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NF κ B Affects Estrogen Receptor Expression and Activity in Breast Cancer through Multiple Mechanisms

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Abstract

Estrogen receptor (ER) and NF κ B are two widely expressed, pleiotropic transcription factors that have been shown to interact and affect one another's activity. While the ability of ER to repress NF κ B activity has been extensively studied and is thought to underlie the anti-inflammatory activity of estrogens, how NF κ B signaling affects ER activity is less clear. This is a particularly important question in breast cancer since activation of NF κ B in ER positive tumors is associated with failure of endocrine and chemotherapies. In this review, we provide an update on the multiple mechanisms by which NF κ B can influence ER activity, including down-regulation of ER expression, enhanced ER recruitment to DNA, and increased transcriptional activity of both liganded and unliganded ER. Additionally, a novel example of NF κ B potentiation of ER-dependent gene repression is reviewed. Together, these mechanisms can alter response to endocrine therapies and may underlie the poor outcome for women with ER positive tumors that have active NF κ B signaling.

Keywords

Breast cancer; estrogen receptor; NF κ B; tamoxifen

Introduction

In the US, breast cancer will affect one in eight women and is second only to lung cancer in cancer-related deaths. Nearly 75% of breast tumors express estrogen receptor α (ER), which remains the most important prognostic factor in breast cancer. ER positive tumors are hormone-dependent and likely to respond to endocrine therapies, such as aromatase inhibitors or ER-targeted therapies, including the selective estrogen receptor modulator tamoxifen or the selective estrogen receptor down-regulator fulvestrant. Endocrine therapy

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is effective at treating both early stage and advanced stage breast cancers, and at preventing breast cancer incidence in women who are at high risk of developing the disease^{1,2}. Despite the demonstrated effectiveness of endocrine therapy drugs, it is estimated that about 50% of ER+ tumors will fail endocrine treatment as a result of de novo or acquired resistance, and will eventually recur³, often as metastatic tumors, which are the main cause of mortality in breast cancer patients (http://cdmrp.army.mil/bcrp/pdfs/bc_landscape13.pdf). Metastatic recurrence in ER+ breast cancer patients can occur years to decades after the primary diagnosis and these recurrent tumors retain ER expression. In fact, greater than 80% of the lymph node metastases and 65–70% of distant metastases arising from ER+ primary tumors retain ER expression^{4,5}. More recently, ER mutations have been identified in hormone-resistant and metastatic breast tumors and these mutations contribute to increased activity and reduced responsiveness to endocrine therapy^{6–10}. Thus, ER remains a central player in progression to advanced stage, therapy resistant, and metastatic disease.

Elucidating the molecular factors and pathways that modulate ER function remains a critical objective in effectively targeting ER in recurrent/resistant breast cancers and thereby reducing breast cancer mortality. Extensive studies have concluded that endocrine therapy failure can be attributed to the following mechanisms: (i) ER expression loss¹¹, (ii) ER crosstalk with other growth factor signaling pathways^{12,13}, (iii) co-activator/co-repressor availability for ER complex formation and DNA accessibility¹⁴, (iv) kinase pathways that phosphorylate ER and other ER-accessory proteins to modify their activity^{15,16}, and (v) non-genomic membrane-initiated ER signaling^{17,18}.

NFκB Activity in ER+ Breast Cancers

One ubiquitous pathway that can influence many, if not all, of the above mechanisms is the nuclear factor κB (NFκB) inflammatory pathway. Studies have shown that a deregulated, or constitutively active NFκB pathway is associated with hormone-independence, and both chemo and endocrine therapy failure^{19–23}. Progression of rat mammary carcinoma cells from an ER+ nonmalignant phenotype to an ER– malignant phenotype was shown to be accompanied by constitutive activation of the NFκB pathway¹⁹. Nuclear expression of NFκB was linked to resistance to neoadjuvant chemotherapy in breast cancer patients with ER+ tumors^{21,23}. More specifically relating to endocrine therapy, studies have shown that: (i) overexpression of an NFκB family member induced aromatase inhibitor resistance in cell lines and its expression was increased in recurrent aromatase inhibitor-resistant breast tumors, as compared to the primary tumors before treatment²², (ii) significantly higher basal NFκB activity and expression were detected in raloxifene resistant cells²⁴, and (iii) high DNA binding activity of NFκB subunits identifies a high-risk subset of ER-positive primary breast cancers destined for early relapse despite adjuvant endocrine therapy with tamoxifen²⁰. Furthermore, studies have shown that repressing NFκB activity can restore sensitivity to ER antagonists in cell-based models of resistance^{25–27}. Together, these findings support a critical role for the NFκB pathway in the development of hormone-independent and/or endocrine resistant ER+ breast tumors.

