Cooperation between Pik3ca and p53 mutations in mouse mammary tumor formation

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Abstract

PIK3CA, which codes for the p110α catalytic subunit of phosphatidylinositol 3’ kinase, is one of the most frequently mutated genes in human breast cancer. Here we describe a mouse model for PIK3CA-induced breast cancer using the ROSA26 (R26) knock-in system, where targeted Pik3ca alleles can be activated through transgenic expression of Cre recombinase. We mated Pik3ca<sup>H1047R</sup> and Pik3ca<sup>wt</sup> knock-in lines with MMTV-Cre transgenics, which express Cre in mammary epithelium. Starting at approximately 5 months of age, female R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre but not control R26-Pik3ca<sup>wt</sup>;MMTV-Cre mice developed mammary tumors, as well as lymphoid and skin malignancies. R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre mammary tumors were typically either adenosquamous carcinoma or adenomyoepithelioma. As p53 is the most commonly mutated gene in breast cancer, we tested for genetic interaction between Pik3ca<sup>H1047R</sup> and p53 loss-of-function mutations in R26-Pik3ca<sup>H1047R</sup>;p53<sup>loxP/+</sup>;MMTV-Cre mice. This led to decreased survival of double mutant animals, which developed lymphoma and mammary tumors with rapid kinetics. Mammary tumors that formed in p53<sup>loxP/+</sup>;MMTV-Cre conditional mutants were either poorly differentiated adenocarcinoma or spindle cell/EMT, whereas R26-Pik3ca<sup>H1047R</sup>;p53<sup>loxP/+</sup>;MMTV-Cre mammary tumors were mostly adenosquamous carcinoma or spindle cell/EMT indicating that double mutant mice develop a distinct spectrum of mammary tumors. Thus, an oncogenic variant of PIK3CA implicated in multiple human breast cancer subtypes can induce a very diverse spectrum of mammary tumors in mice. Furthermore, Pik3ca<sup>H1047R</sup> shows cooperation with p53, which altered the specific tumors that formed. Thus, the two most frequently mutated genes in human breast cancer show cooperation in mammary tumor formation.
Introduction

The PI3K signaling pathway is one of the most frequently mutated pathways in cancer. One common and direct mechanism by which the PI3K pathway is activated in breast, endometrial, colorectal, urinary tract, thyroid and ovarian cancer is through gain-of-function mutations in PIK3CA (1-3). PIK3CA mutations are particularly important in human breast cancer, with activated alleles detected in 25-30% of tumors (4-7). These mutations have been found in ductal carcinoma in situ, suggesting that they play a role in breast tumor initiation (8-10). In addition, they are present at high frequency in estrogen receptor α (ERα) positive, Her2/Neu+ and triple negative breast tumors, suggesting an important role for mutant PIK3CA in etiology of multiple breast tumor subtypes (4). Metaplastic breast cancers have the highest percentage of PIK3CA mutations (11). Importantly, PIK3CA mutations can induce p53-dependent growth inhibition (12) and mutations in both genes occur together in some human breast tumors (13, 14).

PIK3CA point mutations occur most often within two hotspots: the helix and kinase domains (1). H1047R mutations in the kinase domain account for approximately 40% of breast cancer PIK3CA mutant alleles (4). This mutant shows elevated kinase activity (15-17) and is capable of transforming cells in culture (18), including mammary epithelial cells (19, 20). Despite the high frequency, and hence importance of this allele in breast cancer, there is no animal model to test for its role in transformation of mammary epithelium. Here we describe generation of a Cre-mediated system to conditionally express Pik3ca alleles in mouse tissues, and use of this system to test for induction of mammary tumor formation by Pik3caH1047R. We report that ectopic expression of Pik3caH1047R, but not Pik3caWt, induced mammary tumors at high frequency. Furthermore, we have used the knock-in system to test for cooperation between...
Pik3ca and p53 (3), showing that deletion of p53 enhances tumor formation and alters the spectrum of mammary tumor subtypes that form in our Pik3ca$^{H1047R}$ model.
Methods

Generation of Transgenic Mice: Shuttle vectors containing either wildtype or H1047R mutant Pik3ca were subcloned into the pROSA26PA gene-targeting vector (21, 22). pR26-Pik3ca plasmids were linearized with KpnI (Figure 1) and electroporated into mouse embryonic stem cells (mESC). G418 resistant clones were isolated, expanded and screened by Southern blot (probing for the 3’ recombination junction - see below) and PCR (amplifying across the 5’ recombination junction, FP 5’-CGCCTAAAGAAGAGGCTGTG-3’, RP 5’-GAAAGACCGCGAAGAGTTTG-3’). Targeted clones were transiently transfected (Fugene6, Roche 11815091001) with Cre or control vector (pCAGGS-Cre-IRES-puro; pCAGGS-FlpE-IRES-puro (23)). RNA was harvested (RNeasy, Qiagen 74104) and rtPCR performed (SuperScript II, Invitrogen 18064022) using FP in R26 exon1 (5’-CTAGGTAGGGGATCGGGACTCT-3’) and a RP in Pik3ca cDNA (5’-AATTTCCTCGATTGAGGTCTTTTCT-3’). Cre-inducible mESC lines were used for morula aggregation in The Toronto Centre for Phenogenomics (TCP).

