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Priority Report

Cancer Research

Luminal Expression of *PIK3CA* Mutant H1047R in the Mammary Gland Induces Heterogeneous Tumors

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Abstract

The phosphoinositide 3-kinase (PI3K) signaling cascade, a key mediator of cellular survival, growth, and metabolism, is frequently altered in human cancer. Activating mutations in *PIK3CA*, which encodes the α-catalytic subunit of PI3K, occur in approximately 30% of breast cancers. These mutations result in constitutive activity of the enzyme and are oncogenic, but it is not known whether they are sufficient to induce mammary carcinomas in mice. In the present study, we show that the expression of mutant *PIK3CA* H1047R in the luminal mammary epithelium evokes heterogeneous tumors that express luminal and basal markers and are positive for the estrogen receptor. Our results suggest that the *PIK3CA* H1047R oncogene targets a multipotent progenitor cell and, furthermore, show that this model recapitulates features of human breast tumors with *PIK3CA* H1047R. *Cancer Res; 71(13); 4344–51. ©2011 AACR.*

Introduction

Breast cancer is a complex and heterogeneous disease, probably due to the diversity of transforming events and mammary cells in which they occur, as well as to the cross-talk between the transformed epithelium and the surrounding stroma during breast tumorigenesis (1). Definition of the molecular and cellular alterations causing breast tumor heterogeneity should increase our understanding of breast cancer pathogenesis and support the design of optimal therapeutic strategies.

The phosphoinositide 3-kinase (PI3K) pathway, a central regulator of diverse normal cellular functions, is often subverted during neoplastic transformation (2). Mechanisms of activation of the PI3K pathway in cancer include (i) the mutation and/or amplification of *PIK3CA*, the gene encoding the α-catalytic subunit of the kinase (p110α); (ii) the loss of expression of the PTEN phosphatase that reverses PI3K action; (iii) the activation downstream of oncogenic receptor tyrosine kinases; and (iv) the mutation/amplification of Akt. A hyperactive PI3K pathway results in cancer cells that have a competitive advantage by decreasing cell death and increasing cell proliferation, migration, invasion, metabolism, angiogenesis, and resistance to chemotherapy.

PIK3CA is mutated in approximately 30% of human breast cancers with nearly 80% of the mutations occurring at 3 hotspots: E542K (∼4% of human breast cancers) and E545K (∼6%) within the helical domain; and H1047R (∼15%) within the kinase domain of p110α (3–5). These mutations result in a constitutively active enzyme that transforms cells *in vitro* and increases tumorigenicity in xenograft models (6–9). The increase in lipid kinase activity of the mutated p110α makes it a "druggable" target, and several inhibitors have entered phase I/II clinical trials (10, 11). Mutations in *PIK3CA* are more frequent in estrogen receptor (ER)-positive and HER2-positive tumors than in basal-like breast cancers (12). Reported correlations between *PIK3CA* mutations and prognosis are contradictory; studies comprising large numbers of patients have shown a paradoxical correlation of *PIK3CA* mutations with good prognosis (12–15).

PIK3CA mutations have been reported in breast ductal carcinoma *in situ* (DCIS; ref. 16), and the mutation frequencies in pure DCIS, DCIS adjacent to invasive ductal carcinoma (IDC), and IDC (17) are similar. Thus, mutations of *PIK3CA* appear to occur early in breast tumorigenesis. To test the hypothesis that *PIK3CA* mutation initiates mammary tumors, we generated a mouse model conditionally expressing *PIK3CA* H1047R. In this study, we show that the expression of this mutation in the mammary gland induces carcinomas with different phenotypes composed of cells expressing luminal markers or basal markers or both, and a significant number expressing ER.

Materials and Methods

Transgenic mice

We constructed a vector with a transcriptional STOP sequence flanked by *loxP* sites upstream of the 5′-terminally

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hemagglutinin (HA)-tagged human *PIK3CA* cDNA (Addgene) and an *IRES2-EGFP* reporter element (pIRES2-EGFP Vector; Clontech). The resulting *loxP*-STOP-*loxP*-HA-*PIK3CA-IRES2-EGFP* fragment was cloned into a recombination-mediated cassette exchange (RMCE) plasmid. The vector was introduced into the modified Rosa26 locus of Balb/c mouse embryonic stem (ES) cells by RMCE, and the ES cells were used for blastocyst injection (18). Chimeric mice were mated with Balb/c mice, and transgenic mice were identified by genotyping using the primers 5′-TGGCCAGTACCTCATGGATT-3′ and 5′-GCAATACATCTGGGCTACTTCAT-3′. FVB/N-Tg(MMTV-Cre) and FVB/N.B6-Tg(WAPiCre) mice have been described previously (19, 20). Tg(MMTV-Cre) mice have the FVB/N background, and B6-Tg(WAPiCre) mice were backcrossed for 5 generations to an FVB/N background. MMTV-*NeuNT* (strain TG.NK) mice were purchased from Charles River.

