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TITLE: Promotion of Tumor-Initiating Cells in Primary and Recurrent Breast Tumors

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**Title:** Promotion of Tumor-Initiating Cells in Primary and Recurrent Breast Tumors

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**Abstract:**
Breast tumors, like other solid tumors, contain a subset of cells known as tumor initiating cells (TICs). TICs are important in tumor initiation and recurrence, metastasis, and are resistant to radiation and chemotherapy. Our data and work published by others indicate that the transcription factor NF-κB is likely to be important in the generation and maintenance of breast TICs. A major problem in breast cancer therapy is the issue of residual disease where therapy-resistant cancer cells remain dormant only to drive tumor recurrence years later. Here we propose to explore mechanisms whereby NF-κB is activated in breast cancer TICs and recurrent experimental tumors and the roles that IKK/NF-κB play in this process. Our hypothesis is that the IKK/NF-κB pathway is essential for the development and/or maintenance of breast cancer TICs, potentially through the promotion of EMT and the regulation of expression of genes which confer stemness. We hypothesize that inhibition of IKK/NF-κB will reduce or eliminate breast cancer TICs, blocking tumorigenesis. Furthermore, we hypothesize that the activation of NF-κB is an important component in the generation of recurrent diseases derived from residual disease. The Aims are to: (i) Explore the mechanistic roles for IKK/NF-κB in promoting basal-like and Her2+ tumor initiating cells. (ii) Test IKK inhibitors and mTOR inhibitors for effects on tumor growth and TIC phenotypes, (iii) Determine the requirement for the NF-κB, TGFβ and mTOR pathways in promoting the survival and recurrence of residual cancer cells.
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**Attachments:**
- Manuscript: Kendellen et al (in review, Breast Cancer Research)
- Abstract: DOD Era of Hope 2011
INTRODUCTION:

Tumor initiating cells (TICs) are thought to drive the growth and metastatic properties of a tumor (Korkaya et al., 2011). The main goal of this proposal was to determine if the IKK/NF-κB pathway is essential for the maintenance/expansion of basal-like breast cancer TICs and, if so, to determine the signaling pathways that promote this activity. This breast tumor subtype represents a therapeutically difficult cancer (Brenton et al., 2005; Sorlie et al., 2003; Hu et al., 2006; Carey et al., 2006). Experiments have utilized cell-based and animal tumor models. Additionally, we assessed the biological/signaling mechanisms that underlie IKK/NF-κB activity (Hayden and Ghosh, 2004; Karin, 2006; Basseres and Baldwin, 2006) to promote the TIC phenotype. Previously others showed that NF-κB is activated in basal-like breast cancers (Yamaguchi et al., 2009). Currently we have shown that NF-κB is highly active in TICs isolated from different basal-like/triple-negative cancer cell lines. Inhibition of NF-κB (through genetic and drug-based approaches) demonstrates that IKK/NF-κB is required for self-renewal of breast cancer TICs. Importantly, inhibition of NF-κB strongly blocked xenograft tumor formation, consistent with an inhibition of TIC function. Interestingly, we have found that both canonical and non-canonical NF-κB signaling contribute to breast cancer TIC maintenance/expansion. Finally, we showed that certain inflammatory cytokines whose genes are regulated by NF-κB (IL-6, and IL-1) promote the expansion of TICs. These findings have been published (Kendellen et al., Oncogene 2014). We have now extended this studies to include a regulatory link with EZH2, a histone methyltransferase linked with cancer. The completion of our proposed aims (Aims 1 and 2) may lead to new therapeutic options for basal-like breast cancer as well as a better understanding of causative mechanisms in oncogenesis and therapy resistance for this disease.

Residual breast cancer cells have the ability to survive following treatment and linger unrecognized in a presumed dormant state for up to 20 years before re-emerging as recurrent disease. Since recurrent breast cancer is typically fatal, the propensity of residual breast cancer cells to survive therapy and recur is one of the most important determinants of clinical outcome. We (Chodosh --- collaborator) have found that recurrent breast cancers that arise in genetically engineered mouse models frequently undergo EMT. Moreover, we have demonstrated that the EMT transcription factor, Snail, is markedly upregulated in recurrent mammary tumors, and that forced expression of Snail in primary tumors is sufficient to promote mammary tumor recurrence. We have also obtained evidence indicating that the NF-κB and TGFβ pathways are upregulated in recurrent mammary tumors. In aggregate, our findings suggest that the NF-κB and TGFβ pathways may contribute to the reservoir of residual cancer cells that give rise to recurrent breast cancers. The goals of Aim 3 of this proposal are to determine if the NF-κB, TGFβ and mTORC1 pathways are essential for the survival and recurrence of residual cancer cells from TICs.