Southern Blot Analysis: A standard Southern blot protocol was used. Genomic DNA (10μg) was digested with EcoRV/SstI. A ~940bp probe on the 3’ side of the targeted insertion site (CTTGAAAGTGGGCTACTAC to TCAGAGCTTTGAAGTCA in the R26 locus) was 32P-labeled using the Random Primers DNA Labeling System (Invitrogen 18187013). This probe binds an 11kb fragment in non-targeted R26, and a 9kb fragment if the R26 locus is correctly targeted.

Mouse Colony Maintenance: Mice were housed at TCP according to guidelines from the CCAC
Cre strains were genotyped using FP 5’-TCGCGATTATCTTCTATATCTTCAG-3’, RP 5’-GCTCGACCAGTTTAGTTACC-3’. R26-Pik3ca mice were genotyped using primers previously published (22). Multiparous females had three litters. p53<sup>loxP/+</sup> conditional mutant mice (FVB.129-<i>Trp53tm1Brn</i> – MMHCC 01XC2; for simplicity, this strain is labeled throughout as p53<sup>F+</sup>)(24) were obtained from the Mouse Models of Human Cancer Consortium and genotyped by PCR (FP 5’-CACAAAAACAGGTTAAACCAG-3’, RP 5’-AGCACATAGGAGGCAGAGAC-3’). p53 deletion was confirmed using FP 5’-CACAAAAACAGGTTAAACCAG-3’, RP 5’-GAAGACAGAAAGGGGAGGG-3’ to amplify a 612bp fragment (24).

**RT-PCR on Lin<sup>−</sup> Mammary Epithelium:** Mammary glands from young virgin mice were minced and digested in collagenase/hyaluronidase (Stem Cell Technologies 07912) for 4 hours (37°C). Mammary epithelial (lin<sup>−</sup>) cells were isolated using the EasySep kit (StemCell Technologies 19757). RT-PCR was performed as outlined above.

**Tumor Collection:** Mice were humanely sacrificed at end point, and mammary tumors dissected. Humane end point was based on criteria outlined by the CCAC (tumor volume = 1.7 cm<sup>3</sup>, tumor mass = 5% of body weight, or ulcerated tumor). Part of each tumor was fixed in 10% formalin and paraffin-embedded. The remaining tumor was divided into small samples and snap frozen.

**Western Blots:** Tumors and control glands were lysed using a TissueRuptor homogenizer (Qiagen 9001271) in lysis buffer (ProteoJET Lysis Reagent, Fermentas K0301; PhosSTOP, Roche 04906837001; protease inhibitor, Roche 11836170001). 150μg of protein lysates were
analyzed using a standard western blot protocol. Primary antibodies were incubated overnight, and secondary antibodies for 1 hour, both at room temperature. Western blots were quantified using AlphaImager software (Cell Biosciences) and normalized with respect to β-actin expression. Antibodies (used at recommended dilutions): Akt\textsuperscript{pT308} (Cell Signaling CS2965), Akt\textsuperscript{pS473} (Cell Signaling CS927), PTEN (Cell Signaling CS9559), cJun\textsuperscript{pS73} (Cell Signaling CS33427), SGK3\textsuperscript{pS486} (SantaCruz sc33044), ERα (SantaCruz sc71064), Atf3 (SantaCruz sc188), β-catenin (BDTL 610154), CAIX (R&D Systems AF2344), β-actin (abCam ab8226), goat anti-rabbit IgG-HRP (SantaCruz sc2004), goat anti-mouse IgG-HRP (SantaCruz sc2005), donkey anti-goat IgG-HRP (SantaCruz sc2020).

Histology and Immunohistochemistry: 5μm paraffin sections were either stained with hematoxylin and eosin (H&E), or subjected to antigen retrieval in a decloaking chamber (Biocare Medical; SetPoint1 = 125°C, 5min; SetPoint2 = 90°C, 10sec), using epitope-retrieval solution (Biocare Medical BD1000G1 or RV1000G1). Slides were mounted into a Tecan FreedomEvo liquid-handling robot. Immunohistochemistry: Endogenous peroxidases were quenched in 3% H\textsubscript{2}O\textsubscript{2}/methanol (15min, room temperature), then VectaStain ABC kits (Vector Laboratories) were used (PK-6101, PK-4002, PK-6105). Antibodies were incubated 30 minutes at room temperature. DAB substrate kit (Vector Laboratories SK-4100) was used for staining and slides were counterstained in hematoxylin (10sec). Immunofluorescence: Slides were blocked for 30min (DakoCytomation X0909). Primary antibodies were incubated overnight (4°C), secondary antibodies were incubated for 1 hour (room temperature). Slides were mounted with fluorescence mounting medium (DakoCytomation S3023) containing DAPI. Antibodies (used at
recommended dilutions): Gata3 (ProteinTech 10417-1-AP), Cytokeratin 5 (Covance PRB-160P), ERα (SantaCruz sc542), Ki67 (BioCare Medical CRM326), Cytokeratin 8 (Fitzgerald 10R-C177AX), Cytokeratin 14 (Panomics E2624), Cytokeratin 10 (Covance PRB-159P), Vimentin (Cell Signaling CS32322), Desmin (DakoCytomation M0760), N-cadherin (Novus NB200-592), AlexaFluor488 anti-mouse (Invitrogen A21202), AlexaFluor594 anti-rabbit (Invitrogen A21442).

