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The Role of p16 in Controlling Mammary Epithelial Cell Senescence and Brca1-Deficient Tumorigenesis

Alexandria Scott  
*University of Miami, alexandriaccscott@gmail.com*

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UNIVERSITY OF MIAMI

THE ROLE OF P16INK4A IN CONTROLLING MAMMARY EPITHELIAL CELL
SENESCENCE AND BRCA1-DEFICIENT TUMORIGENESIS

By
Alexandria Scott

A DISSERTATION

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Doctor of Philosophy

Coral Gables, Florida
August 2017
UNIVERSITY OF MIAMI

A dissertation submitted in partial fulfillment of
the requirements for the degree of
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THE ROLE OF P16 IN CONTROLLING MAMMARY
EPITHELIAL CELL SENESCENCE AND BRCA1-
DEFICIENT TUMORIGENSIS

Alexandria Scott

Approved:

Xin-Hai Pei, M.D., Ph.D.
Associate Professor of Surgery

Enrique Mesri, Ph.D.
Professor of Immunology

Wassif Khan, Ph.D.
Professor of Immunology

Xiang-Xi Mike Xu, Ph.D.
Professor of Cell Biology

Merce Jorda, M.D., Ph.D.
Professor of Clinical Pathology

Guillermo Prado, Ph.D.
Dean of the Graduate School
A major risk factor of breast cancer is aging. Aging is caused in part by cellular senescence, an important tumor suppressive mechanism that prevents the proliferation of cells at risk of neoplastic transformation. p16INK4A (p16), an inhibitor of CDK4 and CDK6, plays a critical role in controlling cellular senescence in multiple organs. Functional inactivation of p16 by gene mutation and promoter methylation is frequently detected in human breast cancers. However, deleting p16 in mice or targeting DNA methylation within the murine p16 promoter does not result in mammary tumorigenesis. How loss of p16 contributes to mammary cell aging and tumorigenesis in vivo is not fully understood. We reported that senescence was induced in mammary epithelial cells during aging along with increased expression of p16. Loss of p16 abrogated the age-induced senescence in mammary epithelial cells and significantly increased mammary stem cell function. We showed that loss of Brca1, a tumor suppressor, in the mammary epithelium induced senescence with the induction of p16 and a decline of stem cell function, which was rescued by p16 loss. These data identify the role of p16 in suppressing Brca1-deficient function of mammary stem cells. Furthermore, we
found that p16 loss transformed Brca1-deficient mammary epithelial cells and induced mammary tumors, though p16 loss alone was not sufficient to induce mammary tumorigenesis. We demonstrated that loss of both p16 and Brca1 led to metastatic, basal-like, mammary tumors with the induction of EMT and an enrichment of tumor initiating cells. We discovered that promoter methylation silenced p16 expression in most of the tumors developed in mice heterozygous for p16 and lacking Brca1. These data not only identified the function of p16 in suppressing BRCA1-deficient mammary tumorigenesis, but also revealed a collaborative effect of genetic mutation of p16 and epigenetic silencing of its transcription in promoting tumorigenesis. To the best of our knowledge, this is the first genetic evidence directly showing that p16 which is frequently deleted and inactivated in human breast cancers, collaborates with Brca1 controlling mammary tumorigenesis.
DEDICATION

To my grandmothers for their strength, wisdom, love and influence:

W.J.N.
+ 
H.U.T.
+ 
A.R.L.
+ 
V.K.S.
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<tbody>
<tr>
<td>BLBCs</td>
<td>Basal-like breast cancers</td>
</tr>
<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
</tr>
<tr>
<td>BEC</td>
<td>Basal epithelial cell enriched population</td>
</tr>
<tr>
<td>Cdh1</td>
<td>E-cadherin</td>
</tr>
<tr>
<td>Ck14</td>
<td>Cytokeratin 14</td>
</tr>
<tr>
<td>Ck5</td>
<td>Cytokeratin 5</td>
</tr>
<tr>
<td>DAC</td>
<td>aza-2'-deoxycytidine</td>
</tr>
<tr>
<td>EMT</td>
<td>Epithelial to mesenchymal transition</td>
</tr>
<tr>
<td>ER</td>
<td>Estrogen receptor</td>
</tr>
<tr>
<td>FFPE</td>
<td>Formalin fixed paraffin-embedded</td>
</tr>
<tr>
<td>FACS</td>
<td>Flourescence associated cell sorting</td>
</tr>
<tr>
<td>G1</td>
<td>Gap phase 1</td>
</tr>
<tr>
<td>G2</td>
<td>Gap phase 2</td>
</tr>
<tr>
<td>HE</td>
<td>Haematoxylin and eosin staining</td>
</tr>
<tr>
<td>HMECs</td>
<td>Human mammary epithelial cells</td>
</tr>
<tr>
<td>KO</td>
<td>Knock-out</td>
</tr>
<tr>
<td>LEC</td>
<td>Luminal epithelial cell enriched population</td>
</tr>
<tr>
<td>LOH</td>
<td>Loss of heterozygosity</td>
</tr>
<tr>
<td>M</td>
<td>Methylated</td>
</tr>
<tr>
<td>MECs</td>
<td>Mammary epithelial cells</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>MFP</td>
<td>Mammary fat pad</td>
</tr>
<tr>
<td>MGKO</td>
<td>Mammary gland knock-out</td>
</tr>
<tr>
<td>mo</td>
<td>Month</td>
</tr>
<tr>
<td>MS-PCR</td>
<td>methylation-specific PCR</td>
</tr>
<tr>
<td>mt</td>
<td>mutant</td>
</tr>
<tr>
<td>NSG</td>
<td>NOD scid gamma</td>
</tr>
<tr>
<td>p16</td>
<td>p16Ink4a</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI</td>
<td>Propidium iodide</td>
</tr>
<tr>
<td>pRb-Ser608</td>
<td>Rb proteins phosphorylated at Ser608</td>
</tr>
<tr>
<td>q-RT-PCR</td>
<td>Quantitative real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SA-b-gal</td>
<td>Senescence associated B-galactosidase</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation of the mean</td>
</tr>
<tr>
<td>S phase</td>
<td>Synthesis phase</td>
</tr>
<tr>
<td>TF</td>
<td>Tumor-free</td>
</tr>
<tr>
<td>TICs</td>
<td>Tumor initiating cells</td>
</tr>
<tr>
<td>U</td>
<td>Unmethylated</td>
</tr>
<tr>
<td>Vim</td>
<td>Vimentin</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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CHAPTER I
INTRODUCTION

Breast cancer is a heterogeneous disease that is associated with aging

Despite the advancements made at the clinical and laboratory levels, breast cancer remains the most common cancer affecting women in the US, and is the second leading cause of cancer-related deaths among women (BreastCancer.org 2017). Breast cancer risk increases with female gender and inherited genetic mutations[1]. However, the single most important risk factor of breast cancer, and most other cancers, is aging[2]. Currently, the causative cellular and molecular drivers that link the mechanisms of aging with predisposition to tumorigenesis are not fully understood. According to the US census bureau, the elderly population will increase to an estimated 40 million people by the year 2040, which will subsequently increase cancer incidence. Therefore, elucidating the mechanisms that regulate the aging of mammary cells could provide a unique window to explore their paralleling tumorigenicity.

Breast cancer is a broad term used to describe a subset of extremely diverse diseases that arise when tissues in the breast become malignant and invade surrounding tissue. The overall aggressiveness and treatability of the disease is largely dependent on the tumor cell type, which can be highly heterogeneous[3]. Though there have been advancements in breast cancer treatments, certain breast tumor types remain a great therapeutic challenge. The use of adequate in vivo models could aid immensely in understanding how cells are more susceptible to malignant transformation due to aging processes.
Aging is mainly caused by cellular senescence, a barrier to cell transformation and tumorigenesis

Aging is a multifaceted process resulting from the diminished capacity of organisms to maintain homeostasis through time[4]. During organismal aging in both human and mice, the mammary gland endures profound and dynamic restructuring by undergoing age-related lobular involution, a process resulting in the overall structural and functional decline of the mammary epithelium [5, 6]. This age-related decline in mammary function is associated with a reduced risk in breast cancer, and oppositely women with delayed involution have increased breast cancer risk [6, 7]. A recent study examining the age-related changes in the mammary glands of marmosets revealed that tissues from old animals had less abundant ductal and lobular epithelial structures, more loose fibrous tissue and more fat when compared to mammary glands from young animals[8]. Hence, the relationship between aging and cancer is somewhat paradoxical; while aging causes an overall decline in organismal function, there is also an increase in cancer risk. Currently, the factors linking mammary aging with tumorigenesis are not fully understood.

Aging is caused partly by cellular senescence, an important tumor suppressive mechanism that prevents the proliferation of cells at risk of neoplastic transformation[9]. Senescence was first identified by Hayflick and Moorhead after observing an irreversible growth arrest of cultured human cells[10]. This sustained growth arrest is accompanied by other characteristics such as cellular enlargement, flattened and elongated cytoplasms, elevated levels of senescence-
associated beta-galactosidase and upregulation of senescence-associated genes [11, 12]. Cellular senescence can be induced in several tissues and cell types by a variety of stimuli including, oxidative stress, DNA damage, cellular replication and telomere shortening, and activation of oncogenes or loss of tumor suppressors [12].

![Figure 1. The multiple stressors and regulators of senescence (Espín & Serrano, Nature Reviews Molecular Cell Biology, 2014 [12])](image)

Though generally understood as halted cell growth, the process of cellular senescence is extremely complex. The role that senescence plays in development and tumorigenesis is both tissue/cell-specific and context-dependent, and relies heavily on the mechanisms of induction. There are several effector pathways of senescence resulting from the diverse cellular stressors (Fig. 1). Invariably, most cellular effectors of senescence activate two main pathways, the p53 and RB pathways[12, 13]. Consequently, these pathways are among the most commonly mutated in human cancers.
The p16/RB pathway plays a key role in controlling senescence and tumorigenesis

Control of the G1 phase of the cell cycle is intrinsically linked with the maintenance of quiescent and senescent states in stem and somatic cells, respectively, and is primarily controlled by the INK4-CDK4/6-RB pathway (Fig. 2). The INK4 family of cell cycle inhibitors comprising of p16\(^{INK4A}\), p15\(^{INK4B}\), p18\(^{INK4C}\) and p19\(^{INK4D}\) (p16, p15, p18 and p19) inhibit CDK4 and CDK6, leading to the functional inactivation of RB [14-16].

