Title
Understanding the Early Events in Breast Carcinogenesis by Inactivating p16INK4a in Primary Human Mammary Epithelial Cells

Author
Pickering, Curtis Reid

Publication Date
2008-07-21

Peer reviewed|Thesis/dissertation

Understanding the Early Events in Breast Carcinogenesis by Inactivating p16INK4a in Primary Human Mammary Epithelial Cells

by

Curtis Reid Pickering

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Acknowledgements

No biomedical research is performed in isolation, and it is important to recognize the many people who influenced me during the course of my research. I am greatly indebted to my mentors from Michigan State University, Dr. J Throck Watson and Dr. James Trosko. These professors let me get my feet wet in a research lab and provided encouragement and mentoring that helped my career progress to where it is today. I am grateful to the many members of the Tlsty Laboratory during my tenure there. I would specifically like to thank C. Holst, M. Gauthier, H. Berman, K. McDermott and C. Fordyce. G. Benton has been a great business partner and friend. I must also thank Thea Tlsty for giving me the opportunity to grow as a scientist in her laboratory.

I would not be here without the support of my family and friends. Most importantly, this work would not have been possible without the encouragement of my parents and my brother.

Chapter II of this dissertation is a reprint of material originally published in the journal Cancer Research. This material is reprinted with permission from the publisher.
Zhang J*, Pickering CR*, Holst CR, Gauthier ML, Tlsty TD. $p16^{INK4a}$ Modulates p53 in Primary Human Mammary Epithelial Cells. Cancer Res 2006; 66(21): 10325-31.
*These authors contributed equally to this work.
Chapter II lists multiple co-authors. TD Tlsty directed and supervised the research, and CR Holst and ML Gauthier contributed experimental assistance. As noted above, J Zhang and

CR Pickering contributed equally to this work. While J. Zhang initiated these studies, they were completed by CR Pickering, and the resulting manuscript was written by CR Pickering. This work is included as a part of the dissertation of CR Pickering.

Understanding the Early Events in Breast Carcinogenesis by Inactivating p16INK4a in Primary Human Mammary Epithelial Cells

Curtis Reid Pickering

Cancer cells arise from normal cells that have acquired the ability to expand beyond the constraints of a tissue microenvironment. Normal human cells have many mechanisms to prevent carcinogenesis. During the process of carcinogenesis one of the properties a cell must acquire is the ability to evade anti-proliferative signals. Many stimuli cause normal cells to activate a cell cycle arrest. The $p16^{INK4a}$ gene is an important tumor suppressor that activates a cell cycle arrest in response to stimuli like stress, DNA damage, and senescence. Inactivation of $p16^{INK4a}$ occurs in many tumor types and allows the cells to bypass many anti-proliferative signals. Inactivation of $p16^{INK4a}$ is found in about 30% of breast tumors (Rocco and Sidransky, 2001), yet the consequences of that inactivation are not completely understood. We sought to improve our understanding of $p16^{INK4a}$ inactivation in breast carcinogenesis by inactivating $p16^{INK4a}$ in primary human mammary epithelial cells (HMEC). Studying primary human cells allowed us to understand the consequences of $p16^{INK4a}$ inactivation in cells with a wild-type genetic background. Additionally, this system allowed us to examine the role of $p16^{INK4a}$ in early breast carcinogenesis, with a long-term goal of identifying avenues for early detection and preventive intervention of breast cancer. In Chapter II, we describe how inactivation of $p16^{INK4a}$ modulates the levels and functions of another important tumor suppressor gene, p53. The Rb pathway is identified as being necessary for $p16^{INK4a}$ to modulate p53. In Chapter III, we further our understanding of the modulation of p53 by $p16^{INK4a}$ and determine that ATM and the DNA damage response may

be involved. Regulation of cell cycle checkpoints following p16 inactivation is also examined. In Chapter IV, we perform global gene expression analysis to identify new genes and pathways modulated by inactivation of $p16^{INK4a}$ in HMEC. We identify chitinase-3-like-1 as a novel $p16^{INK4a}$ regulated gene that is overexpressed in the basal-like subtype of invasive breast tumors. Chitinase-3-like-1 may be useful as a serum biomarker or therapeutic target for basal-like breast tumors.

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1. Chapter I

Introduction

1.1. Breast Cancer

The American Cancer Society estimated that in the year 2007 there would be 178,000 new cases of invasive breast cancer and 62,000 new cases of *in situ* breast cancer in the United States. They also estimated 40,000 deaths due to breast cancer that same year. Breast cancer is a horrible disease and a public health burden. It is important that we try to improve our understanding of this disease to develop new ways to treat and prevent it.

Breast cancer affects over 200,000 women each year. At least two aspects of breast cancer research and treatment, early detection and molecularly targeted therapeutics, can serve as examples for other cancer fields. For example, breast cancer is one of only a few tumor types that has a cheap, non-invasive method for early detection. Mammography has revolutionized breast cancer detection. Women throughout the world are frequently screened for breast cancer and this has significantly reduced the mortality of breast cancer (Nemec et al., 2007). Although mammography has been very successful for early detection of breast cancer it can still be improved upon.

One major drawback to mammography is that it is not able to differentiate lesions with poor prognosis from more benign lesions. For example, mammography is able to detect malignant breast carcinoma but it also frequently detects ductal carcinoma in situ (DCIS). DCIS is a premalignant breast disease that will recur as invasive breast cancer in about 10% of patients (Kerlikowske et al., 2003). The ability of mammography to detect DCIS has created the problem of overtreatment in breast cancer. Because only a fraction of DCIS lesions will progress to an invasive breast tumor, the clinician and patient are often left with difficult

clinical decisions. How aggressively should one treat a lesion with a 10% chance of progression? A major clinical need is for a more accurate way to predict the prognosis of DCIS. Additionally, although mammography is relatively inexpensive, it requires specialized equipment and skilled radiologists to read the scans. An ideal tool for early detection of breast cancer would be a serum diagnostic. One can imagine a serum-based assay with higher sensitivity and specificity than mammography and more accurate prediction of prognosis. A serum-based assay could be analyzed in a standard clinical laboratory and would not require a skilled radiologist. However, a serum diagnostic for breast cancer will only be useful if it improves upon the sensitivity, specificity or predictive power of mammography.

Another example of the advancement of the breast cancer field is in molecularly targeted treatments. An ultimate clinical goal is to have personalized medicine, where treatments are tailored for each individual case. Breast cancer has taken some of the first steps toward that goal. Breast tumors are traditionally classified with three markers; estrogen receptor (ER), progesterone receptor (PR), and HER2. These three markers can classify many breast tumors into one of three molecular subtypes; luminal (ER/PR positive), HER2 (HER2 positive), or basal-like (ER/PR negative and HER2 negative). These molecular subtypes have also been characterized based on molecular profiling with gene expression arrays. Treatments are currently available to target two out of the three breast cancer subtypes. Luminal tumors are treated with tamoxifen or other hormone therapies. HER2 tumors are treated with Herceptin, which is a monoclonal antibody therapy that targets HER2. Only the basal-like tumors do not have a tailored treatment. While the treatment of breast cancer has taken the first steps toward personalized medicine there is still much room for improvement. Specifically, better

treatments need to be found that target the basal-like subtype of breast cancer. Within the classical subtypes mentioned above, the tumors exhibit heterogeneity, and respond to therapy with different outcomes. Additionally, there are many more subtypes of breast cancer that have not yet been characterized (Kreike et al., 2007). These subtypes need to be studied molecularly and targeted therapeutically.

Our research has focused primarily on understanding the early events in the process of breast carcinogenesis. An improved understanding of the early events in breast carcinogenesis should facilitate the development of better modalities for early detection. This understanding should also facilitate the development of preventive therapeutics, markers for risk assessment, and more targeted therapeutics.

1.2. HMEC System

In order to understand the early events in breast carcinogenesis, we have utilized the human mammary epithelial cell (HMEC) culture system. This model system uses primary human breast epithelial cell populations from disease-free women. These populations of epithelial cells should contain the initiating cells for many types of breast cancers, although the specific identities of these cells are still unknown. Because this system uses human cells from disease-free women it allows for studies into the first events in breast carcinogenesis. An understanding of the first events will shed light on the origins of breast cancer and allow the development of preventive interventions.

This system was originally developed by Martha Stampfer in 1989 (Taylor-Papadimitriou et al., 1989). In this system, primary human mammary epithelial cells are isolated from disease-free tissue (usually spare tissue from reduction mammoplasty surgery) and propagated *in vitro* in specialized culture media. These culture conditions select for the propagation of two specific cell populations, HMEC and variant HMEC (vHMEC) (Brenner et al., 1998; Foster et al., 1998; Huschtscha et al., 1998; Romanov et al., 2001). When the digested tissue is placed in culture the HMEC population proliferates for about 10-15 population doublings (PD) before reaching a proliferative plateau characterized by induction of the cell cycle inhibitor $p16^{INK4a}$ and cell cycle arrest. After a brief period of time (as little as 2 days or as long as 2 weeks) a population of proliferative cells emerges from this plateau and continues proliferating. These cells have been termed vHMEC (previous names include post-selection, post-stasis, and post-G0) and are able to proliferate due to loss of $p16^{INK4a}$ expression caused by promoter DNA hypermethylation (Foster et al., 1998; Huschtscha et al., 1998). This promoter DNA hypermethylation silences expression of the $p16^{INK4a}$ gene. Although the vHMEC have silenced $p16^{INK4a}$ expression they are still non-immortalized and non-tumorigenic. The vHMEC proliferate for an additional 30-50 PD before reaching a second proliferative plateau, characterized by a balancing act between both proliferation and cell death (Romanov et al., 2001).

The HMEC system has proven quite useful in understanding the early events in breast carcinogenesis (Crawford et al., 2004; Holst et al., 2003; McDermott et al., 2006; Reynolds et al., 2006; Romanov et al., 2001). The HMEC have no known consistent genetic alterations and this allows us to use them to understand the consequences of individual genetic changes

during early carcinogenesis. In contrast, tumor cells have many genetic alterations which make it more difficult to understand the contribution of each individual alteration. Additionally, since the carcinogenic process is believed to begin with genetically wild-type cells, the HMEC system allows us to study how the early events in carcinogenesis affect genetically wild-type cells.

The vHMEC, which have silenced $p16^{INK4a}$ expression, are also useful to understand the early events in breast carcinogenesis. vHMEC exhibit a variety of properties found in cancerous cells, including the potential for genomic instability, telomere dysfunction, centrosome dysfunction, and increased expression of cyclooxygenase-2 (COX-2) (Crawford et al., 2004; McDermott et al., 2006; Romanov et al., 2001). However, because vHMEC are non-immortalized, non-tumorigenic, and have few genetic changes in early *in vitro* propagation this system allows us to study the properties of cancer cells in a premalignant context.

Strikingly, the HMEC system has proven useful in identifying markers for early breast cancer lesions. Many of the molecular changes present in vHMEC (i.e. COX-2, $p16^{INK4a}$, phospho-p38) have also been identified in premalignant breast disease (Gauthier et al., 2007). Two specific sets of markers (high $p16^{INK4a}$ and high Ki-67, high COX-2 and high Ki-67) that were identified in vHMEC have recently been shown to identify the basal-like subtype of invasive breast cancer and predict recurrence in ductal carcinoma *in situ* (DCIS) (Gauthier et al., 2007). These markers may soon be used clinically to tailor treatment of DCIS lesions.

This demonstrates the utility of using the HMEC system to study the early events in breast carcinogenesis.

1.3. The Tumor Suppressor p16INK4a

In 1993, the $p16^{INK4a}$ gene was discovered because it coded for a protein that bound to and inhibited CDK4 (Serrano et al., 1993). Because of this ability, it was hypothesized that $p16^{INK4a}$ is an important cell cycle regulator. In 1994, mutations to $p16^{INK4a}$ were identified in melanoma cell lines and familial melanoma, and it was hypothesized that $p16^{INK4a}$ is a tumor suppressor gene (Kamb et al., 1994). Since then, mutations to $p16^{INK4a}$ have been identified in numerous tumor types, and $p16^{INK4a}$ is recognized as an important tumor suppressor gene (Rocco and Sidransky, 2001). The $p16^{INK4a}$ protein acts as a brake on the cell cycle by initiating a G1-phase cell cycle arrest. During carcinogenesis, inactivation of $p16^{INK4a}$ allows cells to proliferate in conditions that would otherwise induce $p16^{INK4a}$ and a cell cycle arrest. $p16^{INK4a}$ function is inactivated in many tumors by a variety of mechanisms including mutation, homozygous deletion, and promoter DNA hypermethylation (Kim and Sharpless, 2006). Additionally, $p16^{INK4a}$ function can be inhibited in tumors by mutation of CDK4 to prevent $p16^{INK4a}$ binding (Helsing et al., 2008; Molven et al., 2005). The frequency of $p16^{INK4a}$ inactivation varies dramatically by tumor type from >70% in pancreatic and esophageal cancers to <10% in sarcoma and renal cell cancer (Rocco and Sidransky, 2001). The route of $p16^{INK4a}$ inactivation can also vary dramatically by tumor type with some tumor types favoring promoter DNA hypermethylation and other types favoring homozygous deletion. In breast cancer, $p16^{INK4a}$ is inactivated in 31% of tumors (Brenner and Aldaz, 1995; Cairns et al., 1995; Rocco and Sidransky, 2001). The primary mode of inactivation is

through promoter DNA hypermethylation (20% of tumors). Additionally, familial mutation of $p16^{INK4a}$ predisposes to melanoma, pancreatic adenocarcinoma and breast cancer (Borg et al., 2000; Ghiorzo et al., 1999).

The intricacies of $p16^{INK4a}$ signaling, the unique aspects of the genomic locus and the phenotypic descriptions of the multiple mouse models for $p16^{INK4a}$ inactivation can be found in the many excellent review articles that have been written about $p16^{INK4a}$ (Gil and Peters, 2006; Kim and Sharpless, 2006; Rocco and Sidransky, 2001; Sharpless, 2004; Sharpless, 2005). Only a few important details will be described here.

$p16^{INK4a}$ functions as a tumor suppressor gene because of its ability to cause a cell cycle arrest (Sharpless, 2004). To induce a cell cycle arrest $p16^{INK4a}$ binds to cyclin-dependent kinase 4 (CDK4) and prevents its binding to CyclinD. This binding inhibits the kinase activity of CDK4 and prevents it from phosphorylating pRb. Phosphorylation of pRb is an important step in G1-phase cell cycle progression. In short, $p16^{INK4a}$ protein prevents pRb phosphorylation and causes the cells to arrest in G1-phase of the cell cycle. An important aspect of this signaling pathway is that $p16^{INK4a}$ signals through pRb. pRb is also an important tumor suppressor gene that is inactivated in many tumors. Interestingly, inactivation of $p16^{INK4a}$ and inactivation of pRb are usually mutually exclusive events. It is very rare to find a tumor cell that has inactivated both $p16^{INK4a}$ and pRb. With respect to cell cycle control, inactivation of $p16^{INK4a}$ and inactivation of pRb are believed to be functionally equivalent, both events allow for bypass of a G1-phase cell cycle arrest. However, with respect to other cellular processes, like DNA replication and chromatin modulation, $p16^{INK4a}$

and pRb have unique functions (Braden et al., 2006; Kotake et al., 2007). It has even been suggested that pRb is necessary for the epigenetic silencing of $p16^{INK4a}$ (Kotake et al., 2007). In breast cancer about 30% of tumors directly inactivate $p16^{INK4a}$ (Rocco and Sidransky, 2001) and about 50% of tumors inactivate pRb (Bosco and Knudsen, 2007). It is not clear if inactivation of $p16^{INK4a}$ and pRb are functionally equivalent in breast cancer. It is intriguing to speculate how the carcinogenic process of each tumor may influence how it inactivates the p16/pRb pathway. Is methylation of $p16^{INK4a}$ an early event in breast carcinogenesis while inactivation of pRb is a later event? Does the cell of origin determine how the p16/pRb pathway is inactivated? The basal-like subtype of breast cancer frequently inactivates pRb and exhibits high levels of $p16^{INK4a}$ (Gauthier et al., 2007), while the estrogen receptor positive subtype often retains functional pRb (Bosco and Knudsen, 2007). Answering these questions would improve our understanding of breast carcinogenesis and potentially identify targets for intervention.

Senescence is a permanent cell cycle arrest induced during the process of cellular aging. Senescence is now recognized as a barrier to carcinogenesis (Braig et al., 2005; Chen et al., 2005; Collado et al., 2005; Michaloglou et al., 2005), and a major role of $p16^{INK4a}$ is to activate and maintain the senescent cell cycle arrest (Sharpless, 2004). It has been documented that $p16^{INK4a}$ levels increase during cellular senescence. This protein is also induced in response to certain cellular stressors, i.e. UV light, and tissue culture. However, because stressful conditions also induce senescence is it not clear if $p16^{INK4a}$ has tumor suppressor functions independent of its role in senescence. Very little is understood about the mechanisms of $p16^{INK4a}$ induction in any circumstance. It is difficult to rapidly induce

endogenous expression of $p16^{INK4a}$. Although stress will induce $p16^{INK4a}$, the induction often occurs over a few days and is compounded by induction of other CDK inhibitors like p21. Because of these experimental hurdles most of the studies on $p16^{INK4a}$ biology have involved exogenous over-expression of the protein. This has led to a significant amount of knowledge about the consequences of over-expression of $p16^{INK4a}$ but very little knowledge about the endogenous induction of $p16^{INK4a}$ expression. Additionally, very few studies (with the mouse models being the major exception) have investigated how inactivation of $p16^{INK4a}$ impacts cells. Studies on familial inactivating $p16^{INK4a}$ mutations have demonstrated an antiproliferative role for $p16^{INK4a}$ in both traditional senescence and oncogene-induced senescence (Brookes et al., 2004; Drayton et al., 2003), but it is important to learn more about the consequences of $p16^{INK4a}$ inactivation because this event frequently occurs in human tumors. The inactivation of $p16^{INK4a}$ often occurs early in the carcinogenic process, and knowledge about the consequences of $p16^{INK4a}$ inactivation could shed new light on the process of carcinogenesis. Thus, $p16^{INK4a}$ signaling could potentially be used to identify targets for early detection or preventive intervention.

1.4. Chitinase-3-like-1

As will be described later, we identified chitinase-3-like-1 (CHI3L1) as a gene modulated by $p16^{INK4a}$. The CHI3L1 gene codes for a protein similar to mammalian chitinase but that lacks chitinase enzymatic activity due to a mutation in the active site (Johansen et al., 2006). (The CHI3L1 protein has many names, including, HC gp39, brp-39, MGP-40, and Chondrex, but it is most commonly called YKL-40. For the purpose of clarity in this dissertation both the gene and protein will be called CHI3L1.) Mammalian chitinases are able to degrade the

polymer chitin, which is found in crustacean shells as well as the surface of pathogenic organisms like fungi and bacteria. Although CHI3L1 does not have chitinase enzymatic activity it is induced by a variety of inflammatory conditions, including irritable bowl disease and rheumatoid arthritis (Kawada et al., 2007). CHI3L1 is secreted by inflammatory cells and can be detected in human serum. While the role of CHI3L1 in inflammation is not well understood, studies suggest that CHI3L1 increases the severity of inflammation. In colitis, CHI3L1 enhances the adhesion and invasion of bacteria to colonic epithelial cells (Kawada et al., 2007), and in asthma, CHI3L1 induces airway hypersensitivity (Kawada et al., 2007). In both of these conditions, inhibition of CHI3L1 reduces inflammation. Importantly for our studies, CHI3L1 is also secreted by a variety of tumor types including breast cancer. CHI3L1 can be detected in the serum of some breast cancer patients (Jensen et al., 2003; Johansen et al., 2003; Johansen et al., 1995). The function of CHI3L1 in carcinogenesis is mostly unknown. It can cause proliferation, invasion, or migration of various cell types, like fibroblasts and endothelial cells (Johansen, 2006). This indicates that CHI3L1 may act as a tumor growth factor or increase angiogenesis. A cellular receptor for CHI3L1 is not known, so the mechanism with which CHI3L1 signals to these cells is unknown. Additionally, CHI3L1 is able to bind to components of the extracellular matrix. CHI3L1 binds heparin-sulfate proteoglycans which can regulate signaling in the extracellular matrix (Fusetti et al., 2003). CHI3L1 also binds type I collagen and regulates the rate of collagen fibril formation (Bigg et al., 2006). These data suggest that CHI3L1 may function to regulate the cellular microenvironment and cell-cell signaling.

1.5. Summary

In summary, we have utilized the HMEC system to improve our understanding of the early events in breast carcinogenesis. We hope that these studies will facilitate the identification of new targets for early detection and preventive intervention of breast cancer. The studies described in this dissertation will investigate the molecular consequences of inactivation of $p16^{INK4a}$. These studies focus on how p16 modulates the tumor suppressor p53, cell cycle regulation, and the DNA damage response. Additionally, we will describe a global screen to identify genes modulated by $p16^{INK4a}$ inactivation and identify new breast cancer biomarkers.

2. Chapter II

p16INK4a modulates p53 in Primary Human Mammary Epithelial Cells.

$p16^{INK4a}$ Modulates p53 in Primary Human Mammary Epithelial Cells

Jianmin Zhang, Curtis R. Pickering, Charles R. Holst, Mona L. Gauthier, and Thea D. Tlsty

Department of Pathology and UCSF Comprehensive Cancer Center, University of California San Francisco, San Francisco, California

Abstract

$p16^{INK4a}$ (p16) and p53 are tumor suppressor genes that are inactivated during carcinogenesis in many tumors. Here we show that p16 gene activity inversely modulates p53 status and function in primary human mammary epithelial cells. Reduced levels of p16 protein stabilize p53 protein through inhibition of proteolytic degradation, and this increase in p53 protein levels enhances the cellular response to radiation, represses proliferation, and transcriptionally activates downstream targets. Stabilization of p53 is mediated through the retinoblastoma/E2F/$p14^{ARF}$/murine double minute-2 pathway. However, we have observed that p16 does not modulate p53 in fibroblasts, indicating a possible cell type–specific regulation of this pathway. (Cancer Res 2006; 66(21): 10325-31)

Introduction

Our understanding of early events in carcinogenesis and how they may influence progression is incomplete. Further understanding of this process is required for the identification of molecular targets for early diagnosis, risk assessment, and preventive therapeutics. Inactivation of tumor suppressor genes is an important step during early carcinogenesis, and$p16^{INK4a}$ (p16) and p53 are two examples of genes that are frequently inactivated in many tumor types including breast carcinomas (1, 2). The inactivation of p16 and p53 can occur through a variety of mechanisms including point mutation, deletion, and epigenetic silencing. In this article, we will use the term "inactivation" or "mutational inactivation" to refer to the many ways through which a cell inactivates the function of p16 or p53. The p16 gene codes for a protein that acts as a cyclin-dependent kinase inhibitor and initiates a G_1-phase cell cycle arrest in response to DNA damage, oncogenic stress, and oxidative stress (1). The p16 gene is transcriptionally induced in response to these cellular stressors. The p53 gene codes for a multifunctional protein that is best known for its function as a transcription factor that initiates either cell cycle arrest or apoptosis in response to DNA damage, oncogenic stress, and oxidative stress (2). These cellular stresses induce p53 activity through a variety of posttranslational modifications that stabilize the protein and modulate its binding to other proteins and DNA. Although p16 and p53 respond to many of the same cellular stresses, their mutational inactivation during carcinogenesis may be temporally separated. Whereas very little is known about the sequence of early mutational events during carcinogenesis in most tumor types, there are some premalignant lesions, such as Barrett's esophagus, where this sequence of events has clearly been shown. In this tissue, inactivation of p53 occurs almost exclusively in cells that have already inactivated p16 (3). Although this sequence of events is known, the mechanistic basis for it is not. It has been postulated that lesions containing inactivated p53 may only be able to expand in the absence of p16 function (3), but it is also possible that inactivation of p16 promotes the inactivation of p53. Additionally, it is unknown if there are any relevant functional interactions between p16 and p53.

