CoA subsequently enters the TCA cycle to produce citrate via ATP citrate lyase (ACL) (Kim, Tchernyshyov et al. 2006) (Ward and Thompson 2012). Therefore, mTORC1-induced HIF-1α leads to inhibition of PDH, resulting in a failure to convert pyruvate to acetyl-CoA (instead pyruvate is diverted to lactate) and blocking glucose carbon incorporation into citrate.

Once glucose carbons are diverted away from TCA, the PI3K/ATK/mTORC1 pathway also promotes anabolic metabolism and biosynthesis of nucleotides, lipids and amino acids. For example, mTORC1-mediated S6K1 activation leads to enhanced pyrimidine synthesis (Ben-Sahra, Howell et al. 2013). During lipid synthesis, AKT may phosphorylate ACL to promote production of cytosolic acetyl-CoA, and mTORC1 can upregulate NADPH generation (Berwick, Hers et al. 2002). Both cytosolic acetyl-CoA and NADPH are necessary for lipid synthesis. Finally, the PI3K/ATK/mTORC1 pathway also facilitates anaplerosis (a process of replenishing TCA cycle intermediates that are important for biosynthesis) and subsequent conversion of intermediates to amino acids (Csibi, Fendt et al. 2013).

1.2.1.1.2 Nuclear functions of PTEN

PTEN localizes to the nucleus where it manifests additional tumor suppressive functions. A major activity of nuclear PTEN is to maintain chromosomal integrity. PTEN maintains proper centromere and kinetochore formations through interactions with centromere-specific binding protein C (CENP-C) (Shen, Balajee et al. 2007). PTEN also assists in repairs of double-stranded DNA breaks through transcriptional upregulation of Rad51 (Shen, Balajee et al. 2007). Another major tumor suppressive function of PTEN involves control of cell cycle progression. Nuclear PTEN induces cell cycle arrest by downregulating cyclin D1 transcription (Radu, Neubauer et al. 2003; Chung, Ostrowski et al. 2006). PTEN also associates with anaphase-promoting complex/cyclosome (APC/C) and promotes APC/C association with APC/C activator protein CDH1, thus enhancing proteolysis by the APC-CDH1 complex, resulting in reduced levels of mitotic determinants and control over cell cycle progression (Song, Carracedo et al. 2011).
1.2.1.3 Alternative PTEN variants

Alternative, longer variants of the PTEN protein (PTEN-long) with an additional 173 N-terminal amino acids, are capable of exiting and functioning outside of the cell (Putz, Howitt et al. 2012; Hopkins, Fine et al. 2013). Further, once secreted into the extracellular environment, PTEN-long can enter neighbouring cells where it functions as a canonical PTEN tumor suppressor to antagonize PI3K signaling.

1.2.1.2 PTEN Loss in Breast Cancer

PTEN expression is altered through several mechanisms including mutation, deletion and transcriptional repression. Germline PTEN mutation leads to the development of PTEN hamartoma tumor syndromes (PHTS), a group of autosomal dominant diseases characterized by hamartoma growth (Hobert and Eng 2009). Cowden syndrome (CS) is the prototypic PHTS. Patients with CS present with multiple hamartomas, most commonly in the skin and gastrointestinal tract, as well as an increased risk of benign and malignant tumor formation in the breast, thyroid, endometrium, colon, rectum, kidney and skin (Blumenthal and Dennis 2008; Riegert-Johnson, Gleeson et al. 2010; Tan, Mester et al. 2012). Among female CS patients, BC is the most common malignancy to develop, with a lifetime risk of 81-85% (Riegert-Johnson, Gleeson et al. 2010; Tan, Mester et al. 2012).

Tumor-associated somatic mutations in PTEN documented in the Catalogue of Somatic Mutations in Cancer (COSMIC) database (3,583 annotated to date) occur throughout the length of PTEN (Catalog of Somatic Mutations in Cancer; Forbes, Beare et al. 2015). Although there are hotspot mutations at specific amino acids, they do not associate with a specific cancer type (Song, Salmena et al. 2012). In BC, PTEN is mutated or lost in 13% of luminal A, 24% of luminal B, 19% in HER2 and 35% in basal-like tumors (The Cancer Genome Atlas Network 2012).

PTEN can be suppressed in BC by aberrant promoter methylation and microRNA-mediated silencing (e.g., miRNAs, including miR-18a and miR-21) (Ma, Kumar et al. 2011; Wang, Lu et al. 2011; The Cancer Genome Atlas Network 2012; Mouw, Yui et al. 2014). In vitro experiments demonstrate that PTEN can be post-transcriptionally regulated by phosphorylation,
ubiquitylation, sumoylation and acetylation. However, the role for each modification remains unclear.

1.2.1.3 Lessons from Mouse Models of PTEN Loss - Requirement for a Second Hit to Induce Aggressive Tumors

Many mouse models have been established to probe the oncogenic role of PTEN loss in BC. To model CS, mice with single Pten allele deletions were created, as loss of both Pten alleles is embryonically lethal (Di Cristofano, Pesce et al. 1998; Suzuki, de la Pompa et al. 1998). Pten heterozygous mice developed some of the CS phenotypes including hyperplasia and/or tumor development in the mammary gland, gastrointestinal tract, thyroid gland and endometrium (Di Cristofano, Pesce et al. 1998; Podsypanina, Ellenson et al. 1999; Stambolic, Tsao et al. 2000). These models develop neoplasms not typically associated with CS including lymphoma, as well as prostate and adrenal gland tumors. Mammary tumors formed much slower, typically at week 30 or later, with a median latency of 50-66 weeks and a penetrance of nearly 50% (Stambolic, Tsao et al. 2000). Mammary tumors were of a relatively well-differentiated and glandular histology, with positive expression of basal cytokeratins (CKs) 5 and 6 (Stambolic, Tsao et al. 2000; Saal, Gruvberger-Saal et al. 2008). In a model for subtle variations in Pten dosage (Pten$^{+/+}$, Pten$^{+/−}$), mice with progressively lower Pten levels demonstrated increasing susceptibility to mammary tumors (Alimonti, Carracedo et al. 2010). When Pten dosage was decreased to 30% of normal level, the majority of mice (75%) developed mammary tumors. These results demonstrate an essential role for PTEN, especially in the breast, where even subtle decreases in Pten levels cause mammary neoplasia.

Models with conditional Pten deletion in the mammary epithelium were particularly illuminating. Mice with homozygous, mammary-specific deletion of Pten driven by MMTV-Cre$^{\text{lineA}}$, MMTV-Cre$^{\text{NLST}}$ and Wap-Cre developed mammary tumors with prolonged median latencies of approximately 300, 792 and 250-456 days, respectively (Li, Robinson et al. 2002; Liu, Voisin et al. 2014; Boelens, Nethe et al. 2016). Histologically, tumors were heterogeneous, ranging from adenomas to adenocarcinomas. Molecularly, and in contrast to human BC, most Pten-null mammary tumors clustered with benign “normal-like” subgroup lesions (Liu, Voisin et al. 2014).
When combined with additional oncogenic mutations, *Pten*-deletion induced mammary tumor formation was drastically enhanced. *Pten* deletion coupled with E-cadherin or p53 loss accelerated mammary tumorigenesis by approximately 4.7 months, 5.4-15.1 months, respectively. As well, *Pten* deletion coupled with Wnt or Her2 overexpression accelerated mammary tumorigenesis by approximately 2.5 months and 5.1 months, respectively (Li, Podsypanina et al. 2001; Schade, Rao et al. 2009; Liu, Voisin et al. 2014; Boelens, Nethe et al. 2016). The spectrum of tumor histology in compound mice also changed to a higher and less-differentiated grade. Further, molecular profiles of compound tumors shifted to aggressive subtypes – luminal when combined with *Her2* overexpression, and claudin-low when combined with *p53* loss (Schade, Rao et al. 2009; Liu, Voisin et al. 2014). Lastly, the frequency of metastasis also increased in models of *Pten*-null when coupled with Her2 overexpression or E-cadherin loss (Schade, Rao et al. 2009; Boelens, Nethe et al. 2016).

In other cancer models, *Pten* deletion alone can lead to rapid tumor formation. For example, *Pten* deletion in T cells lead to lymphoma in as little as 17 weeks (Suzuki, Yamaguchi et al. 2001). In many other tissues, oncogenic cooperation involving *Pten* deletion is commonly seen. Prostate-specific inactivation of murine *Pten* induces benign tumors with elevated senescence after long latency (Chen, Trotman et al. 2005). A secondary mutation in *p53* or *Smad4* caused formation of aggressive, metastatic prostate tumors. *Pten* together with *Her2* amplification or activating *Kras* mutation also drove aggressive prostate tumor formation (Chen, Trotman et al. 2005; Ahmad, Patel et al. 2011; Ding, Wu et al. 2011; Mulholland, Kobayashi et al. 2012). Likewise, in models of von Hippel-Lindau (VHL) disease, pancreatic, skin, lung and biliary tract malignancies, mouse *Pten* inactivation either failed to induce tumors or only elicited organ enlargement or low-grade lesions (Frew, Minola et al. 2008; Iwanaga, Yang et al. 2008; Dankort, Curley et al. 2009; Xu, Ehdaie et al. 2010; Marsh, Davies et al. 2013). An activating oncogenic mutation or tumor suppressor deletion in Vhlh, Smad4, Braf, and K-Ras respectively, was required as a second hit to achieve malignant transformation in these tissues. Together, these experiments suggest that *Pten*-loss can initiate tumors on a continuum of aggressiveness that can further cooperate with other oncogenic mutations to enhance cancer aggressiveness.
1.2.1.4 Mutations in PTEN and PIK3CA are non-redundant

The PI3K pathway is frequently hyper-activated by oncogenic PIK3CA mutations or PTEN loss. Interestingly, such alterations are not necessarily redundant. In BC patients, PTEN deletions and PIK3CA mutations are found to co-exist, with distinct correlations to local recurrence free survival and radiosensitivity (Perez-Tenorio, Alkhori et al. 2007). Analysis of patients with invasive lobular BCs also revealed differential effects of PTEN loss versus PIK3CA mutations on PI3K signaling (Ciriello, Gatza et al. 2015). In mouse models, transcriptional profiling showed that mammary tumors with point mutation in Pi3kca cluster into two groups, with one group closely resembling mammary tumors arising from Pten deletion. Analysis of 18 signaling pathways revealed that despite similar gene expression profiles, Pten-deletion tumors had higher PI3K pathway activity whereas Pik3ca-mutated tumors had higher EGFR pathway activity (Liu, Wang et al. 2016). Finally, important mechanistic distinction exists between PIK3CA mutation and PTEN loss. While tumors with activated PIK3CA mutants are dependent on signaling through its protein product p110α, tumors with PTEN loss are dependent on signaling through a different PI3K subunit, p110β (Wee, Wiederschain et al. 2008).

1.2.2 MicroRNAs (miRNAs)

1.2.2.1 MiRNA Biogenesis

MicroRNAs (miRNAs) are 18- to 24-nucleotide non-coding RNAs that regulate post-transcriptional gene silencing (Esteller 2011; Ha and Kim 2014; Jonas and Izaurralde 2015). Animal miRNAs are synthesized through a multi-step process that begins with transcription by RNA polymerase II to produce long, 60-70 nucleotide, intramolecularly double-stranded RNAs called primary-miRNAs (pri-miRNA) (Lee, Kim et al. 2004; Ha and Kim 2014). Some miRNA genes such as those for miR-143 and miR-145 are located in close proximity with each other and may form a polycistronic transcription unit (Xin, Small et al. 2009). For such multicistronic units, miRNAs from the same cluster are generally co-transcribed, but individual miRNAs may be differentially regulated post-transcriptionally. The mechanism behind asymmetric processing is relatively poorly defined but may include regulation by protein factors and miRNA tertiary structures (Roush and Slack 2008; Chakraborty, Mehtab et al. 2012). Following transcription, pri-miRNAs are cleaved into shorter intermediates called precursor-miRNAs (pre-miRNAs) by RNase III enzyme DROSHA (Lee, Ahn et al. 2003). Pre-miRNAs are then exported into the
cytoplasm, where they are cleaved again by RNase III enzyme DICER. The pre-miRNA is then loaded onto argonaute (AGO) proteins to form an RNA-induced silencing complex (RISC). Finally, one of the strands in the RNA duplex is degraded and the remaining strand becomes a mature miRNA (Ketting, Fischer et al. 2001; Yi, Qin et al. 2003; Kim, Han et al. 2009; Ha and Kim 2014).

1.2.2.2 MiRNA Function

Mature miRNAs contain a 6-8 nucleotide seed sequence at the 5’ end that recognizes target mRNA through complementary miRNA:mRNA pairing (Lewis, Shih et al. 2003; Jonas and Izaurralde 2015). In mRNAs, miRNA-binding sites are usually found in the 3’ untranslated region (UTR) (Lewis, Shih et al. 2003). mRNA binding to miRNA seed sequences triggers translational repression or mRNA degradation, estimated to account for 6-26% and 66-90% of miRNA-mediated target repression in mammalian cells, respectively (Jonas and Izaurralde 2015). Since miRNA seed sequences are rather short, each can affect multiple target mRNAs simultaneously. Consequently, miRNAs are estimated to regulate translation of more than 60% of genes and are involved in a variety of developmental and pathological processes (Friedman, Farh et al. 2009). A full picture of the mechanism surrounding miRNA-induced repression in animal gene expression is still a matter of active research, with data suggesting a combination of mechanisms including cleavage by argonaute (AGO) proteins for perfect miRNA-mRNA complementary pairing, as well as translational block and mRNA decay for partial complementary pairings (Ameres and Zamore 2013).

1.2.2.3 MiRNAs in Breast Cancer

MiRNA dysregulation is associated with many malignancies, including BC. A number of mechanisms can cause aberrant miRNA expression, including 1) epigenetic silencing of miRNA loci by aberrant methylation. Changes in methylation profiles are commonly seen in BC – recent comprehensive analyses found more frequent methylation changes in miRNA promoters than in protein-coding gene promoters (Li, Zhang et al. 2015). Such methylation changes can contribute to cancer development by repressing tumor suppressor miRNAs or activating oncogenic miRNAs. For example, hypermethylation of the miR-200 family of tumor suppressors correlates with reduced miR-200 family expression and activation of EMT-transcriptional inducers such as
Zebral and Zeb2 (Castilla, Diaz-Martin et al. 2012). Additionally, hypermethylation of miR-145 has been found in prostate cancer, non-small cell lung cancer (NSCLC) and some BC cell lines. Clinically, miR-145 hypermethylation is associated with aggressive phenotype, brain metastasis and short survival (Suh, Chen et al. 2011; Donzelli, Mori et al. 2015; Ye, Shen et al. 2015). Aberrant miRNA expression can also be caused by 2) genetic alterations at miRNA loci. Indeed, genome-wide mapping of miRNA genes revealed that over half, including many implicated in BC such as the miR-143/145 cluster, are located in cancer-associated genomic regions or fragile sites, thereby contributing to dysregulated miRNA expressions (Calin, Sevignani et al. 2004; Bertoli, Cava et al. 2015). Beyond mutational or epigenetic changes at miRNA loci, aberrant miRNA expression can also be caused by 3) changes in the miRNA biogenesis pathway. In BC, alterations in expression of DROSHA and DICER have been described, and such changes are also associated with specific subgroups or reduced survival (Dedes, Natrajan et al. 2011; Caffrey, Ingoldsby et al. 2013; Avery-Kiejda, Braye et al. 2014). Finally, 4) miRNA expression can be transcriptionally repressed by tumor-related transcription factors. For example, both TP53 and BRCA1 tumor suppressors have been shown to stimulate miR-145 expression (Spizzo, Nicoloso et al. 2010; Kawai and Amano 2012). Together, these mechanisms can cause specific or global miRNA dysregulation. Indeed, comprehensive RNA expression studies have demonstrated differential miRNA expression across BC subtypes and disease progression (Farazi, Horlings et al. 2011; Dvinge, Git et al. 2013). Recent efforts have also been made to utilize such differential expression for BC diagnosis and treatment. For example, several miRNA-based signatures can be used to diagnose BC, identify subgroups, predict prognosis and therapy response (Iorio, Ferracin et al. 2005; Blenkiron, Goldstein et al. 2007; Lowery, Miller et al. 2009; Dvinge, Git et al. 2013; Gasparini, Cascione et al. 2014; Miller, Clarke et al. 2015). Treatment strategies that target tumor-associated miRNAs have also been explored, with advances made in delivery systems such as nanoparticles (Choi, Silvestre et al. 2014).

1.2.2.4 MiR-143 and miR-145 in Breast Cancer

Mouse miR-143 and miR-145 are separated by ~1.4 KB on chromosome 18 and are derived from the long noncoding RNA MIR143HG (MIR143 host gene, CARMEN) (Xin, Small et al. 2009). This miRNA cluster is conserved across vertebrates and has emerged as an important tumor suppressor. As described above, miR-143 and miR-145 are frequently downregulated in BC
through a variety of mechanisms. Further, low expression of miR-145 in BC is associated with higher proliferative index, larger tumor size, lymph node metastasis, and lack of ERα and HER2 expressions (Iorio, Ferracin et al. 2005; Min, Wang et al. 2014). Functional studies revealed that miR-143 and miR-145 exert tumor suppressive functions through inhibition of proliferation and pluripotency, as well as through promotion of apoptosis and differentiation. Specifically, miR-143 represses oncogenes such as KRAS, ERBB3 and ERK5 (Chen, Guo et al. 2009; Clape, Fritz et al. 2009; Yan, Chen et al. 2014), and miR-145 targets NRAS, MYC, EGFR, IRS-1, OCT4, SOX2, KLF4, RTKN, MUC1, FSCN1, JAM-A, VEGF and others (Sachdeva, Zhu et al. 2009; Wang, Bian et al. 2009; Xu, Papagiannakopoulos et al. 2009; Chiyomaru, Enokida et al. 2010; Gotte, Mohr et al. 2010; Sachdeva and Mo 2010; Zhu, Dougherty et al. 2011; Zou, Xu et al. 2012).