Immunohistochemistry

The following antibodies were used: K14 (Thermo Scientific; catalog no. RB-9020; dilution 1:1,000), K18 (Fitzgerald; catalog no. GP11; dilution 1:500), green fluorescent protein (GFP; Invitrogen; catalog no. A11122; dilution 1:500), ER (Santa Cruz Biotechnology; catalog no. SC-542; dilution 1:1,000), progesterone receptor (PR; Thermo Scientific; catalog no. RM-9102; dilution 1:200), α-smooth muscle actin (α-SMA; Thermo Scientific; catalog no. RB-9010 dilution 1:500), cleaved caspase-3 (Cell Signaling; catalog no. 9661; dilution 1:100), and Ki-67 (Thermo Scientific; catalog no. RM-9106; dilution 1:1,000).

Southern blotting

Genomic DNA from mouse tails was digested with 8 units of AvrII enzyme [New England BioLabs (NEB)] and separated on 1% agarose gel. A DIG-labeled DNA probe targeting the neomycin-resistance cassette was amplified using the PCR DIG Probe Synthesis Kit (Roche) and the primers 5′-ATGGGATCGGCCATTGAACAAGAT-3′ and 5′-CGGCCATTTTCCACCATGATAT-3′.

Reverse transcriptase-PCR

RNA was isolated from mouse tissue using TRIzol (Invitrogen). TaqMan polymerase and appropriate buffers were purchased from NEB. Human *PIK3CA* was detected using the primers 5′-CAGATCCCAGTGTGGTGGTACG-3′ and 5′-CCTCACGGAGGCATTCTAAAGT-3′ and endogenous *gapdh* was detected using the primers 5′-CATCAAGAAGGTGGTGAAGC-3′ and 5′-GGGAGTTGCTGTTGAAGTCG-3′.

Results and Discussion

Expression of *PIK3CA* H1047R in luminal mammary epithelial cells induces carcinomas

To test whether *PIK3CA* H1047R evokes mammary carcinoma, we generated transgenic mice that conditionally expressed this mutation in mammary epithelium. The correct integration of the construct in ES cells conditionally expressing *PIK3CA* H1047R (Fig. 1A) was tested by Southern blotting and PCR (Fig. 1B, left; data not shown). The ES cells were used to generate the H1047R line, and the mutation was confirmed by DNA sequencing (Fig. 1B, right). Next, H1047R animals were crossed to WAPiCre mice in which expression of recombinase Cre was driven by the whey acidic protein (*WAP*) promoter, which is active in alveolar progenitor cells and differentiated secretory luminal cells (20–23). Furthermore, we crossed H1047R animals to mice expressing Cre under the control of the mouse mammary tumor virus (MMTV) long terminal repeat, which results in expression within luminal mammary epithelial cells (19).

Female bitransgenic WAPiCre H1047R mice and littermate controls (WAPiCre) were generated. Mammary glands from WAPiCre H1047R virgin mice had GFP-positive areas indicating expression of the oncogene (Fig. 1C, left). This is consistent with previous studies that reported activity of the *WAP* promoter in a fraction of mammary epithelial cells in virgin mice (21, 23). Examination of whole mounts and hematoxylin and eosin (H&E)-stained sections revealed an average of 5.7 (±2.2) neoplastic lesions in glands from 21- to 24-week-old virgin WAPiCre H1047R mice but not from WAPiCre H1047R virgin mice up to 18 weeks of age or age-matched littermate controls (Fig. 1C, right).

The WAPiCre H1047R and control mice were impregnated to achieve maximal Cre-mediated recombination, and the pups were removed the day after delivery. Although parous WAPiCre mice did not form tumors, WAPiCre H1047R mice developed mammary tumors, on average, 36.8 (±4.9) days after delivery of the pups, corresponding to an age of 140.3 (±6.9) days (Fig. 2A). Bitransgenic MMTV-Cre H1047R mice and littermate controls (MMTV-Cre) were generated and left as virgins. Surprisingly, approximately 75% of the MMTV-Cre H1047R animals died before the age of 4 months. Although we did not identify the cause of death, we consider that leakiness of the *MMTV* promoter causing deleterious H1047R expression in tissues other than the mammary gland was a likely cause (D.S. Meyer and M. Bentires-Alj, unpublished observations). However, approximately 25% of the MMTV-Cre H1047R mice were viable and formed mammary carcinomas, on average, after 214 (±22.6) days, whereas no tumors were detected in MMTV-Cre control mice (Fig. 2B).