In breast carcinogenesis, the earliest sequence of mutational events is poorly understood. DNA hypermethylation of the p16 promoter, which is known to silence gene expression, has been identified in foci of histologically normal breast epithelium (4). In vitro experiments have linked this silencing of p16 to both telomeric and centrosomal dysfunction (4–6). Hence, it has been hypothesized that inactivation of p16 may be a very early event in carcinogenesis that precedes histologic changes within the tissue (4, 7). Inactivation of p16 has not been investigated in the ensuing premalignant breast lesions to any great extent. In contrast, inactivation of p53 is first observed in high-grade ductal carcinoma in situ, a late-stage premalignant lesion (8). Analysis of premalignant lesions such as atypical ductal hyperplasia or low-grade ductal carcinoma in situ has not revealed a significant frequency of p53 mutations (8, 9). In light of these data, we propose that inactivation of p16 precedes inactivation of p53 during early breast carcinogenesis. Mechanistically, we hypothesize that inactivation of p16 increases p53 activity and this increased activity may promote the inactivation of p53. To test this hypothesis, we have used primary human mammary epithelial cells (HMEC) as a model to examine the dynamic interactions between these tumor suppressor pathways.

HMEC can be isolated and propagated in culture from human breast tissue explants from disease-free women (10). These cultures contain two cell populations with distinct proliferative capacities. The majority population, HMEC, cultured under standard conditions, have a life span of 10 to 15 population doublings before reaching a growth plateau characterized by induction of p16 expression and G_1-phase cell cycle arrest (11–13). The minority population, variant HMEC (vHMEC), have an extended life span (30-50 additional population doublings) and become visible when the HMEC population enters the proliferative arrest characterized by elevated p16 levels. The extended proliferative capacity of vHMEC correlates with silenced p16 gene expression due to promoter DNA hypermethylation and the repression of p16 promoter activity (11–13). It has been shown that both cell populations have wild-type (wt) p53 protein as shown by sequencing and functional analysis (14). In this study, we investigate if inactivation of p16 in primary human cells has functional consequences on p53 biology.

Note: Current address for J. Zhang: Department of Neurobiology and Anatomy, University of Utah, Salt Lake City, UT 84132-3401. Current address for C.R. Holst: Genentech, South San Francisco, CA 94000. J. Zhang and C.R. Pickering contributed equally to this work.

Requests for reprints: Thea D. Tlsty, Department of Pathology and UCSF Comprehensive Cancer Center, University of California San Francisco, 513 Pamassus Avenue, HSW 451, Box 0511, San Francisco, CA 94143. Phone: 415-502-6115; Fax: 415-502-6163; E-mail: thea.tlsty@ucsf.edu.

doi:10.1158/0008-5472.CAN-06-1594

Materials and Methods

Cells and cell culture. Primary HMEC and human mammary fibroblasts (HMF) were isolated from reduction mammoplasty samples as previously described (5) and were cultured in modified MCDB 170 (MEGM, Cambrex, Rockland, ME). Specimens from five different individuals, RM9, RM15, RM16, RM21, and RM163, were used in our study. Routine cell culture and the isolation of vHMEC were essentially as described (5). Population doublings were calculated using the following equation: PD = log(A/B) / log 2, where A is the number of cells collected and B is the number of cells plated initially. Human foreskin fibroblasts were isolated from newborn foreskin as previously described (15). All experiments were done two to five times with samples from two to four individuals. HMEC were used at passages 1 to 3, vHMEC were used at passages 2 to 5, normal human fibroblasts were used at passages 2 to 5, and foreskin fibroblasts were used at passages 3 to 6.

Plasmids and retroviral gene transfer. The pBabe-puro retroviral vector construct and human $p16^{INK4a}$ cDNA were a gift from Frank McCormick (UCSF Comprehensive Cancer Center, University of California San Francisco, San Francisco, CA). p16 cDNA was subcloned into pBabe-puro vector between BamH1 and Sal1. The pMSCV retroviral construct encoding the p16-specific short hairpin RNA (sh-p16) under the control of the U6 promoter was generously provided by G. Hannon and S. Lowe (Cold Spring Harbor Laboratories, Cold Spring Harbor, NY). The pBabe-puro retroviral construct encoding dominant-negative p53 (GSE22) was a gift from Andre Gudkov (Department of Molecular Genetics, Lerner Research Institute, Cleveland, OH). The LXSN-E7 construct was provided by Denise Galloway (Cancer Biology, Fred Hutchinson Cancer Research Center, Seattle, WA). Amphotropic retrovirus was produced by transfecting Phoenix-A packaging cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight to 72 hours posttransfection, virus-containing culture medium was harvested and filtered through 0.45 Am syringe filters. Cells were infected by exposing them to virus-containing medium in the presence of 4 Ag/mL Polybrene (Sigma-Aldrich, Milwaukee, WI) for 6 to 10 hours. Cells were maintained in the appropriate medium for 48 hours postinfection, then transferred to medium containing 2 to 4 Ag/mL puromycin (Sigma). Experiments were done 4 to 6 days postinfection to allow selection for cells that exhibit puromycin resistance.

Western blot analysis. Cells were lysed with radioimmunoprecipitation assay buffer or buffer containing 160 mmol/L Tris and 2% SDS supplemented with 1× Complete protease inhibitors (Roche Applied Science, Indianapolis, IN). Protein concentration was determined with bicinchoninic acid (Pierce Biotechnology, Rockford, IL) with bovine serum albumin as the standard (Sigma). Protein from total cell extracts was denatured with 1× loading buffer and then fractionated in gradient (4-20%) polyacrylamide gels (Cambrex) and transferred to Hybond-P (GE Healthcare Bio, Piscataway, NJ) membrane. The following monoclonal antibodies were used to stain the blots: mouse anti-p16 (Ab-4; Lab Vision, Fremont, CA), mouse anti-p53 (DO-1; Santa Cruz Biotechnology, Santa Cruz, CA), mouse anti-p21 (OP64; EMD Biosciences, San Diego, CA), mouse anti-pRb (BD Biosciences, San Jose, CA), mouse anti-E2F1 (Upstate Biotechnology, Lake Placid, NY), mouse anti–murine double minute-2 (MDM2; SMP14, Santa Cruz Biotechnology), mouse anti-h-actin (AC-15; Sigma), and horseradish peroxidase–conjugated goat anti-mouse antibody (Biomedia Corp., Foster City, CA). h-Actin was used as a loading control for all blots. Proteins (5-20 Ag) were analyzed for each sample. Staining was developed with SuperSignal West Pico chemiluminescence detection protocol (Pierce). Images were quantified using ImageQuant software.

Radiation treatment. Cells were plated in 60-mm dishes and irradiated with the indicated dose of g ionizing radiation from a ^{137}Cs source at 281 rad/min. Culture medium was changed following treatment and protein samples were collected 3 hours post ionizing radiation.

Cell cycle analysis. Cells were metabolically labeled with 10 mmol/L bromodeoxyuridine (BrdUrd) for 4 hours before harvest. Cells were isolated by standard trypsinization, resuspended in PBS, and fixed by addition of ice-cold ethanol to a final concentration of 70%. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-BrdUrd antibodies (BD Biosciences; ref. 5). Flow cytometry was done on a FACS-Sorter (BD Biosciences) using CellQuest and Flowjo software for analysis. All analyzed events were gated to remove debris and aggregates. A minimum of 30,000 events were collected for each analysis.

Reverse transcription-PCR. Total RNA samples were isolated with Rneasy Mini Kit (Qiagen, Valencia, CA) as per instructions of the manufacturer. Total RNA (1.5 Ag) was used for the first strand cDNA synthesis with Superscript First-Strand Synthesis System (Invitrogen). Following reverse transcription, 2 AL of each sample were subjected to p53-specific PCR with Expand High Fidelity kit (Roche Applied Science). The PCR products were viewed by running on a 2% agarose gel. The primers for p53 were antisense, 5¶TCAGTCTGAGTCAGGCCCTTC-3 ¶ and sense, 5¶ATGGAGGAGCCGCAGTCAGAT-3 ¶

Real-time PCR. Total RNA samples were isolated with Rneasy Mini Kit (Qiagen) as per instructions of the manufacturer. cDNA was synthesized from 2 Ag of total RNA with TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) as per instructions of the manufacturer. Real-time PCR was done on 5-ng input RNA per reaction containing 1× TaqMan Universal PCR Master Mix (Applied Biosystems) and the appropriate TaqMan probe on the DNA Engine Opticon 2 (MJ Research, Inc., Waltham, MA). $p14^{ARF}$ (Hs00924091_m1) and GUSB (Hs99999908_m1) TaqMan probes were purchased from Applied Biosystems. All samples were analyzed in triplicate on each plate and at least three plates were analyzed. Relative mRNA levels were determined using the relative standard curve method (normalized to GUSB) according to Applied Biosystems User Bulletin #2.[1] The graph represents the average and SD from nine replicates for each sample (three replicates on three plates).

Results

HMEC exhibit an inverse relationship between p16 and p53 protein levels. To investigate the relationship between p16 and p53, we isolated and cultured primary HMEC and did Western blots that examined the protein levels of p16, p53, and the p53 target gene p21. The representative growth curve shown in Fig. 1 illustrates the populations from which protein lysates were collected to compare HMEC to vHMEC. Samples isolated from HMEC during their exponential growth (Fig. 1, sample a) show elevated levels of p16 and reduced levels of the p53 and p21 proteins when compared with vHMEC isolated during their exponential growth (Fig. 1, samples b-e). These data show that elevated levels of p53 and p21 protein correlate with repressed p16 activity due to promoter DNA hypermethylation (11–13).

To determine if p16 activity causally regulates p53 protein levels, we manipulated p16 expression in HMEC. To study the effect of reduced p16 expression, we infected HMEC with a retrovirus containing sh-p16. Expression of sh-p16 in HMEC efficiently repressed p16 expression relative to the control vector (Fig. 2) and resulted in an increase in the protein levels of p53 and the p53 target gene p21 (Fig. 2A). Conversely, to study the effect of increased p16 expression, we infected vHMEC (which lack p16 expression) with a retrovirus containing an expression construct for exogenous wt-p16. Overexpression of exogenous wt-p16 in vHMEC resulted in a decrease in the protein levels of p53 and p21 (Fig. 2A). These data show that p16 activity inversely regulates the protein levels of p53 and its downstream target p21.

p16 activity modulates p53-dependent proliferation. In response to diverse cellular stressors, p53 protein participates in the activation of cell cycle checkpoints or the initiation of apoptosis, thereby limiting the expansion of damaged cells. Our finding that reduced expression of p16 mediates up-regulation of p53 protein led us to query if the up-regulated p53 protein was

[1] http://www.appliedbiosystems.com.

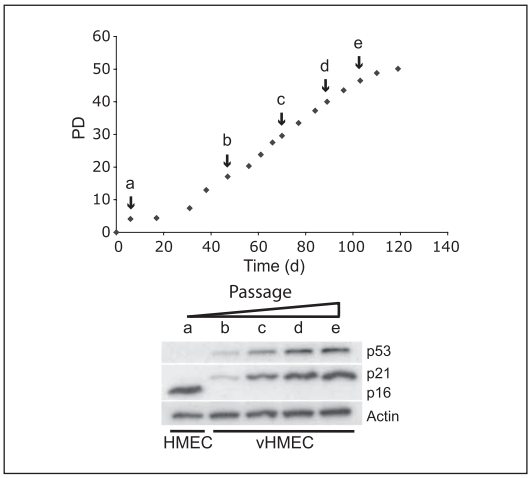

Figure 1. vHMEC, which have silenced p16, exhibit elevated p53 and p21 protein levels. HMEC were cultured and population doublings (PD) were calculated at each passage. vHMEC became visible at about day 10. Protein samples were harvested at the indicated points and Western blots were done for p16, p53, p21, and actin as a loading control.

functional. The elevated p53 protein levels observed in many tumors are strongly correlated with p53 mutations; however, Delmolino et al. (14) have previously shown that p53 in vHMEC retains a wt sequence. To investigate the functionality of the elevated p53 protein levels in vHMEC, we abrogated p53 function and determined if the protein levels of the p53 target genes p21 and MDM2 were reduced. To abrogate p53 function, we infected vHMEC with a retrovirus containing a genetic suppressor element, GSE22, specific for p53. GSE22 codes for a dominant-negative p53 peptide that increases p53 protein levels (Fig. 2B) but inhibits p53-dependent function (16, 17). Expression of GSE22 in vHMEC reduced the levels of the p53 target genes p21 and MDM2, indicating that the elevated p53 protein is necessary for the transcription of p53 target genes (Fig. 2B).

Although p53 function is regulated in many ways, up-regulation of wt p53 is known to limit the expansion of populations of cells by inhibiting cell cycle progression or inducing apoptosis. We sought to determine if the elevated levels of p53 protein in vHMEC limit the expansion of this population by comparing the population doublings of vHMEC and vHMEC expressing GSE22. We found that inactivation of p53 resulted in a population with twice as many cells within a specified time (Fig. 2C). This increased population expansion could be attributed to an increase in proliferation or a decrease in apoptosis. To measure the amount of proliferation, we analyzed the fraction of cells in the S phase of the cell cycle by flow cytometry with BrdUrd incorporation. We found that vHMEC expressing GSE22 have twice as many cells in S phase of the cell cycle compared with vHMEC controls (Fig. 2C, 24% versus 48%). In contrast, the level of apoptosis between vHMEC and vHMEC expressing GSE22 remained unchanged (data not shown). These data indicate that the elevated p53 protein level mediated by the lack of p16 expression reduces proliferation and thereby represses the expansion of the population.

p16 activity modulates a p53-dependent stress response. In response to DNA damage, p53 protein is stabilized and activates cell cycle checkpoints or initiates apoptosis to ensure that damaged cells do not propagate. To determine if inactivation of p16 modulates the response of p53 to DNA damage, we measured the kinetics of p53 protein accumulation in HMEC and HMEC expressing sh-p16 in response to g ionizing radiation. In vector control HMEC, p53 protein levels increased f 4- and 12-fold following exposure to 4 and 10 Gy of ionizing radiation, respectively (Fig. 2D). In contrast, in HMEC expressing sh-p16, p53 protein levels increased f 14- and 20-fold following exposure to 4 and 10 Gy of ionizing radiation, respectively (Fig. 2D). These data indicate that reduced levels of p16 protein produce a more robust activation of p53 in response to ionizing radiation.

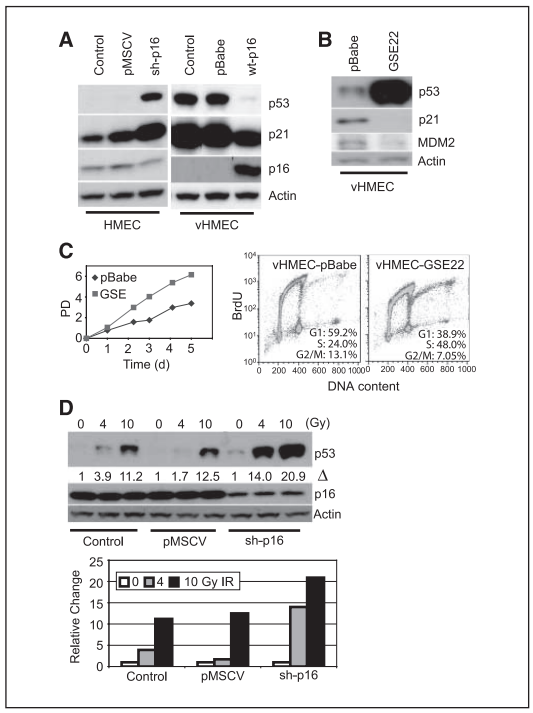

Figure 2. p16 modulates p53 dependent phenotypes. A, HMEC and vHMEC, as indicated, were infected with retroviruses containing pMSCV (vector), sh-p16, pBabe (vector), or wt-p16. Protein samples were harvested 4 to 6 days postinfection and Western blots were done for the indicated proteins. Expression of sh-p16 reduced p16 protein levels and increased p53 and p21 protein levels, and wt-p16 increased p16 protein levels and decreased p53 and p21 protein levels. B, vHMEC were infected with retrovirus containing either pMSCV (vector) or GSE22 (a dominant-negative p53). Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot. Expression of GSE22 in vHMEC decreased the protein levels of the p53 target genes p21 and MDM2. C, vHMEC containing pBabe (vector) or GSE22 were collected and counted on each of 6 consecutive days and population doublings were determined. To determine cell cycle distribution, BrdUrd (BrdU) incorporation and DNA content were analyzed by flow cytometry. Representative cell cycle distributions are listed in the plot. GSE22 increases vHMEC population expansion and increases proliferation. D, control uninfected HMEC and either pMSCV (vector)– or sh-p16-infected HMEC were exposed to 0, 4 or 10 of Gy g-radiation. After 3 hours, protein samples were harvested and p53 and p16 were analyzed by Western blot analysis. Numbers below the p53 blot indicate the relative fold increase in p53 level (D) normalized to the 0 Gy sample. The relative changes in p53 level are also graphed below.

Increased p16 activity decreases p53 protein stability. We sought to determine how p16 modulates p53 protein levels. Because the protein level of p53 is primarily regulated through posttranslational mechanisms that modulate protein stability, we first measured p53 protein half-life in HMEC and vHMEC. We found that the half-life of p53 is twice as long in vHMEC compared with isogenic HMEC (a half life of 3 hours versus 1.5 hours, respectively; data not shown; ref. 14). Because the half-life of p53 was reduced in the cells that express p16, we asked whether p16 promotes proteasome-mediated degradation of p53. We found that inhibition of the proteasome by MG132 increased p53 protein levels in vHMEC overexpressing exogenous wt-p16 (Fig. 3A). Similarly, proteasome inhibition increased p53 protein levels in HMEC expressing endogenous p16 (Fig. 3B). Thus, in both cell populations, the HMEC expressing endogenous p16 and the vHMEC overexpressing exogenous wt-p16, p53 protein levels are regulated by p16 through a proteasome-dependent mechanism. To determine if p53 mRNA expression was also regulated by p16 activity, we measured p53 mRNA levels by reverse transcription-PCR (RT-PCR). In vHMEC overexpressing exogenous wt-p16, p53 mRNA level was minimally altered compared with that in uninfected and vector control cells (Fig. 3C). These data show that p16 expression decreases p53 protein stability predominantly via a proteasome-dependent mechanism.

p16 modulates p53 through the retinoblastoma pathway. To determine the mechanism of how p16 promotes the proteasome-dependent degradation of p53 protein, we took a candidate gene approach. The p16 protein regulates the retinoblastoma (Rb) pathway, which, in turn, can modulate p53 protein stability (18). In short, p16 inhibits the phosphorylation of the Rb protein (pRb), thus allowing pRb to inhibit E2F1. E2F1 activates transcription of $p14^{ARF}$ (p14), Rb, and E2F1. p14 binds and inhibits MDM2 (19). Because MDM2 promotes the proteasome-mediated degradation of p53, a functional link may exist between p16 activity and p53 protein levels. This signaling pathway is depicted in Fig. 4A. To determine if p16 regulates p53 through the Rb pathway, we analyzed

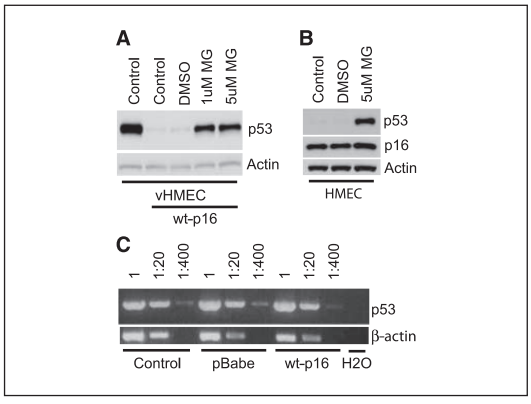

Figure 3. p16 decreases p53 protein stability. A and B, the indicated cells were exposed to the proteasome inhibitor MG132. Proteasome inhibition increases p53 level in both vHMEC expressing exogenous wt-p16 and HMEC expressing endogenous p16. C, RT-PCR analysis of control uninfected vHMEC or vHMEC infected with either pBabe (vector) or wt-p16 shows that wt-p16 does not dramatically alter p53 mRNA level. The PCR templates were diluted as indicated above the lanes. H_2O was used as a negative control.

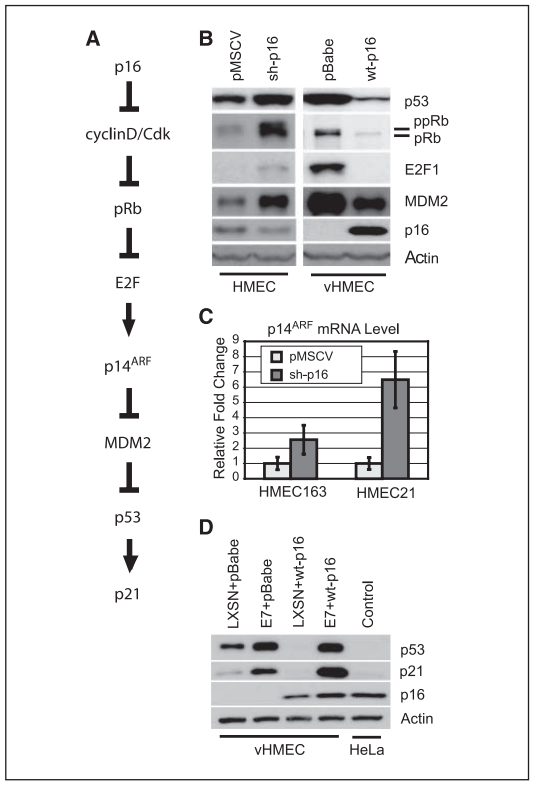

Figure 4. p16 modulates p53 through the Rb pathway. A, simplified diagram of the proposed signaling pathway between p16 and p53. B, HMEC and vHMEC were infected with the retroviruses as indicated. Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot for the indicated proteins. p16 modulates members of the Rb pathway as predicted. MDM2 is also a p53 target. C, TaqMan real-time PCR was done on the indicated samples to determine p14 mRNA level. Expression of sh-p16 increases p14 mRNA level. HMEC163 and HMEC21 are samples derived from two different individuals. D, pBabe (vector)– and wt-p16–expressing vHMEC were infected with retrovirus containing either LXSN (vector) or E7 to inhibit Rb function. E7 expression prevents wt-p16 from decreasing p53 level. HeLa is used as a control.

the major proteins in this pathway. We examined the protein levels of pRb, E2F1, and MDM2 by Western blot in four populations of cells: control HMEC and HMEC expressing sh-p16, as well as control vHMEC and vHMEC overexpressing exogenous wt-p16 (Fig. 4B). In HMEC expressing sh-p16, we observed an up-regulation of phospho-pRb, E2F1, and MDM2 when compared with the vector control HMEC. Conversely, overexpression of wt p16 in vHMEC, which lack p16, results in a down-regulation of phospho-pRb, E2F1, and MDM2. To determine if p16 directly results in the up-regulation of p14 expression, we probed HMEC and HMEC expressing sh-p16 by quantitative PCR. We found that down-regulation of p16 in HMEC leads to an increase in p14 mRNA levels (Fig. 4C).

