Review

Cancer Research

Out-RANKing BRCA1 in Mutation Carriers

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Abstract

Beyond prophylactic mastectomy, there are currently very few options available to *BRCA1* mutation carriers to help reduce their risk of developing breast cancer. An effective prevention therapy therefore remains a pressing area of need. Accumulating evidence points to amplification of the progesterone signaling axis in precancerous tissue from *BRCA1* mutation carriers. Given that RANKL is an important paracrine mediator of hormonal signaling in breast tissue, there has been considerable interest in exploring a potential role for this pathway in oncogenesis. Recent findings indicate that the RANK and NF-κB pathways are aberrantly activated in luminal progenitor cells resident in preneoplastic *BRCA1*$^{mut/+}$ breast tissue. The augmented proliferation of these cells and their predilection for DNA damage suggest that they are prime cellular targets for basal-like cancers arising in *BRCA1* mutation carriers. The end result is a hyperactive pathway, initiated by progesterone and amplified by DNA damage–induced NF-κB signaling, that likely accounts for the susceptibility of *BRCA1*$^{mut/+}$ luminal progenitor cells to oncogenesis and tissue specificity. Specific targeting of this progenitor subset has revealed a compelling new prevention strategy for these and possibly other high-risk women. *Cancer Res; 77(3); 595–600. ©2017 AACR.*

Introduction

Germline mutations in the breast cancer susceptibility gene *BRCA1* confer a significantly increased risk of breast and ovarian cancer. Breast cancers that arise in *BRCA1* mutation carriers are usually early-onset basal-like tumors associated with a poor prognosis (1). Over the past two decades, there has been an exponential increase in our understanding of the biological functions of BRCA1 that include DNA repair, cell-cycle checkpoint control, protein ubiquitylation, and chromatin remodeling (2). However, the cellular mechanisms that culminate in breast carcinogenesis in *BRCA1* mutation carriers remain undefined. An understanding of the mechanisms governing the transition from normal breast epithelium to malignancy is pivotal for the identification of an effective, noninvasive prevention strategy for these high-risk women.

BRCA1 is critical for maintenance of genomic integrity, largely facilitated by its role in homologous recombination (HR)-mediated repair of DNA double-strand breaks (2). In cells deficient in functional BRCA1, homologous recombination–mediated repair is impaired and more error-prone mechanisms such as nonhomologous end joining are employed, resulting in increased chromosomal instability and a higher degree of genomic alterations compared with sporadic breast cancers. Given the requirement for high-fidelity double-strand break repair in all cells to ensure survival, the loss of genomic stability associated with a germline *BRCA1* mutation would be anticipated to promote tumor formation in all tissues. Notably, however, *BRCA1* mutation carriers almost exclusively develop breast and ovarian cancers.

Several hypotheses have been put forward to explain this remarkable tumor specificity. High-fidelity DNA damage repair (DDR) in breast and ovarian tissues has been posited to rely on functional BRCA1 protein, while other tissues may utilize compensatory mechanisms (3). Breast and ovarian tissues have also been speculated to undergo an accelerated rate of loss of heterozygosity at the *BRCA1* locus compared with other tissues, perhaps via enhanced mitotic recombination. Conversely, evidence suggests that loss of the wild-type *BRCA1* allele does not occur in all tumor cells and may not be the rate-limiting tumor-initiating step (4). Many of these hypotheses have primarily focused on findings in cells where both *BRCA1* alleles have been lost, or on events directly preceding this (in the case of an accelerated loss of heterozygosity). However, it seems likely that the tissue specificity associated with *BRCA1*-mutated cancers is facilitated by earlier events initiated in heterozygous *BRCA1* breast tissue. In line with this, Sedic and colleagues (5) recently demonstrated that substantial haploinsufficiency and genomic instability occur in *BRCA1*-mutant breast epithelial cells relative to breast fibroblasts, culminating in premature senescence of epithelial cells and a higher risk of neoplastic transformation. Of note, heterozygous cells also appear to be defective in stalled replication fork repair and/or suppression of fork collapse (6). Cell-intrinsic properties, such as haploinsufficiency for DNA repair, are likely to act in concert with extrinsic influences to promote neoplastic transformation (see below).

