Urothelium-Specific Expression of Mutationally Activated Pik3ca Initiates Early Lesions of Noninvasive Bladder Cancer

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Although approximately 70% of bladder cancers are noninvasive and have high recurrence rates, early-stage disease is understudied. The lack of models to validate the contribution of molecular drivers of bladder tumorigenesis is a significant issue. Although mutations in PIK3CA are frequent in human bladder cancer, an in vivo model for understanding their contribution to bladder tumorigenesis is unavailable. Therefore, a Upk2-Cre/Pik3caH1047R mouse model expressing one or two R26-Pik3caH1047R alleles in a urothelium-specific manner was generated. Pik3caH1047R functionality was confirmed by quantifying Akt phosphorylation, and mice were characterized by assessing urothelial thickness, nuclear atypia, and expression of luminal and basal markers at 6 and 12 months of age. While at 6 months, Pik3caH1047R mice developed increased urothelial thickness and nuclear atypia, progressive disease was not observed at 12 months. Immunohistochemistry showed urothelium maintained luminal differentiation characterized by high forkhead box A1 (Foxa1) and peroxisome proliferator-activated receptor γ expression. Surprisingly, Pik3caH1047R mice subjected to low-dose carcinogen exposure [N-butyl-N-(4-hydroxybutyl)nitrosamine] exhibited no significant differences after exposure relative to mice without exposure. Furthermore, single-sample gene set enrichment analysis of invasive human tumors showed those with mutant PIK3CA did not exhibit significantly increased phosphatidylinositol 3-kinase/AKT pathway activity compared with wild-type PIK3CA tumors. Overall, these data suggest that Pik3caH1047R can elicit early tumorigenic changes in the urothelium, but progression to invasion may require additional genetic alterations. (Am J Pathol 2023, 193: 2133–2143; https://doi.org/10.1016/j.ajpath.2023.07.001)

An estimated 570,000 new cases of bladder cancer (BC) are diagnosed worldwide every year, with about 82,000 new cases and 17,000 deaths occurring in the United States.1,2 Clinically, BC is classified as non–muscle- or muscle-invasive disease, and this distinction is useful in determining whether aggressive treatment is warranted.3 However, classifying BC as noninvasive or invasive can help to distinguish the unique biological characteristics of each as distinct entities and thereby identify appropriate therapies.1–5 Approximately 70% of newly diagnosed BC cases are noninvasive, which include papillary tumors (stage Ta) and carcinoma in situ.4,6 In addition to comprising most diagnoses, noninvasive BC also has a high recurrence rate (up to 70%), which requires long-term monitoring and treatment.7 This is a major contributing factor to the high cost of BC treatment, making BC one of the most expensive cancers to manage.8

Despite the challenges of clinically managing noninvasive BC, most research is focused on invasive disease. One reason for this is the limited availability of models for the
study of early-stage disease. Establishment of early-stage models of BC is vital to furthering the understanding of events leading to tumor initiation and progression and will offer in vivo platforms for the development of novel treatments, while additionally broadening the scope of BC research to increase focus on noninvasive disease. Currently, transgenic and conditional/tissue-specific knockout mouse models provide platforms to test the contribution of genetic alterations (eg, somatic mutation and DNA copy number loss) to tumor development and progression. However, additional clinically relevant models of early-stage BC are required.

The phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit α (PIK3CA) gene encodes the p110α catalytic subunit of phosphatidylinositol 3-kinase (PI3K). Signal transduction following activation of the PI3K/AKT pathway plays an important role in normal cell biology and pathologic states. Although PTEN deletion is common in invasive BC and cooperates with other common genetic alterations in advanced disease, mutations in PIK3CA are more frequently detected and have been identified across all BC stages. Mutations have been found in 20% to 30% of BC cases independent of stage, indicating these mutations are an early event. Although many activating mutations in PIK3CA have been detected in BC, an in vivo model of activating Pik3ca mutations in BC does not exist. Of the most common activating mutations, H1047R is currently available to study in a commercial mouse model. Therefore, this strain was used for the generation of a novel mouse model of BC driven by Pik3ca mutation.