Because these results suggest that wt p16 down-regulates p53 through the Rb pathway, we sought to determine if Rb signaling is necessary for p16 to modulate p53. To inhibit Rb signaling, we retrovirally infected cells with human papilloma virus E7 (E7), which is known to bind all three members of the Rb family (pRb,

p107, and p130) and promote their degradation, thus allowing E2F transactivation of target genes (20). Figure 4D shows that expression of E7 in vHMEC increases p53 and p21 levels above that observed in vector control cells. To determine if p16 down-regulation of p53 and p21 is Rb dependent, we coinfected vHMEC with both wt-p16 and E7. Inactivation of Rb signaling by E7 prevented p16 from down-regulating p53 and p21. We conclude that functional Rb signaling is necessary for p16 to modulate p53 protein stability.

p16 down-regulation of p53 is cell type specific. We have shown that in HMEC, p16 activity regulates p53 protein stability. To determine if this regulation occurs in other cell types, we modulated p16 levels in isogenic HMF. Whereas expression of sh-p16 in HMF decreased p16 protein levels (up to 90%), the p53 and p21 levels were not dramatically altered (Fig. 5A). Similarly, when wt p16 was overexpressed in HMF, p53 and p21 levels did not change significantly (Fig. 5B). To determine if these observations are unique to HMF, we examined human foreskin fibroblasts. Similar to our observations with HMF, p53 levels are not modulated by down-regulation of p16 using sh-p16 (Fig. 5C). We next investigated if inactivation of p16 in HMF sensitized the cells to DNA damage as was observed in epithelial cells. The kinetics of p53 and p21 protein accumulation were examined in HMF and HMF expressing sh-p16 following exposure to 1, 4, 8, and 10 Gy of ionizing radiation. In contrast to our observations in epithelial cells, the kinetics of p53 stabilization are similar in both control and sh-p16 HMF (Fig. 5D). We conclude that p16 does not modulate basal levels of p53 or induced levels of p53 in response to DNA damage in fibroblasts as observed in epithelial cells. Thus, we conclude that regulation of p53 by p16 is cell type specific.

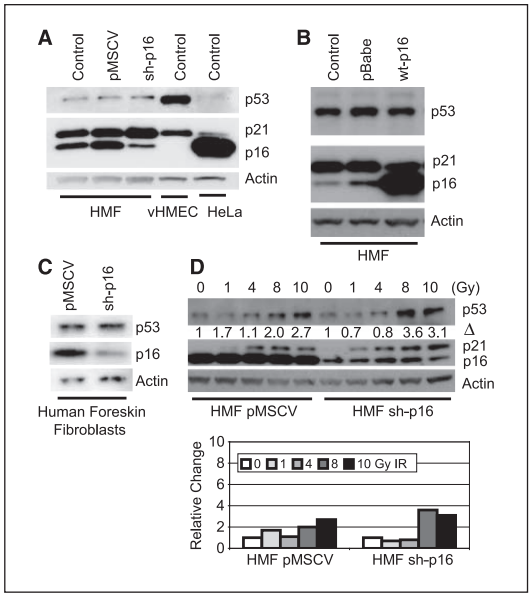

Figure 5. HMF and HMEC differ in their ability for p16 to modulate p53. A and B, HMF were infected with retroviruses containing pMSCV (vector), sh-p16, pBabe (vector), or wt-p16 as indicated. Protein samples were harvested 4 to 6 days postinfection and analyzed by Western blot. vHMEC and HeLa were used as controls. sh-p16 did not alter p53 levels in HMF to a comparable extent as observed in epithelial cells. Likewise, expression of wt-p16 did not alter p53 levels in HMF to a comparable extent as observed in epithelial cells. C, human foreskin fibroblasts were infected with retrovirus containing either pMSCV (vector) or sh-p16. Expression of sh-p16 minimally altered p53 levels in human foreskin fibroblasts. D, HMF infected with either pMSCV (vector) or sh-p16 were exposed either to sham, 1, 4, 8, or 10 Gy of g-radiation. After 3 hours, protein samples were harvested and p53, p21, and p16 were analyzed by Western blot analysis. Numbers below the p53 blot indicate the relative fold increase in p53 level (D) normalized to the 0 Gy sample. The relative changes in p53 level are also graphed below.

Discussion

We have used primary human cells to investigate the consequences of inactivation of the tumor suppressor gene p16 as an early event in carcinogenesis. Our results are of broad interest because they shed new light on the process of carcinogenesis and may provide a mechanism for a sequence of genetic changes that have been observed in many tumors. Our results show that p16 activity inversely modulates p53 protein levels in primary HMEC. Reduced levels of p16 protein increase p53 function, whereas p16 expression reduces p53 protein stability through the Rb pathway. Despite the extensive number of studies on the p16 and p53 pathways, we know of no other study, to date, that has analyzed these two pathways in primary epithelial cells or observed this antagonistic relationship between these two critical tumor suppressors. In fact, this regulation of p53 by p16 has not been observed in other systems. For example, mouse embryonic fibroblasts isolated from p16 knockout mice (21) and human skin fibroblasts isolated from individuals with germ-line mutations in p16 do not exhibit elevated p53 protein levels when compared with controls (22). In concordance, we have shown that reduced p16 expression in human mammary or skin fibroblasts does not increase p53 protein levels. It is not known why this regulation of p53 by p16 does not occur in some cell types, but because it has been observed in HMEC, it may have important implications for the early steps in carcinogenesis in some tumor types. In addition, by analyzing multiple infections of HMEC with retroviruses containing sh-p16, we have observed various levels of p16 inhibition, ranging from >90% to ~50% inhibition, depending on the specific infection, and the p53 level is always dramatically increased (data not shown). In mouse models, p16 has been shown to be haploinsufficient for tumor suppression and we believe that our modest decrease in p16 protein level may mimic haploinsufficiency and show that p16 is haploinsufficient for suppressing p53.

The p16 and p53 pathways are important for the activation of senescence. Senescence is a permanent cell cycle arrest that acts as a barrier to immortalization and tumorigenesis and can be induced by a variety of stimuli, including oxidative stress, DNA damage, oncogene activation, and aging. Although senescence has been studied primarily in vitro, it has recently been shown that senescence is also a barrier to tumorigenesis in vivo (23–26). Senescence markers were identified in premalignant lesions, which supports the hypothesis that bypass of senescence may be an early event in carcinogenesis (26). Whereas p16 and p53 are both involved in the activation of senescence, these proteins seem to function in separate pathways. For example, oncogenic Ras induces a p16-dependent senescence (27) whereas oncogenic phosphatase and tensin homologue (PTEN) induces a p53-dependent senescence (25). Additionally, during the senescence of human skin fibroblasts induced by extended proliferation in culture, two distinct populations of cells are identifiable (28). One cell population has elevated p53 and p21 protein levels, exhibits markers of DNA

damage and shortened telomeres, and does not have elevated p16 protein levels. The other population has elevated p16 protein levels, does not exhibit markers of DNA damage or shortened telomeres, and does not have elevated p53 or p21. The ability of p16 activity to modulate p53 activity adds another layer of complexity to the initiation of senescence and the ability to bypass senescence. Our results suggest that activation of p16-dependent senescence may inhibit p53-dependent senescence. Conversely, bypass of p16-dependent senescence, through inactivation of p16, may promote the activation of a p53-dependent arrest.

The results described here also provide new insight into how inactivation of p16 may be a critical early event that influences subsequent oncogenic events. This study has shown that reduced levels of p16 can increase p53 protein level and functions. This elevated p53 function may allow the cell to compensate for loss of p16-dependent checkpoints; however, sustained up-regulation of p53 activity may also increase the selective pressure to inactivate p53. We have shown that inactivation of p53 in a cell that has already reduced p16 expression provides a proliferative advantage, and inactivation of p16 has previously been shown to promote genomic instability (5, 29). Therefore, inactivation of p16 provides both a selective pressure (elevated p53) and a mechanism (genomic instability) to inactivate p53, and this may be a driving force during carcinogenesis.

We hypothesize that inactivation of p16 may initiate a sequence of events during early carcinogenesis where inactivation of p53 follows inactivation of p16. Although very little is known about the early events in breast carcinogenesis, other organ systems do provide support for this hypothesis. An elegant set of experiments by Maley et al. (3) examined tissue from Barrett's esophagus and determined that inactivation of p53 is found almost exclusively (14 of 15 cases) in cells that have already inactivated p16 and that this subsequent inactivation of p53 predicts progression to esophageal adenocarcinoma (30). These experiments show a sequence of events in Barrett's esophagus where inactivation of p16 precedes inactivation of p53 and subsequent tumor progression. To our knowledge, modulation of p53 by p16 has not been investigated in esophageal epithelial cells, and thus our results may help explain why inactivation of p16 precedes inactivation of p53 in Barrett's esophagus. We suggest that this sequence of events may occur in breast carcinomas and other tumor types (e.g., pancreas and head and neck) because the frequently observed inactivation of p16 may promote the inactivation of p53.

We have shown that reduced levels of p16 protein increase the p53 response to radiation. An implication of these findings is that p16 expression alters the cellular response to genotoxic damage and may modify radiation toxicity. Because foci of cells with silenced p16 have been found in normal human tissues (4) and loss of p16 activity via deletion, mutation, or methylation often occurs during tumor progression (1), these observations could have implications for the response to genomic damage in normal, premalignant, and tumor tissue.

Finally, we have observed that p16 modulates p53 in HMEC but not in fibroblasts. Our data indicate that the signaling pathway between p16 and p53 is context dependent and is regulated by either extrinsic microenvironmental conditions or intrinsic cell type specific factors. If this pathway is regulated extrinsically by microenvironmental conditions, then the conditions that modulate this important pathway need to be identified. The conditions that are known to induce p16 expression include DNA damage, oncogenic stress, and oxidative stress. There are arguments that HMEC are cultured under "stressed" conditions (31, 32), and if the interaction we have observed occurs during stress-induced conditions, it is very relevant to p53 function and the carcinogenic process. It is appreciated that tumors frequently form under conditions that could be equated with "stress" (i.e., chronic injury; ref. 31). It will be interesting to determine if we have identified "stressful" conditions that can dramatically alter the interaction between p16 and p53 signaling and if similar conditions occur in vivo.

If the signaling pathway between p16 and p53 is regulated intrinsically in a cell type specific manner, then results obtained in one cell type may not be directly applicable to others. Because epithelial cells and fibroblasts have very different roles in response to damage and stress, it should not be surprising that key damage and stress response pathways would be regulated in a cell type specific manner. Cell type specific signaling adds an additional layer of complexity to our understanding of carcinogenesis but may provide new directions for therapeutic targets. Additionally, our results in HMEC indicate that inactivation of p16 may increase the selective pressure to inactivate p53. If this effect occurs in epithelial cells but not in fibroblasts, this could partially explain the preponderance of carcinomas (epithelial origin) that occur in humans compared with sarcomas (fibroblast origin).

Acknowledgments

Received 5/2/2006; revised 8/7/2006; accepted 8/25/2006.

Grant support: National Cancer Institute/NIH Institutional Training Grant 5 T32 CA09270 (C.R. Pickering); the Avon Foundation, The Cancer League, Inc., the California Breast Cancer Research Program, Department of Defense, and NIH grants (T.D. Tlsty).

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We thank members of the Tlsty lab for moral support, technical support, and helpful discussions; K. McDermott for critical reading of the mauscript; Dr. Phil Coffino and his lab for shared equipment and expertise; Scott Lowe, Greg Hannon, Frank McCormick, Denise Galloway, and Andre Gudkov for kindly providing reagents; and Karen Chew for acquiring human tissue.

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3. Chapter III

$p16^{INK4a}$ stabilizes p53 through ATM but does not induce a cell cycle arrest or alter certain cell cycle checkpoints.

3.1. Abstract

We have found that $p16^{INK4a}$ modulates p53 through pRb and E2F signaling. It was hypothesized that this signaling also involved $p14^{ARF}$. However, we have found that $p14^{ARF}$ may not be necessary for $p16^{INK4a}$ to modulate p53. Instead, $p16^{INK4a}$ may utilize ATM and the DNA damage response to modulate p53. Additionally, we have found that the modulation of $p16^{INK4a}$ and p53 does not alter cell cycle progression or cell cycle checkpoint control under the conditions reported in this study.

3.2. Introduction

In the preceding chapter we described how inactivation of $p16^{INK4a}$ modulates p53 levels and functions (Zhang et al., 2006). In short, inactivation of $p16^{INK4a}$ increases p53 protein levels by modulating the pRb pathway to increase p53 protein stability. Some p53 functions, like radiation response and cell cycle inhibition, are also increased (Zhang et al., 2006). This phenotype is robust and can be initiated by expression of $p16^{INK4a}$ shRNA in HMEC. While this initial work primarily characterized the phenotype, it left several questions unanswered. In a broad sense, aspects of both the mechanism with which $p16^{INK4a}$ modulates p53 and additional functional consequences of that modulation were still unknown. In this report we address some of these unknowns. We first investigated mechanism by asking if $p14^{ARF}$ is necessary for $p16^{INK4a}$ to modulate p53. The $p14^{ARF}$ locus is an E2F target gene, and its expression is increased by inactivation of $p16^{INK4a}$. Functionally, $p14^{ARF}$ inhibits MDM2, the E3 ubiquitin ligase for p53, and results in a stabilization of p53 protein (Sherr, 2006). It was logical to hypothesize that $p16^{INK4a}$ would modulate p53 through $p14^{ARF}$. However, if we find that $p14^{ARF}$ is not necessary for p53 protein stability, then it is important to identify other proteins which are necessary for modulation of p53 protein stability.

We next investigated the functional consequences of p53 protein stabilization by examining cell cycle control. Because both $p16^{INK4a}$ and p53 are involved in cell cycle regulation, we hypothesized that modulation of $p16^{INK4a}$ and p53 would alter cell cycle progression and cell cycle checkpoint control.

3.3. Results

3.3.1. Partial reduction of $p14^{ARF}$ does not modulate p53 in vHMEC

We have hypothesized that $p16^{INK4a}$ modulates p53 protein levels through $p14^{ARF}$. To test this hypothesis, an shRNA construct to $p14^{ARF}$ was developed. While the $p14^{ARF}$ shRNA only modestly reduces $p14^{ARF}$ levels in RM13 HMEC (30%), this $p14^{ARF}$ shRNA is able to prevent the induction of $p14^{ARF}$ caused by $p16^{INK4a}$ shRNA (Fig 6A). The $p14^{ARF}$ expression levels in cells with both $p16^{INK4a}$ shRNA and $p14^{ARF}$ shRNA are comparable to the $p14^{ARF}$ expression levels in control cells. This demonstrates that the $p14^{ARF}$ shRNA can effectively reduce $p14^{ARF}$ levels.

As shown previously, vHMEC with inactivated $p16^{INK4a}$ have increased expression of $p14^{ARF}$ and increased p53 protein levels when compared to HMEC with functional $p16^{INK4a}$. If $p14^{ARF}$ is necessary for $p16^{INK4a}$ to modulate p53, then it would be predicted that reduced $p14^{ARF}$ levels in vHMEC should cause a reduction in p53 levels. We tested this prediction in samples from RM18, and found that expression of $p14^{ARF}$ shRNA does not modulate p53 levels in vHMEC (Fig 6B). This result refutes our hypothesis and supports the conclusion that other pathways may be operating for $p16^{INK4a}$ to modulate p53.

3.3.2. ATM is necessary for $p16^{INK4a}$ to modulate p53

Once it was determined that reduced levels of $p14^{ARF}$ do not modulate p53, we sought to determine if other proteins are necessary for $p16^{INK4a}$ to modulate p53. We next investigated the role of the DNA damage response. Our previous data suggested that $p16^{INK4a}$ signals

Figure 6

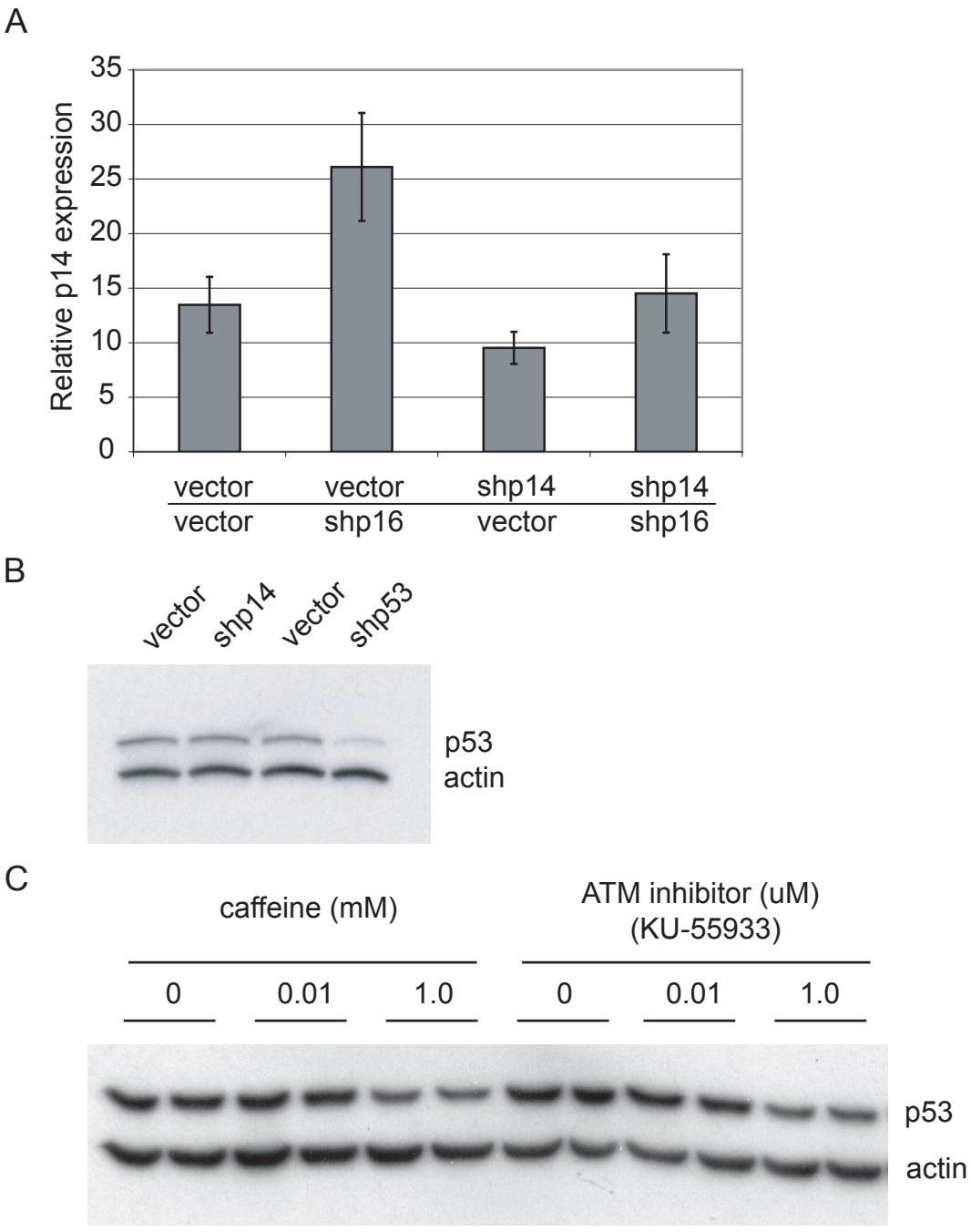


Figure 6: p16 modulates p53 through ATM.
A. Expression of p14 in HMEC cells infected with the indicated constructs. shp14 activity was analyzed in RM13 and 21. RM13 is shown. B. Western blot for p53 demonstrating that shp14 does not modulate p53. Actin is shown as a loading control. This experiment was performed in RM18. C. Western blot for p53 in vHMEC cells exposed to the indicated concentrations of either caffeine or ATM inhibitor for 48 hrs. Each concentration is shown in duplicate. Actin is shown as a loading control. This experiment was performed in RM16, 18, and 21. RM18 is shown.

through pRb and E2F to modulate p53. Other researchers have shown that pRb/E2F dysfunction can induce a DNA damage response and a DNA damage response can stabilize p53 (Pickering and Kowalik, 2006). This lead us to hypothesize that inactivation of $p16^{INK4a}$ might modulate p53 by inducing a DNA damage response. Classically, DNA damage activates the ATM kinase which then phosphorylates and stabilizes p53 (Sherr, 2006). If the DNA damage response is necessary for $p16^{INK4a}$ to modulate p53, then inhibition of the ATM kinase with pharmacological inhibitors should reduce p53 protein levels. Caffeine (which also inhibits the ATR kinase) and an ATM specific inhibitor (KU-55933) were utilized for these experiments. We found that both caffeine and the ATM inhibitor caused a decrease in p53 protein levels in vHMEC from RM16, RM18 and RM21 (Fig 6C and Appendix 3). This supports the hypothesis that ATM and a DNA damage response are necessary for $p16^{INK4a}$ to modulate p53.

3.3.3. Expression of $p16^{INK4a}$ shRNA and stabilization of p53 does not activate a cell cycle arrest

Our previous results (see Chapter 1) suggest that the increased p53 levels caused by inactivation of $p16^{INK4a}$ are functional and inhibit proliferation. We wanted to further investigate the impact of these increased p53 levels on cell cycle progression. Because activated p53 initiates a cell cycle arrest, we hypothesized that if the increased p53 level in cells expressing $p16^{INK4a}$ shRNA contained activated p53, it would inhibit cell cycle progression. RM13 HMEC were infected with different concentrations of lentivirus expressing $p16^{INK4a}$ shRNA (virus:media, 1:19, 1:9, 1:3, 1:1), p16 and p53 protein levels were documented and the population growth curves were collected. It was found that while

various doses of $p16^{INK4a}$ shRNA reduced p16 protein levels by 50% to greater than 90% and increased p53 levels by 2-fold to greater than 10-fold (Fig 7), these altered protein levels did not dramatically change population growth (Fig 8A). Furthermore, all doses of $p16^{INK4a}$ shRNA infection allow for bypass of the $p16^{INK4a}$ induced growth arrest that occurs in control or vector infected cells (Fig 8A). Even a 50% reduction in $p16^{INK4a}$ protein level allows for bypass of senescent signals. We next analyzed the cell cycle profile of parent, vector or $p16^{INK4a}$ shRNA infected cells. Cell cycle profiles were analyzed by flow cytometry for BrdU incorporation and DNA content. Expression of $p16^{INK4a}$ shRNA does not alter the cell cycle profile of HMEC (Fig 8B). This indicates that the elevated levels of p53 do not induce a cell cycle arrest.

3.3.4. Expression of $p16^{INK4a}$ shRNA does not alter cell cycle checkpoint control

We determined whether expression of $p16^{INK4a}$ shRNA modulates the cellular response to DNA damage. It was hypothesized that expression of $p16^{INK4a}$ shRNA would increase p53 levels and cause a more robust cell cycle arrest in response to DNA damage. The cell cycle arrest of HMEC (RM13, RM15, RM18 and RM21) was tested by exposure to doxorubicin (dox, a topoisomerase II inhibitor), tert-butyl hydroperoxide (TBHP, a hydrogen peroxide donor), and UVC (254nm, a DNA crosslinker). Expression of $p16^{INK4a}$ shRNA did not cause any difference in the cell cycle arrest caused by any of these agents (Fig 9 and Appendix 3). Although the cells demonstrated differential sensitivity to each of these agents (i.e. dox reduced the S-phase fraction by 100%, complete cell cycle arrest, while UVC reduced the S-phase fraction by less than 10%), there were no conditions where cells expressing $p16^{INK4a}$

shRNA were more or less sensitive to a cell cycle arrest when compared to the controls (Fig 9 and Appendix 3).

