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# Targeting the mTOR Pathway for the Prevention of ER-Negative Breast Cancer



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## ABSTRACT

Prevention of estrogen receptor (ER)-positive breast cancer is now possible using anti-estrogen drugs; however, this treatment is ineffective against ER-negative breast cancers. In this study, we hypothesized that inhibition of mTOR will suppress the growth of ER-negative and triple-negative breast cancers. To test the hypothesis, we used five ER-negative breast cancer models: MMTV-erbB2, C3 (1)/SV40TAG, p53-null mammary gland-transplant, p53-mutant mammary gland-transplant, and BRCA1<sup>co/co</sup>; MMTV-Cre<sup>+/+</sup>; p53<sup>+/-</sup> mouse models to determine whether the mTOR inhibitor everolimus is effective in preventing growth of ER-negative mammary tumors. Our study demonstrates that everolimus treatment significantly delays mammary tumor formation with varying degree in all five ER-negative mouse models. Everolimus treatment reduces the proliferation, with reduced

phosphorylation of S6 kinase, and induces apoptosis of mammary tumor cells. In some of the p53-mutant mammary gland-transplant mice and C3 (1)/SV40Ag mice, everolimus completely prevents mammary tumor formation. Everolimus treatment also reduces proliferation of normal mammary gland cells. Our results support testing everolimus in clinical trials for the prevention of ER-negative breast cancer in women at high risk of ER-negative breast cancer.

**Prevention Relevance:** Our results show that everolimus delays mammary tumor formation in multiple mouse models, suggesting that mTOR inhibitors will be useful for the prevention of ER-negative and triple-negative breast cancer in humans.

See related *Spotlight*, p. 787

## Introduction

Breast cancer is the second most common cause of cancer-related death in American women (1). Despite improvements in the early detection and treatment of breast cancer, the estimated annual incidence rate of breast cancer in the United States comprises over 275,000 new cases and 40,000 deaths in 2021 (1). These data emphasize the importance of identifying effective preventive agents for preventing breast cancer. A variety of anti-estrogens and aromatase inhibitors have been demonstrated to significantly prevent ER-positive

breast cancer development (2–5). However, these agents do not prevent ER-negative breast cancer, which represents an aggressive disease and carries poor prognoses (6–11). For ER-negative breast cancers that overexpress Her2, treatments can include anti-Her2 antibodies (such as trastuzumab or pertuzumab; ref. 12) or small molecular inhibitors (lapatinib, neratinib, tucatinib; ref. 13) or antibody drug conjugates (14). ER-negative tumors not overexpressing Her2, termed triple-negative breast cancers (TNBC) are typically treated with conventional cytotoxic chemotherapy. However, in a recent clinical trial, the PARP inhibitor olaparib provided a significant benefit over standard therapy in women with a germline BRCA mutation who had HER2-negative metastatic breast cancer (15).

Thus, there is a clear need for the development of new agents with novel mechanisms of action, established efficacy, and minimal toxicity for the prevention of ER-negative and TNBCs. Aberrant activation of the PI3K/Akt/mTOR pathway is involved in the tumorigenesis of breast cancer (16). Accumulating data suggest that mTOR is a crucial mediator of tumor progression and may be a promising target in a significant proportion of patients with breast cancer. In familial and sporadic breast cancers, phosphorylated mTOR is directly associated with positive lymph node status, negative overall survival, and more rapid recurrence (17, 18). IHC analyses have shown that activated mTOR is more frequently observed in TNBC than other subtypes and is associated with poor outcome (19, 20). Deregulation of mTOR has been found in

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TNBCs, and a mutation of the *BRCA1* gene increases the phosphorylation and kinase activity of AKT1 and mTOR signaling pathways (20–22). Preclinical studies indicate that upregulated mTOR expression confers sensitivity to mTOR inhibitors (23).

Inhibition of mTOR with the small molecule everolimus reduces the progression of ductal carcinoma *in situ* to invasive breast cancer in the MMTV-Her2/neu mouse model (24). Everolimus is approved by the FDA to treat advanced-stage, hormone-receptor-positive, HER2-negative breast cancer in postmenopausal women. Further, everolimus is used to treat ER-positive tumors that have become resistant to anti-estrogen therapy. This drug is now being tested in clinical trials for the treatment of ER-negative breast cancer.

The overarching goal of this study is to evaluate the mTOR inhibitor, everolimus, for its cancer preventive efficacy in five separate ER-negative preclinical mouse models, representing a wide variety of genetic and pathologic states of ER-negative and TNBCs: MMTV-erbB2, C3 (1)/SV40 TAG, p53-null mammary gland-transplant, p53<sup>R172H</sup> mutant mammary gland-transplant, and BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> mice. Everolimus treatment significantly delayed the formation of breast cancer in each of these models. Our findings strongly suggest that everolimus is an effective agent for delaying or preventing ER-negative mammary tumor development. These results support testing everolimus in cancer prevention trials in women of high risk of developing breast cancer, such as in African American women with a strong family history of breast cancer, or in women with genetic predispositions to breast cancer, such as women with BRCA1 gene mutations and those with Li-Fraumeni syndrome.

## Materials and Methods

### Bioinformatics analysis

The level of mTOR mRNA expression was obtained from publicly available datasets (25–28) and accessed using the OncoPrint Platform (29). mTOR scores were generated by methods developed by Akbani and colleagues (30, 31), and analyzed with Student *t* test. Phospho-protein expression was obtained from the TCGA RPPA dataset (31). mRNA, mTOR mRNA expression levels, mTOR score, and protein expressions were compared between ER-positive and ER-negative breast cancer samples using Student *t* test. All statistical analysis was performed with Prism 9 (GraphPad).

### Mice

MMTV-erbB2 mice were purchased from the Jackson Laboratory. C3 (1)/SV40 TAG mice were generated by breeding FVB wild-type females with heterozygous C3 (1)/SV40 TAG males. To generate BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> female mice, we mated 129S1 BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> males with BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/+</sup> females. For P53-null mice, the donor and recipient mice were Balb/c p53-null mammary gland mice and Balb/c p53 wild-type mice, respectively (32). In the p53-null model experiment, we

transplanted BALB/c-p53-null mammary epithelium into p53 wild-type BALB/c hosts. To generate p53<sup>R172H</sup> mutant mice, we bred heterozygous Balb/C p53<sup>R172H</sup> mutant males and heterozygous p53<sup>R172H</sup> mutant females. From this breeding we produced p53-mutant/mutant mice, which were used as donor mice. Their mammary glands were removed and transplanted into cleared mammary fat pads of recipient Balb/C p53 wild-type mice. For these experiments, we transplanted BALB/c-p53<sup>R172H</sup> mutant mammary epithelium into p53 wild-type BALB/c hosts.

