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TITLE: Investigating Ductal Carcinoma in Situ Using Noninvasive Imaging of Genetically Engineered Mouse Models

PRINCIPAL INVESTIGATOR: Dr. Sanaz Jansen

CONTRACTING ORGANIZATION: The Geneva Foundation
Tacoma, WA 98402

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Our main goal in this proposal was to investigate the capacity for quantitative in vivo MRI biomarkers to represent underlying molecular signatures of DCIS, using the preclinical genetically engineered mouse (GEM) model framework to our hypotheses. We focused three key molecular pathways—Rb, p53, BRAC1—and whether MRI signatures can identify these molecular subtypes of disease. This report describes our experience in the first six months executing the SOW. We soon discovered limitations in our original GEM modeling strategy suggesting that our proposed studies could not be effectively performed. We therefore designed an alternative GEM modeling strategy that successfully addressed these limitations. We also developed novel MRI techniques to detect, characterize and follow progression of the preinvasive malignancies in the mouse mammary gland at high spatial resolution, and high frequency (rapidly imaging every few days).
Table of Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>05</td>
</tr>
<tr>
<td>Body</td>
<td>11</td>
</tr>
<tr>
<td>Key Research Accomplishments</td>
<td>19</td>
</tr>
<tr>
<td>Reportable Outcomes</td>
<td>19</td>
</tr>
<tr>
<td>Conclusion</td>
<td>19</td>
</tr>
<tr>
<td>References</td>
<td>20</td>
</tr>
<tr>
<td>Appendices</td>
<td>20</td>
</tr>
</tbody>
</table>
**Background:** Improvements in the early detection of breast cancer have yielded a major reduction in mortality because of the downward stage shifting of cancers at initial diagnosis: more women are diagnosed with stage I invasive ductal carcinoma (IDC) and the preinvasive ductal carcinoma *in situ* (DCIS) (Figure 1) now than before screening mammography was widely implemented. DCIS incidence in particular has skyrocketed, from comprising only ~2% of all breast cancers in the 1970’s to ~20-30% now.

Figure 1: The stages of breast cancer. Within the normal mammary duct, an inner layer of luminal epithelial cells and a basal layer of myoepithelial cells are surrounded by a basement membrane. In benign hyperplasia, epithelial cells have proliferated within the duct while retaining a largely normal phenotype. In ductal carcinoma *in situ* (DCIS), the cells appear phenotypically different from normal epithelia — poorly differentiated DCIS more so than well differentiated DCIS — yet are still confined by the basement membrane. DCIS is classified into several subtypes based on its histopathological presentation, including grade, growth pattern and necrosis. Once the neoplastic cells invade beyond a degraded basement membrane into the surrounding breast stroma, they have transitioned to invasive ductal carcinoma (IDC).

Concomitant with these improvements in early detection has been a growing clinical concern that some patients with DCIS may be over-diagnosed and over-treated, as indirect evidence suggests that a significant portion of DCIS lesions may never progress to invasive carcinomas. Unfortunately, there is no way now to identify patients with indolent disease (Table 1). Thus, herein lays *the clinical motivation for this proposal*: to find prognostic biomarkers for DCIS so that indolent and aggressive cancers can be distinguished from one another (if possible), allowing for more tailored patient management.
<table>
<thead>
<tr>
<th>Molecular Markers</th>
<th>Functions</th>
<th>Molecular signatures correlating with increased risk of progression</th>
</tr>
</thead>
<tbody>
<tr>
<td>ER, PR</td>
<td>Steroid receptors</td>
<td>ER− HER2+ ER−/HER2+ ER−/HER2+/Ki-67+</td>
</tr>
<tr>
<td>HER2</td>
<td>Regulates proliferation and apoptosis</td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>Regulates cell-cycle, apoptosis and genomic stability; p53 is an important tumor suppressor</td>
<td>p53+</td>
</tr>
<tr>
<td>Rb/p16 pathway</td>
<td>Regulates cell-cycle; Rb is an important tumor suppressor</td>
<td>Cyclin D− p16+ Ki-67+ COX-2+ p16+/COX-2-/Ki-67+ (DCIS recurrence) p16+/COX-2+/Ki-67+ (invasive recurrence)</td>
</tr>
<tr>
<td>Ki-67</td>
<td>Proliferation marker</td>
<td></td>
</tr>
<tr>
<td>COX-2</td>
<td>Enzyme for prostaglandin synthesis; expressed during inflammatory response</td>
<td></td>
</tr>
<tr>
<td>Akt/PTEN pathway</td>
<td>Regulates proliferation, survival and motility; PTEN is an important tumor suppressor</td>
<td></td>
</tr>
<tr>
<td>BRCA1/2</td>
<td>DNA damage repair</td>
<td></td>
</tr>
<tr>
<td>c-myc</td>
<td>Transcription factor that can activate proliferation; c-myc is a proto-oncogene</td>
<td></td>
</tr>
<tr>
<td>VEGF, vascular patterns</td>
<td>Angiogenesis and vascular markers</td>
<td></td>
</tr>
<tr>
<td>cyclin A, cyclin E, p21, p27</td>
<td>Cell-cycle regulators</td>
<td>p21+</td>
</tr>
<tr>
<td>Bcl-2, Bax, Survivin</td>
<td>Apoptosis regulators</td>
<td>Bcl-2− Survivin+</td>
</tr>
</tbody>
</table>

**Table 1:** Summary of the molecular markers that have been studied in human DCIS. Included are the molecular signatures that have been shown to correlate (even weakly) with an increased risk of subsequent progression in some reports. Note that none of these molecular markers can be used to reliably distinguish indolent and aggressive DCIS. In this proposal, we focus on the p53, BRCA1 and Rb pathways (highlighted).

Our approach in this proposal is to investigate biomarkers of DCIS in a preclinical setting, by developing genetically engineered (GEM) models that develop preinvasive cancers analogous to human DCIS. We further focus on imaging biomarkers, specifically magnetic resonance imaging (MRI) features. Imaging biomarkers have the advantage over other molecular marker (DNA, RN or protein based) of being in vivo and noninvasive. However, they also have the disadvantage of not being directly representative of a molecular signature or pathway. Rather, they have the potential to be surrogate features of underlying molecular pathway aberrations. Therefore, our main goal in this proposal is to investigate the capacity for quantitative in vivo MRI features to represent underlying molecular signatures of DCIS, in the preclinical GEM model framework.

**Hypotheses:** We identified the following hypotheses to be investigated in the Statement of Work (SOW).

**Hypothesis #1.** GEMs provide an experimental framework in which to improve the image-based characterization of molecular subtypes and prognosis of DCIS. GEMs that use different molecular drivers of tumorigenesis have, in context of invasive breast cancer, yielded different molecular subtypes and aggressiveness of disease. Such models
are often used for the development of novel and advanced imaging techniques. We thus contend that a similar paradigm may be applicable for preinvasive cancers—that by using a diversity of molecular drivers it is possible to generate preinvasive neoplasias of different molecular subtypes, which can serve as an excellent test-bed for evaluation of improved imaging strategies.

**Hypothesis #2. Altering the pRb, p53 and BRCA1 pathways individually and in combination within the mammary epithelium will yield different molecular subtypes of preinvasive neoplasias.** We seek to establish and utilize GEMs of different molecular subtypes of DCIS in a homogeneous background. To achieve this, we have selected three drivers that have been shown to be highly relevant to subsets of human breast cancers (Figure 2): pRb, p53 and BRCA1. Aberrant activity of these pathways has also been implicated in studies focusing on DCIS (Figures 3 and 4, Table 2). To disrupt the p53 and BRCA1 pathways we will utilize existing conditional GEM models. To achieve functional inactivation of pRb and family related proteins we have developed a strategy to dominantly interfere with pRb/p107/p130 via expression of the N-terminal SV40 T Ag domain (T121). Employing this strategy to functionally inactivate the pRb family proteins via expression of T121 has been used successfully to generate highly penetrant mouse models of breast and prostate adenocarcinoma, and high grade astrocytoma. Here we propose to target conditional expression of T121 to the luminal, i.e., cytokeratin 18 (K18) positive, mammary epithelia by using the K18 promoter, which has the advantage of being hormone independent.

**Hypothesis #3. Different molecular subtypes of preinvasive neoplasias will exhibit significantly altered MR image-based characterization.** Dynamic contrast enhanced MRI (DCEMRI) is a widely used technique that provides both a morphologic and functional lesion characterization via analysis of contrast media uptake and washout—or kinetic—curves. The rate of contrast uptake and washout reflects the perfusion and diffusion of contrast media from blood vessels into the tumor. Quantitative kinetic parameters are in turn related to vascular properties, such as capillary permeability, surface area and blood flow. In both human and murine studies, DCEMRI parameters of invasive breast cancer have been shown to correlate with tumor biology and even prognosis. We therefore propose to test a similar hypothesis for preinvasive cancers.
Figure 2: p53, Rb and BRCA1 are key tumor suppressor pathways altered in breast cancer. Based on figures from the recent report by the The Cancer Genome Atlas (TCGA) on breast cancer\textsuperscript{12}.
Figure 3: Clinical evidence for the role of p53 in DCIS. Figure from Allred et al\textsuperscript{12} demonstrating that accumulation of p53 correlates with increased heterogeneity of DCIS.
Figure 4: Clinical evidence for role of Rb in DCIS. Figures from Knudsen et al\textsuperscript{14} demonstrating increased recurrence in DCIS patients with Rb deficient lesions. In our proposal, we use expression of T\textsubscript{121} to functionally inactivate the Rb pathway in GEM models.

Table 2: Clinical evidence for the role of BRCA1 in DCIS. Compiling data from screening trials for breast cancer using MRI and mammography finds that only 6% of BRCA1 mutation carriers (MC) have DCIS, compared to 20-30% in the general population.
Scope of this report: This final report represents our progress in addressing these hypotheses during a period of approximately 6 months, from August 1, 2012 to February 5, 2013. The reason for this abbreviated term is that the PI on this grant, Dr. Sanaz Jansen, secured permanent employment in the breast imaging group at the U.S. Food and Drug Administration. Therefore, the PI-directed work on this grant ceased after February 5, 2013, her last day as a postdoctoral fellow at the NCI. This 6 month timeframe spans Tasks 1a-1d and 2a-b in the Statement of Work, which are discussed in detail below.

Overview. Our main goal in this proposal is to investigate the capacity for quantitative in vivo MRI features to represent underlying molecular signatures of DCIS, using the preclinical GEM model framework to test our hypotheses. Our strategy for accomplishing this goal is divided into two Tasks as outlined in the SOW. Task 1 pertains to establishing the GEM models and performing molecular characterization of the preinvasive cancers that arise therein. Task 2 pertains to establishing the magnetic resonance imaging techniques and correlating imaging and molecular features in the GEM models to test Hypothesis #3.

The original GEM modeling strategy is illustrated in Figure 5 below. Here, the K18 promoter is used to drive conditional expression of T121 in the luminal epithelial cells, in a Cre dependent fashion. Expression of T121 inactivates pRb and family proteins, thereby serving as the initiating event for tumorigenesis. In addition, p53 and BRCA1 are conditionally deleted either alone or in combination as further progressing events after Rb inactivation. To achieve focal induction in adult mice, lentiviral-mediated delivery of Cre recombinase was proposed.

Figure 5: Original GEM modeling strategy. The Rb pathway is inactivated via conditional expression of T121 under control of the K18 promoter. The K18 promoter will constrain expression of T121, and thus Rb loss, to
luminal epithelial cells. Conditional models of p53 and BRCA1 loss are also utilized. Focal induction is achieved in adult mice by using direct injection of lentiviral Cre recombinase.

However, early on during this reporting period we realized that there may be substantial flaws with our original GEM modeling strategy. There were two main problems. First, our more recent experience suggested the K18 promoter did not drive conditional T121 expression strongly enough to successfully initiate tumorigenesis via Rb inactivation (Figure 6), even after widespread recombination achieved by crossing with the Wap-Cre mouse strain.

![WAP-Cre induction](image)

Figure 6: K18 promoter driving expression of T121 does not reliably initiate tumorigenesis. We crossed the K18-T121 model to the Wap-Cre mouse strain, to investigate efficiency of tumor initiation after widespread Cre expression. As shown in the pink line above, tumor incidence was very low in this model. We further crossed these mice onto the conditional model of p53 loss (orange line), which again demonstrates low tumor incidence and late onset.