Figure 7

A

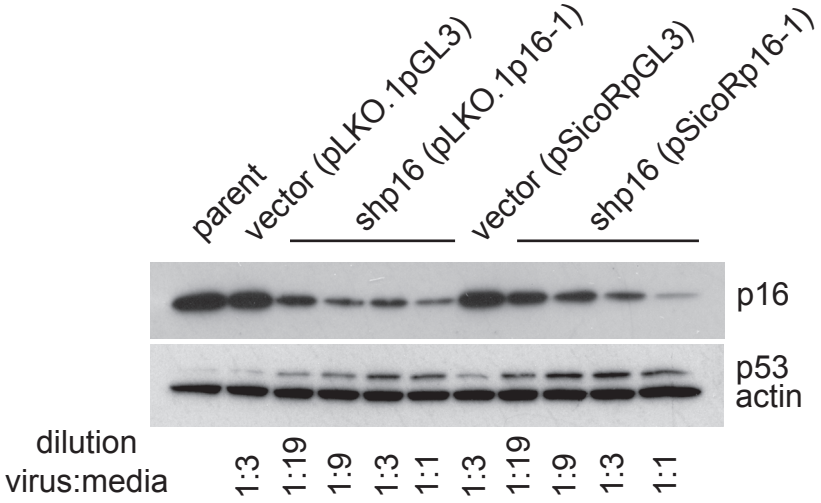
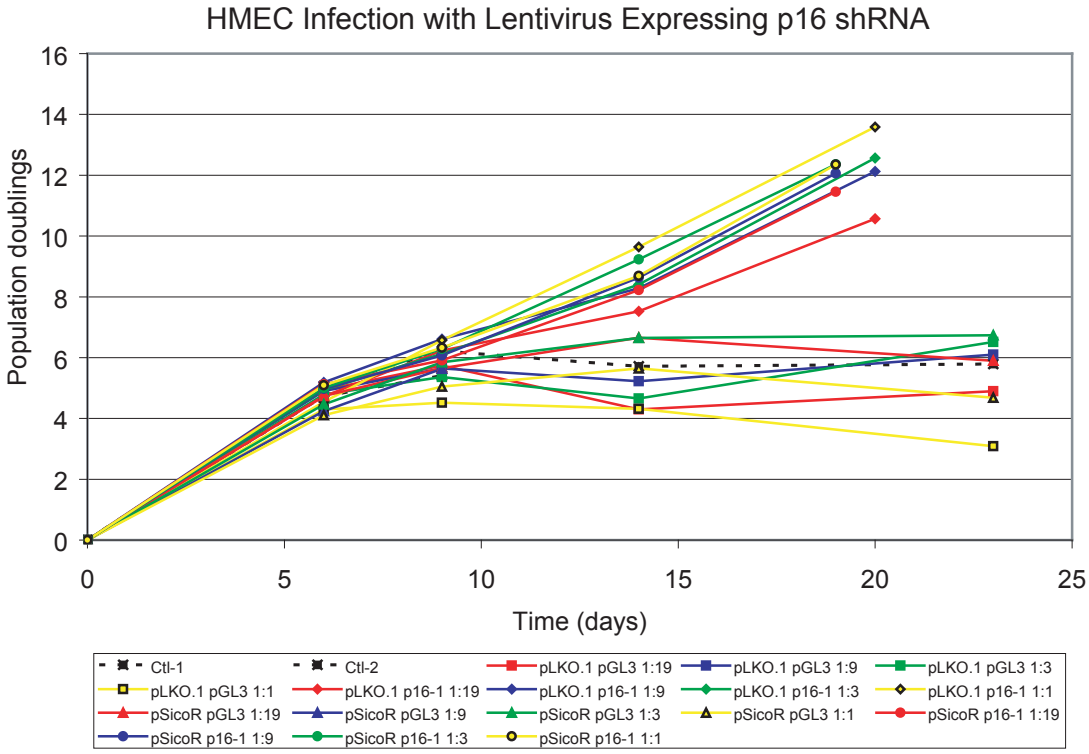


Figure 7: p16 protein levels are reduced by p16 shRNA.
HMEC cells were infected with lentiviruses containing the indicated vector. Various dilutions of the virus were used, as indicated below each sample. Western blots were performed with antibodies against the proteins indicated on the right. Actin was used as a loading control. shp16 activity has been analyzed in samples from at least 4 individuals. RM13 is shown as a representative example.

Figure 8

A



B

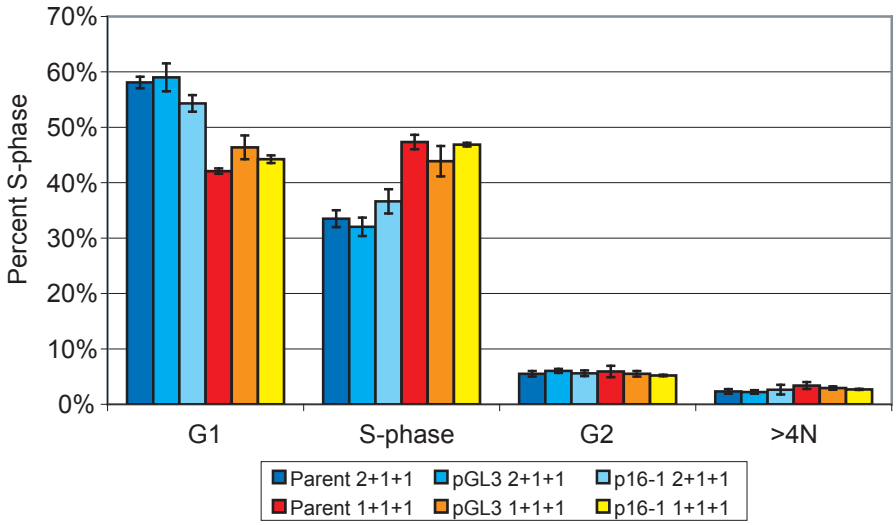


Figure 8: p16 shRNA does not modulate cell cycle.
A. Growth curves of RM13 HMEC cells infected with the indicated virus at the dilution factor shown. B. Cell cycle profile of RM13 HMEC infected with the indicated virus. Cell cycle information was collected by flow cytometry for BrdU incorporation and DNA content. Samples from two infections are shown. This experiment has been performed in samples from at least 3 different individuals.

Figure 9

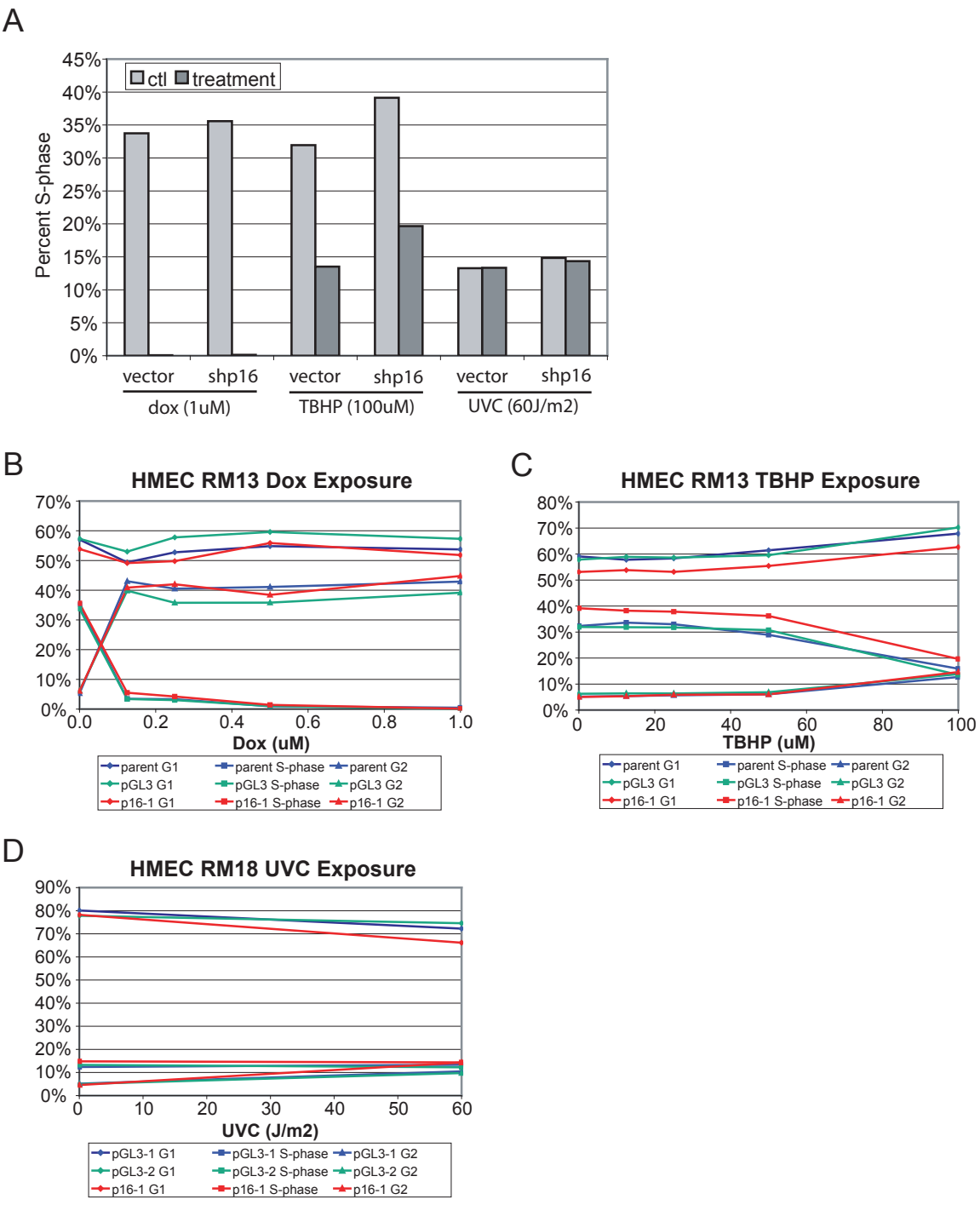


Figure 9: p16 shRNA does not modulate cell cycle checkpoints.
Cell cycle profiles of HMEC infected with the indicated virus and exposed to the indicated DNA damaging agent. Cell cycle information was collected by flow cytometry for BrdU incorporation and DNA content at 24 hrs post-treatment. A. S-phase fraction taken from B, C, and D at the highest dose indicated. B-D. G1, S-phase and G2 cell cycle profiles as indicated. These experiments were preformed in RM13, 15, 18, and 21.

3.4. Discussion

3.4.1. Inactivation of $p16^{INK4a}$ may modulate p53 through ATM instead of $p14^{ARF}$

As shown in Chapter 1, we have found the inactivation of $p16^{INK4a}$ induces p53 levels through the pRb pathway. We hypothesized that this signaling interaction occurred through $p14^{ARF}$. We have now found that $p14^{ARF}$ may not be necessary for $p16^{INK4a}$ to modulate p53. We can not make a definitive conclusion however, because of experimental constraints. It is possible that since the $p14^{ARF}$ shRNA construct used for these experiments does not completely eliminate $p14^{ARF}$ protein, the remaining protein is sufficient to modulate p53. Due to the experimental constraints of working with human cells it is currently difficult to completely eliminate protein expression, thus it may not be possible to get a definitive conclusion for this hypothesis. Chemical inhibitors to $p14^{ARF}$ or dominant negative constructs may help address this issue if they were developed. At this time, the most definitive conclusion is that a reduction in $p14^{ARF}$ protein levels does not reduce p53 protein stability in vHMEC.

Because we could not definitively rule out $p14^{ARF}$ in the regulation of p53 we investigated other proteins involved in the regulation of p53. We examined ATM and the DNA damage response primarily due to anecdotal evidence. Other researchers had shown that modulation of pRb and E2F signaling could induce a DNA damage response in fibroblasts (Pickering and Kowalik, 2006). This led us to hypothesize that modulation of pRb and E2F by $p16^{INK4a}$ might be able to induce a DNA damage response in epithelial cells. Consistent with this hypothesis, preliminary data have suggested that vHMEC exhibit an activated DNA damage response (C. Fordyce, data not shown). Instead of first determining if inactivation of $p16^{INK4a}$

induces a DNA damage response we decided to directly test if inhibition of the DNA damage response would reduce p53 protein levels in vHMEC. We found that two different pharmacological inhibitors of the ATM kinase both reduce p53 levels in vHMEC. This supports the hypothesis that a DNA damage response is necessary for stabilization of p53 in vHMEC. This result makes a variety of testable predictions that we have not yet had the time to completely address. First, genetic inhibition of ATM (i.e. shRNA) should also reduce p53 levels. We have developed an shRNA to ATM. Preliminary data suggests that expression of the ATM shRNA may not reduce p53 levels in vHMEC (data not shown). This result is contrary to our prediction, however because the ATM shRNA does not completely eliminate ATM protein it is possible that the residual ATM activity is sufficient to regulate p53. Second, ATM is a kinase that modulates p53 by phosphorylating the p53 protein (Shiloh, 2001). These phosphorylation sites are well defined and our model predicts that these sites would be differentially phosphorylated in cells with inactivated $p16^{INK4a}$. Lastly, and most importantly, our data suggests that inactivation of $p16^{INK4a}$ induces a DNA damage response. This is easily testable by performing staining or western blots for DNA damage markers or comet assays for DNA damage. Inactivation of pRb and overexpression of E2F1 have been shown to induce a DNA damage response in human fibroblasts (Pickering and Kowalik, 2006), therefore it would not be surprising if inactivation of $p16^{INK4a}$ induces a DNA damage response in epithelial cells. These data are important because DNA damage is now recognized as an early event in carcinogenesis. If inactivation of $p16^{INK4a}$ induces a DNA damage response, then this adds a new role for $p16^{INK4a}$ in the carcinogenic process. Previously, it was thought that inactivation of $p16^{INK4a}$ only allowed cells to bypass certain cell cycle arrests. This perspective describes inactivation of $p16^{INK4a}$ as a conditional event

during carcinogenesis that requires certain conditions before the phenotype is recognized. In contrast, these novel data suggest that inactivation of $p16^{INK4a}$ may be an active event during carcinogenesis that promotes DNA damage and genomic instability.

If these data are confirmed then it will be important to determine how inactivation of $p16^{INK4a}$ activates a DNA damage response. I would hypothesize that altered E2F signaling caused by inactivation of $p16^{INK4a}$ causes an increase in reactive oxygen species that damage DNA. Preliminary data from G. Benton has shown that expression of p16 shRNA causes an increase in reactive oxygen species in HMEC (data not shown). This increase in reactive oxygen species may be caused by replicative stress or some other still unknown mechanism. However, Pickering *et.al.* suggest that the induction of DNA damage by E2F1 expression is not dependent on reactive oxygen species, ATM, NBS, p53, MAD2, or caspases, and is by a different mechanism than the induction of DNA damage by c-MYC which is dependent on reactive oxygen species (Pickering and Kowalik, 2006; Rogoff et al., 2004). These data do not eliminate the possibility that $p16^{INK4a}$/pRb pathway dysfunction modulates DNA replication to induce DNA damage, but they may indicate that identifying the specific details of the mechanism will be difficult.

3.4.2. Inactivation of $p16^{INK4a}$ does not activate a cell cycle arrest or alter select cell cycle checkpoints

Because both $p16^{INK4a}$ and p53 are involved in cell cycle regulation we hypothesized that modulation of both $p16^{INK4a}$ and p53 would alter cell cycle progression. Contrary to this hypothesis we did not observe any modulation of the basal cell cycle by inactivation of

$p16^{INK4a}$. A possible explanation for this contradiction is that the increased p53 levels exactly compensated for the reduced $p16^{INK4a}$ levels. In Chapter I we demonstrated that inactivation of p53 in cells with inactivated $p16^{INK4a}$ increases proliferation (Fig 2). This result indicates that p53 is inhibiting proliferation in these cells. However, because inactivation of $p16^{INK4a}$ does not induce a cell cycle arrest the increased p53 inhibitory function must be balanced by an increase in proliferation caused by inactivation of $p16^{INK4a}$. The p53 protein stabilization is compensating for inactivation of $p16^{INK4a}$. Functional compensation in cellular processes is not novel, and this occurs in many systems, but it has not been shown that p53 can functionally compensate for $p16^{INK4a}$ inactivation. However, because the functions of $p16^{INK4a}$ and p53 are quite drastically different this hypothesis may not be very likely.

We also hypothesized that inactivation of p16 and stabilization of p53 would alter cell cycle checkpoint control, however, we did not observe any alteration in cell cycle checkpoints following inactivation of $p16^{INK4a}$. This result somewhat contradicts our result from Chapter I where we found that inactivation of p16 increased p53 stabilization in response to ionizing radiation (Zhang et al., 2006). There are at least two possible explanations for this contradiction. First, although inactivation of $p16^{INK4a}$ did not alter cell cycle checkpoint control, we may not have tested the proper types of DNA damage or the proper time points. Our experiments with DNA damage utilized only three agents and were all collected at 24 hours post treatment. It is possible that other agents, like ionizing radiation, which we used in Chapter I, or other time points, like 48 hours, may demonstrate a difference between control and $p16^{INK4a}$ shRNA expressing cells. However, preliminary experiments exposing cells to ionizing radiation did not demonstrate a differential cell cycle arrest with inactivation of p16

(Appendix 3). Second, it is possible that inactivation of $p16^{INK4a}$ does not modulate many p53 functions. Although p53 levels are increased by inactivation of p16 these levels may not alter the cell cycle response of p53. If this is true, it will of interest to further investigate the regulation of p53 that is occurring. Post-translational modifications of p53 are a likely to be responsible for any additional regulation. The p53 protein must be activated in order to function properly and protein modifications, like phosphorylation, are often responsible for this activation (Bode and Dong, 2004). Activation of ATM by DNA damage causes phosphorylation of p53 at serine 15. Our data predict that inactivation of $p16^{INK4a}$ should increase p53 serine 15 phosphorylation. Conversely, is something actively inhibiting p53 function? What other factors are necessary to activate this p53? We initiated a few experiments to test this hypothesis. We began to perform some ChIP-on-chip experiments to identify global p53 binding sites in control cells and cells expressing $p16^{INK4a}$ shRNA. These experiments have the potential to identify how inactivation of $p16^{INK4a}$ modulates p53 binding to DNA and its activation of target genes.

3.5. Materials and Methods

3.5.1. Cells and cell culture

Primary HMEC were isolated from reduction mammoplasty samples as previously described and were cultured in modified MCDB 170 (MEGM, BioWhittaker, USA) (Romanov et al., 2001). Specimens from nine different individuals: RM9, RM13, RM15, RM16, RM18, RM21, RM23, RM48, and RM240 were used in our study. Routine cell culture and the isolation of vHMEC were essentially as described (Romanov et al., 2001). Population doublings were calculated using the equation, PD = log (A/B)/log2, where A is the number of

cells collected and B is the number of cells plated initially. All experiments were performed at 2-5 times with samples from 2-4 individuals.

3.5.2. QPCR

Total RNA samples were isolated using Rneasy Mini Kit (Qiagen) as per manufacturer instructions. cDNA was synthesized from 2μg total RNA by using TaqMan Reverse Transcription Reagents (Applied Biosystems N808-0234) as per manufacturer instructions. Real-Time PCR was performed on 5ng input RNA per reaction containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems 4304437) and the appropriate TaqMan probe (Applied Biosystems $p14^{ARF}$ Hs00924091_m1) on the DNA Engine Opticon 2 (MJ Research Inc.). All samples were analyzed in triplicate on each plate and at least 3 plates were analyzed. Relative mRNA levels were determined by using the relative standard curve method (normalized to GUSB) according to Applied Biosystems User Bulletin #2 (www.appliedbiosystems.com).

3.5.3. Western analysis

Cells were lysed with RIPA buffer or Curtis's strong lysis buffer (10mM Tris pH 8.0, 150mM NaCl, 1% Na Deoxycholate, 1% NP-40, 1% SDS, 1mM EDTA) containing 1x Complete protease inhibitors (Boehringer Mannheim). Protein concentration was determined using BCA (Pierce) with BSA as the standard (Sigma). Protein from total cell extracts was denatured with 1x loading buffer and then fractionated in gradient (4-20%) polyacrylamide gels (CAMBREX) and transferred to Hybond-P (Amersham) membrane. The following

monoclonal antibodies were used to stain the blots: mouse anti-p16 (Ab-4 NeoMarkers), mouse anti-p53 (DO-1) mouse anti-β-actin (AC-15 Sigma) and horse-radish peroxidase (HRP)-conjugated goat anti-mouse antibody (Gibco). β-actin was used as a loading control for all blots. 5-20ug of protein was analyzed for each sample. Staining was developed using SuperSignal West Pico chemiluminescence detection protocol (Pierce).

3.5.4. Cell cycle analysis

Cells were metabolically labeled with 10mM bromodeoxyuridine (BrdU) for 4 hours prior to harvest. Cells were isolated by standard trypsinization, resusupended in PBS and fixed by addition of ice-cold ethanol to a final concentration of 70%. Nuclei were isolated and stained with propidium iodide and FITC-conjugated anti-BrdU antibodies (Becton Dickinson, USA). Flow cytometry was performed on a FACS-Sorter (Becton Dickinson), using CellQuest and Flowjo software for analysis. All analyzed events were gated to remove debris and aggregates. A minimum of 30,000 events were collected for each analysis.

4. Chapter IV

Modulation of $p16^{INK4a}$ in primary human mammary epithelial cells identifies CHI3L1 as a $p16^{INK4a}$ regulated gene and a marker of the basal-like subtype of invasive breast cancer.

4.1. Abstract

$p16^{INK4a}$ is an important tumor suppressor gene involved in the early steps of breast carcinogenesis. In order to further understand the role of $p16^{INK4a}$ in early breast carcinogenesis we performed a gene expression array screen in primary human mammary epithelial cells expressing either control shRNA or $p16^{INK4a}$ shRNA. We identified CHI3L1 as a gene regulated by $p16^{INK4a}$ that is a marker of the basal-like subtype of breast cancer. This gene may be useful as a serum biomarker for early breast cancer.

4.2. Introduction

As described in the previous chapters, $p16^{INK4a}$ is an important tumor suppressor gene which is inactivated in many tumor types including breast cancer. The major tumor suppressor function of $p16^{INK4a}$ is thought to be its ability to arrest the cell cycle and induce senescence (Drayton et al., 2003; Kim and Sharpless, 2006; Rocco and Sidransky, 2001; Serrano et al., 1993). However, in human breast epithelial cells inactivation of $p16^{INK4a}$ alters many cellular phenotypes not necessarily linked to cell cycle or senescense including; centrosome regulation, telomere maintenance, COX-2 expression, global DNA methylation, and p53 protein stability (Crawford et al., 2004; McDermott et al., 2006; Reynolds et al., 2006; Romanov et al., 2001; Zhang et al., 2006). These results in the HMEC system have interested us in other roles of $p16^{INK4a}$ that are not directly linked to cell cycle checkpoint control but may be important to tumor suppression. We have used the HMEC system to further understand $p16^{INK4a}$ signaling in the context of early breast carcinogenesis.

In addition to studying $p16^{INK4a}$ signaling in early breast carcinogenesis we would like to identify biomarkers for early detection and prevention of breast cancer. We have used inactivation of $p16^{INK4a}$ in HMEC as a system to identify new biomarkers of early breast cancer. While biomarkers can be used for many endpoints (detection, prognosis, etc.) we were most interested in identifying a secreted protein that could be used as a serum biomarker. There are currently no serum biomarkers used clinically for breast cancer detection (Duffy, 2006).

Over the last few years it has become feasible to perform global assays in primary mammalian cells. One of the most common types of assays is a global gene expression array. These expression arrays enable the examination of the mRNA levels of nearly every gene expressed in a population of cells within a single experiment. This type of experiment is very useful to analyze global changes in gene expression or to identify candidate genes for a particular phenotype. We performed global gene expression array analysis on HMEC with or without inactivation of $p16^{INK4a}$ to examine the consequences of inactivation of $p16^{INK4a}$ on a global scale and further understand $p16^{INK4a}$ signaling in early breast carcinogenesis. This data could be used to identify new signaling pathways modulated by inactivation of p16 or to identify candidate genes to use as biomarkers. Any new biomarkers might be useful to identify cells in early breast carcinogenesis with inactivated $p16^{INK4a}$ signaling. Inactivation of $p16^{INK4a}$ signaling coupled with proliferation has recently been shown to predict outcome in DCIS. An ideal biomarker would be a secreted protein that is detectable in serum. There are currently no serum diagnostic tests utilized for breast cancer detection. In summary, we hypothesized that inactivation of $p16^{INK4a}$ will alter the expression of many signaling pathways including cell cycle regulation and p53, and potentially identify a serum biomarker for breast cancer detection.