**TUNEL:** 5µm paraffin sections were immersed in 2.5µg/mL proteinaseK (2min). Endogenous peroxidases were quenched via 3% H₂O₂ (15min). TUNEL was performed using terminal deoxynucleotidyl transferase (Fermentas EP0161) and biotin-16-UTP (Roche 11093070910). Signal was amplified (ABC, Vector Laboratories PK-6100) and developed (DAB Kit, Vector Laboratories SK-4100); sections were counterstained with methyl green.

**Image Capture:** Images were captured with an AxioCam HRm digital camera (Zeiss) using AxioVision (release 4.6.3) software.
Results

Generation of a Cre-inducible Pik3ca mammary tumor model

To test for the ability of Pik3ca^{H1047R} or wildtype Pik3ca (Pik3ca^{wt}) to induce mammary tumors, we used the Cre-conditional ROSA26 (R26) knock-in system, whereby Cre-mediated deletion of loxP-flanked transcriptional stop sequences allows for tissue-specific expression of either allele (Figure 1A)(21, 22). Mouse embryonic stem cells (mESC) were targeted and screened to identify clones with Cre-inducible transgene expression (Supplemental Figure 1). Cre-responsive mESC clones were then used to generate chimeric mice, and germline transmission of each knock-in allele confirmed. R26-Pik3ca^{H1047R} and R26-Pik3ca^{wt} mice were mated with MMTV-Cre mice, which express Cre recombinase in mammary epithelium. Two different strains of MMTV-Cre were used: MMTV-Cre^{lineA}, which expresses Cre recombinase in mammary epithelium at high efficiency but also in skin and lymphocytes (25) as well as MMTV-Cre^{NLST}, which is less efficient but more mammary restricted in its expression (Supplemental Figure 2)(26, 27). Mammary-inducible transgene expression was observed in R26-Pik3ca;MMTV-Cre mice but not in Cre-negative littermates (Figure 1B).

Female R26-Pik3ca^{H1047R};MMTV-Cre mice from both Cre lines showed reduced survival (Figure 1C, Supplemental Table 1)(note, males were not studied). Some animals died abruptly, whereas most were sacrificed at humane endpoint due to lethargy and impaired breathing or solid tumor growth. Decreased survival was not due to Pik3ca overexpression as R26-Pik3ca^{wt};MMTV-Cre mice remained healthy (Figure 1C). R26-Pik3ca^{H1047R};MMTV-Cre mice started developing mammary tumors at 5 months of age. Mammary tumors were observed in both virgin and multiparous mice using either MMTV-Cre strain. R26-Pik3ca^{H1047R};MMTV-Cre^{lineA} mice reached endpoint more rapidly than R26-Pik3ca^{H1047R};MMTV-Cre^{NLST} mice.
In addition, R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>lineA</sup> mice reached endpoint with a more diverse set of tumor types. 69% of NLST mice and 42% of line A mice had palpable mammary tumors at endpoint (Figure 1D). For this reason, we used MMTV-Cre<sup>NLST</sup> transgenics for subsequent studies. Development of lymphoma/thymoma, skin and other non-mammary tumors was attributed to Cre expression in other tissues (25). Interestingly, survival of R26-Pik3ca<sup>H1047R</sup> mice, without Cre, was reduced in comparison to MMTV-Cre controls (Figure 1C, Supplemental Table 1, p < 1x10<sup>-6</sup>). This was due to development of hamartomas in some R26-Pik3ca<sup>H1047R</sup> animals (data not shown), potentially through spontaneous activation of Pik3ca<sup>H1047R</sup> expression in endothelial cells (28).