In this review, we discuss recent advances in our understanding of cellular and molecular mechanisms that contribute to the initiation of breast tumorigenesis in *BRCA1* mutation carriers. Hormone-driven activation of progenitor cells in the preneoplastic state is likely to be a key initiation event, thus contributing to the tissue specificity of tumors arising in *BRCA1* mutation carriers and providing a rationale for a new chemoprevention strategy.

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Evidence for Enhanced Hormonal Signaling in *BRCA1*-Mutant Tissue

There is accumulating evidence for deregulated hormonal signaling in *BRCA1* mutation carriers. The risk of breast cancer in carriers is substantially higher during pregnancy, when there are high circulating levels of steroid hormones (7), whereas a sharp decline in cancer risk occurs after menopause (8). Moreover, a recent analysis of serum levels of estradiol and progesterone indicated that premenopausal *BRCA1/2* carriers have higher levels of serum progesterone during the luteal phase of the menstrual cycle compared with noncarriers (9). Defects in hormone receptor activity have also been implicated in *BRCA1*-deficient breast epithelial cells, although most of these studies have focused on cells lacking both *BRCA1* alleles. Given that BRCA1 can interact with the progesterone receptor (PR) and estrogen receptor (ER) to repress their transcriptional activity (10), *BRCA1* deficiency would be predicted to deregulate the activity of these receptors in hormonally regulated tissues, such as the breast and ovary. Indeed, disrupted receptor turnover and augmented PR expression have been observed in the mammary epithelium of *Brca1/p53*-deficient mice (11) and in normal tissue adjacent to *BRCA1*-mutated breast tumors (12). Furthermore, treatment of *Brca1*-deficient mice with exogenous progesterone led to increased tertiary branching (13), while the progesterone antagonist mifepristone prevented mammary tumorigenesis in mice lacking functional *Brca1* and *p53* (11). Interestingly, estrogen may also contribute to deregulated hormone signaling. *Brca1*-deficient mouse mammary epithelial cells accumulate reactive oxygen species, but estrogen can overcome oxidative stress–induced cell death in these cells by the induction of NRF2-regulated enzymes (14). Together, these studies suggest that steroid hormone regulation is perturbed in *BRCA1* mutation carriers, creating a milieu in which breast epithelial cells are hyperresponsive to hormonal stimuli.

It is well established that estrogen and progesterone mediate their effects via paracrine mechanisms, thus enabling ER/PR–expressing cells to influence nearby cells lacking these steroid hormone receptors. One of the major target genes of progesterone is receptor activator of nuclear factor kappa-B ligand (RANKL), a member of the TNF superfamily (15). Originally identified in bone tissue as the critical driver of osteoclast formation and activation, RANKL functions via its cognate receptor RANK and can be inhibited by the soluble decoy receptor osteoprotegerin (OPG). RANK and RANKL play critical roles in the mouse mammary gland, orchestrating the formation of alveolar units during pregnancy (16). RANKL is produced by mature luminal cells that express PR (17) and signals to mammary stem (MaSC) and/or luminal progenitor subsets that express RANK on their surface. Interestingly, RANK is exclusively expressed by luminal progenitor cells in human breast tissue (18), whereas in the mouse mammary gland, the receptor is expressed by both MaSCs (19, 20) and ER^-PR^- luminal progenitor cells (21, 22). Notably, the role of RANKL as an effector of hormone signaling has been confirmed in human breast tissue (23).

The RANK–RANKL pathway also plays a key role in hormone-driven mammary tumorigenesis. Overexpression of RANK in mammary epithelium resulted in sustained proliferation and the development of hyperplasia, and accelerated mammary tumor formation following treatment with the carcinogen 7,12-dimethylbenzen(a)anthracene (DMBA) and medroxyprogesterone (MPA; ref. 24). Conversely, ablation of RANK signaling in the mammary epithelium delayed the onset of MPA/DMBA–driven mammary cancer (24, 25). In *BRCA1* mutation carriers, the altered hormone responsiveness of breast epithelial cells is likely to be an early event, prior to cells transitioning to a hormone-independent state.