Because the average age of tumor onset between parous WAPiCre H1047R and virgin MMTV-Cre H1047R mice differs by approximately 75 days (140.3 vs. 214 days), we sought to investigate whether pregnancy accelerates *PIK3CA* H1047R-driven tumorigenesis. To address this question, we compared tumor onset in nulliparous and parous WAPiCre H1047R mice and found tumor onset to occur significantly earlier in parous mice than in nulliparous mice (Fig. 2C). These data show that pregnancy accelerates tumor onset in WAPiCre H1047R mice.

We then comparatively assessed the mechanisms underlying the accelerated tumor onset seen in parous against those in nulliparous WAPiCre H1047R mice. Fluorescence images and Western blot analysis showed enhanced GFP expression in glands from parous mice, indicating an increase in the number of cells that underwent Cre-mediated recombination and thus expressed H1047R (Supplementary Fig. S1). In addition, whole mounts of the involuting glands revealed a dramatic delay in involution in mice expressing *PIK3CA* H1047R compared with control animals (Supplementary Fig. S2A), which is in line with

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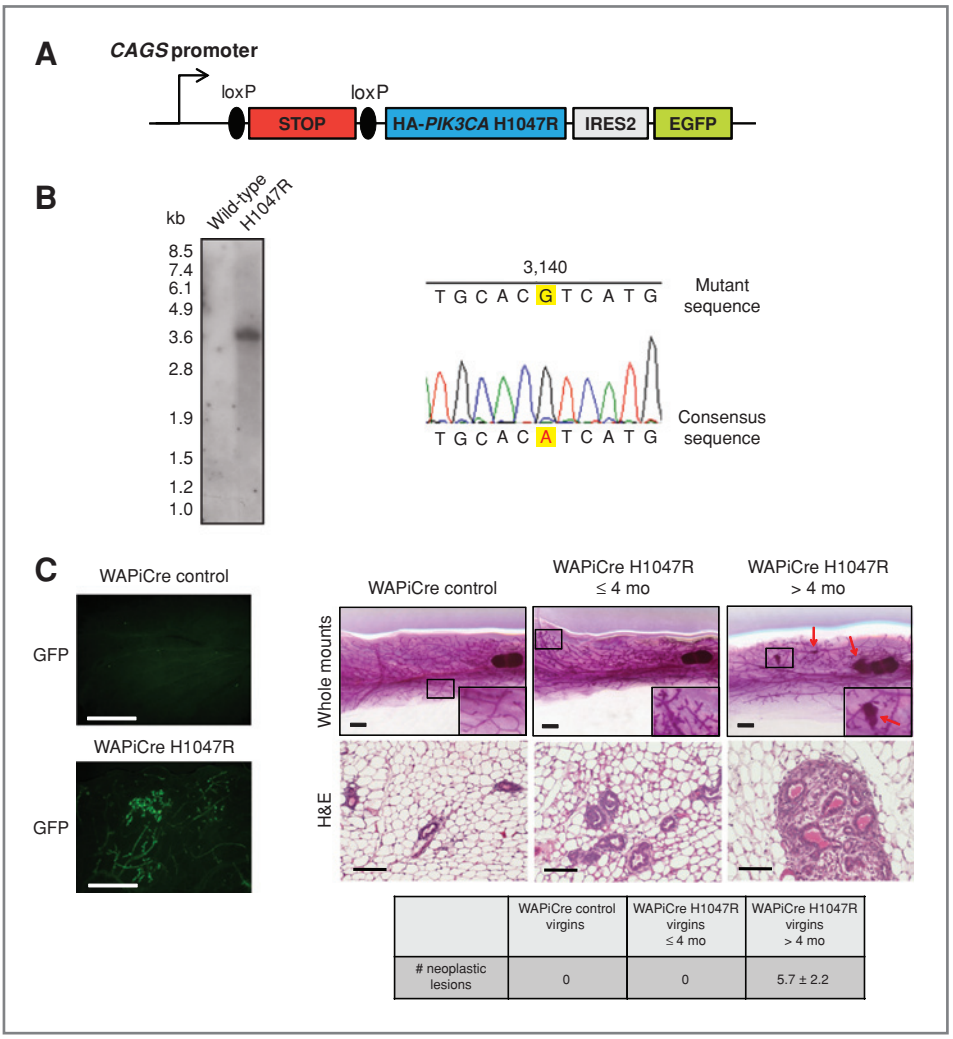