The characteristics of each animal model is detailed in Supplementary Table S1. Virgin animals were used to avoid confounding effects of hormonal surges during pregnancy in all mouse models. All animal experiments were performed in accordance with M.D. Anderson Institutional Animal Care and Use Committee (IACUC)-approved protocols.

### Mammary gland transplantation

Briefly, a small piece of mammary gland (1 mm<sup>3</sup>) from an 8- to 9-week-old female mouse was transplanted into a cleared inguinal fat pads of 3-week-old (10–13 g) p53 wild-type female mice. This procedure results in a successful engraftment rate of over 90% of the recipient mice. Treatments began at 11 weeks, after transplanted mammary fat pads were completely filled, to avoid any potential agent-induced effects on cellular capability to grow and fill the fat pads (33).

### Treatment with everolimus

Everolimus (mg/kg body of weight) was administered via oral gavage for all preclinical experiments in this study. For animal experiments using MMTV-erbB2 mice, we used 20 mice in each group. In animal experiments using C3 (1)/SV40 TAG mice, we used 11 mice in the control group and 12 mice in the treatment group. Everolimus treatment of C3 (1)/SV40 TAG mice started at 2 months of age. In animal experiments with p53-null-mammary gland mice, we used 15 mice per group and with p53-mutant-mammary gland mice, we used 18 mice in each group. Everolimus treatment was begun at 12 weeks of age. In BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>±</sup> mice, we used 18 mice in each group. Everolimus treatment of these mice was started at 4 months of age. In all animal models, Group A control mice were treated with vehicle (sesame oil) and Group B were treated with everolimus.

We started everolimus treatment at different times because time-to-tumor formation is different for each model. For most of the models, treatment was started at 3 months of age. However, in the SV40 model, the mice were treated starting at 2 months of age because the SV40 Tag mice develop mammary tumors much more quickly than do the other mouse models. In all animal experiments, everolimus was administered at either 5 or 2 mg/kg doses, which was given to the mice either two or five times per week. The dose and schedule selection was based on comparable human doses, and to achieve maximum preventive efficacy without inducing any severe toxicities.

### Experimental endpoints

The endpoint of these animal experiments was time to tumor development, compared between groups (primary endpoint). Tumor volume was calculated with the formula  $V = (\text{width}^2 \times \text{length})/2$ , and a palpable tumor was defined as volume equal to or more than  $100 \text{ mm}^3$ . We also measured toxicities such as weight loss, hair loss, and moribund status as secondary endpoint. Statistical evaluation of time to tumor development was completed using the generalized Wilcoxon test. For other comparisons, a Student *t* test was used. A *P* value of  $<0.05$  was considered statistically significant.

### H&E and IHC analysis

Paraformaldehyde (4%) was used to fix mammary glands and tumor tissues followed by embedding in paraffin. Tissue sections were then mounted on slides and processed for either hematoxylin–eosin (H&E) staining or IHC staining. H&E staining was conducted by deparaffinizing  $4 \mu\text{m}$  tissue sections in xylene. Sections were then rehydrated in ethanol and water, followed by incubation in hematoxylin. Samples were destained in water and fixed in acidified alcohol and ammonia. Finally, slides were incubated in eosin for 2 minutes, rinsed in alcohol and xylene, and mounted for evaluation.

For IHC studies,  $4 \mu\text{m}$  tissue sections were deparaffinized and mounted onto slides. The endogenous peroxidase was blocked in 3% hydrogen peroxide buffer. Samples were incubated with the primary antibody Lab Vision anti-Ki67 (Thermo Fisher Scientific), anti-cleaved caspase 3 or anti-phospho S6 (Cell Signaling Technology Inc.), overnight at  $4^\circ\text{C}$  followed by the incubation with biotinylated anti-rabbit antibody (Vector Laboratories, Inc., Burlingame, CA, 1:100) for 30 minutes. Peroxidase activity was visualized with the Vector NovaRED Substrate Kit (PK-6101; Vector Laboratories, Inc.) and the AEC Peroxidase Substrate Kit, 3-amino-9-ethylcarbazole (SK-4200; Vector Laboratories, Inc.). The slides were finally counterstained with hematoxylin and mounted with cover slips.

### Determination of everolimus blood concentration using HPLC-MS analysis

Uncoagulated whole blood was used to measure blood concentration of everolimus using HPLC-MS analysis.

### Effect of everolimus on hematologic and blood chemistry parameters

Complete blood count or other blood parameters was measured using an automated hematology analyzer at MD Anderson core facility.

### Statistical analysis

Comparisons between mRNA, mTOR score, and protein expression were performed with Student *t* test. Time to tumor analyses were estimated by the Kaplan–Meier method and statistically evaluated using the generalized Wilcoxon test. IHC slides were analyzed by comparing percent positivity or Allred

scores using Student *t* test. Differences were considered statistically significant if  $P < 0.05$ .

### Data availability

For mTOR mRNA expression analyses, Minn, METABRIC, ExpO, Tabchy, and Gluck datasets available at <http://www.oncomine.org/> were used. The TCGA RPPA dataset is available through the Broad GDAC (<http://gdac.broadinstitute.org/>). Data supporting the findings are available within the article, and from the corresponding author on reasonable request.