1.2.3 Tumor protein TP53

1.2.3.1 Wildtype TP53

The tumor suppressor TP53 is one of the most frequently mutated genes in cancer – estimated to be altered in more than half of all human tumors (Kandoth, McLellan et al. 2013). The wildtype p53 protein is a transcriptional activator that responds to diverse stress signals such as DNA damage, hypoxia, hyperproliferative signals and nutrient starvation by orchestrating specific tumor suppressive responses including DNA repair, cell cycle arrest, senescence, differentiation and apoptosis (Vousden and Lane 2007; Bieging, Mello et al. 2014).

The p53 protein contains five major domains: 1) an N-terminal transactivation domain required for transcriptional activation, 2) a central proline-rich domain that enables protein-protein interactions, 3) a DNA-binding domain (DBD) that binds to DNA consensus recognition elements in target genes, 4) a tetramerization domain that allows activated p53 to form tetramers, and 5) a C-terminal regulatory domain (Walker and Levine 1996; Weinberg, Freund et al. 2004; Toledo and Wahl 2006).

Mechanistically, stress-response kinases (such as ATM, ATR) phosphorylate and stabilize p53, thereby disrupting its interaction with E3 ligases, MDM2 and MDM4 (Oliner, Pietenpol et al. 1993; Shvarts, Steegenga et al. 1996; Craig, Burch et al. 1999). Stabilized p53 subsequently interacts with transcriptional co-factors, leading to coordinated tumor suppressive responses. For
example, p53 triggers G1 cell cycle arrest by inducing transcription of the gene coding for CDK inhibitor p21 (Macleod, Sherry et al. 1995). p53 activates apoptosis through transactivating genes coding for pro-apoptotic proteins PUMA, NOXA and BAX (Miyashita and Reed 1995; Shibue, Takeda et al. 2003; Chipuk, Bouchier-Hayes et al. 2005). The mechanisms by which p53 dictates cell fate outcome, whether it be cell cycle arrest or apoptosis, are thought to be mediated by a combination of promoter selection as well as stress-response magnitude and duration (Vousden and Prives 2009).

1.2.3.2 Mutant TP53

In cancers, truncation (frameshift or nonsense) mutations result in the loss of p53 protein expression and its tumor suppressive activity. Missense mutations clustered in the DBD of p53 (where six hotspot amino acids have been identified) can block interaction with MDM2/MDM4 and stabilize the protein (Muller and Vousden 2014). These mutations lead to loss of wildtype p53 activity. As p53 functions as a tetramer, these mutant p53s also act as dominant negative inhibitors of wildtype p53 and related proteins including p63 and p73, resulting in gain of novel transforming functions (Gaiddon, Lokshin et al. 2001). Mutant p53s are categorized as either structural mutants (such as R273H in humans, R270H in mice) which affect the global DBD structure, or contact mutants (such as R175 in humans, R172 in mice) which affect the residues that make direct contact with DNA (Olive, Tuveson et al. 2004; Muller and Vousden 2014). Not all p53 mutants are the same. For example, mice harbouring structural or contact p53 mutations develop a distinct spectrum of tumors (Olive, Tuveson et al. 2004).

1.2.3.3 Mutant TP53 in BC

Deletions or mutations in TP53 are frequent amongst all subtypes of BC, with higher frequencies observed in more aggressive forms: 12% in luminal A, 32% in luminal B, 75% in HER2 positive and 84% in basal-like (The Cancer Genome Atlas Network 2012). The type of p53 mutation also varies by subtype, with missense mutations predominating the luminal A, luminal B and HER2 positive groups, whereas both missense and truncation mutations are found at approximately equal percentage in basal-like BC (The Cancer Genome Atlas Network 2012).
1.2.4 Other tumor suppressors in BC

As described in section 1.1.2.6, large scale whole-genome analyses identified 93 genes that are frequently altered in BC, including (in order of decreasing frequency) TP53, PIK3CA, MYC, CCND1, PTEN, ERBB2/HER2, ZNF703/FGFR1, GATA3, RB1 and MAP3K1 (Ellis, Ding et al. 2012; Shah, Roth et al. 2012; Stephens, Tarpey et al. 2012; The Cancer Genome Atlas Network 2012; Lawrence, Stojanov et al. 2014; Nik-Zainal, Davies et al. 2016). Together, these 10 genes account for 62% of all driver mutations. The TP53 and PTEN tumor suppressors are described above. RB1 acts as a transcriptional co-factor that negatively regulates cell proliferation. In resting cells, pRB represses E2F-regulated genes through recruitment of chromatin modifiers, thereby restricting expression of genes required for cell cycle progression and mitochondrial protein translation (Jones, Robinson et al. 2016). At the G1/S transition, pRB is phosphorylated by cyclin-dependent kinases (CDKs). Hyperphosphorylated pRB then dissociates from E2F and allows cell cycle to progress (Sherr 1996; Nevins 2001; Dyson 2016). In BC, RB1 is lost or mutated in 20% of basal-like tumors (The Cancer Genome Atlas Network 2012). Other components of the RB1 pathway, including CCND1 (encodes Cyclin D1), CCNE1 (encodes Cyclin E), CDKN2A (encodes p16 and p14arf), CDK4 and CDK6 are also significantly altered in BC (The Cancer Genome Atlas Network 2012). Similar to PTEN loss, RB1 inactivation often occurs together with TP53 loss in TNBC; in animal models, Rb1 loss cooperates with p53 mutation leading to triple-negative like mammary tumors (Jiang, Deng et al. 2010; Jones, Robinson et al. 2016).

A number of other tumor suppressors are also found to harbor driver mutations in BC, albeit at lower frequencies, including NF1, FBXW7, RUNX1 and CBFB and NF1 encodes a GTPase activating protein (GAP) that converts Ras-GTPase to Ras-GDPase (Cichowski and Jacks 2001; Rad and Tee 2016). Upon NF1 loss, RAS becomes constitutively activated, leading to increased cell proliferation through the RAF/MEK/ERK pathway and enhanced survival through the PI3K/AKT pathway (Cichowski and Jacks 2001). In human BCs, NF1 is mutated in 2% of luminal A, 4% of luminal B and 2% of basal-like BCs (The Cancer Genome Atlas Network 2012). In a mouse transposon screen on a p53<sup>+</sup> background, Nf1 has been identified as a frequent insertion site in mammary tumors (Suarez-Cabrera, Quintana et al. 2017).
FBXW7 encodes an E3 ubiquitin ligase F-box protein that targets a broad network of substrates for degradation, including oncoproteins such as c-Myc, cyclin E and Notch (Davis, Welcker et al. 2014). In BC, FBXW7 is mutated in 2% of cases and FBXW7 promoter methylation is associated with high-grade tumors (Akhoondi, Lindstrom et al. 2010; Davis, Welcker et al. 2014). Additionally, FBXW7 may play important roles in the tumor microenvironment. In mouse models, reduced expression of FBXW7 in bone marrow-derived cells promoted orthotopic breast tumor growth and metastasis (Yumimoto, Akiyoshi et al. 2015). In BC patients, low levels of FBXW7 mRNA in the peripheral blood are associated with poor prognosis (Yumimoto, Akiyoshi et al. 2015).

RUNX1 and its binding partner CBFB are best known as cell fate regulators in hematopoietic stem cells (Okuda, Nishimura et al. 2001). Oncogenic rearrangement of RUNX1 and CBFB are frequently linked to hematopoietic diseases such as acute myeloid leukemia (AML) (Nimer and Moore 2004). Recent studies have implicated mutations in both RUNX1 and CBFB as drivers of luminal BCs. In particular, RUNX1 is mutated in 5% of luminal A, 2% of luminal B and 4% in HER2-positive BCs; CBFB is mutated in 2% of luminal A, 2% of luminal B and 2% of HER2-positive BCs (The Cancer Genome Atlas Network 2012). Mechanistically, RUNX1 acts as a tethering factor that recruits ERα to ER binding sites on target genes (Stender, Kim et al. 2010). RUNX1 also regulates the fate of ER⁺ luminal cells and deletion of RUNX1 leads to profound reductions in ER⁺ mature luminal cells (van Bragt, Hu et al. 2014).

1.3 Thesis rationale and outline

The heterogeneity of BC poses great challenges to our understanding of how myriad oncogenic alterations cooperate to influence cancer development and progression. An effective treatment for BC necessitates not only the identification of oncogenic drivers but also of subtype-specific, functionally cooperating genetic alterations. The hypothesis of this thesis is PTEN loss cooperates with other oncogenes/tumor suppressors to induce distinct BC subtype.

In Aim one, I discovered that the majority of Pten-null mammary tumors (i.e., adenomyoepithelioma, AME) are benign, lacking transplantable TICs that can thrive and form secondary tumors following FACS selection or short-term growth as tumorspheres. However, a rare group of Pten-null mammary tumors with distinct histology (i.e., poorly differentiated
adenocarcinoma, PDA) and similarity to basal-like BC, did contain transplantable TICs. To identify the genetic alterations within PDA tumors that lead to greater transplantability, I undertook comprehensive gene expression and sequencing analyses. Interestingly, I found that miR-143/145 expression is preferentially downregulated in aggressive mouse and human tumors including Pten-null PDAs, basal-like and luminal B human subtypes. Further, enforced disruption of miR-143/145 in nontransplantable Pten-deficient tumors promoted cell proliferation, tumor engraftment, RAS pathway activation and sensitivity to MEK inhibition. This study highlighted cooperation between PTEN loss and miR-143/145 loss in aggressive BC.

In Aim two, I characterized the oncogenic cooperation between Pten deletion cooperated with p53 dominant negative R270H mutation in mammary tumors. I found that Pten\textsuperscript{Δf}:p53\textsuperscript{R270H} mice develop mammary tumors with greater penetrance and shorter latency than single mutant mice. Tumors from Pten\textsuperscript{Δf}:p53\textsuperscript{R270H} mice were diverse and included tumors resembling basal-like and claudin-low-like BC. Pten\textsuperscript{Δf}:p53\textsuperscript{R270H} tumors displayed distinct expression profiles relative to Pten\textsuperscript{Δf}:p53\textsuperscript{Δf} tumors. Finally, Pten\textsuperscript{Δf}:p53\textsuperscript{R270H} tumor cells were more capable of undergoing mesenchymal-to-epithelial transition (MET) at metastatic secondary sites than Pten\textsuperscript{Δf}:p53\textsuperscript{Δf} tumor cells, leading to greater capacity to induce metastatic lesions and morbidity.

Overall, this thesis dissects cancer heterogeneity and distills cooperating oncogenic drivers, showing how different mutations can cooperate with Pten loss to influence different aspects of breast malignancies, including cancer development, progression and response to therapy.
Chapter 2

Materials and Methods

2.1 Animals

Animal protocols were approved by the University Health Network in accordance with guidelines from the Canadian Council of Animal Care. Mice used in this study were on mixed genetic background (FvB, C57BL/6 and 129/sv): WAP-Cre mice were kindly received from Dr. Lothar Hennighausen, NIH, Pten<sup>fl</sup>/mice and were obtained from Dr. Tak Mak, PMCC and p53<sup>R270H</sup> mice generated by Dr. Tyler Jacks, MIT and obtained from the National Cancer Institute (NCI) Mouse Models of Human Cancers Consortium (MMHCC).

2.2 PCR genotyping

Deletion of the Pten<sup>fl</sup> allele was detected by PCR using the following primers: Forward 5’CTCCTCTACTCCATTCTTCCC3’, Reverse 5’ACTCCCAACCAATGAACAAAC3’, where Pten<sup>fl</sup> results in a 335 bp product and wildtype results in a 228 bp product. Pten recombination was detected using the following primers: Forward 5’GTCACCAGGATGCTTCTGAC3’, Reverse 5’ACTATTGAACAGAATCAACCC3’, where Pten<sup>Δfl</sup> resulted in an 849 bp product. For p53<sup>R270H</sup> recombination detection, the following primers were used to yield a wild type band of 290bp and mutant allele of 330bp: wildtype Forward: 5’TTACACATCCAGCCTCTGTGG3’; mutant Forward: 5’AGCTAGCCACCATGGCTTGAGTAAGTCTGCA3’; Reverse: 3’CTTGGAGACATAGCCACACTG3’. To determine the presence of the LSL cassette in p53, the following primers were used to generate a wild type band of 166bp and mutant allele of 270bp: Forward: 5’AGCCTGCCTAGCTTCCTCAGG3’; Reverse: 5’CTTGGAGACATAGCCACACTG3’

2.3 Primary cell isolation and enrichment of lineage-negative (Lin-) cell population

Mammary gland tissue (lymph node removed) or tumor tissues were minced with a sterile razor blade and placed in 100 U/ml collagenase/hyaluronidase solution (StemCell Technology, #07912) for overnight (~15 hours) at 37ºC and 4ºC, respectively. Tumor tissues were also
incubated at 37°C for 30 minutes before processing. Digested tissues were then rinsed with Hanks Balanced Salt Solution (GIBCO) containing 2% FBS (HF) and centrifuged at 350 x g for 10 minutes (mammary tissue) or 1500 rpm for 5 minutes (tumor tissue). For mammary tissue, contaminating red blood cells were lysed in a 1:4 mixture of HF and ammonium chloride (NH₄Cl), further digested with 5mg/ml dispase containing 0.1mg/ml DNaseI (Sigma) and 0.5X trypsin (0.25%), and then passed through a 40 mm cell strainer (BD Falcon). Tumor cells were passed directly through a 40 nm cell strainer without further digestion. Selective depletion of endothelial (anti-CD31, BD PharMingen) and hematopoietic cells (anti-CD45 and anti-TER119, StemCell Technologies) was accomplished at 4°C with magnetic beads using an EasySep™ Mouse Epithelial Cell Enrichment Kit (StemCell Technologies, #19757) and EasySep™ Magnet.

2.4 Tumorsphere culturing in vitro, primary cell line creation and long-term cell labelling

Cell suspensions of lin- mammary tumor epithelial cells were plated onto ultra-low attachment plates (Corning, Costar #3471) in DMEM/F-12 HAM medium (Sigma #D8900) containing 20 ng/ml bFGF (Sigma # F0291), 20 ng/ml EGF (Sigma #E9644), 4 µg/ml of Heparin (Sigma #H4784) and B-27 supplement (1:50 dilution, GIBCO, #17504-044), and cultured at 37°C; 5% CO₂. Spheres were mechanically and enzymatically dissociated weekly in 0.25X (0.05%) Trypsin-EDTA solution (GIBCO #25200) for 3 min at 37°C, followed by gentle trituration by pipette for 1 minute. To create tumor-derived cell lines, tumorspheres were plated on collagen-coated slides and expanded in adherent fashion. To achieve long-term cellular labeling, cells were incubated with SP-DiIC18 (ThermoFisher #D7777) following manufacturer instructions.

2.5 Lentivirus generation and generation of stably expressed and knockdown cell lines.

Lentiviral constructs of mir-143/145 inhibitor and control vectors were kindly gifted by Dr. Aly Kastan, University of British Columbia. To generate lentivirus, mir-143/145 decoy or control vector were cotransfected with second generation lentiviral packaging plasmids (pCMV-R8.74psPAX2 and pMD2.G) into 293T cells by Lipofectamine 2000 following manufacture’s instructions. At 48 and 72 hours post transfection, culture medium was harvested and concentrated by Lenti-X Concentrator (Clontech, #631231). Lentivirus titer was determined by serial dilution of virus-expressed EGFP expression. To create stable lines, mir-143/145 decoy or control lentivirus were incubated with target cells in the presence of polybrene (8ug/ml) for 24
hours. At 72 hours post infection, cells were selected for puromycin or sorted for GFP positive fraction using BD FACS Aria 11-color instruments (Aria GC BRU or Aria II RITT BRV) at 20 PSI (The SickKids-UHN Flow Cytometry Facility).

2.6 Fluorescence-activated cell sorting

Primary mouse mammary epithelial and tumor cells were suspended in HF at 1x10^7 cells/ml and incubated with the following antibodies on ice for 30 minutes: anti-CD49f conjugated with R-phycoerythrin (CD49f-PE, clone GoH3, 1:100, BD Pharmingen), anti-CD24 conjugated with fluorescein isothiocyanate (CD24-FITC, clone M1/69, 1:200, BD Pharmingen) and 2ug/ml Propidium Iodide (BD Pharmingen). Labeled cells were then washed 3x in HF, resuspended in HF at 8 x10^6 cells/ml and kept on ice until analysis. Single (fixed FSC-A/FSC-W ratio) and live cells (PI-negative) were gated for analysis and sorting. Cell sorting experiments were carried out using BD FACS Aria 11-color instruments (Aria GC BRU or Aria II RITT BRV) at 20 PSI (The SickKids-UHN Flow Cytometry Facility) while flow cytometry was performed on a BD FACS Calibur 4-color instrument.

2.7 Orthotopic transplantation and tail vein injection

Cells were transplanted into #4 mammary glands of 3-5 week old immune-deficient NODSCID female mice. Indicated number of cells were resuspended in 10 µl of media and mixed at 1:1 ratio with 10 µl matrigel (BD Bioscience #356234) on ice. Samples (total 20µl) were then injected into mice under isoflurane anesthesia. Mice were monitored for tumor formation for up to six months. In tail vein injections, tumor-derived cell lines were dissociated into single cells and injected (200ul of 1x10^5 cells/100ul resuspended in DMEM media) into the lateral tail vein.