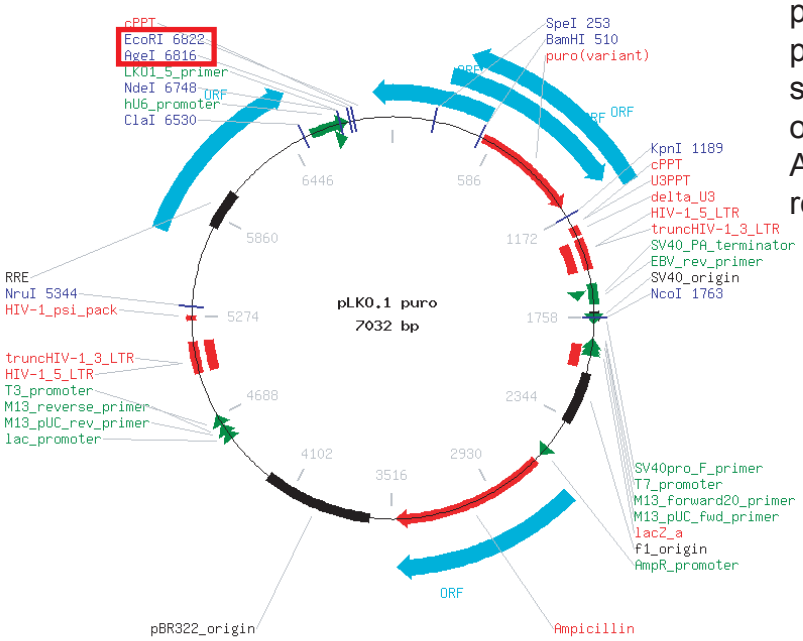
4.3. Results

4.3.1. Cloning $p16^{INK4a}$ shRNA into a lentiviral vector

Our previous experiments (Chapter 1) with shRNA for $p16^{INK4a}$ utilized the p16-1 shRNA sequence in a pBabe-puro retroviral construct. This construct proved sufficient for those experiments. However, as we approached future large-scale expression array experiments we required a new expression system for the p16-1 shRNA. The two main drawbacks to the pBabe-pure system were poor infection frequency (because pBabe-puro will only infect dividing cells) and the necessity for puromycin selection. To improve on this system we utilized a lentiviral shRNA expression system. Lentiviruses infect nearly every cell without a requirement for cell division. Additionally, we desired a system that would allow easy cloning of any shRNA sequence into expression vectors with either puromycin selection or GFP selection. We utilized the pSicoR and pLKO.1 lentiviral vectors (Fig 10). These vectors are based on the same lentiviral system, which means that identical packaging and envelope plasmids can be used for both constructs. Also, cloning shRNA sequences into these vectors is relatively simple. Cloning utilizes synthesized annealed oligos with specific overhangs, which can be designed and cloned for any shRNA sequence in about one week (see appendix 1). This new system allowed us to easily clone the p16-1 shRNA sequence into both the pLKO.1 and pSicoR vectors and have the benefits of lentiviral infection frequencies with either puro or GFP selection. We cloned the p16-1 shRNA sequence into both vectors and found efficient knock-down of $p16^{INK4a}$ RNA levels and protein levels which was equivalent to the knock-down observed with the pBabe-puro vector (Fig 7).

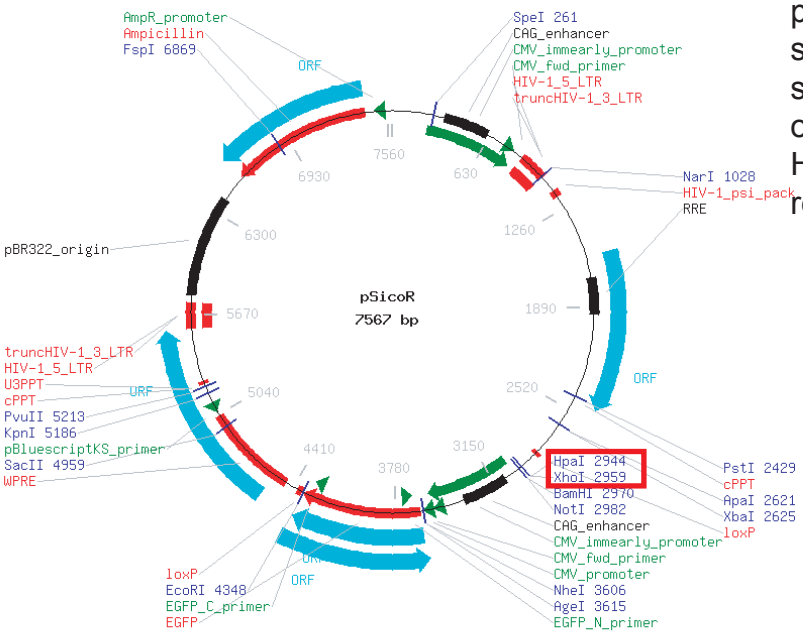
Figure 10

A



pLKO.1 contains puromycin selection. shRNA annealed oligos are cloned with AgeI and EcoRI (see red box).

B



pSicoR contains GFP selection. shRNA annealed oligos are cloned with HpaI and XhoI (see red box).

Figure 10: Lentiviral shRNA expression vectors.
Plasmid maps for the indicated plasmids were derived from the Addgene.org website. Cloning sites for shRNA constructs are indicated with a red box. A. pLKO.1, Addgene plasmid 8453. B. pSicoR, Addgene plasmid 11579.

4.3.2. Shp16 expression arrays

In order to further understand the role of $p16^{INK4a}$ in HMEC we performed global gene expression analysis on HMEC expressing $p16^{INK4a}$ shRNA compared to controls. These experiments required large numbers of HMEC from multiple individuals and became technically feasible with the lentiviral expression system for $p16^{INK4a}$ shRNA described above. HMEC from 6 individuals were infected with vector (pLKO.1pGL3) or shp16 (pLKO.1p16-1) expressing lentiviruses. Successful infections and sufficient cell numbers were obtained from 4 individuals (RM13, RM15, RM18, and RM21) for further analysis (p16 knock-down ranged from 22% to 75% and is shown in Fig 13B). Samples from these 4 individuals were used to make cDNA for expression array analysis on the Affymetrix human U133A Plus 2.0 platform. Hierarchical clustering analysis found that the shp16 expressing samples did not cluster with each other. Instead it was found that the samples (vector and shp16) from each individual clustered with themselves (Fig 11A). This indicates that the changes caused by expression of shp16 are minor when compared to the heterogeneity between samples. Additionally, by analyzing samples from only 4 individuals very few genes had robust statistical changes between vector to shp16 (Fig 11B). By lowering the statistical thresholds the genes that changed most significantly were identified (Table 4-1). Next, pathway analysis was performed based on GO gene classifications (Table 4-2). The most significantly modulated pathways were all cell cycle related. No pathways could be clearly identified independent of cell cycle regulation. Because of our previous work (see Chapters II and III) demonstrating increased p53 protein levels and functions in cells expressing $p16^{INK4a}$ shRNA, the data were analyzed for changes to the p53 pathway. Surprisingly, global gene expression changes to the p53 pathway were not identified in cells expressing $p16^{INK4a}$

Figure 11

A

B

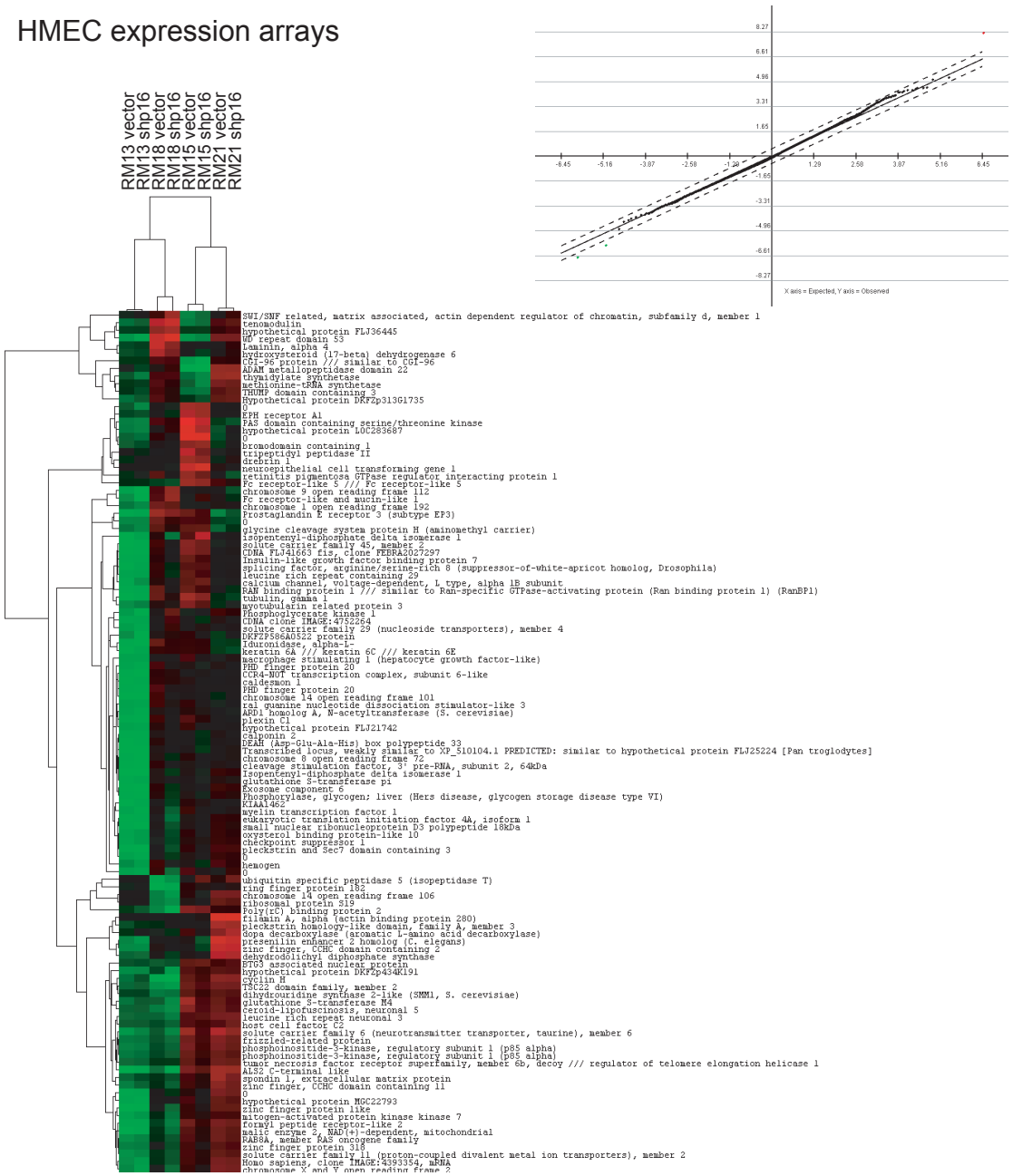


Figure 11: Expression array analysis of HMEC expressing p16 shRNA.
A. Hierarchical clustering of vector or p16 shRNA infected samples from four individuals. The 114 most variable genes were clustered by sample and gene. The dendrograms are shown on the top and left axes. Sample names and gene names are indicated on the top and right axes. Samples from each individual are most similar to themselves. B. SAM analysis comparing vector to p16 shRNA expressing cells. Three genes were significantly changed; PRDX3, PPIA, and an unknown.

Table 1: Genes modulated on p16 shRNA expression arrays

Cell Cycle	Mitosis/ Centrosome	Arrest/p53 /repair	Kinesin	Base Metabolism	Unknown	Other
CCNA2	ASPM	ANLN	ACTA1	APOBEC3B	C10orf3	ANGPTL4
CCNB1	AURKB	BIRC5	KIF11	DCK	C14orf106	ANP32E
CCNB2	BTG3	BRCA1	KIF14	DHFR	C20orf129	APOE
CCNE2	BUB1	BRIP1	KIF15	DUT	C9orf76	HMGA1
CDC2	BUB1B	FANCG	KIF18A	RRM1	DKFZp762E1312	HMGB2
CDC20	CCDC5	GAS2L3	KIF20A	RRM2	FAM54A	HMGN2
CDC25A	CENPA	GMNN	KIF23	TK1	FAM64A	HMMR
CDC25C	CENPF	GTSE1	KIF2C	TYMS	FAM72A	LBR
CDC6	CENPH	NEIL3	KIF4A		FLJ10719	LMNB1
CDC7	CSE1L	RAD51AP1	MASTL		FLJ11029	LYPLA3
CDCA1	DCC1	SSRP1	SYNPO		FLJ20641	NRCAM
CDCA2	DLG7	VRK1			FLJ22624	NUDT21
CDCA3	FKSG14				FLJ23867	PHGDH
CDCA5	HCAP-G				FLJ25416	PKIB
CDCA7	ITGB3BP	**Cancer**	**Immune**	**Histone**	KIAA0101	PLSCR1
CDKN2A	KNTC1	CASC5	CD200	H2AFX	KIAA0286	PPIL5
CDKN2C	KNTC2	DLEU2 /// BCMSUNL	CD59	HIST1H2AD /// HIST1H3D	KIAA1161	PSAP
CDKN3	LUZP5	MAC30	IL8	PHF19	KIAA1524	PSIP1
CDT1	MAD2L1	MYBL1	MICB	SUV39H2	LOC139886	SLC16A3
CLIC3	MLF1IP	NMU	THBD		LOC146909	SSX2IP
DEK	MPHOSPH9	NUDCD1			LOC163233	TFAM
DEPDC1	NEK2	TNFAIP8			MGC16943	TIFA
DNA2L	NPM3		**Ubiquitin**			TMPO
FBXO5	NUSAP1		UBE2C			WHSC1
FOXM1	PLK4		UBE2T			
MCM2	PRC1		UHRF1			
MCM4	SIL		USP1			**Other ?**
MCM5	SPBC25					ATAD2
MCM6	STK6					CHI3L1
MCM7	TTK					CNOT6L
MELK	ZWINT					DONSON
MKI67						DTL
ORC6L						EXOSC8
PBK						FIGNL1
PCNA						KCTD14
Pfs2						LBH
POLE2						LRRN1
PRIM2A						MCFP
PSF1						MOCOS
RACGAP1						NDST1
RBL1						SELM
RFC3						SHCBP1
RFC3						TMEM48

RFC4						
RFC5						
SFRS7						
SGOL2						
SKP2						
SMC2L1						
SMC4L1						
TCF19						
TOP2A						
TOP2A						
TOPBP1						
WEE1						
ZNF367						

Table 2: Gene classifications from the p16 shRNA expression arrays

Category	Term	Count	%	P-Value
GOTERM_BP_ALL	cell cycle	70	34.0%	7.5E-45
GOTERM_BP_ALL	mitotic cell cycle	42	20.4%	2.7E-38
GOTERM_BP_ALL	M phase of mitotic cell cycle	34	16.5%	3.3E-32
GOTERM_BP_ALL	mitosis	33	16.0%	5.6E-31
GOTERM_BP_ALL	cell division	34	16.5%	4.9E-30
GOTERM_BP_ALL	M phase	35	17.0%	8.5E-30
GOTERM_BP_ALL	regulation of progression through cell cycle	47	22.8%	3.1E-29
GOTERM_BP_ALL	regulation of cell cycle	47	22.8%	3.5E-29
GOTERM_BP_ALL	DNA replication	29	14.1%	1.1E-23
GOTERM_CC_ALL	chromosome	31	15.0%	1.3E-21
GOTERM_BP_ALL	DNA metabolism	42	20.4%	1E-19
GOTERM_BP_ALL	cell cycle checkpoint	15	7.3%	5.7E-17
GOTERM_CC_ALL	intracellular non-membrane-bound organelle	55	26.7%	2.2E-16
GOTERM_CC_ALL	non-membrane-bound organelle	55	26.7%	2.2E-16
GOTERM_BP_ALL	DNA-dependent DNA replication	18	8.7%	3.3E-16
GOTERM_CC_ALL	nucleus	91	44.2%	1.9E-15
GOTERM_CC_ALL	spindle	13	6.3%	5.5E-15
GOTERM_CC_ALL	microtubule cytoskeleton	23	11.2%	1.5E-13
GOTERM_BP_ALL	microtubule-based process	19	9.2%	6.1E-13
GOTERM_BP_ALL	biopolymer metabolism	74	35.9%	8.2E-13
GOTERM_BP_ALL	spindle organization and biogenesis	10	4.9%	1.7E-12
GOTERM_MF_ALL	ATP binding	46	22.3%	2.6E-12
GOTERM_BP_ALL	regulation of mitosis	12	5.8%	2.8E-12
GOTERM_BP_ALL	organelle organization and biogenesis	37	18.0%	7.3E-12
GOTERM_MF_ALL	adenyl nucleotide binding	46	22.3%	1.1E-11
GOTERM_CC_ALL	chromosome, pericentric region	11	5.3%	3.7E-11
GOTERM_CC_ALL	intracellular organelle	113	54.9%	4.3E-11
GOTERM_CC_ALL	organelle	113	54.9%	4.4E-11
GOTERM_BP_ALL	cell proliferation	28	13.6%	6.7E-10
GOTERM_BP_ALL	chromosome segregation	10	4.9%	7.8E-10

shRNA even though p53 levels were increased. It is possible that modest gene expression changes in the p53 pathway were not detectable due to our low sample numbers.

4.3.3. Identification of CHI3L1, a basal gene

Because pathway analysis of the expression array data did not reveal dramatically altered pathways, except for pathways related to cellular proliferation, a candidate gene approach was taken for further analysis and validation. A variety of interesting candidate genes were chosen for validation by quantitative real-time PCR. On the expression array CDC6 was increased 1.7-fold, CDKN3A was increased 1.7-fold, RBL1/p107 was increased 1.5-fold, and CHI3L1 was decreased 1.5-fold in HMEC expressing p16 shRNA (Figure 12 and Table 1). We found that the expression array changes observed for CDC6, RBL1/p107, and CDKN3A were confirmed by QPCR in RM13 and RM15 however, the changes in these genes were not confirmed in RM18 and RM21 (Fig 12). The reason for this discrepancy is unknown. However, the expression array changes observed for CHI3L1 were confirmed by QPCR in all 4 RM samples (Fig 13). Additionally, CHI3L1 was found to be reduced in vHMEC when compared to HMEC (10.9-fold), as measured in a previous expression array experiment (Fig 14), and by QPCR in samples from RM18.

Analysis of breast tumor expression array data, published previously by other groups (Chin et al., 2006; Farmer et al., 2005; Richardson et al., 2006; Sotiriou et al., 2003), reveals that CHI3L1 is expressed in the basal-like subtype of invasive breast cancer (Fig 15A-D). The basal-like subtype is most simplistically defined as negative for ER, PR and HER2 (Lu et al., 2008). This subtype has poor prognosis when compared to breast tumors overall. CHI3L1 is

Figure 12

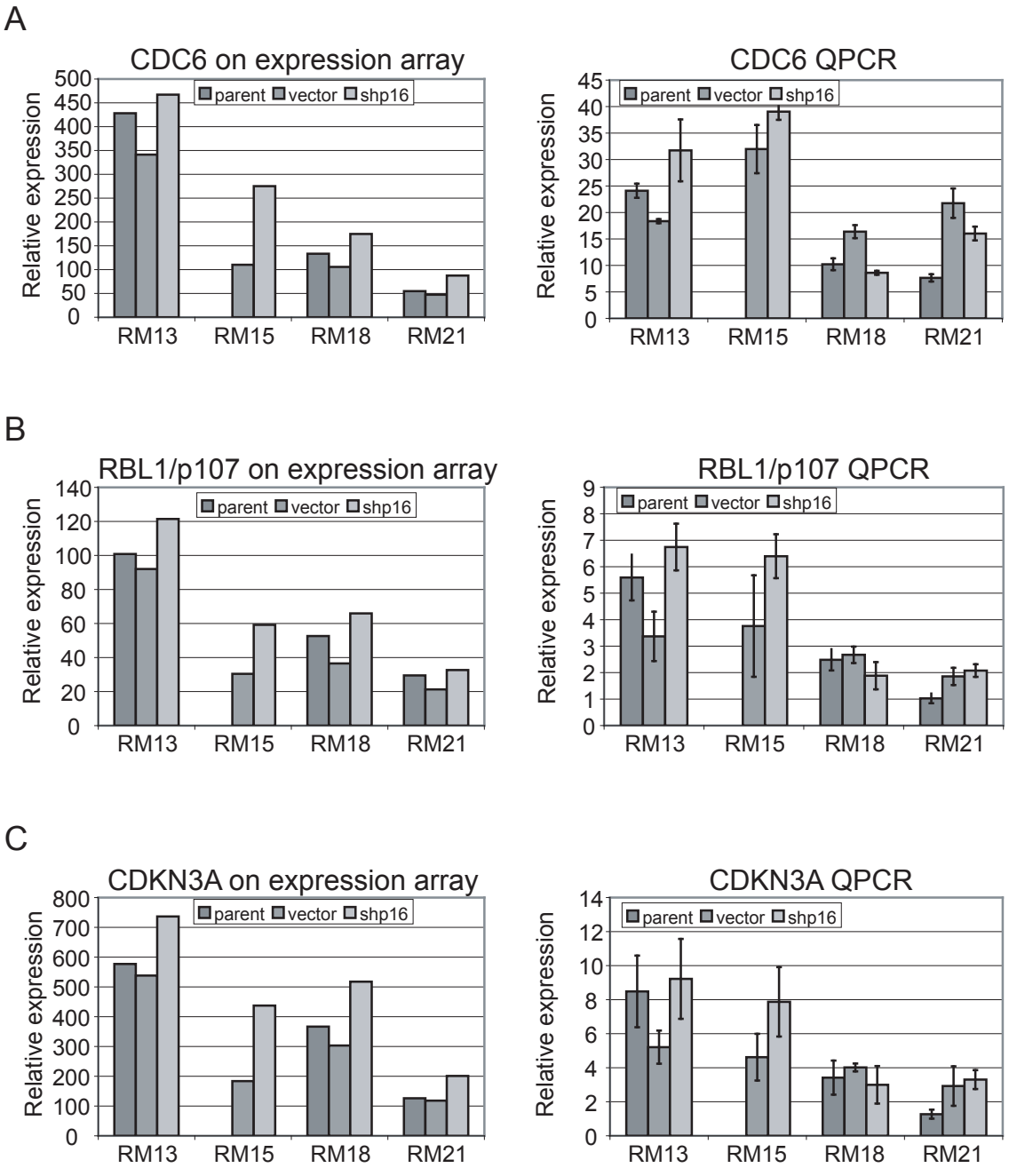


Figure 12: Validation of candidate genes from the expression array analysis.
Relative expression of three genes (A. CDC6, B. RBL1/p107, C. CDKN3A) is shown. Samples were derived from four individuals as indicated in the figure. The graph on the left is data from the expression array and the graph on the right is its validation by QPCR.