Materials and Methods

Mouse Breeding, Genotyping, and Experiments

All animal experiments were performed in accordance with and following approval of the Institutional Animal Care and Use Committee at Pennsylvania State University College of Medicine (Hershey, PA). To generate mice with urothelium- and following approval of the Institutional Animal Care and All animal experiments were performed in accordance with

Mouse Breeding, Genotyping, and Experiments

All animal experiments were performed in accordance with and following approval of the Institutional Animal Care and Use Committee at Pennsylvania State University College of Medicine (Hershey, PA). To generate mice with urothelium-specific expression of one or two mouse Pik3caH1047R mutant alleles, commercially available R26-Pik3caH1047R mice (stock 016977; The Jackson Laboratory, Bar Harbor, ME) were bred with previously described Upk2-Cre mice, which express Cre recombinase in a urothelium-specific manner. This breeding strategy resulted in the development of male and female mice with one (Upk2-Cre/Pik3caH1047R) or two (Upk2-Cre/Pik3caH1047R/Pik3caH1047R) alleles of mutant mouse Pik3ca plus genetic control mice (no Cre and/or R26-Pik3caH1047R alleles).

All mice were genotyped by PCR analysis using the following primers: Upk2-Cre, forward, 5'-CGTACT-GACGTTGGGAGAAT-3'; Upk2-Cre, reverse, 5'-TGCATGATCCTGGATTG-3'; R26-Pik3caH1047R, common (oIMR8545; The Jackson Laboratory), 5'-AAAG TCGCTCTGAGTTGTTAT-3'; R26-Pik3caH1047R, mutant reverse (oIMR8805; The Jackson Laboratory), 5'-GCGA AGAGTTTGTCCCTCAACC-3'; and R26-Pik3caH1047R, reverse (oIMR8546; The Jackson Laboratory), 5'-GGAGGCGGGGAGAATGGGAT-3'. The presence of Upk2-Cre was indicated by a single band at approximately 350 bp. Wild-type and mutant R26-Pik3caH1047R mouse alleles were indicated by bands present at approximately 650 and 340 bp, respectively.

For experiments, one cohort of mice consisting of genetic control and Upk2-Cre/Pik3caH1047R mice with one and two mutant alleles was aged for 6 or 12 months (n = 56), whereas another cohort consisting of genetic control and Upk2-Cre/Pik3caH1047R mice with two mutant alleles was aged for 6 months and then exposed to the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine (BBN; B0938; TCI America, Portland, OR) in their drinking water at a subcarcinogenic concentration of 0.01% for 10 weeks (n = 25). BBN water was changed twice weekly during the 10-week period. Mice were then euthanized for bladder collection.

Immunohistochemistry

Dissected bladders were fixed in 10% formalin for 24 hours, processed, and embedded in paraffin using standard procedures. Sections (5 μm thick) were cut for hematoxylin and eosin and immunohistochemical staining. Immunohistochemistry (IHC) was performed as previously described. Slides were deparaffinized, rehydrated through graded ethanol, and then washed in distilled water. Antigen retrieval was performed using 1% antigen unmasking solution (H-3300; Vector Laboratories, Burlingame, CA) and heating slides in a pressure cooker (CPC-600; Cuisinart, Stamford, CT) for 20 minutes at high pressure. Then, pressure was released in short bursts to prevent boiling and preserve tissue integrity. Slides were cooled to room temperature and washed three times for 10 minutes in phosphate-buffered saline (PBS; pH 7.4). The subsequent incubations were performed at room temperature unless otherwise noted. Endogenous peroxidases were blocked using 1% hydrogen peroxide in methanol, and then washed in distilled water. Antigen retrieval was performed using 1% antigen unmasking solution (H-3300; Vector Laboratories, Burlingame, CA) and heating slides in a pressure cooker (CPC-600; Cuisinart, Stamford, CT) for 20 minutes at high pressure. Then, pressure was released in short bursts to prevent boiling and preserve tissue integrity. Slides were cooled to room temperature and washed three times for 10 minutes in phosphate-buffered saline (PBS; pH 7.4). The subsequent incubations were performed at room temperature unless otherwise noted. Endogenous peroxidases were blocked using 1% hydrogen peroxide in methanol for 20 minutes, and slides were washed again in PBS. Sections were blocked using horse serum (S-2000; Vector Laboratories) diluted in PBS for 1 hour to reduce nonspecific antibody binding and then incubated with primary antibody at 4°C overnight. Primary antibodies used include rabbit anti-phosphorylated Akt (p-Akt; S473; 1:200; 4060; Cell Signaling Technology, Danvers, MA), rabbit anti-p-Akt (T308; 1:200; 13038; Cell Signaling Technology), goat anti-Foxa1 (1:1000; sc-6553; Santa Cruz Biotechnology, Dallas, TX), rabbit anti-peroxisome proliferator-activated receptor γ (Pparγ; 1:200; 2430; Cell Signaling Technology), mouse anti-keratin (Krt)5/6 (1:200; M7237; Agilent, Santa Clara, CA), and mouse anti-Krt14 (1:200; NCL-L-LL002; Leica Biosystem, Buffalo Grove, IL). Following overnight incubation, slides were washed in PBS and incubated in biotinylated secondary antibody (1:200) diluted in PBS containing horse serum for 1 hour. Secondary antibodies used include anti-rabbit and anti-goat