2.8 Histology, immuno-histochemistry and immuno-fluorescence staining

Paraffin sections of 5-µm thickness were dewaxed in xylene and rehydrated in decreasing concentrations of alcohol. After citrate buffer antigen retrieval, sections were blocked for 1 hour and incubated overnight with primary antibody. Antibodies were p53 (1:200 dilution; FL-393; Santa Cruz Biotechnology Inc. #sc-6243;), Ki67 (1:150 dilution; Biocare Medical # CRM325, clone SP6), SMA (1:200 dilution; Novus Biologics #600-531), CK6 (1:200, Covance, #PRB-169B), CK5 (1:20; NeoMarkers, #XM26), K18 (1:200 dilution; Fitzgerald #RDI-PR061028),
and CK14 (1:200 dilution; Panomics #E2624), vimentin (1:50 dilution; Santa Cruz Biotechnology Inc). Secondary biotinylated anti-mouse or anti-rabbit antibodies (Vector) were used at 1:200 dilution. IHC was performed using VECTASTAIN ABC Systems (Vector laboratories). Sections were counterstained with methylene green.

2.9 Western Analysis

Mammary tumor tissues or cultured cells were lysed with ice-cold RIPA buffer (PBS, 1% NP 40, 0.5 % (w/v) sodium deoxycholate, 0.1 % (w/v) sodium dodecyl sulfate (SDS), 1mM phenylmethylsulfonyl fluoride (PMSF), 60 μg/ml aprotinin, 5 mM DTT) in a Dounce homogenizer and immunoblotted as described (Liu et al, 2012). Blots were then incubated at 4°C overnight with antibodies against Ras 1:1000, Cell Signaling Technology #8955), p-erk (Thr202/Tyr204, Cell Signaling #9101), tubulin (1:3000, Cell Signaling Technology), Erk (1:1000, Cell Signaling Technology, #9102) diluted with 1% BSA in PBS, followed by anti-rabbit IgG HRP antibodies (1:3000, Cell Signaling) for 1 hour at room temperature. Chemiluminescence was detected using Super Signal West Dura (Pierce).

2.10 RNA isolation, real-time PCR and microarray

Total RNA was extracted using TRIZOL reagent (Invitrogen, Burlington, ON, Canada). Microarray analysis was carried out using Affymetrix Mouse Gene 1.0 ST with 500 ng of total RNA (Centre for Applied Genomics, Hospital for Sick Children, Toronto). Microarray data were normalized using RMA method via Partek and log2-transformed gene expression values obtained as described (Liu, Voisin et al. 2012). To compare our mouse models with human BC and other published models, median-centered values of our mouse microarray data were integrated with GSE18229 (human BC) and GSE3165 (mouse models) by “Distance Weighted Discrimination” (DWD). Subtypes of human BCs in GSE18229 were pre-determined with PAM50 and the claudin-low signature (Herschkowitz, Simin et al. 2007; Prat, Parker et al. 2010; Liu, Voisin et al. 2014). The DWD-integrated samples were classified by unsupervised hierarchical clustering (complete linkage) using shared intrinsic genes, the basal, luminal, and mesenchymal gene sets (Herschkowitz, Simin et al. 2007), and the claudin-low signature (Prat, Parker et al. 2010). Values of gene expression were log2-transformed, median-centered, and visualized as heatmaps by Java TreeView.
2.11 Quantitative RT-PCR
RNA was extracted with mirVana™ miRNA Isolation Kit (ThermoFisher, #AM1560), following manufacturer’s instructions. miR-143 and miR-145 expressions were quantified using Taqman primers (ThermoFisher, #4427975) relative to 18s (ThermoFisher, #4333760).

2.12 DNA copy number and exome sequencing
Mouse SurePrint G3 1M arrays were analyzed at The Centre for Applied Genomics (operated by the Hospital for Sick Children) using tail DNA pooled from WapCre:Pten\textsuperscript{flo/flo} mice as reference and Partek® Genomics Suite™ (Partek). Exome sequencing was performed on Illumina HiSeq at McGill University and Genome Quebec Innovation Centre.

2.13 Bioinformatics
Microarray analysis with mouse tumor models was carried out using Affymetrix Mouse Gene 1.0 ST with 500 ng of total RNA isolated by double Trizol extractions (Centre for Applied Genomics, Hospital for Sick Children, Toronto). Microarray data were normalized using RMA method via Partek software and log2-transformed gene expression values obtained.

2.14 Gene Set Enrichment Analysis
Gene expression data were analyzed using GSEA Preranked method from the Broad Institute (version 2.2.0, (Subramanian, Tamayo et al. 2005)) with parameters set to 1000 gene-set permutations. Genes were ordered using the “limma” package in R to obtain t-values corresponding to each pair-wise comparison with Benjamini and Hochberg's method to control the false discovery rate. The gene-sets included in the GSEA analyses were obtained from MSigDB containing BioCarta, KEGG, Matrisome, Pathway Interaction Database, Reactome, SigmaAldrich, Signaling Gateway, Signal Transduction KE, & SuperArray gene sets (c2, v5.0) and Gene Ontology (GO) databases (c5, v5.0), updated on March 2012 (http://www.broadinstitute.org/gsea/downloads.jsp). An enrichment map (version 1.1 of Enrichment Map software (Merico, Isserlin et al. 2011), was generated by Cytoscape (version 2.8.3) for each comparison using enriched gene-sets with a nominal p-value < 0.005, FDR < 0.1, and overlap coefficient set to 0.5.
2.15 Preswick Drug Screening

HTS of 238 compounds targeting 154 different kinases were screened using a robotic Biomek FX liquid handler equipped with a pintool for automated compound dispensing. Assays were carried out in a 384-well format, using 300 cells/well. Compounds, resuspended in DMSO as 1mM stock solutions, were added in a volume of 200 µl to 3µM final concentration. Cell viability was monitored by incubation with Alamar Blue (Life Technologies, #DAL1025) at 10x dilution for 4 hours, and read by a FLUOstar Optima reader (BMG Labtech) at 590nm emission wavelength with 530-560nm excitation wavelength. As a reference for 100% activity, each plate included 32 wells with cells treated with vehicle only, and background was measured with media in the absence of cells.

2.16 MTT viability assay

Cells were seeded in 96-well plates and treated the following day. At 3 days post-treatment, 20 µL of 2 mg/mL MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide, Sigma-Aldrich) were added and incubated for 4 h. MTT/media solution was aspirated and replaced with DMSO and cell viability read after 5 minutes incubation.

2.17 Additional Statistical Analysis

Chapter 3 Parametric paired samples were analyzed by Student t-test; non-parametric paired samples by MannWhitney test. Significance of comparing multiple samples was calculated using ANOVA and the Tukey test for post hoc analysis. Kaplan-Meier and Survival analysis were performed using GraphPad Prism, and p-value calculated using the Wilcoxon method. Differences between values were considered statistically significant at p< 0.05. TIC frequency and 95% confidence intervals were calculated using L-Calc (www.stemcell.com).
Chapter 3

Results presented in this chapter have been submitted for publication:

Note:
- Dr. Jeff C. Liu and Dr. Rajwinder Lehal contributed to Table 3-1
- Dr. Jeff C. Liu, Dr. Giovanna Pellecchia, Dr. Veronique Voisin and Dr. Dong-Yu Wang contributed Figures 3-5A-C, 3-6, 3-7, 3-10E-G, 3-14
- Dr. YoungJun Ju contributed Figure 3-12F-G

3 microRNA-143/145 loss cooperates with Pten-deficiency to promote an aggressive subgroup of basal-like breast cancer

3.1.1 Abstract

The tumor suppressor PTEN is frequently inactivated in breast and other cancers; yet, germ-line mutations in this gene induce non-malignant hamartomas, indicating dependency on additional cooperating events. Here we show that most tumors derived from conditional deletion of mouse Pten in mammary epithelium are highly differentiated and lack transplantable tumor initiating cells (TICs) capable of seeding new tumors following orthotopic injection of FACS-sorted or tumorsphere cells. A rare group of poorly differentiated tumors did harbour transplantable TICs. These transplantable tumors exhibited distinct histology and molecular classification, signaling pathways, chromosomal aberrations and mutational landscape, as well as reduced expression of microRNA-143/145. Stable knockdown of miR-143/145 conferred tumorigenic potential upon poorly transplantable Pten-deficient tumor cells through a mechanism involving induction of RAS signaling, leading to increased sensitivity to MEK inhibition. In humans, combined PTEN/miR-145 deficiency occurs primarily in basal-like breast cancer leading to poor clinical outcome. These results underscore selective pressure for combined loss of PTEN and miR-145 in aggressive forms of breast cancer, and a need to identify and prioritize these tumors for aggressive therapy.

3.2 Introduction

Precision medicine would benefit from identification and targeting of not only oncogenic alterations but also cooperating oncogenic events. Breast cancer (BC) is a highly heterogeneous disease, comprising Estrogen Receptor alpha (ERα)-positive and HER2/ERBB2/NEU-positive
tumors, as well as triple-negative BCs (TNBCs) that do not express ERα, HER2 or the progesterone receptor. Molecular classification identifies luminal A and luminal B subtypes, which overlap with ERα+ BC, and basal-like, claudin-low/mesenchymal-like and other subtypes, which overlap with TNBC (Perou, Sorlie et al. 2000; Herschkowitz, Simin et al. 2007; Prat, Parker et al. 2010; Prat, Parker et al. 2012). Recent exome and whole genome sequencing, copy number variation, mRNA and proteomic analyses of thousands of BC samples representing all major subtypes identified 93 affected oncogenes and tumor suppressors, only nine of which are frequently altered including PTEN (phosphatase and tensin homolog deleted on chromosome ten) (Curtis, Shah et al. 2012; The Cancer Genome Atlas Network 2012; Nik-Zainal, Davies et al. 2016). PTEN is lost in 25-35% of luminal B and TNBC through multiple mechanisms including promoter silencing and microRNA-mediated suppression (Salmena, Carracedo et al. 2008; The Cancer Genome Atlas Network 2012).

Accumulating evidence suggests that the effect of PTEN loss is context-specific. In PTEN Hamartoma Tumor Syndromes (PHTS) like Cowden and Bannayan-Riley-Ruvalcaba, inherited autosomal mutations in PTEN lead to non-cancerous tumor like growths called hamartomas (Hobert and Eng 2009). In mouse models, prostate-specific inactivation of Pten induces benign tumors with increased senescence, which is bypassed by concomitant inactivation of the tumor suppressor p53 (Chen, Trotman et al. 2005; Ahmad, Patel et al. 2011). While Pten deletion in mouse mammary epithelium leads to tumor formation (Liu, Voisin et al. 2014), little is known about the tumorigenic potential of these lesions or of cooperating oncogenic alterations that induce malignant transformation. Identification of such cooperating oncogenic alterations may uncover a new subgroup of patients that may benefit from specific targeted therapy.

PTEN, a lipid and protein phosphatase, is primarily tethered to plasma and endosomal membranes where it antagonizes the Phosphatidylinositol 3-kinase (PI3K) pathway by dephosphorylating the lipid second messenger phosphatidylinositol 3,4,5-trisphosphate (PIP₃) (Salmena, Carracedo et al. 2008; Naguib, Bencze et al. 2015), thereby controlling a host of cellular processes including proliferation, survival and migration. In addition, nuclear localized PTEN regulates centromere stability, DNA-damage response, cell-cycle progression and cellular senescence (Shen, Balajee et al. 2007; Hanahan and Weinberg 2011; Song, Carracedo et al. 2011). Alternative variants (PTENlong and PTENα) are secreted or exported into the

MicroRNAs (miRNAs) are 18-24 nucleotide non-coding RNAs that target multiple mRNAs by interfering with protein translation, and often, though not always, with mRNA stability (Esteller 2011). Mouse miR-143 and miR-145 are separated by ~1.4 kb on chromosome 18 and are derived from the long noncoding RNA MIR143HG (MIR143 host gene, CARMEN) (Xin, Small et al. 2009). This miRNA cluster is conserved across vertebrates and has emerged as an important tumor suppressor involved in regulation of proliferation, metastasis, pluripotency, differentiation and angiogenesis (Cui, Wang et al. 2014). In BC, low miR-145 expression is associated with a high proliferative index, larger tumor size, lymph node metastasis, and lack of ERα and HER2 expressions (Iorio, Ferracin et al. 2005; Min, Wang et al. 2014). However, the oncogenic context in which miR-143/145 is lost, and cooperating oncogenic alterations are not fully understood.

To define the role of PTEN in BC and identify its cooperating oncogenic partners, we hereby interrogated mouse models with targeted deletion of Pten in mammary epithelium. We discovered that Pten inactivation induces indolent mammary tumors that, contrary to multiple other mouse models of BC, lack tumorigenic potential following orthotopic transplantation of sorted tumor cells or of tumorsphere cells transiently propagated under non-adherent conditions. Intriguingly, a group of rare Pten-deficient tumors with features of basal-like BC did harbour highly transplantable TICs. These transplantable tumors exhibited reduced expression of miR-143/145. Knockdown of miR-143/145 in poorly-tumorigenic Pten-deficient tumors conferred tumor-initiation potential through activation of Ras signaling. A subset of basal-like BC patients exhibits reduced expression of both PTEN and miR-145, and poor clinical outcome. Our results underscore an important cooperation between PTEN and miR-145 loss, and suggest that such tumors should be identified and targeted as a distinct subgroup.
3.3 Results

3.3.1 Most Pten-deficient mammary tumors are not transplantable following cell sorting or short-term propagation as tumorspheres

To determine the effect of Pten loss on BC, we deleted Pten using a floxed allele (Pten\textsuperscript{f/f}) (Suzuki, de la Pompa et al. 1998) and the deleter lines MMTV-Cre\textsuperscript{NLST}, which targets stem/bi-potent progenitor cells, or WAP-Cre, which targets CD24-positive, pregnancy-identified stem cells/alveolar progenitors (Wagner, Boulanger et al. 2002; Jiang, Deng et al. 2010). As we previously described, tumors from these models were heterogeneous, consisting primarily of adenomyoepithelioma (AME) and other well-differentiated subtypes (Liu, Voisin et al. 2014). Formation of secondary tumors following engraftment into host mice is a major hallmark of tumor aggressiveness (Kreso and Dick 2014). To identify tumor initiating cells (TICs), we performed FACS sorting and transplantation assays. In these experiments, mammary tumors were dissociated into single cells using collagenase digestion, lineage depletion, sorted on the basis of specific cell surface markers, serially diluted, mixed with matrigel and transplanted into the mammary gland of young, immuno-compromised mice. In previous studies, we identified TICs in mouse models of Her2/Neu, Wnt1, mammary-specific Rb-loss, p53-loss, Rb/p53- and Pten/p53-double mutants (Liu, Deng et al. 2007; Liu, Voisin et al. 2014; Jones, Robinson et al. 2016).

Remarkably, transplantation of as many as tens of thousands MMTV-Cre:Pten\textsuperscript{f/f} (n=8) or WAP-Cre:Pten\textsuperscript{f/f} (n=7) tumor cells sorted into single 7AAD\textsuperscript{−} (total live), CD24\textsuperscript{+}:Sca1\textsuperscript{+} or CD24\textsuperscript{+}:CD49f\textsuperscript{+} populations failed to induce secondary tumors (Table 3-1). These experiments were performed side by side with other models such as MMTV-Her2/Neu, which readily gave rise to secondary tumors even when only a few hundred sorted cells were transplanted into mammary fat pads of immunocompromised Rag1\textsuperscript{−/-} recipient mice. Injection of small pieces of MMTV-Cre:Pten\textsuperscript{f/f} or WAP-Cre:Pten\textsuperscript{f/f} tumors, or 10\textsuperscript{4} cells from collagenase-digested lysate (crude) or lineage depleted (Lin\textsuperscript{−}) cells did induce secondary tumors after 10-12 weeks (Table 3-1A). The latter tumors maintained the same histology as the primary tumors from which they were derived (not shown). The failure of sorted Pten\textsuperscript{Δf} tumor cells to seed new tumors was unlikely due to immune-rejection because secondary tumors did not develop even after injecting sorted cells from 6 independent MMTV-Cre:Pten\textsuperscript{f/f} tumors into divergent immuno-compromised
mice including Rag1\(^{-}\), SCID/Beige, NOD/SCID, NOD/SCID/\(\gamma\)C\(^{-}\), Rag2\(^{-}\)/\(\gamma\)C\(^{-}\) or young MMTV-Cre:Pten\(^{ff}\) recipient mice (self/original background; Table 3-1B).

We also generated tumorspheres from \(Pten\)-deficient tumors by seeding lineage-depleted (Lin\(^{-}\)) tumor cells onto ultra-low attachment plates in serum-free media (Liu, Deng et al. 2007; Jiang, Deng et al. 2010). Tumorspheres were observed after ~7 days and could be propagated through light trypsinization. Transplantation of 8 independent MMTV-Cre:Pten\(^{ff}\) and WAP-Cre:Pten\(^{ff}\) tumorspheres also failed to produce secondary tumors. Thus, for most or all \(Pten\)-deficient
Table 3-1 Most *Pten*-deficient mammary tumors are not transplantable following cell sorting or short propagation as tumorspheres

A. TIC frequency in WAP-Cre:*Pten*<sup>fl/fl</sup> and MMTV-Cre:*Pten*<sup>fl/fl</sup> mammary tumors following orthotopic transplantation of indicated cells into NOD/SCID mice

B. TIC frequency of *Pten*-deficient mammary tumor following cell sorting and orthotopic transplantation into same (self) or indicated strains of immune-comprised mice
## A

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</tr>
<tr>
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<td>7 / 16 6 / 26</td>
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</tr>
<tr>
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<tr>
<td>Scal</td>
<td>--</td>
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<td>--</td>
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## B

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<td>0 / 2</td>
<td>0 / 2</td>
</tr>
</tbody>
</table>
mammary lesions, TICs can survive only when transplanted as bulk, but lose tumorigenic potential when transplanted as single cells following cell sorting or propagation as non-adherent tumorspheres.