Identification of Candidate Cells of Origin

Tissue obtained from *BRCA1* mutation carriers who underwent a prophylactic mastectomy offers a unique opportunity to explore cellular mechanisms that are altered in the preneoplastic phase. Earlier studies suggested that an ER^- stem/progenitor population was perturbed in these carriers (26). With the advent of flow cytometric fractionation of epithelial subsets resident in human breast tissue and their biological characterization, it has been possible to pinpoint an aberrant luminal progenitor subpopulation in preneoplastic tissue from *BRCA1* mutation carriers (27). In a complementary study using human breast tissue, Proia and colleagues showed preferential transformation of *BRCA1*-mutated luminal cells compared with basal cells (28). Moreover, in *Brca1*-deficient mouse models, conditional deletion of *Brca1* from the luminal subset predisposed mice to tumors that resembled basal-like tumors (29). Nevertheless, the question remains: why are luminal progenitors more susceptible to oncogenesis than other cell types? Recent studies have indicated that the answer may lie in activation of the RANK and NF-κB pathways, as discussed below (18, 30, 31).

RANK and NF-κB Activation Mark Perturbed Luminal Progenitors in *BRCA1*-Mutant Tissue

Interrogation of a potential link between hormone-mediated RANKL signaling and *BRCA1*-associated tumorigenesis revealed an expanded population of progesterone-responsive $RANK^+$ luminal progenitor cells in the preneoplastic phase. $RANK^+$ cells were highly proliferative and exquisitely sensitive to DNA damage (both stalled fork repair and irradiation) in the haploinsufficient state compared with other breast epithelial cell types, likely setting the stage for neoplastic transformation (18). Strikingly, the $RANK^+$ but not $RANK^-$ subset shared a molecular profile more closely aligned with basal-like breast tumors than any other subtype (18). These findings suggest that $RANK^+$ luminal progenitors are a likely target population for malignant transformation in *BRCA1*-mutant breast tissue (Fig. 1A).

In a parallel study, the aberrant growth properties of $BRCA1^{mut/+}$ luminal progenitors were linked to DDR-induced activation of NF-κB (30). Double-stranded DNA breaks are known to activate the ataxia–telangiectasia (ATM) checkpoint kinase and the NF-κB essential modulator (NEMO), resulting in activation of NF-κB signaling (32). Earlier studies established a crucial link between the RANK–NF-κB signaling axis and proliferation: RANKL was shown to signal through IκB kinase-α (IKKα) to NF-κB, leading to upregulation of cyclin D1 and cell-cycle progression (33). Thus, activation of NF-κB in *BRCA1*-mutant tissue would be predicted to result in expansion of the $RANK^+$ subset and accounts for the observed 2-fold increase (18). RANKL–RANK signaling may also be triggered by alternative pathways, such as p38 MAPK, PI3K/Akt, and Id2-mediated downregulation of p21.