	WAPiCre control virgins	WAPiCre H1047R virgins ≤ 4 mo	WAPiCre H1047R virgins > 4 mo
# neoplastic lesions	0	0	5.7 ± 2.2

Figure 1. Targeted expression of mutant p110α in luminal mammary epithelial cells. A, schematic of the construct used for generating transgenic mice conditionally expressing *PIK3CA* H1047R. The *PIK3CA* cDNA is flanked by a floxed STOP cassette upstream and an IRES2-EGFP reporter element downstream. Expression of *PIK3CA* H1047R is driven by a chicken β-actin (*CAGS*) promoter. B, Southern blotting of genomic DNA from wild-type (WT) and *PIK3CA* H1047R mice (left) and sequencing of genomic DNA from H1047R transgenic mice harboring an A–G mutation at nucleotide 3140 (right). C, left, fluorescence images of glands from virgin WAPiCre control and virgin WAPiCre H1047R mice showing GFP expression; right, representative images of mammary glands from WAPiCre control mice (left), WAPiCre H1047R virgin mice between 12 and 18 weeks of age (middle), and WAPiCre H1047R virgin mice between 21 and 24 weeks of age (right). Images show whole-mount preparations (top) and H&E-stained sections (bottom). The red arrows indicate neoplastic lesions. Inserts show the indicated areas at higher magnification. Table (bottom) shows quantification of neoplastic lesions. Scale bars, 1 mm (whole mounts, fluorescence images); 100 μm (H&E images).

previous reports of a delayed involution when the PI3K pathway is hyperactivated (24, 25). Immunostaining for cleaved caspase-3 revealed a reduction in the number of apoptotic cells in involuting glands from WAPiCre H1047R mice compared with control mice, suggesting that reduced cell death is the cause of the delayed involution (Supplementary Fig. S2B and C). Our results suggest, therefore, that the acceleration of tumor onset is most likely due to an increase in the number of cells expressing *PIK3CA* H1047R in parous glands and to impaired cell death in involuting glands with the H1047R mutation. Indeed, pregnancy-induced proliferation could facilitate the acquirement of further genomic alterations and, therefore, accelerate tumorigenesis.

Analysis of RNA and proteins from WAPiCre H1047R and MMTV-Cre H1047R-induced tumors confirmed that mutant *PIK3CA* was expressed in the bitransgenic mice (Fig. 3A and B). In addition, tumors from both WAPiCre H1047R and MMTV-Cre H1047R mice showed 3-fold and 8-fold higher phospho-Akt (p-Akt) levels than mammary tumors from the MMTV-NeuNT model, respectively. In contrast, activation of the Erk1/2 pathway in *PIK3CA* H1047R tumors tended to be weaker than in tumors from MMTV-NeuNT mice (Fig. 3C).

Our results show that luminal expression of *PIK3CA* H1047R induces mammary tumor formation. This is consistent with the observation that conditional expression of mutant *PIK3CA* H1047R in type II lung alveolar epithelial cells causes lung adenocarcinomas in transgenic mice (26) and suggests that this mutation plays a causal role in epithelial cancers.

WAPiCre H1047R and MMTV-Cre H1047R–evoked mammary tumors are heterogeneous

To gain insight into significant pathophysiologic features, 22 WAPiCre H1047R and 21 MMTV-Cre H1047R-induced mammary tumors were characterized histologically. MMTV-Cre H1047R-caused tumors showed multiple adenomyoepitheliomas, with clusters of well-delineated polypoid tumors composed of a mixture of glandular epithelium and interstitial fusiform cells having abundant polar cytoplasm (Fig. 4A, top left). Similar tumors have been reported in MMTV-Cre/Pten$^{fl/fl}$/ErbB2^{KI} mice, suggesting that an activated PI3K pathway mediates this histotype (27).