![Figure 2. The p16/RB pathway](Piepkorn, Journal of the American Academy of Dermatology, 2000 [16])

In the absence of p16, Cyclin D binds to CDK4/6 and initiates a series of phosphorylation events, directly phosphorylating RB thus inducing a conformational change, rendering RB inactive. The now, inactive, hyperphosphorylated, RB releases its grip on E2F which goes on to transcribe other genes necessary for the next phase of the cell cycle. However, when p16 inhibits Cyclin D from binding CDK4, this phosphorylation event does not occur, maintaining RB in its hypophosphorylated, inactive state.

Unlike other INK4 family proteins, p16 is not expressed during early development, but is markedly increased with aging and senescence [17, 18]. Early studies have provided an extensive body of in vitro work suggesting that p16 has
a cell autonomous role in senescence in human T-lymphocytes, prostate epithelial cells and mammary epithelial cells[19-21]. Furthermore, experiments in mice with a germline deletion of p16 reveal that p16 controls the aging of stem/progenitor cells in compartments of the brain, pancreatic islets and blood by inducing senescence [22-24]. The physiological role of p16 in controlling mammary cell senescence remains to be determined.

Functional inactivation of the p16/RB pathway is a common event in the development of most types of cancers[25], and also causes the loss of quiescent and senescent states of cells[26]. Importantly, p16 is deleted in ~50% of breast cancer cell lines, and p16 inactivation by DNA methylation occurs in ~30% of human breast cancers [25, 27, 28]. Notably, overexpression of p16 has also been identified in some cases of breast cancer, and is often correlated with invasive, aggressive disease[29, 30]. p16 inhibits CDK4/6, inactivating RB, whereas, RB loss, in turn, stimulates p16 expression[14, 31]. Furthermore, loss of function of RB by LOH is detected in 20-40% breast cancers, ER-negative breast cancer in particular, in which p16 expression is enhanced[32]. In a study examining the expression of p16 and RB in human estrogen receptor-negative breast cancer cell lines, not only were p16 and RB determined to be inversely correlated but loss of p16 was associated with increased stem cell characteristics and resistance to chemotherapy [33]. Therefore, overexpression of p16 in breast cancers is very likely resulted from the loss of function of RB and often viewed as the last attempt by the tumor cell to halt progression as a result of the p16-RB pathway feedback loop.
In this study, I was determined to examine how loss of p16 leads to mammary tumorigenesis in vivo which is not fully understood because deleting p16 in mice or targeting DNA methylation within the murine p16 promoter does not result in mammary tumorigenesis but rather, these mice develop lymphomas and sarcomas with long latency [34-36]. How/if loss of p16 collaborates with other tumor inducing mechanisms to contribute to mammary tumorigenesis in vivo is not fully understood.

Loss of BRCA1 is associated with invasive breast cancers but induces senescence in mice

90% of breast cancers are sporadic and develop with no known genetic predisposition [37]. Of the 10% of breast cancers remaining, half are caused by heterozygous germline deletion of BRCA1 [38-40]. Women harboring a BRCA1 mutation have an 80% chance of developing breast cancer in their lifetime [41]. Brca1 is involved in a multitude of cellular processes and pathways, and functions in maintaining genomic instability by promoting efficient repair of DNA double-strand breaks which has been viewed as its most important property in tumor suppression [42].
BRCA1 deficiency in human and mouse mammary epithelial cells activates both the p16-RB and p53 pathways, inducing premature senescence [43-45]. Consistently, complete allelic deletion of Brca1 in mice leads to premature aging and embryonic lethality, and heterozygous germline deletion of Brca1 or specific deletion of Brca1 in mouse mammary epithelial cells leads to blunted ductal morphogenesis [46]. Mice harboring Brca1 deficiency in mammary epithelial cells...
rarely develop mammary tumors as about 10% of Brca1+/− or Brca1ff;MMTV-cre mice develop mammary tumors by 18 months of age [43, 44, 47-49]. Loss of p53 or its downstream target, p21, in vivo as well as knockdown of p16 or its downstream target, Rb, in vitro partially rescues the premature senescence of Brca1-deficient cells [43-45, 47, 48, 50], suggesting that disruption of the p53 and p16-Rb pathways are required to overcome Brca1-deficiency induced senescence and induce breast cancers. Indeed, inactivation of the p53 pathway enhances mammary tumor incidence and shortens the time of tumor onset in Brca1-deficient mice [47, 48, 51, 52]. It remains elusive whether inactivation of the p16-Rb pathway is required for Brca1-deficient tumorigenesis.

**Mammary epithelia originate from a common mammary stem cell**

During early development, the mammary gland emerges in the embryonic placode and gradually enters the underlying mesenchyme eventually forming a highly dynamic, bi-layered, branching structure [53]. Each branch is composed of epithelial cell layers derived from two main lineages: luminal and basal/myoepithelial. Luminal cells constitute the inner lumen while basal cells surround the lumen and constitute the outer layer that is adjacent to the basement membrane. Histologically, luminal and basal cells can be identified by their distinct morphology and respective cytokeratin markers. Luminal cells are cuboidal in shape and typically express cytokeratins 8 and 7 while basal cells are thin and elongated and typically express cytokeratins 5 and 14(Fig. 4, [54]). The structure of luminal and basal cells is directly related to their function. The luminal lineage is
comprised of ductal and alveolar cells which produce and secrete milk respectively. Basal cells are contractile in function and help to further facilitate the secretion of milk.

Organized within the mammary epithelial tree is a hierarchy of highly heterogeneous cell types including stem, progenitor and terminally differentiated cells. The first evidence of the regenerative capacity of mammary cells was appreciated when Daniel et al. 1987 demonstrated that any portion of the mammary gland is capable of regenerating a fully functional gland when transplanted into a host mouse with a cleared mammary fat pad [55]. Subsequent studies revealed that a single multipotent mammary cell is capable of forming an entire fully functioning mammary gland [56]. Though there has been a great deal of effort to purify putative mammary stem cells to resident homology, the precise temporal and spatial location of such cells depends heavily on the animal model used and remains controversial to date. Linage tracing studies coupled with fluorescence-activated cells sorting (FACs) have identified sub- populations of

Figure 4. Luminal and basal mammary epithelial cells
The mammary gland consists of two main epithelial cell types, luminal and basal. Luminal cells constitute the inner layer of the ducts and consistently express Ck8 (red). Basal cells surround the luminal cells and consistently express Ck5 (green).
mammary cells enriched with stem/progenitor-like properties [57]. There is currently no single model that identifies the mammary stem cell hierarchy. There is however a simplified, general, idea of cells within the mammary cell hierarchy [58]. Multipotent MaSCs give rise to more differentiated progenitor cells of the luminal and basal lineage which give rise to downstream differentiated luminal cells of the ductal and alveolar compartments, and basal cells (Fig. 5 [58]). Understanding the great heterogeneity and complexity of normal mammary cells, provides insight into the diverse cellular properties of breast cancer.

![Figure 5. Mammary epithelial cell hierarchy. (Visvader and Clevers, Nature Cell Biology 2016 [58])](image)

**Breast cancer is a heterogeneous disease**

Breast cancer is highly heterogeneous with tumors that are both phenotypically and pathologically distinct with regards to their aggressiveness and responsiveness to treatment. Breast cancer is comprised of three major subtypes: HER2-positive, luminal, and basal-like cancers (BLBCs) [59]. Luminal breast cancers are ER-positive and tend to have a good prognosis and respond well to hormone therapy. BLBCs are ER-negative and more metastatic with few
BLBCs are highly heterogeneous and are generally classified by expression of bona fide normal basal/myoepithelial cell markers such as cytokeratins 5, 14, and 6, smooth-muscle. Of the subtypes of breast cancer, BLBCs are the most distinct with a greater degree of genomic complexity owning to their vast heterogeneity. Functional loss of BRCA1 by germline or somatic mutation or by promoter methylation is associated with more than one-third of basal-like breast cancers and cell lines. Notably, most human BLBCs with functional loss of BRCA1 have dysfunctional p16-RB and p53 pathways. However, most genetic studies of Brca1 in mice co-mutate Brca1 with one of the genes in the p53 pathway. It remains poorly understood whether p16 is involved in Brca1-deficient MEC senescence and tumorigenesis.

Statement of purpose

I was determined to elucidate the role of p16 in mammary epithelial cell aging and tumorigenesis by answering the following questions: Does p16 regulate the age-induced senescence of mammary epithelial cells and if so, would loss of p16 abrogate the aging phenotype? Similarly, does p16 regulate the premature senescence caused by Brca1-deficiency, and does loss of p16 mitigate Brca1-deficiency induced senescence leading to mammary tumorigenesis? How loss of p16 and Brca1 induces mammary tumorigenesis is not understood and is the focus of this study.
I hypothesized that during aging or Brca1 deficiency, expression of p16 is increased leading to the senescence of mammary cells. Functional loss of p16 abrogates senescence and leads to the malignant transformation of Brca1-deficient MECs.

Figure 6. Schematic outline of hypothesis. I hypothesized that during aging or Brca1 deficiency, expression of p16 is increased leading to the senescence of mammary cells. Functional loss of p16 abrogates senescence and leads to the malignant transformation of Brca1-deficient MECs.
CHAPTER II

MATERIALS AND METHODS

Mice

The generation of $p16^{-/-}$,$Brca1^{f/f}$;MMTV-Cre and $Brca1^{f/f}$;MMTV-Cre has been described previously [35, 44]. $Brca1^{f/f}$ and $Brca1^{+/-}$ mice were obtained from the NCI Mouse Repository. Tg(MMTV-Cre)4Mam and $p16$ mutant mice were obtained from the JAX lab, and gifted by our collaborators, Dr. Norman Sharpless respectively [34].