## Results

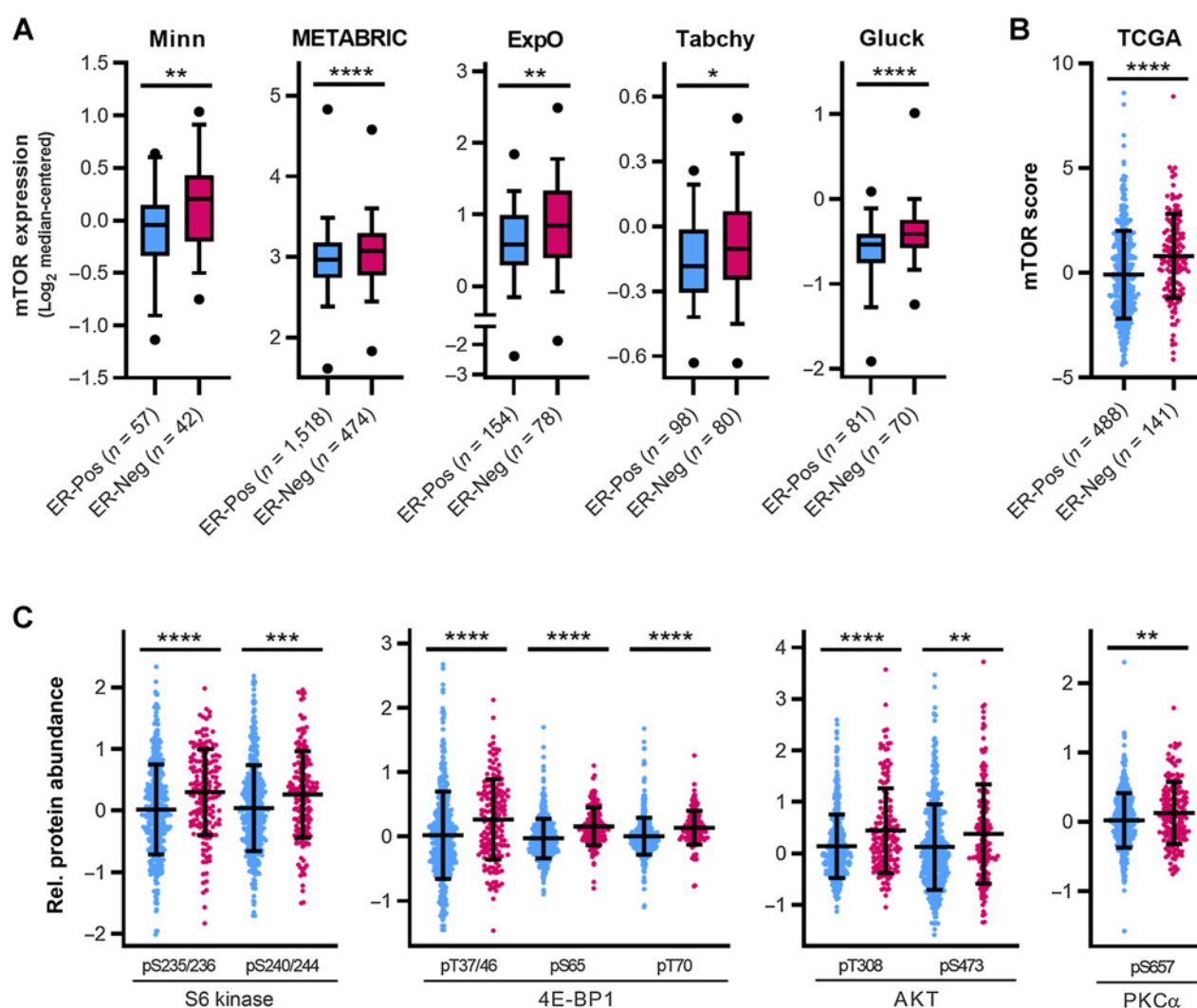
### mTOR expression and activity are increased in ER-negative breast cancers

We sought to determine how the expression of mTOR differs between ER-negative and ER-positive tumors. Using publicly available datasets (25–28, 34, 35), we determined that mTOR mRNA expression is significantly increased in ER-negative, as compared with ER-positive, breast cancer patient samples (Fig. 1A). To determine if the increased mRNA expression of mTOR led to an increase in mTOR pathway signaling, we compared relative mTOR pathway activity using the mTOR signature generated by Akbani and colleagues (30, 31) with TCGA breast data. Using this signature, we determined that ER-negative breast cancers exhibited a higher mTOR score, and therefore higher mTOR pathway activity, than did ER-positive breast cancers (Fig. 1B). To further confirm this finding, we additionally examined the abundance of mTOR target protein phosphorylation using publicly available TCGA RPPA data (31). mTORC1 associated proteins S6 Kinase and 4E-BP had significantly higher phosphorylation, as did the mTORC2-associated proteins AKT and PKC $\alpha$  (Fig. 1C). Collectively, these data indicate that mTOR expression and activity are increased in ER-negative breast cancers.

### Cancer preventative activity of everolimus in MMTV-erbB2 mice

To investigate the cancer preventative effect of everolimus on ER-negative and triple negative breast cancers, we performed several *in vivo* experiments using various transgenic animal models. We began by using MMTV-erbB2 mice.

We treated MMTV-erbB2 mice with sesame oil (Group A,  $N = 20$ ) or with everolimus (5 mg/kg; Group B,  $N = 20$ ), 5 days a week (Fig. 2A). Our results showed that everolimus significantly delayed tumor formation in MMTV-erbB2 mice ( $P < 0.0001$ , Fig. 2B). In MMTV-erbB2 mice, everolimus reduced tumor incidence and was associated with an increase in median survival time from 240 to 410 days. At day 365, when all mice in control group died, only half of the mice treated with everolimus had developed tumors. Long term treatment ( $>150$  days) of everolimus was very well tolerated, with only slight weight gain (average  $<10\%$ ) observed in the treatment group mice. An effective cancer preventative agent should minimize the risk of developing tumors while, and at the same time, not causing any side effects. Therefore, we performed



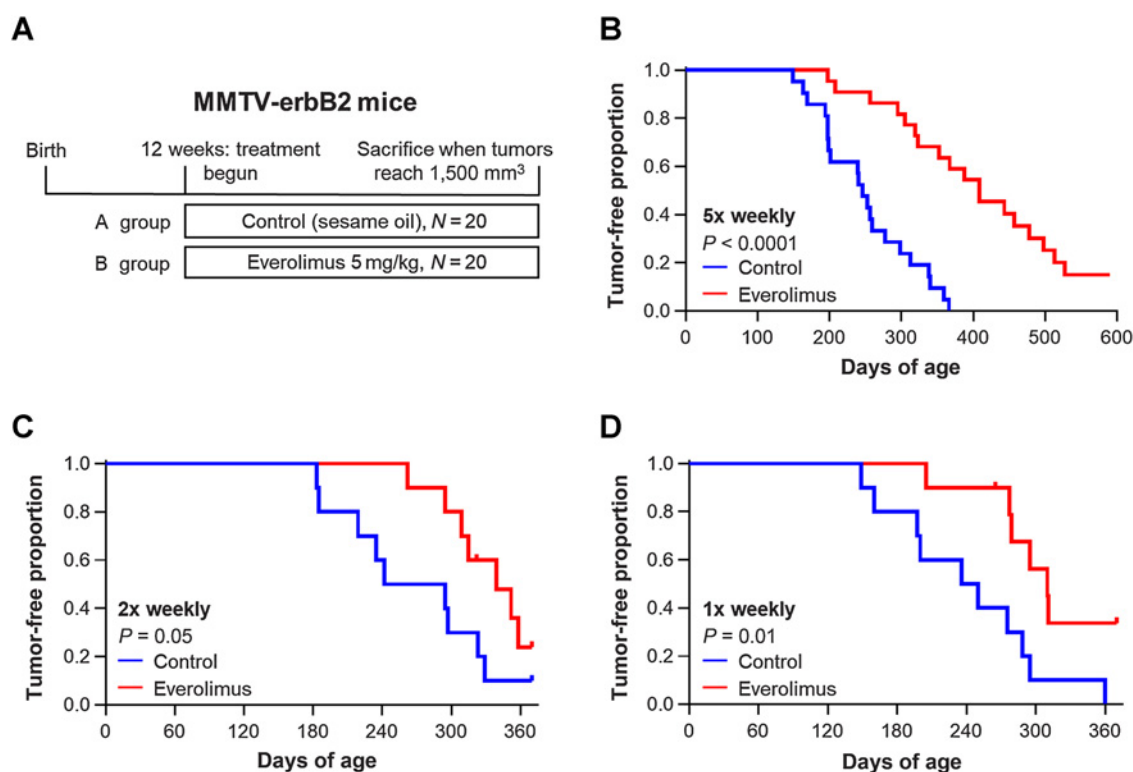
**Figure 1.** High mTOR expression correlates with ER-negative breast cancer. **A**, Expression of mTOR mRNA in publicly available datasets, compared between ER-positive and ER-negative breast cancers. **B**, Comparison of mTOR score between ER-positive and ER-negative breast cancer samples in the TCGA dataset. **C**, Protein expression of the phosphorylated mTOR target proteins S6 Kinase, 4E-BP1, AKT, and PKC $\alpha$  in the TCGA dataset. mTOR expression and scores were compared with Student *t* test (\*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$ ; \*\*\*\*,  $P < 0.0001$ ).