**Pik3ca and p53 mutations cooperate in vivo**

To test for cooperation between Pik3ca and p53, we mated R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice with Cre-conditional p53 mutants (24). Most R26-Pik3ca<sup>H1047R</sup>;p53<sup>f/+;MMTV-Cre<sup>NLST</sup></sup> mice died early with lymphoma/thymoma (Figure 2A). On autopsy, some had small mammary tumors (Figure 2C left). Lymphoma/thymoma occurred at a much lower rate in R26-Pik3ca<sup>H1047R</sup>;p53<sup>f/+;MMTV-Cre<sup>NLST</sup></sup> p53 heterozygous mice, which also showed significantly reduced survival as compared to R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup>, p53<sup>f/+;MMTV-Cre<sup>NLST</sup></sup>, and Cre negative control mice (Figure 2A, Supplemental Table 2). To test whether Pik3ca and p53 show an additive or synergistic interaction, we simulated a Kaplan-Meier survival curve by combining single mutant cohorts, such that as any R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> or p53<sup>f/+;MMTV-Cre<sup>NLST</sup></sup> mouse reached endpoint, it was scored as an event within one cohort. R26-Pik3ca<sup>H1047R</sup>;p53<sup>f/+;MMTV-Cre<sup>NLST</sup></sup> double mutant mice showed significantly decreased survival (p = 2.79x10<sup>-5</sup>) compared to this hypothetical cohort indicating synergy between Pik3ca<sup>H1047R</sup> and p53 gene loss. Synergistic interaction was also evident when considering only
mice that reached endpoint from mammary tumors (Supplemental Figure 3). p53 deletion was observed in R26-Pik3ca<sup>H1047R</sup>;p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> and p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> tumors (Figure 2B). 92% of p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> and 80% of R26-Pik3ca<sup>H1047R</sup>;p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> mice had mammary tumors (Figure 2C). Many mice had mammary tumors in more than one gland. These were treated as distinct lesions. Histological analysis revealed that mammary tumors from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice were typically either adenosquamous carcinoma or adenomyoepithioma (Figure 3A, Supplemental Figure 4). Adenosquamous tumors contained cystic regions composed of laminar keratin lined with squamous epithelium as well as adjacent glandular elements. Some tumors had squamous nodules or necrosis, and several had invasive margins. Expansile tumors were also observed. Adenomyoepitheliomas contained a mixture of glandular epithelium and interstitial fusiform cells with abundant polar cytoplasm. These myoepithelial stromal cells represented between 10% to 80% of the tumor. Isolated lung metastasis were observed in rare R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice (data not shown). Mammary tumors from p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> mice were either spindle/epithelial-mesenchymal transition (EMT) type or poorly differentiated adenocarcinoma as previously reported (Figure 3B, Supplemental Figure 4)(29-31). Spindle tumor cells had fusiform nuclei and polar cytoplasm. Swirling patterns and necrotic areas were observed. Poorly differentiated adenocarcinomas were composed of solid sheets of cells with little tissue architecture and some necrosis. Cells had large, pleomorphic nuclei and dark staining cytoplasm. Interestingly, mammary tumors that formed in R26-Pik3ca<sup>H1047R</sup>;p53<sup>fl/+</sup>;MMTV-Cre<sup>NLST</sup> double mutant mice were typically either spindle/EMT or adenosquamous carcinoma (Figure 3C, Supplemental Figure 4). Radial scar and poorly differentiated adenocarcinomas were also observed, although at a lower frequency (Figure 3C, Supplemental Figure 4). Radial scar tumors were small and had a
stellate outline composed of dense connective tissue and a center with distorted neoplastic glands. Gross metastasic lesions were not observed in R26-Pik3ca<sup>H1047R</sup>;p53<sup>F/+</sup>;MMTV-Cre<sup>NLST</sup> mice (data not shown).

**Akt activation in PIK3CA<sup>H1047R</sup> and p53 mutant mammary tumors**

To test for PI3K pathway activation, lysates from representative tumors were analyzed for Akt phosphorylation, as well as for expression of PTEN and phospho-c-Jun (S73). Western blot signals were normalized to β-actin protein expression, and compared to levels in mammary lysates from a non-tumor bearing 74-week old R26-Pik3ca<sup>H1047R</sup> virgin female. Adenosquamous carcinomas from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice showed elevated accumulation of phospho-Akt<sup>S473</sup>, PTEN and phospho-cJun<sup>S73</sup> (Figure 4). Adenosquamous carcinomas from R26-Pik3ca<sup>H1047R</sup>;p53<sup>F/+</sup>;MMTV-Cre<sup>NLST</sup> showed increased PTEN and phospho-cJun<sup>S73</sup> expression. Surprisingly, however, no change in phospho-Akt was observed in these tumors. The reason for this is not clear, perhaps representing a change in PI3K signaling such as a reduced dependence on Akt activation (32). Alternatively, the wildtype sample used for normalization in this experiment was whole mammary gland lysate. This tissue contains a large amount of insulin-responsive fat, and may show higher basal levels of Akt activation than mammary epithelium. We therefore tested additional adenosquamous tumors from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre mice for phospho-Akt and used lineage-depleted mammary epithelium as control. Indeed, phospho-Akt<sup>T308</sup> and phospho-Akt<sup>S473</sup> were significantly elevated in 12 adenosquamous carcinomas from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre mice (Supplemental Figure 5).

