LETTER TO THE EDITOR

Differential efatutazone's impact on mammary neoplasia dependent upon *Brca1* dose

Dear Editor,

The impact of oral peroxisome proliferator-activated receptor gamma agonist (PPARG) efatutazone exposure through diet on mammary preneoplasia and cancer development was evaluated in genetically engineered mice carrying one vs two disrupted *Brca1* alleles targeted to mammary epithelial cells in the background of germline *Trp53* (*p53*) haploinsufficiency. *Brca1*^{WT/f111/Cre/p53+/-} and *Brca1*^{f111/f111/Cre/p53+/-} female mice were randomly assigned to receive efatutazone-containing diet (30 mg/kg) or equivalent diet without efatutazone at age 2 months. Efaturazone exposure significantly increased the percentage of mice with tertiary branching (Fig. 1A). Palpable mammary cancers appeared significantly earlier in *Brca1*^{f111/f111/Cre/p53+/-} as compared to *Brca1*^{WT/f111/Cre/p53+/-} mice, independent of efaturazone exposure (Fig. 1B). Efaturazone significantly reduced mammosphere development in *Brca1*^{f111/f111/Cre/p53+/-} mice (Fig. 1C, D and E). The significant reduction in numbers and prevalence of hyperplastic alveolar nodules (HANs) was more profound in *Brca1*^{WT/f111/Cre/p53+/-} as compared to *Brca1*^{f111/f111/Cre/p53+/-} mice (Fig. 1F and G). Palpable subcutaneous lipomas appeared in both efaturazone cohorts beginning at age 7 months, significantly shifting the reason for killing in the two different models (Fig. 2A). Fewer *Brca1*^{WT/f111/Cre/p53+/-} mice were killed for palpable mammary gland cancer than *Brca1*^{f111/f111/Cre/p53+/-} mice. Most mice developed one mammary cancer with a range of histology. Four mice developed two palpable mammary cancers: three *Brca1*^{f111/f111/Cre/p53+/-} mice (two control and one efaturazone cohort) and one *Brca1*^{WT/f111/Cre/p53+/-} mouse (control cohort). Most mammary cancers were triple negative (Fig. 2B, C, D, E, F and G). The two estrogen receptor alpha (ER)- and progesterone receptor (PR)-positive mammary adenocarcinomas that appeared were unique to the *Brca1*^{f111/f111/Cre/p53+/-} efaturazone cohort (Fig. 2H and I).

The study was prompted by a previous investigation demonstrating that efaturazone exposure beginning at

age 4 months in *Brca1*^{f111/f111/Cre/p53+/-} mice, a model which typically generates a range of histology of triple-negative mammary cancers, significantly reduces mammary hyperplasia and induces more differentiated mammary cancers including some ER-positive adenocarcinomas (Nakles et al. 2013). Questions raised by the earlier study included whether or not mice carrying a disruption in only one *Brca1* allele (Alothman et al. 2017) might be more responsive to efaturazone and the possibility that earlier administration would be more effective in reducing mammary cancer development (Dong 2013). PPARG has been reported as detectable in over 90% of triple-negative breast cancers developing in women who carry a *BRCA1* mutation (Heublein et al. 2017).

Significant differences in efaturazone response were found between the two models. Both models are derived from the same breeding colony with one vs two disrupted *Brca1* alleles discriminating between them. The proportion of control mice that developed mammary cancer by age 12 months was not significantly different between the two genotypes; however, the models did exhibit statistically significantly different time-courses of mammary carcinogenesis. Efaturazone was delivered at the same age in both models, raising the possibility that the significant differences in response found between the two models was due to the timing of the intervention relative to the course of mammary carcinogenesis, rather than the underlying genetic difference in full-length *Brca1* expression. Even the significant difference in mammosphere development between the models could be related to timing as the published literature suggests that mammosphere development from cancer cells is more profoundly affected by a PPARG agonist as compared to non-cancer cells. Results could argue that for efaturazone to be maximally effective, it may need to be initiated at very early stages of carcinogenesis. Theoretically to test that hypothesis efaturazone could be started even