additional experiments in MMTV-erbB2 to minimize this weight gain side effect by reducing everolimus treatment to twice and once per week. Twice per week treatment of everolimus reduced tumor incidence and was associated with an increase in median survival time from 242 to 340 days ( $P = 0.05$ , Fig. 2C). Once per week treatment of everolimus reduced tumor incidence and was associated with an increase in median survival time from 236 to 310 days ( $P = 0.01$ , Fig. 2D). Both twice and once per week treatment with everolimus reduced toxicities compared with 5 days per week treatment. Our results indicated that everolimus treatment of MMTV-erbB2 significantly ( $P < 0.0001$  for five times weekly,  $P = 0.05$  for twice weekly, and  $P = 0.01$  for once weekly treatments, respectively) delayed tumor development in all three schedules.

### Tumor formation was delayed in multiple models of ER-negative and triple-negative mouse breast cancer by everolimus

We next tested the ability of everolimus to prevent breast cancer in four additional models of ER-negative breast cancer: C3 (1)/SV40 TAG mice; p53-null mammary gland mice; BRCA1<sup>co/co</sup>; MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> mice, and p53<sup>R172H</sup> mutant mice.

Transgenic mice with C3 (1)/SV40 TAG expression in mammary glands develop ER-negative tumors that histologically resemble aggressive TNBCs. The atypical lesions progress to high grade mammary intraepithelial neoplasia (MIN) beginning at roughly 12 weeks of age, following which MIN progress into invasive carcinoma at about 16 weeks of age. All control mice died at five months of age. We treated C3 (1)/SV40 TAG



**Figure 2.**

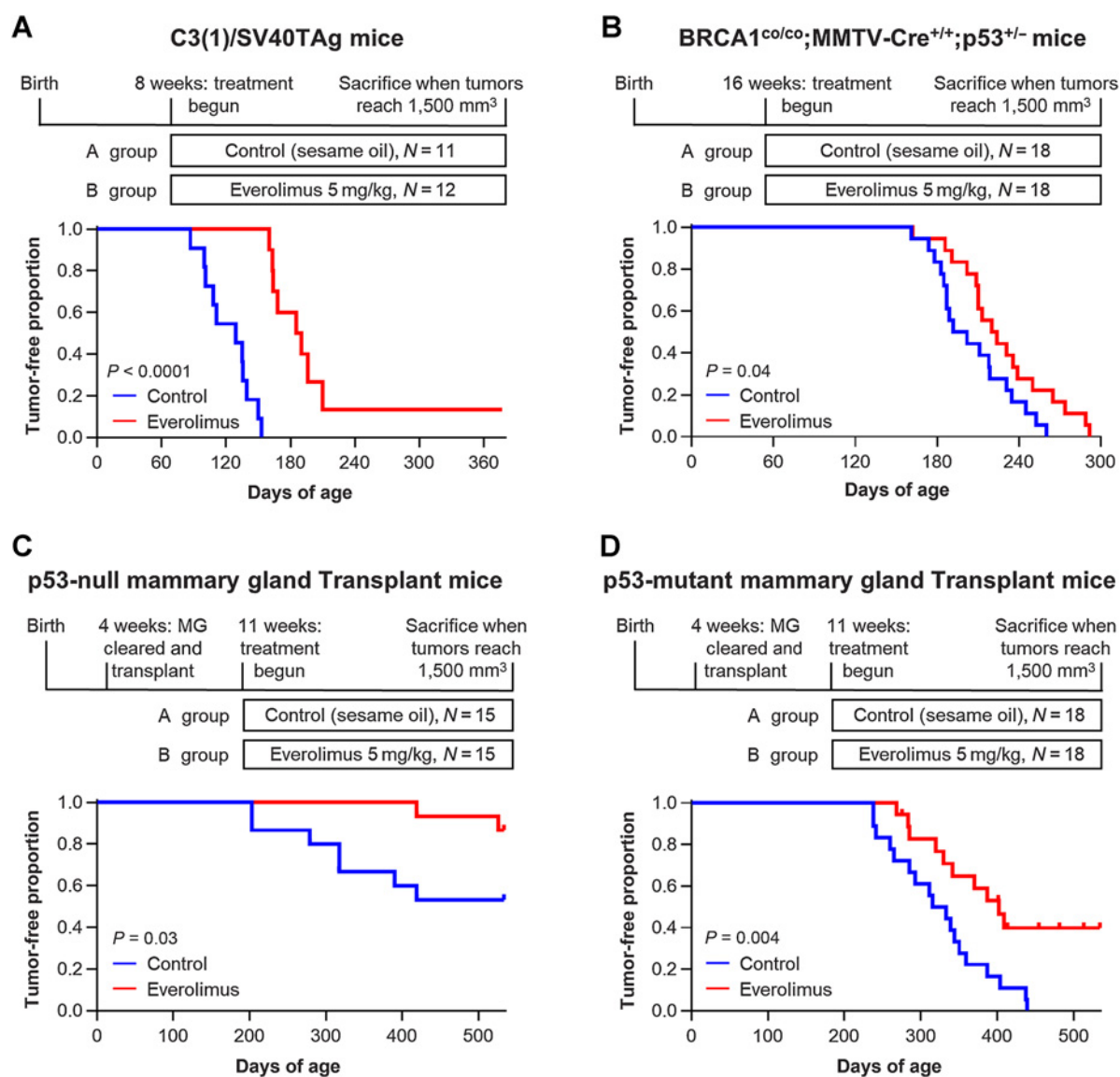
Treatment with the mTOR inhibitor everolimus inhibits development of mammary gland tumorigenicity in MMTV-erbB2 mice. **A**, Treatment scheme: 3 months old female MMTV-erbB2 mice were randomly assigned to receive either vehicle (sesame oil,  $N = 20$ ) or everolimus (5 mg/kg,  $N = 20$ ) by oral gavage (0.1 mL). Mice were observed daily for toxicity, and tumor growth was measured biweekly. **B**, MMTV-erbB2 mice were treated with vehicle or everolimus five times a week, represented with tumor-free interval curves measured using a Kaplan-Meier plot ( $P = 0.0001$  using the Wilcoxon test). **C**, MMTV-erbB2 mice were treated with vehicle or everolimus two times a week, represented with tumor-free interval curves measured using a Kaplan-Meier plot ( $P = 0.05$  using the Wilcoxon test). **D**, MMTV-erbB2 mice were treated with vehicle or everolimus once per week, represented with tumor-free interval curves measured using a Kaplan-Meier plot ( $P = 0.01$  using Wilcoxon test).