Second, our recent *in vitro* and *in vivo* studies suggested that lentiviral-mediated Cre delivery was a very inefficient method for achieving recombination (see Task 1c below). Therefore, the weak initiating event (due to the K18 promoter) coupled with poor recombination efficiency (due to lentiviral delivery of Cre) combined to strongly indicate our original GEM modeling strategy would not yield penetrant mouse models of DCIS.

In light of these potential challenges, we proposed an alternative GEM modeling strategy, illustrated in Figure 7 below. This new strategy was submitted to the DOD as a modification to our proposal (see Appendix A), and was approved.
Figure 7: Alternative GEM modeling strategy. The Rb pathway is again inactivated via conditional expression of T121, but this time under control of the MMTV promoter. The MMTV promoter also constrains expression of T121, and thus Rb loss, to luminal epithelial cells. The same conditional models of p53 and BRCA1 loss are also utilized. Induction is achieved in adult mice by crossing with Wap-Cre transgenic mice, and subsequent pregnancy/lactation required to activate the Wap promoter (and therefore recombination). While tumor induction is no longer focal, it is penetrant. See Appendix B for more details.

This alternative strategy targets the same molecular pathways as the original strategy, including using T121 to inactivate pRb. However, it addresses the two problems above by using (i) the MMTV promoter to drive T121 expression, and (ii) the Wap-Cre strain to achieve efficient recombination during pregnancy and lactation. These models were developed by our collaborator, Dr. Karl Simin from the University of Massachusetts. His thorough characterization of these models (published in Kumar et al\textsuperscript{15}, included in Appendix B) underscores the most important feature of the alternative GEM modeling strategy: it yields a series of highly penetrant mouse models of breast cancer and DCIS (Figure 8).
Figure 8. Alternative GEM modeling strategy achieves penetrant tumor initiation with varying latency depending on genotype. Figure from Kumar et al\textsuperscript{15} characterizing tumor initiation and progression in different genotypes depending on which pathways (Rb, p53, BRCA1) are altered. For example, the solid red line represents inactivation or loss of all three pathways. This genotype is the most aggressive. In comparison, the solid green line represents loss of Rb and p53, which is less aggressive than the triple hit model. We propose to perform an image-based characterization of these different genotypes, to determine whether the MRI features of preinvasive malignancies correlate with molecular signatures. See Appendix B for more details.

Therefore, by using this alternative GEM modeling strategy we have identified an effective model system in which to test our hypotheses. We propose to perform an image-based characterization of these different genotypes, to determine whether the MRI features of preinvasive malignancies correlate with molecular signatures.

\textbf{Statement of Work (SOW).} The following Tasks from the SOW are applicable to the abbreviated reporting period and are presented below highlighted in grey. All tasks were completed, but not necessarily with the expected results.

\textbf{Task 1. Establish relevant inducible GEM model systems for preinvasive breast cancer by disruption of the pRb, BRCA1 and p53 pathways singly and in combination.} We seek to establish conditional GEMs reflecting different molecular drivers of tumorigenesis. To accomplish this, we have selected three pathways that are relevant to subsets of human breast cancer: pRb, p53 and BRCA1. We hypothesize that alteration of these pathways alone and in combination will generate a spectrum of molecular subtypes of preinvasive malignancies, including possibly those that are more indolent vs. aggressive (i.e., more slowly vs. rapidly progressing to invasive carcinoma). To achieve focal induction of tumorigenesis—which is desirable from an imaging perspective to facilitate accurate lesion detection and tracking—lentiviral mediated delivery of Cre recombinase into mammary epithelia will be performed.

\textit{1a. Obtain regulatory approval (Months -3 to 0).} Upon notice of proposal approval we will commence the regulatory process.

Completed. Regulatory approval to generate sufficient numbers of mice (both original GEM models and alternative GEM models) and conduct the studies outlined in Task 1 was granted by the NCI-Frederick Animal Care and Use Committee, the NCI-Frederick Institutional Biosafety Committee and also through the DOD ACURO process.
1b. **Generate study mice (Months -3 to 2).** All of the mice needed to generate our study genotypes are already part of the Van Dyke stock colony. We will begin the breeding schema upon notice of proposal approval. A total of 5 genotypes will be studied, all on the FVB background inbred strain. Completed. All of the original GEM model strains were generated in a homozygous state and breeder pairs established. In addition, breeder pairs for the alternative GEM model strains were obtained from our collaborator, Dr. Karl Simin, and re-derivation procedures commenced in order to house the mice as part of the Van Dyke colony and expand the cohort.

1c. **Optimize viral delivery of Cre recombinase via intraductal injection into Cre reporter mice (Months 1-3).** A total of 30 mice (RosaYFP) will be used. Lentiviral-Cre recombinase at different titers and volumes will be injected into the nipple of inguinal glands. The dose that consistently delivers the highest recombination efficiency will be used for the remainder of the study. Completed. As described in more detail below, we developed a new intraductal injection technique and evaluated recombination efficiency in vitro and in vivo for two different lenti-Cre constructs (pBob-CAG-iCre-SD and rrl-CMV-Cre-SD), packaged by two different institutions (SBI and the Salk Institute) at approximately 10^9 PFU/ml. Our in vitro results identified the most effective lenti-Cre construct as the pBOB-CAG-iCre-SD packaged by the Salk Institute. However, in vivo evaluation of this construct injected into the mammary glands of RosaYFP Cre reporter mice demonstrated poor recombination efficiency, thereby necessitating a change in plan to the alternative GEM modeling strategy.

**New no-incision intraductal injection technique:** Previously reported intraductal injection techniques involve a y-incision along the lower belly of the mouse, holding the cut skin taught, injecting into the #4 and #9 inguinal gland nipples, and then using wound clips to close the incision. Such injection techniques do not permit experimental manipulation of the animal for approximately 10 days due to the necessity of waiting for the wound to heal. In addition, there is potential for harm to the animal during and after the y-incision intraductal injection procedure. To avoid these limitations, we developed a no-incision intraductal injection technique wherein the y-incision is avoided and injections are performed directly into the #4 and #9 inguinal gland nipples (Figure 9). We optimized this technique so that it was successful approximately 60% of the time when injected in 8-10 week old mice (matching previous reported performance using the y-incision method). A total volume of 10-20 μl could be injected without damaging the ductal tree. This no-incision approach has the added advantage of being less harmful to the animals, and allowing experimental manipulation (e.g., MRI) to occur immediately after injection, rather than waiting 10 days for removal of wound clips.

![Figure 9: No-incision intraductal injection technique.](image)

Blue dye was injected into the #9 inguinal nipple of a 10 week old FVB mouse (left). Ex vivo examination demonstrates intact ductal tree (right).
**Lenti-Cre:** We evaluated two different lenti-Cre constructs (pBob-CAG-iCre-SD and rrl-CMV-Cre-SD), packaged by two different institutions (SBI and the Salk Institute) at approximately $10^9$ PFU/ml. Our *in vitro* results identified the most effective lenti-Cre construct as the pBOB-CAG-iCre-SD packaged by the Salk Institute. Figure 10 shows the map for this construct.

![Map of pBOB-CAG-iCre-SD construct](image.png)

**Figure 10:** Map of pBOB-CAG-iCre-SD construct. The CAG promoter is used to drive expression of self-deleting Cre.

**In vitro evaluation of lenti-Cre recombination efficiency:** Recombination efficiency of the different lenti-Cre constructs was evaluated in *RosaYFP* MEFS (cultured at 200 MOI) at one and two weeks post infection. Analysis of YFP expression was performed demonstrating that the pBOB-CAG-iCre-SD construct packaged by the Salk Institute exhibited the highest recombination efficiency (Figure 11).
**Figure 11. Improved recombination efficiency using the pBOB-CAG-iCre-SD construct.** RosaYFP MEFS were cultured and infected with 200 MOI of each construct. MEFS were examined at 1 and 2 weeks post infection for YFP expression (i.e., evidence for recombination).

**In vivo evaluation of lenti-Cre recombination efficiency:** Inguinal mammary glands of n=14 8-10 week old RosaYFP Cre reporter mice were injected with 10μl of the pBOB-CAG-iCre-SD (~10^7 PFU) and were examined at 4 weeks and 2 months post injection for analysis of YFP expression i.e., evidence of recombination. YFP expression by IHC was not detected. Therefore, in vivo evaluation of the most promising lenti-Cre candidate demonstrated at most very poor recombination efficiency when injected into adult mammary glands. These results prompted us to revisit our original plan and design an alternative GEM modeling strategy which avoids the use of lenti-Cre for recombination.

**1d. Evaluate optimized dose in study mice (Months 3-6).** A total of 30 mice will be used. Lentiviral-Cre will be delivered via intraductal injection and recombination will be monitored by PCR of genomic DNA and by IHC for T121 expression.

Completed. Using the no-incision intraductal injection technique developed in Task 1c, we injected n=22 mice from the original GEM model with 10μl of the pBOB-CAG-iCre-SD (~10^7 PFU). Excised mammary glands were examined by IHC at 2 weeks, 4 weeks and 2 months post injection to evaluate for T121 expression, and therefore Rb inactivation. T121 protein expression was not visualized, further confirming the poor recombination efficiency achieved by lentiviral mediated Cre delivery in the adult mouse mammary gland.
**Task 2.** Perform clinically relevant magnetic resonance imaging characterization of preinvasive neoplasias and correlate with genotype and molecular features.

### 2a. Obtain regulatory approval (Months -3 to 0).
Upon notice of proposal approval we will commence the regulatory process.

Completed. Regulatory approval to conduct the studies outlined in Task 2 was granted by the NCI-Frederick Small Animal Imaging Program, NCI-Frederick Animal Care and Use Committee, the NCI-Frederick Institutional Biosafety Committee and also through the DOD ACURO process.

### 2b. Effect of virus injection on MRI of the normal mammary gland (Months 3-6).
A total of 10 mice of the FVB inbred strain will be used. Right side inguinal glands will be injected with lentiviral Cre and compared with left side uninjected glands on MRI.

Completed. The right inguinal glands of n=10 8-10 week old FVB mice were injected with 10μl of the pBOB-CAG-iCre-SD (~10^7 PFU) using the no-incision intraductal injection technique. These mice were imaged 19 days later using a **noncontrast T_1 3D MRI protocol** (T1 FFE, 3D, TR/TE 26/2.9ms, in plane resolution=0.2 mm, slice thickness=0.4 mm, field of view (FOV)=30×20×9 mm, number of excitations (NEX)=8, flip angle (FA)=25°, no respiratory gating). There were no qualitative or quantitative (SNR) differences found in the MRI presentation of the right (injected) and left (uninjected) inguinal glands. Therefore, injection with lentiviral-Cre does not result in a long-term measurable difference in the MRI presentation of the normal mouse mammary gland.

### 2c. Sensitivity/specificity study and DCEMRI (Months 7-24).
The same mice from Task 1e will be used. Mice will be imaged with rapid noncontrast T_1 3D MRI and high temporal resolution dynamic contrast enhanced MRI (DCEMRI). The rapid noncontrast T_1 3D images will be compared with pathologic evaluation to calculate the sensitivity/specificity of MRI. The DCEMRI data will be analyzed using several clinically relevant quantitative parameters.