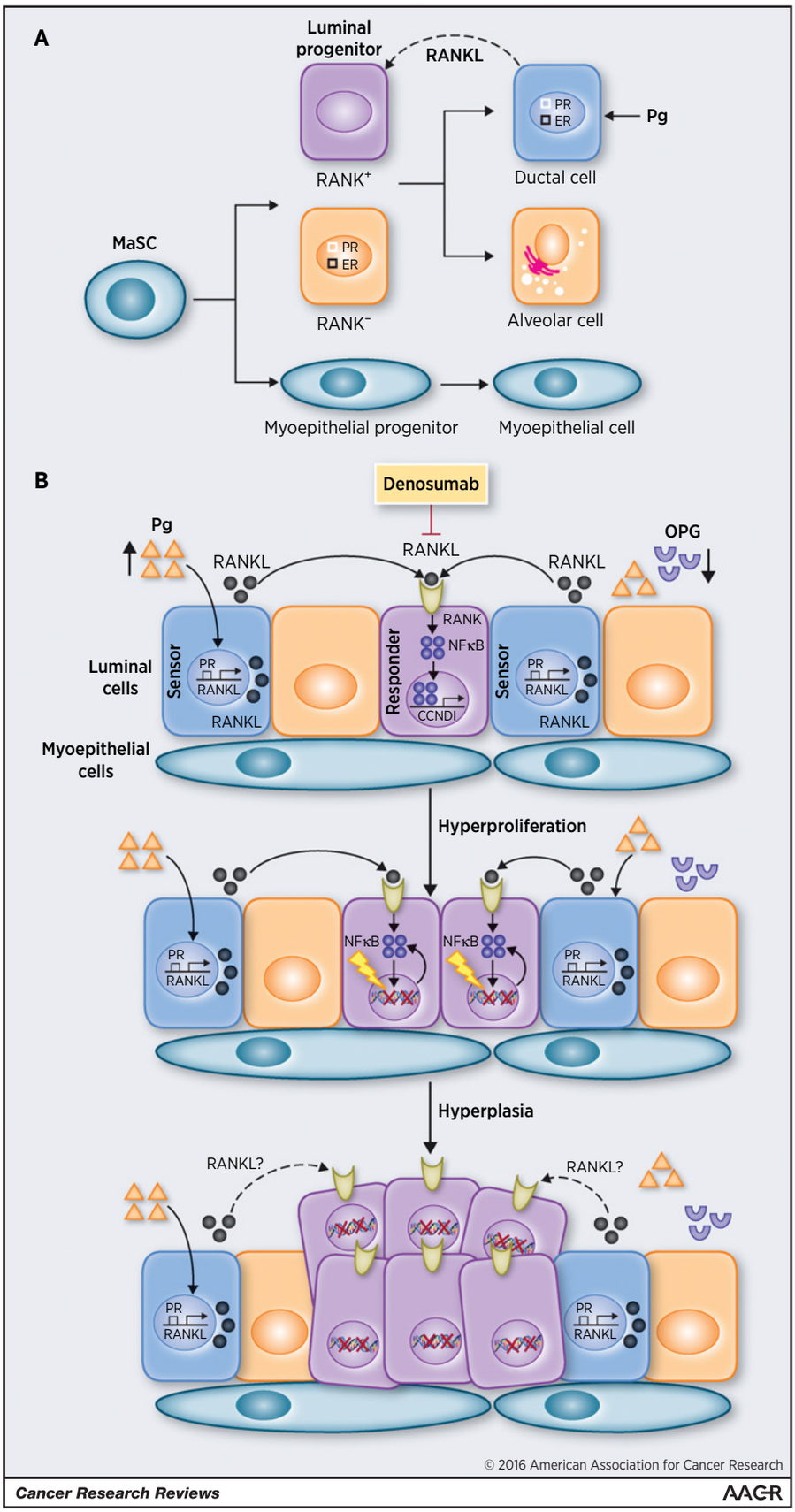

Figure 1.
Schematic model of breast oncogenesis in young premenopausal *BRCA1*$^{mut/+}$ mutation carriers. **A,** Simplified model of the human breast epithelial differentiation hierarchy. RANK^{+} luminal progenitors lack hormone receptor expression but can receive mitogenic signals from progesterone via the secretion of RANKL from mature ductal cells. **B,** Model showing potential cellular and molecular mechanisms that may drive hyperplasia in *BRCA1* mutation carriers. Progesterone binds to its cognate receptor on mature ductal cells in *BRCA1*$^{mut/+}$ breast tissue, stimulating the secretion of RANKL, which then binds and activates RANK on hormone receptor-negative luminal progenitors. This results in the activation of pro-proliferative pathways, such as NF-κB–induced cyclin D1 expression. Hyperactivation of this pathway in response to amplified hormonal signaling leads to enhanced proliferation and exerts undue pressure on the DNA repair machinery. Compromised DNA repair and consequent genomic instability lead to the acquisition of potentially deleterious mutations and the activation of NF-κB via ATM:NEMO complexes. Once RANK signaling initiates the DDR in BRCA1$^{+/-}$ cells, the alternative p52 pathway is activated, resulting in a sustained response that further exacerbates genomic instability by triggering the proliferation of RANK^{+} cells. Hyperplasia is likely to persist in the absence of hormonal influence, but the cells remain responsive to RANKL stimulation. Pg, progesterone; CCND1, cyclin D1.