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antibodies (BA-1000 and BA-9500, respectively; Vector Laboratories). Specific antibody binding was visualized using the Vectastain Elite ABC Peroxidase kit (PK-6100; Vector Laboratories), according to manufacturer’s protocol, with diaminobenzidine (K346811-2; Agilent) as the chromogen. Sections were then counterstained with hematoxylin and mounted with Cytoseal (8310-4; Thermo Scientific, Waltham, MA). This procedure was modified for mouse primary antibodies for use with the Mouse on Mouse Immunodetection Kit (BMK-2202; Vector Laboratories), according to manufacturer’s protocol. For p-Akt antibodies, Tris-buffered saline with Tween and Tris-buffered saline were used for washes and diluent, respectively, in place of PBS throughout the procedure.

Urothelial Thickness and Histologic Scoring

Urothelial thickness was measured using cellSens Entry 1.13 imaging software (Olympus, Center Valley, PA) on hematoxylin and eosin sections. Five measurements of the urothelium were taken and averaged per bladder, then graphed using GraphPad Prism software version 9 (GraphPad Software Inc., San Diego, CA). Statistical significance of urothelial thickness for 6- and 12-month aging mouse groups was determined via application of the Tukey multiple-comparisons tests using GraphPad Prism. Statistical significance of urothelial thickness for the comparison of 6-month—old mice with and without BBN exposure was determined by applying two-way analysis of variance tests both with and without interaction using R software version 4.2.0 (The R Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org). All tissues were examined and scored by a dedicated genitourinary pathologist (J.I.W.) in a blinded manner. For nuclear atypia, atypia was defined as presence of nuclear enlargement with nuclear contour irregularity. An atypia score of 0 to 2 was assigned to describe the extent of nuclear atypia observed, with 0 meaning no atypia; 1, patchy atypia; and 2, extensive atypia seen throughout the urothelium. For p-Akt IHC stains, expression levels were scored by calculating the H-score. The H-score was determined by multiplying percentage of positive cells × intensity of staining (from 0 to 3). Box plots were generated, and Kruskal-Wallis tests were applied to compare the H-scores of p-Akt across mouse groups (controls, one mutant Pik3ca allele, and two mutant Pik3ca alleles) using R software version 4.2.0.