3.3.2 A fraction of Pten-deficient mammary tumors with distinct histology contains resilient TICs

To understand the basis for scarcity of sorted TICs in Pten-deficient mammary tumors, we focused on WAP-Cre:Pten^fl/fl mice, which gave less heterogeneous types of tumors than MMTV-Cre:Pten^fl/fl mice. Tumor biopsies were fixed for histology or dissociated into single cell suspension, cultured as tumorspheres and then either frozen for future analysis or engrafted into recipient mice to detect TICs. Remarkably, after assessing tumorspheres from 21 independent tumors, we identified TICs in two. After decoding the histology of these tumors, we discovered that all non-transplanted tumors were AMEs, whereas both transplantable tumors were poorly differentiated adenocarcinomas (PDAs; Figure 3-1A). We then retrospectively screened tumors/tumorspheres for PDA histology and identified 6 additional PDAs. Transplantation of each of these 6 tumors gave rise to secondary tumors (Figure 3-1B). Deletion of the Pten allele was confirmed by PCR analysis of transplantable and non-transplantable tumors (Figure 3-1C). In all, ~81% of the tumors were non-transplantable AMEs, 3.85% were non-transplantable tumors of mixed histology, and 6.41% were transplantable PDA (Figure 3-1D). Critically, PDAs could be serially transplanted, with each successive generation giving rise to tumors with exceedingly short latency (Figure 3-1E-F, p<0.001).

There were no gross phenotypic differences between tumorspheres derived from PDAs versus AMEs (Figure 3-1G, n=3). In flow cytometry profiles, PDA tumor cells contained a prominent CD24⁺:CD49⁺ double positive fraction, while AME tumor cells showed both a double positive fraction and a CD24⁻:CD49⁺ single positive fraction fraction and a CD24⁺:CD49⁺ single positive fraction (Figure 3-1H). In addition, IHC analyses showed PDA tumors to be highly proliferative (Ki67⁺), and negative for ERα and HER2, suggestive of TNBC-like tumors (Figure 3-1I, Figure S1). Furthermore, PDAs were generally negative for the differentiation markers cytokeratin 14 (K14) and 18 (K18), but positive for cytokeratin 5 (K5, basal BC marker; 83% of n=6) and vimentin (EMT marker, 50% of n=6), respectively. In contrast, AMEs were less proliferative,
ERα positive and HER2 negative (Figure 3-11, Figure 3-2AB). In addition, AMEs exhibited high levels of both K14 and K18, and lower expression of K5 and vimentin, indicative of luminal

Figure 3-1 A small fraction of Pten-deficient mammary tumors is transplantable

A. Representative histology of adenomyoepithelioma (AME) and poorly differentiated adenocarcinoma (PDA) tumors arising from WAP-Cre:Pten^{fl/fl} mice.
B. Summary of tumor initiating potential of AMEs and PDAs.
C. Detection of Pten gene deletion in AME and PDA tumor cells by PCR using primers specific for the Cre-excised Pten^{fl} allele.
D. Distribution of tumor types (%) in WAP-Cre:Pten^{fl/fl} mice: AMEs, PDAs as well as mixed types (other). n=120.
E. Kaplan-Meier mammary tumor-free curves for WAP-Cre:Pten^{fl/fl} mice. Median survival=378 days, n=289.
F. Kaplan-Meier mammary tumor-free curves for secondary and tertiary tumors arising from PDAs. PDA tumor cells were serially transplanted; tumor latency was significantly shortened with each generation (p<0.001, Wilcoxon Test). Secondary: n=37, median survival=81.5 days, Tertiary: n=18, medial survival=35.5 days.
G. Representative images of tumorspheres from AME and PDA tumors showing no obvious morphological differences.
H. Flow cytometry profiles of AMEs and PDAs using the CD24 and CD49f cell surface markers. AME tumors contain both a CD24 low / CD49f high, and a CD24 high / CD49f high populations. PDA tumors exhibit a single CD24 high / CD49f high cell population.
I. Immunohistochemistry analyses of AMEs and PDAs for ERα, basal (K14, K5), luminal (K18), mesenchymal (vimentin) and proliferation (Ki67) markers. AMEs are ERα positive, HER2-negative, exhibit varied expression of K5 and vimentin, and high levels of differentiation markers K14 and K18. In contrast, PDA tumors are negative for ERα and HER2, with varied expression of K5 and vimentin, high levels of Ki67 and K18, and very low K14 expression. n=9.
like tumors. Consistent with the ERα positive status of AME tumors, they grew much faster *in vitro* in the presence of β-Estradiol (Figure 3-2C). Thus, *Pten*-deficient PDAs were highly proliferative poorly-differentiated TNBC-like tumors with a high frequency of transplantable TICs. In contrast, AMEs were highly-differentiated, ERα positive luminal like tumors that lack TICs that can seed new tumors in host mice after FACS selection or non-adherent growth.

### 3.3.3 Transplantable PDA but not non-transplantable AME cells continue to proliferate following orthotopic transplantation

To trace the fate of AME and PDA cells following transplantation, we labeled tumor cells with the lipophilic carbocyanine dye SP-DiIC$_{18}$(3). This dye fluoresces weakly in water but is induced to strongly fluoresce over a sustained period of time when incorporated into cellular membranes (Figure 3-3A-B). SP-DiIC$_{18}$(3)-labeled AME and PDA tumor cells were followed for over 46 days post injection (p.i.). By day 3 p.i., AME cells formed ductal structures expressing K14 and K18 (Figure 3-3C-D). However, these ductal structures failed to maintain proliferation and were cleared by day 18 p.i. (Figure 3-3C, Figure 3-4). This indicates that AME cells are unable to colonize foreign transplant sites, possibly due to dependency of ERα$^+$ tumor cells on estrogen or permissive microenvironment (Al-Hajj, Wicha et al. 2003; Sfomos, Dormoy et al. 2016), and instead undergo attrition over 18 days. In contrast, PDA cells formed multi-focal growth by day 3 p.i. These foci were highly proliferative relative to AME cells (Figure 3-3E, p<0.02; Figure 3-4) and continued to grow into full-fledged secondary tumors by day 46 p.i. (Figure 3-3C).

### 3.3.4 Distinct genetic alterations including reduced expression of microRNA145 in *Pten*-deficient PDAs versus AMEs revealed through analysis of mRNA and miRNA profiling, copy number variation and mutation

To molecularly classify AMEs and PDAs, we compared their transcriptomic landscape to other mouse models using an intrinsic BC signature and unsupervised hierarchical clustering (Herschkowitz, Simin et al. 2007). Normal mammary gland tissues and MMTV-Her2/Neu tumors were included as internal controls (Figure 3-5A-B). Most AMEs clustered with wild-type mammary glands. In contrast, transplantable PDAs clustered with the much more aggressive model of MMTV-Her2/Neu mouse mammary tumors. However, we note that the *Pten*-deficient PDAs do not express HER2 (Figure 3-1I). We also performed clustering analysis with human BC samples. Whereas AMEs grouped together with “normal”-like BC, most PDAs clustered
with basal-like human BC. We attribute the distinct clustering with mouse Her2/Neu vs human basal-like tumors to the intrinsic difficulty in batch comparison and interspecies analyses. More importantly, however, our classification analysis is consistent with the more aggressive nature of Pten-deficient PDAs and their higher tumor initiating potential in xenograft assays as compared to AMEs.

We next sought to pinpoint the oncogenic networks that cooperate with Pten loss to induce aggressive PDAs. Gene Set Enrichment Analysis (GSEA) visualized with “Functional Enrichment Maps” (Merico, Isserlin et al. 2010), revealed that pathways associated with immune response, migration and myogenesis were downregulated in PDAs as compared to AMEs (Figure 3-5C, Figure 3-6). Similar results were observed following g:Profiler analysis, where pathways associated with immune response, migration, adhesion and organ system development were differentially regulated (Figure 3-7).

Array comparative genomic hybridization (aCGH) revealed that Pten-null AMEs and PDAs exhibited relatively quiet genomes with similar landscapes of gains and losses (Figure 3-5D). An exception is chromosome 12, which we identified as a hotspot for differential DNA copy number aberrations. Overall, only 13 genes showed significant copy number changes in PDA compared to AMEs. These included gains in Ano2, Klra1, Atp6v1d and Ncapg2, and losses in Lyrm4, Pnp2 and Prune2, and putative genes Gm694, Igh-A (1g2), Gm16844, Igh, Ighg and V00821 (Figure 3-5D, p<0.05, differential threshold 25%; Figure 3-8). Notably, mouse insertional mutagenesis screens indicate Ano2 and Atp6v1d are cancer driver genes (Forbes, Beare et al. 2015). In humans, Ano2 shows copy number gains in 1.26% and overexpression in 4.53% of breast carcinomas (Forbes, Beare et al. 2015). Klra1 displays copy number gains in 1.15% of cases. Further analysis of these alterations in combination with Pten loss is required to assess their effect on PDA formation and TIC frequency.

To identify mutations in coding sequences, we performed exome sequencing on seven independent PDAs and three AMEs. This analysis identified PDA specific, recurrent mutations in ubiquitin ligases Cul9 and Cbl, microtubule motor Kif6, autophagy regulators Tfëb and Tbc1d5 and others (Figure 3-5E). The tumor suppressor Cul9 is a component of an E3 ubiquitin ligase that regulates subcellular localization of p53 and maintains genome integrity (Pei, Bai et
al. 2011; Li, Pei et al. 2014). The ubiquitin ligase Cbl attenuates RTK signaling by mediating ubiquitination and degradation of activated RTKs. However, point mutations in Cul9 occurred at
Figure 3-2 IHC and proliferation analysis of Pten-deficient mammary tumors

A. Induced expression of estrogen receptor α (ERα) in AME vs PDA tumors. p=0.0005
B. Induced expression of Ki67 in PDAs vs AMEs. p=0.0031
C. In vitro growth curves of independent AME tumor cell lines incubated with indicated concentrations of β-estradiol. *p<0.05, **p<0.01; ***p<0.001
Figure 3-3 AME tumor cells undergo attrition and fail to proliferate following orthotropic transplantation

A. Representative images of tumor cells 5 days post labelling with SP-DiIC18.

B. Representative images of a mammary gland 18 days post injection (p.i.) with SP-DiIC18-labelled tumor cells. Arrow indicates site of injection. Fluorescent dye is retained over time in vivo.

C. Representative histological images of mammary glands injected with AME or PDA tumor cells over 46 days. Injected PDA cells formed multi-focal growth at day 3 p.i., which continued to grow (arrows) while AME cells formed ductal structures at day 3 p.i., which disappeared by 18 p.i.

D. IHC analysis of basal (K14) and luminal (K18) differentiation markers in ductal-like structures transiently formed by injected AME cells.

E. Quantification of phospho-H3 positive cells following cell transplantation, showing increased proliferation of PDA but not AME cells.
Figure 3-4 Proliferation and apoptosis analysis of AME and PDA cells following orthotopic transplantation into NOD/SCID mice
Shown are representative IHC images of phospho-H3, Ki67 and activated caspase 3 of mammary glands injected with AME or PDA cells at indicated time points.
the C-terminus, downstream of known functional domains, whereas the point mutations in \textit{Cbl} are not found in equivalent locations in human cancers; a mutation prediction software (Pmut) suggests that none of these changes are likely pathological (\textbf{Figure 3-9}). Mouse insertional mutagenesis screens identified Tmcc1, Cbl, Tfeb Tango6, Fam35a and Tbc1d5 as cancer driver genes (Forbes, Beare et al. 2015). In humans BC, Lyrm4, Tmcc1, Cul9, Fam35a, Garl, Kif6, Slc29a1 and Tbc1d5 are overexpressed in more than 4% of cases (Forbes, Beare et al. 2015). We note that no mutations on the RAS pathway such as \textit{Nf1}, \textit{Dusp4}, \textit{Rasal1}, \textit{Rasal2} or \textit{Rnd1}, or in \textit{p53} have been detected.

Importantly, RNA profiling identified miR-145 and miR-143 to be downregulated by 7.4 and 1.7 folds in PDAs relative to AMEs, respectively (\textbf{Figure 3-10A}, \(p<0.0001\)). Q-RT-PCR confirmed that miR-145 was also downregulated 6.3 fold in PDA vs AME tumorspheres (\textbf{Figure 3-10B}, \(p=0.03\)). MiR-143 and miR-145 are known tumor suppressors affecting various malignancies including BC (Iorio, Ferracin et al. 2005; Iorio, Visone et al. 2007; Liu, Sempere et al. 2009; Dvinge, Git et al. 2013). We therefore investigated the effects of combined loss of miR-143/145 and \textit{Pten} loss in human BC as well as mouse mammary tumors.

\subsection{3.3.5 miR-145-low/PTEN-low tumors represent a distinct subgroup of human breast cancer}

To evaluate the effects of miR-143- and miR-145-loss on human BC, we initially used a cohort of 181 patients with miRNA and mRNA expression data (GSE19783). In this cohort, miR-143 was downregulated in all major subtypes including basal-like (\(p<0.01\)), HER2/ERBB2 (\(p<0.05\)), luminal A (\(p<0.0001\)) and luminal B (\(p<0.0001\)) compared to normal-like tumors. In contrast, miR-145 was preferentially downregulated in basal-like (\(p<0.01\)) and luminal B (\(p<0.05\)), which are the subtypes in which PTEN is most commonly lost (The Cancer Genome Atlas Network 2012) (\textbf{Figure 3-10C-D}).

To determine which BC subtype expresses low levels of both \textit{PTEN} and miR-145, we analyzed RNA and miRNA data from a cohort of over 1300 patients (EGAS00000000122) (Dvinge, Git et al. 2013). \textit{PTEN} is often deregulated through methylation and miRNA-mediated silencing, and its mRNA expression is the primary determinant of PTEN protein levels in BC (Saal, Johansson et al. 2007; Salmena, Carracedo et al. 2008; The Cancer Genome Atlas Network 2012). We therefore assessed PTEN status at the RNA level. We used two-fold change from median
expression as the “cut-off” level for high- and low-expression categories of PTEN and miR-145.
Figure 3-5 Pten-deficient mammary PDAs and AMEs are molecularly distinct

A. Cluster analysis of AMEs and PDAs using an intrinsic gene signature in comparison with other mouse models of BC. PDA n=8, AME n=13

B. Cluster analysis of AMEs and PDAs using an intrinsic gene signature in comparison with human BC samples. PDA n=8, AME n=13

C. Selected GSEA pathways enriched in PDAs (red) versus AMEs (blue) visualized using Cytoscape Enrichment Map. Green lines connect overlapping pathways. Size of circles corresponds with levels of enrichment (normalized enrichment score) whereas thickness of lines corresponds with degree of overlap. p<0.001, FDR<0.001. GAG – Glycosaminoglycan; PK, activation of protein kinases. PDA n=8, AME n=13

D. ArrayCGH showing frequency of copy number alterations in PDAs vs AMEs. Differential threshold 25%. Sig. genes: genes with significant copy number variations between PDAs and AMEs. PDA n=5, AME n=5

E. Selected mutations enriched in PDAs versus AMEs identified by exome sequencing. Threshold: genes with ≥3 mutations in PDA tumors. PDA n=7, AME n=3
Figure 3-6 GSEA reveals distinct pathways in PDAs versus AMEs

Complete GSEA of AMEs (blue) versus PDAs (red) using canonical pathways (c2.all, v4.0, Broad Institute) visualized using Cytoscape Enrichment Map. Green lines connect overlapping pathways. Size of the circles corresponds with levels of enrichment (normalized enrichment score) whereas thickness of lines corresponds with degree of overlap. p<0.001, FDR<0.001.
P value < 0.001; FDR Q Value < 0.001
Node size proportional to gene-set size
Jaccard Overlap Combined Cut-off: 0.275
Test used: Jaccard Overlap Combined Index (k constant = 0.5)

Node colour intensity proportional to enrichment score
Figure 3-7 g:Profiler analysis of AMEs versus PDAs. p<0.05
Figure 3-8 aCGH analysis showing distinct genetic alterations in PDAs versus AMEs. Shown is a list of genes with significant copy number changes in PDAs versus AMEs including chromosomal location and frequency of alterations. Region indicates largest boundaries of CN changes.
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Figure 3-9 Schematic representation of Cul9 (a) and Cbl (b), and relative location (asterisks) of mutations identified in PDAs
A  
Cul9

B  
CBL

**A**

N  
Cul17  
APC10  
Cullin  
IBR  
*  
C

**B**

N  
TKB domain  
RING  
Proline-rich  
UBA/LZ  
*  
*  
*  
C

E2467  
E2467  
E2467  
E2467  

G848D S873T A895T  
G848D S873T A895T  
S873T
Significant correlations between PTEN and miR-145 expression were seen in all subtypes, with the strongest correlation observed in basal-like BC (Figure 3-10E). Basal-like BC also contained a significantly higher percentage of tumors with both low PTEN and miR-145 expression (7.3%; Figure 3-10F).

To determine if PTEN-low/miR-145-low expression affects clinical outcome, we analyzed BC cohorts (GSE19536 and GSE19783) with known PTEN and miR-145 levels as well as disease-free survival data. Patients with PTEN-low/miR-145-low had a significantly worse disease-free survival (DFS) compared to those with PTEN-high/miR-145-low, indicating that PTEN-low/miR-145-low tumors represent a distinct group of aggressive BCs (hazard ratio - HR=2.21; p=0.0127; Figure 3-10G). These results imply that PTEN status significantly affects prognosis only in miR-145-low patients. However, no statistical difference was observed between PTEN-low BC with low vs high miR-145 expression (not shown), suggesting that in human BC, other genes can be altered together with PTEN-loss to promote aggressive BC. We conclude that PTEN-low/miR145-low BCs represent a distinct group of aggressive tumors that should be identified and treated as a unique disease.