KEYWORDS: breast cancer, tumor initiating cells, cancer recurrence, epithelial mesenchymal transition, NF-kappaB, IKK (IkappaB kinase), tumor xenografts, tumor cell self-renewal, EZH2

PROJECT SUMMARY:

Aim 1 (Directed by Dr. Baldwin).

---Explore the involvement of NF-κB in promoting epithelial-mesenchymal transition as a regulatory mechanism in promoting TIC phenotype. Experimental approaches: inhibition of IKK/NF-κB in bulk cultures and CD44+ TICs, immunoblotting of breast cancer TICs for markers of EMT.

---Determine signaling pathways, important to breast cancer TICs, that promote the EMT phenotype and the upregulation of NF-κB. Experimental approaches: isolation of CD44+ cells, siRNA knockdown experiments, co-immunoprecipitation experiments related to TAK1/TAB/IKK interaction, immunoblotting for EMT markers.
---Characterize the NF-κB-dependent gene expression profile in breast cancer cell TICs. Determine if NF-κB promotes chemoresistance of TICs. Experimental approaches: RNA isolation from bulk cultures and CD44+ and CD44- cells (some of which will be inhibited for IKK/NF-κB), RT-PCR analysis to measure candidate RNAs, treatment with doxorubicin, cell death assays, tumorsphere assays. Timeline:
---Analyze the involvement of cytokines/growth factors that promote the TIC phenotype in an NF-κB-dependent manner. Experimental approaches: cytokine inhibition (neutralizing antibodies, receptor inhibitors), CD44+ cell isolation, immunoblotting, RNA analysis, tumorsphere assays.
---Explore the involvement of mTOR and Stat3 in promoting TIC phenotypes, dependent on IKK and NF-κB-dependent mechanisms. Experimental approaches: CD44+ isolation from breast cancer cells, inhibitor studies, immunoblotting, tumorsphere assays.

Aim 2 (Directed by Dr. Baldwin)
---Analyze the ability of IKK (and potentially other inhibitors) to block growth of tumor xenografts derived from basal-like and Her2+ breast cancer cells, focused on effects of inhibitors on TICs. Experimental approaches: establish breast cancer cell xenograft tumors (derived from 3 breast cancer cell lines), treat xenograft tumors (10 control and 10 experimental tumors each year) with inhibitors (IKK inhibitor and potentially others derived from work in Aim 1), determine if tumor growth is inhibited potentially through induction of cell death, determine if TICs are targeted by the inhibitor as measured through TIC markers including CD44+ cells, EMT markers, and angiogenesis.
---Characterize in situ models of basal-like and Her2+ relative to responses to inhibitors and effects on TICs. Experimental approaches: C3Tag and Her2+ transgenic animal genotyping [animals are maintained in the Lineberger tumor models core facility], analyze 10 control and 10 treated animals with inhibitors described above, monitor tumor growth over two weeks of treatment with a focus on cell death analysis, effects on TICs, angiogenesis, and cell proliferation. As described above, if another inhibitor shows significant effects in Aim 1, this inhibitor will be pursued in this aim.

Statement of Work/Aim 3 (Work performed by Dr. Chodosh -- collaborator/W81XWH-12-10-0177 -Univ. Pennsylvania)
--- Using conditional transgenic mouse models for Her2/neu-driven breast cancers, characterize TGFβ and NF-κB-related signaling pathways in residual cancer cells and recurrent breast cancers. Experimental approaches: immunoblotting and immunofluorescence for markers of EMT, NF-κB activity, Akt activity, mTORC1 activity, Stat3, and IL-6 expression in primary tumors, recurrent tumors, and residual disease. We are collaborating with Dr. Chodosh on these goals.

KEY RESEARCH ACCOMPLISHMENTS:

>>Regarding Aim 1

(Pt1) Explore the involvement of NF-κB in promoting epithelial-mesenchymal transition as a regulatory mechanism in promoting TIC phenotype. Progress: We have published (see Kendellen et al., Oncogene 2014) that both canonical and non-canonical NF-κB promote the breast cancer TIC phenotype. In Figure 5a of Kendellen (see attached) we showed that blocking NF-κB expression suppressed the EMT marker vimentin and blocking NF-κB suppressed the ability of TGFβ (known to induce EMT) to induce self-renewal (Figure 5b – Kendellen et al, attached). We showed (see that blocking canonical NF-κB (using the super-repressor form of IκBκ) strongly blocked xenograft tumor growth (using a limiting dilution experiment), which is consistent with the role of NF-κB in TIC function.