Figure 13

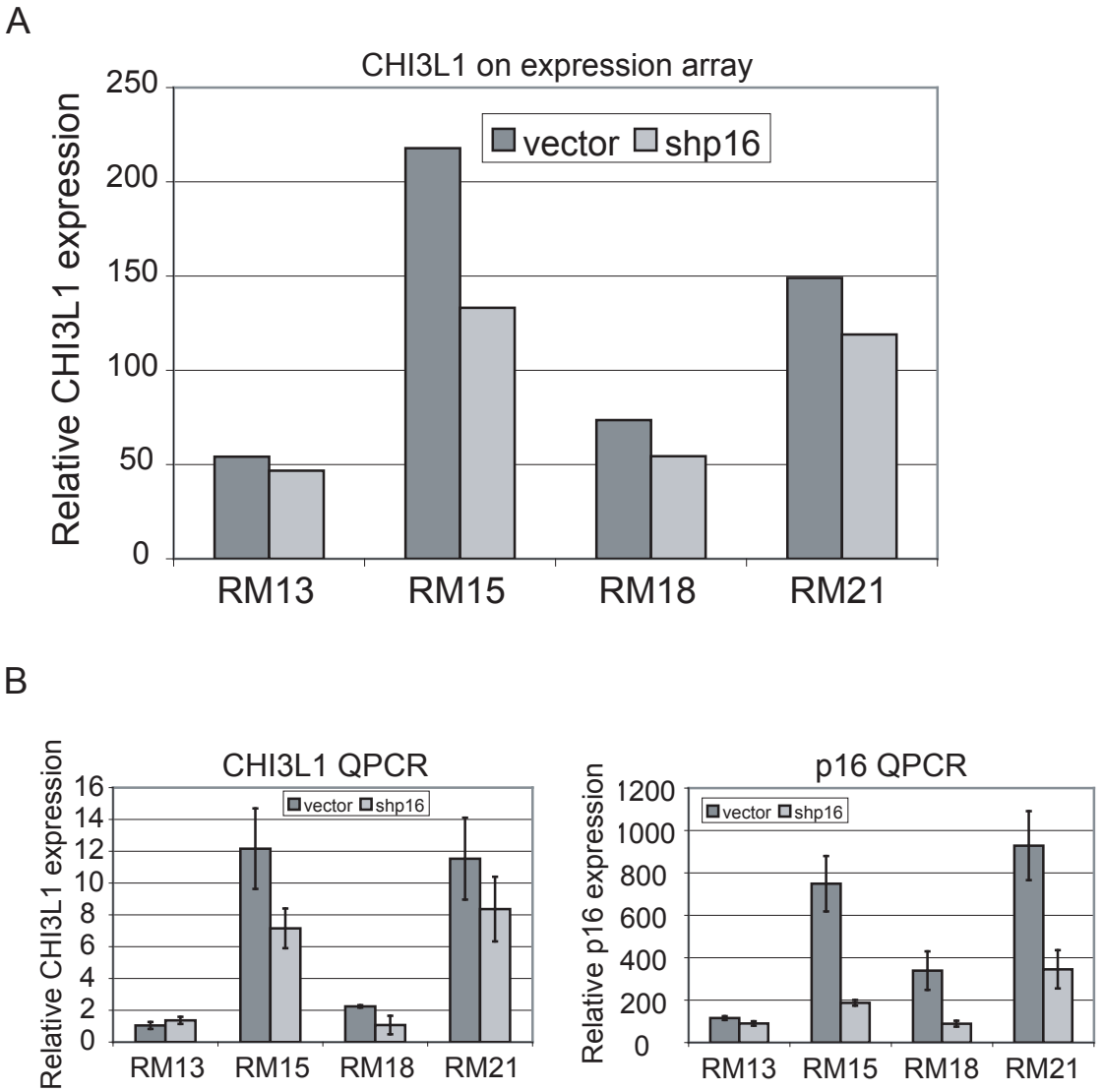
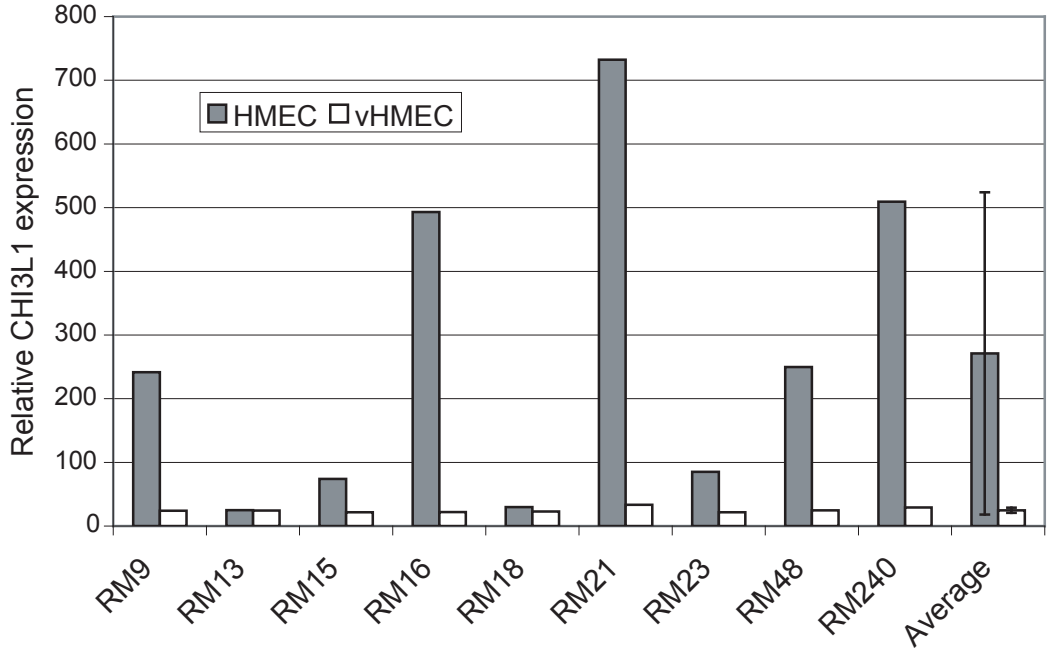


Figure 13: Validation of CHI3L1 from the expression array analysis.
Relative expression of the indicated genes is shown. Samples are also as indicated. A. CHI3L1 expression from the expression array. B. Validation of CHI3L1 (left graph) and p16 (right graph) expression by QPCR.

Figure 14

A



B

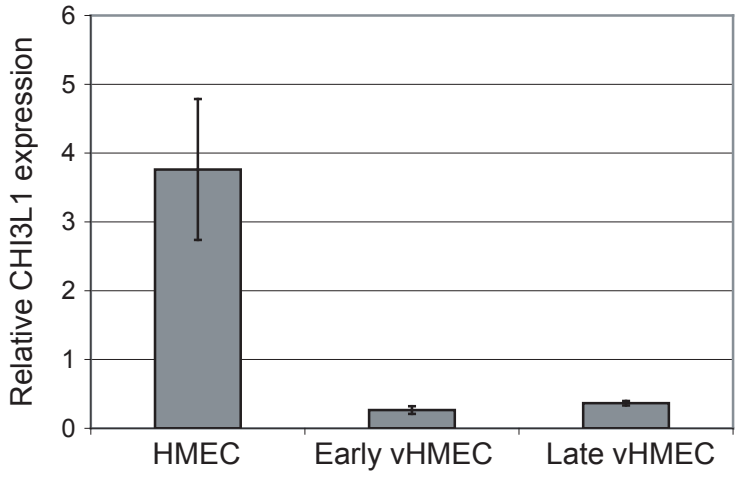


Figure 14: CHI3L1 levels are reduced in vHMEC compared to HMEC.
A. Expression of CHI3L1 in HMEC and vHMEC cells from 9 individuals as indicated. The Average expression and standard deviation are shown on the far right. Data was derived from an expression array experiment. B. Expression of CHI3L1 in RM18 HMEC compared to early and late passage vHMEC. Analysis was performed by QPCR.

Figure 15

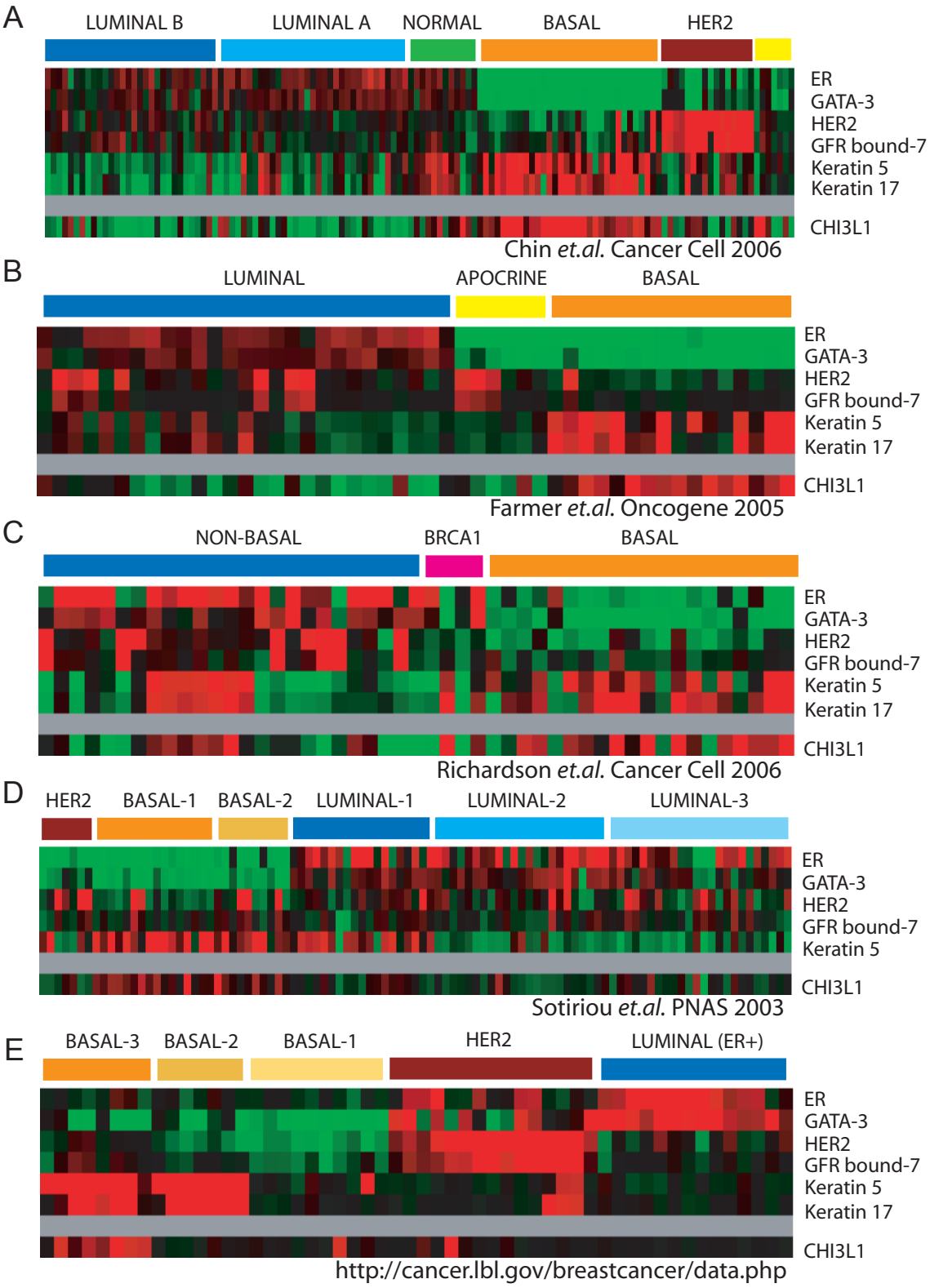

A
LUMINAL B
LUMINAL A
NORMAL
BASAL
HER2
ER
GATA-3
HER2
GFR bound-7
Keratin 5
Keratin 17
CHI3L1
Chin et.al. Cancer Cell 2006
B
LUMINAL
APOCRINE
BASAL
ER
GATA-3
HER2
GFR bound-7
Keratin 5
Keratin 17
CHI3L1
Farmer et.al. Oncogene 2005
C
NON-BASAL
BRCA1
BASAL
ER
GATA-3
HER2
GFR bound-7
Keratin 5
Keratin 17
CHI3L1
Richardson et.al. Cancer Cell 2006
D
HER2
BASAL-1
BASAL-2
LUMINAL-1
LUMINAL-2
LUMINAL-3
ER
GATA-3
HER2
GFR bound-7
Keratin 5
CHI3L1
Sotiriou et.al. PNAS 2003
E
BASAL-3
BASAL-2
BASAL-1
HER2
LUMINAL (ER+)
ER
GATA-3
HER2
GFR bound-7
Keratin 5
Keratin 17
CHI3L1
http://cancer.lbl.gov/breastcancer/data.php

Figure 15: CHI3L1 is a marker of basal-like breast tumors.
Heat maps of expression array data on breast tumor cells were compiled from published sources as indicated below the data. Breast cancer subtypes were defined in the original publications and are labeled above the heat map. ER and GATA-3 are shown as markers of the luminal subtype. HER2 and GFR bound-7 (growth factor receptor bound 7) are shown as markers of the HER2 subtype. Keratin 5 and keratin 17 are shown as markers of the basal-like subtype. A-D. Breast tumor samples. E. Breast cancer cell lines.

one of the defining genes of the basal-like subtype. CHI3L1 is also highly expressed in a basal-like subtype of breast cancer cell lines (Fig 15E).

4.3.4. Modulation of CHI3L1 by $p16^{INK4a}$

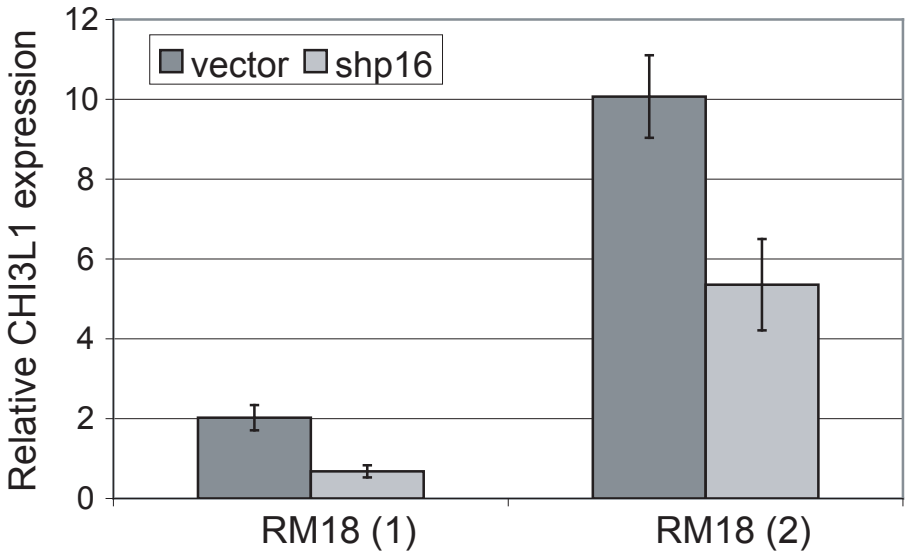
These expression array data suggest that CHI3L1 is regulated by $p16^{INK4a}$. To further examine this question we modulated $p16^{INK4a}$ levels and examined CHI3L1 expression (Figs 13 and 16). Expression of $p16^{INK4a}$ shRNA in RM18 HMEC reduced CHI3L1 expression while expression of wild-type $p16^{INK4a}$ in RM18 vHMEC increased CHI3L1 expression. This indicates that CHI3L1 is regulated by $p16^{INK4a}$.

4.3.5. Modulation of CHI3L1 by p53

Because $p16^{INK4a}$ is not a known transcription factor and thus not likely to directly modulate CHI3L1 expression, we sought to determine if $p16^{INK4a}$ modulated other proteins to regulate CHI3L1. Based on our previous studies into the modulation of p53 by p16 we decided to investigate if $p16^{INK4a}$ modulates CHI3L1 through p53. We hypothesized that p53 inhibits CHI3L1 levels. To investigate this, a genetic suppressor element, GSE22, which acts as a dominant negative to p53, was utilized to inhibit p53 function (Ossovskaya et al., 1996). Expression of the p53 GSE22 in vHMEC caused an increase in CHI3L1 levels (Fig 17). Additionally, expression of a p53 shRNA reduced p53 levels in vHMEC and increased CHI3L1 levels. In these two experiments, CHI3L1 levels were increased by reduced p53 function (Fig 17). These preliminary results indicate that $p16^{INK4a}$ might regulate CHI3L1 through p53.

Figure 16

A



B

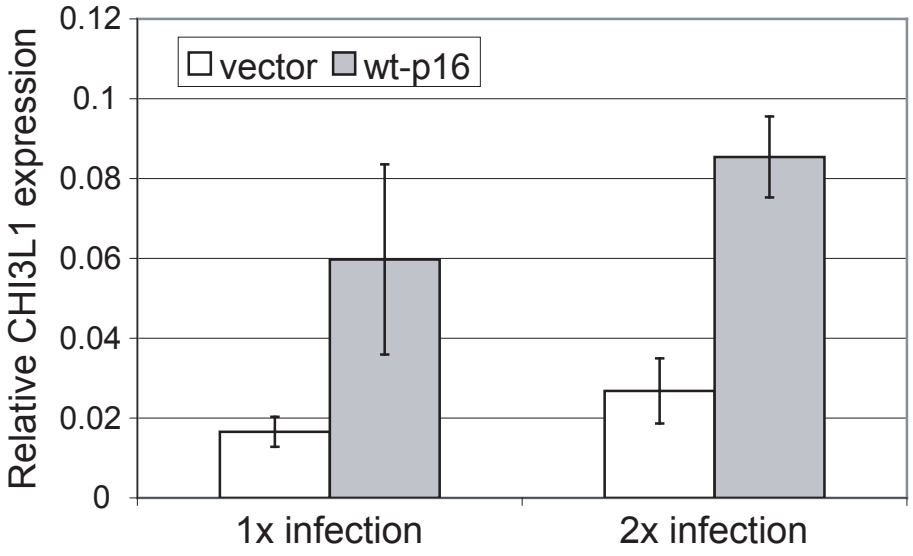


Figure 16: p16 modulates CHI3L1.
A. Expression of CHI3L1 as measured by QPCR in HMEC cells infected with lentivirus expressing either control vector or shp16 vector as indicated. Replicate experiments in RM18 are shown. B. Expression on CHI3L1 as measured by QPCR in RM18 vHMEC cells infected with retrovirus expressing either control vector or wt-p16 vector as indicated. Samples were infected either once (1x) or twice (2x) with the indicated viruses. Cells infected twice demonstrated a higher level of p16 expression than those infected only once. This experiment was performed in RM18 and 15.

Figure 17

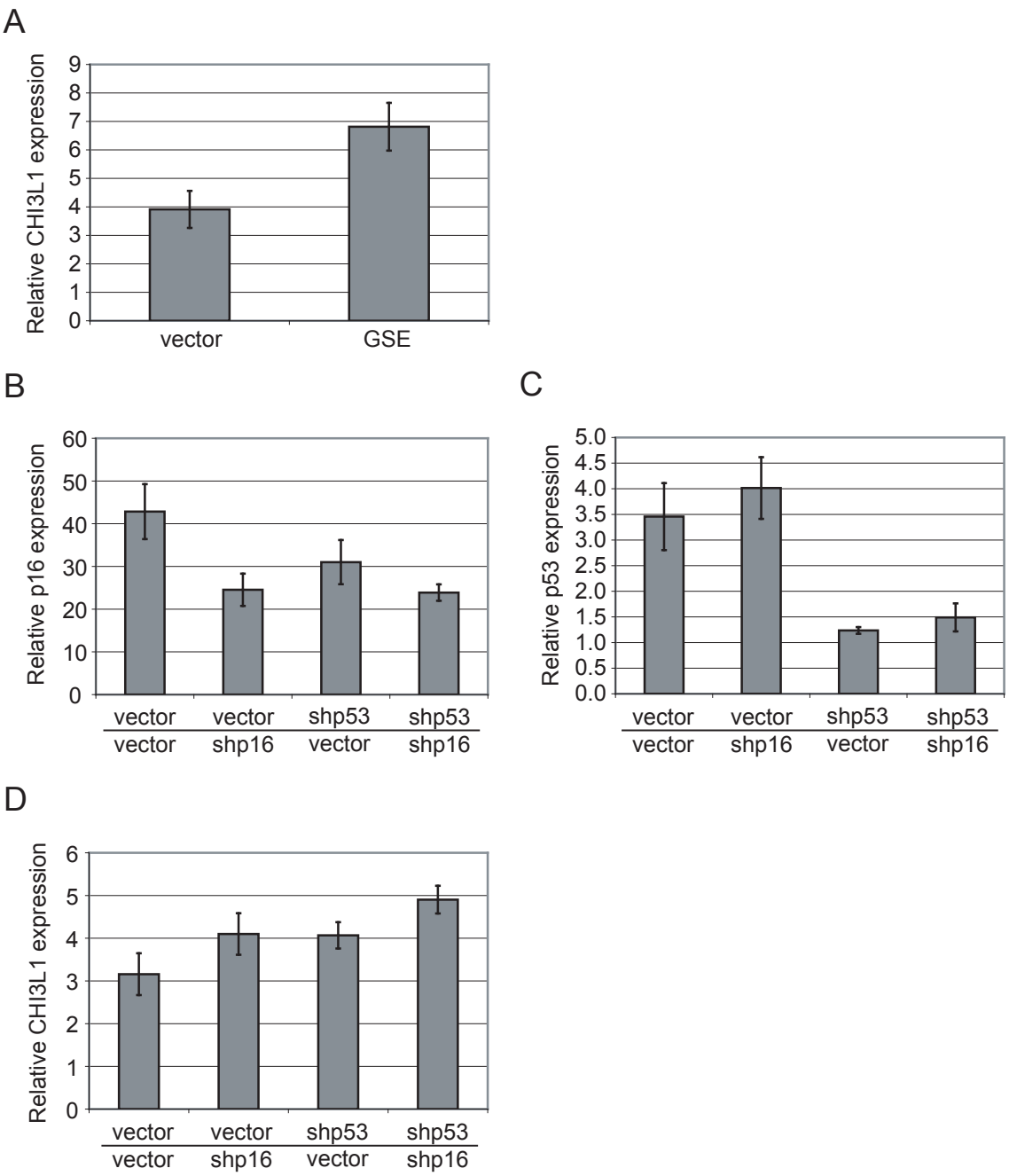


Figure 17: CHI3L1 expression may be modulated by p53.
Relative expression of the indicated gene as determined by QPCR. A. RM16 vHMEC cells were infected with retrovirus expressing either control vector or p53 genetic suppressor element (GSE) vector. B-D. RM13 HMEC cells were infected with lentiviruses expressing the indicated combinations of vectors. B. Expression of p16 as a control for p16 shRNA. C. Expression of p53 as a control for p53 shRNA. D. Expression of CHI3L1. This individual (RM13) has unique regulation of CHI3L1, p16 shRNA does not decrease CHI3L1. No conclusion can be made about its regulation by p53.

If reduced $p16^{INK4a}$ levels induce p53 to inhibit CHI3L1 expression then the modulation of CHI3L1 by $p16^{INK4a}$ is dependent on p53. Therefore, co-expression of shRNA to both $p16^{INK4a}$ and p53 should cause an increase in CHI3L1 levels. This result would indicate that p53 signals downstream of $p16^{INK4a}$. This experiment has been performed in samples from 2 different individuals, RM13 and BC164, however the results must still be considered preliminary. The first experiment did not reveal a convincing result because it was performed in samples from the wrong individual, RM13. RM13 was a poor choice because CHI3L1 is not modulated by $p16^{INK4a}$ in RM13 (Fig 13). For some unknown reason, regulation of CHI3L1 is different in RM13 when compared to other RMs. It is possible that this differential regulation is due to the low basal levels of $p16^{INK4a}$ in this RM. The second experiment, performed in BC164, did not validate our hypothesis. We did not observe an increase in CHI3L1 levels by p53 shRNA in BC164 vHMEC (data not shown). Additionally, double infection of BC164 HMEC with lentiviruses expressing both p16 and p53 shRNA exhibited reduced levels of CHI3L1 (data not shown). This result is consistent with a p53 independent regulation of CHI3L1 by $p16^{INK4a}$. The regulation of CHI3L1 by p53 must be examined in additional RMs before a definitive conclusion can be made about its regulation by p53.