Task 2c commences at Month 7 of the SOW, and therefore falls outside of the 6 month reporting period. Nonetheless, some progress was made in this subtask with regards to imaging technique development. As noted above, our original GEM modeling strategy did not yield effective mouse models of DCIS due to an insufficient initiating event. Without an effective mouse model, imaging technique development is not possible. Therefore, after obtaining DOD ACURO approval via a modification request (see Appendix A) we turned to the C3(1) SV40 Tag mouse model of breast cancer for imaging technique development; see the draft manuscript in Appendix C for more details. To summarize the study, we developed a high resolution, multi-modality strategy for imaging preinvasive mammary neoplasias in the C3(1) SV40 Tag GEM model. We performed high resolution MRI on a 3Tesla clinical scanner, using noncontrast T_1 and T_2 weighted acquisitions and dynamic contrast enhanced MRI. Preinvasive lesions were readily identified on noncontrast T_1 and T_2 weighted acquisitions. Subsequently, MRI-directed ultrasound was performed and an agar grid was used to correlate MRI with ultrasound and histology. We found that both MRI and ultrasound were able to detect preinvasive neoplasias with high sensitivity (over 90% in the posterior inguinal mammary glands). This study established an imaging strategy for monitoring mouse models of DCIS, with rapid imaging acquisitions in two different modalities. This work sets the stage for combining advanced imaging and mouse modeling techniques to gain relevant insights on image-based biomarkers of DCIS. We also performed a pilot serial imaging study in n=10 C3(1) Tag mice to demonstrate the feasibility of using noncontrast T1 3D MRI to follow lesion development over time (over two months, imaging the mice every few days). Our plan was to apply these imaging strategies to the alternative GEM models, so as to achieve our goal of investigating the capacity for quantitative in vivo MRI features to represent underlying molecular signatures of DCIS. Note that the MRI datasets acquired from the C3(1) Tag mice were compiled into a publically available database in the Mouse Mammary collection of the National Biomedical Imaging Archive (NBIA) to facilitate sharing of our results with the larger breast cancer research community.
KEY RESEARCH ACCOMPLISHMENTS

The following key research accomplishments were achieved during the abbreviated 6 month reporting period:

- **New no-incision intraductal injection technique** was developed and optimized. With an over 60% success rate, this new approach matches the reported efficacy of the y-incision intraductal injection technique, while minimizing the duress to the mouse and expanding the opportunity for experimental manipulation to occur shortly after injection. Although the lentiviral mediated delivery of Cre recombinase proposed in the original GEM modeling strategy was found to be inefficient, the no-incision intraductal injection technique can be used for a wide range of purposes. It has been shared with several of our very interested collaborators.

- **New MRI and multimodality imaging techniques** for preinvasive mouse mammary cancer were developed and optimized (see Appendix C). This includes highly sensitivity (over 90%) and rapid (~5 minute acquisition) screening MRI techniques for detection preinvasive malignancies in a GEM model.

REPORTABLE OUTCOMES

The following reportable outcomes were achieved during the abbreviated 6 month reporting period:

- **New database** of mouse mammary gland MR images, uploaded to the National Biomedical Imaging Archive (https://wiki.nci.nih.gov/display/CIP/Mouse+Mammary). The Mouse Mammary Collection consists of 25,998 MR images of mouse mammary glands. It has been placed on the web-accessible NBIA to facilitate sharing of our research findings with the larger community.

- **Manuscript** draft in preparation, summarizing the efficacy of MRI for detection and characterization of preinvasive mammary cancers in mice (see Appendix C).

- **New employment**. The PI on this grant accepted a new permanent position with the breast imaging group at the U.S. Food and Drug Administration in February 2013. Obtaining this DOD Postdoctoral Award was an important qualification for the new position, as it underscored her interdisciplinary training and expertise in medical physics, breast cancer biology and animal modeling.

CONCLUSION

Our main goal in this proposal was to investigate the capacity for quantitative *in vivo* MRI features to represent underlying molecular signatures of DCIS, using the preclinical GEM model framework to our hypotheses. We were particularly interested in three key molecular pathways—Rb, p53, BRAC1—and whether MRI signatures can identify these molecular subtypes of disease. Early on in our endeavors as we executed the Tasks outlined in the SOW, we discovered limitations in our original GEM modeling strategy suggesting that our proposed studies could not be effectively performed. We therefore designed an alternative GEM modeling strategy that successfully addressed these limitations. We also developed novel MRI techniques to detect, characterize and follow progression of the preinvasive malignancies in the mouse mammary gland at high spatial resolution, and high frequency (rapidly imaging every few days). Although a new employment opportunity resulted in the duration of this award being cut short, the progress made in the first six months of this award, including the pitfalls and solutions, was exciting and informative.
REFERENCES


APPENDICES

The following appendices provide relevant supplementary information to what has been presented in this report.

- Appendix A: Approved modification request submitted to the DOD proposing our alternative GEM modeling strategy
- Appendix B: Published article from the group of our collaborator, Dr. Karl Simin, providing additional
information on our alternative GEM modeling strategy

- Appendix C: Manuscript draft on imaging technique development in C3(1) SV40 Tag mice
Modification request for BC112281: *Investigating ductal carcinoma in situ using noninvasive imaging of genetically engineered mouse models.*

**Background/Justification**

The key scientific element in Specific Aim #1 is to establish mouse models of preinvasive breast cancer by disrupting the *Rb, BRCA1* and *p53* pathways singly and in combination. We contend that these genetic events will produce diverse preinvasive mammary carcinomas that can be studied with noninvasive imaging (in Specific Aim #2). Based on our extensive experience in many tissue types, inactivation of the *Rb* tumor suppressor pathway is likely the critical initiating event for tumorigenesis; additional loss of *BRCA1* and *p53* are likely progressing events. We proposed to utilize two novel tools for implementing these genetic events in the mouse:

- **Using T121 protein to inactivate the Rb family for tumor initiation.** Inactivation of the Rb tumor suppressor family (which includes Rb, p107, p130) is a critical step for cancer initiation. We use a novel protein T121 to functionally inactivate all three family members, which is a much more efficient route to Rb inactivation than genetic deletion. We proposed to use a keratin 18 (K18) promoter to target conditional T121 expression to luminal epithelial cells (*K18-eGT\(_{121}\)tg/+ mice). 
- **Using viral-Cre mediated tumor induction.** The proposed mouse models are conditional, meaning that the genetic mutations occur only after exposure to Cre recombinase. In this way, the timing of malignant transformation can be experimentally controlled. We proposed to deliver Cre recombinase using lentiviral vectors. This has the advantage of inducing tumorigenesis focally, which would greatly improve our capacity to image single lesions over time.

However, recent evidence obtained in the past several months suggests that our initially proposed strategy may not efficiently induce tumor initiation:

- **Limitation 1: Using the K18 promoter to express T121 is inefficient for tumor initiation.** *K18-eGT\(_{121}\)tg/+ mice* only weakly express T121 in mammary epithelial cells, and thus may not inactivate the Rb family sufficiently for tumor initiation.
- **Limitation 2: Using viral-Cre is inefficient for tumor induction.** Lentiviral delivery of Cre recombinase inefficiently induces recombination and thus tumorigenesis. Indeed, this was an anticipated pitfall we acknowledged in our proposal: “Technically, the most significant potential pitfall is that the lentiviral-Cre may not be sufficiently effective to induce tumorigenesis... Given our desire for focal induction, we will first evaluate other viral vectors for delivery and subsequently try a mammary specific Cre line, such as WAP-Cre transgenic mice.”

These recent technical developments may significantly impact the efficacy of our initially proposed strategy, as it will delay or perhaps even preclude a tumor phenotype from developing.

**Modification request**

We thus propose the following modifications that will ensure a much higher likelihood of success in testing our scientific hypotheses:

- **Modification 1: Use MMTV-eGT\(_{121}\)tg/+ mice, demonstrated to efficiently express T121 and yield mammary tumor initiation.** These mice, developed by our collaborator Dr. Karl Simin, use the MMTV promoter to target conditional T121 expression to luminal mammary epithelial cells. We plan to obtain from Dr. Simin either live mice or frozen materials for rederivation. Dr.
Simin has successfully used these mice to initiate mammary tumorigenesis, including in the context of additional BRCA1 and p53 loss (results are now in press, PLoS Genetics). Therefore, we are highly confident that MMTV-eGT\textsubscript{121}tg/+ mice will efficiently yield tumor initiation, thereby mitigating Limitation #1 noted above.

- **Modification 2: Use other Cre delivery strategies demonstrated to be effective in the mammary gland.** As stated in our proposal, in addition to evaluating other lenti-Cre constructs, we will utilize mammary specific Cre lines for genetic recombination, specifically WAP-Cre and K14Cre, which are widely utilized in mouse models of breast cancer (Liu et al. PNAS 2007, Li et al. Cancer Cell 2007). In doing so, we will mitigate Limitation #2 noted above.

- **Modification 3: Concurrent imaging technique development with C3(1)SV40Tag mice.** Specific Aim #2 of our proposal involves a significant component of imaging technique development prior to the image-based characterization of our T121-based mouse models. The heretofore described limitations and modifications imply that it may be some time before we can use the T121-based models for this purpose. To facilitate timely completion of our aims according to the SOW, we thus propose to use the C3(1)SV40Tag transgenic mouse model of breast cancer (Green et al, Oncogene 2000) for imaging technique development. In this way, once we have implemented Modifications 1 and 2 we will be ready to proceed with the image-based characterization of our T121-based mouse models.

We are not revising the scientific purpose, methodology or evaluation outlined in the original proposal. Importantly, the targeted genetic events are not changed—Rb pathway inactivation, BRCA1 loss and p53 loss. Rather, these minor modifications alter some of the technical methods used to achieve the same scientific ends. These technical modifications will put us in a much stronger position to test our scientific hypotheses.
Cooperativity of \( Rb \), Brca1, and \( p53 \) in Malignant Breast Cancer Evolution

Prashant Kumar\(^1\), Malini Mukherjee\(^2\), Jacob P. S. Johnson\(^1\), Milan Patel\(^1\), Bing Huey\(^3\), Donna G. Albertson\(^3\), Karl Simin\(^1\*)

\(^1\) Department of Cancer Biology, University of Massachusetts Medical School, Worcester, Massachusetts, United States of America, \(^2\) Department of Pediatric Hematology/Oncology, Texas Children’s Cancer Center, Baylor College of Medicine, Houston, Texas, United States of America, \(^3\) Helen Diller Family Comprehensive Cancer Center, University of California San Francisco, San Francisco, California, United States of America

Abstract

Breast cancers that are “triple-negative” for the clinical markers ESR1, PGR, and HER2 typically belong to the Basal-like molecular subtype. Defective \( Rb \), \( p53 \), and \( Brca1 \) pathways are each associated with triple-negative and Basal-like subtypes. Our mouse genetic studies demonstrate that the combined inactivation of \( Rb \) and \( p53 \) pathways is sufficient to suppress the physiological cell death of mammary involution. Furthermore, concomitant inactivation of all three pathways in mammary epithelium has an additive effect on tumor latency and predisposes highly penetrant, metastatic adenocarcinomas. The tumors are poorly differentiated and have histologic features that are common among human \( Brca1 \)-mutated tumors, including heterogeneous morphology, metaplasia, and necrosis. Gene expression analyses demonstrate that the tumors share attributes of both Basal-like and Claudin-low signatures, two molecular subtypes encompassed by the broader, triple-negative class defined by clinical markers.


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* E-mail: karl.simin@umassmed.edu

Introduction

The dire need for more effective treatments for aggressive breast cancers has motivated intensive investigations into their cellular and molecular etiology. Breast cancers classified as “triple-negative” by clinical diagnostic markers (ESR1, PGR, and HER2 negative) are heterogeneous in their clinical behavior, morphology, and molecular biology. Triple-negative breast cancers (TNBC) typically express the Basal-like molecular signature, thus TNBC and Basal cancer classifications are frequently used interchangeably. However, they are not completely synonymous [1,2]. TNBCs also include the Claudin-low molecular subtype [3], which is characterized by greatly reduced expression of intercellular junction components and by activation of molecular pathways associated with epithelial-to-mesenchymal transition (EMT), cancer stem cells, and the immune response [4].

Histologically, most triple-negative breast cancers are invasive ductal carcinomas, but TNBCs also include the metaplastic, medullary, and adenosquamous histologic special types, distinctive morphologies that are prevalent among Claudin-low tumors [4]. TNBCs are insensitive to endocrine therapy and HER2 antagonists, but they are sensitive to chemotherapy. Nevertheless, long-term patient outcomes are poor due to high rates of relapse and acquired chemoresistance [5,6]. Mouse models that mimic the complexity of TNBC will be invaluable tools for defining the diverse cellular biology and behavior of these tumors and for rigorously triaging new drug candidates.

Basal-like breast cancers often simultaneously inactivate three tumor suppressors that are infamous for their roles in familial cancers: \( Rb \) (\( RB1 \)) [7], \( p53 \) (\( TP53 \)) [8], and \( Brca1 \) [9]. \( p53 \) is mutated in 20–30% of human breast cancers and defective pathway intermediates also increase breast cancer risk [10]. Moreover, nearly all Basal-like cancers with \( Brca1 \) mutation have concomitant \( p53 \) mutation [11]. Germline \( Brca1 \) mutation predisposes early-onset breast cancers that are often triple-negative and that correlate with Basal-like tumors in microarray analyses [9]. \( Brca1 \) mutation accounts for nearly half of familial breast cancers (OMIM 604370), but \( Brca1 \) is also down-regulated in sporadic breast tumors without germline mutation [12]. The overall effect of \( Brca1 \) loss is likely pleiotropic. The best studied function of \( Brca1 \) is in orchestrating DNA double-strand break repair through homologous recombination or non-homologous end-joining. The importance of defective DNA repair in breast cancer is underscored by the observation that all known genes associated with inherited forms of the disease safeguard genomic integrity [13]. \( Brca1 \) also functions as a transcription factor [14] that appears to be required for the differentiation of stem/progenitor cells into mature luminal cells [15]. This finding is consistent with the recent characterization of \( Brca1 \)-associated tumors as aberrant luminal progenitor cells [16].