(Pt2) Determine signaling pathways, important to breast cancer TICs, that promote the EMT phenotype and the upregulation of NF-κB. Progress: As described above, Kendellen et al (see attached) showed that both non-canonical and canonical NF-κB activation drives the TIC phenotype of breast cancer
cells. For example, see Figures 2 and 3 in that publication. Figure 4f (in that publication) shows that TAK1 activity (an upstream marker of IKK activity in the canonical is active in the CD44+ subset of TIC cells. Figure 4d shows that non-canonical NF-κB activity, as measured in processing of p100 to p52 is higher in CD44+ cells.

In new work for this subaim, we have shown that the EZH2 pathway is important for breast cancer TICs. In Figure 1 (below), EZH2 knockdown and EZH2 inhibitor blocks tumorsphere/self-renewal of breast cancer cells. EZH2 knockdown was approximately 80% effective (not shown). The work used two breast cancer cell lines (SUM-149 and MDA-MB-231). It was shown that EZH2 promotes breast cancer TICs (Chang et al., 2011) and that EZH2 interacts with RelA and RelB to promote gene expression in breast cancer cells (Lee et al., 2011). We have confirmed these results. We now have found that NF-κB drives the expression of EZH2 (see figure 2 below left) – thus EZH2 may function in the TIC phenotype downstream of canonical NF-κB.

In figure 3 shown immediately below, knockdown of EZH2 blocks expression of RelB in basal-like and claudin-low breast cancer cell lines. This result places EZH2 upstream of RelB expression. Inducible shRNA blocks tumor growth. Thus a role for EZH2 in promoting breast cancer TIC phenotype can be partly explain by the control of RelB expression.

(Pt3) Characterize the NF-κB-dependent gene expression profile in breast cancer cell TICs. Determine if NF-κB promotes chemoresistance of TICs. Progress: We showed in Fig. 6a and 6b in Kendellen et al. (see attached) that NF-κB regulates the expression of several cytokines in breast cancer cells. Important these cytokines can promote tumorspheres/self-renewal (see Fig. 6c in Kendellen et al.). We
are currently studying the effects of chemotherapy treatment (cell-based) on survival of differentiated cells and on TICs. This work will be accomplished in year 2.

(Pt4) **Analyze the involvement of cytokines/growth factors that promote the TIC phenotype in an NF-κB-dependent manner.** Progress: As described above, Fig. 6c in Kendellen et al (attached) shows that NF-κB-regulated cytokines function to promote the TIC phenotype. In new work, we are working to show that tumor-associated macrophages promote the breast cancer TIC phenotype through secretion of cytokines (regulated by NF-κB). In vitro naïve macrophages are activated by breast cancer cells to release inflammatory cytokines. We hypothesize, based on our published work, that these cytokines will promote the TIC phenotype.

(Pt5). **Explore the involvement of mTOR and Stat3 in promoting TIC phenotypes, dependent on IKK and NF-κB-dependent mechanisms.** Progress: We have initiated experiments analyzing a role for Stat3 in breast tumor initiating cells, linking with potential control by EZH2. Results are too preliminary to report at this time.

>>Regarding Aim 2 goals:

(Pt1). **Analyze the ability of IKK (and potentially other inhibitors) to block growth of tumor xenografts derived from basal-like and Her2+ breast cancer cells, focused on effects of inhibitors on TICs.**

Progress: We showed blocking NF-kB activation by expression of the super-repressor form of IκBa blocked breast cancer tumor xenograft growth (see Kendellen et al., 2014) and see Table 1.

We also found that a pharmaceutical grade IKK inhibitor (cmpdA- Bayer Pharmaceuticals) blocks the self-renewal capacity of breast cancer TICs (see Fig. 2f, Kendellen et al., attached). We have found that Thioridazine (an FDA approved drug that is known to block dopamine receptor signaling and to block MALT1, a factor upstream of IKK) strongly reduces self-renewal of breast cancer TICs. **Fig. 4 below** shows that increasing doses of thioridazine (1µM, 5µM, and 10µM) blocks self-renewal/tumorspheres of the SUM-149 breast cancer cells. At the immediate left is a figure that shows that knockdown of MALT1 (known to be blocked by thioridazine) suppresses the TIC phenotype in SUM-149 breast cancer cells. We are currently testing the IKK inhibitor and thioridazine on breast tumor xenografts.