Elevated levels of phospho-Akt<sup>T308</sup> and phospho-Akt<sup>S473</sup> were observed in lysates from adenomyoepithelioma lesions in R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice, as well as in lysates from R26-Pik3ca<sup>H1047R</sup>;p53<sup>F/+</sup>;MMTV-Cre<sup>NLST</sup> spindle/EMT tumors. Interestingly, elevated
levels of phospho-AktT308 and phospho-AktS473 were also seen in lysates from spindle/EMT as well as poorly differentiated adenocarcinomas from p53f/+;MMTV-CreNLST mice. Elevated levels of PTEN expression and of phospho-c-Jun accumulation were observed in each mammary tumor type studied. In the case of PTEN, this is likely a result of PI3K-pathway induced phosphorylation and stabilization of PTEN, a negative feedback loop which functions to decrease PIP3 levels (33). Also, increased phospho-c-Jun is associated with elevated PI3K pathway activation (34). Finally, it has been suggested that activation of other PDK1 substrate kinases, such as SGK3, may play an important role downstream of PIK3CA gene mutation in breast cancer (32). We therefore analyzed SGK3 phosphorylation in these tumors. Phospho-SGK3S486 was not elevated in any of the tumor types analyzed (Supplemental Figure 6).

**PIK3CAH1047R mice form multiple mammary tumor subtypes**

PIK3CA gene mutations are found in ERα positive, HER2/Neu positive and in TNT cancers (4-7). To further define mammary tumor subtype in our Pik3ca model, we stained representative tumors for expression of luminal and basal cell keratins, keratin 8 (K8) and keratin 14 (K14), respectively (Figure 5, Supplemental Figure 7). Glandular regions of R26-Pik3caH1047R;MMTV-CreNLST adenosquamous carcinomas contained cells expressing either keratin. Mammary architecture was maintained in glandular regions, in that K14+ cells surrounded layers of K8+ cells (Figure 5A middle). K8+ cells in these regions also expressed Gata3, albeit at a level lower than in adjacent normal glands (data not shown) (35). The glandular epithelium in R26-Pik3caH1047R;MMTV-CreNLST adenomyoepitheliomas also stained positive for both keratins (Figure 5A right). K14+ expression was noted in interstitial fusiform cells surrounding glandular type structures (data not shown). Spindle tumors from p53f/+;MMTV-CreNLST mice had few K8 or K14 positive cells (Figure 5B left). In contrast, cells
in poorly differentiated adenocarcinomas from p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} mice were either mostly K14\textsuperscript{+} (Figure 5B middle) or mostly K8\textsuperscript{+} (Figure 5B right). Some double negative regions and regions with double positive cells were also observed (data not shown). Adenosquamous carcinomas from R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} and R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} mice showed the same staining pattern (Figure 5C left). Similarly, K14 and K8 staining in spindle/EMT (Figure 5C middle) and poorly differentiated adenocarcinomas (Figure 5C right) from R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} and p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} mice was indistinguishable (note, right panel of Figure 5C shows a tumor region with many K14\textsuperscript{+}/K8\textsuperscript{+} double positive cells; such regions were also frequently observed in poorly differentiated adenocarcinomas from p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} mice as described above). K5 immunostaining closely matched that observed for K14 in each case (data not shown).

ER\alpha expression was noted in R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} (Figure 5D) and R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} (data not shown) adenosquamous carcinomas, although nuclear staining appeared reduced in most tumor cells as compared to that observed in normal ducts (Figure 5D inset). R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} adenomyoepitheliomas also contained nuclear ER\alpha\textsuperscript{+} cells (Figure 5D right). In contrast, less signal was observed in other tumor types. To better quantify ER\alpha expression, western blot analysis was performed on tumor lysates (Figure 5D bottom). Adenosquamous carcinomas (from R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} and R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST}) as well as adenomyoepitheliomas from R26-Pik3ca\textsuperscript{H1047R};MMTV-Cre\textsuperscript{NLST} mice expressed similar levels of ER\alpha as in the control gland. In contrast, ER\alpha expression was decreased in p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} poorly differentiated carcinomas, R26-Pik3ca\textsuperscript{H1047R};p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} spindle/EMT tumors and trended towards decreased expression in p53\textsuperscript{f/+};MMTV-Cre\textsuperscript{NLST} spindle/EMT tumors (Figure 5D).
Next, we stained for the EMT marker N-cadherin (N-Cad) and, in some tumors, for desmin (Des) or vimentin (Vim) (Figure 6, Supplemental Figure 8)(27, 36, 37). R26-Pik3caH1047R;MMTV-CreNLST adenosquamous carcinomas had scattered N-Cad+ cells and Vim+ cells in squamous regions (Figure 6A). R26-Pik3caH1047R;MMTV-CreNLST adenomyoepitheliomas had many N-Cad+ cells and some Des+ cells in non-glandular regions (Figure 6B). In contrast, N-Cad+ cells were observed throughout spindle/EMT tumors from p53f/+;MMTV-CreNLST and R26-Pik3caH1047R;p53f/+;MMTV-CreNLST mice (Figure 6C, left). Both also had rare Des+ cells. Adenosquamous carcinomas from R26-Pik3caH1047R;p53f/+;MMTV-CreNLST animals had some N-Cad+, but no Des+ cells. Finally, poorly differentiated adenocarcinomas from p53f/+;MMTV-CreNLST mice showed no evidence of EMT.