Thus, it appears that NF-κB is activated in a two-pronged manner: by incumbent DNA damage as well as progesterone-mediated activation of the RANK pathway. Interestingly, luminal progenitor cells present in normal mammary tissue utilize canonical NF-κB signaling (30). In both human and mouse *BRCA1*-deficient cells, however, the noncanonical pathway can also be aberrantly activated. The DDR that results from proliferation-induced replication stress appears to perpetuate p65 phosphorylation and p52/RelB NF-κB activation in both the heterozygous and nullizygous states (30). Indeed, p100/p52, ATM, and IKKα were shown to be essential for the sustained growth factor–independent proliferation of *Brca1*-deficient luminal progenitors. It is noteworthy that the alternative NF-κB pathway is longer acting than the canonical one, as it is not subject to autoinhibition by IκBα. Moreover, Sau and colleagues (30) showed that NF-κB inhibition *in vivo* was able to markedly reduce γH2AX foci in *Brca1*$^{-/-}$ mammary glands, consistent with an additional role for NF-κB downstream of DNA damage in RANK^{+} progenitors. The activation of NF-κB by the RANK signaling pathway likely exacerbates genomic instability by stimulating the proliferation of RANK^{+} cells. NF-κB–mediated proliferation may be self-perpetuating (due to the DDR), as it can persist in the absence of further hormone stimulation (permitting factor-independent colony formation *in vitro*; ref. 30), but nevertheless remains highly responsive to progesterone-induced RANK signaling. NF-κB activation may also confer a survival advantage to genetically unstable RANK^{+} cells through the activation of antiapoptotic genes.

Impact of Systemic Factors on RANK Pathway Activation

There are additional features associated with *BRCA1* haploinsufficiency that are likely to result in persistent activation of the RANKL/NF-κB pathway in mutant tissue. *BRCA1* mutation carriers exhibit amplified steroid hormone signaling as a consequence of deregulated ER/PR expression and higher circulating levels of progesterone, which would promote stimulation of RANK^{+} cells through RANKL in the luteal phase of the menstrual cycle. In addition, *BRCA1* mutation carriers were recently reported to have lower serum OPG levels (34). As OPG is the decoy receptor for RANKL and plays a crucial role in modulating RANK activation, reduced OPG levels would be anticipated to trigger amplification of the RANKL signaling pathway in breast epithelium. Together with the striking haploinsufficiency of BRCA1$^{+/-}$ RANK^{+} cells for executing DNA repair and the ensuing damage-induced activation of NF-κB, the end result is likely to be a genetically unstable population of cells in *BRCA1*$^{mut/+}$ breast tissue (Fig. 1B).

Inhibition of RANK–RANKL Signaling as a Prevention Strategy

The possibility that RANKL–RANK blockade might represent a novel prevention strategy for *BRCA1* mutation carriers has recently been explored in mouse models. Such a strategy would remove the initializing mitogenic signal and prevent further cyclical stimulation of the DDR/ATM/NF-κB pathway in RANK^{+} cells. Genetic ablation of RANK in the mammary epithelium of *Brca1*/*p53*–deficient mice under the control of either the *WapCre*^{C} or *K5* promoter substantially delayed the onset of tumors and hyperplasia, respectively (31). A reduced number of lesions was reported in the *MMTV-Cre;Brca1*$^{fl/fl}$ model (31). In complementary work to recapitulate a prevention study in young *BRCA1*$^{mut/+}$ women, significantly delayed tumor onset and reduced hyperplasia were observed in MMTV-cre/*Brca1*$^{fl/fl}$/*p53*$^{+/-}$ mice treated with either the RANKL inhibitor OPG-Fc or a neutralizing anti-RANKL antibody during the preneoplastic phase (18).

Importantly, RANKL inhibition showed efficacy in the human tissue setting. In 3D human breast organoid cultures derived from preneoplastic *BRCA1*-mutant tissue, progesterone-induced proliferation was significantly attenuated by denosumab, a human-specific RANKL inhibitor (18). This blocking antibody is currently in clinical use for osteoporosis and the prevention of skeletal-related events in patients with bone metastases (35). Preliminary data from a preclinical window study ("BRCA-D"; ACTRN12614000694617) to evaluate the biological effects of denosumab on breast tissue biopsies from *BRCA1* mutation carriers revealed that proliferation was markedly reduced by short-term treatment (18). RANKL inhibition may be even more effective when initiated at a younger age, allowing less time for the accumulation of DNA lesions (including *p53* mutations, which are common in established BRCA1-associated tumors) and DDR-activated NF-κB activity. Although direct targeting of NF-κB may also have efficacy as a preventive therapy, there could be significant toxicity and immunosuppression associated with DMAPT or other agents owing to the plethora of roles that NF-κB holds in biological processes, including immunity and inflammation. By targeting RANKL rather than NF-κB, a more restricted subset of cells will be inhibited. Furthermore, other pathways besides NF-κB may promote proliferation of RANK^{+} cells. Therefore, the targeting of RANKL may prove more efficacious for breast cancer prevention.