(Pt2). **Characterize in situ models of basal-like and Her2+ relative to responses to inhibitors and effects on TICs.** Progress: We will be analyzing two animal models to test inhibitors of IKK/NF-κB on the growth of these tumors and on the potential that they target TICs. These models are the C3Tag model for generating basal-like breast tumors and the use of patient-derived breast tumors. Both of these models are immediately available to us. Our animal protocol is now approved, and
we will initiate experiments in this direction right away. Additionally, we are planning to cross the C3Tag mouse model for basal-like cancer onto mice carrying floxed alleles for IKK, RelA, and RelB to use genetic approaches to test our hypotheses. Given the results with thioridazine, we will test this compound on the two different breast tumor models.

>>Regarding Aim 3 goals.

We have provided Dr. Chodosh reagents needed to analyze the research goals under his direction. He will describe progress for this Aim in his part of the progress report.

REPORTABLE OUTCOMES:
--RelA regulates EZH2 in breast cancer cells, and EZH2 regulates RelB to promote breast cancer TICs. Knockdown of EZH2 blocks tumor growth.

--Naïve monocytes are activated by breast cancer cells to release inflammatory cytokines to further activate NF-κB in breast cancer cells.

--Thioridazine blocks breast cancer TICs.

CONCLUSIONS: NF-κB (both canonical and non-canonical pathways) plays important roles in promoting the breast cancer tumor initiating phenotype. Inhibition of NF-κB in these cells strongly blocks tumor growth, consistent with the hypothesis that NF-κB is driving tumor formation through the TIC mechanism. Evidence indicates that inflammatory cytokines (controlled by NF-κB) promote breast cancer TICs and that tumor-associated macrophages promote the TIC phenotype. Experiments are currently focused on the mechanisms whereby NF-κB is activated in breast cancer cells, the roles that NF-κB plays in the TIC phenotype, and whether inhibiting this pathway (pharmacologically) will have a significant impact on breast tumor growth/survival. EZH2 appears to play an important role in regulating the breast cancer TIC phenotype, via its regulation of RelB. FDA-approved thioridazine may be a treatment for basal-like/triple-negative breast cancer via its ability to block TICs.

PUBLICATIONS/MEETING ABSTRACTS:

--Kendellen et al., Oncogene 2014. Demonstration that canonical and non-canonical NF-κB promote that breast cancer TIC phenotype. See attached.

PERSONNEL RECEIVING PAY:
--Albert S. Baldwin (PI)
--Cortney Lawrence (Postdoctoral Research Associate)
--Jose Roques (Research Technician)

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Tumor-initiating cells (TICs) are a sub-population of cells that exhibit a robust ability to self-renew and contribute to the formation of primary tumors, the relapse of previously treated tumors and the development of metastases. TICs have been identified in various tumors including those of the breast, and are particularly enriched in the basal-like and claudin-low subtypes of breast cancer. The signaling pathways that contribute to the function and maintenance of TICs are under intense study. We explored the potential involvement of the nuclear factor-κB (NF-κB) family of transcription factors in TICs in cell lines that are representative of basal-like and claudin-low breast cancer. NF-κB was found to be activated in breast cancer cells that form tumorspheres efficiently. Moreover, both canonical and non-canonical NF-κB signaling is required for these cells to self-renew in vitro and to form xenograft tumors efficiently in vivo using limiting dilutions of cells. Consistent with this fact, canonical and non-canonical NF-κB signaling is activated in TICs isolated from breast cancer cell lines. Experimental results indicate that NF-κB promotes the function of TICs by stimulating epithelial-to-mesenchymal transition and by upregulating the expression of the inflammatory cytokines interleukin-1β and interleukin-6. The results suggest the use of NF-κB inhibitors for clinical therapy of certain breast cancers.

Keywords: NF-κB; basal-like breast cancer; tumor-initiating cells; EMT; IL-6; IL-1β

INTRODUCTION

Tumors are comprised of a heterogeneous population of cells including bulk epithelial tumor cells, inflammatory cells and sub-population of cells termed cancer stem cells or tumor-initiating cells (TICs). The primary characteristic of TICs is their ability to self-renew, which is measured in vitro by the formation of spheroid cellular structures termed tumorspheres. In addition, TICs exhibit elevated motility and invasiveness in vitro that correlates with high metastatic potential in vivo and are frequently radio- and chemo-resistant. Importantly, TICs are thought to drive the progression of primary tumors, promote tumor recurrence and stimulate the development of metastases at distant sites. The importance of TICs in the clinical outcome of breast cancer is evidenced by the observation that an increase in their abundance following initial systemic treatment correlates with worse prognosis. TICs have been observed in multiple subtypes of human breast cancer and are particularly enriched in the basal-like and claudin-low subtypes.