Adenosquamous carcinomas from R26-Pik3caH1047R;MMTV-CreNLST single or Pik3caH1047R;p53f/+;MMTV-CreNLST double mutant mouse also contained cells expressing a marker of differentiated squamous epithelium, cytokeratin 10 (K10). K10+ cells were typically found lining keratinized cysts (Figure 6C right, Supplemental Figure 8). Atf3 has been implicated in squamous cell carcinoma (38) and accumulated to widely variable levels in our mammary tumors. For example, some R26-Pik3caH1047R;MMTV-CreNLST tumors (adenosquamous carcinomas and adenomyoepitheliomas) and some p53f/+;MMTV-CreNLST poorly differentiated carcinomas expressed Atf3. Expression was not observed in other tumor types (Supplemental Figure 9). Mammary tumors of all genotypes appeared less bloody than MMTV-Wnt1 control tumors. This was typically associated with less vasculature and, in most cases, with elevated expression of carbonic anhydrase IX (CAIX), an indirect marker of hypoxia (Supplemental Figure 9) (39). Interestingly, elevated β-catenin levels, comparable to levels in MMTV-Wnt1 tumors, were observed in both major tumor types from R26-Pik3caH1047R;MMTV-CreNLST mice.
and in poorly differentiated adenocarcinomas in p53^{f/+};MMTV-Cre^{NLST} mice, suggesting that Wnt signaling may be activated in each case (Supplemental Figure 9).

To quantify cell proliferation and apoptosis in each tumor type, we performed Ki67 immunohistochemistry and TUNEL analysis respectively (Table 1, Supplemental Table 3). Each tumor type examined had very low levels of TUNEL positive nuclei (<1% in all cases). This result is not surprising given that PI3K pathway activation and p53 gene deletion are both associated with enhanced cell survival. In contrast, the percentage of Ki67 positive nuclei varied with genotype and tumor type. Mammary tumors from R26-Pik3ca^{H1047R};MMTV-Cre^{NLST} mice had the highest proportion of Ki67 positive nuclei, while p53^{f/+};MMTV-Cre^{NLST} tumors had the lowest. In tumor type analysis, adenosquamous carcinomas had the highest Ki67 index while poorly differentiated carcinomas had the lowest.

Discussion

PIK3CA is commonly mutated in human breast cancer. The PIK3CA locus is also amplified in some breast cancers (6, 40, 41). To test for initiation of mammary tumor formation by PIK3CA alleles we generated syngeneic R26-Pik3ca^{H1047R} and R26-Pik3ca^{wt} knock-in mice. Each strain carried a Cre-inducible allele of mouse Pik3ca, and each was crossed to MMTV-Cre mice to activate expression within mammary epithelium. Starting at 5 months, R26-Pik3ca^{H1047R};MMTV-Cre females developed mammary tumors at a high frequency. Importantly, R26-Pik3ca^{wt};MMTV-Cre mice did not. Thus, low-level ectopic expression of p110α^{H1047R}, but not p110α^{wt}, is sufficient to initiate mammary tumor formation in mice. This result is consistent with identification of H1047R mutations in premalignant lesions (8-10). We did not observe any gross abnormalities in glands of young R26-Pik3ca^{H1047R};MMTV-Cre^{NLST}
mice, likely due to inefficient Cre-deletion in this line. It will be important to define early responses to PIK3CA<sup>H1047R</sup> gene activation in our model, perhaps using line A or through ex vivo, Cre-induced, gene activation and transplantation.

PIK3CA mutations occur in all major subtypes of human breast cancer (4-7, 11). Our mouse model of Pik3ca<sup>H1047R</sup>-induced breast cancer develops adenosquamous carcinoma and adenomyoepithelioma of the mammary gland at high frequency. Both tumor types were ERα<sup>+</sup>. Both also contained luminal and myoepithelial keratin positive cells, suggesting that the cell of origin had bilineage potential. As PIK3CA and TP53 are the two most commonly mutated genes in human breast cancer, and mutations in both occur together in many tumors, we tested for cooperation between these genes in our model. Indeed, we observed reduced survival of R26-Pik3ca<sup>H1047R</sup>;p53<sup>f</sup>;MMTV-Cre<sup>NLST</sup> double mutant animals. Cooperation between Pik3ca<sup>H1047R</sup> and p53 loss-of-function mutation is in contrast to the situation observed in MMTV-myrPik3ca mice, where mammary tumor formation is enhanced by expression of CDK4<sup>R24C</sup> but not by deletion of one p53 allele (42). In our model, apoptosis was very low in all of the tumors analyzed. In contrast, the proliferation rate varied significantly from one tumor type to another, tracking more closely with tumor type than with genotype. Thus, the observed cooperation between pik3ca<sup>H1047R</sup> and p53 loss-of-function mutation is not at the level of apoptosis or proliferation. Indeed, data presented in Figures 2A and 3C indicate that pik3ca<sup>H1047R</sup> and p53 mutations cooperate in mammary tumor initiation and interact to control mammary tumor type. In total, on wildtype and p53 mutant backgrounds, we observed 5 distinct types of mammary tumors in our PIK3CA model: adenosquamous carcinoma, adenomyoepithelialomas, spindle/EMT tumors, poorly differentiated adenocarcinomas and radial scar type lesions. While some of these tumor types are quite rare in humans, poorly differentiated adenocarcinomas are
common, as are spindle/EMT tumors (Basal B tumors in humans). In addition, adenosquamous carcinomas are metaplastic, a tumor type in humans with a high frequency of PIK3CA mutations (11). This result is consistent with the wide spectrum of human breast cancers found to have PIK3CA mutations. In addition, our diverse collection of tumors includes ERα+ tumors as well as TNT-type tumors. This result contrasts with tumor models using Neu, Wnt, Myc or Polyoma middle T, each of which induce a very specific mammary tumor type in mice (43).

