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Review

Cancer Prevention Research

NF-κB at the Crossroads of Normal Mammary Gland Biology and the Pathogenesis and Prevention of *BRCA1*-Mutated Breast Cancer

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Abstract

Recent studies have shown that progesterone receptor (PR)–expressing cells respond to progesterone in part through the induction of the receptor activator of NF-κB ligand (RANKL), which acts in a paracrine manner to induce expansion of a RANK-expressing luminal progenitor cell population. The $RANK^+$ population in human breast tissue from carriers of *BRCA1* mutations ($BRCA1^{mut/+}$) as well as the luminal progenitor population in *Brca1*-deficient mouse mammary glands is abnormally amplified. Remarkably, mouse $Brca1^{+/-}$ and human $BRCA1^{mut/+}$ progenitor cells are able to form colonies *in vitro* in the absence of progesterone, demonstrating a hormone-independent proliferative capacity. Our research has demonstrated that proliferation in *BRCA1*-deficient cells results in a DNA damage response (DDR) that activates a persistent NF-κB signal, which supplants progesterone/RANKL signaling for an extended time period. Thus, the transcriptional targets normally activated by RANKL that promote a proliferative response in luminal progenitors can contribute to the susceptibility of mammary epithelial cells to *BRCA1*-mutated breast cancers as a consequence of DDR-induced NF-κB. Together, these latest findings mark substantial progress in uncovering the mechanisms driving high rates of breast tumorigenesis in *BRCA1* mutation carriers and ultimately reveal possibilities for non-surgical prevention strategies. *Cancer Prev Res; 11(2); 69–80.* ©*2017 AACR.*

The Mammary Gland Contains Stem and Progenitor Cells that Are Cells of Origin for Breast Cancer Subtypes

In 1998, Kordon and Smith showed that a single cell was able to regenerate a ductal lobular outgrowth with complete luminal and myoepithelial cells (1). FACS based on cell surface receptor immunoreactivity is used to enrich stem/progenitor populations within the mammary gland. Primitive mammary stem cells have been functionally defined in transplantation assays as able to regenerate an entire mammary gland in mice. Transit amplification of progenitors results in subpopulations that include luminal progenitor cells (LP) and basal (myoepithelial) progenitors (2). Primitive luminal progenitor cells are thought to give rise to hormone receptor–positive and negative ductal progenitors and ultimately mature ductal cells. Evidence from lineage-tracing studies suggests that the hormone receptor–negative alveolar lineage may derive from either a common luminal progenitor or from the basal progenitor pool (reviewed in refs. 2, 3).

On the basis of their gene expression profile, human breast cancers have been categorized into five molecular subtypes: luminal A, luminal B, receptor tyrosine protein kinase erbB-2 (ERBB2) positive, basal-like, and normal-like (4). Although the mammary stem cell genomic signature is most aligned with the normal-like subtype, the luminal progenitor signature more closely resembles the basal-like subtype (5). Subpopulations of luminal progenitors are also proposed to be the target of transformation in luminal A, luminal B, and the ERBB2-positive breast cancers (6). Approximately 15% of all breast cancers belong to the basal-like, and more than 90% of *BRCA1*-associated breast cancers belong to this subtype (7).

Breast Cancer–Associated Gene 1

The *BRCA1* gene was originally mapped in 1990 and cloned in 1994 (8). Mutations in one copy of *BRCA1* gene in the germline lead to the hereditary breast and ovarian cancer syndrome, which is characterized by early-onset cancers and lifetime risks of up to 80% by the age of 70. The BRCA1 protein plays an important role in maintaining global genomic stability. Single or double DNA strand breaks (SSB or DSB) can occur during normal cellular replication or during exposure to

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genotoxic compounds. During replication in S-phase, the genome is particularly susceptible to SSBs that are converted into DSBs. In S-phase, BRCA1 has a role in the high fidelity process of homology-directed DNA repair where it acts as a scaffold protein to assemble a cohort of other DNA repair proteins, including BRCA2, Rad50, and Rad51, to the DSB (9). In the presence of a deleterious mutation in carriers coupled to loss of the normal allele of *BRCA1*, HR is dysfunctional, and DSBs can result in chromosome rearrangements and genomic instability leading to cellular transformation.

More recently, BRCA1 has also been found to have a critical role in the protection of stalled replication forks in an HR-independent manner (10). Replication fork progression is frequently impaired as a consequence of DNA breaks caused by exogenous (such as UV) or endogenous (such as reactive oxygen species) factors or nucleotide depletion. If left unprotected, the meiotic recombination 11 protein (MRE-11) can recognize and degrade stalled replication forks, inducing DNA damage and chromosome instability. BRCA1, together with the Fanconi anemia group D2 protein (FANCD2), BRCA2, and Rad51 can protect stalled forks to prevent nucleotide degradation during replication stress (10). Thus BRCA1 has a critical role in maintaining global genomic stability during normal cell replication and under stressed conditions.

BRCA1 also plays a role in the differentiation of the mammary glands through gene regulation at both the transcriptional and epigenetic levels (11) in response to ovarian hormones (12). Gene expression profiling showed that depletion of BRCA1 upregulates the genes involved in proliferation but downregulates those associated with differentiation (13). Indeed, an important target of BRCA1 is the estrogen receptor (ER) gene, which is positively regulated by BRCA1 through direct DNA binding (14).