3.3.6 Functional cooperation between miR-143/145 and Pten loss promotes engraftment of non-transplantable Pten-deficient mammary tumors

To determine the effect of miR-143/145 expression on Pten-deficient tumors, we knocked-down these miRNAs using a lentiviral miRNA decoy (sponge) (Venner et al., 2013). To obtain efficient transduction, tumorspheres derived from six independent AME tumors were cultured under adherent conditions as monolayer cells, transduced with lenti-miR-143/145 decoy, sorted for GFP+ cells and then subjected to proliferation assays in vitro and/or fat pad engraftment to assess TIC activity (Figure 3-11A). Although this experimental scheme increased the transplantation potential of some AME tumors (see below), it was critical because of exceedingly low levels of lentiviral transduction of tumorsphere cells. Under monolayer conditions, 72% knockdown was achieved on average for miR-143 and 66% for miR-145 (Figure 3-11B). These levels of miR-143/145 suppression in AME cell lines led to increased proliferation in vitro compared to empty vector treated cells (Figure 3-11C, Figure 3-12A).
In xenograft assays, miR-143/145 knockdown in three of six independent AME lines (AME#1, AME#2 and AME#3) led to a significantly higher incidence of secondary tumor formation and
Figure 3-10 Low miR-145 expression in PTEN-deficient BC leads to poor clinical outcome

A. Microarray analyses of miRNA levels in PDAs versus AMEs. Both miR-143 and miR-145 are significantly downregulated in PDAs.

B. Quantitative real-time PCR confirms miR-145 downregulation in tumorspheres derived from PDAs. *p=0.03.

C. Analysis of miR-143 expression in human BC database. Mir143 levels are downregulated in all BC subtypes compared to normal-like breast tissue. *p<0.05, **p<0.01, ***p<0.0001.

D. Analysis of miR-145 expression in human BC database. Aggressive basal-like and luminal B cancers show downregulated miR-145 expression. *p<0.05, **p<0.01.

E. Significant correlation between low PTEN and miR-145 gene expression, primarily in basal-like (0.33) as well as other BC subtypes. p<0.01.

F. Percentage of tumors with low PTEN and low miR-145 expression, showing highest prevalence in basal-like BC relative to all other subtypes.

G. Kaplan-Meier disease-free survival analysis showing patients with PTEN-low and miR-145-low have poorer prognosis than patients with PTEN-high & miR-145-low. Wilcoxon method, p=0.0127.
decreased latency/mammary tumor-free survival (Figure 3.11D–E, p<0.025). In another line (AME#4), miR-143/145 knockdown decreased latency (p=0.0101), though not the incidence of tumor formation. A fifth line (AME#5) had intrinsic high incidence and low latency, and miR-143/145 knockdown did not further accelerate tumorigenesis, whereas AME#6 was refractory to the miR-143/145 decoy. This diversity in tumor engraftment rate/latency compared with the initial AMEs is likely due to selection of aggressive variants following under adherent conditions (Figure 3.11A). Nonetheless, our results demonstrate that miR-143/145 knockdown converted 4 of 6 un-transplantable or poorly-transplantable Pten-deficient cultures to readily transplantable tumors.

In contrast to the glandular appearance of primary AMEs, secondary tumors arising after engraftment of miR-143/145 knockdown or control cells were invariably poorly differentiated (Figure 3.11F). In the 4 AME tumor cell lines where miR-143/145 depletion led to increased tumor initiating potential (i.e. AME#1–#4), miRNA decoy treatment also resulted in lower expression of the differentiation markers K14 and K18 relative to the control lentivirus treated groups (Figure 3.13B). In addition, only one of the six AME derived cell lines (AME#1) showed strong nuclear staining for p53, suggestive of stabilizing p53 mutation (Figure 3.11E, G). Importantly, in all AME-derived tumors, p53 status was consistent between decoy-treated and control-treated groups. Therefore, the enhanced tumorigenesis of miR-143/145 decoy transduced AME cells was not due to acquisition of p53 mutations. In summary, miR-143/145-knockdown cooperated with Pten loss to promote tumor initiation potential with poorly differentiated histology.

3.3.7 MiR-143/145 loss cooperates with Pten-deficiency at least in part by inducing RAS signalling

MiR-143 and miR-145 target a large number of genes in various biological contexts including K-RAS and N-RAS (Cordes, Sheehy et al. 2009; Wang, Bian et al. 2009; Xu, Papagiannakopoulos et al. 2009; Gotte, Mohr et al. 2010; Zou, Xu et al. 2012; Su, Liang et al. 2014; Yan, Chen et al. 2014; Gao, Miao et al. 2016; Zhao, Kang et al. 2016; Zheng, Sun et al. 2016). To determine the mechanism through which miR-143/145 loss cooperates with Pten deficiency to promote aggressive mammary tumors we performed the following analysis. First, we identified predicted targets for both miR-143 and miR-145 using 12 miRNA target prediction software packages (Figure 3.15-E) (Dweep and Gretz 2015). Specifically, we identified genes that (1) are
Figure 3-11 Disruption of miR-143/145 in AMEs promotes proliferation and tumor engraftment

A. Schematic illustration of the experimental design used to analyze effects of miR-143/145 knockdown in AME cells.

B. Quantitative RT-PCR analysis of miR-143 and miR-145 levels following knockdown with lentiviral miRNA decoy. miRNA levels relative to 18s. Results are shown as means ± SD; n≥6.

C. Representative *in vitro* growth curves of AME tumor cell lines transduced with miR-143/145 decoy showing significant increased growth in vitro. Results are shown as means ± SD; n≥6

D. A representative Kaplan-Meier overall survival curve of an AME tumor cell line (#1) transduced with the miR-143/145 decoy or control vector following orthotopic transplantation into NOD/SCID mice.

E. Control and miR-143/145 decoy expressing AME cell lines were injected into fat pads of NOD/SCID mice. The table summarizes tumor incidence and median survival of six independent transduced AME lines, showing higher tumor incidence and significantly shortened latency after miR-143/145 knockdown relative to empty vector.

F. Representative histology of a primary AME tumor (#1, top) and secondary tumors induced by miR-143/145 decoy or vector alone, both showing PDA morphology.

G. IHC analyses of p53 in PDA and AME primary tumors, and secondary tumors induced by miR-143/145 decoy.
A

AME tumor-spheres ➔ AME monolayer ➔ Transduce with lentiviral-miR-143/145 Decoy ➔ FACS for GFP+ cells ➔ Proliferation assay ➔ Fat pad injection

B

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<th>p53 status (IHC)</th>
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C

Relative cell number vs. Day 1, 2, 3, 4

D

Percent survival vs. Latency (Days)

E

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<th>Treatment</th>
<th>Tumor incidence</th>
<th>Median survival (days)</th>
<th>p53 status (IHC)</th>
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</table>

F

H&E images of primary tumor

G

p53 staining images: Positive control, PDA, AME, AME#1+miR decoy, AME#1+empty vector, AME#4+miR decoy, AME#4+empty vector
predicted targets of miR-143 or miR-145 by at least 6 of the 12 programs, and (2) emerge as predicted targets in human, mouse and rat. We reasoned that only robust miRNA-target interactions would emerge following such rigorous criteria. Using this approach, we identified 61 miR-143 and 98 miR-145 predicted and conserved targets. Second, we submitted our predicted target lists for DAVID Functional Annotation Bioinformatics Microarray Analysis to identify pathways targeted by miR-143/145 (Huang da, Sherman et al. 2009; Huang da, Sherman et al. 2009). Strikingly, RAS-associating domain pathways involved in Ras signaling and other GTPases (Ponting and Benjamin 1996) were by far most significantly enriched among the predicted targets of miR-143 and miR-145 (Figure 3-12A). In parallel, we performed pathway activity analysis on 18 signaling pathways as defined by Gatza el al., (Gatza, Lucas et al. 2010). Of these 18 pathways, four: RAS, TGFβ, TNFα and IFNα, were significantly altered in PDAs relative to AMEs (Figure 3-14). Importantly, Ras signaling was elevated 1.522 fold (Figure 3-12B; p=0.0018). Thus, Ras pathway is a major target of miR-143/145 and it is elevated in PDAs expressing low levels of these miRNAs.

To determine the effect of miR-143/145 knockdown on Ras signaling we used phospho-Erk as a readout for Ras pathway activation. In AME cell lines, miR-143/145 knockdown led on average to a two-fold increase in phospho-Erk levels (Figure 3-12C, p=0.0445), suggesting elevated Ras signaling activity is at least partially responsible for enhanced tumorigenicity in miRNA decoy-treated AME cells. Next, using immortalized HC11 mammary epithelial cells, we examined phospho-Erk levels upon knockdown of Pten, miR-143/145 or both. Pten was knocked-down using a lentivirus-shRNA vector leading to on average 64% reduction in protein expression (Figure 3-13C). In single treatment groups, knockdown of Pten or miR-143/145 increased phospho-Erk levels relative to non-infected control treatment by an average of 2.5 fold (p>0.5) and 3.3 fold (p<0.01), respectively (Figure 3-12D). Combined Pten plus miR-143/145 depletion led to a 3.1 fold increase in phospho-Erk levels relative to single Pten knockdown (p<0.001), and 2.2 fold increase relative to single miR-143/145 knockdown (p<0.5) (Figure 3-12D and Figure 3-13E). Combined knockdown of Pten plus miR-143/145 also induced cell growth relative to knockdown of each gene alone (Figure 3-12E, Figure 3-13D). Thus, deficiency in Pten or miR-143/145 alone elevates cell growth but combined loss of both genes is needed for robust proliferation, and this may underlie their cooperation during PDA formation in vivo.
We next asked whether elevated phospho-Erk signaling in PDAs or in AMEs following miR-143/miR-145 knockdown altered sensitivity to the MEK inhibitor Selumetinib. We generated drug response curves for four independent PDAs, AMEs or AME cell lines stably transduced with miR-143/145 decoy or empty vectors (16 lines in total). Remarkably, PDAs and miR-143/145-knocked-down AMEs were significantly more sensitive to Selumetinib than the parental AMEs or AMEs transduced with empty vector (Figure 3-12F). In contrast, no significant differences in response to the PI3K inhibitor BYL719 (Furet, Guagnano et al. 2013) were observed across cell lines (Figure 3-12G). Thus, MEK inhibitors may represent a promising treatment option for BC patients with low-PTEN and low-miR-143/145 expression.

To further investigate the effect of miR-143/145, we overexpressed miR-143 and miR-145 through a single lentiviral vector and assessed its impact on RAS signaling. Enforced miR-143/145 expression in Pten-deficient cells derived from a PDA tumor reduced levels of Ras proteins (K-Ras and N-Ras) (Figure 3-15A-B, D; p=0.0470) and Erk1/2 phosphorylation (Figure 3-15C-D, p=0.0387).

Next, we assessed the effects of miR-143/145 over-expression on levels of predicted and known Ras pathway targets. Figure 3-15E-F shows the workflow and Venn diagrams generated to identify conserved and predicted targets of miR-143 (13 genes) and miR-145 (14 genes) on the Ras pathway, that are also differentially regulated between PDAs and AMEs. Of these 27 genes, we selected 6 genes (Mapk3, Efnal1a, Efnal1b, Epha2, Gng7, Rasa11) as well as 4 additional known miR143/145 targets (Nras, Kras, Rreb1, Fli1) for Q-RT-PCR. We observed both significant (Mapk3, Efnal1a and Epha2a) and trend toward significant (Nras, p=0.0694; Rreb1, p=0.0869; Kras, p=0.0838) inhibition of some of the Ras pathway genes following miR-143/145 overexpression (Figure 3-15G and Figure 3-13F). We conclude that the miR-143/145 cluster cooperates with Pten-loss both in mouse and human BC, at least in part, by inducing RAS signaling through multiple genes on this pathway (Figure 3-16).

3.4 Discussion

We report that most Pten-deficient mammary tumors are highly differentiated and lack TICs that can give rise to secondary tumors in recipient mice following FACS selection or sphere growth. This is a stark contrast to previous findings by our group and others in which TICs have been
Figure 3-12 miR-143/145 knockdown cooperates with *Pten* loss by inducing phospho-Erk, leading to increased sensitivity to MEK inhibitors

A. DAVID Functional Annotation Bioinformatics Microarray Analysis of pathways predicted to be targeted by miR-143 and miR-145.

B. Significant induction of Ras pathway activity in PDA vs AMEs tumors. p=0.0018. Activities of all 18 signaling pathways are shown in Figure 3-14.

C. miR-143/145 knockdown results in elevated phospho-Erk levels. n≥6, p=0.0445.

D. Representative Western blot analysis for phospho-Erk following miR-143/145 and Pten knockdown in immortalized mammary epithelial HC11 cells.

E. In vitro growth curves of HC11 cells transduced with miR-143/145 decoy, Pten shRNA or both.

F-G. Selumetinib or BYL719 dose response curves for PDA, AME, miR-143/145-decoy treated AME and control AME cell lines by MTT assay. *p< 0.05; **p< 0.01.
Figure 3-13 miR-143/145 knockdown cooperates with Pten loss by activating the Ras pathway

A. In vitro growth curves of independent AME tumor cell lines transduced with miR-143/145 decoy.

B. Epithelial marker analysis of AME and PDA tumors for ERα, K18 and K14.

C. Representative Western Blot analysis of Pten knockdown by lentiviral shRNA.

D. In vitro growth curves of HC11 cells transduced with miR-143/145 decoy, Pten shRNA or both.

E. Western blot quantification showing elevated phospho-Erk levels in HC11 cells treated with both miR-143/145 decoy and Pten shRNA compared to cells transduced with only miR-143/145 decoy, or only Pten shRNA.

F. Quantitative RT-PCR analysis of indicated predicted miR-143/145 targets following miR-143/145 overexpression.
**A**

- Relative cell number vs. Day 1, Day 2, Day 3, Day 4 for Vector and miR decoy groups.
  - p < 0.001 for Vector and miR decoy at Day 1, p < 0.01 at Day 2, and p < 0.05 at Day 3.

**B**

- ERα, K18, K14 expression levels in AME+ and Empty Vector groups.

**C**

- Pten shRNA and Empty vector groups.
  - Pten and Tubulin expression levels.

**D**

- Relative cell number across Day 1, Day 2, Day 3, Day 4 for PTEN shRNA+miRNA Decoy, miRNA Decoy, PTEN shRNA, Empty Vector groups.
  - p < 0.022, p < 0.043, p < 0.043 for PTEN shRNA+miRNA Decoy, miRNA Decoy, PTEN shRNA respectively.

**E**

- Double infection vs. Pten shRNA and Double infection vs. miR Decoy table.
  - Values for different conditions.

**F**

- Relative Expression for Efnal1, Flp1, Gng7, Rasal1 under Empty Vector and miR overexpression conditions.
Figure 3-14 Distinct levels of activation of 18 signalling pathways in AMEs vs PDAs. * denotes significant differences. Red – induction in PDAs; green – induction in AMEs.
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Figure 3-15 miR-143/145 overexpression targets the Ras pathway

A. Quantitative RT-PCR analysis of miR-143 and miR-145 levels following lentiviral transduction of miR-143 and miR-145.

B. Representative Western blot analysis for Ras (KRas plus NRas) following miR-143/145 overexpression.

C. Representative Western blot analysis for phospho-Erk following miR-143/145 overexpression.

D. miR-143/145 overexpression leads to reduced Ras expression (n=5, p=0.0470), and phospho-Erk levels (n=5, p=0.0387).

E. Workflow used to identify potential targets and pathways regulated by miR-143/145.

F. Venn diagram of predicted targets of miR-143 (13 genes) and miR-145 (14 genes), which are conserved, on the Ras pathway and differentially regulated between Pten-deficient AME and PDA tumors. Genes enriched in AMEs are highlighted in green; those enriched in PDAs in red.

G. Quantitative RT-PCR analysis of predicted miR-143/145 targets Mapk3, Efna1a, Epha2, Nras, Kras and Rreb1 following miR-143/145 overexpression. *p<0.05, one tail t-test; **p<0.002, two tail t-test; #p<0.09, 1-tail t test.
Figure 3-16 Models of oncogenic cooperation between PTEN deficiency and miR-143/145 loss

A. A model of oncogenic cooperation between miR-143/145 and Pten loss in mouse mammary tumors, leading to highly tumorigenic, transplantable TICs. Additional alterations such as those identified by aCGH and exome sequencing may further cooperate with Pten-miR143/145-loss to induce aggressive PDAs. PTEN loss promotes cancer via PIP3-dependent and PIP3-independent mechanisms.

B. A model of oncogenic cooperation between miR-143/145 and PTEN loss in human BC. miR-143/145 and other genetic alterations induce RAS signaling. In both mouse and human BC, the miR-143/145 locus targets additional pathways, not only RAS signaling, and therefore PTEN-low/miR143/145-low TNBC patients should be identified and treated as a distinct group.
A  MOUSE

Pten-deficient non-transplantable AMEs

High
miR-143/145

RAS

PI3K

PTEN loss

PIP2

PIP3

Benign tumors lacking transplantable TICs

Low
Expression Level

Pten-deficient transplantable PDAs

Low
miR-143/145

RAS

PI3K

PTEN loss

PIP2

PIP3

Agressive tumors with transplantable TICs

B  HUMAN

PTEN-loss, miR-143/145-low TNBC

miR-143/145 loss

Other alterations

RAS Pathway

PTEN loss

Agressive TNBC with poor prognosis
readily identified in many mammary tumor models (Liu, Deng et al. 2007; Vassilopoulos, Wang et al. 2008; Zhang, Behbod et al. 2008; Liu, Voisin et al. 2014; Jones, Robinson et al. 2016; Wang, Liu et al. 2016). One possible explanation is that AME tumor cells are dependent on estrogen signaling, thus unable to survive and proliferate in recipient mice without exogenous $\beta$-estradiol. Indeed, luminal BC cells require $\beta$-estradiol pellets or injection into milk ducts to grow in a mouse host (Al-Hajj, Wicha et al. 2003; Sflomos, Dormoy et al. 2016). Freshly isolated single cell suspensions, but not sorted cells or tumorspheres from $Pten$-deficient AMEs may provide a permissive milieu required for their growth. However, other possibilities such as tumor-stroma (e.g. fibroblast) interaction may be required for survival of most $Pten$-deficient TICs in recipient mice. Importantly, we identified a rare group of $Pten$-deficient mammary tumors with features of basal-like BC that do harbour robust TICs. These tumors express low levels of miR-143/145 compared with the non-tumorigenic lesions. Knockdown of miR-143/145 in the non-transplantable tumors induced RAS pathway activity and conferred tumor initiation potential. Importantly, we found that BC patients with low PTEN and miR-143/145 expression show poor clinical outcome. Thus, these patients should be identified and prioritized for aggressive therapy.