The generation of the $p16^{-/-}$,$Brca1^{f/f}$;MMTV-Cre mouse was previously described[72, 73]. Briefly, to generate the $p16^{-/-}$,$Brca1^{f/f}$;MMTV-Cre mouse, $p16^{-/-}$ and MMTV-Cre mice were crossed to yield $p16^{+/-}$;MMTV-Cre mice, which were then crossed to yield $p16^{-/-}$;MMTV-Cre mice (Fig.7 A, B and C). Separately, $Brca1^{+/-}$ and $Brca1^{f/f}$ were crossed to yield $Brca1^{f/f}$ mice (Fig.7D and E). $p16^{-/-}$
MMTV-Cre mice and Brca1f/- mice were crossed to yield the genotypes necessary to generate all appropriate controls (generation not shown), and ultimately the p16-/-;Brca1f/-;MMTV-Cre genotype mice (Fig. 7F). Virgin Brca1f/f;MMTV-Cre and Brca1f/-;MMTV-Cre (Brca1MGKO) mammarys express <20% of Brca1 protein and mRNA relative to the levels in Brca1f/++;MMTV-Cre, indicating depletion of Brca1 in the mammary epithelia[44]. Virgin female mice were used in the study unless otherwise specified. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of North Carolina and University of Miami.

**Histopathology, Immunohistochemistry, Immunofluorescence & Western Blotting**

Histopathology, Immunohistochemistry (IHC), and Immunofluorescence (IF) were performed as previously described [43, 44]. Briefly, freshly isolated mammary tissues were fixed in 10% Neutral Buffered Formalin for 24-48 hours and then embedded in paraffin wax into tissue-blocks. Blocks were then cut into 5μm sections, placed in a 45°C water bath, and transferred to histology slides to dry overnight. Histology slides were then deparaffinized/rehydrated and washed with ddH₂O and PBS for IHC or PBS/0.2% Triton-X-100 (PBS-T). Sections were then boiled in 10mM of sodium citrate buffer (pH 6.0) for 30 minutes for antigen unmasking. Sections were then washed with ddH₂O and PBS/PBS-T and blocked for 1 hour at RT. The blocking solution was removed and the diluted primary antibody was added to sections and incubated at 4°C overnight in a humidified chamber. After incubation with the primary antibody, slides were washed in
PBS/PBS-T and the diluted secondary antibody was applied and incubated for 30 minutes at RT. For IHC, after the secondary antibody was removed and washed with PBS, an ABC reagent was applied using the Elite Vectastain ABC HRP Kit (Vector Laboratories), and incubated for 30 minutes. The ABC reagent is removed and slides are washed in PBS. A freshly made 1X DAB solution (ThermoScientific) is added to slides for 30-60 seconds, and monitored closely under the microscope for development of color from antibody, and then placed in ddH₂O. Slides were then counterstained with Hematoxylin. For IF, after secondary antibody was removed, slides were incubated with DAPI. Slides were then dehydrated (for IHC) and coverslipped. Primary antibodies used are as follows: Ki67 (Novocastra Laboratories, Newcastle upon Tyne, UK), γH2AX, phosphor-RB (Cell Signaling), CK14 (Thermo Scientific), ER, p16, p53 (Santa Cruz Biotechnology), Vim, Twist (Abcam), Cdh1 (BD Biosciences), CK5, (Covance prb-160p). Mammary tumors in which at least two EMT markers (decreased Cdh1, increased Vim or Twist) were detected in >2% tumor cells are defined as EMT+ tumors, as we previously described[44].

For western blot analysis, tissue was lysed in RIPA supplemented with 1mM of dithiothreitol, 1X protease inhibitor, 1mM of phenylmethane sulfonyl fluoride, 5mM of NaF and 1mM of Na₂VO₄. 50μg of protein for each sample were run on a 10% SDS-page gel and transferred to a .25μm nitrocellulose membrane. Membranes were blocked in 5% BSA for 1 hour at RT, and then incubated overnight at 4°C with antibodies against p53 (Santa Cruz Biotechnologies), and GAPDH (Thermo Fisher Scientific). Dilutions were used as suggested by the
manufacturer. The IACUC (Institutional Animal Care and Use Committee) at the University of Miami approved all procedures.

**RNA Isolation and Quantitative PCR**

Quantitative PCR (Q-RT-PCR) was carried out as previously described [43, 44]. Briefly, total RNA was extracted by an RNA easy kit per the manufacture’s protocol (Qiagen, Valencia, CA, USA). cDNA was synthesized with random hexamers provided by the Quantitect Transcription Kit (Qiagen). The cDNA was added to a Q-RT-PCR mixture that contained 1X sybr green PCR master mix (Applied Biosystems) and 1μM of gene-specific forward and reverse primers, respectfully. Assays were performed on the C1000 Thermal Cycler CFX96 Real Time System (Bio Rad). The expression level of each gene was normalized with a house keeping gene, GAPDH.

**Table 1. List of primer sets for Q-RT-PCR**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
</table>
| *Pai-1* (Mouse) | F: TCAGAGCAACAA-GTTCACAAGTACAC TGAG  
|         | R: CCCACTGTCAAGGCTCCATCACTT-GCCCCCA     |
| *Nrg1* (Mouse)   | F: CATGGTGCAACATAGCGAATGGCC             
|         | R: CCACAATATGCTCAGTGGAGATG              |
| *Igfbp2* (Mouse) | F: GGGTGCCAAAACACCTCAG                  
|         | R: AGGTTGTACGGCCATGC                    |
| *Mmp13* (Mouse)  | F: GGTCCCAAACGAACCTCACTACA             
|         | R: CCTTGAACGTCACTCACTAGGAGAAGC         |
| *Gata3* (Mouse)  | F: GCTGACGGAAGAGGTGGACGTACT            
|         | R: TGGTGAGTGGGAGGTACCT                  |
| *Elf5* (Mouse)   | F: GGACTCCGTAACCCATAGCA                
|         | R: TACTCGTCGAGCAGAATTG                 |
**p16INK4A** (Mouse)

<table>
<thead>
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<th>F: CCGA CCTTTGTCGTAC</th>
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<tr>
<td>R: GAGAAGTAGTGGG GTCCTC</td>
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**Mammary cell dissociation, cell culture, colony formation and mammosphere assays**

After mechanical dissociation, the tissue was processed as previously described [43, 44]. Briefly, primary mammary epithelial cells were generated from either age-matched controls of WT, p16−/−;Brca1f/−;MMTV-Cre, p16−/−, and Brca1f/−;MMTV-Cre mice. For 2D culture, all cells were maintained in DMEM/F12 media supplemented with 4μg/mL Insulin, 10ng/mL epidermal growth factor, 10 ng/mL fibroblast growth factor, 4mg/mL hydrocortisone, 1X bovine pituitary extract, 1X Glutamax, 1X pen/strep and 10% fetal bovine serum. For purification of mammary epithelial cells, freshly isolated mammary glands were dissected after the removal of lymph nodes and placed into a 100mm petri dish (p100) containing a digestion solution consisting of DMEM/F12 supplemented with 10x hyaluronidase/collagenase, 5% fetal bovine serum and 1x pen/strep. The p100 dish containing digestion solution and mammary gland tissue was placed into a cell culture hood and minced into fine pieces with micro-surgery scalpels. The minced tissue and digestion solution was then placed into a 15mL conical tube and rotated at 37°C for 1.5 hours. The solution was then pelleted at 450g for 5 minutes and the supernatant was discarded. The pellet was re-suspended in .25% trypsin to ensure complete dissociation. To deactivate the trypsin, the mammary cell suspension was placed in 15mL of HBSS supplemented with 2% fetal bovine serum (HF). The solution was then re-suspended in NH₄HCl and pelleted at 450g for 7 minutes, and the
supernatant was discarded. The cell pellet was re-suspended with DNAse/dispase after which 10mL of HF is added. Cells were then pelleted at 450g for 5 minutes and re-suspended with DMEM/F12 supplemented with pen/strep.

For the mammosphere assay freshly isolated primary mammary epithelial cells (20,000 cell per well) were cultured for two weeks in ultra-low attachment 24 well-plates with DMEM/F12 supplemented with 1X B27, 20ng/mL epidermal growth factor and 20ng/mL fibroblast growth factor. Mammosphere number and size were counted under a Lecia Microscope.

For the colony formation assay, freshly isolated primary mammary epithelial cells (20,000 cells per well) were cultured onto 24 well-plates coated with matrigel in DMEM/F12 supplemented with 10ng/mL epidermal growth factor, 10ng/mL fibroblast growth factor, 4ug/mL insulin, 0.5ug/mL hydrocortisone, 1x pen/strep and 10% fetal bovine serum. After 24 hours, the media was replaced with DMEM/F12 supplemented with all previous components excluding fetal bovine serum. After two weeks, the number and size of colonies was counted under a Lecia microscope.

**Mammary Transplantation and mammary tumor cell transplantation assays**

For mammary transplantation assays, 2 x 10⁶ mammary cells isolated from mice of indicated genotypes were injected into the mammary fat pads (MFPs) of precleared 3-week old NSG mice. After eight weeks, the resultant outgrowths were dissected and stained with carmine alum for wholemount analysis. The percentage of total ductal outgrowth into the MFP was quantified as described[74]. For mammary tumor cell transplantation, 1 x 10⁶ tumor cells were transplanted into
MFPs of NSG mice as previously described [44]. Four weeks post-transplantation, newly generated tumors were dissected and analyzed.

**Flow Cytometry**

For fluorescence associated cell sorting (FACs) analysis, freshly isolated mammary epithelial cells were blocked with CD16/32 Fc (gamma) III/II receptor antibody (BD Pharmingen) and then stained with anti-CD29-FITC (Biolegend, San Diego, CA) anti-CD24-PE (BD Pharmingen), biotinylated and APC-conjugated CD45, CD31 and TER119 (BD Phamingen) antibodies, and Violet dye (Invitrogen). For multicolor staining of cell surface antigens (CD45, CD31 and TER119), secondary antibody SAV APC was added (BD Pharningen). Cells were re-suspended in HF and counted. Cell analysis and sorting was performed using the BD FACs Aria-II, and BD LS-Fortessa HTS, respectively. Subsequent data analysis was performed using “Kaluza Analysis Software” (Beckman Coulter, Life Sciences).