Progesterone Regulates Progenitor Cell Proliferation through NF-κB

Mammary stem and progenitor cells are highly responsive to steroid hormones. Progesterone targets progesterone receptor (PR)–positive mammary epithelial cells to induce proliferation in a cyclin D1–dependent manner (15). In addition, progesterone induces the synthesis and release of the receptor activator for NF-κB ligand (RANKL). Paracrine RANKL, released from PR^+ luminal cells, induces the expansion of the RANK-expressing progenitor cell compartment in the mouse mammary gland. RANKL functions by binding to its receptor RANK, expressed on PR^- stem and progenitor cells (15–17), thereby activating the transcription factor, NF-κB. RANK signaling promotes the transcription of numerous genes involved in proliferation, including cyclin D1, resulting in expansion of the mammary gland and ductal side branching during mammary gland development (18, 19).

NF-κB not only regulates the transcription of genes involved in proliferation, but also many genes controlling apoptosis/survival and the secondary immune response and plays a critical role in cancer biology (20). Extracellular signal–mediated induction of NF-κB occurs through the TNFα family of receptors and ligands that include RANK and RANKL. Activation of the RANK receptor can activate both types of NF-κB signaling called the canonical pathway and the alternative pathway (21). The canonical NF-κB pathway typically consists of a p65/RelA;p50 heterodimer whose nuclear translocation is stimulated by the inhibitor of κB kinase (IKKα/IKKβ/NEMO) complex, which results in the degradation of the cytoplasmic inhibitor, IκBα. This releases p65/p50, which then moves into the nucleus. The proteins p52 and RelB constitute the alternative NF-κB pathway, which is dependent on IKKα-mediated phosphorylation for its activation and nuclear localization (reviewed in ref. 22). Interestingly, NEMO and IKKα can also form a functional IKK complex able to activate the alternative NF-κB pathway (23). Once in the nucleus, NF-κB dimers bind κB enhancer sequences in target genes and regulate transcription through the recruitment of coactivators or corepressors.

NF-κB Drives Normal Mammary Cell Proliferation and Contributes to Tumorigenesis

NF-κB is an integral player in the normal postnatal morphogenesis of the mammary gland. NF-κB activity is high during pregnancy, decreases during lactation, and increases again during involution. This pattern demonstrates the contrasting regulatory roles of NF-κB during the pregnancy (proliferative) and involution (apoptotic) phases of the mammary gland (24, 25). Mice deleted for *Rank* or *Rankl* had normal mammary gland development after birth; however, mammary glands showed increased apoptosis and failed to form lobuloalveolar structures during pregnancy (26). Transgenic expression of mammary gland *Rank* in mice prevents development of a functional mammary gland, showing increased proliferation during pregnancy, impaired differentiation, and decreased expression of the milk protein, β-casein. Moreover, constitutive overexpression of RANK resulted in the development of hyperplasias at advanced age (27). Importantly, mice lacking the catalytic domain of IKKα show a lactation defect similar to *Rank*-null mice (18), suggesting that the alternative NF-κB pathway plays a key role in the response to RANKL in the mammary gland.

Altogether, these data show that RANK/NF-κB signaling is a critical regulator of mammary epithelial cells in the luteal phase and during pregnancy, whereas dysregulation is associated with failure of cell differentiation. As a consequence of its important participation in cyclin D1 and RANKL regulation, progesterone is now recognized as

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having a central role in the pathophysiology of mammary neoplasia (28).

Several studies have shown that NF-κB is activated in breast cancers, and this activation is increased with hormone independence. NF-κB was found constitutively active in the nucleus of human breast cancer cells, while no detectable levels were found in normal cells (29). We and others have shown that the canonical NF-κB pathway appears to be most active in the ER^+ and $HER2^+$ breast cancers (30, 31). Moreover, we have demonstrated that transgenic inhibition of NF-κB by the stable "superrepressor" inhibitor of κB ($IκBα^{SR}$) increases the latency and decreases the incidence of carcinogen-induced mammary adenomas and carcinomas (32). NF-κB can also regulate epithelial-to-mesenchymal transition (EMT), a key process in breast cancer progression (33). More recently, it was demonstrated that both the canonical and alternative NF-κB pathways are required for the self-renewal and proliferation of tumor-initiating cells through the stimulation of genes involved in EMT (34). A direct link between alternative NF-κB activation and breast cancer initiation was established using a mouse mammary p100/p52 transgenic model wherein mice showed a delay in mammary gland development and reduction in ductal branching during pregnancy. Instead, these mice had thickening of primary ducts, loss of epithelial cell organization, and hyperplasia (35). RelB has also been found to be increased in $ERα^-$ breast cancer. Indeed, RelB is necessary to maintain the mesenchymal phenotype of $ERα^-$ breast cancer through the direct transcriptional activation of *BCL2* (36). In an analysis of microarray data from 249 human breast tumors, we found that p100/p52 and RelB gene expression is predominantly expressed in TNBC (32). Consistent with this, another study showed that p100/p52 and RelB expression is higher in *BRCA1*-mutated tumors compared with other subtypes (37). The association of NF-κB with the development of basal-like tumors is also supported by identification of EMT-associated gene targets. NF-κB induces the mesenchymal-specific gene vimentin and suppresses the expression of epithelial-specific gene E-cadherin by inducing the expression of Snail (38) and the transcription factors ZEB1 and ZEB2 (39) and Slug (40, 41). Moreover, NF-κB has been shown to be critical for maintenance of the mesenchymal phenotype as blocking of NF-κB results in a partial MET (39). Transcription of the *ESR1* gene is also negatively regulated by RelB (42), which would prevent differentiation into hormone-sensing cells.