The pair-wise cooperativity of \( Brca1 \) and \( p53 \) in mammary tumorigenesis has been studied in mouse models [17]. We and others have investigated the impact of combined \( Rb \) and \( p53 \) inactivation on mammary tumorigenesis \emph{in vivo} [18–20]. We showed that following \( Rb \) perturbation \emph{in vivo}, tumor progression is limited largely by \( p53 \)-dependent apoptosis, and that loss of the second \( p53 \) allele in \( Mft121+/p53^{−/−} \) tumors is likely a prerequisite...
for mammary tumor progression [19], since the vast majority of tumors lose the wild type p53 allele during tumorigenesis. In the context of a brain carcinoma model initiated by T121, apoptosis appears to be the critical function of the normal p53 allele that is the target of selective pressure [21].

Our motivation to combine Rb inactivation with Brca1 and p53 mutation derives, in part, from the observation that the Rb gene is among the most frequently deleted loci in Brca1/p53-mutated mouse tumors [22], indicating that Rb is a critical barrier to tumor progression. Rb pathway inactivation is strongly associated with human triple negative breast cancers. A cardinal feature of basal-like breast cancers is the abundant expression of the “proliferation cluster” genes [7], which include many E2F-regulated genes that are de-repressed following Rb inactivation. In human breast cancers, reduced pRb activity correlates with higher tumor grade [23], but also predicts improved chemotherapy responsiveness [24]. The Rb gene itself is mutated in breast cancer [25], and recent genominc studies have indicated an overrepresentation of mutations within pRb-binding sites of human gene regulatory domains [26].

In this study we show that mammary tumors caused by inactivation of the pRb family (pRbf) of proteins (pRb, p107, p130), together with Brca1 and p53 inactivation, mimic several aspects of the most aggressive forms of breast cancer, including rapid tumor progression, poor differentiation, distant metastasis, necrosis, metaplasia, and genomic instability. Our findings illustrate the compounding effect of acquiring multiple tumor suppressor mutations during tumor evolution and underscore the distinct requirements of each of these canonical tumor suppressor proteins.

Results

Conditional T121 expression in mammary epithelium

We constructed the MFT121 (MMTV-Floxed-eGFP-T121) transgene to conditionally inactivate the pRb family (pRbfl) of pocket proteins in mammary epithelium (Figure 1A). The T121 protein is an amino-terminal fragment of the SV40 large T antigen that perturbs pRbfl activity and predisposes tumorigenesis in a range of tissues [27]. While Rb inactivation alone is sufficient to induce mammary tumors [20], the shorter latency of TgWAP-T121 tumors indicates there is functional redundancy or compensation by the related pocket proteins p107 or p130 [19]. In the MFT121 model, the MMTV-LTR promotes mammary-specific transgene expression (Figure 1B). The approach of inactivating pRbf, Brca1, and p53 specifically in mammary epithelial cells via the Wap-Cre transgene [28] enabled us to avoid the appearance of lymphomas [20] or sarcomas [29]. Wap-Cre excised the LoxP-eGFP-stop-LoxP reporter cassette and initiated T121 expression in ductal and alveolar luminal epithelial cells (Figure 1C). Virgin glands of TgWap-T121; WAP-Cre mice appeared normal. Lactating glands (WAP-Cre-induced) showed reduced alveolar density, similar to the gland atrophy phenotype in the TgWap-T121 model, which was associated with apoptosis caused by T121-induced proliferation [19] and lactation defects observed in WAP-Cre; Rbfl/fl; p107/fl; mice [20].

Concomitant pRb, p53, and Brca1 inactivation significantly accelerates tumor onset

Multiparous TgMFT121; TgWap-Cre mice remained tumor-free for more than a year after Cre-induction, but mice that were either...
heterozygous or homozygous for a conditional p53 allele [30] (TgMFT121; TgWAP-Cre; p53+/− or TgMFT121; TgWAP-Cre; p53−/−) developed mammary tumors with 100% penetrance and clear evidence of p53 haploinsufficiency (Figure 1D, p < 0.0001, log-rank test). Heterozygous p53 mice developed tumors with a median latency of 299 days, while p53 homozygous mice had a median tumor latency of 150 days. These results are consistent with previous findings that p53 activity is rate limiting for mammary tumor progression initiated by Rb inactivation [18–20]. In contrast to the high penetrance we previously found in our TgWAP-T121 breast tumor model [19], the present studies indicate that the TgMFT121 transgene only partially inhibits Rbβ pathways, possibly owing to the reduction in the transgene gene copy number following Cre excision. Similar dosage effects were observed in a conditional T121 transgenic model of astrocytoma [31].

Breαl mutation dramatically accelerated tumor onset in mice with Rbβ/p53 inactivation (Figure 1D, p < 0.0005). Only a single mouse (n = 13), doubly defective for Rbβ and Breαl activity (TgMFT121; TgWAP-Cre;Breαlfl/fl), developed mammary tumors at 386 days following Cre induction by multiple pregnancies. In contrast, 100% (n = 10) of TgMFT121; Breαlfl/fl p53−/−, TgWAP-Cre mice (hereafter, TBP) developed mammary tumors with a median latency of only 47 days. Thus, a single pregnancy was sufficient for 100% TBP tumor penetrance. Once palpable, TBP tumors grew rapidly to 1500 cc within several days (data not shown). Therefore, inactivation of the three canonical tumor suppressors showed additive effects on tumor latency, since TBP mammary tumors developed significantly faster than did pair-wise combinations.

Enhanced survival and increased proliferation rates cause rapid tumor progression in pRBβ/p53/Breαl-perturbed epithelium

To investigate the early effects of tumor suppressor inactivation, we examined mammary gland biopsies (n = 5 mice) from time points (0, 2, 6 weeks) following forced weaning at Day 1 parturition. At the earliest time point, T121 expression alone was sufficient to cause benign, hyperproliferative lesions (Figure 2A, 2B). T121-expressing cells showed a higher Ki67 index than did cells without T121, as expected (p < 0.0063, Figure 3P). Combinations with Breαl and p53 mutation caused higher grade, premalignant lesions (Figure 2C–2F). Tall columnar epithelia of darkly staining cells and papillary tufting (Figure 2C) were characteristic of the disrupted epithelial morphology. Pleomorphic, faintly staining nuclei with prominent nucleoli were common among T121 expressing cells (Figure 3F).

The combined inactivation of pRbβ and p53 also dramatically suppressed the physiologic cell death of mammary involution (Figure 3I, 3N), although pRbβ perturbation alone had little effect (Figure 3B, 3G, 3L). It was shown that p53 mutation alone delayed involution by several days [32]. Here, we observed that the combined inactivation of pRbβ and p53 blocked involution through six weeks (Figure 3I, 3N). Biopsies from mice harboring Breαl mutation (Figure 3C, 3J) showed extensive cellular debris from dead or dying cells within their lumen. The dual loss of pRbβ and Breαl activities (TgMFT121; TgWAP-Cre;Breαlfl/fl) triggered elevated cell death that was p53-dependent (p < 0.0001, Figure 3Q). The combined loss of pRbβ, Breαl, and p53 activities accelerated tumor progression, indicated by frank tumors that appeared by six weeks (Figure 3O). However, when measured at the earliest time point, neither an increased Ki67 proliferation index (Figure 3P) nor a decreased cell death index (Figure 3Q) presaged faster onset of TBP tumors compared to Rbβ/p53 tumors. Thus, full transformation occurred in only a minority cell population, which likely reflects the requirement for additional collaborating oncogenic events.

Histopathologic features of tumors with conditional inactivation of pRbβ, p53, and Breαl

T121/p53 (TP) and TBP mice developed high grade mammary adenocarcinomas with heterogeneous phenotypes indicative of highly perturbed differentiation, summarized in Table 1. Tumors of both genotypes showed mixed solid and glandular morphologies. More TBP mice than TP mice (13/14 vs. 8/16 cases, p = 0.0169, two-tailed Fisher’s exact) developed solid tumors that were largely devoid of glandular architecture (Figure 4A). All solid tumors showed a mixture of pushing and infiltrative borders, and nearly half of the tumors were vascularized (Figure 4A). All carcinomas expressed varying levels of luminal epithelial markers, including Keratin-8 (Figure 4E, 4G, 4K) and E-Cadherin (Figure 4I, 4L). These markers were abundant in well-differentiated tumor regions but were greatly diminished or absent in poorly differentiated areas (Figure 4E, 4I, 4K, 4L). Nests of carcinoma cells variably expressed basal/myoepithelial lineage markers (Keratin-5, -14, Figure 4E, 4K).

Both TP and TBP mice (4/16 vs. 1/14 cases, p = 0.3359) developed carcinosarcomas (Figure 4C), also known as “EMT” (Epithelial to Mesenchymal Transition) tumors [33]. These tumors are comprised of faintly-staining, fusiform, spindleoid cells and are characteristic of p53 mutant mouse. The histology of hematoxylin and eosin stained carcinosarcomas is relatively homogeneous and is visibly distinct from carcinomas with spindloid metaplasia that showed biphase carcinomatous and spindleoid morphologies (Figure 4B, 4G). Among both carcinosarcomas and metastatic tumors, we observed dual expression of mesenchymal (Vimentin) and epithelial (Keratin-8) markers (Figure 4G), which are mutually exclusive lineage markers in the normal gland. Dual expression is also a feature of human Claudin-low [4] and mouse EMT tumors [33]. Poorly differentiated spindleoid cells along invasive tumor fronts showed either dual expression (Figure 4G) or greatly diminished epithelial marker expression (Figure 4J–4L).

In both TP and TBP tumors, we observed squamous metaplasia that was characterized by keratin nests (Figure 4M) and high expression of Keratin 6 (Figure 4N), a marker of progenitor cells that is expanded in Wnt-1-induced mammary tumors. Four of fourteen cases (29%) of TBP tumors, but zero cases of TP tumors (p = 0.0365), included whorls of spindleoid cells that resembled myoepithelial cells (not shown). Finally, central necrosis, a hallmark feature of human BRCA1-mutated tumors, was present in twelve of fourteen TBP cases, a greater proportion than the eight of sixteen T121/p53 cases (p = 0.0577), indicating that the selective pressure of cell death persists even in the absence of p53 activity.

Distant metastases were observed in the lungs of both TP (4 of 14 cases) and TBP (3 of 6 cases) mice (Figure 4P), which is noteworthy because relatively few transgenic mammary tumor models metastasize. Among mouse models that do metastasize, most are derivatives of the PyMT model, which more closely resembles human Luminal subtype tumors than Basal-like tumors [3]. Metastases were not evident in the sternum, liver, or spleen of these mice. A primary cell line established from a TBP tumor formed secondary tumors following serial transplantation into syngeneic (FVB) mammary fat pads (Patel, unpublished), confirming the malignant capacity of TBP tumors.

In summary, TBP tumors displayed heterogeneous histology, including high grade, central necrosis, metaplasia, pushing boarders, and metastasis, features that are characteristic of human BRCA1-mutated and Claudin-low breast tumors.
Global gene expression analysis

The majority of the \( T_{\text{G}MFT121} \) mouse tumors (76%) co-segregated with human Basal-like breast cancers by hierarchical clustering of the top \( \sim 1000 \) most variable genes in a combined data set of mouse (\( n = 135 \)) and human (\( n = 337 \)) tumor expression profiles (Figure 5, Figure S1, File S2). Using a similar approach that we reported previously \cite{3}, we assayed global transcript levels by microarray using tumors derived from TBP (\( n = 8 \)) and TP mice (\( n = 9 \)), and we compared them directly to tumors and normal tissue from other mouse models \cite{3} and patient samples \cite{4}. Two TP tumors clustered with human tumors that showed a mixture of the PAM50 molecular subtypes that were assigned by Prat and colleagues \cite{2010}. The two remaining TP tumors clustered with Claudin-low subtype human tumors.