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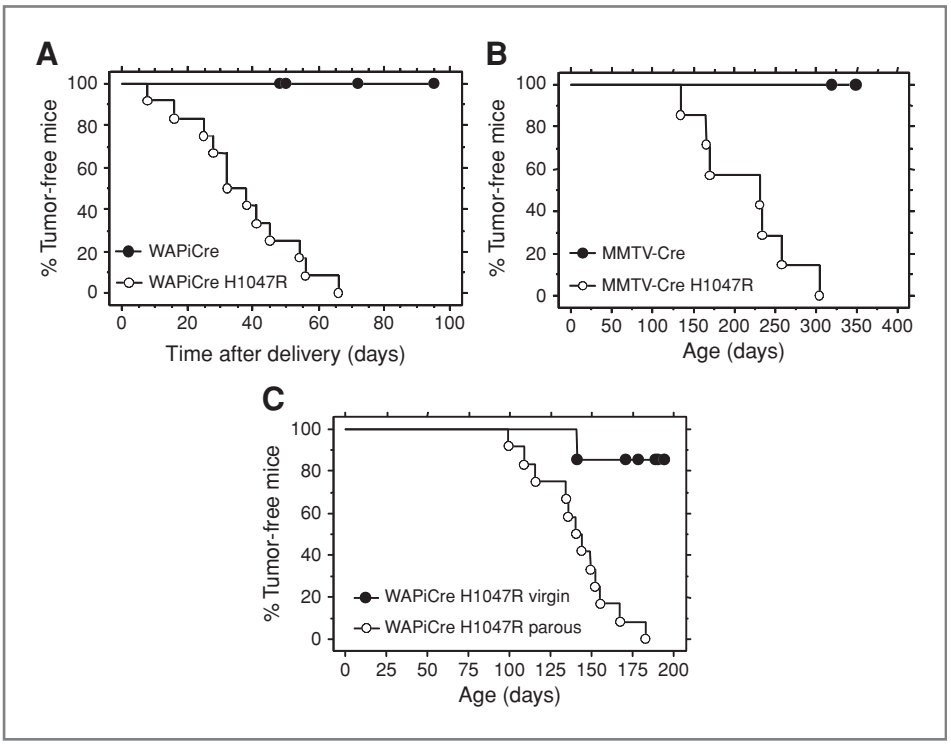

Figure 2. WAPiCre H1047R and MMTV-Cre H1047R mice develop mammary tumors. A, Kaplan–Meier curves showing tumor onset in bitransgenic WAPiCre H1047R mice ($n = 12$) and WAPiCre littermate controls ($n = 7$). The mice were impregnated and the pups removed from the mothers the day after delivery. Bitransgenic animals developed palpable tumors, on average, 36.8 (±4.9) days after delivery, corresponding to age 140.3 (±6.9) days. B, Kaplan–Meier curves showing tumor onset in double transgenic MMTV-Cre H1047R mice ($n = 7$) and MMTV-Cre littermate controls ($n = 8$). MMTV-Cre H1047R mice developed palpable tumors, on average, within 214 (±22.6) days. C, Kaplan–Meier curves showing tumor onset in virgin WAPiCre H1047R ($n = 7$) and parous WAPiCre H1047R mice ($n = 12$). Parous animals developed palpable tumors, on average, at 140.3 (±6.9) days and all animals had at least 1 tumor within 183 days of age. In contrast, by 170 days, only 1 out of 7 virgin WAPiCre H1047R mice developed a tumor (at 141 days). The difference in tumor latency between parous and virgin animals is statistically significant ($P = 0.0006$).

In contrast, the WAPiCre H1047R mice formed a more diverse spectrum of tumors with 5 distinct histotypes. The most prevalent tumor phenotypes found are adenosquamous carcinomas (54.6%) and adenomyoepitheliomas (22.7%). Furthermore, adenocarcinomas with squamous metaplasia (13.6%) and adenocarcinomas (9.1%) were observed, albeit at lower frequencies (Fig. 4A and B). All the glands surrounding the tumors displayed diffuse adenocarcinomatosis with invasive periductal cords of neoplastic epithelial cells in dense connective tissue (Fig. 4A, top right).

To further characterize H1047R-induced carcinomas, tumors were stained for luminal cytokeratin 18 (K18), basal/myoepithelial cytokeratin 14 (K14), and myoepithelial α-SMA markers, as well as for ER and PR. Notably, approximately 18% to 26% of the tumor cells of the adenomyopitheliomas from both transgenic mice expressed ER and approximately 16% expressed PR in the luminal cells (Fig. 4A; Supplementary Table S1; Supplementary Fig. S3A). In addition, other tumor histotypes contained ER-positive cells although at lower frequencies (<5%; Fig. 4A; Supplementary Table S1). WAPiCre- and MMTV-Cre H1047R tumors were positive for both luminal K18 and basal K14. The relative tumor area positive for K14 was approximately 15% in adenomyoepitheliomas and adenocarcinomatosis, whereas in the other phenotypes it ranged between 26% and 43%. The percentage of K18-positive tumor area was 25% in adenocarcinomatosis and ranged between 36% and 45% in the other tumor histotypes (Fig. 4A; Supplementary Table S1). Although the majority of tumor cells expressed either K18 or K14, some cells were positive for both K14 and K18 (Fig. 4C). As expected, the K14-positive cells within WAPiCre- and MMTV-Cre H1047R adenomyoepitheliomas were also α-SMA-positive (Fig. 4A). In contrast, the K14-positive cells within adenosquamous carcinomas observed in WAPiCre H1047R mice were largely negative for α-SMA, a characteristic of human metaplastic breast cancer in which *PIK3CA* is mutated in approximately 50% of cases (28). Interestingly, all tumors showed very low rates of apoptosis (0.2%–1.4%; Supplementary Table S1; Supplementary Fig. S3A), most likely due to the antiapoptotic effect of an activated PI3K pathway. Furthermore, we found the percentage of Ki-67–positive cells to be lower in adenomyoepitheliomas and adenocarcinomatosis than in all other tumor phenotypes (Supplementary Table S1; Supplementary Fig. S3A).