NF-κB Is Activated by DNA Damage

In addition to its activation by ligands through receptor-mediated signaling, NF-κB is also induced in response to genotoxic stresses. Following irradiation or chemotherapeutic drugs that cause double-strand DNA breaks, the ATM kinase is activated and phosphorylates H2AX, leading to the recruitment of several other proteins involved in DNA damage response (DDR; 43). Early evidence demonstrated that ATM activation in response to genotoxic stimuli was also instrumental in the induction of NF-κB (44). Subsequent studies revealed that activation of ATM in response to DNA double-strand breaks was necessary for the phosphorylation of NEMO, which resulted IKK activation resulting in an atypical mechanism of inducing NF-κB activity (45). Although cells heterozygous for *BRCA1* are competent for most functions, including HR, they demonstrate significant genomic instability (46) and are hypersensitive to genotoxic stress (47). Consistent with this, NF-κB can also be induced by replication stress (45). Indeed, $BRCA1^{+/-}$ cells are susceptible to replication fork stalling and collapse (10). Thus, replication stress, coupled to stalled fork instability is likely a critical factor precipitating DNA damage in *BRCA1* mutation carriers.

Abnormal Progenitor Cells in *BRCA1* Mutation Carriers: Basal Breast Cancer Originates in the Luminal Progenitor Population

As discussed, BRCA1 expression is required for normal differentiation of ER^- stem/progenitor cells to luminal cells by modulating critical pathways determining cell fate. Consequently, knockdown of BRCA1 in primary breast epithelial cells leads to increased expression of the stem/progenitor cell marker aldehyde dehydrogenase 1 (ALDH1) and a decrease in cells expressing luminal epithelial markers and the ERα. Although normal control mammary lobules are marked with $ER^+/ALDH^-$ cells, remarkably, sections of breast tissue from women with germline *BRCA1* mutations contain entire lobules displaying loss of heterozygosity that express ALDH1 but not the ER (48).

Examination of the mammary progenitor cell populations from *BRCA1* mutation carriers, including basal stem/progenitor, luminal progenitor, and mature luminal cells, showed that these glands contain an expanded luminal progenitor population. Intriguingly, unlike normal progenitor cells, the $BRCA1^{mut/+}$ luminal population was able to proliferate and form colonies *in vitro* in the absence of several media supplements, including progesterone (49). Thus, $BRCA1^{mut/+}$ cells must possess an intrinsic mechanism that conveys the ability to grow for extended periods without hormonal stimulation. The gene expression profiles of ostensibly normal luminal progenitor cells from breast tissue heterozygous for a *BRCA1* mutation and the basal breast cancer signature are highly similar suggesting for the first time that basal breast cancers may arise from an (early) luminal progenitor rather than the mammary stem/bipotent subpopulation. Indeed, specific deletion of *Brca1* in mouse mammary epithelial luminal progenitors using a luminal keratin promoter to drive expression of the cre-recombinase in $Tp53^{-/+}$ mice results in

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tumors that phenocopy human *BRCA1* breast cancers and non-*BRCA1* basal breast cancers. In contrast, *Brca1* deficiency mediated by a basal keratin promoter driving cre results in tumors that have basal breast cancer gene expression profiles but are not histologically similar to *BRCA1*-deficient or the majority of sporadic basal-like human breast tumors (50). This finding was also supported by a study by Bai and colleagues (2013) showing that *BRCA1* deficiency resulted in a luminal to basal transformation of luminal progenitors resulting in basal mammary cancers in mice (51).

Further studies (52) reported that the transcriptional repressor Slug that participates in EMT is abnormally expressed in *BRCA1*$^{mut/+}$ mammary tissue. Slug is required for promoting a basal-like phenotype in progenitor and cancer cells, making it a key effector of the gene expression program that may ultimately contribute to the transformed basaloid phenotype.

The Progesterone/RANKL Axis and Breast Cancer Risk in BRCA1-Deficient Mammary Luminal Progenitors

The PR antagonist, RU486 (mifepristone), has been shown to prevent mammary cancer in Brca1/Tp53–deficient mice (53). As noted above, breast cancers arising in this population typically belong to the ER/PR and HER2$^-$ basal subtype. Recent work by Nolan and colleagues (54) has shown that RANK$^+$ cells from *BRCA1*$^{mut/+}$ mammary glands are highly proliferative and have a molecular signature resembling basal breast cancer. Women treated with the RANKL inhibitor denosumab prior to biopsy showed reduced Ki67$^+$ cells. They also demonstrated that mammary outgrowths from transplanted *Brca1*$^{-/-}$;*Tp53*$^{+/-}$ mammary cells developed fewer tumors with longer latency than controls when mice were treated with denosumab. A contemporaneous study (55) showed similar results in *Brca1*$^{-/-}$ mice. Interestingly, the enriched luminal progenitor population from *BRCA1*$^{mut/+}$ carriers expressed a higher level of RANK$^+$ cells compared with normal controls, and benign hyperplastic mammary tissue from the *Brca1*$^{-/-}$;*Tp53*$^{+/-}$ mice also expressed high levels of RANK. Together, these two studies support the potential for RANKL inhibition as a viable new option for preventative treatment for women with *BRCA1* mutations.