**PI/Brdu assay**

MECs isolated from mice were cultured in DMEM/F12, with 10% fetal bovine serum, 10 μg/mL insulin, 10 ng/mL epidermal growth factor, and 10 μg/mL hydrocortisone. For cell cycle analysis, MECs were pulse-labeled with bromodeoxyuridine (BrdU) for 15 hours. Cells were then stained with propidium iodide (PI) and an antibody against bromodeoxyuridine (BrdU) and analyzed via flow cytometry as described [75].
**SA-β-gal assay**

Freshly isolated mammary cells were grown to 30-50% density for two days. Media was aspirated and cells are washed 1X with PBS. Cells are then fixed in 0.2% glutaraldehyde (Sigma) for 5 minutes and then washed 2X with PBS. Cells were stained with a freshly made beta-gal solution filtered through a 0.2 micron syringe filter: 40mg/ml beta-gal (Sigma), 5M NaCl, 1M MgCl2, 100mM K3Fe(CN)6, 100mM K4Fe(CN)6 (Sigma) and 1M NaPi, pH 6.0. The plate was covered with foil to protect from light with a moistened KimWipe inside of the foil packet to avoid evaporation. Cells were stained for 6-24 hours at 37°C. For SA-β-gal staining of mammary tissue, sections were processed as previously described [18]. Whole mammary tissues were flattened thinly onto histology slides. Tissues were then fixed for 15 minutes in 0.5% glutaraldehyde/PBS (PH 6.0). After fixation, slides were washed with PBS and stained overnight at 37°C with the beta-gal solution as explained above. Stained tissues were then washed with PBS and imbedded in paraffin wax and made into tissue blocks. Blocks were then cut in to 5μm sections placed on histology slides and counterstained with Nuclear Fast Red (Vector Laboratories) and cover-slipped.

**LOH and methylation analysis**

For LOH analysis, genomic DNA extracted from micro-dissected \( p16^{+/−};\text{Brca1}^{\text{MGKO}} \) mammary tumor cells was analyzed with primers amplifying the wild-type (WT) and knock-out (KO) allele of \( p16 \) as previously described [34, 43]. For methylation analysis, genomic DNA from \( p16^{+/−};\text{Brca1}^{\text{MGKO}} \) mammary tumors and tumor-free mammary tissues of the same mice was treated with bisulfide according to the
manufacture’s protocol (EZ DNA Methylation™ Kit) and analyzed for $p16$ methylation with specific primers amplifying the unmethylated or methylated allele as described[34]. In addition, $p16$ mRNA levels in primary $p16^{+/−};Brca1^{MGKO}$ mammary tumor cells treated with a methylation inhibitor DAC (Sigma-Aldrich) at the indicated concentrations for 72 hours, were analyzed by Q-RT-PCR.

**Human breast cancer samples**

Formalin fixed paraffin-embedded (FFPE) human breast cancer samples lacking patient-identifying information were obtained from the Tissue Bank Core Facility at the University of Miami. All samples obtained were non-treated invasive breast cancers with known ER status. The expression of $BRCA1$ in tumors was determined by microdissection-based RNA extraction and Q-RT-PCR as we previously described[44].

**Statistical analysis**

All data are presented as the mean ± SD for at least three repeated individual experiments for each group unless otherwise specified. Statistical analyses of mRNA expression, histopathology quantifications, cell assay quantifications and tumor volume were performed using a two-tailed Student’s t-test. Statistical analysis of tumor incidence was performed using a two-tailed Fisher’s exact test. Statistical analysis of mammary tumor-free survival was performed using a Log-rank (Mantel-cox) test. P<0.05 was considered statistically significant. Statistical analyses were conducted using Microsoft Excel and GraphPad Prism 5.
CHAPTER III
RESULTS

Increased expression of p16 is associated with age-induced senescence in mammary glands

We determined the expression of p16 in both young (2-4-month-old) and old (12-24-month-old) virgin mammary glands in wild-type (WT) mice. We found that mRNA levels of p16 along with senescence markers, Pai-1, Mmp13, and Igfbp2 were increased in old mammary glands relative to young (Fig. 8A), suggesting that expression of p16 increases with age-induced senescence. Notably, immunohistochemical staining of virgin mammary glands revealed that p16 was predominantly expressed in the MECs of the old mice, whereas, it was hardly detected in those of the young (Fig. 8B). Consistently, mammary epithelia from aged mice exhibited significantly fewer Ki67-positive cells and a stronger positive staining of senescence-associated β-galactosidase (SA-β-gal) than young epithelia (Fig. 8C, D).

Figure 8. p16 expression is increased in the mammary during age-induced senescence
(A) mRNA expression of mammary tissue was analyzed by Q-RT-PCR in WT young (2-4-month-old) and old (12-24-month-old) virgin mice. Data are expressed as the mean ± SD from triplicates of each two separate mice. (B-E) Representative immunohistochemical staining of p16 (B), Ki67 (C) and γH2AX (E) in three mice per group, and SA-β-gal assay in two animals per group (D), in mammary glands. The percentages of Ki67-positive cells in (C) were calculated from cells situated in clear duct/gland structure, and the results represent the mean ± SD of three animals per group. Note the significant decrease of Ki67-positive cells and strong p16 and SA-β-gal staining in MECs from old mammary glands.
DNA damage accumulates during age-induced senescence in several tissues [76-78]. To explore the age-related increase of DNA damage in mammary epithelial cells, we determined the expression of γH2AX, a marker for DNA double-strand breaks [79]. We found that on average, a positive MEC was detected in every 10-15 old glands while γH2AX-positive cells were rarely seen in young glands (Fig. 8E), confirming enhanced DNA damage in old MECs. Taken together, these data suggest that expression of p16 is increased during aging, along with the senescence of MECs and increased DNA damage.

Previous studies have shown that the accumulation of adult stem cells undergoing senescence can contribute to tissue degradation during aging [80, 81]. We wanted to determine if mammary stem cell function does decline with age and if p16 regulates this function. We examined mammosphere forming potential for primary MECs and found a decreased number of mammospheres formed by MECs from old mice than those from young mice (145.0 ± 73.9 vs 200.0 ± 40.6, p = 0.369, Fig. 9A), though it was not statistically significant. We then transplanted primary MECs into the cleared mammary fat pads (MFP) of NSG recipient mice. Wholemount staining of MFPs showed that the percentage of the fat pad filled from outgrowths reconstituted with old MECs was less compared to young MECs (% of fat pad filled: 30.3% ± 19.6 vs 40.3% ± 19.7, p >0.05, Fig. 9B and C), though we failed to obtain statistical significance which is likely due to micro-environmental factors in the mammary stroma that are specific to aging and affect mammary stem cell function in a cell non-autonomous manner [82].
Figure 9. There is a marginal decline in mammary stem cell function with age

(A) Mammary cells freshly isolated from both old and young mice were analyzed by mammosphere assay (20,000 cells per 24-well plate). After 2 weeks the number of spheres larger than 20µm was quantified. Bar graph, mean ± SD from duplicates of each three separate mice. (B) Freshly isolated mammary cells from both WT young and old mice were transplanted into the cleared MFPs of NSG mice. The mammary glands were dissected after 8 weeks and analyzed for the percent of mammary fat pad filled. The bar graphs represent the mean ± SD of both groups (N=4 for young WT and N=8 for old). (C) Representative mammary gland wholemounts transplanted with MECs of the indicated ages.
Loss of p16 rescues age-induced senescence of MECs

Given that senescence in MECs correlates with the increased expression of p16, we hypothesized that p16 plays an important role in mediating this process. We determined the role of p16 loss in controlling mammary cell senescence during aging. We found that p16 loss drastically reduced the expression of senescence markers (Fig. 10A) and enhanced MEC proliferation (Fig. 10B and C, Ki67-positive cells in old mice, 14.65% in p16<sup>−/−</sup> vs. 5.7% in WT, p<0.05). These data indicate that loss of p16 rescues the age-induced senescence of MECs.

Figure 10. Loss of p16 rescues the age-induced senescence of MECs
(A) mRNA expression was analyzed by Q-RT-PCR from virgin mammary tissue of old mice of the indicated genotypes. Q-RT-PCR data are expressed as the mean ± SD from duplicates of each of three separate mice in each genotype. (B) Results for the quantification of Ki67-positive MECs. Results represent the mean ± SD of three animals per group. (C) Representative immunohistochemical staining of Ki67 in age-matched mammary glands.
Loss of p16 increases mammary stem cell function

We then determined the role of p16 loss in the regulation of mammary stem cell function. We found that MECs from $p16^{-/-}$ mice produced significantly more and larger colonies than WT cells (155.8 ± 30.6 vs 63.3 ± 8.9, for colonies greater than 20 µm, 76.8 ± 6.0 vs 27.8 ± 6.0, for colonies greater than 50 µm, 30.6 ± 4.7 vs 9.9 ± 2.4, for colonies greater than 100 µm, p<0.05, Fig. 11A and B). Correspondingly, MECs from $p16^{-/-}$ mice formed more and larger mammospheres than those from WT cells at similar ages (sphere number: 280.4 ± 30.9 vs 145.5 ± 28.3, p<0.05, sphere size: 136.0 µm ± 37.7 vs 71.7µm ± 15.6, p< 0.05, Fig. 11C).

We then performed transplantation assays for MECs isolated from WT and $p16^{-/-}$ mice and found that the percentage of mammary fat pad filled of outgrowths formed from transplanted $p16^{-/-}$ MECs was greater than those from WT cells (Fig. 11D and E), indicating increased reconstitution potential in old $p16^{-/-}$ MECs.

Taken together, these results suggest that p16 loss enhances mammary stem cell function.

Figure 11. Loss of p16 increases mammary stem cell function

(A-C) Mammary cells freshly isolated from both WT and $p16^{-/-}$ old mice were analyzed by colony formation and mammosphere assays. The number of colonies and spheres larger than 20µm was quantified. The bar graphs represent the mean ± SD from duplicates of each three separate mice. (B) Representative colony formation assays formed from MECs of the indicated genotypes. (D) Mammary cells isolated from old mice of the indicated genotypes were transplanted into the cleared MFPs of 3-week-old NSG mice. The mammary glands were dissected after 8 weeks and analyzed for the percentage of mammary fat pad filled. The bar graph represents the mean ± SD of N = 8 for WT and N = 4 for $p16^{-/-}$ mice examined for each group respectively. (E) Representative mammary gland wholemounts transplanted with MECs of the indicated genotypes.
Loss of p16 increases the luminal epithelial cell enriched population

Previous studies identified multiple sub-populations in the highly heterogeneous mammary cell hierarchy [83, 84]. FACs analysis combined with lineage tracing studies identified distinct Lin⁻ sub-populations based on CD29 and CD24 expression [85]. The CD24⁺CD29⁻ sub-population is composed predominately of luminal cells (LCs) and is enriched with luminal progenitors, whereas the CD24⁺CD29⁺ sub-population comprises the basal cells (BCs) and is enriched with basal progenitors and mammary stem cells. Considering that MEC proliferation decreased during aging and was rescued by loss of p16, we wanted to further examine the role of p16 loss in the respective mammary sub-populations.