4.3.6. Function of CHI3L1

In general, the functions of CHI3L1 are poorly understood, although, some functions have been identified in various systems (Johansen, 2006). CHI3L1 is able to promote proliferation in some cell types, including synovial fibroblasts. We asked if CHI3L1 could modulate

proliferation in a variety of breast epithelial cells. To test this, a lentiviral over-expression construct for CHI3L1 was developed and vHMEC were infected with virus containing either the empty vector (pWP1) or the CHI3L1 over-expression vector (pWP1CHI3L1) (Fig 18A). Growth curves demonstrate that over-expression of CHI3L1 does not alter proliferation of vHMEC (Fig 18B). Similarly, infection of the HCC38 breast cancer cell line to over-express CHI3L1 did not modulate proliferation (Fig 18C). Two shRNA constructs (CHI-1 and CHI-4) were developed to reduce CHI3L1 expression, although only one (CHI-4) efficiently reduced CHI3L1 levels (Fig 18A). The HCC70 breast cancer cell line, which expresses CHI3L1, was infected with lentiviruses that express either a control shRNA (pLKpGL3) or an shRNA toward CHI3L1 (pLKCHI-1 or pLKCHI-4). Expression of CHI3L1 shRNA did not modulate the proliferation of the HCC70 cells (Fig 18C). Additionally, a few preliminary experiments have been performed to examine the effect of CHI3L1 on other cells types. We have found that purified CHI3L1 does not increase the migration of HUVEC cells in a trans-well assay in contradiction to the results from Malinda, *et.al* (Malinda et al., 1999). Preliminary data also suggests that conditioned media collected from CHI3L1 over-expressing cells does not modulate the proliferation or motility of breast fibroblasts (data not shown).

Figure 18

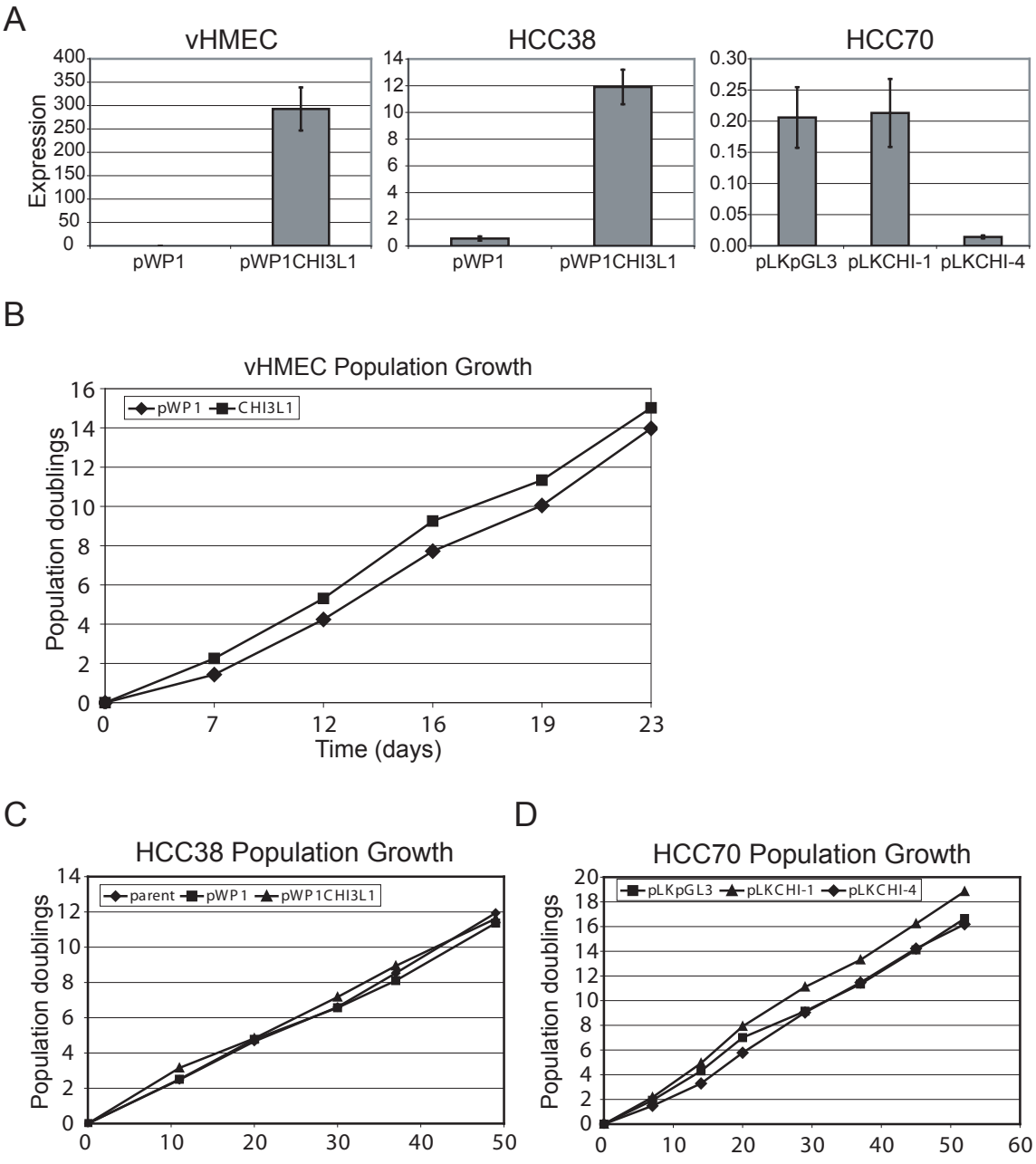


Figure 18: CHI3L1 does not regulate proliferation of breast epithelial cells.
The indicated cells (vHMEC, HCC38 or HCC70) were infected with either control lentivirus (pWP1 or pLKpGL3), lentivirus expressing CHI3L1 (pWP1CHI3L1), or lentivirus expressing shRNA designed against CHI3L1 (pLKCHI-1 or pLKCHI-4). Only one of the CHI3L1 shRNA efficiently reduced CHI3L1 expresssion (pLKCHI-4). A. Control experiment demonstrating overexpression or knock-down of CHI3L1 by the vectors. B. Growth curve of RM18 vHMEC cells infected with control or CHI3L1 virus. C. Growth curve of HCC38 cells infected with control or CHI3L1 virus. D. Growth curve of HCC70 cells infected with control or CHI3L1 shRNA virus.

4.4. Discussion

4.4.1. Cloning

An important step in our research on HMEC was the transition to the pLKO.1 and pSicoR lentiviral vectors for expression of shRNA constructs. These vectors have many advantages including; ease of cloning, high infection frequency, and multiple section markers. These vectors made it possible to perform our expression array experiments and have proven critical for many other projects as well. The ease of cloning has allowed us to design shRNA constructs to many genes and utilize them in primary human cells.

4.4.2. Shp16 expression arrays-general conclusions

These shp16 expression array studies were designed to improve our understanding of $p16^{INK4a}$ signaling in HMEC and identify markers for early detection of breast cancer. In many ways our expression array results were disappointing. Our sample numbers were not sufficient for robust statistical analysis. This lack of statistical power made it difficult to identify pathways and genes modulated by $p16^{INK4a}$. However, we were able to identify CHI3L1 as a gene regulated by p16 that may be important in early breast cancer and is a potential serum biomarker.

4.4.3. Shp16 expression arrays-pathways and genes

Pathway analysis of the expression array data identified the cell cycle as the primary pathway modulated by $p16^{INK4a}$. This result was not surprising because the $p16^{INK4a}$ protein inhibits

cell cycle progression. Since $p16^{INK4a}$ is known to regulate the cell cycle we attempted to identify cell cycle independent functions of $p16^{INK4a}$. During this analysis an inherent difficulty in this line of research was realized. In order to identify genes that are independent of the cell cycle it is necessary to first identify all cell cycle genes and then exclude them from the analysis. While databases have been made to classify genes into pathways these databases are not sufficient to eliminate all cell cycle genes. The only way to identify all cell cycle genes would be to experimentally modulate the cell cycle and perform expression array analysis. Our experimental setup did not include these controls. This difficulty with the analysis can be demonstrated with an example from our expression array data. GO database analysis identified a statistically significant enrichment for genes linked to the cytoskeleton as being modulated by $p16^{INK4a}$ shRNA. Is the cytoskeleton independent of the cell cycle? One can hypothesize that modulation of the cell cycle will also modulate the cytoskeleton because proliferative cells need to reorganize the cytoskeleton. Although we had hoped to identify cell cycle independent functions of $p16^{INK4a}$ we soon realized that our experimental setup did not allow for it. Future gene expression array experiments would need to be performed in which the cell cycle was modulated independent of $p16^{INK4a}$. This setup would enable the elimination of cell cycle genes from the analysis and facilitate the identification of cell cycle independent functions of $p16^{INK4a}$.

Additionally, pathway analysis of the shp16 expression arrays did not identify genes in the p53 pathway. This was surprising because we had previously shown $p16^{INK4a}$ shRNA expression increases p53 protein levels and functions. We had expected to find modulation of many p53 target genes following expression of $p16^{INK4a}$ shRNA. The inability to identify p53

pathway modulation may be due to the low sample numbers and reduced statistical power. An alternate hypothesis is that although $p16^{INK4a}$ modulates p53 it may only modulate some p53 target genes. Detailed analysis of the expression of numerous p53 target genes would be necessary to clarify this hypothesis.

As mentioned above, the low sample numbers hindered the identification of genes modulated by $p16^{INK4a}$. The expression changes identified for three out of four candidate genes on the gene expression arrays were not confirmed by QPCR. While the changes in gene expression observed on the arrays can not be considered definitive, these data can be used as a discovery tool for the identification of genes modulated $p16^{INK4a}$. For example, these data were able to identify CHI3L1 as a $p16^{INK4a}$ modulated gene and potential serum marker for breast cancer.

4.4.4. CHI3L1

These expression array experiments demonstrated that expression of CHI3L1 is reduced by $p16^{INK4a}$ shRNA. It was then validated that $p16^{INK4a}$ expression regulates CHI3L1 expression. The specific details of this regulation are still unknown. $p16^{INK4a}$ is not a known transcription factor so it must signal through other proteins to modulate CHI3L1. Because $p16^{INK4a}$ modulates E2F signaling it is possible that E2F regulates CHI3L1, however E2F binding sites are not present in the CHI3L1 promoter and expression array studies have not previously identified CHI3L1 as an E2F regulated gene (data not shown). We hypothesize that p53 inhibits CHI3L1 expression. Although the CHI3L1 promoter does not contain a p53 binding site it has been shown that modulation of p53 can modulate CHI3L1 expression (Junker et al., 2005). Some of our data support this hypothesis, since expression of $p16^{INK4a}$ shRNA

increases p53 function and reduces CHI3L1 levels. Preliminary data also suggest that reduced p53 levels in vHMEC lead to an increase in CHI3L1 levels. However, our initial experiments to inhibit both $p16^{INK4a}$ and p53 in HMEC did not confirm a p53 regulation of CHI3L1. Further experiments are being performed to address this question.

An alternate hypothesis about the regulation of CHI3L1 is also suggested from our data, epigenetic silencing of CHI3L1 in vHMEC. The levels of CHI3L1 in vHMEC from different RMs are uniformly low and near the limit of detection by QPCR, suggesting possible silencing by DNA hypermethylation. Any modulation of CHI3L1 levels (2-fold increase) that we observed in vHMEC (either by wt-p16 or p53) was relatively minor when compared to the levels observed in HMEC (20-fold higher). Additionally, exposure of vHMEC to the DNA methytransferase inhibitor 5-aza-2-deoxycytidine caused an increase in CHI3L1 levels (Appendix 4). This increase in CHI3L1 levels could simply be caused by regulation by $p16^{INK4a}$ which is silenced by DNA methylation in vHMEC and induced by 5-aza-2-deoxycytidine treatment, but it could also indicate epigenetic regulation of CHI3L1 itself. Analysis of the CHI3L1 promoter region by using the UCSC genome browser (genome.ucsc.edu) did not reveal any CpG islands in the CHI3L1 promoter that could serve as sites of DNA hypermethylation. The lack of CpG islands in the CHI3L1 promoter suggests either global silencing of the CHI3L1 genome region or the silencing of a transcription factor that regulates CHI3L1. It is attractive to speculate that pRb is involved in epigenetic regulation of CHI3L1. The pRb protein binds to the promoter DNA of some genes and recruits chromatin modifying factors. The pRb protein levels in vHMEC are increased when compared to HMEC (K. McDermott, data not shown). These elevated pRb levels may

cause it to bind to the promoter of CHI3L1 and modulate the surrounding chromatin to silence the expression of CHI3L1. Additionally, in basal-like breast tumors, where CHI3L1 expression is high, pRb protein levels are often very low. These low levels of pRb may prevent silencing of CHI3L1 and allow increased expression. Genetic manipulation of pRb in the HMEC system could test these hypotheses.

The factors responsible for transcriptional regulation of CHI3L1 in HMEC are still unknown. It is possible that a transcription factor that regulates CHI3L1 would exhibit similar expression patterns to CHI3L1 and could be identified in gene expression array experiments. The promoter of CHI3L1 has bHLH, ets, and SP1 binding sites (Rehli et al., 2003). Analysis of the HMEC-vHMEC expression array data and the vector-shp16 expression array data identified ID4, a bHLH transcription factor, as a potential regulator of CHI3L1 expression due to its similar expression patters. However, ID4 is a dominant-negative transcription factor, which makes it unlikely to regulate CHI3L1. The expression array data also identified EHF, ets homologous factor, as a transcription factor with similar expression to CHI3L1, but EHF is a transcriptional repressor so it is unlikely to regulate CHI3L1. So far we have not been able to identify a candidate transcription factor for CHI3L1 based on the gene expression array data.

Although CHI3L1 expression is reduced in cells with reduced $p16^{INK4a}$ function, CHI3L1 levels are elevated in the basal-like subtype of breast tumors. It is unknown how CHI3L1 is regulated in breast tumor cells, but our data suggest a few possible hypotheses. First, $p16^{INK4a}$ may regulate CHI3L1. Basal-like breast tumors have elevated $p16^{INK4a}$ expression when

compared to other breast tumors, which is consistent with a $p16^{INK4a}$ regulation of CHIL1 (Gauthier et al., 2007). However, basal-like breast tumors also exhibit pRb pathway dysfunction, and pRb pathway dysfunction usually functionally recapitulates $p16^{INK4a}$ loss. The increased $p16^{INK4a}$ levels are considered non-functional because the $p16^{INK4a}$ is unable to signal through pRb. If the high levels of $p16^{INK4a}$ in basal-like breast tumors modulate CHI3L1, then it suggests a novel pRb independent function of $p16^{INK4a}$. This could be examined by reducing $p16^{INK4a}$ levels in a basal-like breast tumor cell line with high $p16^{INK4a}$ expression, pRb pathway dysfunction and high CHI3L1 levels. Our second hypothesis is that p53 may be regulating CHI3L1. Basal-like breast tumors have the highest frequency of p53 mutations among breast tumors (Langerod et al., 2007). These p53 mutations would prevent p53 from inhibiting CHI3L1 expression. This is difficult to study experimentally because of the diverse spectrum of 53 mutations that modulate p53 function in many different ways (Wasielewski et al., 2006). A preliminary analysis of p53 mutations in breast cancer cell lines did not identify any p53 mutations that correlated with CHI3L1 levels, and many cell lines with p53 mutations exhibited very low CHI3L1 expression (data not shown). A third hypothesis is that CHI3L1 chromatin is regulated by pRb. If pRb modulates the chromatin around CHI3L1 to silence its expression, then the low levels of pRb that are found in basal-like breast tumors may de-repress CHI3L1. Lastly, it is possible that CHI3L1 is regulated independent of $p16^{INK4a}$ or p53 in breast tumors. It may have been fortuitous that we identified CHI3L1 in our expression array screen, while a completely different regulation of CHI3L1 may occur in breast tumors.

Our experiments into the functions of CHI3L1 in breast cancer have so far been inconclusive. Others have demonstrated numerous functions for CHI3L1 including; proliferation, migration, and invasion (Johansen, 2006). However each of these phenotypes has only been demonstrated in certain cell types. We are performing experiments to examine these functions in HMEC, fibroblasts, and endothelial cells. It is necessary to analyze both epithelial and non-epithelial cell types because it is possible that CHI3L1 is secreted by breast epithelial tumor cells but functions to modulate stromal cells or angiogenesis. We found that over-expression of CHI3L1 in breast epithelial cells did not modulate proliferation or motility of those epithelial cells (Fig 18B-D). Preliminary data in breast fibroblasts did not identify any modulation of proliferation; however the levels of CHI3L1 in the conditioned media were not analyzed and could have been below a functional level.

4.4.5. vHMEC as a model for the basal-like subtype of breast cancer

The HMEC model system is useful for studies into the early events in breast cancer. Recently, many of our results have pointed to a new utility for the HMEC system, studying basal-like breast cancer. Expression array analysis has demonstrated that HMEC and vHMEC are most similar to the basal-like subtype of invasive breast cancer. Trivially it could be hypothesized that this was due to the expression of a few “basal” genes in these primary cells. However, our most recent data suggest a much deeper connection between the HMEC system and the basal-like subtype. Work by Gauthier and Berman *et.al.* demonstrated that vHMEC can be used to identify new markers of the basal-like subtype (Gauthier et al., 2007). More importantly, these markers were found in DCIS samples and predicted

recurrence. Additionally, these markers ($p16^{INK4a}$, COX-2, Ki-67) were functionally relevant to the tumor, and it may be possible to target these pathways therapeutically.

Our $p16^{INK4a}$ shRNA expression arrays in HMEC have also identified a marker of the basal-like subtype of breast cancer, CHI3L1. This gene was found in many published expression array studies but its role in the basal-like subtype has not been recognized. This gene may be functionally relevant to the basal-like subtype and may be useful as both a marker and a therapeutic target. These data suggest that the HMEC system may improve our understanding of the basal-like subtype and be useful to identify markers of this subtype. Additionally, the HMEC system may shed new light on basal-like breast tumor signaling pathways that can be targeted therapeutically.

4.4.6. CHI3L1 future directions

The most immediate clinical utility of CHI3L1 in breast cancer may be as a serum marker for basal-like breast tumors. CHI3L1 is secreted by tumors and is detectable in human serum. A subset of breast cancer patients has high serum levels of CHI3L1. One report suggests that the high CHI3L1 subset is enriched for ER negative tumors when compared to the low CHI3L1 subset (75% vs 24%) (Johansen et al., 2003). This affirms the hypothesis that CHI3L1 can be used to detect basal-like breast cancers, which are ER negative. If these results are confirmed by others then CHI3L1 may prove useful as a serum marker for the basal-like subtype of breast cancer. Although CHI3L1 may identify the basal-like subtype it will not identify other subtypes of breast cancer. In order for CHI3L1 to have clinical utility it will need to be combined with other serum markers to detect the other breast cancer

subtypes. A few other serum markers have been studied in breast cancer, however they have been rejected clinically due to low sensitivity (Duffy, 2006). The recent understanding of breast cancer subtypes may allow these markers to be salvaged. While a single marker may have low sensitivity, a combination of markers that each detects a unique subtype (basal-like, luminal and HER2) can have clinical utility. We are initiating a study to investigate this possibility. CHI3L1 will be used to detect the basal-like subtype. HER2 may be used to detect the HER2 positive subtype (Duffy, 2006). Another marker (CEA or CA15.3) may be used to identify the luminal subtype. This approach opens the possibility of a serum based diagnostic for breast cancer detection.

CHI3L1 function in breast cancer needs to be investigated further. CHI3L1 is likely to influence the microenvironment or angiogenesis. While CHI3L1 does not have enzymatic activity to digest chitin it may still be targeted therapeutically. Monoclonal antibodies could be used to target CHI3L1. MedImmune is currently developing this type of therapy toward CHI3L1 in asthma. This type of therapy may prove useful in breast and other cancers. The receptor for CHI3L1 is also not known. It is important to identify the CHI3L1 receptor, which could be targeted a therapeutically.

4.5. Materials and Methods

4.5.1. Lentiviral cloning and expression

For cloning of shRNAs, synthesized oligos were designed, synthesized, annealed, and cloned into the pSicoR and pLKO.1 vectors according to the protocol on the website of the Dr. T. Jacks laboratory (http://web.mit.edu/jacks-lab/protocols/pSico.html) and as described in Appendix 1. The pSicoR vector was a gift from Dr. M. McManus. The pLKO.1 vector was purchased from Sigma (SHC001). shRNA sequences are as follows: p16 GACCGTAACTATTCGGTGCGTTGGGCAG, p53 GACTCCAGTGGTAATCTAC, pGL3 CTTACGCTGAGTACTTCGA, p14 GAACATGGTGCGCAGGTTC, CHI-1 GGACAGGTATATAAAGGAA, CHI-4 GGAGCCAAACATCCTACAA.

For CHI3L1 expression, CHI3L1 was purchased from Origene (SC125328) in the pCMV6 vector. CHI3L1 was removed from the pCMV6 vector with NotI and blunted with T4 polymerase. CHI3L1 was then cloned into the pWP1 lentiviral expression vector with PmeI. pWP1 was kindly provided by Dr. D. Trono.

Lentivirus constructs were packaged in 293T cells for viral propagation. Cells were infected with lentiviral supernatant diluted with media and containing polybrene (8ug/mL, Sigma) for 1-4 hours.

4.5.2. Expression arrays

Total RNA samples were isolated using Rneasy Mini Kit (Qiagen) as per manufacturer instructions. RNA was amplified by using the MessageAmp II-Biotin Kit (Ambion) as per manufacturer instructions. Amplified RNA was labeled by using the enzo BioArray High

Yield RNA transcript labeling kit (Affymetrix) as per manufacturer instructions. Microarray hybridization was performed at the J. David Gladstone Institutes Core Genomics Laboratory on the U133A Plus 2.0 arrays (Affymetrix). Expression array analysis was performed with RMA Express (http://rmaexpress.bmbolstad.com/) and BRB Arraytools (http://linus.nci.nih.gov/BRB-ArrayTools.html). Hierarchical clustering analysis was performed by using Cluster 3.0 (http://bonsai.ims.u-tokyo.ac.jp/~mdehoon/software/cluster/software.htm). Clustered data was visualized by using Java Treeview (http://jtreeview.sourceforge.net/).

Published expression array data (see Fig 15) was collected from the indicated references, supplemental data, or the NCBI GEO database (http://www.ncbi.nlm.nih.gov/geo/). Samples were arranged as described by the authors and heat maps were generated with Java Treeview.

4.5.3. Cells and cell culture

Primary HMEC were isolated from reduction mammoplasty samples as previously described and were cultured in modified MCDB 170 (MEGM, BioWhittaker, USA) (Romanov et al., 2001). Specimens from nine different individuals: RM9, RM13, RM15, RM16, RM18, RM21, RM23, RM48, and RM240 were used in our study. Routine cell culture and the isolation of vHMEC were essentially as described (Romanov et al., 2001). Population doublings were calculated using the equation, PD = log (A/B)/log2, where A is the number of cells collected and B is the number of cells plated initially. All experiments were performed at 2-5 times with samples from 2-4 individuals. HCC38 and HCC70 cell lines were purchased from ATCC and maintained in RPMI1640 with 5% FBS, 1.25g glucose, and 0.11mg/mL sodium pyruvate.