Increased RANKL production by BRCA1-deficient PR$^+$ epithelial cells could coordinately increase proliferation *in vivo* to promote an expanded luminal progenitor population. However, increased RANKL in *BRCA1* deficiency has not been reported nor does this account for the ability of these cells to continue to proliferate and form colonies *in vitro* in the absence of progesterone. As discussed above, signals transduced by the DDR can activate NF-κB. Given that BRCA1 is a critical DNA repair protein and is involved in protection of the stalled replication fork, we investigated whether or not *BRCA1*-deficient cells demonstrate abnormal activation of NF-κB signaling (56). We assessed the activation of this pathway in mammary progenitors and found that p65/RelA is transiently activated by phosphorylation at ser536 following BRCA1 knockdown while p52/RelB is persistently activated in a subset of luminal progenitors with either homozygous or heterozygous deletion of *BRCA1*. Numerous lobular structures in sections from *BRCA1* mutation carriers showed high levels of p52 compared with normal mammary tissue. Knockdown of both p100/p52 and the inhibitor of kappaB kinase, IKKα, revealed that factor-independent *in vitro* proliferation required these NF-κB pathway constituents. We found that the DDR protein, ataxia telangiectasia-mutated (ATM), was phosphorylated accompanied by P-H2AX foci in human cell lines expressing siBRCA1 and mouse mammary progenitors deficient in Brca1. Activated ATM is required for NF-κB activation by the DDR (44, 57). The involvement of the DDR was further substantiated by demonstrating that hormone-independent luminal progenitor colony formation required ATM (56). In addition progesterone-stimulated proliferation resulted in a marked enhancement of DNA damage foci in *Brca1*$^{-/-}$ mouse mammary glands in our study as confirmed in subsequent studies (54, 55).

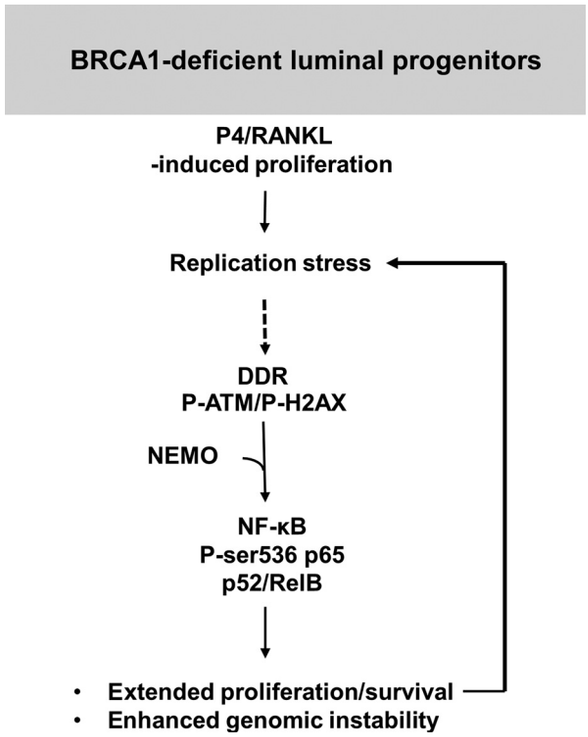

Figure 1.

BRCA1 deficiency leads to enhanced proliferation of RANK$^+$ cells: diagram showing the sequence of events following progesterone (P4)-induced RANKL-mediated stimulation of RANK$^+$ luminal progenitor cells. *BRCA1* deficiency increases replication fork stalling and reduces fork protection leading to a DDR to initiate repair. The DDR simultaneously activates NF-κB through an ATM/NEMO–dependent pathway, thus establishing a proliferation–DDR–NF-κB feed-forward cycle.

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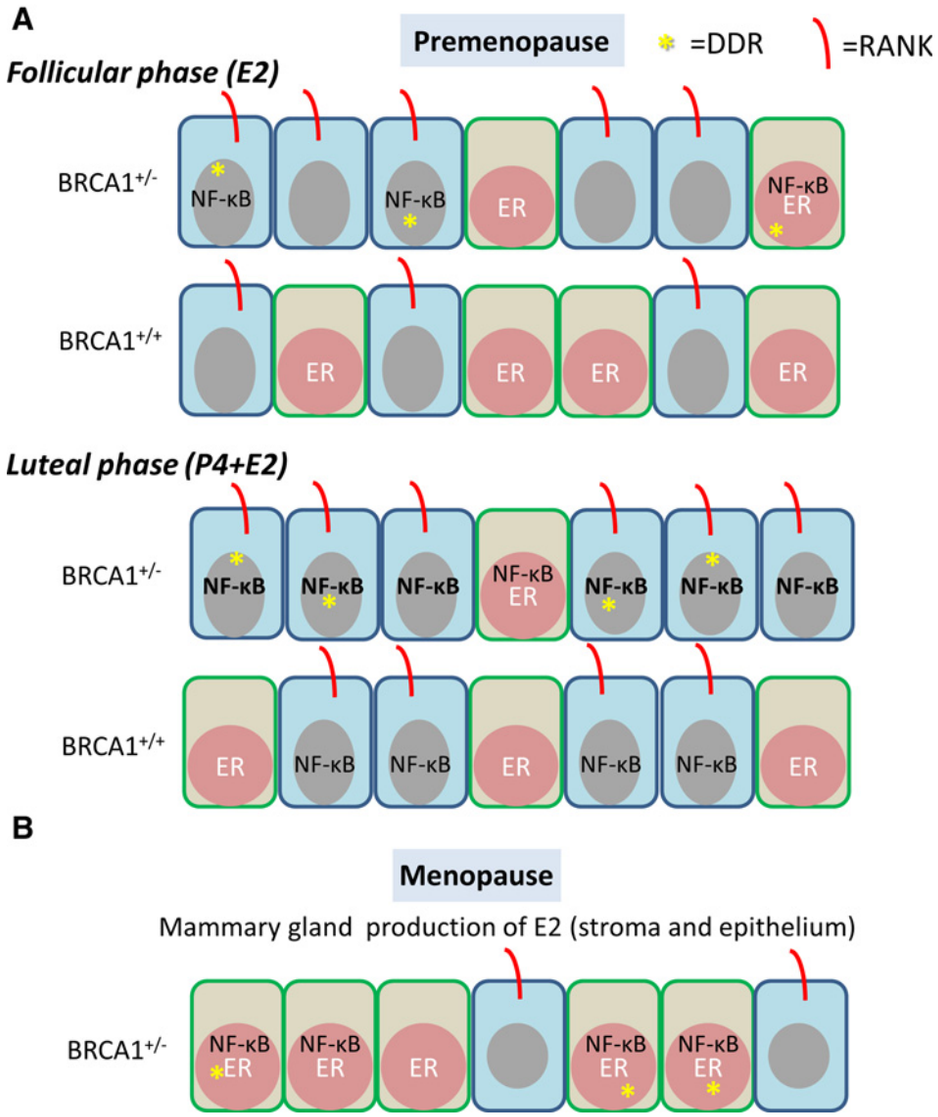

Figure 2.