What is the basis for lowered miR-143/145 expression in mouse PDAs and human basal like BC? Several related possibilities exist. (i) Mutations in miRNA biogenesis and processing may cause global deregulation in miRNA levels (Esteller 2011), preferentially effecting miR-145 in the cell-of-origin of $Pten$-deficient tumors. (ii) Genetic and epigenetic alterations at the miR-143/145 locus may change its expression. (iii) Distinct mammary epithelial cell populations may express differing levels of miRNAs, and aggressive PDAs may originate from cells with intrinsically low miR-143/145 expression. Indeed, data mining of miRNA expression from Fu et al (Fu, Rios et al. 2015), suggest that miR-145 may be differentially expressed in basal vs luminal mouse mammary cell lineages. However, in preliminary analyses with mouse $Pten$-deficient mammary epithelial cells, we observed no difference in miR-143 or miR-145 expression in FACS-purified basal, luminal, luminal progenitor or mature luminal mammary epithelial cells. Moreover, no copy number changes were seen at the miR-143/145 locus by aCGH analysis in PDAs vs AMEs. Additional analysis is required to address this issue.
Other mouse models also show that secondary mutations are required for a *Pten*-deficiency to achieve full malignancy. As noted, prostate-specific inactivation of *Pten* induces benign tumors with increased p53-dependent senescence (Chen, Trotman et al. 2005; Ahmad, Patel et al. 2011). Likewise, in models of biliary tract malignancies and von Hippel-Lindau (VHL) disease, murine *Pten* inactivation either fails to induce tumors or elicits low-grade lesions (Frew, Minola et al. 2008; Marsh, Davies et al. 2013). Patients with Cowden syndrome exhibit multiple hamartomas most commonly in skin and gastrointestinal tract, and an increased risk of benign and malignant tumor formation in the breast, thyroid, endometrium, colorectal, kidney and skin. Among female Cowden patients, BC is the most common malignancy, with a lifetime risk of 81-85% (Riegert-Johnson, Gleeson et al. 2010; Tan, Mester et al. 2012). Our results suggest that tumor progression to full malignancy in Cowden patients may similarly involve miR-143/145 loss or RAS pathway activation. Unfortunately, no expression profiling is publically available to test this hypothesis. However, our results strongly encourage such studies because identification of cooperating oncogenic alterations in these PTEN-mutant tumors may allow its targeting together with PI3K pathway inhibitors as a low dose prophylactic therapy.
Chapter 4

4 Targeted Pten deletion plus p53-R270H mutation in mouse mammary epithelium induces aggressive claudin-low and basal-like breast cancer

Results presented in this chapter have been published:


Note:
- Dr. Jeff C Liu contributed Figures 4-4 and 4-5A

4.1 Abstract

4.1.1 Background

Triple-negative breast cancer (TNBC), an aggressive disease comprising several subtypes including basal-like and claudin-low, involves frequent deletions or point mutations in TP53, as well as loss of PTEN. We previously showed that combined deletion of both tumor suppressors in the mouse mammary epithelium invariably induced claudin-low-like TNBC. The effect of p53 point mutation plus Pten deletion on mammary tumorigenesis and whether this combination can induce basal-like TNBC in the mouse are unknown.

4.1.2 Methods

WAP-Cre:Pten\textsuperscript{f/f}:p53\textsuperscript{lox.stop.lox.R270H} composite mice were generated in which Pten is deleted and a p53-R270H mutation in the DNA-binding domain is induced upon expression of Cre-recombinase in pregnancy-identified alveolar progenitors. Tumors were characterized by histology, marker analysis, transcriptional profiling [GEO-GSE75989], bioinformatics, high-throughput (HTP) FDA drug screen as well as orthotopic injection to quantify tumor-initiating cells (TICs) and tail vein injection to identify lung metastasis.
4.1.3 Results

Combined Pten deletion plus induction of p53-R270H mutation accelerated formation of four distinct mammary tumor types including poorly differentiated adenocarcinoma (PDA) and spindle/mesenchymal-like lesions. Transplantation assays revealed highest frequency of TICs in PDA and spindle tumors compared with other subtypes. Hierarchical clustering demonstrated that PDA and spindle tumors grouped closely with human as well as mouse models of basal and claudin-low subtypes, respectively. HTP screens of primary Pten\(^\Delta\):p53\(^\Delta\) vs. Pten\(^\Delta\):p53\(^{R270H}\) spindle tumor cells with 1120 FDA-approved drugs identified 8-azaguanine as most potent for both tumor types, but found no allele-specific inhibitor. A gene set enrichment analysis revealed increased expression of a metastasis pathway in Pten\(^\Delta\):p53\(^{R270H}\) vs. Pten\(^\Delta\):p53\(^\Delta\) spindle tumors. Accordingly, following tail vein injection, both Pten\(^\Delta\):p53\(^{R270H}\) spindle and PDA tumor cells induced lung metastases and morbidity significantly faster than Pten\(^\Delta\):p53\(^\Delta\) double-deletion cells, and this was associated with the ability of Pten\(^\Delta\):p53\(^{R270H}\) tumor cells to upregulate E-cadherin expression in lung metastases.

4.1.4 Conclusions

Our results demonstrate that WAP-Cre:Pten\(^{\text{ff}}\):p53\(^{\text{lox,stop,lox_R270H}}\) mice represent a tractable model to study basal-like breast cancer because unlike p53 deletion, p53R270H mutation in the mouse does not skew tumors toward the claudin-low subtype. The WAP-Cre:Pten\(^{\text{ff}}\):p53\(^{\text{lox,stop,lox_R270H}}\) mice develop basal-like breast cancer that is enriched in TICs, can readily form lung metastasis, and provides a preclinical model to study both basal-like and claudin-low TNBC in immune-competent mice.

4.2 Introduction

Breast cancer is a highly heterogeneous disease comprising Estrogen Receptor alpha (ER\(\alpha\))—positive and HER2/ERBB2/NEU-positive subtypes as well as triple-negative breast cancers (TNBCs) that do not express ER\(\alpha\), HER2 or the progesterone receptor. TNBC can be further divided into several additional groups including basal-like and mesenchymal/claudin-low (Lehmann, Bauer et al. 2011; Banerji, Cibulskis et al. 2012; Curtis, Shah et al. 2012; The Cancer Genome Atlas Network 2012; Prat, Adamo et al. 2013). These latter subtypes contain mutations or deletion in the tumor suppressor p53 in 60-80% of cases (The Cancer Genome Atlas Network...
2012). In addition, 30% of TNBC also show loss of expression of the tumor suppressor Phosphatase and TEnsin homolog deleted in chromosome 10 (PTEN). PTEN codes for a phosphatase that converts phosphatidylinositol (3, 4, 5)-trisphosphate (PIP3) into phosphatidylinositol (4, 5)-disphosphate (PIP2), thereby antagonizing phosphatidylinositol-3 kinase (PI3K) pathway activation (Cully, You et al. 2006; Hopkins, Hodakoski et al. 2014; Stambolic 2015). On the basis of frequency at which each gene is disrupted alone, combined loss of p53 and Pten in TNBC is calculated at about 18-24%. Indeed, using Pten RNA expression and p53 pathway activity it was estimated that 24.4% of TNBCs are Pten-low, 65.6% are p53-activity low, and 18.7% are both Pten-low and p53-pathway activity low (Liu, Voisin et al. 2014).

The generation of mice with mutations that occur in breast cancer offers a window into the mechanisms of tumor initiation, progression and dissemination within immune-competent mice, and provide preclinical models to test for potential new therapies. Deletion of p53 in the mouse induces diverse tumors as well as claudin-low-like TNBC (Herschkowitz, Zhao et al. 2012). We recently showed that combined deletion of p53 and Pten via MMTV-CreNLST or WAP-Cre drivers induces almost exclusively claudin-low TNBC-like tumors (Liu, Voisin et al. 2014). This suggests that p53 deletion in the mouse promotes mesenchymal-like cancer, which is further accelerated by disruption of Pten.

To generate a model for basal-like Pten/p53 mutant TNBC, we here tested the effect of deleting Pten and expressing p53R270H mutation in the DNA-binding domain (DBD) (Olive, Tuveson et al. 2004). This and similar mutations in p53 DBD were shown to act as dominant-negative or gain-of-function alleles that accelerate metastasis through multiple mechanisms (Powell, Piwnica-Worms et al. 2014). Targeted expression of p53R270H in the mammary epithelium was reported to induce divergent tumors (Wijnhoven, Speksnijder et al. 2007). Here we show that WAP-Cre:Pten^f/f:p53^{lox,stop,lox_R270H} composite mice develop spindle-like and poorly differentiated adenocarcinoma (PDA) as well as other subtypes. Using microarray profiling, we found that the spindle and PDA clustered with claudin-low and basal-like breast cancer (BLBC), respectively, and metastasized to the lung following tail-vein injection significantly faster than tumors from WAP-Cre:Pten^f/f:p53^f/f double deletion mice. Thus, WAP-Cre:Pten^f/f:
p53<sup>lox.stop.lox_R270H</sup> mice provide a preclinical model to study aggressive Pten/p53-mutant basal-like and claudin-low TNBC.

4.3 Results

4.3.1 Targeted Pten deletion plus expression of a p53-R270H mutant in mammary epithelium accelerate mammary tumorigenesis

WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>lox.stop.lox_R270H/+</sup> composite female mice (WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>lsl_R270H/+</sup> for short) developed mammary tumors with significantly shorter latency than female WAP-Cre:Pten<sup>fl/fl</sup> or WAP-Cre:p53<sup>lsl_R270H/+</sup> animals (Figure 4-1A). Deletion of Pten and the loxP-stop-loxP cassette was confirmed by PCR (Figure 4-1B). Histology analysis revealed that WAP-Cre:p53<sup>lsl_R270H/+</sup> mice developed three major tumor types: spindle-like, PDA and mixed histotypes (Figure 4-1C). Tumor distribution in WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>lsl_R270H/+</sup> mice was more similar to WAP-Cre:Pten<sup>fl/fl</sup> mice including spindle-like, PDA, squamous carcinoma, adenomyoepitheliomas (AME) and mixed tumors. However, the ratio of these tumors varied (Figure 4-1C). The spindle and PDA tumors were highly proliferative relative to other types as judged by Ki67 expression and expressed low levels of the myoepithelial cytokeratin marker, K14 (Figure 4-1D). Spindle tumors did not express the luminal marker, K18, whereas the PDAs showed low/variable expression of this protein. We specifically stained PDA and spindle-like tumors for basal keratins K5 and K6, which are hallmarks of BLBC. We found that five of seven PDA were positive for K5, and four of seven were positive for K6. For spindle tumors, three of three were positive for both K5 and K6 (Figure 4-1E).

Prior to Cre-mediated recombination, the non-recombined p53<sup>lsl_R270H</sup> gene acts as a null (−) allele, and p53<sup>lsl_R270H/wt</sup> mice are functionally p53<sup>−/−</sup> in all tissues (including mammary epithelium) (Olive, Tuveson et al. 2004). To determine if the p53<sup>lsl_R270H</sup> allele was induced through deletion of the Lox-stop-lox cassette via WAP-Cre, we immunostained tumor sections for p53, which is stabilized by the p53<sup>R270H</sup> mutation, leading to high expression. Some tumors were uniformly positive, indicative of Cre-recombination and p53<sup>R270H</sup> expression; others were negative or contained areas of p53-positive cells alongside p53-negative cells, likely reflecting clonal evolution (Figure 4-2A-D). We focused our analysis (including IHC in Figure 4-1) on tumors with high p53 expression (i.e. expressing the p53<sup>R270H</sup> allele) in large areas or throughout the tumor (e.g. Figure 4-2C).
Figure 4-1 Pten deletion plus p53-R270H point mutation cooperate to accelerate mammary tumor formation.

A. Kaplan-Meier mammary tumor-free curves for WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>wt/wt</sup> (n = 16; median latency - 413 days), WAP-Cre:Pten<sup>wt/wt</sup>:p53<sup>R270H/wt</sup> (n = 39; median latency - 507 days), and WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>R270H/wt</sup> (n = 75; median latency - 291 days) mice. Statistical significance by Wilcoxon method: Pten versus p53, p = 0.0118; Pten/p53 vs. Pten, p < 0.0001; Pten/p53 vs. p53, p < 0.0001.

B. Detection of conditional Pten deletion and p53 mutant allele by PCR.

C. Distribution of tumor types (%) in WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>wt/wt</sup> (left; n = 13), WAP-Cre:Pten<sup>wt/wt</sup>:p53<sup>R270H/wt</sup> (middle; n = 9), and WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>R270H/wt</sup> (right; n = 76) mice.

D. Histology of the four major tumor types from WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>R270H/wt</sup> mice. Original magnification 40×.

E. Representative IHC analysis of basal differentiation marker (K14), luminal differentiation marker (K18) and proliferation marker Ki67 in the major tumor types from WAP-Cre:Pten<sup>fl/fl</sup>:p53<sup>R270H/wt</sup> mice.

F. Representative expression of basal differentiation marker (K5 and K6) in PDA (n = 7) and spindle (n = 3) tumors. IHC immunohistochemistry, PCR polymerase chain reaction, PDA poorly differentiated adenocarcinoma.
A. Mammary Tumor Survival (%) vs. Latency (Days)

- WAP-Cre: Pten^wt/wt: p53^wt/wt
- WAP-Cre: Pten^fl/fl: p53^R270H/wt
- WAP-Cre: Pten^fl/fl: p53^R270H/wt

B. Western Blot
- pten^M
- p53^R270H
- p53^wt

C. Pie Charts
- WAP-Cre: Pten^fl/fl: p53^wt/wt
- WAP-Cre: Pten^wt/wt: p53^R270H/wt
- WAP-Cre: Pten^fl/fl: p53^R270H/wt

- Mixed
- Squamous
- Well differentiated (adenomyoepithelioma)
- Spindle
- Poorly differentiated

D. Histological Staining
- Squamous
- Well differentiated
- Spindle
- Poorly differentiated

E. Immunohistochemistry
- K14
- K18
- K67

F. K5
- Spindle
- Poorly differentiated
Figure 4-2 Analysis of mutant p53 accumulation in tumor cells.

A-C. p53 IHC of WAP-Cre:Ptenc^{fl/fl}:p53^{R270H/wt} tumors showing lack of p53 accumulation, indicating the p53^{R270H} allele was not activated in (A). Arrows and dashed outlines indicate a region with strong p53 accumulation; arrowheads and dotted outlines to regions with weak or no p53 accumulation (B). Note strong nuclear accumulation of mutant p53 in tumor cells (C).

D. Summary of mutant p53 accumulation in the major tumor types in WAP-Cre:Ptenc^{fl/fl}:p53^{R270H/wt} mice: mixed, n = 9; squamous, n = 4; AME, n = 9; spindle, n = 12; PDA, n = 6. AME adenomyoepithelioma, IHC immunohistochemistry, PDA poorly differentiated adenocarcinoma.
4.3.2 Frequent tumor initiating cells (TICs) in poorly differentiated adenocarcinomas and spindle-like Pten$^{Δf}$:p53$^{R270H}$ tumors

One measure for tumor aggressiveness is the frequency of tumor-initiating cells (TICs), capable of inducing secondary tumors following transplantation into recipient mice (Magee, Piskounova et al. 2012; Kreso and Dick 2014). Pten$^{Δf}$:p53$^{R270H}$ tumors from each of the four different subtypes were dissociated to obtain single cells, cultured as tumorspheres in ultra-low attachment plates in serum-free media supplemented with EGF and FGF, passaged once, and then 1000 or 3500 cells were injected orthotopically into recipient female mice (Figure 4-3A). While squamous and AME gave undetectable or low TIC frequencies of 1/4481, respectively, the spindle tumors showed TIC frequency of 1/925 and the PDA tumors an even higher frequency that was not calculatable because all recipient mice developed secondary lesions (Figure 4-3B). Thus, Pten$^{Δf}$:p53$^{R270H}$ spindle and PDA tumors exhibit high TIC frequency, indicative of aggressive cancer. Interestingly, histology analysis of secondary tumors revealed dominant spindle histology even when primary lesions were from AME (4/4) or PDAs (11/16) (Figure 4-3C), suggesting that they arose from rare mesenchymal/spindle subclones within these lesions.

4.3.3 Poorly differentiated and spindle-like Pten$^{Δf}$:p53$^{R270H}$ tumors cluster with known mouse models of basal-like and claudin-low breast cancer

To molecularly classify the Pten$^{Δf}$:p53$^{R270H}$ spindle and PDA tumors, we performed unsupervised hierarchical clustering on six spindle and eight PDA tumors using an extended intrinsic breast cancer signature (Herschkowitz, Simin et al. 2007; Liu, Voisin et al. 2014). To compare gene expression across platforms we used the DWD algorithm (Benito, Parker et al. 2004). We included several normal mammary glands and MMTV-Her2/Neu tumors as internal controls. Cluster analysis grouped the normal glands and tumors with published wild-type mammary gland and MMTV-Her2/Neu tumors, respectively, thereby validating our normalization procedure (Figure 4-4A). Comparing our mammary tumors to 13 other mouse models of breast cancer (Herschkowitz, Simin et al. 2007), most (4/6) Pten$^{Δf}$:p53$^{R270H}$ spindle tumors clustered with other models of spindle breast cancer including certain p53-deficient, Brac1/p53-deficient and 7,12-dimethylbenzanthracene (DMBA)-induced mammary tumors (Figure 4-4A). We
attribute this incomplete consistency (4/6 rather than 6/6) to intra-tumor heterogeneity and the distinct biopsies taken for bioinformatics vs. histology. In contrast, all PDA (8/8) as well as two

Figure 4-3 High frequency of TICs in PDA and spindle tumors.