The NFκB family consists of five members, namely, p65 (RelA), RelB, c-Rel, p50, and p52, which form homo- and heterodimers to activate transcription of target genes regulating host

inflammatory and immune responses, as well as cellular growth and survival. The canonical NF κ B pathway consists of p65 and p50 transcription factors, which are held in the cytoplasm by an inhibitor protein, I κ B α . Upon stimulation by inflammatory cytokines, such as TNF α , IL-1 β , or other factors, the I κ B kinase (IKK) complex, consisting of IKK α , IKK β and the scaffolding protein NEMO, is activated. This leads to phosphorylation and proteasomal degradation of I κ B α . As a result, p65/p50 factors are liberated and can translocate to the nucleus, where they bind the NF κ B response elements (NF κ B-RE). Phosphorylation of p65 by IKKs and other kinases at various sites, including serines S486 and S536, has been shown to be important for its transcriptional activity²⁸. The alternative, non-canonical pathway involves membrane-initiated signaling, which activates IKK α , followed by subsequent phosphorylation and processing of p100 into p52, which then leads to nuclear translocation of the p52-RelB heterodimer.

Within the breast tumor microenvironment, activation of the NF κ B pathway is both a cause and effect of local inflammation. Inflammation as an instigator and driver of aggressive ER+ breast cancer phenotypes has been reviewed elsewhere^{29,30}. Briefly, activation of NF κ B transcription factors drives breast cancer aggressiveness by promoting tumor cell survival, proliferation, migration, invasion, angiogenesis, cancer stem cell properties, and resistance to therapy^{31,32}. Hence, understanding how NF κ B specifically affects ER activity and function may provide the tools to overcome endocrine resistance in patients.

Studies on the interaction between ER and NF κ B in breast cancer cells led to the paradigm that ER represses NF κ B activity, which is one of the proposed mechanisms underlying estrogen's anti-inflammatory activity in premenopausal women^{33,34}. The molecular mechanisms by which this occurs have been thoroughly reviewed previously³⁵. However, work from our laboratory has shown that ER and NF κ B also extensively work together to synergistically up-regulate genes associated with luminal B breast tumors and poor response to endocrine therapy³⁶, suggesting that other mechanisms may be involved in progression of ER+ disease. In the following sections, an update on the molecular mechanisms by which the NF κ B pathway affects ER expression and functional activity is provided and the clinical implications of these mechanisms in aggressive ER+ breast cancer are discussed.

NF κ B Represses ER Expression

Generally, there is an inverse correlation between the expression and activation profiles of ER and NF κ B in breast cancer tumors and cell lines^{19,37,38}. This inverse relationship has been attributed to the several mechanisms by which NF κ B can repress ER expression. The first mechanism was proposed by Belguise and Sonenshein who have shown that the serine/threonine protein kinase C θ (PKC θ) represses the levels of ER in breast cancer cells³⁹. PKC θ , which induces NF κ B activity and regulates c-Rel-driven mammary tumors in mice, also activates AKT, which in turn phosphorylates the transcription factor FoxO3a to cause its export from the nucleus. FoxO3a has been shown to bind to two upstream forkhead elements in the ER promoter and to activate transcription of the gene⁴⁰. Thus, PKC θ can inhibit ER expression through inactivation of FoxO3a.

The second mechanism of ER repression was proposed by Wang and colleagues, who have shown that the NF κ B subunit RelB inhibits ER expression in breast cancer cells⁴¹. The levels of RelB are inversely correlated with the status of ER in breast cancer cells and the transactivation of RelB promoter by NF κ B and AP-1 is reported in ER negative but not ER positive breast cancer cells. RelB stimulates the expression of the zinc finger repressor B-lymphocyte-induced maturation protein (BLIMP1), a process mediated by the interaction of the Rel B target Bcl2 with Ras in the mitochondrial membrane. BLIMP1, in turn, can bind to the ER promoter and repress ER expression⁴².

A third mechanism involving the Enhancer of Zeste Homolog2 (EZH2) has been recently suggested³⁰. EZH2 is a core member of the polycomb repressor complex 2 which induces the di and tri-methylation of histone 3 residue 27 (H3K27) and hence represses gene expression⁴³. An inverse correlation between EZH2 and ER is observed in primary breast tumors. Silencing EZH2 in MCF-7 cells significantly enhances ER expression⁴⁴. It has been reported that the cytokine TNF α activates the expression of EZH2 in an NF κ B dependent manner⁴⁵, leading to the possibility that NF κ B inhibits ER expression via EZH2³⁰.