We performed FACS analysis for primary MECs in old mice and found that the percentage of LCs, were increased in p16⁻/⁻ mice (WT: 29% vs p16⁻/⁻: 38%, Fig. 12A and B) Notably, CD24⁺CD29⁺ cells, showed very little variation in p16⁻/⁻ mice compared to WT (WT: 4% vs p16⁻/⁻: 7%, Fig. 12A and B). These results suggest that loss of p16 leads to an increase in the percentage of LCs. Since the cell number of the BC population is low, the significance of the role of p16 loss on the percentage of BCs remains to be determined.

In order to corroborate the increase of LCs following p16 loss, we analyzed the expression of senescence marker, Pai-1, in the LC and BC populations in old mice. Of the senescence markers, Pai-1 was the most reliable as it could consistently be detected in the respective populations. Consistent with the increased percentage of LCs in p16⁻/⁻ mice, we found that loss of p16 resulted in the decrease of Pai-1 in LCs while there was no clear change of Pai-1 in BCs which
could explain the lack of variation in the percentage of BCs in $p16^{-/-}$ mice compared to WT (Fig. 12C). These data are consistent with our initial finding that p16 is predominantly expressed in luminal epithelial cells (Fig. 8B) and that p16 loss increases Ki67, mainly in the luminal cell lineage (Fig. 10C). Consistently, we previously demonstrated that germline deletion of $p18$ led to the expansion of LCs and subsequent luminal tumorigenesis[86].

Figure 12. Loss of p16 expands the luminal cell-enriched population
(A) Representative experiment of mammary cells analyzed by flow cytometry.
(B and C) Results represent the mean S.D. of experiments from three and two independent pairs of mice respectively.
Deletion of \textit{Brca1} in the mammary epithelium results in senescence with an increase of p16 expression

We and others previously demonstrated that heterozygous germline deletion of Brca1 in mice induces senescence in MECs [43, 72] though the molecular and cellular basis controlling Brca1-deficiency induced senescence is not fully understood. To directly determine the role of loss of function of Brca1 in controlling the senescence of MECs, mammary stem cell function, and tumorigenesis, we used \textit{Brca1}^{f/-};MMTV-Cre (\textit{Brca1}^{MGKO}) mice, in which MMTV-cre is active in virgin epithelia and mammary glands from these mice express <5% of the Brca1 protein and mRNA relative to the levels in \textit{Brca1}^{+/+} mice as we previously described[44].

We compared p16 expression in \textit{Brca1}^{MGKO} mice with age-matched WT animals and found a substantial increase of p16 in \textit{Brca1}^{MGKO} mammarys relative to WT counterparts (Fig. 13A). Consistently, mRNA levels of \textit{p16} and senescence markers were increased in \textit{Brca1}^{MGKO} mammarys relative to WT (Fig. 13B). These data suggest that specific depletion of Brca1 in the mammary epithelium induces senescence with increased p16 expression.
Figure 13. Brca1 depletion in the mammary epithelium leads to senescence with increased p16 expression
(A) Representative immunohistochemical staining of p16 in mammary glands of WT and Brca1<sup>MGKO</sup> mice in three mice per group. (B) mRNA expression of mammary tissue was analyzed by Q-RT-PCR in WT and Brca1<sup>MGKO</sup> mice. Data represent the mean ± SD from duplicates of 2 mice per genotype.
Depletion of *Brca1* in the mammary epithelium leads to a decline in mammary stem cell function

We performed colony formation and mammosphere assays for primary MECs and found that the number of *Brca1*<sup>MGKO</sup> colonies was significantly reduced when compared to colonies from WT MECs (13.3 ± 3.3 vs 32.7 ± 1.7, for colonies greater than 50 µm, 6.0 ± 0.5 vs 15.8 ± 0.8, for colonies greater than 100 µm, p<0.05, Fig. 14A and B). Correspondingly, *Brca1*<sup>MGKO</sup> mammospheres were reduced relative to WT counterparts (sphere number: 91.7 ± 21.2 vs 205.9 ± 12.5, p<0.05, sphere size: 36.2 ± 8.3 µm vs 80.2 ± 8.6 µm p<0.05, Fig. 14C and D). We then transplanted primary MECs into the MFPs of NSG mice and found a significant reduction in the percentage of mammary fat pad filled from transplanted *Brca1*<sup>MGKO</sup> MECs when compared to WT cells (Fig. 14E and F). Together, these data suggest that Brca1 loss in mammary epithelium results in premature senescence of MECs with a functional decline of mammary stem cells, and an increase of p16 expression.

**Figure 14. Brca1 depletion in the mammary epithelium leads to a functional decline of mammary stem cells**

(A-D) Mammary cells from age-matched WT and *Brca1*<sup>MGKO</sup> mice were isolated and analyzed by colony formation (A) and mammosphere (C) assays. Colonies and spheres larger than 20 µm were quantified. (B & D) Bar graph, mean ± SD from duplicates of each two separate mice per genotype. (E) Representative mammary outgrowths from transplanted MECs of age-matched WT and *Brca1*<sup>MGKO</sup> mice. The mammary glands were dissected after 8 weeks and analyzed for the percentage of mammary fat pad filled. (F) The bar graph represents the mean ± SD of N = 8 for WT and N = 5 for WT and *Brca1*<sup>MGKO</sup> mice respectively.
p16 loss ameliorates the senescence and decline in mammary stem cell function caused by Brca1 deficiency

Considering that loss of p16 stimulates proliferation and mitigates senescence in MECs during aging, and that Brca1 loss induces senescence with an increase of p16 expression, we were inspired to determine the role of p16 in controlling Brca1 deficiency-induced senescence and tumorigenesis. To this end, we generated $p16^{-/-};Brca1^{MGKO}$ mice on a Balb/c-FVB-B6 mixed background. We found that the percentages of Ki67-positive MECs in $p16^{-/-}$ and $p16^{-/-};Brca1^{MGKO}$ mice (24.28 ± 2.1 and 24.06 ± 4.3) were significantly higher than those in WT mice (6.39 ± 1.3), which in turn were significantly higher than the percentage in $Brca1^{MGKO}$ (2.20 ± 0.2) mice (Fig. 15 A and B).

![Figure 15. Loss of p16 rescues the proliferative decline of Brca1-deficient MECs](image)

(A) Representative immunohistochemical staining of Ki67 in mammary glands of the indicated genotypes. (B) Results represent the mean ± SD of three animals per group.
We then directly examined Rb phosphorylation in MECs of different genotypes by using an antibody specifically recognizing Rb proteins phosphorylated at Ser608 by CDK4 and CDK6 [87, 88], the functional targets of p16 (Fig. 16A). A consistent increase of pRb-Ser608 phosphorylation was detected in \( p16^{-/-} \) mammary epithelia (9.86 ± 6.6% in WT to 21.64 ± 5.4% in \( p16^{-/-} \), Fig. 16B), confirming the activation of CDK4 and/or CDK6. Consistent with our previous finding [43], 3.42 ± 5.0% pRb-Ser608 positive MECs were detected in \( Brca1^{MGKO} \) females, which is significantly less than those in WT counterparts. However, \( p16^{-/-};Brca1^{MGKO} \) mice had a significant higher percentage of positive MECs (20.69 ± 7.2%, Fig. 16B) than \( Brca1^{MGKO} \) mice. Together with the data derived from Ki67 staining, these results indicate that loss of p16 stimulates CDK4 and/or CDK6.

Figure 16. Loss of p16 rescues the decrease of phosphorylated Rb in Brca1-deficient MECs
(A) Representative immunohistochemical staining of pRB-Ser608 in mammary glands of the indicated genotypes. (B) Results represent the mean ± SD of two animals per group.
activity toward the Rb protein in MECs, increasing their proliferation and rescuing
the proliferative decline observed in Brca1-deficient MECs.

To further consolidate the role of loss of p16 and Brca1 in the regulation of
MEC senescence, we isolated and cultured primary MECs from virgin mice. We
observed that Brca1MGKO MECs exhibited a large and flattened shape, a typical
morphology of cellular senescence, while MECs from the other genotypes of mice
were smaller and spindle-shaped (Fig. 17A). Notably, most Brca1MGKO MECs
exhibited strong, peri-nuclear staining of SA-β-gal whereas only a small population
of WT MECs and very few p16−/− and p16−/−;Brca1MGKO MECs showed positive
staining (Fig. 17B and C).

Figure 17. Loss of p16 rescues the senescent morphology of Brca1-deficient
MECs
(A) MECs of the indicated genotypes were isolated and cultured to analyze cell
morphology. (B) SA-β-gal assay of MECs of the indicated genotypes. (C) Results
represent the mean ± SD of triplicates per genotype.
We then directly tested the role of p16 in Brca1MGKO mediated cell cycle regression. We pulse-labeled primary MECs with bromodeoxyuridine (BrdU) for 15 hours and performed FACS analysis. Brca1MGKO MECs had increased G1 and decreased S phase cells relative to their WT counterparts (G1 phase cells, 29% vs 15%; S phase cells, 60% vs 68% Fig. 18). Importantly, MECs from p16⁻/⁻ or p16⁻/⁻;Brca1MGKO mice displayed similar BrdU incorporation rates, 81% for p16⁻/⁻ and 79% for p16⁻/⁻;Brca1MGKO, which were significantly higher than their WT counterparts (Fig. 18). These data suggest that loss of Brca1 induces senescence in MECs, which is rescued by p16 loss.

Figure 18. Loss of p16 rescues cell cycle arrest in Brca1-deficient MECs
Primary MECs of the indicated genotypes were pulse-labeled with bromodeoxyuridine (BrdU) for 15 hours and analyzed by FACS. Note the increase of Brca1MGKO MECs in G1 and the decrease of cells in S phase relative to WT, and the decrease of p16⁻/⁻;Brca1MGKO MECs in G1 and increase of cells in S phase relative to Brca1MGKO.
Lastly, we determined the function of p16 loss in the Brca1-deficiency induced functional decline of mammary stem cells. We found a significant increase in mammosphere-forming potential from $p16^{-/-};Brca1^{MGKO}$ (212.0 ± 18.0 ) when compared to $Brca1^{MGKO}$ MECs (72.0 ± 15.5, Fig. 19A). We performed transplantation assays and found a drastically increased the percentage of mammary fat pad filled from outgrowths of transplanted $p16^{-/-};Brca1^{MGKO}$MECs relative to $Brca1^{MGKO}$ counterparts (Fig. 19B). These data indicate that p16 loss rescues the decline of mammary stem cell function caused by Brca1 insufficiency.