A. Schematic illustration of the experimental design used to analyze TIC frequency in the four major tumor types.

B. Tumor-initiating cell (TIC) frequency in the major tumor subtypes in WAP-Cre:Ptenc1/1:p53R270H/HWT mice, showing high frequency in PDA and spindle tumors.

C. Histology of secondary tumors showing a selection for spindle-like tumors. For PDAs - 5/16 secondary tumors remained PDAs, and the rest (11/16) became spindle. For AME tumors, 4/4 secondary were all spindle-like. All primary spindle tumors gave spindle secondary tumors.

AME adenomyoepithelioma, PDA poorly differentiated adenocarcinoma
A

1. Isolate tumor cells
2. Freeze tumor cells
3. Thaw tumor cells
4. Grow as tumorspheres
5. Passage
6. Regrow as tumorspheres
7. Trypsinize
8. Count cells
9. Fat pad injection

B

<table>
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<th>Histological Type</th>
<th>3500 Cells Injected</th>
<th>1000 Cells Injected</th>
<th>N</th>
<th>TIC frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td>Squamous</td>
<td>0/4</td>
<td>0/4</td>
<td>2</td>
<td>Low&lt;&lt; 1/4481</td>
</tr>
<tr>
<td>Well differentiated</td>
<td>--</td>
<td>4/12</td>
<td>3</td>
<td>1/4481</td>
</tr>
<tr>
<td>Spindle</td>
<td>4/4</td>
<td>13/20</td>
<td>6</td>
<td>1/925</td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>4/4</td>
<td>12/12</td>
<td>3</td>
<td>High &gt;&gt; 1/925</td>
</tr>
</tbody>
</table>

C

<table>
<thead>
<tr>
<th>Primary tumor</th>
<th>Secondary tumor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poorly differentiated</td>
<td></td>
</tr>
<tr>
<td>Well Differentiated</td>
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</tr>
<tr>
<td>Spindle</td>
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</table>
spindle-like tumors clustered most closely, albeit on a separate branch, with SV40 large T antigen-induced tumors, which were previously shown to resemble human basal-like breast cancer (Herschkowitz, Simin et al. 2007).

4.3.4 Poorly differentiated and spindle-like Pten<sup>Δf</sup>:p53<sup>R270H</sup> tumors cluster with human basal-like and claudin-low breast cancer

We next compared our Pten<sup>Δf</sup>:p53<sup>R270H</sup> tumors with the five human molecular breast cancer subtypes: ERα+ (luminal A and luminal B), HER2, basal-like and claudin-low breast cancer as well as normal-like tumors (Herschkowitz, Simin et al. 2007). Despite the difficulty with interspecies analysis, four mouse Pten<sup>Δf</sup>:p53<sup>R270H</sup> spindle tumors clustered with human claudin-low breast cancer, whereas two clustered with BLBC. Four of the eight Pten<sup>Δf</sup>:p53<sup>R270H</sup> PDAs clustered with human basal-like breast tumors, whereas four other PDAs clustered with HER2+ breast cancer (Figure 4-4B). We note that in contrast to the original Herschkowitz et al. article, in which MMTV-Her2/neu mouse tumors clustered with human luminal breast cancer (Herschkowitz, Simin et al. 2007), in our classification, the Her2/Neu tumors clustered with human HER2+ breast cancer.

Next, we used a claudin-low signature developed by Prat et al. (Prat, Parker et al. 2010) and the GSE18229 dataset to classify our Pten<sup>Δf</sup>:p53<sup>R270H</sup> tumors with 397 human BC samples. The data was integrated by DWD and unsupervised clustering done by complete linkage analysis. Again, the four mouse spindle tumors, but not the other (outliers) spindle or the eight PDA, clustered with human claudin-low BC (Figure 4-4C). Thus, despite some variations due to difficulties with batch correction across different platforms and interspecies analysis as well as intra-tumor heterogeneity, most Pten<sup>Δf</sup>:p53<sup>R270H</sup> spindle tumors and some of the PDAs clustered with human claudin-low or BLBC, respectively (Herschkowitz, Simin et al. 2007).

4.3.5 Spindle-like Pten<sup>Δf</sup>:p53<sup>R270H</sup> mutant and Pten<sup>Δf</sup>:p53<sup>Δf</sup> deletion mammary tumors cluster closely together

We directly compared expression of basal, luminal and mesenchymal markers, as defined by Herschkowitz et al., (Herschkowitz, Simin et al. 2007) in WAP-Cre:Pten<sup>Δf</sup>:p53<sup>Δf</sup> mutant spindle and PDA tumors as well as WAP-Cre:Pten<sup>Δf</sup>:p53<sup>Δf</sup> double-deletion spindle tumors (Liu, Voisin et al. 2014). Classification analysis with these markers grouped the four Pten<sup>Δf</sup>:p53<sup>R270H</sup>
Figure 4-4 Molecular classification of PD and spindle WAP-Cre:Pten$^{fl/fl}$:p53$^{R270H/wt}$ mammary tumors.

A. Cluster analysis of spindle and PD WAP-Cre:Pten$^{fl/fl}$:p53$^{R270H/wt}$ mammary tumors using an intrinsic gene signature in comparison with 13 other mouse models of breast cancer.


C. Cluster analysis of WAP-Cre:Pten$^{fl/fl}$:p53$^{R270H/wt}$ mammary tumors with human claudin-low (green) and basal-like (basal) BC using the Prat et al. claudin-low signature. BC breast cancer, PDA poorly differentiated adenocarcinoma
spindle and nearly all (10/11) Pten\(^{Δf}\)\(:p53^{Δf}\) spindle tumors together with human claudin-low BC, whereas six of eight Pten\(^{Δf}\)\(:p53^{R270H}\) PDA grouped together with human BLBC (Figure 4-5A).

Heat map showing expression of claudins, mesenchymal and epithelial to mesenchymal transition (EMT) markers in Pten\(^{Δf}\)\(:p53^{R270H}\) and Pten\(^{Δf}\)\(:p53^{Δf}\) spindle tumors is shown (Figure 4-5B). With the exception of two Pten\(^{Δf}\)\(:p53^{R270H}\) outliers, all tumors exhibited low claudin expression (claudin 1, 3, 7) and high expression of EMT markers such as Twist2 and Zeb2. In contrast, N-cadherin (cdh2) was higher in Pten\(^{Δf}\)\(:p53^{Δf}\) double-deletion compared with Pten\(^{Δf}\)\(:p53^{R270H}\) mutant, whereas Met gene expression was relatively higher in mutant cells, suggesting that these tumors are not equally committed to EMT (see below).

4.3.6 FDA-approved drug screens reveal similar sensitivity of Pten\(^{Δf}\)\(:p53^{R270H}\) and Pten\(^{Δf}\)\(:p53^{Δf}\) claudin-low-like tumors to 8-azaguanine but no differential sensitivity to other drugs

In TNBC, p53 is disrupted by deletions or by mutations in the DBD (The Cancer Genome Atlas Network 2012). Identification of drugs that can efficiently kill both tumor types or selectively target p53-deletion vs. p53-mutant-driven breast cancer are therefore of great interest. To identify such drugs, we set up a repurposing screen to identify FDA-approved drugs with common or differential effects on Pten\(^{Δf}\)\(:p53^{R270H}\) vs. Pten\(^{Δf}\)\(:p53^{Δf}\) tumors. For these screen, we established and compared three spindle-like lines from Pten\(^{Δf}\)\(:p53^{R270H}\) and from Pten\(^{Δf}\)\(:p53^{Δf}\) primary tumors. HTP robotic screens were performed on 1120 FDA-approved drugs (Prestwick library). The top three drugs that most efficiently suppressed all six tumor lines were the purine analog, 8-azaguanine, the imidazole antifungal agent, miconazole, and the dopamine antagonist, thiethylperazine malate (Figure 4-6A). Despite growing interest in the effect of dopamine antagonists on cancer stem cells (Sachlos, Risueno et al. 2012), we suspect that the effects of miconazole and thiethylperazine are not specific because multiple other imidazoles and dopamine inhibitors present in our FDA-approved library had much lower, often agonistic effects on tumor growth (Discussion). Sensitivity of Pten\(^{Δf}\)\(:p53^{Δf}\) and Pten\(^{Δf}\)\(:p53^{R270H}\) tumor cells to 8-azaguanine was validated on the six lines revealing IC\(50\) of 0.53 μM (Figure 4-6B, top; Discussion).

Tumors driven by a p53 DBD mutation are addicted to its continuous expression (Freed-Pastor, Mizuno et al. 2012; Weissmueller, Manchado et al. 2014; Alexandrova, Yallowitz et al. 2015).
Figure 4-5 Molecular clustering of spindle-like $\text{Pten}^\Delta_r : p53^{R270H}$ and $\text{Pten}^\Delta_r : p53^\Delta_f$ mammary tumors.

A. Hierarchical clustering using basal, luminal and mesenchymal genes showing that $\text{Pten}^\Delta_r : p53^{R270H}$ and $\text{Pten}^\Delta_r : p53^\Delta_f$ spindle tumors grouped together with human claudin-low breast cancer, whereas $\text{Pten}^\Delta_r : p53^{R270H}$ PDA and two of the spindle tumors grouped with human basal-like breast cancer.

B. Heat map showing expression of EMT genes in spindle-like $\text{Pten}^\Delta_r : p53^{R270H}$ and $\text{Pten}^\Delta_r : p53^\Delta_f$ tumors. EMT epithelial to mesenchymal transition, PDA poorly differentiated adenocarcinoma.
Addiction is at least in part due to mutant p53-mediated transcriptional activation of the mevalonate pathway, leading to increased sensitivity to statins, which inhibit 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase, the enzyme that produces mevalonate (Freed-Pastor, Mizuno et al. 2012). Importantly, depletion of mutant p53 in these tumors leads to cell demise. This was observed by knocking down p53 mutant expression via RNA interference or through HSP90 inhibitors, which destabilize mutant p53. Accordingly, we found that knocking down p53R270H protein expression via lenti-shRNA significantly reduced growth of primary PtenΔf:p53 R270H tumor cells (data not shown). However, selective inhibition of p53-mutant vs. p53-deleted TNBC may be achieved not only by destabilizing mutant p53 but also by targeting distinct oncogenic genes/pathways that cooperate with each p53 allele (i.e. mutation vs. deletion). We therefore asked whether any of the 1120 FDA-approved drugs had differential inhibitory effect on PtenΔf:p53Δf vs. PtenΔf:p53 R270H tumor cells. Surprisingly, our screens identified two statins (fluvastatin and simvastatin) (Figure 4-6A), as well as four other drugs (thiostrepton, podophyllotoxin, parbendazole, mebendazole) as having differential effects on PtenΔf:p53 R270H vs. PtenΔf:p53Δf. Upon robotic re-screens, PtenΔf:p53Δf showed increased sensitivity relative to PtenΔf:p53 R270H tumor cells to low concentrations of fluvastatin and simvastatin but not to the other drugs. This mild increase in sensitivity to statins was not reproducible or robust enough to show significance in subsequent validation experiments (p = 0.3341) (Figure 4-6B; bottom). Taken together, using highly similar PtenΔf:p53Δf vs. PtenΔf:p53 R270H claudin-low like tumor cells we found no robust differences in sensitivity to 1120 FDA-approved drugs (see Discussion).

4.3.7 Claudin-low-like and basal-like PtenΔf:p53 R270H tumor cells have higher metastatic potential than PtenΔf:p53Δf spindle mammary tumor cells

Results from our cluster analysis and HTP screens suggested that despite the similarity, PtenΔf:p53 R270H and PtenΔf:p53Δf spindle tumors exhibited variances. To explore such differences further, we performed pathway analysis using GSEA. Pathways enriched in the PtenΔf:p53Δf spindle tumors (blue in Figure 4-6C) were associated with cell proliferation and DNA repair/Brca, whereas pathways induced in PtenΔf:p53 R270H spindle tumors (red) were related to metastasis and other pathways (heat map of selected genes Figure 4-6D-F).
Figure 4-6 HTP screens and GSEA reveal similarities and differences between Pten^Δf:.p53^{R270H} and Pten^{Δf}.p53^{Δf} mammary tumors.

A. Average response of three independent primary tumor lines from WAP-Cre:Pten^{fl/fl}.p53^{R270H/wt} mice vs. three independent primary tumor lines from WAP-Cre:Pten^{fl/fl}.p53^{fl/fl} mice to 1120 FDA-approved small molecules. Highlighted are C (no cells negative control), 8-azaguanine, miconazole, thiethylperazine malate, fluvastatin and simvastatin.

B. Dose–response curves for 8-azaguanine (average IC50 = 0.53 μM; top) and fluvastatin (IC50 = 0.52 μM for mutant lines; IC50 = 0.34 μM for deletion lines; bottom) using MTT viability assay.

C. GSEA showing selected pathways enriched in claudin-low-like WAP-Cre:Pten^{fl/fl}.p53^{R270H/wt} (red) versus pathways enriched in WAP-Cre:Pten^{fl/fl}.p53^{wt/wt} (blue) tumors. Green lines connect overlapping pathways. Proliferation and metastasis pathways are highlighted.

D-F. Heat map showing expression of metastasis (D), proliferation (E) and DNA repair (F) genes in Pten^Δf:.p53^{R270H} vs. Pten^Δf:.p53^{Δf} spindle-like tumors. HTP high-throughput, GSEA, gene set enrichment analysis.
To functionally test the latter observation, we determined the metastatic potential of primary Pten$^{Δf}$:p53$^{R270H}$ spindle-like and PDA relative to primary Pten$^{Δf}$:p53$^{Δf}$ spindle tumor cells using tail vein injection assays. Although this assay does not monitor invasion and intravasation through the basal membrane into a blood or lymphatic vessel – it does measure for the ability of tumor cells to survive in the circulating system, extravasate and form macrometastases in distal sites, a rate-limiting step in the metastatic cascade. Following engraftment, mice were closely monitored for breathing difficulty, weight loss and sickness. Using these criteria, end points following transplantation of Pten$^{Δf}$:p53$^{R270H}$ spindle and PDA tumor cells were significantly shorter than for Pten$^{Δf}$:p53$^{Δf}$ double-deletion tumors (Figure 4-7A). Upon autopsy, lungs from all engrafted mice contained macroscopic nodules, which were confirmed as lung metastases by histological examination (Figure 4-7B-C). Like the TIC assay (Figure 4-3), the lung metastases from spindle tumors invariably shared the same spindle-shaped morphology as the parental tumors from which they were derived, whereas primary PDAs gave rise to metastases that showed either PDA or spindle morphology (Figure 4-7D-E). These observations point to subclonal diversity/intra-tumor heterogeneity and more efficient metastasis by Pten$^{Δf}$:p53$^{R270H}$ spindle tumor cells.

Primary Pten$^{Δf}$:p53$^{Δf}$ spindle tumors and their metastases showed near complete absence of the epithelial marker E-cadherin. In contrast, while Pten$^{Δf}$:p53$^{R270H}$ primary spindle tumors were largely negative for E-cadherin, lung metastases expressed significantly more of this transmembrane glycoprotein than primary lesions; PDA primary tumors were positive for E-cadherin to varying degrees, and this was largely preserved in metastatic counterparts (Figure 4-7F-G). While E-cadherin expression increased in Pten$^{Δf}$:p53$^{R270H}$ metastases, expression of the mesenchymal marker vimentin was reduced (Figure 4-7G). Together these results suggest that increased metastatic potential of Pten$^{Δf}$:p53$^{R270H}$ relative to Pten$^{Δf}$:p53$^{Δf}$ tumors may be attributed at least in part to their ability to undergo partial mesenchymal to epithelial (MET) conversion, which is required for efficient colonization and growth at distal sites (Brabletz 2012).

4.4 Discussion

We show that targeted deletion of the tumor suppressor Pten together with expression of a p53 DBD mutant, R270H, in mammary epithelium via WAP-Cre induces diverse mammary tumor subtypes including PDA and spindle tumors. Intra- and inter-species cluster analysis classified
Figure 4-7 WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} spindle and PDA tumor cells exhibit higher metastatic potential than WAP-Cre:Pten^{fl/fl}:p53^{fl/fl} spindle tumor cells.

A. Kaplan-Meier survival curve of NOD/SCID female mice following tail vein injection with indicated primary tumor cells showing that p53R270H mutation accelerated metastatic formation relative to p53 deletion. Mice were euthanized when moribund. Statistical significance by Wilcoxon method: spindle Pten^{fl/fl}/p53^{fl/fl} vs. spindle Pten^{fl/fl}/p53^{R270H}, p = 0.0004; spindle Pten^{fl/fl}/p53^{fl/fl} vs. PDA Pten^{fl/fl}/p53^{R270H}, p = 0.0012; spindle Pten^{fl/fl}/p53^{R270H} vs. PDA Pten^{fl/fl}/p53^{R270H}, p = 0.16.

B-E. Representative whole lung (B) and H&E images (C) of mice injected with WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} mammary tumor cells at end point. Arrowheads indicate metastatic lesions. Representative H&E images of (D) primary spindle and (E) primary PDA WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} mammary tumors and their metastatic counterparts. Note that one of the metastatic lesions from the PDA had spindle-shaped morphology (bottom right).

F. Representative images of E-cadherin expression in primary WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} and WAP-Cre:Pten^{fl/fl}:p53^{fl/fl} mammary tumors and lung metastases.