mice with sesame oil ( $N = 11$ ) or with 5 mg/kg everolimus ( $N = 12$ ) five days a week, and followed the mice for time to tumor development. Our results showed that everolimus significantly delayed tumor formation in C3 (1)/SV40 TAG mice ( $P < 0.0001$ , **Fig. 3A**). In these mice, everolimus reduced tumor incidence and was associated with an increase in median survival time from 129 days to 185 days. At day 153, when all mice in control group died, none of the mouse treated with everolimus had developed tumors. Long term treatment of everolimus was not associated with any toxicities in C3 (1)/SV40 TAG mice in everolimus group. Our results showed that long term treatment with everolimus completely prevented tumor formation in 27% (3 out of 11) of the C3 (1)/SV40 TAG mice.

To investigate the cancer preventive efficacy of everolimus in a TNBCs rodent model representing *BRCA1* gene mutation carriers, we selected *BRCA1* mice with a conditional knockout of *BRCA1*, coupled with a mutation in *Tp53*. These mice develop triple-negative mammary tumors at approximately 23 weeks of age, making them an ideal model to test new cancer preventive drugs for women at high risk of TNBC with *BRCA1* mutations. We treated *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;

*p53*<sup>+/-</sup> mice with either sesame oil (Group A,  $N = 18$ ) or with 5 mg/kg everolimus (Group B,  $N = 18$ ), five a week. Our results showed that everolimus significantly delayed tumor formation in *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;*p53*<sup>+/-</sup> mice ( $P = 0.04$ , **Fig. 3B**). In *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;*p53*<sup>+/-</sup> mice, everolimus reduced time to tumor formation and was associated with an increase in median survival time from 192 days to 220 days. Long-term treatment of everolimus was not associated with any toxicities in *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;*p53*<sup>+/-</sup> mice in everolimus group. To test cancer preventive efficacy of everolimus using lower dose of everolimus, we tested two and a half fold lower—2 mg/kg opposed to 5 mg/kg of everolimus and also instead of treating 5 days a week treatment everolimus was treated twice per week. *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;*p53*<sup>+/-</sup> mice were treated with either sesame oil (Group A,  $N = 18$ ) or with this lower concentration of 2 mg/kg everolimus (Group B,  $N = 18$ ) twice a week. Our results showed that everolimus significantly delayed tumor formation in *BRCA1*<sup>co/co</sup>;*MMTV-Cre*<sup>+/+</sup>;*p53*<sup>+/-</sup> mice ( $P = 0.01$ ) without any visible toxicities (Supplementary Fig. S1A). In this experiment, everolimus reduced tumor incidence and increased median survival time from 185 to 231 days. Our result indicated that everolimus was



**Figure 3.**

Treatment with the mTOR inhibitor everolimus delays development of mammary gland tumorigenicity in multiple ER-negative breast cancer models. **A**, Top: Treatment scheme: At 8 weeks of age, female C3(1)SV40 Tag mice were randomly assigned to receive either vehicle (sesame oil;  $N = 11$ ) or everolimus (5 mg/kg;  $N = 12$ ) by oral gavage (0.1 mL). Bottom: Preventive efficacy of everolimus in the C3(1)SV40 Tag mouse model. Mice were treated with vehicle or everolimus five times per week. **B**, Top: Treatment scheme: At 16 weeks of age, female BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/-</sup>;p53<sup>+/-</sup> mice were treated with vehicle (sesame oil) or everolimus (5 mg/kg) by oral gavage (0.1 mL) five times per week ( $N = 18$ ). Bottom: Preventive efficacy of everolimus in the BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/-</sup>;p53<sup>+/-</sup> mouse model. Mice were treated with vehicle or everolimus five times per week. **C**, Top: Treatment scheme: At 4 weeks of age, p53-null mammary glands were implanted in recipient mice. Beginning at 11 weeks of age, mice received vehicle (sesame oil) or everolimus (5 mg/kg) by oral gavage (0.1 mL) five times per week ( $N = 15$ ). Bottom: Preventive efficacy of everolimus in the p53-null (-) mammary gland mouse model. Mice were treated with vehicle or everolimus five times per week. **D**, Top: Treatment scheme: At 4 weeks of age, p53<sup>R172H</sup> mutant mammary glands were implanted in recipient mice. Beginning at 11 weeks of age, mice received vehicle (sesame oil) or everolimus (5 mg/kg) by oral gavage (0.1 mL) five times per week ( $N = 18$ ). Bottom: Preventive efficacy of everolimus in the p53<sup>R172H</sup> mutant gland mouse model. Mice were treated with vehicle or everolimus five times per week. For all experiments, time-to-tumor incidence was represented with tumor-free internal Kaplan-Meier plots, and significance was determined with generalized Wilcoxon tests. A  $P$  value of <0.05 was considered statistically significant.

able to significantly delay tumor progression in BRCA1<sup>co/co</sup>; MMTV-Cre<sup>+/-</sup>;p53<sup>+/-</sup> mice, both at 5 and at 2 mg/kg with no apparent toxicities.

To check the cancer preventive efficacy of everolimus on the p53-null mammary gland mouse model (32), we treated

p53-null mammary gland mice with either sesame oil (Group A,  $N = 15$ ) or with 5 mg/kg everolimus (Group B,  $N = 15$ ), 5 days a week. Our results showed that everolimus significantly delayed tumor formation in p53-null mammary gland mouse ( $P = 0.03$ , Fig. 3C). The vehicle control mice treated with

sesame oil developed mammary tumors in 46% of transplanted mammary glands at 420 days posttransplantation, whereas mice treated with 5 mg/kg everolimus developed mammary tumors in only 6% of the transplanted mammary glands at the same time point. We did not observe any noticeable toxicities in this model.