Proposed effects of *BRCA1* deficiency on relative subsets of luminal progenitor cells in premenopausal and postmenopausal mammary tissue. **A,** In normal premenopausal women, E2 levels rise in the follicular phase and activate a subset of ER^+ cells to divide. *BRCA1* deficiency would increase the likelihood of a DDR in the ER^+ population and subsequent activation of NF-κB. However, ER^+ cells may have lower proliferative capacity than $RANK^+$ cells in premenopausal glands and represent a smaller percentage. In the luteal phase, progesterone (P4) stimulation of paracrine RANKL results in proliferation of $RANK^+$ cells, which leads to a DDR in *BRCA1*-deficient cells and enhanced activation of NF-κB (denoted in bold type), followed by further proliferation. This leads to an expansion of the $RANK^+$ population in the $BRCA1^{mut/+}$ gland, which persists beyond the cycle into the next as evidenced by the increased $RANK^+$:ER^+ ratio in the follicular phase. **B,** After menopause, the proportion of ER^+ cells increases in normal (not shown) and *BRCA1*-deficient mammary tissue, and proliferation is induced by E2, which may be higher than normal as a consequence of loss of BRCA1 repression of the aromatase gene (see text and Fig. 3 for details).

Treatment of MMTV-cre;$Brca1^{fl/fl}$ mice with a water soluble derivative of the natural product of the feverfew weed, parthenolide, called DMAPT (dimethyl-amino parthenolide), prevented the recovery of $Brca1^{-/-}$ hormone-independent colony-forming cells. DMAPT is an NF-κB inhibitor and a differential inducer of reactive oxygen species in cancer cells, but has little effect on normal cells (58, 59). Importantly, dosing of mice with DMAPT resulted in prolonged inhibition of factor-independent growth lasting over 4–5 estrus cycles. Consistent with both DDR-activated NF-κB and RANKL/RANK signaling *in vivo*, the majority of human $BRCA1^{mut/+}$ mammary glands showed marked lobular expression of nuclear p52 NF-κB. Thus, the aberrant hormone-independent proliferative capacity of *Brca1*-deficient luminal progenitor cells initially induced by the progesterone/RANKL axis is linked to replication-associated DDR, where proliferation of mammary progenitors is perpetuated by feed-forward damage-induced, autologous NF-κB signaling (Fig. 1).

A reduced serum level of the endogenous antagonist of RANKL, osteoprotegerin (OPG) has also been proposed as a mechanism underlying abnormal proliferation in the mammary glands of *BRCA1* mutation carriers. In support of OPG levels as a contributing risk factor, serum OPG from a limited number ($n = 18$) of *BRCA1/2* mutation carriers (60) and from the general population ($n = 78$; ref. 61) have been negatively correlated with breast cancer incidence. Low OPG levels have been associated with increased proliferation in primate mammary tissues (62). Both serum and mammary OPG levels were reduced in progesterone-treated animals in the latter study, suggesting that serum levels are representative of mammary tissue levels; however, serum levels in patients do not appear to vary with the menstrual cycle (62) indicating that cyclic

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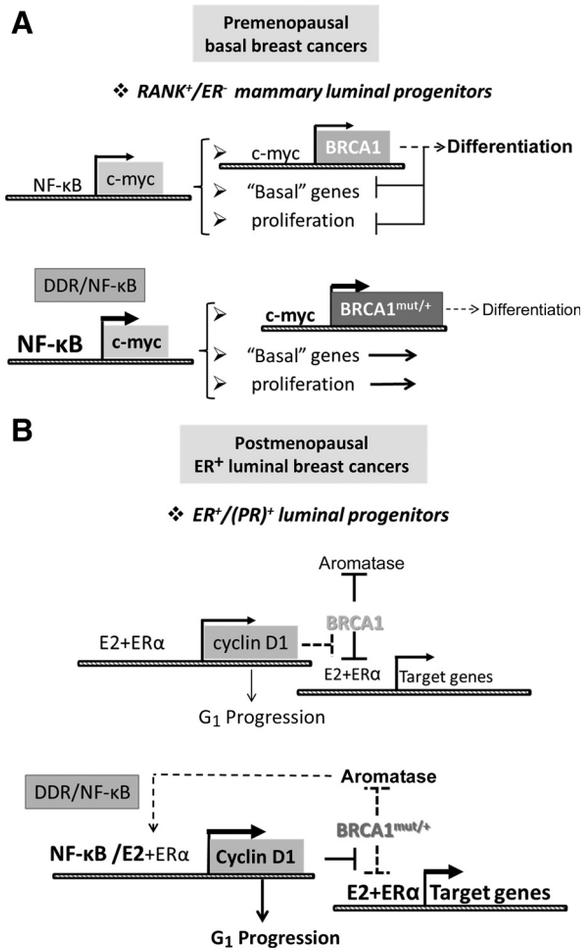

Figure 3.