It is noteworthy that RANKL levels are reduced by both oophorectomy and tamoxifen (18). There is circumstantial evidence to suggest that tamoxifen, a selective estrogen receptor modulator, could have efficacy for the prevention of breast cancer in *BRCA1* mutation carriers. However, uptake of tamoxifen prevention has been poor in the clinic, and given reports that endometrial cancer risk may be amplified in *BRCA1* mutation carriers, it seems unlikely that tamoxifen prevention will be formally evaluated in a prevention study for mutation carriers.

Concluding Remarks and Future Directions

An effective preventive therapy for hormone receptor–negative breast cancer is currently lacking. Emerging data point to RANKL inhibition as a promising prevention strategy for *BRCA1* mutation carriers. Although preliminary data from clinical samples in *BRCA1* mutation carriers indicate that short-term denosumab treatment may be effective (18), the true potential of denosumab as a preventive therapy (either delaying or preventing breast cancer) awaits a large randomized international clinical trial. Denosumab has been extensively investigated in the clinic, although mainly in postmenopausal women. Low-dose therapy has been shown to have an acceptable safety profile, and serious adverse events are uncommon. Osteonecrosis of the jaw (ONJ) and atypical fractures have emerged as rare but serious side effects, although this has typically been observed in patients who receive prolonged high-dose therapy (including following major dental procedures). It will be essential to establish whether denosumab has a favorable safety profile in young, premenopausal women. If "repurposing" denosumab were to prove safe and effective in

"buying time" for young *BRCA1* mutation carriers before considering a double mastectomy, it could potentially have broader applicability for other women at increased genetic risk, including *BRCA2* mutation carriers or other women from high-risk families. Given that RANKL levels drop precipitously in serum and breast tissue in the postmenopausal setting (18), it will also be imperative to ascertain whether postmenopausal *BRCA1* mutation carriers could also benefit from RANKL blockade and whether this strategy also ameliorates ovarian cancer risk. As a corollary, RANKL inhibition should help to abrogate the loss in bone mineral density that accompanies oophorectomy. As risk-reducing bilateral salpingo-oophorectomy (RRSO) is an important prevention strategy for ovarian cancer, we speculate that a sequential approach comprising RANKL inhibition (which would delay the need for mastectomy) could be followed by RRSO at a suitable age to minimize both breast and ovarian cancer risk.

Although single-nucleotide variants in the human *RANK* locus were recently reported to be associated with a modified breast cancer risk in *BRCA1* mutation carriers (31), this will require validation in larger cohorts and evidence of their functional relevance. It will also be important to explore a role for RANKL inhibition beyond prevention to the treatment of established breast cancer, given that RANK expression appears to be retained in a significant proportion of tumors arising in *BRCA1* mutation carriers and in a subset of sporadic triple-negative cancers (18). Indeed, RANKL inhibition was found to synergize with docetaxel chemotherapy in a *BRCA1*-deficient patient-derived xenograft model of triple-negative breast cancer (18). This question may be addressed in the upcoming GeparX neoadjuvant clinical trial (NCT02682693). It is also noteworthy that improved disease-free survival was recently reported in the ABCSG-18 trial, in which postmenopausal women received low-dose denosumab with an aromatase inhibitor as adjuvant therapy for hormone receptor–positive breast cancer (36). Finally, as NF-κB hyperactivation in *BRCA1*-mutant breast cancer cells appears to be associated with an immune signature (37), exploration of combinatorial strategies that target the immune checkpoint inhibitors may also be warranted.

Disclosure of Potential Conflicts of Interest

G.J. Lindeman reports receiving other commercial research support from Amgen and a consultant/advisory board member for an Amgen Breast Cancer Advisory Board. J. Visvader reports receiving other commercial research support from Amgen. No potential conflicts of interest were disclosed by the other author.

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