G. Expression levels of epithelial (E-cadherin) and mesenchymal (vimentin) markers in primary WAP-Cre:Pten^{fl/fl}:p53^{R270H/wt} and WAP-Cre:Pten^{fl/fl}:p53^{fl/fl} mammary tumors and lung metastases. H&E hematoxylin and eosin, PDA poorly differentiated adenocarcinoma
most PDA and spindle tumors as basal-like and claudin-low like TNBC, respectively. In stark contrast, double-deletion tumors from WAP-Cre:PTEN^f/f:p53^f/f mice invariably cluster with human claudin-low breast cancer (Liu, Voisin et al. 2014). Claudin-low-like TNBC were also found when Pten and p53, or Rb plus p53 were homozygously deleted via MMTV-Cre (Jiang, Deng et al. 2010; Liu, Voisin et al. 2014), or when the MET oncogene was overexpressed in mammary epithelium together with homozygous p53 deletion (Knight, Lesurf et al. 2013). Indeed, these articles showed that homozygous loss of p53 alone induces many, though not exclusively, claudin-low-like tumors, indicating that deletion of this tumor suppressor in the mouse directs tumorigenesis toward this subtype. The Pten^Δf:p53^R270H PDAs expressed the basal keratins K5 and K6, clustered with mouse models of BLBC and with human BLBC samples, exhibited high TIC frequency, and readily formed lung metastases following tail vein injections. Thus, WAP-Cre:PTEN^f/f:p53^lsl_R270H/+ mice offer a new model to study basal-like as well as claudin-low Pten/p53-deficient TNBC. Our results also suggest that WAP-Cre:p53^lsl_R270H/+ rather than WAP-Cre:p53^f/f mice should be used to model human breast cancer in conjunction with mutations in other breast cancer drivers.

PTEN^Δf:p53^Δf and PTEN^Δf:p53^R270H tumors provide the means to compare histologically indistinguishable tumors with either mutation of deletion of p53. We therefore performed FDA-approved drug screens to identify compounds that can kill both tumors or preferentially target tumors driven by p53 deletion or mutation. We found that 8-azaguanine, a purine analog, efficiently killed both PTEN^Δf:p53^Δf and PTEN^Δf:p53^R270H tumors. Sensitivity to 8-azaguanine may reflect high cell proliferation or low levels of guanine deaminase, which can convert this purine analog to a noncytotoxic metabolite (8-azaxanthine) (Meyers and Shin 1981). 8-azaguanine incorporation via hypoxanthine guanine phosphoribosyl transferase (HGPRTase) leads to inhibition of purine nucleotide synthesis. It may also be toxic due to its incorporation into RNA (Nelson, Carpenter et al. 1975). Despite some benefits to leukemia patients, adverse dermatological reactions, nausea and vomiting limit its use (Colsky, Meiselas et al. 1955). Additional analysis is required to assess possible benefits, if any, of 8-azaguanine either alone or in combination with PI3K antagonists or other inhibitors for Pten/p53-deficient TNBC. Notably, in a kinome screen of PTEN^Δf:p53^Δf tumors we identified eEF2K antagonists as better inhibitors than AKT and PI3K inhibitors (Liu, Voisin et al. 2014). Such pathway-specific drugs are much more likely to have a large therapeutic window as compared to cytotoxic drugs like 8-
azaguanine. Additional screens with both \( \text{Pten}^\Delta f : \text{p53}^\Delta f \) and \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumors as well as with human Pten/p53-deficient breast cancer lines using large libraries of divergent compounds may uncover new potent drugs for this aggressive cancer subtype.

Contrary to published results on increased sensitivity of mutant p53 to statins (Freed-Pastor, Mizuno et al. 2012), we observed consistent but mild and statistically insignificant increase in sensitivity of \( \text{Pten}^\Delta f : \text{p53}^\Delta f \) tumor cells to HMG-CoA inhibitors relative to \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumor cells. One explanation for this discrepancy may be that although p53 mutation alone increases sensitivity to statins by inducing the mevalonate pathway, tumors that develop in the absence of p53 (p53 deletion) may increase sensitivity to statins through other mechanisms. Indeed, we found that a signature that predicts sensitivity to statins (Goard, Chan-Seng-Yue et al. 2014) is elevated in \( \text{Pten}^\Delta f : \text{p53}^\Delta f \) relative to \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumor cells (not shown). We note that the FDA-approved drug library we used here does not include the HSP90 inhibitor geldanamycin or its more potent derivatives, which destabilize mutant p53. However, we found that knockdown of \( \text{p53}^{R270H} \) protein impeded cell growth (not shown) indicating that \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumor cells are addicted to continuous expression of mutant p53.

Despite – and perhaps because of – the inter- and intra-tumor heterogeneity seen in WAP-Cre: \( \text{Pten}^{lif} : \text{p53}^{lif} \text{R270H}^{+/+} \) mice, they offer certain advantages over WAP-Cre: \( \text{Pten}^{lif} : \text{p53}^{lif} \) lif mice. First, they develop PDA/basal-like tumors in approximately 10% of mice and spindle/claudin-low-like tumors in approximately 30% of mice. Tumor-bearing animals can be biopsied to determine histology and then treated with candidate drugs, allowing their effect to be tested on diverse tumor types with similar oncogenic initiation events (Pten loss; p53 mutation). Alternatively, as described herein, tumors can be harvested, frozen, classified and then transplanted to obtain multiple xenografts with uniform tumor subtype. Together, the WAP-Cre: \( \text{Pten}^{lif} : \text{p53}^{lif} \text{R270H}^{+/+} \) and WAP-Cre: \( \text{Pten}^{lif} : \text{p53}^{lif} \) lif mice model the spectrum of p53 gene aberrations and Pten loss seen in human TNBC.

Using GSEA and functional analysis we showed that \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumor cells are more metastatic than \( \text{Pten}^\Delta f : \text{p53}^\Delta f \) tumors and this was correlated with induction of E-cadherin in lung metastases from \( \text{Pten}^\Delta f : \text{p53}^{R270H} \) tumor cells. P53 mutations in the DBD were shown to increase metastasis through induction of EMT via several routes including sequestration of p63,
stabilization/activation of SLUG/SNAIL, induction of the mir130b-Zeb1 axis, TWIST and SLUG transcription factors and other mechanisms (Senoo, Pinto et al. 2007; Godar, Ince et al. 2008; Adorno, Cordenonsi et al. 2009; Muller, Caswell et al. 2009; Wang, Wang et al. 2009; Powell, Piwnica-Worms et al. 2014). Our study suggests that once disseminating mutant p53 tumors home at distal sites, they more readily undergo MET, which enhances colonization, the dominant rate-limiting step of the metastatic cascade (Brabletz 2012; Ocana, Corcoles et al. 2012; Tsai, Donaher et al. 2012).

4.4.1 Limitations

A limitation of this study is the use of tail-vein injection assays to model metastatic spread. This assay involves injection of mammary tumor cells directly into the circulation in order to evaluate the ability of injected cells to form metastatic lesions (Mohanty and Xu 2010). Tail-vein assays thus fail to recapitulate the earlier local invasion and intravasation steps of the metastatic cascade (Saxena and Christofori 2013). A more accurate model would be to monitor the ability of cells from a primary tumor to spontaneously metastasize. If needed, primary tumors can be surgically resected to prolong survival and allow sufficient time for disseminated tumor cells to establish metastatic lesions. I tried to create a spontaneous metastatic model with xenografts of Pten^Δf:p53^R270H mammary tumor cells in NOD SCID mice. I removed primary tumors to prolong survival. However, tumors often recurred within a week of surgery and further optimization is required.

4.5 Conclusions

WAP-Cre:Pten^Δf:p53^lox,stop,lox_R270H/+ mice described herein together with WAP-Cre:Pten^Δf:p53^lox,stop,lox_R270H/+ mice (Liu, Voisin et al. 2014), provide powerful preclinical models to interrogate and decipher the genetic cooperation that drives primary basal-like and claudin-low TNBC development and metastatic spread/colonization, as well as identify and test for novel therapeutics that target these aggressive breast cancers.
Chapter 5

5 Future Directions

5.1 Cooperation between PTEN-deletion and miR-143/145-loss

As described in Chapter 3, I found most Pten-null mammary tumors to be well-differentiated (AMEs), which generally did not form secondary tumors following transplantation. Intriguingly, a group of rare Pten-null tumors with distinct histology (PDAs) did harbor highly transplantable TICs. In comparing these two tumor types, I identified low expression of tumor suppressor miR-143/145 as a critical factor that determined transplantability of Pten-null tumor cells. Furthermore, enforced knockdown of miR-143/145 in Pten-null tumor cells induced RAS pathway activation, promoted secondary tumor engraftment and enhanced sensitivity to MEK inhibition. In human BC, patients with low PTEN/miR143-145 are preferentially found in the basal-like subtype, and these patients had a poor clinical outcome. This data highlights cooperation between PTEN loss and miR-143/145 loss in aggressive BC.

A critical future direction is to determine if dual targeting of PI3K pathway and miR-143/145 can result in sustained and robust therapeutic response for patients with low-PTEN/low-miR143/145 basal-like BCs. To answer this question, we can utilize preclinical models of basal-like and TN patient derived xenografts (PDXs) (Ding, Ellis et al. 2010; Zhang, Cohen et al. 2014). PDXs that harbor PTEN deletion and miR-143/145 loss can be identified and treated with compounds that target the PI3K pathway (such as LY294002) and restore miR-143/145 tumor suppressor expression and function. Several approaches can be taken to restore miR-143/145. First, since a primary function of this miRNA cluster is to downregulate the Ras pathway, we can explore using Ras pathway inhibitors. Indeed, I showed in Chapter 3 that MEK inhibitors (i.e., selumetinib) were particularly effective against cells with repressed miR-143/145 levels. A second approach is to replace miR-143/145 through delivery of miRNA expressing vectors or stabilized oligonucleotides (Zhang, Wang et al. 2013). Similar approaches utilizing lipid-based nanoparticles for systemic miR-143/145 delivery have been shown to induce pancreatic tumor inhibition in mice (Pramanik, Campbell et al. 2011). A third approach is to conduct high
throughput compound screens in miR-143/145-deficient cells to identify novel molecules that can mimic miR-143/145 function (Haefliger, Prochazka et al. 2016). If PDXs respond to such dual targeting of PI3K and miR-143/145, we can then assess this treatment strategy in patients.

This study focused on the consequences of miR-143/145 inhibition in AME tumor cells, thereby modelling the effects of a second oncogenic hit once a Pten-null mammary tumor has become fully established. An important question to explore is how miR-143/145 inhibition cooperates with Pten deletion to affect earlier stages of tumor initiation. Two approaches can be used to answer this question. The first is to isolate and transduce Pten-null mammary epithelial cells (MECs) with miR-143/145 decoy ex vivo, transplant the resulting cells into immunocompromised mice and monitor for tumor formation. Other groups have published similar experimental workflows and reported 4-93% infection efficiencies and secondary tumor formation (Welm, Dijkgraaf et al. 2008). However, in my experience, achieving high levels of MECs transduction efficiency is challenging (data not shown) and requires further optimization. I also tried to simplify the experimental process by delivering lentivirus directly into WAP-Cre:Pten^fl/fl mammary glands via intraductal injections, but with minimal success (data not shown) (Du, Podsypanina et al. 2006).

The second approach is to develop genetically modified mice harbouring mammary-specific deletions of both Pten and miR-143/145. Other groups have created mice with single miR-143 or -145 deletions and mice with combined deletions (Xin, Small et al. 2009). These mice are viable and show defects in smooth muscle phenotypic switching during vascular disease. Composite triple mutant mice (i.e., Pten/miR-143/145-deletions) can be created by breeding with mammary specific WAP-Cre:PTEN^fl/fl mice. Cohorts of triple mutant mice can then be monitored for differences in tumor latency and penetrance. At endpoint, tumors can also be analyzed for histology, TIC frequency, molecular subtype and oncogenic pathway activation (such as Ras). Finally, if appropriate, these mice can be used as a model for drug discovery targeting PTEN-low/miR-143/145-low BCs. Based on this study, I expect that triple mutant mice will develop tumors with poorly-differentiated histology, high TIC frequency and elevated Ras signaling.

Pten-null tumor cells only lost transplantability when processed into single cells (such as post FACS isolation). When groups of tumor cells were left intact (such as in tumor chunks or crude
digests), these cells were capable of forming secondary tumors in immunocompromised mice, suggesting that tumor-associated stromal components may help to maintain or promote tumorigenicity. Indeed, in a model of squamous cell carcinoma of the skin, transplantation of FACS-isolated tumor cells similarly did not lead to secondary tumors (Lapouge, Beck et al. 2012). However, when these cells were co-transplanted with tumor-associated stromal components, secondary tumors did form. I initiated a similar approach to co-transplant Pten-null mammary epithelial tumor cells with tumor associated endothelial cells, hematopoietic cells and fibroblasts. I found that when tumor epithelial cells were co-transplanted with endothelial or hematopoietic cells, secondary tumors did not form (data not shown). However, I did not investigate the effects of co-transplanting tumor cells with tumor-associated fibroblasts. If further analyses showed that tumors can form under these conditions, this would indicate that tumor microenvironment is crucial for maintaining tumorigenicity of Pten-null tumor cells. Additional analyses can then delineate oncogenic mechanisms associated with tumor-stromal interactions and identify potential therapeutic targets. Notably, since breast carcinoma-associated fibroblasts (CAFs) are more genetically stable and rarely demonstrate copy number variations, therapies targeting CAFs may help overcome treatment issues associated with BC heterogeneity (Qiu, Hu et al. 2008).

5.2 Cooperation between PTEN-deletion and p53 mutation

TNBCs display frequent alterations in established tumor suppressors such as PTEN and TP53 (The Cancer Genome Atlas Network 2012). However, the functional cooperations of these alterations are not well defined. I developed a mouse model with mammary specific deletion of Pten and dominant negative point mutation in Trp53. I investigated the combined oncogenic effects of PtenΔf:p53R270H, and highlighted differences in oncogenic cooperativity to those found in PtenΔf:p53Δf tumors. Major findings include: composite PtenΔf:p53R270H mice develop diverse mammary tumors including basal-like and claudin-low-like. In contrast, the overwhelming majority of mammary tumors arising from p53Δf and PtenΔf:p53Δf mice are claudin-low tumors (Knight, Lesurf et al. 2013; Liu, Voisin et al. 2014), suggesting that p53 deletion in the mouse mammary gland shifts tumor formation towards a claudin-low subtype. Additionally, PtenΔf:p53R270H claudin-low tumors appeared to be less committed to EMT as they generally exhibited both a smaller degree of enrichment in EMT markers (such as N-cadherin and Zeb1).
and a smaller degree of downregulation in claudins and E-cadherin. This more flexible phenotype translated into greater metastatic potential as Pten\[^\alpha\_f\].p53\[^R270H\] tumor cells were more capable of undergoing MET at distant sites than were Pten\[^\alpha\_f\].p53\[^\alpha\_f\] tumors cells. Collectively, this study demonstrated differences between Pten\[^\alpha\_f\].p53\[^R270H\] and Pten\[^\alpha\_f\].p53\[^\alpha\_f\] in tumor spectrum, molecular subtype and metastatic progression, but not sensitivity to therapy.

I conducted preliminary analyses to determine the status of miR-143/145 expression in Pten\[^\alpha\_f\].p53\[^R270H\] and Pten\[^\alpha\_f\].p53\[^\alpha\_f\] tumors. Using gene expression data, I found miR-143/145 levels to be highest in normal mammary gland tissues and lowest in Pten\[^\alpha\_f\].p53\[^\alpha\_f\] tumors (data not shown). This indicates that similar to Pten-null mammary tumors (see Chapter 3), low levels of miR-143/145 is also associated with aggressive Pten- and p53- inactivated/mutated tumors. Additionally, since Pten\[^\alpha\_f\].p53\[^\alpha\_f\] mice are models of Claudin-low BC subtype (Liu, Voisin et al. 2014), we can also identify those Claudin-low tumors that express low levels of miR-143/145 and determine if 1) Ras pathway is activated, 2) patients experience worse clinical outcome, and 3) tumors are more responsive to MEK inhibitors.

p53 status may affect therapeutic response to chemotherapies. Preclinical studies showed that wildtype p53 impairs response to doxorubicin, an anthracycline (Jackson, Pant et al. 2012). However, a large Phase III study did not find p53 status to be predictive of selective sensitivity to an anthracycline-based versus a taxane-based chemotherapy (Bonnefoi, Piccart et al. 2011). It is important to understand how p53 may affect treatment response in patients with both Pten- and p53- inactivation/mutations. Here, we conducted a HTP drug screen to identify differential inhibitory effects in Pten\[^\alpha\_f\].p53\[^R270H\] vs. Pten\[^\alpha\_f\].p53\[^\alpha\_f\] tumor cells (See 4.3.6). In a screen of 1120 FDA-approved drugs, we found modest but insignificant differences. Future studies can conduct more comprehensive HTP screens using small molecule, peptide and peptidomimetic libraries. Indeed, our lab has previously used a small molecule kinase screen to identify eukaryotic elongation factor-2 kinase (eEF2K) as a promising candidate for TNBCs with high AKT pathway activity (Liu, Voisin et al. 2014).

My analyses focused on the oncogenic cooperation between Pten deletion and p53 R270H mutant. However, a much greater variety of PTEN mutations are observed in cancers, including point mutations that selectively inactive specific PTEN functional domains (see 1.2.1).
Therefore, to fully understand the cooperation between PTEN and p53 in aggressive BCs, it would be important to develop mammary tumor models harbouring different combinations of \textit{Pten}-domain inactivation with \textit{p53}^{R270H}. Using these models, we can determine if selective inactivation of various PTEN domains (such as the phosphatase or PTEN-long domain) results in different oncogenic cooperation with mutant \textit{p53}^{R270H}. Specifically, we can monitor such mice for differences in tumor spectrum, molecular subtype and metastatic potential. Importantly, these models can be used to interrogate differential sensitivity to anti-cancer inhibitors in order to develop tailored treatment strategies. For example, cells lacking nuclear PTEN are particularly sensitive to DNA damage therapies due to inability to repair double-stranded DNA breaks (Bassi, Ho et al. 2013). Therefore, patients with nuclear-excluded PTEN mutants may especially benefit from genotoxins. But would patients harboring both nuclear excluded PTEN and \textit{p53}^{R273H} mutation (human equivalent of mouse \textit{p53}^{R270H} mutation) also respond in a similar manner? Thus, these composite models can be used to identify effective treatment strategies against aggressive \textit{p53}^{R273H} BCs with various PTEN domain-inactivated mutants.


Catalog of Somatic Mutations in Cancer. from cancer.sanger.ac.uk.