The effect of \( \text{Bra}c1 \) mutation on TP tumors was more evident when we focused our analysis on the mouse specimens alone (Figure 6, File S3). 56% of the TP tumors (5 of 9) clustered with mouse tumors that we previously showed resemble human Luminal tumors \cite{3}. \( \text{Bra}c1 \) mutation shifted the tumor phenotype (\( p = 0.0529 \), Fisher’s exact). All eight TBP tumors segregated with mouse tumors, including other \( \text{Bra}c1 \)-mutant models that more closely resemble human Basal-like TNBC (Figure 6A). TBP tumors and the other Basal-like mouse tumors expressed low levels of luminal markers and high levels of both Proliferation and Basal cluster transcripts, including \( \text{Keratins-14, -6b, -17} \) (Figure 6C). In contrast, the TP tumors that clustered with Luminal-B-like tumors (Figure 5A, blue box) showed higher expression of luminal marker genes that correlate with the estrogen pathway target \( \text{Xbp1} \) (Figure 5A). Interestingly, TBP tumors were distinct from most other Basal-like mouse tumors in their elevated expression of a subset of Claudin-low signature genes \cite{3,4}, including \( \text{Snail1, Tgfbi, Dtr, and Timp1} \) (Figure 6C).

Four TP tumors (44%) did not segregate with Luminal-like tumors. This finding is consistent with previous reports by us and
Figure 3. Combined inactivation of pRbf and p53 causes a durable block in involution. Lactating mammary epithelium of Cre-negative control mice (A, F, K) and T121-expressing mice (B, G, L) involute normally (A–E: 0 wks, F–J: 2 wks, K–O: 6 wks). T121-expressing mice have reduced alveolar density. Cell death and debris were abundant in TB glands (arrows panels C, J). TP glands failed to involute (N) and persisted as highly cystic glands. Frank tumors were present by 6 wks in TBP mice. An invasive adenocarcinoma fills the field of panel O. Measured at 0 wks, T121 increased the Ki67 index but without added effect by Brca1 and/or p53 loss (P). Combined inactivation of pRbf and Brca1 increased TUNEL-positive cells, which was suppressed by p53 loss (Q, p<0.0001). For each genotype n = 5 mice. Original magnification of each panel was 200×.

Table 1. Characteristics of TP and TBP tumors.

<table>
<thead>
<tr>
<th>Histologic Feature</th>
<th>TgMFT&lt;sub&gt;T121&lt;/sub&gt;:Brca1&lt;sup&gt;f/f&lt;/sup&gt;, p53&lt;sup&gt;f/f&lt;/sup&gt;</th>
<th>TgMFT&lt;sub&gt;T121&lt;/sub&gt;: p53&lt;sup&gt;f,+&lt;/sup&gt;</th>
<th>TgMFT&lt;sub&gt;T121&lt;/sub&gt;: p53&lt;sup&gt;f/f&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 14 (%)</td>
<td>n = 11</td>
<td>n = 16</td>
</tr>
<tr>
<td>Solid*</td>
<td>13 (93)</td>
<td>6 (55)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Glandular</td>
<td>4 (29)</td>
<td>2 (18)</td>
<td>6 (38)</td>
</tr>
<tr>
<td>Spindloid metaplasia</td>
<td>9 (64)</td>
<td>4 (36)</td>
<td>5 (31)</td>
</tr>
<tr>
<td>Carcinomasarcoma</td>
<td>1 (7)</td>
<td>3 (27)</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Squamous Metaplasia</td>
<td>2 (14)</td>
<td>2 (18)</td>
<td>4 (25)</td>
</tr>
<tr>
<td>Myoepithelioma*</td>
<td>4 (29)</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Cribriform</td>
<td>0 (0)</td>
<td>1 (9)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Invasion</td>
<td>9 (64)</td>
<td>4 (36)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Apoptosis/Necrosis&lt;sup&gt;1&lt;/sup&gt;</td>
<td>12 (86)</td>
<td>7 (64)</td>
<td>8 (50)</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>1 (7)</td>
<td>1 (9)</td>
<td>5 (31)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>5 (36)</td>
<td>5 (45)</td>
<td>7 (44)</td>
</tr>
</tbody>
</table>

Significant at p<0.05.
*two-tailed.
†one-tailed Fisher’s exact.
Many tumors showed multiple, mixed morphologies; therefore, the percentage reflects the proportion of tumors showing the phenotype within each genotype. We saw no differences due to the initial p53 allele status (f/+ vs. f/f). We combined these classes (n = 11+16=27) for statistical comparison to tumors with Brca1 mutation (n = 14).

doi:10.1371/journal.pgen.1003027.g003
Figure 4. Perturbed differentiation in TBP tumors. Poorly differentiated tumors with solid morphology were most common (A), with and without vascularization (white arrows) or central necrosis (asterisk). Other tumors retained remnant glandular architecture (B, white arrows). Metaplastic cells invaded adjacent muscle and stroma (B, black arrow). Homogeneous spindloid cells of a carcinosarcoma entrap carcinomatous cells (C, 100×). Keratin-8 (Krt8, green) and Keratin-5 (Krt5, red) immunolabeling of luminal and myoepithelial cells, respectively (D, E, K). Greatly reduced expression of both Krt5 and Krt8 (E dashed lines, and K arrow). DAPI staining (blue) indicates the high cellularity of the region devoid of epithelial markers in panel E (F). Metaplastic tumor cells (G) with dual staining of Krt8 (red) and the mesenchymal marker Vimentin (green), or reduced Krt...
staining (K). Abundant E-cadherin (CDH1, green) in normal adjacent (H) or well-differentiated tumor (I). Reduced or absent CDH1 along invasive edge (J). E-cadherin staining (K). Abundant E-cadherin (CDH1, green) in normal adjacent (H) or well-differentiated tumor (I). Reduced or absent CDH1 along invasive edge (J).

others that Rb/p53 tumors can also resemble TNBC and the Claudin-low molecular phenotype [3,18,20]. A single TP tumor clustered among the previously designated Group II tumors (Figure 6A, yellow box), which are the paradigm cases of the Claudin-low subtype [3]. In addition, a single TP tumor clustered with tumors in a group that we refer to as the Claudin-low molecular phenotype [3,18,20]. A single TP tumor is one of the most frequently detected loci among Brea1/p53-mutated mouse tumors [22].

Pathway analysis

The similarity between TPB tumors and human Claudin-low and Basal-like cancers was also evident from pathway analysis of up-regulated genes of each of the three tumor types (Figure 7A, File S1). We queried the KEGG (Kyoto Encyclopedia of Genes and Genomes) and GO (Gene Ontology) databases with lists of genes that were differentially expressed by TPB tumors (see Methods) and by human Claudin-low and Basal-like cancers [4]. Cytokine, chemokine, and MAPK signaling pathways ranked highly among both Claudin-low and TPB tumors. Pathways that are enriched in cancers of diverse origins ranked highly in both Basal-like and murine TPB tumors.

The GO terms associated with the respective tumor types were consistent with the enriched KEGG pathways. Cell-cycle progression (GO:0007049, p = 2.43551E-59) and DNA repair (GO:0004484, p = 6.95081E-22) dominate the list of functions enriched in Basal-like tumors (File S1). Similarly, regulation of cell proliferation (GO:0042127, p = 6.01E-13) is among the top terms for TPB tumors. The three top scoring, inter-related GO terms for TPB tumors are regulation of developmental process (GO:0050793, p = 7.50E-16), organ morphogenesis (GO:0009887, p = 3.53E-14), and tissue development (GO:0009988, p = 1.36E-13). These GO terms are reflective of the enrichment of the Wnt, ErbB, TGF-β, and VEGF signaling pathways identified by KEGG pathway analysis. Claudin-low tumors are enriched for wound (GO:0006911, p = 4.29939E-66) and inflammatory responses (GO:0006954, p = 1.26817E-30), which are also among the top functions associated with TPB tumors (7.37E-13 and 6.46E-12, respectively).

CGH analysis

Given the requirement for BRCA1 in DNA damage repair and centrosome regulation, we tested the hypothesis that TPB tumors harbor more genomic copy number aberrations (CNAs) than do TP tumors with intact Brea1. We enumerated CNAs by counting “copy number transitions,” the number of changes in the CGH profile from one copy number level to another that occur within chromosomes [34]. Unexpectedly, we found no statistically significant difference (p = 0.8374) in the mean number of CNAs between TPB tumors (n = 8) and TP tumors (n = 10) using array-based comparative genomic hybridization (aCGH).

The low multiplicity of TPB and TP tumors (1-3 per mouse) and their latency indicate that combinations of pRbf, Brca1, and p53 pathway perturbations are not sufficient for malignant transformation in our models. To identify potentially collaborating oncogenic events, we manually curated loci with copy number changes (see Methods). In nine tumors (50%), we observed recurrent losses of large, variable regions spanning chr4 and chr10 (Figure 8). Both chromosomes harbor many potential tumor suppressors, including regulators of cell death, such as Tn2d1, Up111, Tgf73, Df2a, Runx3, Lck, Dho234, Faf1, Pax7, and Casp9, and effectors of cell death, such as Cal18a1, Gadd45b, Dapk3, and Casp14. Among all the tumors assayed (n = 18), we identified nearly five-hundred loci (Table S9 in File S1) with potential copy number gains. Approximately half of the genes are included on curated lists of cancer-associated genes, including the Cancer Gene Census (Sanger Institute) and the KEGG Pathways in Cancer. We observed focal amplification of several canonical proto-oncogenes, including e-Myc amplification (log2ratio = 3.64, p < 0.0001) in a single TP tumor, H-ras amplification in two of ten TP tumors, and K-ras amplification in two of eight TBP tumors. Pathway analysis of these five-hundred putative collaborating genes revealed enrichment of several signaling pathways, including the MAP Kinase, Focal Adhesion, Wnt, and ErbB pathways (Table S10 in File S1).

Discussion

Here we report a highly penetrant engineered mouse model of TNBC. Our previous work showed that when pRbf and p53 are simultaneously perturbed in mammary epithelium, adenocarcinomas develop with long latency, suggesting a requirement for additional oncogenic events. However, these mouse tumors displayed only limited chromosomal copy number aberrations [19]. Because genomic instability is a hallmark of malignant transformation [35], especially among BRCA1 familial cancers [36] and aggressive sporadic breast cancers [37], we hypothesized that Brea1 mutation would accelerate the tumor development we observed following dual inactivation of pRbf and p53. Our results show that concomitant inactivation of all three tumor suppressor pathways in mammary epithelium has an additive effect on tumor latency and predisposes highly penetrant, malignant carcinomas. Although Brea1 inactivation accelerated tumorigenesis compared to TP tumors, we observed no statistical difference between chromosomal copy number transitions in tumors with or without Brea1, despite the extensive CNAs observed by others in Brea1/p53 tumors [22].

The pRbf-regulated cell cycle network is frequently disrupted in TNBC tumors [7,26,37,38], and the Rb locus is among the most frequently lost in Brea1/p53 mouse tumors [22], indicating that there is strong selective pressure for Rb pathway inactivation. We speculate that direct inactivation of pRbf by T121 may allow TBP cells to escape this rate-limiting barrier of transformation without accruing numerous chromosomal aberrations. Thus, in the context of defective pRbf and p53 function, tumor progression may be unrelated to the proportion of the genome altered by copy number alterations. It will be important to determine the effect of Brea1 loss on the abundance and identities of somatic mutations that are not detectable by CGH.

The importance of p53 mutation in breast cancers is well documented and is confirmed in the present study. The dual inactivation of pRbf and Brea1 caused markedly increased cell death that was reduced by p53 mutation. p53-independent cell death likely remains a significant barrier to tumor progression among TBP tumors and may account, in part, for the observed loss of genomic regions that harbor cell death regulatory genes, most notably on chr4 where the p53 paralog p73 resides. Identifying the genomic alterations that are conserved across species will be useful
in evaluating the impact of the myriad of CNAs observed in breast cancers and may help to explain the heterogeneity of TNBCs. Brcal mutation not only accelerated tumor development but also shifted the tumor spectrum. Whereas T121/p53 mouse tumors often resembled the Luminal-B molecular subtype breast cancers, which show relatively abundant expression of luminal epithelial cell differentiation markers, TBP tumors consistently shared features of Basal-like and Claudin-low molecular subtypes. Others have argued that Basal-like and Claudin-low gene expression signatures reflect progenitor and stem cell phenotypes, respectively [4,16], consistent with a role for Brcal in mediating stem/progenitor cell maturation [15]. Loss of BRCA1 activity may also alter tumor phenotype through deregulation of the EMT inducer SLUG [39].