Proposed model of oncogenic gene regulation by NF-κB in premenopausal and menopausal mammary epithelial cells. **A,** NF-κB induces transcription of c-myc, which induces proliferation and also activates the *BRCA1* promoter, which is important to facilitate differentiation by repressing basal genes and attenuating proliferation. In *BRCA1*-deficient cells the DDR-enhanced NF-κB activity (bold) would increase c-myc expression; however, mutations in *BRCA1* can render the protein unable to mediate its function in differentiation. **B,** After menopause, ER^+ cells can be induced to proliferate by E2 in part by the induction of cyclin D1. Cyclin D1 is also an NF-κB target gene, and the resulting DDR/NF-κB axis could further enhance this signaling. In addition, *BRCA1* deficiency may increase breast production of E2 as a consequence of increased aromatase expression. See text for details.

variation in progesterone levels in women is insufficient to alter OPG expression. Although it is not clear why OPG levels would be reduced in $BRCA1^{mut/+}$ patients, the significant inverse association between luteal progesterone level and serum levels of OPG in *BRCA1* carriers may be a function of the previously reported increased progesterone levels in these patients (63). However, complicating the correlation between low OPG levels and higher breast cancer risk is data showing that progesterone levels are lower in those carriers of mutations with a high hazard ratio, which would presumably reduce RANKL production and as well as mitigate any hormonal effects on OPG (62). Interestingly, a study of a large cohort (>2,000) of breast cancer cases has shown that higher serum concentrations of OPG were associated with increased incidence of ER^- breast cancers (64), which is the predominate histologic subtype that develops in *BRCA1* mutation carriers. Taken together, the interplay between OPG expression and hormone levels in *BRCA1* mutation carriers is likely to be complex and may be dependent on specific mutations in this gene.

On the basis of hormone levels, the different phases of the menstrual cycle and menopause involve proliferation of different subsets of mammary epithelial cells. During the follicular phase when β-estradiol (E2) levels are increasing, at least a subset of ER^+ cells may undergo proliferation, which would be expected to induce a DDR resulting in activation of NF-κB in $BRCA1^{mut/+}$ patients (Fig. 2A). Although in the normal gland, the ratio of $RANK^+$ to $RANK^-$ luminal progenitor cells is predicted to increase in the luteal phase and be reduced during the follicular phase of the menstrual cycle, this ratio appears to be greater in $BRCA1^{mut/+}$ mammary glands relative to normal glands in random cycling patients (56) presumably as a consequence of autologous NF-κB activity and prolonged proliferation. In ovariectomized or postmenopausal *BRCA1* mutation carriers, progesterone levels are dramatically lower (Fig. 2B). Low progesterone levels would presumably obviate hormonally stimulated RANKL production by PR^+ mammary epithelial cells and ultimately reduce the transformation of the $RANK^+$ population. Although this suggests that premenopausal progesterone is a significant risk factor of breast cancer in *BRCA1* mutation carriers, the benefit of oophorectomy is not clear with several studies showing no benefit while others demonstrate significant or marginal benefit (ref. 65 and references therein). Nevertheless, as described above, the progesterone-independent growth of $BRCA1^{mut/+}$ luminal progenitors *in vitro* indicates the presence of an intrinsic defect in these presumptive breast cancer precursors (49), which might account in part for the variable effectiveness of oophorectomy on risk reduction.

Interestingly, similar to the non–*BRCA1*-mutated breast cancers, postmenopausal *BRCA1* carriers tend to develop more ER^+ breast cancers (66, 67). The proportion of $ERα^+$ cells increases after menopause such that the majority of epithelial cells in some lobules express the ERα (68). Aromatase is the key enzyme involved in E2 synthesis by conversion of testosterone to E2 and indirectly by conversion of androstenedione to estrone, which is a substrate for 17β-hydroxysteroid dehydrogenase-mediated conversion to E2. Although systemic E2 and progesterone levels dramatically decrease after menopause, levels of E2 in breast tissue have been reported to increase as a consequence of increased activity of aromatase (69) predominantly in adipose cells (70), which increase as a proportion of the

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mammary stroma with age. Although low abundance in the premenopausal breast, ER^+ proliferating cells significantly increase with age (71–73). BRCA1 is an inhibitor of aromatase (*CYP19*) transcription (74, 75) and also interferes with transcriptional activation mediated by the ERα (76). Thus, BRCA1 deficiency may render at least some mutation carriers more likely to produce increased levels of estradiol in the postmenopausal breast, which would stimulate the ERα in responding cells with a reduced level of BRCA1-mediated transcriptional interference.

NF-κB Target Genes Are Oncogenes Expressed in Basal and Luminal Breast Cancers

Although NF-κB regulates many genes, two key target genes in the mammary gland that relate to both ductal and lobuloalveolar morphogenesis may play different roles in proliferation and tumorigenesis in the pre- and postmenopausal $BRCA1^{mut/+}$ mammary gland. The *c-MYC* gene is induced by NF-κB (7) and normally plays a role in both proliferation and differentiation as overexpression induces precocious lobuloalveolar development and lactation (77). As depicted in Fig. 3A, c-MYC also mediates the induction of the *BRCA1* gene (78), which is critical for normal differentiation of luminal progenitor cells into mature luminal cells. BRCA1 in turn attenuates the transforming function of c-MYC (79) and forms a complex with the c-MYC protein that represses genes associated with the basal phenotype (80). Therefore, it is possible that haploid *BRCA1* may be insufficient to mediate differentiation as a response to NF-κB–driven c-MYC expression, instead permitting further proliferation without differentiation. Indeed, c-MYC is overexpressed in both *BRCA1*-deficient premalignant mammary glands and tumors (81) and is a component of the basal mammary tumor signature (80). It is important to note that deleterious mutations may also alter the ability of BRCA1 to regulate gene expression necessary for normal differentiation of mammary luminal progenitor cells. NF-κB would contribute directly, simultaneously increasing proliferation and survival signaling within this cell population. Together, these factors contribute in different ways to the accumulation of genomically unstable luminal progenitors, ultimately leading to a high risk of breast cancer.