The nuclear factor-κB (NF-κB) family of transcription factors contains five members, p65 (RelA), RelB, c-Rel, p105/p50 and p100/p52. In most cells, NF-κB proteins exist as hetero- and homo-dimers in the cytoplasm bound to a class of inhibitory proteins called IκB (inhibitor of κB). In response to a wide variety of cellular stimuli, NF-κB becomes active through one of two pathways. In the canonical pathway, NF-κB activation depends on the IκB kinase complex (IKK), which contains two catalytic subunits, IκKα and IκKβ, and a regulatory subunit, IκKγ or NEMO. Upon stimulation, IκBα is phosphorylated at Ser32/36 by IKK in a manner that requires IκKγ, which results in the degradation of IκBα and the release of the p65-p50 dimer to accumulate in the nucleus. Phosphorylation of p65 at Ser536 by IKK is also important for its activity. Separately, the non-canonical NF-κB pathway is regulated by an IκKα homodimer. In this cascade, RelB-p100 heterodimers are processed to RelB-p52 heterodimers in a manner that depends on IκKα. In the nucleus, NF-κB dimers activate genes including those involved in cell cycle regulation (for example, cyclin D1), suppression of apoptosis (for example, Bcl-2 and Bcl-xL) and inflammation (for example, cytokines such as interleukin (IL)-6 and IL-8). Activation of NF-κB is strongly associated with oncogenesis, as it is known to promote the oncogenic phenotype through processes including cell proliferation, inflammation, cell invasion and suppression of apoptosis. Consistent with this fact, both canonical and non-canonical NF-κB signaling is activated in human breast cancer cell lines and primary breast tumors. Recently, IKK/NF-κB was shown to be important in TICs isolated from HER2 + breast cancer. Others have observed that NF-κB functions to promote proliferation in basal-like breast cancer cells. Here, we have explored a potential role for NF-κB in TIC function in cells derived from basal-like and claudin-low breast cancer cells. Specifically, we show that NF-κB signaling is more highly activated in breast cancer cell lines that undergo efficient self-renewal. Moreover, inhibition of either canonical or non-canonical NF-κB signaling blunts the self-renewal of human breast cancer cells in vitro. Inhibition of NF-κB also reduces the formation of xenograft tumors in the mammary fat pads of nude mice in vivo. Mechanistically, we provide evidence that NF-κB promotes the function of TICs through stimulation of epithelial-to-me...
mesenchymal transition (EMT) and the production of inflammatory cytokines that are encoded by NF-κB target genes. Collectively, these data demonstrate that canonical and non-canonical NF-κB signaling have critical roles in the function of TICs derived from basal-like and claudin-low subtypes of breast cancer cells.

RESULTS
NF-κB signaling is preferentially activated in tumorsphere-forming breast cancer cells

Cell lines representing the basal-like subtype (SUM149) and claudin-low subtype (MDA-MB231) of breast cancer were utilized to investigate the role of NF-κB in the function of TICs. A hallmark of breast cancer TICs is the ability to drive the formation of spheroid structures termed tumorspheres (or mammospheres) in serum-free culture, which reflects the ability of these cells to self-renew in vitro (reviewed in Charafe-Jauffret et al. and Pastrana et al.28). It was observed that both SUM149 and MDA-MB231 cells efficiently form tumorspheres over at least three cycles of culture (Figure 1a). It was then determined whether the ability of basal-like and claudin-low cancer cells to form tumorspheres correlates with the level of basal NF-κB activation in the bulk population. Importantly, both p65 and IκBz are preferentially phosphorylated in SUM149 and MDA-MB231 cells that form tumorspheres efficiently compared with MCF10A cells that form tumorspheres less efficiently (Figures 1a and b).29

Canonical NF-κB signaling is required for basal-like breast cancer cells to efficiently self-renew in vitro