Figure 1

Efatutazone exposure starting at age 2 months significantly reduced mammary preneoplasia. (A) Bar graphs comparing percentages of *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} mice demonstrating tertiary branching on fourth (inguinal) mammary gland whole mounts (WMs) following exposure to efatutazone or control diet. * $P < 0.05$, Fisher's exact test, one-sided. WM number (n) analyzed, age (months (m), mean $\pm$ s.e.m.) and weight (grams (g), mean $\pm$ s.e.m.) for each cohort: *Brca1*^{WT/fl11}/Crep53^{+/-} Efaturazone (E): $n = 12$ (9.0 ± 0.7 m, 38.0 ± 1.9 g). Control (C): $n = 12$ (10.1 ± 0.5 m, 36.9 ± 2.4 g). *Brca1*^{fl11/fl11}/Crep53^{+/-} E: $n = 12$ (8.4 ± 0.3 m, 40.3 ± 2.2 g). C: $n = 11$ (8.9 ± 0.3 m, 40.6 ± 2.4 g). WMs prepared at the time of necropsy for experimental endpoints designated in IACUC-approved protocol. All available fourth gland WMs analyzed. Mean ages and weights at necropsy were not statistically significantly different between cohorts. (B) Kaplan-Meier survival plot of all *Brca1*^{WT/fl11}/Crep53^{+/-} mice on efaturazone (green) and control (blue) and *Brca1*^{fl11/fl11}/Crep53^{+/-} mice on efaturazone (red) and control (black) diets enrolled in study. *Log-rank test for trend chi-Square $P = 0.0005$ between both *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} cohorts. Cohort numbers (n), median age (m) from survival plot: *Brca1*^{WT/fl11}/Crep53^{+/-} E: $n = 17$ (12.1 m). C: $n = 15$ (12.1 m). *Brca1*^{fl11/fl11}/Crep53^{+/-} E: $n = 17$ (9.1 m). C: $n = 19$ (9.2). Censored points for death due to non-palpable-tumor-related health reasons prior to age 12 months indicated by vertical ticks (skin ulcer, rectal prolapse, leg paralysis, shaking, lymphoma, found dead). Median survival was not statistically significant between efaturazone and control cohorts for either genotype. (C) Representative phase-contrast images of mammospheres (indicated by black arrows) that formed from primary mammary epithelial cells isolated from *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} on E and C diets. Images taken at 4x. Scale bar = 0.1 mm. (D) Box and whisker plots (mean and range) illustrating a reduction in mammosphere numbers forming from primary mammary epithelial cells isolated from efaturazone-exposed *Brca1*^{fl11/fl11}/Crep53^{+/-} mice as compared to *Brca1*^{fl11/fl11}/Crep53^{+/-} mice on control diet. * $P < 0.05$ Mann-Whitney U test, two-tailed. Twelve wells plated and analyzed per mouse. Cohort numbers (n), mean age (m) and weight (g): *Brca1*^{WT/fl11}/Crep53^{+/-} E: $n = 7$ (4.2 ± 0.0 m, 31.5 ± 1.5 g). C: $n = 6$ (3.9 ± 0.1 m, 34.6 ± 1.8 g). *Brca1*^{fl11/fl11}/Crep53^{+/-} E: $n = 4$ (3.9 ± 0.1 m, 32.3 ± 1.7 g). C: $n = 5$ (4.2 ± 0.0 m, 37.3 ± 2.9 g). Mammosphere numbers forming from *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} mice on control diet were significantly lower than numbers forming from MMTV-Cre (Cre)^c and wild-type (WT)^{a,d} mice on standard facility diet. * $P < 0.05$ Kruskal-Wallis Test, Dunn's multiple comparison. MMTV-Cre: $n = 3$ (4.2 ± 0.0 m, 31.2 ± 5.1 g). WT: $n = 3$ (5.2 ± 0.0 m, 25.6 ± 0.6 g). (E) Box and whisker plots (mean and range) illustrating that mammospheres derived from efaturazone-exposed *Brca1*^{fl11/fl11}/Crep53^{+/-} were significantly smaller than those obtained from efaturazone-exposed *Brca1*^{WT/fl11}/Crep53^{+/-} mice. * $P < 0.05$ Kruskal-Wallis Test, Dunn's multiple comparison. Wells and cohorts analyzed were the same as those analyzed for mammosphere numbers (Panel D). (F) Scatter plots (mean $\pm$ s.e.m. indicated) comparing numbers of HANs per WM from *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} mice following exposure to efaturazone or control diet. * $P < 0.05$, Mann-Whitney U test, one-tailed. WMs and cohorts analyzed were the same as those analyzed for tertiary branching (Panel A). (G) Bar graphs comparing percentages of *Brca1*^{WT/fl11}/Crep53^{+/-} and *Brca1*^{fl11/fl11}/Crep53^{+/-} mice demonstrating HANs on WM following exposure to efaturazone or control diet. * $P < 0.05$, Fisher's exact test, one-sided. WMs and cohorts analyzed were the same as those analyzed for tertiary branching (Panel A).