To determine cancer preventive efficacy of everolimus in p53<sup>R172H</sup> mutant mice, we transplanted p53<sup>R172H</sup> mammary epithelium into p53 wild-type recipient mice as described in Materials and Methods. We used female p53<sup>R172H</sup> mammary gland as mammary gland-transplant donors and female p53 wild-type/wild-type mice (which had their mammary fat pads cleared) as recipient mice. We treated p53<sup>R172H</sup> mammary gland-transplant mice with sesame oil (Group A,  $N = 18$ ) or with 5 mg/kg everolimus (Group B,  $N = 18$ ) 5 days a week. Our results showed that everolimus significantly delayed tumor formation in p53<sup>R172H</sup> mammary gland mice ( $P = 0.004$ , Fig. 3D). Most importantly, our results showed that long term treatment everolimus completely prevented tumor formation in 44% (8 of 18) of the p53<sup>R172H</sup> mutant mammary gland transplant mice. In p53<sup>R172H</sup> mammary gland mice, everolimus treatment reduced tumor incidence and was associated with an increase in median survival time from 311 to 400 days. Long-term treatment of everolimus was associated with slight (10%) body weight loss in 3 of 18 mice after 32 weeks of treatment. We also tested everolimus at a concentration two and a half-fold lower, 2 mg/kg compared with 5 mg/kg. P53-mutant/mutant mammary gland mice were treated with sesame oil (Group A,  $N = 18$ ) or with 2 mg/kg everolimus (Group B,  $N = 18$ ) 5 days a week. Our results showed that everolimus significantly delayed tumor formation in p53<sup>R172H</sup> mutant mammary gland mice

( $P = 0.04$ ) without any visible side effects (Supplementary Fig. S1B). Everolimus reduced tumor incidence and increased in median survival time from 311 to 368 days. Our results indicate that both 5 and 2 mg/kg everolimus treatment was able to significantly delay tumor progression in p53-mutant/mutant mice with no apparent toxicities.

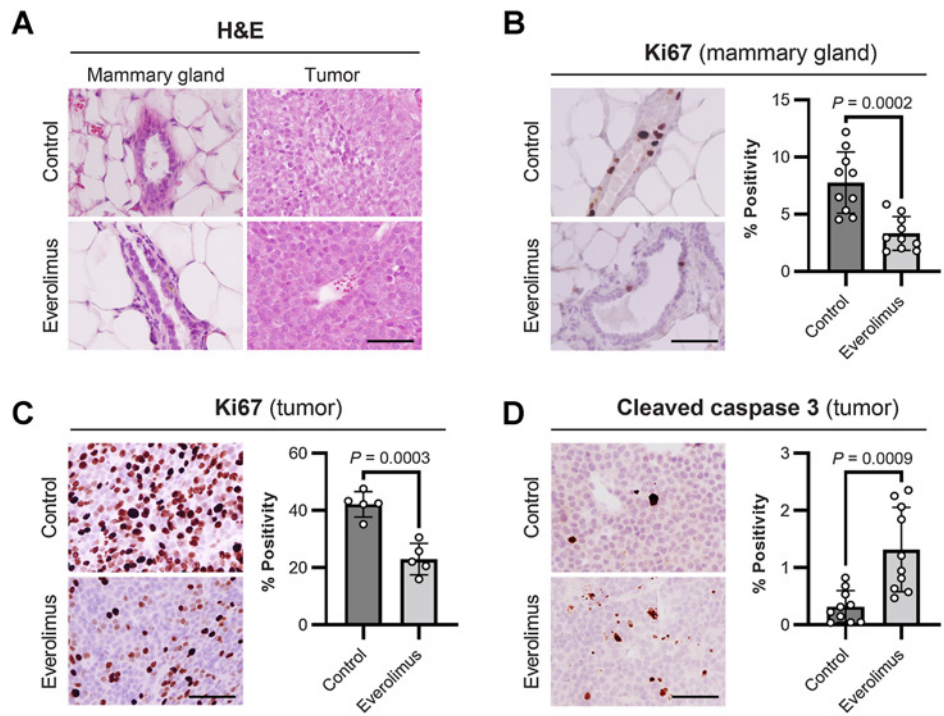
To determine receptor status (ER- $\alpha$  and HER2) of the tumors which arose in vehicle and everolimus-treated mice, we performed IHC of ER- $\alpha$  and HER2 expression in all five mouse models. (see Supplementary Table S2). Our results showed that in p53-null mammary gland transplant, C3 (1)/SV40 Tag, and BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> mice 90% of the tumors were ER-negative. Eighty percent of the tumors that arose in the p53<sup>R172H</sup> mutant mice were ER-negative, whereas 20% of these tumors were strongly ER-positive. Sixty percent of tumors that arose in the MMTV-erbB2 mice expressed ER, whereas 40% of the tumors were ER-negative. All of the tumors that arose in the MMTV-erbB2 mice were strongly HER2 positive, whereas all the tumors that arose in the other mouse models were HER2 negative.

We also compared the frequency of ER-positive tumors in the vehicle- or everolimus-treated mice. There was no clear difference in frequency of ER-positive tumors between treatment groups; however, in the MMTV-erbB2 mice and in the p53<sup>R172H</sup> mutant mammary gland mice, the strongly ER-positive tumors were seen only in the vehicle-treated mice.

We also tested multiple doses and schedules of everolimus in different mouse models to determine whether toxicity is reduced while still maintaining preventive efficacy (Supplementary Table S3). When we analyzed the efficacy of everolimus in MMTV-erbB2, BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>±</sup>

**Figure 4.**

Effect of the mTOR inhibitor everolimus on histopathology and IHC of Ki67 on MMTV-erbB2 mice. **A**, Mammary tissue sections of MMTV-erbB2 mice, treated with everolimus or vehicle, were stained with hematoxylin-eosin. Representative images of mammary glands (left) and tumors (right) are shown (5 mg/kg; five times per week). **B**, Representative images of Ki67 staining of MMTV-erbB2 mammary glands treated with everolimus or control (left; 5 mg/kg; five times per week), and quantified by Ki67% positivity (right;  $n = 5$ ). **C**, Representative images of Ki67 staining of MMTV-erbB2 tumors treated with everolimus or control (left), and quantified by Ki67% positivity (right;  $n = 5$ ; 5 mg/kg; five times per week). **D**, Representative images of cleaved caspase-3 staining of MMTV-erbB2 tumors treated with everolimus or control (left), and quantified by cleaved caspase percent positivity (right;  $n = 5$ ; 5 mg/kg; five times per week). Percent positivities were compared with Student  $t$  test, and  $P < 0.05$  was considered statistically significant. All scale bars represent 100  $\mu$ m.





and p53<sup>R172H</sup> mutant mice, our results showed that low and less frequent doses were associated with no toxicity, but still showed delays in tumor formation. We also measured the effect of long-term everolimus treatment on body weight. Our results showed that long-term everolimus treatment has no significant effect on body weight in any of these mouse models (Supplementary Fig. S2).