Graphical Representation of Mutations and ssGSEA of TCGA Data

Gene-level RNA-sequencing read counts for The Cancer Genome Atlas (TCGA) BC cohort were downloaded from the Genomic Data Commons. After restricting to protein-coding genes, the edgeR R package was applied to compute gene-level log counts-per-million reads mapped values as input, the GSVA R package that performs gene set variation analysis (GSVA) was used to calculate single-sample gene set enrichment analysis (ssGSEA) scores based on the Hallmark PI3K/AKT/mammalian target of rapamycin (MTOR) gene set that represent PI3K/AKT/MTOR pathway activity levels in each sample. For follow-up ssGSEA, missense hotspot mutations recognized as oncogenic within other genes of the PI3K/AKT pathway were identified within TCGA data set using cBioPortal (https://www.cbioportal.org, last accessed October 25, 2022), and samples with these mutations were removed. Such mutations were identified in the following genes: FGFR3, ERBB2, ERBB3, HRAS, NRAS, PTEN, and AKT1. Kruskal-Wallis and Wilcoxon rank-sum tests were applied to compare the ssGSEA scores across groups of interest (normal tissue, Pik3CA wild-type tumors, and Pik3CA mutant tumors). A lollipop plot displaying the locations of Pik3CA mutations in the TCGA BC cohort was generated using the maftools R package and gene mutation information in the public mutation annotation format (MAF) file containing results produced by the Multi-Center Mutation Calling in Multiple Cancers project. These analyses were performed using R software version 4.2.0.

Results

Upk2-Cre/Pik3caH1047R Mice Express Increased Levels of Nuclear p-Akt

A novel mouse model of early urothelial tumorigenesis was developed by crossing a previously described Upk2-Cre mouse line with a commercially available R26-Pik3caH1047R mouse line. This resulted in Upk2-Cre/Pik3caH1047R mice, a new strain that constitutively expressed one or two alleles of Pik3caH1047R in a urothelial-specific manner (Figure 1A). Cre is expressed in the luminal and intermediate cells, allowing for the stop codon (flanked by loxp sites) preceding the Pik3ca mutant gene to be excised, resulting in the expression of Pik3caH1047R.

Following PI3K activation, Akt is phosphorylated at residue T308, then additionally phosphorylated at residue S473. Because p-Akt can be found in the cytoplasm and nucleus, both cytoplasmic and nuclear staining was assessed. Most notably, nuclear staining of S473 was significantly increased in the mutant mice compared with controls at 6 and 12 months of age (P = 5.26 × 10−4 and P = 3.39 × 10−4, respectively; Kruskal-Wallis test). Although significant increases in cytoplasmic T308 or S473 staining were not observed, it is interesting that cytoplasmic T308 staining was observed in umbrella and intermediate cells in which the Upk2 promoter driving Cre is active. Nuclear staining of T308 was not observed. Overall, these data confirm that expression of Pik3caH1047R activates downstream signaling in vivo.
Expression of \( \text{Pik3ca}^{H1047R} \) in Urothelial Cells Results in Hyperplasia and Nuclear Atypia

Morphologic analysis showed 6- and 12-month—old mice with one or two alleles of \( \text{Pik3ca}^{H1047R} \) developed urothelial hyperplasia, as indicated by increased urothelial thickness (Figure 2A). To quantify the extent of hyperplasia in the \( \text{Upk2-Cre/Pik3ca}^{H1047R} \) mice, urothelial thickness (in micrometers) was measured at 6 and 12 months of age (Figure 2B). At 6 months, urothelial thickness was significantly increased in mice with one or two alleles of \( \text{Pik3ca}^{H1047R} \) compared with controls.
At 12 months of age, urothelial thickness was also significantly increased in mice with one or two alleles of Pik3caH1047R compared with controls ($P < 1.0 \times 10^{-4}$ and $P < 5.0 \times 10^{-4}$, respectively; Tukey multiple-comparisons test). Atypia was also increased in mice aged 12 months with one or two alleles of Pik3caH1047R compared with control mice ($P < 0.0001$ and $P = 0.0005$, respectively; Tukey multiple-comparisons test). Urothelial thickness is also significantly increased in mice aged 12 months with one and two alleles of Pik3caH1047R compared with controls ($P = 0.0001$ and $P < 0.0001$, respectively; Tukey multiple-comparisons test). Urothelial thickness was progressive, with mutant mice developing thicker urothelium at 12 months of age.

Nuclear atypia (defined as presence of nuclear enlargement with nuclear contour irregularity) was also observed in Upk2-Cre/Pik3caH1047R mice (Table 1). To quantify nuclear atypia, a score of 0 to 2 was assigned to describe the extent of atypia observed (0 indicates no atypia; 1, patchy atypia; and 2, extensive atypia seen throughout the urothelium). At 6 months of age, atypia was clearly increased in mice with one or two alleles of Pik3caH1047R compared with controls. Atypia was also increased in mice aged 12 months of age with one or two alleles of Pik3caH1047R compared with controls. Notably, atypia was observed largely within the luminal and intermediate layers of the urothelium, which were targeted by the Upk2 promoter driving Cre, coinciding with p-Akt described in Figure 1.