![Figure 19. Loss of p16 rescues the decline in mammary stem cell function induced by Brca1-deficiency](image)

(A) Mammary cells freshly isolated from WT, $Brca1^{MGKO}$, $p16^{-/-};Brca1^{MGKO}$ and $p16^{-/-}$ mice were analyzed by mammosphere assay. After 2 weeks the number of spheres larger than 20µm were quantified. Bar graph, mean ± SD from duplicates of each three separate mice. (B) Mammary cells isolated from mice of the indicated genotypes were transplanted into the cleared MFPs of 3-week-old NSG mice. The mammary glands were dissected after 8 weeks and analyzed for the percentage of mammary fat pad filled. The bar graph represents the mean ± SD of N = 8 for WT, N = 5 for $Brca1^{MGKO}$, N = 4 for $p16^{-/-};Brca1^{MGKO}$ and N = 4 for $p16^{-/-}$ mice examined for each group respectively. (C) Representative mammary gland wholemounts.
Loss of p16 transforms Brca1-deficient MECs and induces mammary tumors

45% (n=20) of p16⁻/⁻ mice developed lymphoma and sarcoma in 24 months, which is consistent with previous reports [34, 35] (Fig. 20A and Table 2). Of the nine p16⁻/⁻ tumors, two were detected in the mammary gland and were highly composed of lymphoma cells, as evidenced by their lymphocyte-like morphology, positivity for CD45 and CD31 by FACS analysis and negativity for Cdh1, an epithelial cell marker, by IHC (Fig. 20A, B and C). Interestingly, FACS analysis revealed that 1.7%-3.5% of the total tumor cell population was negative for CD45 and/or CD31 respectively (Fig. 20B), and IHC showed that less than 4% of sporadic tumor cells were epithelial-like and positively stained with Cdh1 (Fig. 20C), indicating that this tumor was comprised predominantly of lymphoma cells and that a small portion of cells originated from the mammary epithelium. These results confirm the predominant role of p16 in suppressing the development of lymphoma and sarcoma, and suggest that mammary tumorigenesis in p16 null mice may be masked by lymphomas and sarcomas.

Figure 20. Histological and molecular characterization of tumors developed in p16⁻/⁻ and p16⁻/⁻;Brca1MGKO mice
(A) Representative H.&E. of primary tumors in mammary glands of the indicated genotypes. (B) Tumor cells from the mammarys of p16⁻/⁻ and p16⁻/⁻;Brca1MGKO mice were analyzed by flow cytometry for CD31 and CD45. (C) Representative immunostaining of Cdh1.
Table 2. Spontaneous tumor development in WT and mutant female mice

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a, Brca1MGKO, Brca1fr;MMTV-Cre or Brca1fr;MMTV-Cre mice.
b, one mouse was 24 months of age, and the remaining mice were 11-20 months of age.
c, one mouse was 24 months of age, and the remaining mice were 11-20 months of age.
d, two mice developed tumors in mammary glands composed of lymphoma cells and 1%-4.0% epithelial-like cells respectively.
e, one mouse was 23 months of age, and the remaining mice were 11-19 months of age.
f, four mice developed mammary tumors at 18, 18, 19 and 20 months of age, respectively. One mouse developed two different mammary tumors at two separate mammary glands. One mouse was 23 months of age, and the remaining mice were 14-20 months of age. One mouse was a breeder. Mammary tumor incidence, p16+/-;Brca1MGKO vs p16+/-, P=0.103; p16+/-;Brca1MGKO vs Brca1MGKO, P=0.082.
g, five mice developed mammary tumors at 11, 12, 16, 17 and 20 months of age, respectively. Two mice developed two different mammary tumors at two separate mammary glands. One mouse was 20 months of age, and the remaining mice were 11-17 months of age. One mouse was a breeder. Mammary tumor incidence, p16+/-;Brca1MGKO vs p16-/-, P=0.0006; p16+/-;Brca1MGKO vs Brca1MGKO, P=0.026; p16mt;Brca1MGKO (p16+/-;Brca1MGKO and p16+/-;Brca1MGKO) vs Brca1MGKO, P=0.022.
h, lung metastasis from primary mammary tumors was detected in 3 mice, whose ages were 18, 18 and 20 months of age, respectively.
i, lung metastasis from primary mammary tumors was detected in 2 mice, whose ages were 17 and 20 months of age, respectively.
j, one mouse developed an ovarian tumor at 24 months of age.
k, nine mice developed sarcoma or lymphoma.
l, one mouse developed lymphoma at 15 months of age.
m, three mice with mammary tumors also developed lymphomas.
n, one mouse developed pancreatic carcinoma at 16 months of age, and three mice with mammary tumors also developed lymphoma, sarcoma, lung adenoma, and hepatocellular carcinoma respectively.
We then followed the mammary tumor development in older mice and found that 63% of the $p16^{-/-};Brca1^{MGKO}$ mice (n=8) and 44% of $p16^{+/+};Brca1^{MGKO}$ mice (n=9) developed mammary tumors at 11-20 months and 16-23 months, respectively (Table 2), whereas no $p16^{-/-}$, $p16^{+/+}$ or $Brca1^{MGKO}$ mice did so at similar ages (Table 2). Median mammary tumor-free survival time in $p16^{mt};Brca1^{MGKO}$ mice (including $p16^{-/-};Brca1^{MGKO}$ and $p16^{+/+};Brca1^{MGKO}$ mice) was 18 months (Fig. 21). These results indicate that haploid or complete loss of p16 transforms Brca1-deficient mammary epithelial cells and induces mammary tumors.

![Figure 21. p16;Brca1 double-mutant mice develop mammary tumors](image)

(A) Mammary tumor-free survival curve. $p16^{mt};Brca1^{MGKO}$ includes: $p16^{+/+};Brca1^{MGKO}$ and $p16^{-/-};Brca1^{MGKO}$ mice. $p16^{mt}$ includes: $p16^{+/+}$ and $p16^{-/-}$ mice. Log-rank (Mantel-cox) test: p<0.05
Depletion of p16 and Brca1 leads to aggressive basal-like mammary tumors

All mammary tumors developed in \( p16^{mt};Brca1^{MGKO} \) mice were positively stained with basal markers, Ck5 and Ck14 in 2-75% of total tumor cells (Table 3 and Fig. 22A and B). Additionally, 56% of the \( p16^{mt};Brca1^{MGKO} \) mammary tumors (n=9) metastasized to the lung in 17-20 months (Fig. 22B, bottom right panel and Table 3).

**Table 3.** Characterization of mammary tumors developed in mutant mice

<table>
<thead>
<tr>
<th>Mouse ID</th>
<th>Age (mo)</th>
<th>Genotype</th>
<th>Mammary tumor pathology</th>
<th>Ck5</th>
<th>Ck14</th>
<th>ERα</th>
<th>Lung metastasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1255</td>
<td>18</td>
<td>( p16^{+/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma with scirrhous pattern</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>1279</td>
<td>12</td>
<td>( p16^{+/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1288</td>
<td>16</td>
<td>( p16^{-/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma</td>
<td>+</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1347</td>
<td>19</td>
<td>( p16^{-/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma with central necrosis</td>
<td>+</td>
<td>++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1479</td>
<td>11</td>
<td>( p16^{+/-};Brca1^{MGKO} )</td>
<td>A: Adenomyoepithelioma</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: Adenocarcinoma with scirrhous pattern</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1497</td>
<td>20</td>
<td>( p16^{+/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma with cribriform pattern</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>1509</td>
<td>18</td>
<td>( p16^{+/-};Brca1^{MGKO} )</td>
<td>A: pleomorphic carcinoma</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>B: Adenocarcinoma with central necrosis</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>1558</td>
<td>20</td>
<td>( p16^{-/-};Brca1^{MGKO} )</td>
<td>Adenocarcinoma with central necrosis</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td>1618</td>
<td>17</td>
<td>( p16^{-/-};Brca1^{MGKO} )</td>
<td>A: pleomorphic carcinoma</td>
<td>++</td>
<td>+++</td>
<td>-</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>B: Adenocarcinoma with central necrosis</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\( a \) mo, month  \( b \) -: <2%, +: 2-25%, ++: 25-50%, +++: 50-75%, ++++: >75%
Figure 22. p16;Brca1 double-mutant basal-like tumors develop lung metastasis

(A) Immunofluorescence staining of mammary tumors in mouse 1479 with Ck14 and Ck5. The boxed areas in the left panel are enlarged in the middle and right panels. Note the majority of tumor cells are positive for either Ck5 or Ck14. (B) Immunofluorescence and H.E. staining of mammary tumor in a p16++;Brca1MGKO mouse (1509) at 18 months of age. Note this tumor was comprised predominately of Ck5 and Ck14+ cells (middle panel) and developed a distant metastasis (M) to the lung (right panel), which also comprised of Ck14+ cells (inset in the right panel).
**p16;Brca1** double-mutant tumors display inter- and intra-tumoral heterogeneity

Mammary tumors developed in $p16^{mt};Brca1^{MGKO}$ mice were very aggressive and displayed typical morphological characteristics of highly malignant features (increased necrosis, spindle cells, nuclear-cytoplasm ratio, and mitotic indices) (Fig. 23). 18% of the $p16^{mt};Brca1^{MGKO}$ mice (n=17) developed two distinct mammary tumors in two separate mammary glands, demonstrating the ability of these mice to develop both intra- and inter-tumoral heterogeneity (Fig. 23).

![Figure 23. Characterization of inter- and intra-tumoral heterogeneity in mammary tumors](image)

Representative H.E staining of a tumor developed in the right 3rd mammary gland (A – C), and a tumor in the left 3rd mammary gland (D), in a $p16^{−/−};Brca1^{MGKO}$ mouse (1509). Note the different cell types in mammary tumors of this mouse.
**p16;Brca1** double-mutant basal-like tumors exhibit decreased luminal gene expression and activated EMT

Mammary tumors developed in \( p16^{mt};Brca1^{MGKO} \) mice expressed 44% *Gata3*, and 54% *Elf5*, both of which are genes associated with luminal cell differentiation, relative to the tumor-free mammary tissues of the same mouse (Fig. 24), confirming Brca1-deficiency impaired luminal differentiation during tumorigenesis, as we previously demonstrated [43, 44].