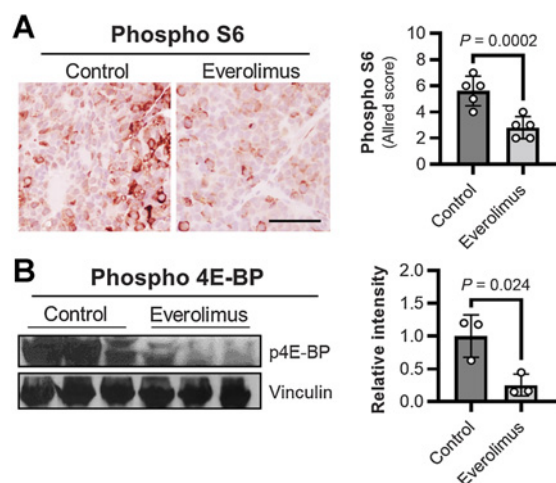
### Effect of everolimus on biomarker expression

To investigate the mechanism of tumor growth suppression by everolimus, we performed analysis of several biomarkers. First, we investigated the effect of everolimus on premalignant lesions using H&E staining in the MMTV-erbB2 breast cancer model. We began everolimus treatment at 3 months, at which time mice had no palpable tumors. Although our primary endpoint was time to tumor formation, we also collected contralateral mammary glands to observe any changes in the mammary gland at the time of sacrifice. In the MMTV-erbB2 transgenic model, our H&E analysis showed that there were no significant changes in the contralateral mammary glands when we compared the control group to the everolimus group (Fig. 4A). Moreover, there was no difference in tumor morphology between control and treatment groups (Fig. 4A). We also investigated the effect of everolimus on morphologic changes (Supplementary Figs. S3–S6) in mammary glands and tumors from p53-null mammary glands (Supplementary Fig. S3A), C3 (1)/SV40 TAg (Supplementary Fig. S4A), and BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> (Supplementary Fig. S5A) and p53<sup>R172H</sup> mutant (Supplementary Fig. S6A) models. Our results showed that everolimus has no effect on morphologic

changes in mammary gland and tumor H&E in any of these models.

To investigate the mechanism by which everolimus suppressed tumor development, we measured the effect of everolimus treatment on Ki67, a marker of tumor proliferation, and cleaved caspase-3, a marker of apoptosis in the MMTV-erbB2 model. As shown in Fig. 4B, treatment with everolimus significantly reduced mammary gland Ki67 positivity by nearly 60% (from 7.7% to 3.3%,  $P = 0.0002$ ). Everolimus also significantly reduced tumor cell proliferation by approximately 50% (from 42% to 23%,  $P = 0.0003$ ; Fig. 4C). We also determined whether everolimus treatment could induce apoptosis by measuring the proportion of cells positive for cleaved caspase-3. As demonstrated in Fig. 4D, apoptosis was significantly induced in the everolimus treatment group ( $P = 0.0009$ ). The percentage of cells undergoing apoptosis in tumor cells was approximately four-fold higher in everolimus treated mice when compared with control. We also measured the effect of everolimus treatment on the rate of normal mammary gland proliferation by analyzing Ki67 in p53-null mammary gland transplant glands (Supplementary Fig. S3B), C3 (1)/SV40 TAg mammary glands (Supplementary Fig. S4B), BRCA1<sup>co/co</sup>; MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> mammary glands (Supplementary Fig. S5B), and p53<sup>R172H</sup> mutant mice (Supplementary Fig. S6B). Our results showed that everolimus significantly reduce proliferation in the normal mammary gland in MMTV-erbB2 ( $P = 0.002$ ), and p53-mutant mammary glands ( $P = 0.05$ ) models; however the other three models the reduction of mammary gland proliferation was not statistically significant. Proliferation rates of tumors were also measured by Ki67 IHC from p53-null mammary gland model ( $P = 0.006$ , Supplementary Fig. S3C), C3 (1)/SV40 TAg ( $P = 0.0002$ , Supplementary Fig. S4C), BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> ( $P = 0.048$ , Supplementary Fig. S5C), and p53<sup>R172H</sup> mutant mice ( $P = 0.02$ , Supplementary Fig. S6C) mouse models. Our results showed everolimus significantly reduced tumor proliferation in the tumors of all of these mouse models. In addition, we examined whether everolimus induced apoptosis in our ER-negative and triple-negative models by measuring percent positivity of cleaved caspase-3 in tumors collected at the time of sacrifice. Our results showed that everolimus induced apoptosis in the tumors of p53-null mammary gland ( $P < 0.0001$ , Supplementary Fig. S3D), C3 (1)/SV40 TAg ( $P < 0.0001$ , Supplementary Fig. S4D) BRCA1<sup>co/co</sup>;MMTV-Cre<sup>+/+</sup>;p53<sup>+/-</sup> ( $P = 0.003$ , Supplementary Fig. S5D), and p53<sup>R172H</sup> mutant mice ( $P = 0.003$ , Supplementary Fig. S6D) models. Our results showed everolimus significantly induced apoptosis and reduced proliferation in all of these mouse mammary tumor models and played an important role in delaying tumor incidence and tumor growth.

To further delineate the mechanism of growth suppression induced by the everolimus treatment, we measured mTOR downstream target genes phospho-S6 and phospho-4E-BP. To determine the proportion of positive cells for S6 phosphorylation, we performed IHC analysis using tumor sections from



**Figure 5.**

Everolimus inhibits phosphorylation of mTOR target protein S6 and 4E-BP. **A**, The phosphorylation of the mTOR target protein S6 was measured by IHC staining using an anti-phospho-S6 antibody and quantified using the Allred scoring system ( $P = 0.0002$ ). **B**, Western blot analysis of phospho-4E-BP and vinculin (left), and quantification of phospho-4E-BP expression (right,  $P = 0.03$ ) are shown. All scale bars represent 100  $\mu\text{m}$ .

control and everolimus treatment groups in MMTV-erbB2 mouse model (Fig. 5A). There was a significant reduction of phospho-S6 expression in the tumors of the everolimus treated group compared with the control group ( $P = 0.0002$ , Fig. 5A). In addition to the MMTV-erbB2 mouse model, we also determined the level of S6 phosphorylation in the  $BRCA1^{co/co};MMTV-Cre^{+/+};p53^{+/-}$  mouse model after everolimus treatment (Supplementary Fig. S5E). Similarly, there was a significant suppression of phospho S6 in the tumors of the everolimus treated group compared with the control group ( $P = 0.008$ ). We also checked the level of an additional downstream target of mTOR, phospho-4E-BP, in MMTV-erbB2 tumor lysates with Western blot analysis (Fig. 5B). Our results showed a significant reduction of phospho-4E-BP level in the everolimus treatment group, compared with control ( $P = 0.024$ ). These findings demonstrate a significant association between everolimus treatment and suppression of mTOR protein phosphorylation targets S6 and 4E-BP, thus confirming the on-target effects of everolimus in mammary tumors.

#### Determination of everolimus blood concentration using HPLC-MS analysis

We next sought to determine everolimus concentrations in the blood of mice. We used 129S1 and Balb/c mice, the background strains of our transgenic mice, and treated them with everolimus (1, 5, or 10 mg/kg) once a week for 4 weeks. Uncoagulated whole blood was used to measure blood concentration of everolimus using HPLC-MS analysis. Our result showed that everolimus concentration increased in a dose-dependent manner in both 129S1 and Balb/c mice (Supplementary Table S4).