Table 1  A Summary of Nuclear Atypia Scores of Upk2-Cre/Pik3caH1047R Mice at 6 and 12 Months of Age

<table>
<thead>
<tr>
<th>Atypia score</th>
<th>6 Months</th>
<th>12 Months</th>
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<tr>
<td></td>
<td>Control</td>
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<td>0</td>
<td>0 (0)</td>
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<tr>
<td>1</td>
<td>10 (77)</td>
<td>1 (8)</td>
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<tr>
<td>2</td>
<td>3 (23)</td>
<td>11 (92)</td>
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Data are given as number (percentage) in each group. At 6 months, atypia is increased in mice with one (n = 12) and two (n = 4) alleles of Pik3caH1047R compared with controls (n = 13). Atypia is also increased in mice aged 12 months with one (n = 13) and two (n = 6) alleles of Pik3caH1047R compared with control mice (n = 8).

(P = $1.0 \times 10^{-4}$ and $P < 1.0 \times 10^{-4}$, respectively; Tukey multiple-comparisons test). At 12 months of age, urothelial thickness was also significantly increased in mice with one or two alleles of Pik3caH1047R compared with controls ($P < 1.0 \times 10^{-4}$ and $P = 5.0 \times 10^{-4}$, respectively; Tukey multiple-comparisons test). Urothelial thickness was progressive, with mutant mice developing thicker urothelium at 12 months of age.
The Urothelium of Upk2-Cre/Pik3caH1047R Mice Exhibits High Expression of Luminal Differentiation Markers

To determine whether the Upk2-Cre/Pik3caH1047R mice exhibited a luminal or basal profile, a set of luminal (Foa1 and Pparγ) and basal (Krt5/6 and Krt14) differentiation markers was used for IHC analysis (Figure 3). Overall, mice with one or two alleles of Pik3caH1047R maintained a high level of Foa1 and Pparγ expression, similar to urothelium of genetic controls at 6 and 12 months of age. Also, mutant mice expressed low levels of Krt5/6 and Krt14, with Krt5/6 limited to the basal layer and Krt14 expressed in a rare subset of basal cells, as is consistent with normal mouse urothelium.20 These observations suggest Upk2-Cre/Pik3caH1047R mice retained a luminal expression profile similar to that observed in human early-stage BC.

Upk2-Cre/Pik3caH1047R Mice Do Not Exhibit Enhanced Susceptibility to the Carcinogen BBN

To determine the impact of Pik3caH1047R expression on sensitivity to a bladder-specific carcinogen, 6-month-old control and Upk2-Cre/Pik3caH1047R+/+ mutant mice were exposed to a subcarcinogenic concentration of BBN (0.01% via drinking water for 10 weeks). Mutant mice exposed to BBN showed thicker urothelium compared with control mice exposed to BBN (Figure 4A). To separately assess the effects of Pik3caH1047R mutations and BBN treatment, quantitative measurements of urothelial thickness were plotted for control and mutant mice, both with and without BBN treatment (Figure 4B). A two-way analysis of variance model with interaction was fit using factors for Pik3caH1047R mutations and BBN treatment. The interaction term was not significant ($P = 0.3941$), suggesting that the effect of BBN

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**Figure 3** Luminal differentiation markers forkhead box A1 (Foa1) and peroxisome proliferator-activated receptor γ (Pparγ) are highly expressed in Upk2-Cre/Pik3caH1047R mouse urothelium. Immunohistochemical staining of luminal (Foa1 and Pparγ) and basal [keratin (Krt) 5/6 and Krt14] markers of urothelial differentiation in Upk2-Cre/Pik3caH1047R mice aged 6 and 12 months. High expression of luminal markers Foa1 and Pparγ is maintained in the urothelium of mice with one and two alleles of Pik3caH1047R. In addition, basal markers Krt5/6 and Krt14 remain low in expression. Scale bars = 20 μm.
PIK3CA Mutation Is Not Associated with Increased Pathway Activation in Invasive Human Bladder Cancer