The CGH analysis of our mouse tumors revealed CNAs consistent with mutations observed in genomic surveys of human breast cancers [40,41]. Similar to the studies of human tumors, we saw increased copy numbers of known oncogenic driver genes, including myc, egfr, crebbp, jak1, H-ras, and K-ras, as well as enrichment of pathways implicated in tumor progression, including the WNT signaling pathway, regulation of actin cytoskeleton, focal adhesion, cell shape, and mobility proteins. Fewer investigations have focused on genetic deletions and cancer development mechanisms. We also found decreased copy numbers of known tumor suppressors, including map2k, ppp2r, and pten. Given the strong similarities between our mouse model and aggressive human breast cancers, the TBP model provides an invaluable preclinical platform to identify and assess potential therapeutics for aggressive and chemoresistant breast cancer subtypes [42,43].

Materials and Methods

Ethics statement

This study was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health.

Derivation of MFT121 transgenic mice

The LoxP-eGFP-Stop-LoxP cassette and T121-encoding DNA were cloned into EcoRI HindIII sites of MMTV-SV40-Bssk (Addgene plasmid 1824). The LoxP-eGFP-Stop-LoxP cassette was provided courtesy of the T. Jacks lab. Resulting and subsequent generation MFT121 transgenic mice were identified by PCR amplification of a 215-bp fragment using the oligo pair: 5'-GCATCCAGAAGCCTCAAAG-3' and 5'-GAATCTTTGCAGCTAATGGACC-3' complementary to the T121 sequence. Cre transgenic mice were identified using the oligo pair: 5'-TGATGAGGTTCGCAAGAACC-3' and 5'-CCATGAGTGAACGAACCTGG-3'. The cycling profile was 94°C for 2 min., 35 cycles of 94°C for 20 sec., 62°C for 45 sec., and 72°C for 45 sec.; the final incubation of 72°C was for 2 min. We established five TgMFT121 founder transgenic lines, though three lines failed to express the eGFP reporter. We describe here our studies of the single mouse line with higher eGFP expression in virgin mammary glands. eGFP expression was also evident in salivary glands and foot pads in this line (data not shown).
Transgenic breeding strategies

*TgMFT*121;*TgWAP-Cre* mice were mated to *p53* conditional allele mice (*p53f/f*) [44]. *p53* genotypes were determined by PCR using two reactions: neomycin primer 5' TAGCTCAGAGCCGCTTGC-3', *p53* primer 5'-TATACTCAGAGCCGCTTGC-3', 525-bp product; the endogenous *p53* allele; substituting 5'-ACAGCGTGGTGGTACCTTAT-3' for the neo primer, 475-bp product. Cycling parameters were the same as they were for the *T121* reaction. We produced female mice with the genotypes *TgMFT121;TgWAP-Cre;p53f/+* and *TgMFT121;TgWAP-Cre;p53f/f*, and female littermates served as controls. To study the effect of *Brca1* loss, *TgMFT121;TgWAP-Cre* mice were mated to *Brca1f/f*, *p53f/f* mice [44]. *Brca1* genotypes were determined by PCR using two reactions. We generated female mice with the genotypes *TgMFT121;TgWAP-Cre;Brca1f/+*, *p53f/+* and *TgMFT121;TgWAP-Cre;Brca1f/f*, *p53f/f* with nontransgenic (Cre negative) littermate controls for each cohort. Pregnancy induced WAP-Cre transgene expression. Parturition of the first litter was designated as Day 1 for all aging studies. Matings with *TgMMTV-Cre* mice (Line F) yielded small litter sizes; therefore, experiments reported here employed *TgWap-Cre* unless otherwise indicated.

Histopathology and apoptosis assays

A portion of each mammary sample was fixed overnight in 10% phosphate-buffered formalin, transferred to 70% ethanol, and then embedded in paraffin. Samples were sectioned for 10 successive layers at 5-µm intervals and stained with hematoxylin and eosin for histopathologic examination, as described previously. Apoptosis levels were assessed using the terminal deoxynucleotidyl transferase–mediated dUTP-biotin nick end labeling (TUNEL) method with standard protocols. Differences in apoptosis levels between mice with different genotypes were evaluated by the *t* test (*p* < 0.05 was deemed statistically significant).

Immunostaining

Immunohistochemical analysis was performed using formalin-fixed paraffin sections. Antigen retrieval for all antibodies was done by boiling the slides in citrate buffer (pH 6.0) for 15 min. Antibodies were α-cytokeratins 8/18 (Ker8/18, 1:450 Progen, GP11), α-cytokeratin 5 (K5, 1:3000, Covance, PRB-160P), smooth muscle actin (SMA; 1:1000, mouse A2537; Sigma, St. Louis, MO), anti-phosphorylated histone H3 (1:100, rabbit 06-570; Upstate, Waltham, MA) and SV40
(monoclonal Ab2, 1:100; Oncogene, Cambridge, MA). All immunofluorescence reactions were done using AlexaFluor-conjugated secondary antibodies (AlexaFluor 488 and 594, Molecular Probes). Slides were counterstained with 4,6-diamidino-2-phenylindole (DAPI) using Hardset Mounting Media (Vector Laboratories).

Microarray analysis

We compared microarray profiles of T121/p53 (n = 9) and TBP (n = 8) tumors to published microarray profiles (n = 152) using two-way hierarchical clustering (centroid linkage) of 866 “intrinsic genes” [3,45]. Total RNA was collected from end-stage tumors.
RNA was purified using the Qiagen RNeasy Mini Kit according to the manufacturer’s protocol using 20–30 mg tissue. RNA integrity was assessed using the RNA 6000 Nano LabChip by Bioanalyzer (Agilent). Two micrograms of total RNA were reverse transcribed, amplified, and labeled with Cy5 using a Low RNA Input Amplification kit (Agilent). Common reference RNA consisted of total RNA harvested from equal numbers of C57Bl6/J and 129 male and female Day 1 pups (courtesy of Dr. Cam Patterson, UNC). Reference RNA was reverse transcribed, amplified, and labeled with Cy3. The amplified sample and reference were co-hybridized overnight to Agilent Mouse Oligo Microarrays (G4121A). They were then washed and scanned on an Axon GenePix 4000B scanner, analyzed using GenePix 4.1 software, and uploaded into the UMD database (https://genome.unc.edu/) where Lowess normalization is automatically performed. All data were submitted to GEO (GSE34479). The genes for all analyses were filtered by: 1) requiring intensity values in both channels to have a mean Lowess normalized intensity of >10, 2) Values being reported in >70% of the samples, and 3) the absolute value of the log2 of the ratio of Channel 2/Channel 1 for at least three arrays having to be >1.6. Hierarchical clustering was performed using Cluster v3.0 and displayed using JavaTreeview v1.0.8.

We identified 871 differentially expressed TBP transcripts using SAM implemented in BRB-ArrayTools (R. Simon and the BRB-ArrayTools Development Team, NCI; Table 1; FDR 0.0485, delta 0.92931). Gene ontology analyses were performed using the FatiGO tool (Babelomics ver. 4.2, babelomics.bioinfo.cipf.es). Mouse gene symbols were converted to human EntrezIDs using Agilent annotations and the Mouse Genome Informatics database of The Jackson Laboratory for comparisons. The Fisher’s exact
two-tailed test was used to determine significance of GO (biological process [levels 3–9]) and KEGG pathway terms. Terms with p<0.05 are reported.

Combined murine and human expression data sets

For the mouse tumor data set, IDs for 21,670 unfiltered probes were retrieved (GSE34479). Human EntrezIDs were assigned to orthologous genes as above. Mean values of redundant mouse probes were calculated, missing values were imputed, the columns were standardized to N(0,1), and the rows were median centered using R (ver. 2.10.0). For the human tumor data, we downloaded the file “UNC27arraydata_imputedCollapsed.txt” [4] from the UNC MicroArray Database (genome.unc.edu). The two data sets were corrected for systemic biases using Distance Weighted Discrimination [46]. The combined data set was used for centroid linkage hierarchical clustering analysis.

CGH analysis

We performed array CGH essentially as previously described [47]. Briefly, genomic DNA was isolated from tumors, fluorescently labeled, and competitively hybridized with wt DNA spotted BAC (Bacterial Artificial Chromosomes) arrays. All data were submitted to GEO (GSE40925). We used the HaarSeg algorithm with default parameters implemented in waviCGH (wavi.bioinfo.cipf.es) for chromosomal segmentation of mutations and for CNA calling. We manually curated BAC clones spanning putative CNAs with a conservative tumor:normal DNA threshold of log2ratio > 0.5 or <-0.5. Genes mapping to the BAC clones were identified using the National Center for Biotechnology Map Viewer and the Jackson Laboratory Mouse Genome Database. Gene lists were compared to the Cancer Gene Census (Sanger Institute), the KEGG Pathways in Cancer, Atlas Genetics Oncology, and the Michigan Molecular Interactions database. For the pathway analysis, we used the SAM algorithm implemented in BRB-Array Tools to identify differentially expressed genes (TBP_SAM) among TBP tumors versus the rest of the mouse tumors in the data set. We interrogated KEGG (TBP_KEGG) and GO (TBP_GO_BP) databases using the FatiGO algorithm implemented in the Babelomics (ver. 4.2) suite of bioinformatics tools (babelomics.bioinfo.cipf.es). We compared these results to the differentially expressed genes reported by Prat et al. (2010) of human Claudin-low (CL_KEGG, CL_GO_BP) and Basal-like (Basal_KEGG, Basal_GO_BP) tumors. We also used the FatiGO tool in the Babelomics 4.3.0 integrative platform with parameters set for the Fisher’s exact two-tailed test to determine the most alternately expressed KEGG pathways among the amplified genes determined by CGH analysis.

Supporting Information

Figure S1 Hierarchical clustering of the cross-species analysis summarized in Figure 5.

(PDF)

File S1 Tumor annotations (Table S1 Tumor_Descriptors). Differentially expressed genes defined by SAM analysis of TBP tumors (S2 TBP_SAM). Gene Ontology (GO) terms enriched among tumor TBP, Basal, and Claudin-low subtypes (S3 TBP_GO_BP, S4 Basal_GO_BP, and S5 CL_GO_BP). KEGG pathways enriched among the tumor subtypes (S6 TBP_KEGG, S7 Basal_KEGG, S8 CL_KEGG). Among all tumors assayed (n = 18), we identified nearly five-hundred loci with potential copy-number gains (log2ratio > 0.5, Table S9 putative_gained_CHG-_loci). Approximately half of the genes are previously defined oncogenes represented in oncogene lists including the Cancer Gene Census (Sanger Institute) and the KEGG Pathways in Cancer. The KEGG Pathways enriched among putative gained genes (Table S10 CGH_Gain_KEGG). [XLSX]

File S2 JavaTreeview data files of the cross-species clustering analysis shown in Figure 5.

(ZIP)

File S3 JavaTreeview data files of the mouse tumor clustering analysis shown in Figure 6.

(ZIP)

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Author Contributions

Conceived and designed the experiments: PK MM KS. Performed the experiments: PK MM MP KS. Analyzed the data: PK MM [PS] KS. Contributed reagents/materials/analysis tools: PK KS DGA BH. Wrote the paper: JPSJ KS.

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Ductal carcinoma *in situ*: magnetic resonance and ultrasound imaging in mouse models of breast cancer

Running head: MRI and ultrasound of murine DCIS

S A Jansen¹, L Ileva², L Riffle², L Lu¹, M Anver³, J D Kalen² and T Van Dyke¹º.