These data show that luminal expression of *PIK3CA* H1047R can induce mammary tumors expressing the basal marker K14. To exclude the possibility that luminal *PIK3CA* H1047R induces expression of paracrine factors that transform basal cells, we analyzed K14-positive cancer cells for GFP expression

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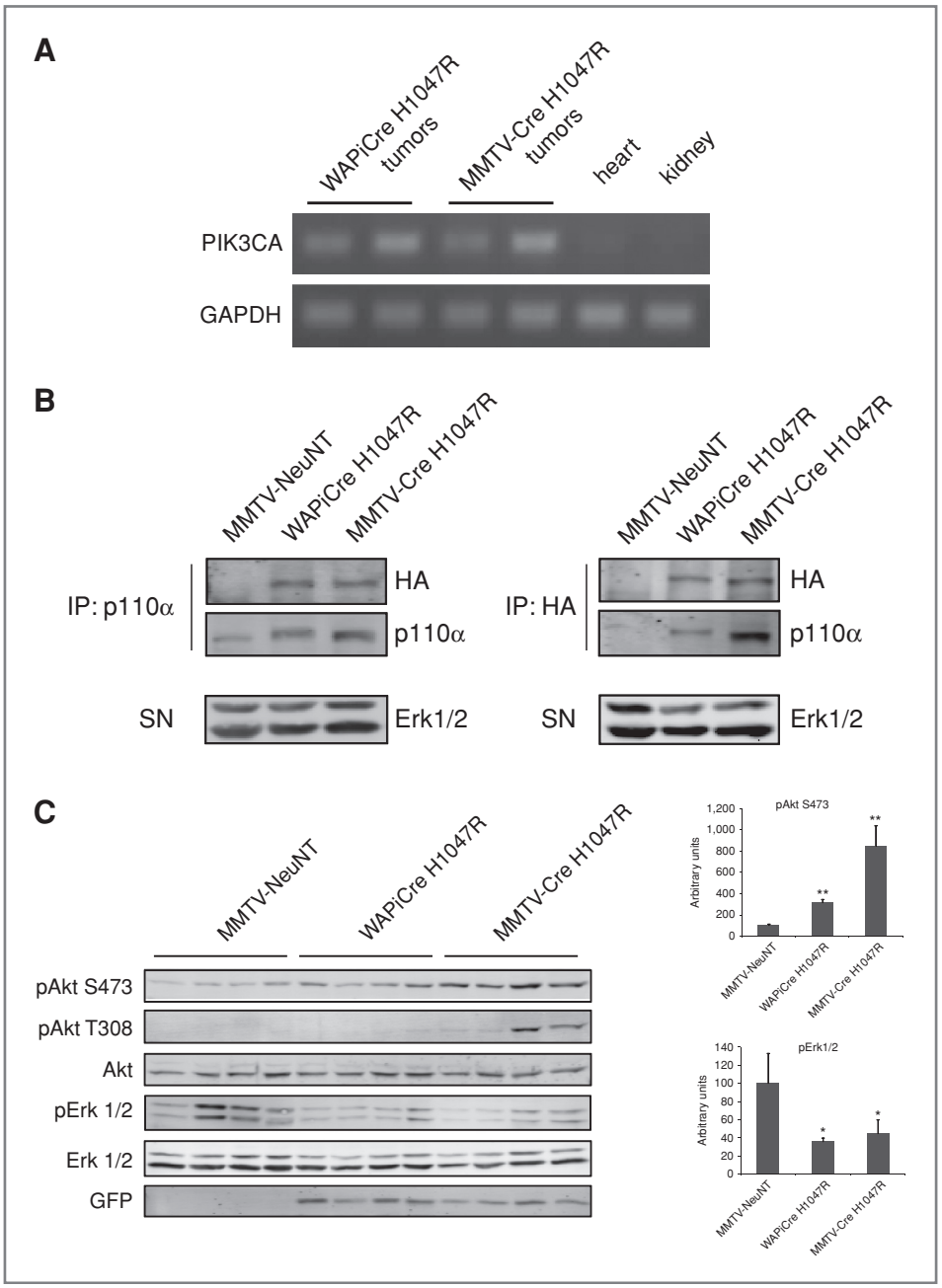