The impact of PIK3CA mutation in human BC was investigated using TCGA BC cohort, primarily composed of invasive tumor samples. A lollipop plot highlighted E542K, E545K, and H1047R as the three most common mutations (E542K, n = 18; E545K, n = 28; and H1047R, n = 6) (Figure 6A). ssGSEA was then performed to determine the impact of mutant PIK3CA on PI3K/AKT/MTOR pathway activation. Tumors with mutant PIK3CA, tumors with wild-type PIK3CA, and normal tissue samples were compared (P = 0.0158; Kruskal-Wallis test) (Figure 6B). Follow-up Wilcoxon rank-sum pairwise tests indicated that the scores of tumors with wild-type PIK3CA were similar to those of normal tissue (normal versus PIK3CA wild type, P = 0.29). However, the scores of tumors with mutant PIK3CA were significantly higher than those of tumors with wild-type PIK3CA and normal tissue (PIK3CA wild type versus PIK3CA mutant, P = 0.011; and normal versus PIK3CA mutant, P = 0.030). In addition, tumors with E542K, E545K, and H1047R mutations were highlighted for visual comparison and appeared to have similar pathway activation levels to each other. Further ssGSEA was then conducted on a restricted sample set in which samples that harbored oncogenic hotspot mutations within other genes of the pathway (FGFR3, ERBB3, HRAS, NRAS, PTEN, and AKT1) were removed (Figure 6C). In this analysis, no significant differences were observed among the groups (P = 0.211; Kruskal-Wallis test), and Wilcoxon rank-sum P values were as follows: normal versus PIK3CA wild type, P = 0.312; normal versus PIK3CA mutant, P = 0.110; and PIK3CA wild type versus PIK3CA mutant, P = 0.189. Tumors with E542K, E545K, and H1047R mutations were again highlighted for comparison, and pathway activation levels remained comparable to each other. Overall, these data suggest that PIK3CA mutations do not play a significant role in pathway activation in invasive BC.

Discussion

Activating mutations within the PI3K/AKT pathway are common in many cancers, making pathway components attractive therapeutic targets. More specifically, mutations within PIK3CA are frequent in several cancer types.
These mutations appear to be an early event in BC development as they are frequently detected in early-stage disease. The three most common activating PIK3CA mutations in BC are E542K, E545K, and H1047R. These mutations occur in the helical (E542K and E545K) and kinase (H1047R) domains and increase cell motility and migration in normal human urothelial cells. However, a transgenic mouse model of activating mutations in Pik3ca in BC does not currently exist. Coupled with the relative lack of research and availability of models for early-stage BC, this is a significant gap in the field. To address this, a Upk2-Cre/Pik3caH1047R mouse model was generated, which is the first model to express mutant Pik3ca within the urothelium, thus providing a novel model of BC tumorigenesis.

Upk2-Cre/Pik3caH1047R mice were bred and genotyped to identify mice carrying Upk2-Cre and one or two alleles of R26-Pik3caH1047R. To verify that the PI3K/Akt pathway was being activated in these mice, IHC was conducted for p-Akt at residues T308 and S473 (Figure 1). Following PI3K activation, Akt is partially activated by phosphorylation at residue T308, then an additional phosphorylation at residue S473 allows for full activation of Akt, making these residues appropriate markers of pathway activation. Akt has cytoplasmic as well as nuclear targets and, thus, staining was assessed for both subcellular locations. Results showed nuclear expression of S473 was significantly increased in the mutant mice at 6 and 12 months of age, indicating pathway activation by Pik3caH1047R. Although increased cytoplasmic T308 and/or S473 expression was expected, its absence may be indicative of scoring or sampling bias. For example, p-Akt appeared to be more prevalent in the urothelium at the neck of the bladder compared with the dome, which may have introduced variability contributing to sampling bias. Although nuclear expression of T308 was not observed, this was not surprising. T308 is the first residue phosphorylated and only induces partial activation of the pathway; therefore, it may not be sustained long enough for nuclear translocation. Overall, the clear increase in nuclear expression of S473 verified the functionality of mutant Pik3ca in these mice.