![Figure 24. p16\( ^{mt};Brca1^{MGKO} \) mammary tumors have decreased luminal gene expression](image)

(A) Q-RT-PCR analysis for \( p16^{mt};Brca1^{MGKO} \) (\( p16^{+/+};Brca1^{MGKO} \) and \( p16^{-/-};Brca1^{MGKO} \)) mammary tumors and their corresponding tumor-free mammary tissues from same mice. Data are expressed as the mean ± SD of three mice.

All mammary tumors derived from \( p16^{mt};Brca1^{MGKO} \) mice were stained positively for vimentin (Vim), a mesenchymal marker, and Twist, an EMT transcription factor (Fig. 25, Table 2). These data indicate that depletion of both p16 and Brca1 results in basal-like mammary tumors with activation of EMT, which is consistent with our previous finding that deletion of *Brca1* activates EMT in mammary tumorigenesis[44].
The pathology $p16^{mt};Brca1^{MGKO}$ tumors resembles that of human ER- breast tumors

We screened 43 human invasive breast cancers and selected 10 ER-negative samples with the lowest $BRCA1$ mRNA expression as previously described[44]. We compared tumor pathology and expression of CK5 and CK14 in these samples with mouse mammary tumors. We noticed that both the tumor cell morphology and expression pattern of CK5 and CK14 in $p16;Brca1$ double mutant mouse mammary tumors resembled human basal-like breast cancers that were ER-negative and expressed low BRCA1 (Fig. 26).
Figure 26. Characterization of ER-negative human breast cancers and *p16;Brca1* double-mutant mouse mammary tumors
Representative CK5 (A-F) and H.E staining (G-L) of ER-negative human invasive breast cancers (A-C, G-I) and mammary tumors developed in *p16;Brca1* double-mutant mice (D-F, J-L) are shown. Note the similarity of tumor cell morphology and CK5 staining between human breast cancers and mouse mammary tumors.
p16<sup>mt;Brca1<sup>MGKO</sup> tumor cells exhibit increased DNA damage

Given the function of Brca1 in DNA damage repair, we also evaluated the role of Brca1 loss in inducing DNA damage in tumor development. We determined the expression of γH2AX, a marker for DNA double-strand breaks, in spontaneous tumors from mutant mice. Since p16 single-mutant mice only developed lymphoma and sarcoma, we compared γH2AX expression in these tumors with mammary tumors. We found that the number of γH2AX-positive cells in p16<sup>mt;Brca1<sup>MGKO</sup> tumors was significantly greater than in p16 single-mutant tumors (4.5% ± 2.5% vs 0.84% ± 0.36%, p<0.05, Fig. 27), indicating a significant increase of cells with DNA damage in Brca1-deficient tumors.

Figure 27. p16;Brca1 double-mutant mammary tumors exhibit DNA damage
Representative immunostaining of γH2AX. The percentages of γH2AX-positive cells were calculated from >800 cells for each tumor. For p16 mutant (p16<sup>mt</sup>) tumors, results from a sarcoma and two lymphomas were pooled. For p16<sup>mt;Brca1<sup>MGKO</sup> tumors, results from a p16<sup>+/−;Brca1<sup>MGKO</sup> mammary tumor and two p16<sup>−/−;Brca1<sup>MGKO</sup> mammary tumors were pooled. Results represent the mean ± SD.
Together, these results suggest that depletion of both p16 and Brca1 induces metastatic aggressive, malignant, basal-like tumors that have an activated EMT program and enhanced DNA damage.

**p16 and Brca1 double-mutant tumor cells are transplantable**

We transplanted $1 \times 10^6$ cells from a $p16^{-/-};Brca1^{MGKO}$ mammary tumor, and $4 \times 10^6$ cells from a $p16^{+/-};Brca1^{MGKO}$ mammary tumor, respectively, into MFPs of NSG mice (three recipients per primary tumor). We found that $p16^{mt};Brca1^{MGKO}$ tumor cells regenerated significantly larger tumors than $p16^{-/-}$ cells with the same number of cells in the same time period (Fig. 29) Further analysis revealed that the generated $p16^{mt};Brca1^{MGKO}$ tumors resembled the pathology of the primary tumors and were positive for Ck14 recapitulating the phenotype observed in primary mammary tumors (Fig. 28A). These data suggest that $p16;Brca1$ double-mutant mammary tumors are enriched with mammary tumor initiating cells.
Tumors regenerated, like $p16^{-/-}$ primary tumors, were predominantly composed of lymphoma cells mixed with Cdh1-positive and Ck14-negative tumor cells (Fig. 29A, B and C). The Cdh1-positive cells in the regenerated tumors accounted for < 5% of total tumor cells, however, they aggregated together and formed a larger mass of epithelial-like tissue than in primary tumors (Fig. 29C). This result suggests that $p16$ loss stimulates luminal epithelial cell proliferation, possibly contributing to the development of premalignant lesions. These data are also consistent with our finding that loss of $p18$, another Ink4 family cell cycle inhibitor, promotes luminal epithelial cell proliferation and induces luminal tumorigenesis [86].

**Figure 28. Regenerated $p16;Brca1$ compound mutant tumor growth**

1x10⁶ cells from the tumors developed in the mammary glands of $p16^{-/-}$ and $p16^{-/-};Brca1^{MGKO}$ mice were transplanted into the left and right inguinal MFPs of female NSG mice, respectively. Generated tumor volume was measured 4 weeks after transplant. Results are representative of the mean ± SD of three tumors of each genotype.
Figure 29. \textit{p16;Brca1} double-mutant tumor cells are enriched with TICs and capable of generating basal-like mammary tumors.

(A) Representative H.E. and immunostaining of Ck14 (B) and Cdh1 (C) of regenerated mammary tumors.
Expression of \textit{p16} is lost in \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} tumors due to promoter methylation

We determined the expression of \textit{p16} in \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} mammary tumors. We found that \textit{p16} mRNA expression was retained in one tumor (number 1255), but clearly reduced in the other tumors when compared with tumor-free mammary tissues from the same mouse (Fig. 30A). \textit{p16} mRNA levels in tumor 1255 were 8.5-fold more than tumor-free mammary tissues, which could be caused by a reduction in the function of the Rb or p53 pathways[31, 89]. We performed loss of heterozygosity (LOH) analysis for DNA extracted from \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} mammary tumors and found that the remaining WT allele of \textit{p16} was retained in all four tumors (Fig. 30B). These results suggest that epigenetic silencing of \textit{p16}, likely by promoter methylation as observed in DMBA-induced \textit{p16}^{+/−} lymphomas and sarcomas[34], plays an important role in \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} mammary tumorigenesis. To determine the methylation status of the \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} mammary tumors, we performed methylation-specific PCR (MS-PCR) and detected methylation of the \textit{p16} promoter in three tumors with reduced expression of \textit{p16}, but not in tumor 1255 in which \textit{p16} expression was not decreased (Fig. 30C). Notably, we also detected \textit{p16} promoter methylation in a tumor-free, but premalignant lesion-containing mammary tissue in the mouse 1497 (Fig. 30C), which explains why there was not a significant decrease in \textit{p16} expression between tumor vs tumor-free tissues in mouse 1497 (Fig. 30A). We isolated and cultured primary cells from \textit{p16}^{+/−};\textit{Brca1}^\text{MGKO} mammary tumors and found that \textit{p16} mRNA levels were significantly increased in the 1347 tumor cells treated with 5-
aza-2'-deoxycytidine (DAC), a methylation inhibitor, but not in the 1255 tumor cells (Fig. 30D), confirming the findings derived by MS-PCR in tumors. These results suggest that epigenetic silencing of p16 by promoter methylation plays an important role in the development of, at least, some of the p16⁺⁻;Brca1MGKO mammary tumors.

**Figure 30. Promoter methylation silences p16 expression in p16⁺⁻;Brca1MGKO tumors**

(A) mRNA expression of p16 in p16⁺⁻;Brca1MGKO mammary tumors was determined by Q-RT-PCR. Corresponding tumor-free mammary tissues from the same mice were used as controls. Note the control mammary tissue for mouse 1497 tumor was from a premalignant lesion of the same mouse. Data are expressed as the mean of triplicate experiments. (B) LOH analysis of the p16 gene in p16⁺⁻;Brca1MGKO mammary tumors. Ear DNA was used as a control. (C) Bisulphite-treated DNA from mammary tumors (T) or tumor-free mammary tissues (TF) from the same mice was analyzed for methylation of p16. U, unmethylated. M, methylated. A normal mammary gland from a WT mouse was used as a control. Note, no methylated p16 was detected in the mammary tumor developed in mouse 1255 in which the p16 mRNA level was not reduced relative to tumor-free mammary tissue of the same mouse. (D) p16 mRNA analysis in primary mammary tumor cells after treatment with DAC at 0, 0.5, or 5µM for 72 hours.
**p16;Brca1 mutant tumors have somatic p53 mutations**

We found that 56% (5/9) of p16\(^{-/-}\);Brca1\(^{MGKO}\) and p16\(^{+/-}\);Brca1\(^{MGKO}\) mammary tumors displayed strong p53 by western blot (Fig. 31A). We sequenced the p53 gene and identified missense mutation, R270P (Fig. 31B). Mouse R270, equivalent to human codon R273, is a mutational hotspot in p53 that is known to inactivate p53 function in breast cancers [90, 91]. Taken together this data suggest that p16;brca1 mutant tumors also have somatic p53 mutations.

![Figure 31. p53 mutation in p16;Brca1 mutant tumors](image)

(A) Western blot of tissues from mammary tumors of the respective genotypes. (B) Identification of p53 mutation in p16\(^{-/-}\);Brca1\(^{16};\text{MC}\)(1279). DNA sequence of WT p53 (bottom panel) with the corresponding sequence of amino acids (top panel)
CHAPTER IV
DISCUSSION

**p16 controls age-related senescence in MECs**

Aging is a complex physiological process equipped with fail-safe mechanisms that protect organisms from developing cancer while also leading to the functional deterioration of several tissues and cell types [92]. The fact that aging is the single most important risk factor of breast and several other cancers, introduces a unique window of opportunity to elucidate the mechanisms that mediate aging, yet also play an important role in tumor development. We wanted to understand whether p16, a well-established tumor-suppressor and mediator of aging, controls mammary epithelial cell senescence and tumorigenesis in vivo. We therefore examined the role of sequential and premature-aging by comparing old with young WT mice, and age-matched WT and Brca1MGKO mice, respectively. We studied the role of p16 in mammary cell senescence by examining the loss of function of p16 in old mice (p16^-/-) and in Brca1-deficient mice (p16^-/-;Brca1MGKO).