¹Mouse Cancer Genetics Program, National Cancer Institute, Frederick National Laboratory for Cancer Research, Building 560 Room 32-24, Frederick MD 21702

²Small Animal Imaging Program, Laboratory Animal Sciences Program, SAIC-Frederick, Inc., Frederick National Laboratory for Cancer Research, P.O. Box B, Frederick, MD 21702

³Pathology/Histotechnology Laboratory, SAIC-Frederick, Frederick National Laboratory for Cancer Research
ABSTRACT

The earliest stage of breast cancer, ductal carcinoma in situ (DCIS), is a remarkably heterogeneous disease with diverse molecular and prognostic subtypes. Mouse models of breast cancer offer an effective experimental framework in which to investigate DCIS, and biomedical imaging can provide potential insights into the biology of these models. In this study, we develop a high resolution, multi-modality strategy for imaging preinvasive mammary neoplasias in two mouse models of DCIS. We performed high resolution magnetic resonance imaging (MRI) on a 3Tesla clinical scanner, using noncontrast T1 and T2 weighted acquisitions and dynamic contrast enhanced MRI. Preinvasive lesions were readily identified on noncontrast T1 and T2 weighted acquisitions. Subsequently, MRI-directed ultrasound was performed and an agar grid was used to correlate MRI with ultrasound and histology. We found that both MRI and ultrasound were able to detect preinvasive neoplasias with high sensitivity (over 90% in the posterior inguinal mammary glands). This study has established an imaging strategy for monitoring mouse models of DCIS, with rapid imaging acquisitions in two different modalities. We have also introduced a new method for correlating MRI with ultrasound and histology. This work sets the stage for combining advanced imaging and mouse modeling techniques to gain relevant insights on image-based biomarkers of DCIS.

Keywords: DCIS, breast cancer, MRI, ultrasound, mouse models
INTRODUCTION

Ductal carcinoma in situ (DCIS) is a preinvasive breast cancer wherein neoplastic cells remain confined within the milk duct basement membrane. DCIS is a nonobligate precursor to invasive ductal carcinoma (IDC) and comprises 20-30% of newly diagnosed breast cancers in the US (Kerlikowske, 2010). It is a remarkably heterogeneous disease with distinct histological and molecular subtypes. These include nuclear grade (low, intermediate or high), gene-expression profiling patterns (luminal A, luminal B, basal or ERBB2) and alterations in regulators of cellular proliferation, apoptosis and genomic stability such as HER2, Rb and PTEN (Allred, 2010, Lari and Kuerer, 2011). Perhaps most relevant are the subtypes of DCIS with more aggressive growth, i.e., lesions that differentiate based on risk of progression to invasive carcinoma (Allegra et al., 2010). Indirect evidence suggests that a significant portion of DCIS lesions will never progress to an invasive, life-threatening carcinoma (Erbas et al., 2006). However, reliable identification of this subset of women with indolent disease who may benefit from less aggressive therapeutic interventions is not currently possible based on known histological or molecular characteristics (Allegra et al., 2010).

As a complementary approach, the capacity for in vivo imaging to provide a noninvasive characterization of DCIS biological and physical properties is an important goal (Jansen, 2011). Clinically, DCIS presents as calcifications on x-ray mammography in a variety of shapes and distributions (D’Orsi, 2010). On dynamic contrast enhanced MRI (DCEMRI) DCIS exhibits a distinctive nonmass-like morphology, with a diversity of distributions and internal enhancement patterns as described by the Breast Imaging and Reporting Data System (BIRADS) lexicon (Jansen et al., 2007). The contrast media uptake and washout, or kinetic, curves for DCIS usually exhibit a plateau or washout shape (Jansen et al., 2007). Breast ultrasound is a widely performed clinical imaging technique, most often utilized in a mammogram- or MRI-directed manner to further evaluate detected abnormalities (Abe et al., 2010). DCIS typically presents as a hypoechoic lesion with irregular shape and indistinct margins on ultrasound (Cho et al., 2011). Despite the widespread use of imaging in the management of DCIS, the association between image-based features and the underlying molecular and prognostic subtypes of DCIS is not well
understood. Studies of women with DCIS are hampered by several challenges including applying consistent imaging techniques in large cohorts of women, obtaining access to tissue specimens, and performing accurate radiologic-pathologic correlation.

Mouse models provide an appealing alternative experimental framework for investigating image-based biomarkers of DCIS because they, in principle, do not suffer from these limitations. As a further advantage, in genetically engineered mouse models (GEMMs), distinct genetic alterations can be targeted to the mammary epithelium in an experimentally controlled manner. These, in turn, have the potential to generate different molecular and prognostic subtypes of preinvasive cancer that can form the foundation for investigating imaging correlations (Van Dyke and Jacks, 2002). However, if mouse models are to serve as a test-bed for developing image-based molecular and prognostic biomarkers of DCIS, several technical advances are required. First, for the purpose of studying molecular, metabolic or physical imaging signatures, highly sensitive anatomic imaging techniques are essential for accurate lesion detection and localization. Second, it is critical to develop multimodality co-registration techniques that can register images with one another and with histology. In this study, we set out to address these goals. We have developed and validated an MRI and ultrasound imaging approach for characterizing preinvasive neoplasias in two mouse models of DCIS.
MATERIALS AND METHODS

Animals

All procedures were carried out in accordance with our institution’s Animal Care and Use Committee approval. Twenty-two mice from commonly utilized xenograft (n=5) and GEMM (n=17) models of DCIS were selected. In MCF10DCIS xenograft mice, human cells were injected into the inguinal mammary gland #4 of 8-10 week old athymic nude mice and DCIS-like lesions developed after ~3 weeks (Miller et al., 2000, Hu et al., 2008). In the C3(1) SV40 Tag GEMM, mammary intraepithelial neoplasia (MIN), an analog to human DCIS, developed at ~12 weeks of age while invasive ductal carcinoma developed by ~16 weeks of age (Green et al., 2000, Maroulakou et al., 1994). Animals were anesthetized prior to imaging experiments, and anesthesia was maintained during imaging at 1.5% isoflurane. The temperature, heart rate and respiration rate were monitored with data taken every minute, and the respiration rate was used to obtain gated images when necessary. All four inguinal mammary glands were imaged, numbered #4,5 (left side), and #9,10 (right side). Glands #4 and #9 extend from the intramammary lymph node to the spine, and will be referred to as the posterior inguinal glands. Glands #5 and #10 extend down the belly of the animal, and will be referred to as the anterior inguinal glands (Figure 1).

Imaging protocols and co-registration

The experimental workflow is illustrated in Figure 1, consisting of preparation and placement of the agar grid, MRI, MRI-directed ultrasound, and euthanization for subsequent tissue processing and histological evaluation.

Agar grid: A fine polyethylene mesh ~ 65 mm × 30 mm in size with 3 mm spacing was embedded in partially deuterated agar and wrapped around each mouse during imaging. Using Sharpie markers, the grid edges were marked on the skin of the mouse. In prior work, this grid has been shown to produce a two-dimensional coordinate system on MRI that allows for correlation with histology (Jansen et al., 2008)
and for registration of serial MRI scans (Jansen et al., 2009a). In the present study, the agar grid was used to co-register MRI with ultrasound and histology.

**MRI protocols:** Imaging was performed on a 3.0T Philips Achieva scanner. Mice were positioned in a volume coil so that the inguinal intramammary lymph nodes were in the center of the coil. Initially, a $T_1$ weighted acquisition with fat suppression was performed ($T_1$ FFE, 3D, TR/TE 26/2.9ms, in plane resolution=0.2 mm, slice thickness=0.4 mm, field of view (FOV)=30×20×9 mm, number of excitations (NEX)=8, flip angle (FA)=25°, no respiratory gating) followed by a $T_2$ weighted scan with fat suppression ($T_2$ TSE, 2D, TR/TE=4000/65 ms, in plane resolution = 0.17 mm, slice thickness=0.5 mm, FOV=30×30×12 mm, NEX=2, number of slices =24, with respiratory gating). On a subset of n=9 C3(1)SV40 Tag mice, dynamic contrast enhanced MRI (DCEMRI) ($T_1$ FFE DCE, 3D, TR/TE=13/3ms, in plane resolution =0.23 mm, slice thickness=1.0 mm, NEX=2, FA=20°, temporal resolution= 17 secs, duration =17 min 42 sec, no respiratory gating) was obtained before, during and after a single-dose injection of gadopentetate dimeglumine (Magnevist; Berlex, Wayne, NJ) via a tail vein catheter at a dose of 0.2 mmol/kg.

**MRI-directed ultrasound:** Ultrasound was subsequently performed to further evaluate MR findings. To correlate MRI and ultrasound, the grid coordinates of MRI-detected lesions were noted on the MRI, and then transferred to the skin of the mouse (Figure 1). These skin markings were subsequently used to guide placement of a 40 MHz ultrasound transducer for the acquisition of B-mode images on a Vevo2100 imaging system (VisualSonics Inc, Toronto Canada).

**Correlation of MRI with histology**

To evaluate the sensitivity of MRI and ultrasound methods for detecting preinvasive lesions, hematoxylin and eosin (H&E) stained sections of imaged mammary glands were obtained (5-micron thick H&E sections every 50 microns) and evaluated by a veterinary pathologist (MA). Each gland (#4,5,9,10) was separately classified into one of four categories: (i) normal, (ii) preinvasive carcinoma with/without microinvasion, (iii) invasive and preinvasive carcinoma, and (iv) predominantly invasive carcinoma. MR
images were reviewed by a post-doctoral fellow (SAJ) with 6 years of experience analyzing MRI of the mouse mammary gland, and each gland (#4, 5, 9, 10) was classified into the same categories, using the morphologic descriptors nonmass and mass to identify preinvasive and invasive regions, respectively (Jansen et al., 2009a, Jansen et al., 2008). The MRI assessment was blinded to the pathological assessment. MR images were spatially correlated with H&E sections using the agar grid as detailed in prior work (Jansen et al., 2008) and the sensitivity of MRI was calculated using the histologic evaluation as the reference standard. Sensitivity was compared in the posterior and anterior inguinal glands to determine regional variability in the efficacy of MRI.

**Image Analysis**

*mBIRADS*: T₁ FFE images were analyzed according to an adapted version of the human BIRADS lexicon (ACR, 2003), referred to as the mouse BIRADS or mBIRADS lexicon. Initially the *type* of the lesion was classified as ‘mass’ or ‘nonmass’. Prior work has shown that the ‘nonmass’ descriptor reliably identifies preinvasive lesions in the C3(1) SV40 Tag model (Jansen et al., 2008). For nonmass lesions, additional descriptors were the *distribution* (‘linear’, ‘ductal’ or ‘segmental’) and the *pattern* (‘homogeneous’, ‘clumped’ or ‘stippled’).

**SNR and CNR.** All data analysis was performed using software written in IDL (Research Systems, Inc., Boulder, CO, USA). A postdoctoral fellow (SAJ) with six years of experience analyzing MRI of preinvasive and invasive mammary cancers traced the following regions of interest (ROI), confirmed by the histologic evaluation: invasive carcinoma, preinvasive carcinoma, lymph node, normal mammary gland (NMG) and muscle. The signal-to-noise ratio (SNR) and the contrast-to-noise ratio (CNR) relative to NMG tissue and muscle were calculated on T₁ FFE and T₂ TSE as follows:

\[
SNR = \frac{\overline{S}}{\sigma_{\text{noise}}}, \quad CNR_{\text{NMG}} = SNR_{\text{ROI}} - SNR_{\text{NMG}}, \quad CNR_{\text{muscle}} = SNR_{\text{ROI}} - SNR_{\text{muscle}}
\]

where \(\overline{S}\) is the average signal intensity in the ROI and \(\sigma_{\text{noise}}\) was averaged from the standard deviations of signal intensities measured in a 5×5 mm ROI drawn in the corners of the image.
Contrast kinetics. Signal intensity vs. time, or kinetic, curves were generated from T₁ FFE DCE data by manually tracing an ROI around lesions. These curves were analyzed qualitatively and classified as persistent, plateau or washout as described by the BIRADS lexicon (ACR, 2003). In addition, two compartment pharmacokinetic modeling was performed and parameters K<sup>trans</sup> and v<sub>e</sub> calculated, using software as described previously (Turkbey et al., 2010).

Statistical analysis

The SNR, CNR<sub>NMG</sub> and CNR<sub>muscle</sub> of lymph nodes, invasive and preinvasive carcinomas on T₁ FFE and T₂ TSE pulse sequences were compared using the Student’s t-test. The sensitivity of MRI and MRI-directed ultrasound in the posterior vs. anterior inguinal glands was compared using the Fishers exact test. For both tests, a p value < 0.05 was considered to be statistically significant.
RESULTS

Images of preinvasive cancers from representative GEMM and xenograft mice are shown in Figure 2. The MIN lesions in C3(1) mice arise *de novo* within an intact mammary fat pad, surrounded by normal tissue. Conversely, DCIS arising in MCF10DCIS mice appears as larger palpable lesions that are not embedded within an intact mammary gland. Given that the imaging presentation of human DCIS is more akin to the GEMM, i.e., a typically nonpalpable lesion embedded in the normal breast, the remainder of our analysis was focused on MIN lesions in C3(1) SV40 Tag mice.