Since a linear correlation between ER levels and the risk of recurrence has been reported⁴⁶, the ability of NF κ B to repress ER may be clinically relevant. As noted previously, constitutive NF κ B activity has been observed in a subset of ER+ tumors that respond poorly to tamoxifen, aromatase inhibitors, and chemotherapy and is associated with shortened disease-free survival and increased metastatic recurrence^{19–22}. Similarly, the luminal B subtype of ER+ breast cancers is less responsive to endocrine therapy and is associated with late recurrence, metastasis, and reduced patient survival^{47,48}. Studies have demonstrated that Luminal B tumors exhibit lower expression of ER and ER-related genes compared with luminal A tumors^{47,49–51}, thus it is possible that NF κ B may contribute to the luminal B subtype of breast cancers by repressing ER expression and subsequent activity.

NF κ B Influences ER Interaction with DNA

ER is a ligand-dependent transcription factor that regulates gene expression through interaction with genomic DNA motifs known as the estrogen response elements (ERES), half-EREs, or through protein-protein interactions with other transcription factors including SP1⁵², AP1⁵³, Pitx1⁵⁴, and Runx1⁵⁵. ER genomic binding requires the recruitment of pioneering factors, including FOXA1, which interacts with ER binding sites prior to cell activation to create an epigenetic landscape conducive for ER binding and transcriptional activation^{56–58}. Interference with FOXA1 expression levels, alters the genomic recruitment of the ER to regulatory sites, and modulates the transcriptional potential of ER in breast cancer cells and defines how breast cancer tumors respond to endocrine-directed therapies. Interestingly, ER has been shown to interact cooperatively with p65, a member of the NF κ B family, and proteomic studies have identified the protein complex of ER and p65 on DNA, suggesting a potential pioneering function for NF κ B. Electrophoretic mobility shift assays demonstrate the ER can supershift an oligonucleotide containing just NF κ B motifs, while p65 supershifts an oligonucleotide containing just an ERE, further demonstrating that ER and p65 exist in a protein complex on DNA⁵⁹.

While the full impact of ER and p65 interaction remains to be elucidated through genome-wide studies, the existing experimental data suggests that the NF κ B pathway modulates the recruitment of ER to regulatory sites. For example, NF κ B activation greatly increases ER recruitment to an ERE upstream of the PTGES gene⁶⁰ and to BIRC3 and ABGC2 promoters, both of which contain an ERE adjacent to one or more NF κ B motifs^{61,62}. This enhancement of ER occupancy is accompanied by recruitment of p65 and an increase in the acetylation on histones H3 and H4, which correlates with enhancement of mRNA production by NF κ B signaling. Pharmacological inhibition of the NF κ B pathways blocks the cytokine-dependent recruitment of p65 and the enhancement of ER recruitment. Mutational analysis of either the ERE or NF κ B motif demonstrates a requirement for each motif in the regulation of the BIRC3 and ABGC2 promoters by E2 or NF κ B signaling, respectively, and mutation in either eliminates the cooperative crosstalk between the two pathways. Therefore, activation of both pathways seems to stabilize an ER/p65 complex on target gene promoters, which results in synergistic mRNA transcription.

NF κ B Affects ER Transcriptional Activity

Activation of the NF κ B pathway has been shown to modulate the transcriptional potential of the estrogen receptor in both in vivo and in vitro experimental models. Genome-wide expression studies in human breast cancer cells have shown that while inflammatory cytokines repress the ability of ER to activate a subset of target genes, the majority of the E2 regulated genes affected by NF κ B activation are in fact increased³⁶. This observation is most likely a result of enhanced recruitment of ER/p65 and associated coactivators to the promoter and regulatory regions of target genes.

In addition, ER can also be activated upon cytokine-induced NF κ B signaling in the absence of E2. The inflammatory cytokines IL-1 β and TNF α activate the transcriptional potential of the ER in cell based models^{59,63,64}, albeit with less potency than E2 signaling. For example, TNF α treatment of endothelial cells results in ERK 1/2-dependent phosphorylation of S118 in the AF1 region of ER, which is required to activate an ERE-dependent reporter⁶³. TNF α treatment also induces expression of classic ER-dependent target genes in Ishikawa cells, which is abrogated by knockdown of ER or alanine mutation of S118. ER phosphorylated at S118 is detected at the promoters of estrogen-regulated genes⁶⁵. S118 phosphorylation enhances the sensitivity of the receptor to estradiol and increases binding of coactivators, such as SRC3⁶⁶ and CBP/p300⁶⁷. In contrast, S118 phosphorylation can also decrease the affinity of ER to tamoxifen and reduce the binding of tamoxifen bound receptor to DNA⁶⁸. Therefore, the potential for ER activation via cytokine signaling may contribute to endocrine therapy failure via two distinct mechanisms, i) by preventing the repressive effects of tamoxifen and ii) activating the unliganded receptor in women taking aromatase inhibitors.