NF-κB can collaborate with the ERα in the induction of the cyclin D1 gene in the mammary gland (18, 82), which is critical for lobuloalveolar morphogenesis during pregnancy (83). Cyclin D1 also functions to block BRCA1-mediated ERα transcriptional repression (84). Interestingly, high expression of cyclin D1 is a feature of the ER^+ luminal breast cancers that develop in DNA repair–deficient *BRCA2* mutation carriers (85, 86). Therefore, by promoting cyclin D1 expression in BRCA1-deficient cells, autologous NF-κB activity activated by the DDR in the postmenopausal gland could contribute and synergize with E2 to promote proliferation in *BRCA1*-deficient ER^+ mammary epithelial cells (Fig. 3B). This activity would be exacerbated by the reduced levels of functional BRCA1, defective in the ability to repress ERα-mediated transcription.

Another potential protumorigenic consequence of a DDR-activated NF-κB response in the mammary gland is the induction of the cytokine IL6. NF-κB has been shown to activate the miRNA-processing factor Lin28 to reduce repression of *IL6* promoter by Let-7 (87). IL6 is expressed in breast cancers, and serum levels can be correlated with poor prognosis (88, 89). Importantly, IL6 also participates in a feed-forward inflammatory signaling loop, which promotes further NF-κB and JNK2 activation. JNK2 phosphorylation of heat shock transcription factor-1 (HSF1) facilitates epigenetic change at the IL6 promoter resulting in transactivation by NF-κB (90). The ensuing IL6 expression further activates NF-κB (87). Exposure of mammary stem cells to IL6 results in expansion of primary progenitor cells as assayed by the formation of secondary mammospheres and resistance to hypoxia (91). Notably, IL6 expression was found exclusively in basal breast cancers in the latter report. On the basis of this information, it is tempting to speculate that *BRCA1*-deficient mammary LPs, by virtue of DDR-activated NF-κB may establish an IL6 feedback loop that contributes to the perpetuation of proliferative signaling.

Perspective on the Breast as a Target of Transformation in *BRCA1/2* Mutation Carriers and Implications for Prevention

Although *BRCA1* mutation has been associated with pancreatic and colorectal cancers, the level of risk does not approach that of breast or ovarian cancers (92, 93). Deleterious *BRCA1* mutations resulting in a DDR perpetuate NF-κB signaling in $RANK^+$ mammary luminal progenitor cells, which are programmed to proliferate in response to NF-κB downstream of RANKL. This renders mammary luminal epithelial cells subject to the pathogenic proliferative activation by NF-κB. In contrast, in certain cells in a stimulus-dependent manner, NF-κB can promote cell death (94). Even within the mammary gland, the response to activation of NF-κB in luminal progenitors is the opposite to that of differentiated alveolar cells that undergo cell death during involution in response to the TNF-like weak inducer of cell death (TWEAK)-stimulated NF-κB (95). Thus, by promoting proliferation/DNA synthesis in BRCA1-deficient/mutation-prone progenitor cells, NF-κB functions as both a tumor initiator and promoter in the mammary gland.

Significant advances in identifying and characterizing the mammary gland cell hierarchy in the past decade have provided major insight into the origins and subtypes of breast cancers. We have shown that *BRCA1* mutation–associated breast cancers represent a subgroup of

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basal breast cancers whose inherent deficiency in DNA repair promotes not only genomic instability but also proliferative signaling that enhances the risk of transformation (56). In premenopausal women, the progesterone/RANKL/RANK axis initiates the proliferation/DNA damage cycle in $RANK^+$ cells resulting in basal breast cancers. In the postmenopausal breast, E2 produced in the breast parenchyma and stroma becomes the dominant hormone. E2-induced proliferation of ER^+ cells deficient for BRCA1 would also generate a DDR resulting in the activation of NF-κB, which in turn would augment or synergize with E2/ERα–mediated transcription enhancing the possibility for transformation in the ER^+ progenitor population. Collectively, the results described above inform potential new approaches to the management of *BRCA1* mutation carriers. Young, premenopausal patients wishing to postpone mastectomy might opt instead for chemoprevention using denosumab to block RANKL stimulation of progenitor proliferation. Alternatively, transient blockade of NF-κB during the luteal phase of the menstrual cycle might be employed to inhibit RANKL signaling as well as the ongoing propagation of NF-κB activity resulting from DNA damage. On the basis of the long-term effects on aberrant LPs in mice (57), DMAPT treatment might only be required at 2- to 3-month intervals. Such a regime would mitigate issues associated with the systemic suppression of NF-κB for extended periods. On the basis of the potential for a proliferation-associated DDR and subsequent NF-κB activation, DMAPT or another NF-κB inhibitor might also be effective as a prevention strategy in the postmenopausal setting. However, a switch from denosumab to a selective ER modifier, such as tamoxifen, may be more appropriate for chemoprevention in postmenopausal individuals, where E2 is likely to have the predominate proliferative influence on ER^+ cells. Going forward, evidence establishing IL6 as a downstream target of NF-κB in the aberrant proliferation in $BRCA1^{mut/+}$ glands could support the use of tocilizumab (a humanized anti-IL6 receptor antibody) as an alternative biologic agent (96).