A total of 60 MIN lesions were histologically confirmed, of which MRI detected 53 for an overall sensitivity of 88% (53/60). The sensitivity of MRI was higher in the posterior inguinal glands compared to the anterior inguinal glands, although this difference was not statistically significant ($p > 0.2$). Similarly, the sensitivity of MRI-directed ultrasound was significantly higher in the posterior glands, at 97%, compared to the anterior inguinal glands, at 37% ($p<0.0001$) (Table 1).

Qualitative analysis (Table 2) of lesion morphology according to the mBIRADS lexicon revealed that the predominant distribution of nonmass MIN lesions was segmental, with a homogeneous pattern. Quantitatively (Figure 3), MIN lesions presented with a significantly higher SNR than NMG on $T_1$ FFE (at 35±7 vs. 18±2, respectively) and $T_2$ TSE (at 29±8 vs. 13±3, respectively) ($p < 0.0001$), and a significantly lower SNR compared to lymph nodes and invasive tumors ($p<0.001$). Although MIN lesions exhibited higher SNR on $T_1$ FFE than on $T_2$ TSE ($p < 0.0002$), both MIN and invasive tumors achieved comparable $\text{CNR}_{\text{NMG}}$ on $T_1$ and $T_2$ weighted acquisitions ($p > 0.2$) and significantly higher $\text{CNR}_{\text{muscle}}$ on $T_2$ TSE ($p < 0.0001$). DCEMRI was obtained for 15/53 MRI-detected MIN lesions. Analysis of kinetic curve shape demonstrated that 20% (3/15) exhibited minimal enhancement, while 47% (7/15) displayed a plateau curve shape and 20% (3/15) a washout curve shape. For the n=12 enhancing MIN, the average $K_{\text{trans}}$ and $v_e$ values were 0.11±0.09 min$^{-1}$ and 0.47 ±0.22, respectively.
DISCUSSION

Our results suggest that from an imaging perspective the C3(1) SV40 Tag GEMM more faithfully mimics human DCIS compared to the commonly used MCF10DCIS xenograft model. In GEMMs, preinvasive cancers arise stochastically within pre-existing mammary ducts and are surrounded by the mammary gland microenvironment including an intact immune system. Although intramammary fat pad injections of MCF10DCIS cells did not achieve this phenotype, recently developed xenograft models may do so. For example, DCIS patient-derived cells that are injected directly into the murine ductal system via the nipple may prove to be more effective models of DCIS (Valdez et al., 2011, Behbod et al., 2009).

Previous studies have shown that on small bore small animal imaging systems at high magnetic field strength (4.7 T and 9.4 T), it is possible to detect preinvasive cancers in transgenic mice using a proton density/moderately $T_1$ weighted pulse sequence (Gradient Recalled Echo, TR/TE ~1000/5.5 ms) (Jansen et al., 2008, Jansen et al., 2011). Our study complements this prior work in several important ways. We have implemented pulse sequences that are more similar to clinically acquired $T_1$ and $T_2$ weighted breast MRI, on a 3T clinical MRI scanner. In doing so, we have established a new $T_1$ weighted fast-field echo imaging approach for preinvasive murine neoplasias that matches or exceeds the sensitivity, SNR and $CNR_{NMG}$ of prior techniques, in half the imaging time. Finally, we found that the $CNR_{NMG}$ of preinvasive neoplasia on $T_2$ TSE was comparable to $T_1$ FFE, whereas in prior studies $T_2$ weighting was not reported to be as effective (Jansen et al., 2008).

Human DCIS is typically detected with $T_1$ FFE after the administration of a gadolinium-chelated based contrast agent (Kuhl et al., 2007). Conversely, we have found that murine MIN can be visualized on $T_1$ FFE and $T_2$ TSE with high $CNR_{NMG}$ without contrast medium. The mechanisms responsible for this important discrepancy are not clear. Given the likelihood that the magnetic properties of neoplastic epithelial cells/ducts may be similar in mice and women at 3T, it may be differences in the tumor microenvironment that yield a different imaging phenotype. For example, neoplastic ducts in the mouse are distributed sparsely within a fat pad, whereas in women they may be in close proximity with normal TDLUs embedded within an intralobular stroma. In performing a contrast kinetic characterization of
MIN lesions at 3T we found some qualitative and quantitative similarities with human disease, including exhibiting a predominantly plateau or washout curve shape (Jansen et al., 2007) and comparable values of $K^{\text{trans}}$ and $v_e$ (Furman-Haran et al., 2005). However, our DCEMRI methods were limited and further improvements are required, including increased population size and temporal resolution, to better compare the contrast enhanced phenotype of murine and human preinvasive neoplasias.

Molecular and metabolic imaging is highly desirable to better reveal biological diversity in mouse models of DCIS. Indeed, in previous work by Abbey et al., FDG-PET imaging was used to characterize metabolic changes during breast cancer progression in an allograft mouse model of DCIS (Abbey et al., 2004, Abbey et al., 2006). However, molecular imaging techniques often suffer from poor spatial resolution and anatomic localization, and could thus benefit from concomitant anatomic imaging for lesion detection and localization. Our results set the stage for such an approach, as we have established rapid, high resolution and highly sensitive anatomic imaging techniques in two different modalities, MRI and ultrasound. Furthermore, in carefully mapping out the lesions in this study, we found improved sensitivity in the posterior inguinal glands compared to anterior inguinal glands, likely due to partial volume effects near the belly of the mouse.

For multimodality imaging it is also essential to develop co-registration methods that can accurately correlate diverse images with one another other and with histology. This is particularly relevant for small preinvasive mammary cancers that are not palpable or visible. Prior work in MRI-ultrasound correlation has been developed for palpable lesions. For example Loveless et al. developed an iterative closest point algorithm method to co-register MRI and ultrasound images using palpable tumors ($\sim 100\text{mm}^3$) arising in a xenograft model of breast cancer (Loveless et al., 2009). The novel approach we have established in this study utilizes the agar grid as a two dimensional coordinate system that can accurately co-register in vivo images and histology of sub-millimeter cancers.

Ultimately we are interested in investigating the capacity for image-based biomarkers to identify molecular or prognostic subtypes of human DCIS, using GEMMs as a model system. In this study, we have added to the imaging toolkit that can be used to characterize murine preinvasive neoplasia by
developing new clinically relevant MRI techniques at 3 Tesla, adding ultrasound characterization and establishing a new multi-modality correlation strategy. In future work, application of more advanced imaging techniques, such as diffusion weighted MRI and contrast enhanced ultrasound, can yield additional quantitative measures of lesion biology and physiology. However, in addition to improved imaging techniques it is also essential to utilize more diverse GEMMs of DCIS. Imaging investigations of murine MIN have been limited to models wherein viral proteins (e.g., SV40 Tag (Jansen et al., 2008, Jansen et al., 2011, Jansen et al., 2009b) and PyMT (Abbey et al., 2006, Namba et al., 2006, Abbey et al., 2004)) induce aggressive tumorigenesis by concurrently altering several molecular pathways (Green et al., 2000). However, these models do not capture the full complexity of human DCIS. More advanced GEMMs can target individual pathways known to be altered in DCIS, including Rb (Simin et al., 2004), HER2 (Moody et al., 2002) and PTEN (Dourdin et al., 2008) in a cell type and temporally controlled manner. It is by combining advanced imaging approaches with sophisticated mouse modeling techniques that insights on image-based biomarkers of DCIS are likely to follow.
ACKNOWLEDGEMENTS

We thank Dr. Esta Sterneck for use of the C3(1) SV40 Tag mice, Dr. Peter Choyke for useful discussions and comments on the manuscript, and Dr. Yuxi Pang for assistance with the DCEMRI analysis software.

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**TABLES**

*Table 1:* Sensitivity of MRI and MRI-directed ultrasound for MIN in different gland regions of C3(1) SV40 Tag mice.

<table>
<thead>
<tr>
<th></th>
<th>Number of MIN histologically</th>
<th>Sensitivity of MRI</th>
<th>Sensitivity of MRI-directed ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td>All inguinal glands</td>
<td>60</td>
<td>88% (53/60)</td>
<td>72% (38/53)</td>
</tr>
<tr>
<td>Posterior inguinal glands</td>
<td>33</td>
<td>94% (31/33)</td>
<td>97% (30/31)</td>
</tr>
<tr>
<td>Anterior inguinal glands</td>
<td>27</td>
<td>82% (22/27)</td>
<td>36% (8/22)</td>
</tr>
</tbody>
</table>
Table 2: Qualitative mBIRADS features for MIN lesions in C3(1) SV40 Tag mice.

<table>
<thead>
<tr>
<th></th>
<th>MIN No. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Type</strong></td>
<td></td>
</tr>
<tr>
<td>Mass</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Nonmass</td>
<td>53 (100)</td>
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<tr>
<td><strong>Distribution</strong></td>
<td></td>
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<tr>
<td>Linear</td>
<td>15 (28)</td>
</tr>
<tr>
<td>Ductal</td>
<td>11 (21)</td>
</tr>
<tr>
<td>Segmental</td>
<td>27 (51)</td>
</tr>
<tr>
<td><strong>Pattern</strong></td>
<td></td>
</tr>
<tr>
<td>Homogeneous</td>
<td>23 (43)</td>
</tr>
<tr>
<td>Stippled</td>
<td>20 (38)</td>
</tr>
<tr>
<td>Clumped</td>
<td>10 (19)</td>
</tr>
</tbody>
</table>
FIGURE CAPTIONS

Figure 1. Multimodality imaging workflow. A. Initially, the agar grid is wrapped around the mouse and grid edge positions marked on the skin of the mouse. B. MRI of one axial cross-section through the mouse (dotted line from A) revealing an intramammary lymph node (red arrow). The agar grid can be seen on MRI (arrows) and thus the grid coordinates of the lymph node noted. C. After removing the mouse from the imaging coil, the location of the MRI detected lesion is marked on the skin of the mouse to guide placement of the ultrasound transducer. D. Ultrasound imaging is performed to locate and evaluate the MRI-detected lesion. E. Finally, the animal is euthanized and glands are excised for histological analysis. Shown are the posterior inguinal glands #4 and 9 (outlined in blue), the anterior inguinal glands #5 and #10 (outlined in black) and the intramammary lymph nodes circled in orange.

Figure 2. Representative noncontrast T₁ FFE, T₂ TSE, ultrasound and H&E images from a normal gland (top row), MIN lesion in the C3(1) SV40 Tag GEMM (middle row, thin arrows) and DCIS lesion in the MCF10DCIS xenograft model (bottom row, thin arrows). Intramammary lymph nodes are identified by arrowheads, while an invasive tumor (middle row) is indicated with a gray arrow.

Figure 3. The SNR, CNR, and CNR of lymph nodes, MIN and invasive tumors in C3(1) SV40 Tag mice on noncontrast T₁ FFE and T₂ TSE acquisitions.
Figure 1. Example multimodality imaging workflow. A. Initially, the agar grid is wrapped around the mouse and grid edge positions marked on the skin of the mouse. B. MRI of one axial cross-section through the mouse (dotted line from A) revealing an intramammary lymph node (red arrow). Because the agar grid is MR-visible, the grid coordinates of the lymph node are noted. C. After removing the mouse from the imaging coil, the location of the MRI detected lesion is marked on the skin of the mouse to guide placement of the ultrasound transducer. D. Ultrasound imaging is performed to locate and evaluate the MRI-detected lesion. E. Finally, the animal is euthanized and glands are excised for histological analysis. Shown are the posterior inguinal glands #4 and 9 (outlined in blue), the anterior inguinal glands #5 and #10 (outlined in black) and the intramammary lymph nodes circled in white.
**Figure 2.** Representative noncontrast T\textsubscript{1} FFE, T\textsubscript{2} TSE, ultrasound and H&E images from a normal gland (top row), MIN lesion in the C3(1) SV40 Tag GEMM (middle row, thin arrows) and DCIS lesion in the MCF10DCIS xenograft model (bottom row, thin arrows). Intramammary lymph nodes are identified by arrowheads.
Figure 3. The SNR, CNR_{NMG} and CNR_{muscle} of lymph nodes, MIN and invasive tumors in C3(1) SV40 Tag mice on T₁ FFE and T₂ TSE acquisitions.
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