We tested for PI3K pathway activation in major mammary tumor types that developed in our PIK3CAH1047R model mice. To this end we analyzed Akt phosphorylation, which was elevated in each tumor type. The modest level of Akt activation noted was somewhat surprising, although this was related to the use of whole mammary gland control tissue rather than lin-mammary epithelium for normalization (Supplemental Figure 5). In any case, Akt-independent transformation has been noted in some breast tumors with a PIK3CA mutation (32), and other PDK1 substrates are thought to play a role in this case. Indeed, transgenic mice expressing activated alleles of Akt1 from the MMTV LTR do not form mammary tumors, highlighting the importance of other PI3K pathways in breast cancer (44, 45). Future studies will be required to define the importance of specific PIP3-responsive kinases and pathways in our Pik3caH1047R model.

By using the Cre-conditional knock-in system, we can compare mammary tumor formation in response to distinct alleles of Pik3ca, each expressed at precisely the same level and in the same cell types. For example, we can now directly compare mammary tumors in our R26-Pik3caH1047R model with tumor formation in mice expressing helical domain mutants of Pik3ca. As our system is Cre-inducible, we can also compare mammary tumor formation in R26-Pik3caH1047R;MMTV-CreNLST mice and in Ptenfr;MMTV-CreNLST mice, where PI3K pathway...
activation will occur in the same cell of origin but through a distinct mechanism. The importance of cell of origin can now be probed in our system as transgenic mice are available to activate Pik3ca alleles in luminal committed cells (WAP-Cre) as well as in bipotential cells expressing Keratin 14 (K14-Cre). Finally, this model can be used to identify genetic and cellular events associated with progression/metastasis, to screen for novel therapeutic targets in Pik3ca\(^{H1047R}\) mammary tumor cells \textit{ex vivo} and to develop preclinical data on PI3K pathway inhibitors for treatment of breast cancer.
Acknowledgments: We thank Neil Adams, Farrah Awan, Adrian Cozma, Zhe Jiang, Tao Deng, Hui Qin Li, Jodi Garner, Marina Gertsenstein, Alex Manno, Brenda Cohen and Aaron Kucharczuk for advice and/or technical support; Drs. C.-C. Hui, Andras Nagy, Janet Rossant and Brian Ciruna for advice/reagents, as well as Dr. Jean Andrey for statistical advice. Finally, we are grateful to Dr. Robert Cardiff for comments on mouse tumor pathology.
References
Table 1: Ki67 and TUNEL data for R26-Pik3ca<sup>H1047R</sup>;p53<sup>f/+</sup>;MMTV-Cre<sup>NLS1</sup> mouse mammary tumors.

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Figure Legends

Figure 1. *R26-Pik3ca^{H1047R};MMTV-Cre* mice develop tumors in the mammary gland, skin and lymphocytic tissue. *A*, Conditional knock-in mice were generated by recombining mouse *Pik3ca* cDNAs (preceded by a loxP-flanked transcriptional stop cassette) into the R26 locus (22). *B*, Lineage depleted (lin⁻) mammary epithelium from R26-Pik3ca;MMTV-Cre mice expressed their respective transgene, while cells from Cre⁻ littermates did not. *C*, Kaplan-Meier survival curves for R26-Pik3ca^{H1047R};MMTV-Cre female mice. *D*, Cause of death data for R26-Pik3ca^{H1047R};MMTV-Cre lineA (left) and R26-Pik3ca^{H1047R};MMTV-Cre^{NLST} (right) mice. “Other” includes mice that died without obvious tumors (for example, with dystocia) or mice that could not be examined due to decomposition. MGT – mammary gland tumors, L/T – lymphoma/thymoma, ST – skin tumors.

Figure 2. *Pik3ca^{H1047R} and p53 deletion show cooperation in mice*. *A*, Kaplan-Meier survival curves. *B*, p53 deletion in tumor DNA was confirmed via PCR (diagnostic 612bp fragment). *C*, Cause of death data for R26-Pik3ca^{H1047R};p53^{f/+};MMTV-Cre^{NLST} (left), p53^{f/+};MMTV-Cre^{NLST} (middle), and R26-Pik3ca^{H1047R};p53^{f/+};MMTV-Cre^{NLST} (right) mice. MGT – mammary gland tumors, L/T – lymphoma/thymoma.