In addition to ERK-induced S118 phosphorylation, the non-canonical NF κ B pathway kinase, IKK α , can also influence estrogen-mediated transcription through S118 phosphorylation in breast cancer cells. E2 treatment leads to the recruitment of IKK α , ER, and the coactivator AIB1/SRC-3 to the promoters of ER target genes where transcriptionally active complexes are formed⁶⁹. Phosphorylation of histone H3 with the subsequent acetylation of H3 and H4 and phosphorylation of RNA polymerase II are observed and

IKK α kinase activity is important for these events. siIKK α inhibited E2 mediated ER S118 phosphorylation and reduced E2 mediated enhancement of the coactivator AIB1/SRC-3 phosphorylation at serine 857. The biological relevance of this finding was manifested when IKK α proved to be critical for E2 mediated proliferation of breast cancer cells. In fact, the major influence of IKK α on estrogen-stimulated cell cycle progression is through the regulation of cell cycle genes, including cyclin D1, E2F1, and E2F1 target genes^{69,70}.

In addition to ER phosphorylation, inflammatory signaling by IL-1 β can override the ability of tamoxifen to repress E2-dependent transcription⁷¹. Upon binding to ER, tamoxifen induces a protein conformation that allows ER to interact with corepressors, such as NCoR1 (Nuclear Receptor Corepressor 1), which leads to the formation of a repressive complex containing histone deacetylases and other epigenetic regulators⁷². As a consequence, histones associated with ER target genes are deacetylated and transcription of these target genes is repressed⁷³. IL-1 β treatment results in dismissal of the NCoR1 complex from ER target promoters and this dismissal is the proposed mechanism by which pro-inflammatory cytokines disable the repressive capacity of tamoxifen⁷¹. It remains to be discovered whether IL-1 β alters the binding affinity of tamoxifen to the ER.

Post-transcriptional Activity

MicroRNAs (miRs) are small non-coding RNAs that function as post-transcriptional negative regulators of their mRNA targets. Deregulation of many of these miRs has been associated with tumorigenesis in various cancers. Since the first report in 2005 of miR deregulation in breast cancer, numerous studies have established miRs as important mediators of breast cancer initiation, resistance to therapy, progression and metastasis^{74,75}. We have recently shown that ER and NF κ B work together to repress expression of two miR-181 family members⁷⁶. miR-181a and b are arranged in a bicistronic fashion within an intron of the miR-181A1 host gene (HG). Treatment of breast cancer cells with E2 and TNF α led to a more rapid and robust down-regulation of miR-181A1 HG expression, as well as mature miR-181a1 and b1 levels, when compared to either E2 or TNF α alone. This down-regulation was shown to be both ER and NF κ B-dependent and represents the first example of cooperative crosstalk between ER and NF κ B resulting in gene repression. Beyond a few well-established molecular mechanisms of positive crosstalk highlighted above, the molecular mechanisms of synergistic repression remain elusive, illustrating the complex multi-faceted nature of the ER-NF κ B interaction.

One important consequence of miR-181 down-regulation by ER and NF κ B is that it represents an amplification loop by which ER and NF κ B can further up-regulate expression of their target genes. We have shown that PHLDA1 is up-regulated at both the transcriptional and post-transcriptional levels by ER and NF κ B and that PHLDA1 is a direct target of miR-181a and b in breast cancer cells⁷⁶. Thus, by down-regulating miR-181, ER and NF κ B's effects on PHLDA1 are further enhanced. Interestingly, a number of other synergistically up-regulated ER-NF κ B crosstalk genes are also either direct or putative targets of miR-181³⁶. The functional consequences of this amplification loop remain to be fully explored, but initial studies indicate a potential role in promoting the cancer stem-like cell properties of anchorage independent growth and self-renewal⁷⁶. Given the correlation

between cancer stem-like cells and therapy resistance, the ER-NF κ B-miR-181 gene regulatory axis may also have important clinical implications.

Together, this extensive body of work suggests that ER and NF κ B work together in a concerted manner to coordinate gene expression through a wide variety of direct transcriptional mechanisms, as well as indirect post-transcriptional mechanisms. This complex interplay attests to clinical importance and therapeutic potential of these two pleiotropic transcription factors in breast cancer.

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Abbreviations

ABCG2	ATP-binding cassette sub-family G member 2
BIRC3	baculoviral IAP Repeat Containing 3
BLIMP1	B-lymphocyte-induced maturation protein
E2	17 β estradiol
ER	estrogen receptor
ERE	estrogen response element
EZH2	enhancer of zeste homolog 2
FoxA1	forkhead box A1
FoxO3a	forkhead box O3
NCoR1	nuclear receptor corepressor 1
NFκB-RE	NF κ B response element
NFκB	nuclear factor κ B
PKCθ	protein kinase C θ
PTGES	prostaglandin E synthase

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Highlights

- NF κ B signaling down-regulates ER expression
- NF κ B and ER bind cooperatively to regulatory regions to increase transcription
- NF κ B affects ER activity through phosphorylation and ligand-independent activation
- NF κ B can potentiate ER-dependent gene repression