#### Effect of everolimus on hematologic and blood chemistry parameters

To determine whether long-term treatment of everolimus had any significant effect on complete blood count or other blood parameters, we collected whole blood and plasma from the long-term treatment of everolimus on  $BRCA1^{co/co};MMTV-Cre;p53^{+/-}$  mice (5 mg/kg, twice weekly for 4 weeks). Our results showed that long-term treatment of everolimus had no significant effects on complete blood counts (white cells, red cells, or platelets; Supplementary Fig. S7) or on other blood parameters (Supplementary Fig. S8). Our results suggest that long-term treatment of everolimus has no observable hematologic adverse effects and is safe for treatment.

## Discussion

In this study, we investigated the cancer preventive effect of everolimus using several different mouse models of breast cancer, which represent a wide spectrum of ER-negative and TNBCs. The MMTV-erbB2 model represents a predominantly ER-negative breast cancer with Her2 amplification. As a traditional model of TNBC, we used the C3 (1)/SV40 TAG mouse

model. p53-null and p53-mutant mammary gland transplant mouse models represent a hallmark of TNBCs—the lack of p53 wild-type functionality. TNBC tumors can also harbor *BRCA1* gene mutations, especially prevalent in women with a family history of breast cancer; this set of patients with breast cancer was represented by the  $BRCA1^{co/co};MMTV-Cre^{+/+};p53^{+/-}$  mouse model. Therefore, in this study, we have taken a comprehensive approach to investigate the prevention of various types of ER-negative and TNBCs by everolimus, using genetically altered animal models. Our studies show everolimus prevented or delayed mammary tumor development in each of these models, and that long-term treatment of everolimus was well tolerated by the mice.

In this study, we demonstrated for the first time that everolimus can completely prevent tumor development in a portion of C3 (1)/SV40 TAG and  $p53^{R172H}$  mutant mice. Biomarker analysis of resulting tumors showed that everolimus treatment significantly reduced proliferation, induced apoptosis, and inhibited phosphorylation of mTOR target S6 in our MMTV-erbB2 and  $BRCA1^{co/co};MMTV-Cre^{+/+};p53^{+/-}$  mouse models. Among all ER-negative and triple-negative models tested, everolimus treatment was the least preventative in the  $BRCA1^{co/co};MMTV-Cre^{+/+};p53^{+/-}$  mouse model, most likely due to the mutations in both *Tp53*- and *BRCA1* genes, compared with other models which are driven by the alteration of single oncogene or tumor suppressor gene. Variations in everolimus sensitivity may also be due to differential expression of other tumor suppressors relevant to other ER-negative murine models, such as PTEN and inositol polyphosphate-4-phosphatase type II B (INPP4B; refs. 36, 37). Hatem and colleagues have previously shown that everolimus sensitivity in TNBC PDX models depends on activation of various tumor suppressors and oncogenic responses (38).

The PI3K/AKT/mTOR pathway is critical in multiple normal cell processes, and alteration of this pathway has been implicated in the progression of cancers (39, 40). The small molecule inhibitor of mTOR, everolimus, is effective against different cancers, including breast cancers, renal cell carcinomas, hepatocellular carcinomas, as well as other cancers (41). Everolimus delayed tumor onset and progression of tumor development in a transgenic mouse model of ovarian cancer (42). The mTOR inhibitor rapamycin has previously been shown to inhibit pancreatic cancer growth in the Pan02 murine pancreatic cancer model (43). In a recent study, treatment with everolimus inhibited progression from ductal carcinoma *in situ* to an invasive ductal carcinoma by inhibiting MMP9 activity (24). Breast cancers often increase expression of the PI3K/AKT/mTOR signaling pathways through molecular changes, such as overexpression of tyrosine kinases, downregulation of PTEN, or activation of PI3K and AKT (12, 16–19, 21, 22, 44–46). Activation of the mTOR pathway has also been observed in ER+ cancers resistant to endocrine therapies. This led to the BOLERO-2 trial, which demonstrated the combination of everolimus and exemestane

was superior to exemestane alone, leading to approval of this drug combination for patients with estrogen receptor positive metastatic breast cancer (47, 48). Everolimus is now commonly used in combination with hormonal drugs such as exemestane, letrozole, or tamoxifen to manage endocrine therapy resistance.

In addition to efficacy, everolimus is also known to cause deleterious side effects in humans. The most common toxicities of everolimus are stomatitis, rash, fatigue, diarrhea, infections (49). These side effects are typically mild and tolerable; however, many patients need dose reductions or discontinuation due to these side effects (50). Our studies suggest that lower doses of everolimus (lower than the 10 mg standard human dose) may be more tolerable but still efficacious. In addition our studies of 2×/week doses show that there is still modest cancer preventive efficacy with this intermittent dose without any significant toxicities.

Our study showed that the mTOR inhibitor everolimus is capable of delaying and, in some mice, completely preventing, tumor development in various types of ER-negative breast cancer mouse models. Importantly, everolimus was well tolerated in all five breast cancer models, making it a good candidate for ER-negative breast cancer prevention trials. Collectively, our results suggest that long-term treatment with everolimus is well tolerated and is a safe treatment option for the prevention of ER-negative, triple-negative and/or *TP53*-mutant breast cancer. Hence, this study suggests that everolimus is a promising candidate for ER-negative breast cancer prevention trials for high-risk patients. However, although everolimus can prevent cancer development, resistance may evolve after long-term treatment. Therefore, to achieve an even greater delay in tumorigenesis it may be necessary to combine everolimus with other targeted drugs. Collectively, this study provides the foundation for future clinical trials combining everolimus with one or more targeted therapies, including PARP inhibitors, immune check-point inhibitors, and new generation rexinoids for high-risk individuals to further define the effect of everolimus treatment and prevention on various ER-negative or TNBCs.

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## Authors' Contributions

**A. Mazumdar:** Conceptualization, resources, data curation, software, formal analysis, supervision, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing. **W.M. Tahaney:** Conceptualization, data curation, formal analysis, validation, investigation, visualization, writing—original draft. **J.L. Hill:** Formal analysis. **Y. Zhang:** Data curation. **S. Ramachandran:** Resources, formal analysis. **J. Kawedia:** Resources, formal analysis. **J. Qian:** Resources, formal analysis. **A. Contreras:** Resources, data curation, formal analysis. **M.I. Savage:** Resources, validation, writing—original draft, writing—review and editing. **L.A. Vornik:** Conceptualization, writing—review and editing. **S. Sei:** Conceptualization, resources. **A. Mohammed:** Conceptualization, resources, funding acquisition. **P.H. Brown:** Conceptualization, resources, data curation, formal analysis, supervision, funding acquisition, validation, investigation, visualization, methodology, writing—original draft, project administration, writing—review and editing.

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