Figure 3. Tumors from WAPiCre H1047R and MMTV-Cre H1047R mice express mutant *PIK3CA*. A, RT-PCR showing expression of *PIK3CA* H1047R in WAPiCre H1047R and MMTV-Cre H1047R mammary tumors but not in heart or kidney tissues of a WAPiCre H1047R animal. B, expression of exogenous p110α as indicated by p110α-immunoprecipitation (IP) from MMTV-NeuNT, WAPiCre H1047R, and MMTV-Cre H1047R tumor lysates using anti-p110α (left) or anti-HA antibodies (right). C, immunoblotting of mammary tumor lysates from the indicated genotypes using the specified antibodies (left) and quantification of pAkt S473 and pErk1/2 signals (right). *, not significant; **, $P < 0.01$; SN: supernatant.

by immunostaining and fluorescence-activated cell sorting (FACS). There was a significant overlap between K14 and GFP expression (Fig. 4D; data not shown), suggesting that some K14-positive cancer cells within the WAPiCre H1047R and MMTV-Cre H1047R tumors resulted from expression of the oncogene in luminal cells. This supports the emerging notion that certain tumors with basal characteristics arise from luminal cells (29, 30).

Taken together, our results show that luminal expression of *PIK3CA* H1047R evokes mammary tumors, recapitulating the heterogeneity of human breast cancer. These results lead to major conclusions. The finding that *PIK3CA* H1047R causes ER- and PR-positive tumors suggests that PI3K activity expands ER-positive mammary cells, consistent with the presence of *PIK3CA* H1047R mutations in human ER-positive tumors (4).

The presence of cancer cells expressing luminal and basal markers in WAPiCre- and MMTV-Cre H1047R–evoked tumors suggests, in both models, that multipotent progenitor cells are the targets of H1047R-mediated transformation. The *WAP* promoter is active in a multipotent progenitor population, the parity-identified mammary epithelial cells (PI-MEC)—present in nulliparous mice, and expanded after pregnancy (21, 23). Tumors that developed in WAPiCre H1047R nullipar-