We reported that during aging, mammary epithelial cells undergo senescence along with increased expression of p16. Furthermore, loss of p16 attenuated MEC senescence and enhanced mammary stem cell function. p16 is not expressed during early development, but is markedly increased during aging and senescence in most organs or cells[17]. Our finding that p16 increases with age in MECs, is consistent with a previous study revealing increased p16 expression in the breasts of older women [93] establishing a strong link between
p16 expression and mammary aging. Until now, the functional role of p16 in MEC aging and senescence has been largely unknown. Our data provide novel in vivo evidence demonstrating that p16 effectively induces MEC senescence during aging.

It has been shown that loss of p16 in mice rescues the functional decline of adult stem/progenitor cells in old mice in multiple tissues and cell types [22-24]. Though we detected a marginal reduced reconstitution potential in aged MECs, we failed to obtain statistical significance. This may be mainly resulted from micro-environmental factors in the mammary stroma that are specific to aging and affect mammary stem cell function in a cell non-autonomous manner [94]. Wu et al. noted observing a decrease in the self-renewal potential of mouse stem/progenitor cells and found increased p16 expression in the mammary stem cell niche[8]. The role of aging in mammary stem cells remains to be determined. Importantly, we demonstrated that loss of p16 does stimulate the reconstitution potential of MECs in aged mice, confirming the role of p16 in increasing mammary stem cell function in aging mammary glands.

**Germline deletion of p16 in mice expands the luminal cell-enriched population but does not induce mammary tumors**

The function of p16 in breast cancer suppression has been extensively studied and confirmed in human breast cancer samples and cell lines [25, 95]. However, the role of p16 in suppressing mammary tumorigenesis in vivo is elusive. Previous findings [34-36] that mice lacking p16 or targeting DNA methylation within the p16 promoter rarely develop mammary tumors suggests that p16 loss alone is
not sufficient for mammary tumorigenesis *in vivo*. We reported that loss of p16 significantly increased the LC population consistent with our previous studies that depletion of *p19* in mice stimulates mammary luminal cell proliferation [75] and deletion of *p18* in mice stimulates luminal progenitor cell proliferation, leading to mammary luminal tumorigenesis [86]. Furthermore, Deletion of both Rb1 and p107 in mouse mammary epithelia cells (MECs) induces luminal tumors[96] solidifying the important function of the INK4/Rb pathway in mammary luminal proliferation and tumorigenesis. Uniquely, of the four INK4 genes, only p16 is frequently deleted and inactivated in breast cancers, while p18 expression is significantly downregulated ([14, 25, 27, 28, 67]. While we did not detect a mammary tumor in *p16*−/− mice, two of the nine lymphomas were detected in the mammary gland with a small portion of poorly-differentiated, luminal-like mammary epithelial cells, suggesting that p16 loss may contribute to the development of premalignant lesions. The function of p16 loss in *p16*−/− mice is most likely masked by their tendency to develop lymphomas and sarcomas.

**p16 loss rescues mammary epithelial cell senescence and decline in stem cell function caused by Brca1-deficiency**

We found that disruption of Brca1 in mammary epithelium resulted in the senescence of MECs with an increase of p16 expression and the decline of stem cell function, which was also rescued by p16 loss. These data identify the role of p16 in suppressing MEC senescence and the Brca1-deficient function of mammary stem cells. It has only been demonstrated in a few systems including the hematopoietic and skeletal muscle systems in which stem cell functional decline
can be resulted from endogenous DNA damage that is accumulated with age and genetic deficiency in DNA damage repair[97-100]. Determining whether the function of stem cells in solid organs is also limited by deficiency in DNA damage repair is difficult due to the lack of suitable models.[101, 102]. BRCA1 deficiency causes chromosomal abnormalities, leading to the activation of DNA-damage checkpoint pathways and premature senescence and aging [45, 47, 48, 50]. Our finding that the reconstitution potential of MECs from Brca1-deficient mice was significantly reduced relative to WT counterparts, suggests that deficiency in DNA damage repair is also associated with the functional decline of mammary stem cells, one type of solid organ stem cells. In line with these data, it was shown that deletion of BRCA1 in HMECs increases genetic instability and premature senescence [103, 104]. Furthermore, deletion of BRIIP1, a DNA helicase that interacts directly with BRCA1 during DNA repair, in HMECs impaired normal acinus formation [105]. These studies taken together with our data suggest that deficiencies in DNA damage repair caused by Brca1 deficiency are associated with the decline in mammary stem cell function.

The finding that loss of p16 rescues the senescence of MECs caused by Brca1 deficiency suggests that p16 blocks these cells from entering an active cell cycle. These results indicate that, in addition to the p53-p21 pathway that is activated by BRCA1 loss [47, 48, 50], p16 is also a critical downstream target of BRCA1 in controlling mammary cell proliferation and senescence. It was recently reported in vitro that BRCA1 knockdown enhances the association of BRG1, a chromatin-remodeling factor that interacts with BRCA1, with the promoters of p16
and p21, leading to activation of their transcription and senescence[50]. More recently, it was found that human mammary epithelial cells from BRCA1-mutation carriers exhibit senescence, which is triggered by RB pathway activation[45]. Since deregulation of p53 alone induces DNA damage, \( p16;Brca1 \) compound mutant mice and cells offer a unique opportunity to investigate the role of Brca1 in DNA damage repair under a genetically intact p53 background.

The functions of BRCA1 have been linked with multiple pathways. It has also been reported that BRCA1 deficiency in MECs impairs stability and activation of Nrf2, a key transcription factor in regulating antioxidant response, and leads to the accumulation of reactive oxygen species (ROS), along with the increase of p16[106-108]. Interestingly, accumulation of ROS has been associated with cellular senescence [109], and Nrf2 activation restores ROS levels in Brca1-deficient MECs[106, 107]. These findings suggest that BRCA1 deficiency induced premature senescence in MECs, at least, partially resulted from accumulation of ROS. Our results that loss of p16 rescues the MEC senescence caused by Brca1 deficiency suggests that p16 loss may allow Nrf2 levels to accumulate in Brca1-deficient cells and suppress ROS, which remains to be determined.

**p16 protects Brca1-deficient MECs from malignant transformation**

The Rb family proteins consist of RB, p107, and p130, which are also frequently deleted and inactivated in breast cancers and are downstream targets of INK4 proteins [14, 25, 27, 28, 67]. RB is a major target for genomic disruption in \( BRCA1 \) mutant human breast cancers and loss of both RB and BRCA1 is a feature of basal-like breast cancers[67, 69, 96]. Here, we report that loss of p16 not only
rescues senescence in MECs caused by Brca1 depletion but also leads to mammary tumorigenesis though p16 loss alone is not sufficient to induce spontaneous mammary tumorigenesis.

Unexpectedly, \( p16^{+/-};Brca1^{MGKO} \) mice also developed mammary tumors. Though the remaining WT allele of \( p16 \) was retained in all \( p16^{+/-};Brca1^{MGKO} \) mammary tumors, the \( p16 \) promoter was methylated and \( p16 \) mRNA was significantly reduced in most of these tumors, indicating that silencing of \( p16 \) by promoter methylation plays a role in the development of, at least, some of the \( p16^{+/-};Brca1^{MGKO} \) mammary tumors. In line with these results are the clinical findings that methylation of BRCA1 and \( p16 \) is frequently detected in sporadic breast cancers and has a predictive value for tumor recurrence[110]. Together, our data not only support the function of \( p16 \) in suppression of Brca1-deficient mammary tumorigenesis, but also indicate that genetic mutation of \( p16 \) cooperates with epigenetic silencing of its transcription to promote tumorigenesis.

\textbf{\( p16;Brca1 \) compound mutant mammary tumors are poorly differentiated basal-like tumors with activated EMT}

\( BRCA1 \) mutation has long been associated with basal-like breast tumors though, not completely understood [111]. Interestingly, we found that while loss of \( p16 \) transformed Brca1-deficient MECs into basal-like tumor cells, depletion of Brca1 caused a dramatic increase of \( p16 \) expression, and subsequent decrease of Ki67, mainly in the luminal cell compartment and not the basal, suggesting a preferential role of Brca1 and \( p16 \) collaborating to control luminal cell proliferation. This data is also consistent with our findings demonstrating that loss of \( p16 \)
increases the LC population while we observed no significant change in the percentage of BCs[86]. Furthermore, we previously demonstrated that depletion of Brca1 in p18 null mice converts luminal tumors into basal like tumors and activates EMT [43, 44].

We previously found that while heterozygous germline deletion of Brca1 (Brca1<sup>+/−</sup>) reduced the expression of some genes associated with luminal epithelial differentiation, combined deletion of p18 and Brca1<sup>+/−</sup> significantly decreased their expression[44]. These data along with the observation that all tumors developed in the p16<sup>mut</sup>;Brca1<sup>MGKO</sup> mice were basal-like, inspired us to determine the expression luminal-related genes in these tumors. Our finding that expression of luminal genes was decreased in the mammary tumor tissues of p16<sup>mut</sup>;Brca1<sup>MGKO</sup> mice when compared to non-tumor mammary tissue of the same mice, further substantiates the combined role of INK4 and Brca1 in inducing basal-like tumors.

Our findings that p16<sup>mut</sup>;Brca1<sup>MGKO</sup> tumors have enhanced EMT is consistent with our previously reported data[44]. We found that 59% of p16<sup>mut</sup>;Brca1<sup>MGKO</sup> tumors had strong p53 staining, and identified a functional missense mutation, R270P. Most, if not all genetic mouse models of Brca1-deficiency use a co-mutation of p53 to overcome Brca1-deficiency induced senescence and induce tumorigenesis[47, 48, 51]. However, mutation of p53 alone activates EMT and contributes to the basal-like subtype [112-114] which could mask the contributions Brca1 and p16 loss in basal-like tumor development. Our finding that some, but not all, mammary tumors have strong p53 staining offer a
unique mouse model that can explore the combined role of p16 and Brca1 loss in basal-like mammary tumorigenesis with or without p53 mutation.
References