To inhibit NF-κB, SUM149 cells were stably infected with a retrovirus expressing either an empty vector or IκBz-SR (a modified form of IκBz that cannot be phosphorylated) and selected with puromycin (Figure 2a). To assay for self-renewal, 100 SUM149 cells expressing empty vector or IκBz-SR were plated in serum-free media on low-adhesion plates and the number of tumorspheres was determined 5 days later. Importantly, cells in which NF-κB was inhibited by expression of IκBz-SR formed threefold fewer tumorspheres than control cells, expressing an empty vector (Figure 2b). To confirm these results, an RNA interference knockdown approach was utilized. SUM149 cells were stably infected with an empty lentiviral vector, a lentivirus encoding a scrambled short hairpin RNA (shRNA) or a lentivirus encoding a shRNA construct targeting p65 or IκBz (Figure 2c). Subsequently, self-renewal of these cells was assayed through tumorsphere formation as described above. Importantly, knockdown of either p65 or IκBz resulted in a statistically significant, approximately fivefold reduction in the ability of SUM149 cells to form tumorspheres (Figure 2d). Notably, expression of IκBz-SR, or knockdown of IκBz or p65 does not alter the growth of SUM149 cells as assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide (MTT) assay (Supplementary Figures 1a and b). In addition, further analysis demonstrated that tumorspheres were arising from single cells (data not shown). This indicates that any variation in the formation of tumorspheres is due to reduced self-renewal, rather than an inhibition of cell growth or cytotoxicity. Interestingly, shRNA-based knockdown of Iκκs (Figure 2c) induced a statistically significant, approximately fivefold reduction in the self-renewal of SUM149 cells (Figure 2d) without altering overall cell growth (Supplementary Figure 1b). Treatment with an Iκκ inhibitor (compound A30) blocked both basal and tumor-necrosis factor-α-induced phosphorylation of p65, (Figure 2e) and led to a reduction in tumorsphere formation that was very similar to the results obtained with shRNA knockdown of different Iκκ and NF-κB subunits (Figure 2f). These data demonstrate that canonical NF-κB signaling, driven by Iκκ, promotes self-renewal in basal-like breast cancer cells (also see below).

In addition to the breast cancer cells analyzed above, we analyzed the role of NF-κB in the claudin-low representative cell line SUM159 (reviewed in Prat and Perou31 and Prat et al.32). Notably, similar results were obtained in SUM159 cells compared with SUM149 cells. Specifically, inhibition of Iκκ with compound A blocked NF-κB activity as measured by inhibition of phosphorylation of p65 and IκBz (Supplementary Figure 2a). In addition, and consistent with the findings using basal-like breast cancer cells, compound A blocked the formation of tumorspheres derived from SUM159 cells (Supplementary Figure 2b).

Non-canonical NF-κB activity is required for breast cancer cells to self-renew in vitro

As shown above, Iκκ contributes to the self-renewal of basal-like breast cancer cells. Iκκ typically acts in non-canonical NF-κB signaling (reviewed in Ghosh and Karin15 and Hayden and Ghosh16), although it has recently been reported to promote non-canonical NF-κB signaling in breast cancer cells of the HER2+ subtype.33 Knockdown with p100/p52 small interfering RNA (siRNA) efficiently reduced the precursor 100 kDa component and almost completely eliminated the processed p52 subunit (Figure 3a). Consistent with the Iκκ knockdown results (Figures 2c and d), a statistically significant reduction in tumorsphere formation...
NF-κB promotes the self-renewal of breast cancer cells in vivo. In vivo, self-renewal is assayed by measuring the ability of cells to establish primary tumors when injected at limiting dilutions, which is specifically associated with the function of TICs.1,2,34,35 To directly test the role of NF-κB in the self-renewal of basal-like breast cancer cells in vivo, SUM149 cells expressing empty vector or IkBα-SR at low (10^2 cells/100 μl) or high (10^6 cells/100 μl) density were prepared. These cells were injected into the mammary fat pad of nude mice and monitored as described in the methods. In these experiments, cells in which NF-κB signaling was deficient exhibited both delayed tumor onset and reduced overall tumor size (Table 1). Specifically, while the high density of SUM149 cells expressing empty vector formed palpable tumors at 6 weeks and reached an average tumor volume of 138 mm^3 by 10 weeks, the high density of SUM149 cells expressing IkBα-SR did not form palpable tumors until week 8 and these tumors maintained a significantly smaller size (11 mm^3) at week 10 (Table 1). Importantly, the low density of SUM149 cells expressing empty vector formed palpable tumors at week 9 and these tumors continued to increase in size at a significant rate (Table 1). Conversely, the low density of SUM149 cells expressing IkBα-SR did not form tumors (Table 1). These data demonstrate that NF-κB is required for xenograft-generated tumorigenesis in a context (limiting dilutions of cells, reviewed in Charafee-Jauffret et al.2) that depends significantly on self-renewal.