Our finding that the DDR in *BRCA1* mutation carriers activates persistent NF-κB signaling may also have implications in high-risk individuals with mutations in other DNA repair genes, including *BRCA2* and *PALB2* (97), where replication stress-induced DNA damage might extend hormone-initiated proliferation and exacerbate genomic instability in DNA repair–deficient mammary progenitor cells. Germline *BRCA2* mutations are associated with an approximate breast cancer risk of 55% by age 70 (98). Lifetime risk associated with *PALB2* mutation has been estimated at 35% (99). Both proteins participate in the response to replication stress to facilitate fork recovery, participate in homologous recombination to prevent genomic instability (100). *BRCA2* mutation carriers more typically develop $ER^{+/}$ luminal B cancers (ref. 101 and refs therein), suggesting that they originate in a different subset of LPs than those primarily affected by *BRCA1* mutations. In the human gland, the FACS-enriched LP population can be further subdivided into $RANK^+$ and $RANK^-$ cells where basal breast cancers are thought to arise from the $RANK^+/ER^-$ subset of LPs (16). wt*BRCA1* alleles are expected to facilitate the maintenance of differentiated luminal cells (102). Importantly, the expression of BRCA1 is reduced in $RANK^-$ LPs relative to $RANK^+$ LPs (54). Thus, $ER^-/RANK^+$ LPs may differentiate normally in *BRCA2* mutation carriers. Cells originating from within the spectrum of $Sca1^+/RANK^-$ LPs that contains a subpopulation of ER^+ cells (16) may be the most probable candidates as a target population for transformation resulting in ER^+ luminal cancers in *BRCA2* mutation carriers (2).

Although luminal B breast cancers are ER^+, they are more aggressive than luminal A tumors and are often resistant to endocrine therapies (103). Many studies, including our own (104–106), have demonstrated antagonistic signaling between the ERα and NF-κB. Indeed, basal breast cancers lacking the ERα express higher levels of NF-κB compared with luminal cancers (32, 107). NF-κB activation in conjunction with the pioneering factor, FOXA1, stimulates binding of the ERα to a novel cistrome (108), which was associated with better prognosis in breast tumors. However, several laboratories have reported that estrogen and TNF signaling through NF-κB can synergize in promoting proliferation in breast cancer cells (109, 110). A subset of ERα-regulated genes and genes not normally regulated by estrogen alone that are associated with survival are induced by TNFα or estrogen and combinations thereof in MCF-7 breast cancer cells. Comparison with breast cancer signatures demonstrated that constituents of this gene set positively coregulated by the ERα and NF-κB are significantly enriched in the luminal B signature (111). Furthermore, the inhibitor of κB kinase-α (IKKα) and the ERα directly interact and knockdown of IKKα potently reduces the transcription of proliferation-associated genes including *CCD1* (cyclin D1) and *c-MYC* (112). Thus, NF-κB, induced in response to the DDR in BRCA2-deficient cells, could contribute to tumorigenesis in $ER\alpha^+$ cells through the cooperative transactivation of key proto-oncogenes and/or survival factors. Given the significant differences between the molecular phenotypes of the breast cancers that develop in *BRCA1* and *BRCA2* mutation carriers, prudent approaches to prevention for $BRCA2^{mut/+}$ patients would include estrogen receptor–targeted agents such as tamoxifen as an inhibitor of the primary stimulus for proliferation of ER^+ LPs. DDR-activated NF-κB might also be considered as an intermittent target to prevent extended proliferation and block cooperative gene transactivation between the ERα and NF-κB or IKKα.

The Fanconi anemia protein PALB2 serves as a link between BRCA1 and BRCA2 during HDR at DNA breaks (113, 114) and is emerging as clinically relevant familial breast cancer gene. BRCA2 interacts with the C-terminus of

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PALB2 (115), while association with BRCA1 occurs in the N-terminal regions of PALB2 (113, 114). Overall about 30% to 40% of *PALB2*-mutated tumors are triple negative, while 60% to 70% are ER^+ (116, 117). Interestingly, a familial *PALB2* mutation that prevents BRCA1 interaction is associated with ER^+ breast cancers (118). The nature of the mutation in *PALB2* could therefore determine whether the ultimate deficiency in replication fork stabilization or HDR resembles a *BRCA1* or a *BRCA2* mutation phenotype. From the standpoint of hormone-based prevention, a lack of information on the specific breast cancer subtypes that develop across a broader range of *PALB2* mutation carrier families makes rational decisions regarding the targeting the progesterone/RANKL axis versus the ERα for prevention more challenging. However, basing chemoprevention on the breast cancer diagnosis and use of endocrine therapy to treat previous disease in first-degree relatives when available might be a reasonable strategy. It is feasible that breast cancer–associated mutations in *PALB2* would also elicit a DDR resulting in NF-κB activation and affording abnormal proliferative capacity to the affected subpopulation(s) of LPs. NF-κB may then be a viable target in these individuals that ostensibly would be independent of the nature of the mutation.

As with any intervention, neither hormonal nor agents targeting NF-κB signaling pathways are without potential side effects. Therefore, the estimated risk-to-benefit ratio must be taken into account and that associated with mutations in *PALB2*, for example, is considerably less than that for *BRCA1* or *BRCA2* mutations. Nevertheless, family histories might provide a more individual risk profile that could enter into decision making regarding prophylactic therapy. Although long-term prevention clinical trials are difficult and expensive, the high risk associated with *BRCA1/2* mutations makes this population a strong candidate for chemoprevention and patient compliance. As mastectomy is the only current form of primary prevention, window of opportunity trials in the months before scheduled surgery or core biopsy (the latter as described for preliminary assessments of denosumab; ref. 54) could provide a unique opportunity for assessing intermediate endpoints such as a reduced proliferative index on noncancerous breast sections. Application of mechanism-based primary prevention strategies in these high-risk populations ultimately has the potential to avoid the often intractable complexities of treating cancer.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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