4.5.4. QPCR

Total RNA samples were isolated using Rneasy Mini Kit (Qiagen) as per manufacturer instructions. cDNA was synthesized from 2μg total RNA by using TaqMan Reverse Transcription Reagents (Applied Biosystems N808-0234) as per manufacturer instructions. Real-Time PCR was performed on 5ng input RNA per reaction containing 1x TaqMan Universal PCR Master Mix (Applied Biosystems 4304437) and the appropriate TaqMan probe (Applied Biosystems p16 Hs99999021_m1, cdc6 Hs00154374_m1, cdc25A Hs00948005_m1, RBL1/p107 Hs00161234_m1, CHI3L1 Hs00609691_m1) on the DNA Engine Opticon 2 (MJ Research Inc.). Relative mRNA levels were determined by using the relative standard curve method (normalized to GUSB) according to Applied Biosystems User Bulletin #2 (www.appliedbiosystems.com).

4.5.5. Western analysis

Cells were lysed with RIPA buffer or Curtis's strong lysis buffer (10mM Tris pH 8.0, 150mM NaCl, 1% Na Deoxycholate, 1% NP-40, 1% SDS, 1mM EDTA) containing 1x Complete protease inhibitors (Boehringer Mannheim). Protein concentration was determined by using the BCA assay (Pierce) with BSA as the standard (Sigma). Protein from total cell extracts was denatured with 1x loading buffer and then fractionated in gradient (4-20%) polyacrylamide gels (CAMBREX) and transferred to Hybond-P (Amersham) membrane. The following monoclonal antibodies were used to stain the blots: mouse anti-p16 (Ab-4 NeoMarkers), mouse anti-p53 (DO1), mouse anti-β-actin (AC-15 Sigma) and horse-radish

peroxidase (HRP)-conjugated goat anti-mouse antibody (Gibco). β-actin was used as a loading control for all blots. 5-20ug of protein was analyzed for each sample. Staining was developed using SuperSignal West Pico chemiluminescence detection protocol (Pierce).

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6. Appendix 1

Lentivirus Short-hairpin RNA Cloning Instructions

Cloning Steps:

Choose lentivirus cloning vector
- pSicoR-GFP
- pLKO.1-puro

Digest/CIP treat vector
Design shRNA oligos
Anneal oligos
Ligate oligos and vector
Grow and purify DNA
Test digest and Sequence insert
Make virus

Choose Lentivirus Cloning Vectors:

pSicoR-GFP
Vector used by Tyler Jacks lab (MIT) and Michael McManus lab (UCSF)
Digest with HpaI and XhoI

pLKO.1-puro
Vector used by The RNAi Consortium—Broad Institute, Harvard, MIT, etc.
shRNA library available from Openbiosystems or Sigma
Digest with AgeI and EcoRI

Digest/CIP Treat Vector:

For pSicoR	For pLKO.1
1 μg vector DNA	1 μg vector DNA
2 μL NEB buffer 4	2 μL NEB buffer 1
1 μL HpaI	1 μL AgeI
1 μL XhoI	1 μL EcoRI
H2O to 20 μL	H2O to 20 μL

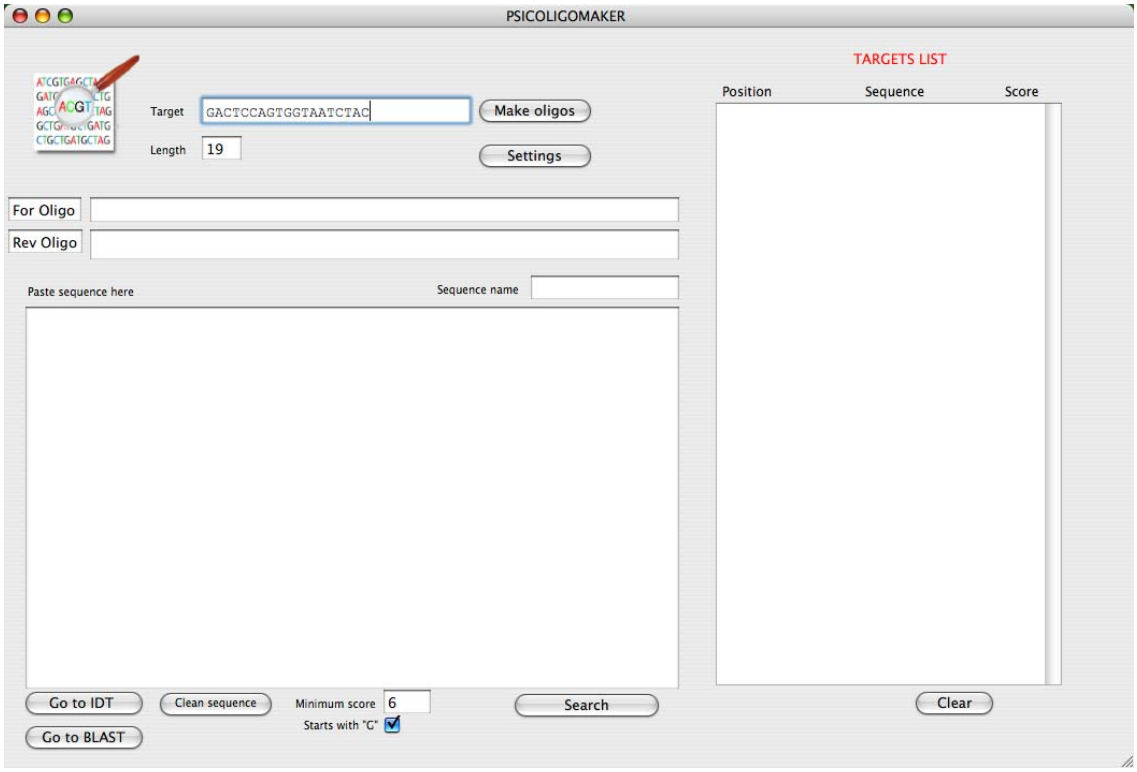
Incubate 37^0 1 hr
Add 0.5 mL CIP
Incubate 37^0 1 hr
Gel purify and elute in 50 uL

Design Short-hairpin Oligos:

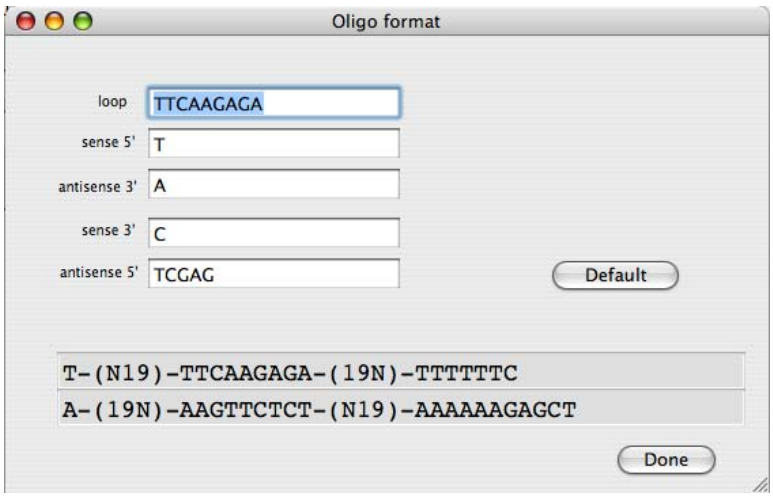
Find 19-mer sequence to target gene
- Tip—Use sequences that are verified in publications

Use the program psicoligomaker 1.5 from Tyler Jacks lab to design oligos
http://web.mit.edu/jacks-lab/protocols/pSico.html
Directions below:

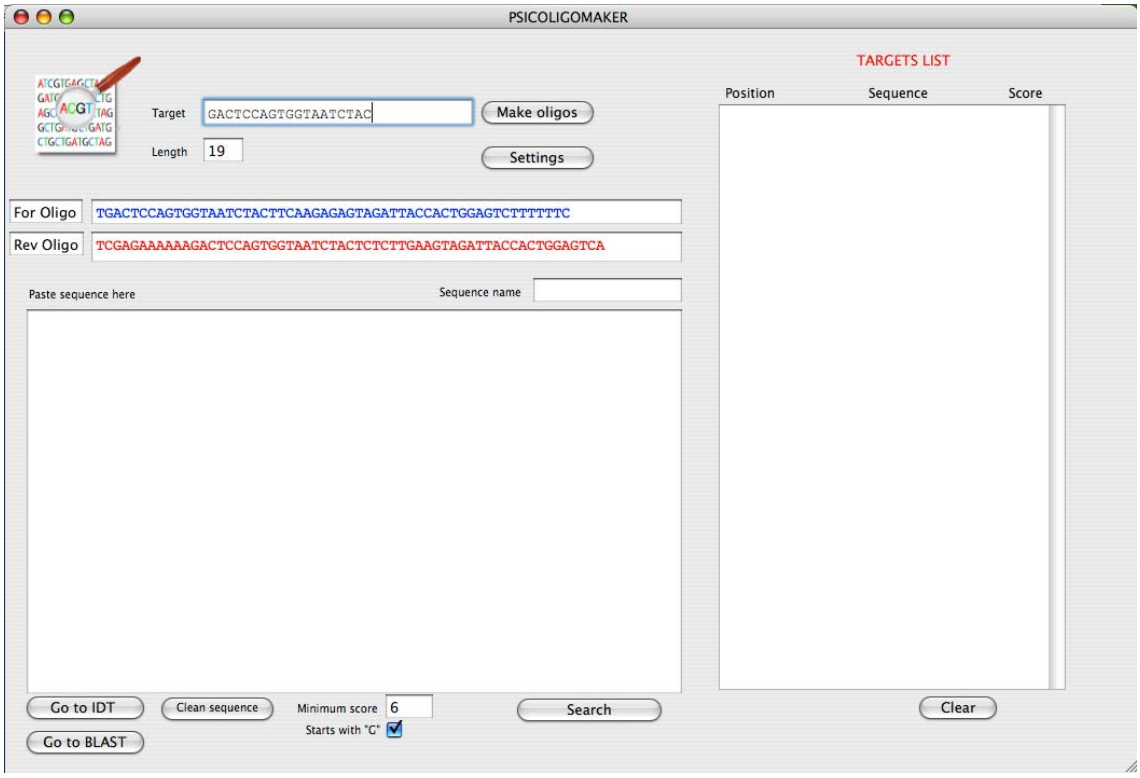
For pSicoR:
Type 19-mer gene-specific sequence in “Target”



Click “Settings”

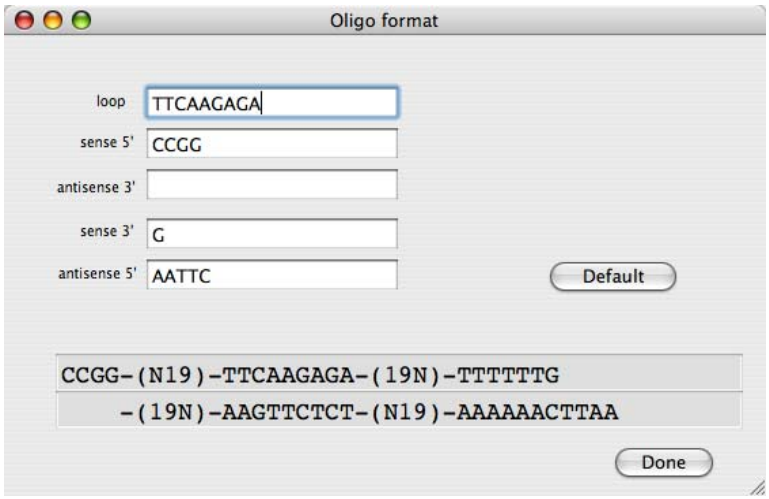


Use defaults
loop = TTCAAGAGA
sense 5’ = T
antisense 3’ = A
sense 3’ = C
antisense 5’ = TCGAG
Click “Done”
Click “Make oligos”



The “For Oligo” is your forward oligo
The “Rev Oligo” is your reverse oligo
Order the oligos, 50nmol, 5’phosphorylated

For pLKO.1:
Type 19-mer gene-specific sequence in “Target”
Click “Settings”



Change end sequences
loop = TTCAAGAGA
sense 5' = CCGG
antisense 3' =
sense 3' = G
antisense 5' = AATTC
Click "Done"
Click "Make oligos"
The "For Oligo" is your forward oligo
The "Rev Oligo" is your reverse oligo
Order the oligos, 50nmol, 5'phosphorylated

Anneal Oligos:

Resuspend oligos at 1nmol/μL

Annealing solution
1 μL of diluted forward oligo (1:10 dilution of stock)
1 μL of diluted reverse oligo (1:10 dilution of stock)
5 μL of 10x NEB buffer 2
43 μL ddH2O

Incubate 4 min at 95^0C
Incubate 10 min at 70^0C in 1L H2O and allow the water to cool to RT

Ligate Oligos and Vector:

Use Roche Rapid Ligation Kit

1 μL of annealed oligos (ctl no oligo and dilutions 1:10, 1:100, 1:1000)
2 μL of digested, CIP treated, gel purified vector
2 μL 5x DNA dilution buffer
10 μL 2x ligation buffer

1 μL ligase

Incubate at RT for 1-24 hrs

Grow and Purify DNA:

Transform DH5α with ligation mix

Thaw DH5α on ice
Add 2-5 uL of ligation mix to 20-50 uL DH5α
Incubate on ice 20 min
Heat shock at 42^0C for 30-60 sec
Incubate on ice 10 min
Add transformed bugs to 500 μL LB (no amp)
Shake at 37^0C for 30-60 min
Plate 200 μL on LB amp 100 plates
Incubate at 37^0C overnight

Pick colonies
Grow up in 3 mL of LB (100 μg/mL amp)
Mini-prep DNA

Test Digest and Sequence Insert:

Test digest

For pSicoR	For pLKO.1
7 μL mini-prep DNA	7 μL mini-prep DNA
1 μL NEB buffer 1	1 μL NEB buffer 1
	0.1 μL BSA
1 μL XhoI	1 μL AgeI
1 μL XbaI	1 μL SpeI

Incubate at 37^0C 1 hr
Test on 2% agarose gel

Expected result

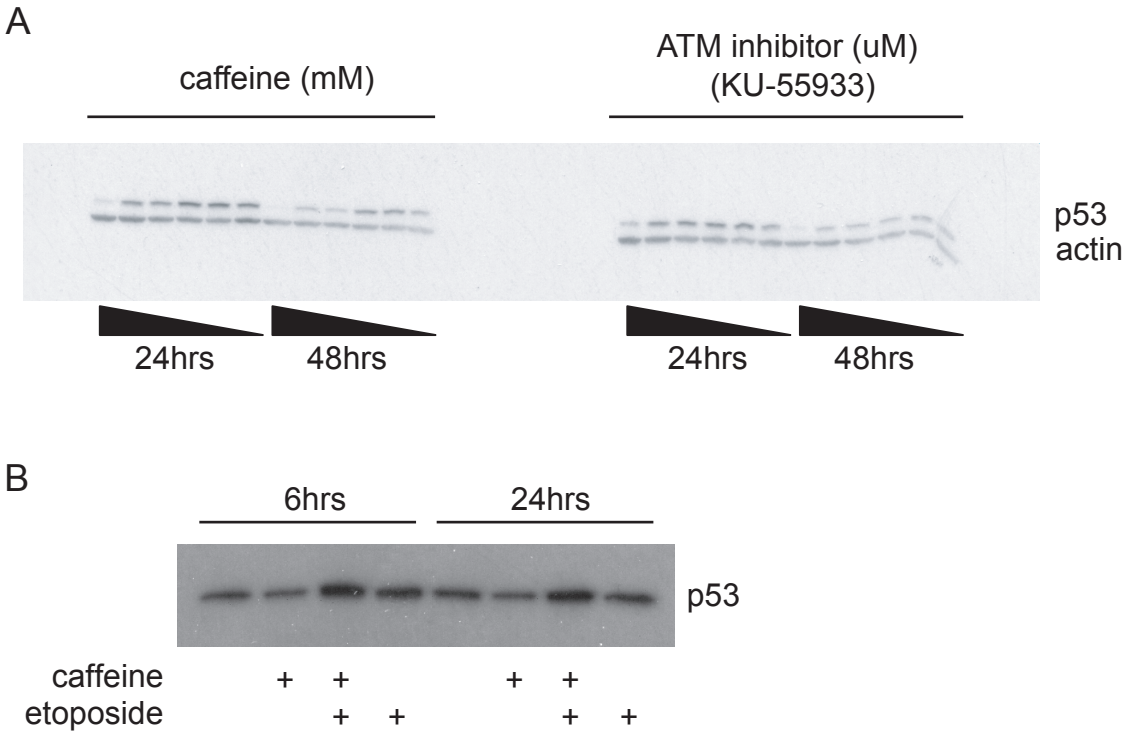
For pSicoR	For pLKO.1
350 bp to 400 bp shift if insert is correct	Loss of 750 bp band if insert is correct (note: oligos should destroy AgeI site, however if sense 5' oligo starts CCGGT AgeI site is not destroyed and there will be a 750 bp to 780 bp shift if the insert is correct)

Sequencing primers:

pSicoR 5' TGCAGGGGAAAGAATAGTAGAC

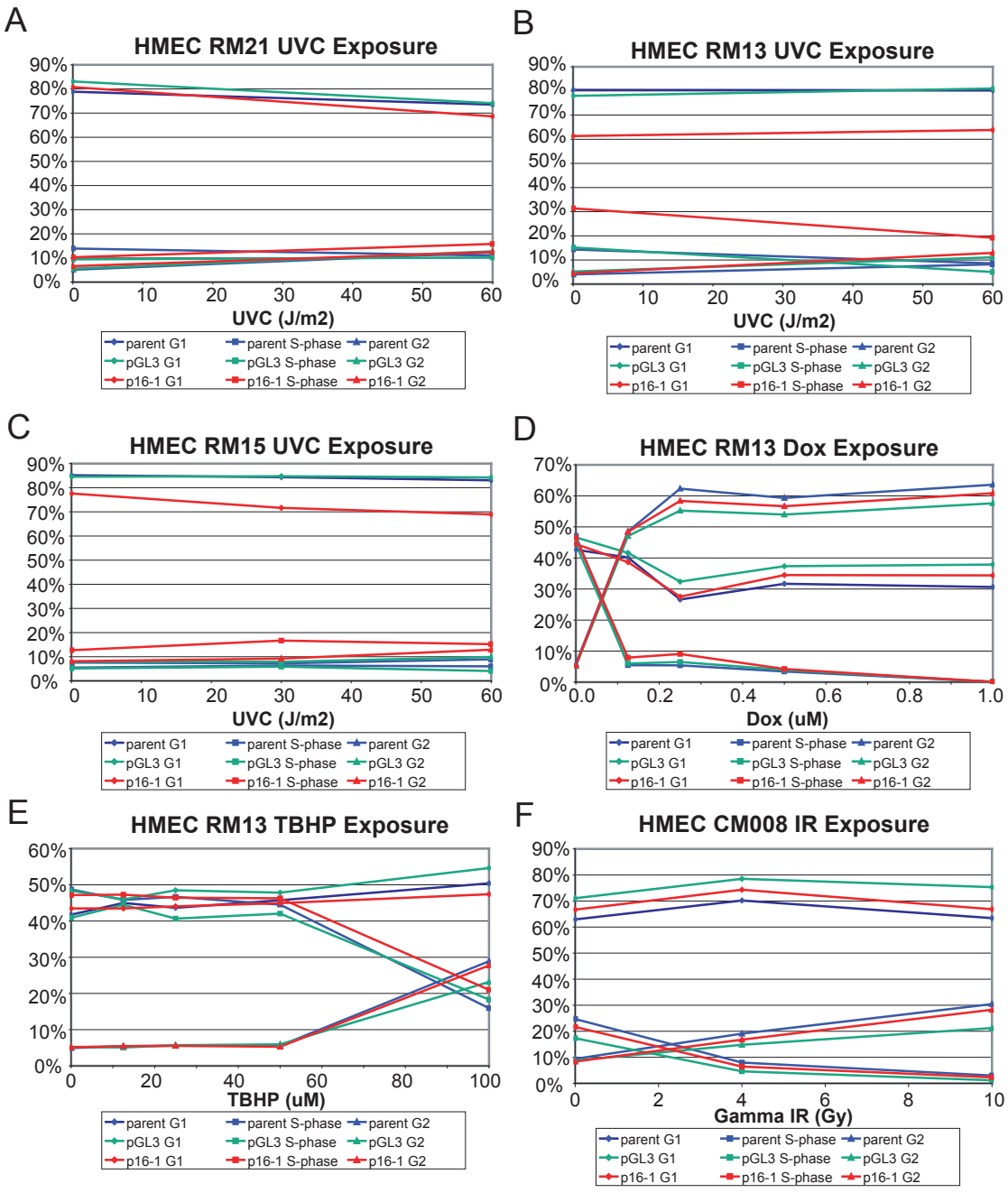
pLKO.1 5’ GACTATCATATGCTTACCGT

7. Appendix 2



Appendix 2: p16 modulates p53 through ATM.
Repeat experiments from Figure 6. A. RM21 vHMEC treated with caffeine or KU-55933 for the indicated times, concentrations decrease left to right (5, 1, 0.5, 0.1, 0.01, or 0 mM caffeine and 5, 1, 0.1, 0.01, or 0 uM KU-55933). Western blot for p53 and actin is shown. B. RM16 vHMEC treated with caffeine (2mM), etoposide (10uM), or both for the indicated time. Western blot for p53 is shown.

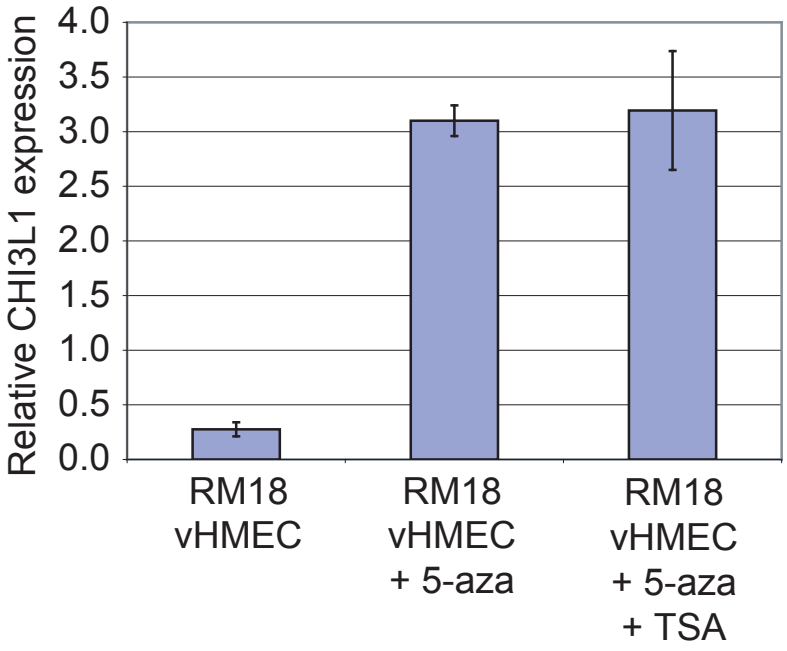
8. Appendix 3



Appendix 3: p16 shRNA does not modulate cell cycle checkpoints.
Repeat experiments from Figure 9. Cell cycle profiles of HMEC infected with the indicated virus and exposed to the indicated DNA damaging agent. Cell cycle information was collected by flow cytometry for BrdU incorporation and DNA content at 24 hrs post-treatment. A. RM21 treated with UVC. B. RM13 treated with UVC. C. RM16 treated with UVC. D. RM13 treated with Dox. E. RM13 treated with TBHP. F. CM008 treated with gamma IR.

9. Appendix 4

A



Appendix 4: CHI3L1 expression is increased by treatment with 5-aza-2-deoxycytidine. Expression of CHI3L1 as measured by QPCR in RM18 vHMEC treated with 5-aza-2-deoxycytidine (5-aza) and tricostatin A (TSA).

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