Canonical and non-canonical NF-κB are activated in breast cancer TICs and required for maintenance of TICs in the bulk population

The cell surface profile most commonly associated with breast cancer TICs is CD44^+CD24^-/CD0,7,13,34,36–38 although CD44^+, EpCAM^+ or ALDEFLUOR-positivity (which depends on the activity of the enzyme ALDH1) are also indicative of this subpopulation of cells.12,26,34–43 After examining the various cell surface profiles previously reported to enrich for TICs (data not shown), we chose to analyze CD44^+ cells, which could be identified, isolated and, more importantly, self-renewed efficiently (see below). By calculating the percentage yield from the isolation protocol, bulk populations of both the SUM149 (Figures 4g and h) and MDA-MB231 cell lines (data not shown) were found to contain ~10% CD44^+ cells. This percentage is lower than earlier reports,2,12,42 although different groups have reported a range of the proportion of TICs even within the same cell line, particularly when different cell surface markers are utilized.12,42

Although the bulk population and CD44^- cells each contain only a small proportion of cells that are positive for CD44, nearly all the cells in the CD44^-isolated population robustly express CD44 (Figure 4a). This confirms that the isolation protocol successfully
enriches for CD44+ cells. Notably, CD44+ cells form tumorspheres significantly more efficiently than CD44− cells (Figure 4b), which demonstrates that the CD44+ isolation protocol enriches for TIC function. Given that NF-κB is required for the self-renewal of TICs (Figures 2 and 3), the activation of NF-κB was assessed in lysates from breast cancer TICs (CD44+ cells) compared with lysates isolated from the bulk population of cells and non-TICs (CD44− cells). Importantly, phosphorylation of p65 (Figure 4c) and levels of p52 (Figure 4d) are detected in CD44+ cells in both SUM149 and MDA-MB231 cells, indicating that both canonical and non-canonical NF-κB signaling is activated in breast cancer TICs. As before, the role of NF-κB in TICs of the claudin-low SUM159 cells was also assessed. Specifically, CD44+ cells isolated from SUM159 cells also exhibit elevated NF-κB activity, as measured by phosphorylation of p65 (Supplementary Figure 2c).

To determine if pathways upstream of NF-κB are also activated in breast cancer TICs, phosphorylation of IKKα and IKKβ was assessed by immunoblot of cellular lysates prepared from the bulk population of SUM149 cells and its TIC and non-TIC counterparts. In support of the concept that NF-κB activation is IKK-dependent in these cells, an increased signal for phosphorylated IKKα/β was observed in TICs isolated from SUM149 cells (Figure 4e). This fact is also consistent with evidence that canonical and non-canonical NF-κB pathways are important in TICs (Figures 2, 3, 4c and d).

Finally, the activation of TAK1, a kinase that is an upstream activator of IKK,44 was examined. Similar to IKK, phosphorylation of TAK1, which is indicative of its activation,45 was enriched in CD44+ cells (Figure 4f).

Given that NF-κB is preferentially activated in breast cancer TICs (Figures 4a–f) and required for their self-renewal in vitro and in vivo (Figures 2 and 3 and Table 1), it was determined whether NF-κB is important in the maintenance of TICs in the bulk population of basal-like breast cancer cells. To this end, the percentage of TICs in the bulk population of SUM149 cells was determined in the presence or absence of overexpression of IkBα-SR. The resulting data showed that inhibition of NF-κB reduced the percentage of CD44+ cells by ~50% (Figure 4g). Similarly, stable knockdown of p65, IKKβ or IKKα each reduced the percentage of CD44+ cells by ~50% (Figure 4h). Taken together, these data demonstrate that both canonical and non-canonical...
NF-κB signaling is preferentially activated in breast cancer TICs, consistent with observations from TICs in the bulk population of cells (Figures 2 and 3), and that NF-κB is important for the maintenance of the TIC population.

NF-κB promotes expression of markers of EMT in TICs and transforming growth factor-β-induced self-renewal

EMT is a process by which an epithelial cell releases from the basement membrane and transforms into a spindle-like, mesenchymal cell expressing vimentin and fibronectin (reviewed in Kalluri and Weinberg46). EMT has been demonstrated to promote the self-renewal of at least immortalized breast cancer cells.36,37 However, this has not been examined in specific subtypes of breast cancer cells. NF-κB is one of the multiple signaling pathways implicated in the regulation of EMT, as activation of NF-κB is required for EMT that occurs during Ras-driven transformation.47 Thus, we hypothesized that NF-κB promotes the function of breast cancer TICs by stimulating EMT. Expression of mesenchymal markers was analyzed in the bulk population of SUM149 or MDA-MB231 cells, and in CD44+ and CD44− cells. Both vimentin and fibronectin were detected in the bulk population as well as CD44+ and CD44− cells (data not shown). Importantly, expression of mesenchymal markers depends on NF-κB, as vimentin expression is decreased in the bulk population of SUM149 and MDA-MB231 cells expressing IκBα-SR (Figure 5a). To extend these results, knockdown of the RelA/p65 NF-κB subunit reduced vimentin and fibronectin expression in SUM149 cells (Supplementary Figure 3).