Histologic analysis was then conducted to characterize this model. At 6 and 12 months of age, mutant mice developed progressive hyperplasia. The thickness of the urothelium was significantly increased compared with controls (Figure 2). Nuclear atypia (Table 1) was also clearly increased in mutant mice compared with controls and was concentrated within the superficial and intermediate cell populations, as was similarly observed with p-Akt staining. As these are the cell populations in which the Upk2 promoter is active, it was anticipated that the expression of Pik3caH1047R would have the greatest effect in these cell populations. This specificity of the Upk2-Cre system is a major strength of this study. To increase the number of urothelial cell layers targeted by Pik3caH1047R, an alternative approach would be to use intravesical delivery of tamoxifen to activate inducible Cre systems, such as UBC-CreERT2 and Shh-CreERT2. This approach would limit the impact of activating Pik3ca mutations outside of the bladder while enabling future studies to determine the impact of mutant Pik3ca expression within a greater number of cell populations. Future studies could also include prolonged aging of these mice to determine whether Pik3ca mutation leads to the development of noninvasive BC.

These morphologic changes seen in the mutant mice suggest a role for activating Pik3ca mutations in urothelial tumorigenesis and support the use of PI3K inhibitors as a targeted treatment option for a subset of patients with noninvasive BC. Treatment with PI3K inhibitors is promising and currently used for cancers, such as breast and lung cancers. In fact, studies suggest patients with PIK3CA mutations have better response rates to PI3K inhibitors compared with those without PIK3CA mutations in breast cancer. Studies also suggest specific PIK3CA mutations may lead to differential responses to PI3K inhibitors; in particular, patients with H1047R mutations had better response rates compared with patients with other or no mutations in PIK3CA in various cancers.
could include use of this model to determine the treatment efficacy of PI3K inhibitors in noninvasive BC with Pik3ca mutations. This model could also be used for studying downstream signaling events as well as therapies targeted toward other components of the pathway, such as AKT and MTOR.

As BC can primarily be classified into luminal and basal molecular subtypes, a set of luminal (Foxa1 and Pparγ) and
basal (Krt5/6 and Krt14) urothelial differentiation markers was used to characterize the Upk2-Cre/Pik3ca<sup>H1047R</sup> mice. Mutant mice maintained high expression of Foxa1 and Pparγ and relatively low expression of Krt5/6 and Krt14, similar to control mice (Figure 3), indicating a model of luminal BC tumorigenesis consistent with expression seen in most human noninvasive BCs.

To also assess the contribution of Pik3ca<sup>H1047R</sup> to carcinogen susceptibility, control and mutant mice with two Pik3ca<sup>H1047R</sup> alleles after 10 weeks of BBN exposure were compared with mice not exposed to BBN. Surprisingly, a significant difference in urothelial thickness or nuclear atypia was not observed (Figure 4 and Table 2). This could in part be due to variability induced by BBN. In addition, mutant Pik3ca mice did not show increased sensitivity to BBN as differences in IHC staining for Foxa1, Pparγ, Krt5/6, and Krt14 were not observed between mutant and control mice (Figure 5). Future studies could include the use of different concentrations or exposure times to BBN to elucidate a possible impact of Pik3ca mutation on susceptibility to carcinogen.

As PIK3CA mutations are seen across all stages of BC, the impact of these mutations in invasive human BC was additionally investigated using a publicly available TCGA BC cohort (Figure 6). A lollipop plot highlighted E542K, the impact of these mutations in invasive human BC was susceptibility to carcinogen.

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In conclusion, these data describe Upk2-Cre/Pik3ca<sup>H1047R</sup> mice as a novel in vivo model of luminal BC tumorigenesis. As PIK3CA mutations are commonly detected in early-stage BC, this model can be useful in understanding the impact of PI3K/AKT pathway activation in BC tumorigenesis as well as studying the use of targeted therapies, including PI3K inhibitors, in noninvasive BC treatment.

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Disclosure Statement

None declared.

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