Figure 2

Impact of efatutazone exposure on mammary cancer development in *Brca1*^{WT/f111/Cre/p53+/-} and *Brca1*^{f111/f111/Cre/p53+/-} mice. (A) Stacked bar graphs comparing numbers of mice euthanized for tumor burden with lipoma alone, lipoma and mammary cancer, or mammary cancer alone. **P* < 0.05, 2 × 3 Fisher's exact. Number (*n*) of mice and age (m, mean ± s.e.m.) for each cohort: *Brca1*^{WT/f111/Cre/p53+/-} E: *n* = 8 (9.8 ± 0.7 m). C: *n* = 7 (10.4 ± 0.7 m). *Brca1*^{f111/f111/Cre/p53+/-} E: *n* = 13 (8.5 ± 0.4 m). C: *n* = 10 (9.0 ± 0.4 m). (B) Stacked bar graphs comparing distribution of mammary cancer histology identified in the palpable mammary cancers. Number (*n*) of mice and age (m, mean ± s.e.m.) for each cohort: *Brca1*^{WT/f111/Cre/p53+/-} E: *n* = 3 (7.7 ± 0.7 m). C: *n* = 7 (10.4 ± 0.7 m). *Brca1*^{f111/f111/Cre/p53+/-} E: *n* = 11 (8.6 ± 0.4 m). C: *n* = 10 (9.0 ± 0.4 m). Representative hematoxylin and eosin images of triple negative mammary adenocarcinoma (C), anaplastic carcinoma (D and F), sarcomatoid carcinoma (E and G) histology from palpable mammary cancers that developed in *Brca1*^{WT/f111/Cre/p53+/-} and *Brca1*^{f111/f111/Cre/p53+/-} mice on efatutazone or control diet. Insets to right show representative images of ER (top), PR (middle) and HER2 (bottom) IHC. (H and I) Representative images of the histology and ER, PR and HER2 IHC of the two palpable ER+/PR+ mammary adenocarcinomas that developed in *Brca1*^{f111/f111/Cre/p53+/-} mice on efatutazone diet. Arrows indicate representative mammary cancer cells demonstrating nuclear-localized ER and PR staining. Inset IHC images taken at 40× (C, D, E, F and G). Larger images taken at 20× (C, D, E, F, G, H and I). Scale bar = 0.1 mm.

before the end of puberty; however, here we identified a toxicity, lipoma development, prominent in this study but not found when efatutazone exposure was started at age 4 months (Nakles *et al.* 2013). PPARG activation previously has been suggested as a component of lipoma pathophysiology (Koppen & Kalkhoven 2010). The study indicates that developmental age at exposure may also play a role. Finally, we cannot exclude the possibility that germ-line *Trp53* haploinsufficiency also contributed to the susceptibility of the mice for lipoma development.

For preneoplasia reduction, *Brca1*^{WT/f111/Cre/p53+/-} mice were more sensitive than *Brca1*^{f111/f111/Cre/p53+/-} mice, but this did not translate to absolute mammary cancer prevention. The possibility of a parallel significant reduction in mammary cancer appearance could not be fully assessed due to the unexpected appearance of the palpable lipomas, found uniquely in this study when efatutazone was initiated at the younger age. This necessitated killing for tumor burden, required by the Institutional Animal Use and Care approved protocol, prior to the scheduled endpoint for evaluation of mammary cancer prevalence (age 12 months) in a significant proportion of the *Brca1*^{WT/f111/Cre/p53+/-} mice. In contrast, mammary tumor and lipoma development appeared temporally coincident in the *Brca1*^{f111/f111/Cre/p53+/-} mice that exhibit earlier mammary cancer development. The appearance of lipomas in *Brca1*^{f111/f111/Cre/p53+/-} mice did not compromise assessment of mammary cancer prevalence, which was unchanged by efatutazone exposure.

An alternative or contributing hypothesis for the significant differences found would be a differential action of efatutazone based on the presence or absence of full-length *Brca1*. *Brca1*^{WT/f111/Cre/p53+/-} mice still retain one functional *Brca1* allele, and mammary cancer development occurs without loss of the second *Brca1* allele (Allothman *et al.* 2017), as occurs in a portion of women carrying *BRCA1* mutation (Roy *et al.* 2011). PPARG agonists are reported to increase *BRCA1* expression *in vitro* in human breast cancer cells and *in vivo* in mammary glands of FVB mice (Subbaramaiah *et al.* 2012). Perhaps significantly, efatutazone is reported to be effective in delaying progression and inducing differentiation in a xenograft model of triple-negative ductal carcinoma *in situ* (MCFDCIS) with intact *BRCA1* and *p53* alleles (Ory *et al.* 2018).