Figure 3. Mammary tumor pathology in *R26-Pik3ca^{H1047R};p53^{f/+};MMTV-Cre^{NLST}* mice. *A*, Mammary tumors in R26-Pik3ca^{H1047R};MMTV-Cre^{NLST} mice are mostly adenomyoepitheliomas [AME] (left) or adenosquamous carcinomas [ASC] (middle). *B*, p53^{f/+};MMTV-Cre^{NLST} mice develop mammary spindle cell tumors (left) or poorly differentiated adenocarcinomas [PDA].
C, R26-Pik3ca<sup>H1047R</sup>;p53<sup>E+</sup>;MMTV-Cre<sup>NLST</sup> mice develop mostly spindle cell tumors (left) or ASCs (middle). Some developed radial scar [RS] or PDAs (right). Scale bars = 50μm.

Figure 4. Akt activation in mammary tumors from Pik3ca<sup>H1047R</sup> model mice. A, Western blot analysis of PI3K-pathway components and phospho-c-jun. B, Western blot signals were quantified and normalized with respect to β-actin. Mean fold-increase compared to mammary lysate from a control mouse was calculated after normalization (bars: group mean values, error bars: +/- standard error, asterisks: significant increase in expression [one-sided one sample T-test, α = 0.05, * p < 0.05 and ** p < 0.1]). Yellow dashed line – protein expression in a normal mammary gland, AME – adenomyoepithelioma, ASC – adenosquamous carcinoma, PDA – poorly differentiated adenocarcinoma.

Figure 5. Expression of luminal and basal differentiation markers and ERα in Pik3ca<sup>H1047R</sup> model mammary tumors. A, Normal mammary ducts (left) express cytokeratin 14 (K14) and cytokeratin 8 (K8). Glandular regions of adenosquamous carcinoma [ASC] (middle) or adenomyoepithelioma [AME] (right) from R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> mice. B, Spindle cell tumor (left) and poorly differentiated adenocarcinomas [PDA] (middle, right) from p53<sup>E+</sup>;MMTV-Cre<sup>NLST</sup> mice. C, Glandular region of ASC (left), spindle cell tumor (middle), and PDA (right) from R26-Pik3ca<sup>H1047R</sup>;p53<sup>E+</sup>;MMTV-Cre<sup>NLST</sup> mice. D, Immunohistochemistry for estrogen receptor α (ERα) in R26-Pik3ca<sup>H1047R</sup>;MMTV-Cre<sup>NLST</sup> ASCs (top left, top middle) and AMEs (top right) compared to normal mammary epithelium (inset). Western blot analysis for ERα (bottom left). Western signals were quantified as described above (bottom right) (bars: sample mean, error bars: +/- standard error, asterisks: significant increase [one-sided one sample test, α = 0.05, * p < 0.05 and ** p < 0.1]).
T-test, $\alpha = 0.05$, * $p < 0.05$ and ** $p < 0.1$). White scale bars = 40$\mu$m, black scale bars = 20$\mu$m.

Yellow dashed line – protein expression in a normal mammary gland.

**Figure 6.** Epithelial-Mesenchymal Transition in mammary tumors from Pik3ca$^{H1047R}$ model mice. *A*, R26-Pik3ca$^{H1047R}$;MMTV-Cre$^{NLST}$ adenosquamous carcinomas express N-cadherin (*left*) and vimentin (*right*). *B*, R26-Pik3ca$^{H1047R}$;MMTV-Cre$^{NLST}$ adenomyoepitheliomas express N-cadherin (*left*) and desmin (*right*). *C*, Spindle tumors express N-cadherin (*left*); adenosquamous carcinomas express cytokerain 10 (*right*). Scale bars = 40$\mu$m.
FIGURE 2

A

Survival

Time (days)

n=7 n=13 n=10

- R26-Pik3ca

- R26-Pik3ca

- R26-Pik3ca

- R26-Pik3ca

- p53

- p53

- p53

- p53

B

p53

PDA

Censored Data

612bp

C

n=7

n=13

n=10

L/T with MGT, 14%

L/T with MGT, 8%

L/T no MGT, 8%

L/T no MGT, 8%

MGT, 84%

MGT, 80%

MGT, 80%
FIGURE 4

A

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<th>Normal Mammary Gland</th>
<th>MMTV-Wnt1 (n=2)</th>
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<th>R26-Pik3caH1047R;MMTV-CreNLST, AME (n=4)</th>
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<th>p53f/++;MMTV-CreNLST, Spindle (n=4)</th>
<th>R26-Pik3caH1047R;p53f/++;MMTV-CreNLST, ASC (n=3)</th>
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B

- R26-Pik3caH1047R;MMTV-CreNLST, ASC
- R26-Pik3caH1047R;MMTV-CreNLST, AME
- p53f/++;MMTV-CreNLST, PDA
- p53f/++;MMTV-CreNLST, Spindle
- R26-Pik3caH1047R;p53f/++;MMTV-CreNLST, ASC
- R26-Pik3caH1047R;p53f/++;MMTV-CreNLST, Spindle

Fold Change Protein Expression, with Respect to a Normal Mouse Mammary Gland
Cooperation between Pik3ca and p53 mutations in mouse mammary tumor formation

Jessica R Adams, Keli Xu, Jeff C. Liu, et al.

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