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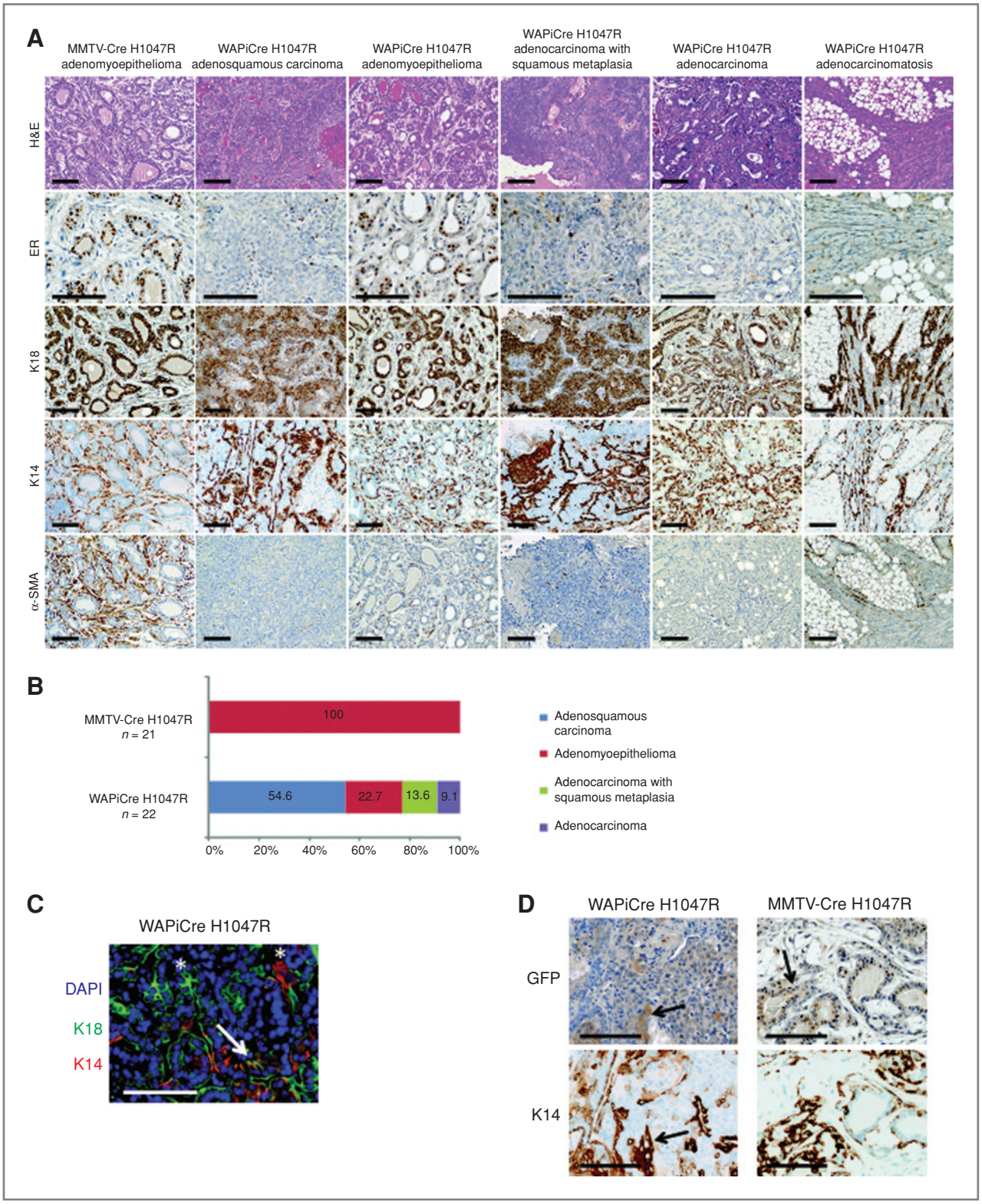

Figure 4. WAPiCre H1047R- and MMTV-Cre H1047R–evoked tumors express basal markers. A, H&E-stained sections and immunostainings for ER, K18, K14, and α-SMA from MMTV-Cre H1047R adenomyoepithelioma and different WAPiCre H1047R tumor histotypes as indicated. B, relative prevalence of adenosquamous carcinoma (blue), adenomyoepithelioma (red), adenocarcinoma with squamous metaplasia (green), and adenocarcinoma (purple) among MMTV-Cre H1047R- and WAPiCre H1047R–evoked tumors. C, fluorescence image of 4',6-diamidino-2-phenylindole (DAPI) staining (blue) and immunostaining of K18 (green) and K14 (red) in a WAPiCre H1047R tumor section. The arrow indicates K14/K18 double-positive cells. *, K14 and K18 single-positive cells. D, images of immunostaining for GFP and K14 in WAPiCre H1047R (left) and MMTV-Cre H1047R tumor sections (right). Arrows indicate areas of K14/GFP double-positive cells. Scale bars, 100 μm.

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ous mice most likely derived from PI-MECs because this is the cell population that expresses WAP-driven Cre in glands from nulliparous mice (21). Recently, PI-MECs were shown to be the target of MMTV-NeuNT-driven carcinogenesis (21, 31). Therefore, it is plausible that PI-MECs are the cells of origin of cancer in both WAPiCre- and MMTV-Cre H1047R–evoked tumors; however, at this stage, we cannot completely exclude the possibility that expression of *PIK3CA* H1047R in more differentiated cells also contributes to tumor formation in these models.

The observation of different histotypes between WAPiCre H1047R- and MMTV-Cre H1047R-derived tumors has several possible explanations. First, WAPiCre H1047R, but not MMTV-Cre H1047R, mice underwent pregnancy. Second, it is possible that the cellular targets of MMTV and WAP are overlapping but not congruent.

The fact that tumors from MMTV-Cre H1047R, but not MMTV-NeuNT, mice express K14 (Supplementary Fig. S3B) suggests a model in which *PIK3CA* H1047R transforms multipotent progenitors, allowing differentiation along the luminal and basal lineages. In contrast, the *NeuNT* oncogene favors luminal differentiation resulting in K18-positive, but not K14-positive, tumors. An alternative and more interesting explanation is that the *MMTV* promoter is active in differentiated luminal cells and H1047R causes their dedifferentiation to multipotent progenitor cells, which then give rise to K14- and/or K18-positive cancer cells. This would suggest a role for *PIK3CA* H1047R in cancer cell plasticity, a hypothesis that merits testing.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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