Replicated in this investigation was the novel appearance of ER+/PR+ mammary adenocarcinomas in the efatutazone-exposed *Brca1*^{f111/f111/Cre/p53+/-} mice. While a low-incidence event in both this and the prior study (Nakles *et al.* 2013), it did appear in two independent

and temporally distinct experiments. Results support the possibility that activation of the PPARG pathway can contribute to the appearance of more differentiated mammary cancers retaining expression of ER and PR. None of the cancers expressed pathophysiological levels of Erb-B2 receptor tyrosine kinase 2 (ErbB2/Her2) and the majority of cancers in all groups were triple negative, consistent with previous studies in these and related models (Nakles *et al.* 2013).

Recognizing the fact that efatutazone was not protective of mammary cancer development, even as it significantly reduced preneoplastic growth, prompts the question of whether or not combining the drug with a second agent such as an antihormonal, a retinoid X receptor RXR ligand, TNF superfamily member 11 (TNFSF11)/RANKL inhibition or agents affecting the cell cycle such as cyclin-dependent kinase (CDK)4/6 inhibitors would be more effective. Time-course studies in which interventions are initiated at different ages in models with different temporal patterns of carcinogenesis on a prospective basis may help to further define the points during carcinogenesis when specific preventive agents can be effective. Direct comparison of the mechanistic action of efatutazone in *in vitro* and *in vivo* models with different gene dosages of *Brca1* and *Trp53* intact could establish if there are specific roles for their respective proteins in moderating response to a PPARG agonist.

All animal procedures were performed in accordance with federal guidelines and were approved by the Georgetown University Institutional Animal Care and Use Committee. Generation of mouse cohorts, dietary interventions (F3028, rodent diet, grain-based 1/2-in pellets without or with efatutazone (30mg/kg), Bio-Serv, Frenchtown, NJ, USA) and histology were performed as previously described (Nakles *et al.* 2013, Allothman *et al.* 2017). Pathology was read blindly by a board-certified pathologist (B V K). NanoCulture Plates (ORGANOGENIX Inc., Kanagawa, JP) were used to assess 3D mammosphere formation in serum-free EpiCult-B Mouse media (STEMCELL Technologies, Vancouver, CA, USA) (Arai *et al.* 2016, Allothman 2018). Immunohistochemical (IHC) procedures: ER (primary antibodies: ab75635, 1:50, overnight 4°C, Abcam; 06-935, 1:100, 1 h, room temperature (RT), Millipore Sigma), PR (sc538, 1:350, 1 h, RT, Santa Cruz Biotechnology, Inc.), HER2 (ERBB2) (2165, 1:100, overnight 4°C, Cell Signaling). Before primary antibody exposure, heat-induced epitope retrieval was performed by immersing tissue sections at 98°C for 20 min in 10 mM citrate buffer (pH 6.0) with 0.05% Tween (ER, PR) or 98°C for 20 min in 0.5 M EDTA

buffer (pH 8.0) (HER2) followed by treatment with 3% hydrogen peroxide and 10% normal goat serum (10 min each). Following primary antibody exposure, slides were exposed to an HRP-labeled polymer (EnVision+ System-HRP Labeled Polymer Anti-Rabbit, K4003, Dako, 30 min) and 3'-Diaminobenzidine (DAB) (Dako, 5 min). Cancers were read as ER+/PR+ when nuclear localization of both proteins was present. Two independent primary antibodies were used to test ER nuclear localization. Positive (human ER+/PR+/HER2-amplified breast cancer) and negative (no primary antibody) controls were performed with each individual IHC experiment. Statistical analyses were performed using GraphPad Prism and the VassarStats: Statistical Computation Web Site. $P < 0.05$ was considered significant.

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Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this article.

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Author contribution statement

Study conception and design (S J A, P A F), mouse husbandry (P A F, W W), data procurement (S J A, W W, S C, B V K, E S D-C, P A F), data analysis (S J A, S C, P A F), writing manuscript (S J A, P A F), review and editing manuscript (S J A, W W, S C, B V K, E S D-C, P A F).

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