Transforming growth factor-β (TGFβ) is a well-characterized inducer of EMT and has been shown to promote the self-renewal of at least immortalized breast cancer cells.36 To first confirm that TGFβ promotes self-renewal of basal-like breast cancer cells, 100 SUM149 cells expressing empty vector were plated on low-adhesion plates in serum-free media and treated every other day for 7 days with 10 ng/ml TGFβ or vehicle control. Such treatment induced a statistically significant, threefold increase in the number of tumorspheres formed by these cells (Figure 5b), suggesting that EMT is important for the function of TICs in basal-like breast cancer.

Figure 4. Canonical and non-canonical NF-κB signaling is preferentially activated in TICs and required for the maintenance of TICs in breast cancer cells. (a) Fluorescence-activated cell sorting analysis of the indicated populations of SUM149 cells stained with CD44-APC. (b) Quantification of tumorspheres formed by 100 cells from the indicated cell populations of SUM149 or MDA-MB231 cells. (c) Phosphorylation of p65 as a marker of activation of canonical NF-κB signaling in the indicated populations of SUM149 and MDA-MB231 cells. (d) Cleavage of p100 to p52 as a marker of activation of non-canonical NF-κB signaling in the indicated populations of SUM149 and MDA-MB231 cells. (e) Phosphorylation of IKKα and IKKβ in the indicated populations of SUM149 cells. (f) Phosphorylation of TAK1 in the indicated populations of SUM149 cells. (g, h) Percentage of TICs isolated from SUM149 cells stably expressing IκBα-SR (g) or the indicated shRNA constructs (h).
with exogenous IL-8 failed to promote the formation of tumorspheres in control SUM149 cells (Figure 6c). Consistent with the results described above, addition of an IL-6 receptor antagonistic antibody suppressed tumorsphere formation ~ 25% and addition of recombinant IL-1β receptor antagonist suppressed tumorsphere formation ~ 30% (Supplementary Figure 4). As IL-1β and IL-6 are known NF-κB target genes and were upregulated by NF-κB in SUM149 cells (Figures 6a and b), we investigated whether NF-κB promotes self-renewal by inducing their expression and secretion. Specifically, we tested whether treatment with IL-1β or IL-6 can rescue the ability to self-renew in SUM149 cells in which NF-κB is inhibited. To do so, the above experiment was also performed using SUM149 cells expressing IκBα-SR. As before (Figure 2b), expression of IκBα-SR rescued the ability of untreated SUM149 to form tumorspheres (Figure 6c). Notably, treatment with either IL-1β or IL-6, but not IL-8, partially rescued the ability of IκBα-SR-expressing cells to form tumorspheres (Figure 6c). These data indicate that IL-1β and IL-6 promote self-renewal of basal-like breast cancer cells downstream of NF-κB.

**DISCUSSION**

Most solid tumors, including those of the breast, are characterized by a hierarchy of cells including a sub-population of cells that can self-renew and give rise to the differentiated cells that comprise the bulk of the tumor. These TICs promote tumor initiation, cellular motility and invasiveness, tumor recurrence, and are typically radio- and chemo-resistant. As such, characterizing the functional and phenotypic differences between the bulk population of cancer cells and TICs is critical in understanding tumorigenesis and gaining insight into new approaches for cancer therapy. The transcription factor NF-κB is widely implicated in a variety of oncogenic mechanisms in both hematological malignancies as well as solid tumors, including cancer cell proliferation, survival and metastasis. Here, we have shown the involvement of NF-κB in promoting TICs in a basal-like breast cancer cell line, SUM149, and two claudin-low lines, MDA-MB231 and SUM159. Interestingly, both canonical and non-canonical NF-κB appear to be important in promoting TICs in these cancer cell lines. It will be interesting to determine if distinct or overlapping functions of these two pathways are operative in the maintenance of TICs. Work by others has indicated the association of NF-κB activity with other TICs. For example, prostate cancer TICs exhibit increased canonical NF-κB activity. Also, published work indicates that canonical NF-κB signaling is important in TICs in the HER2+ breast tumor subtype.

One study utilized inhibitors that are not specific to NF-κB to suggest an involvement of NF-κB in promoting MCF7 breast cancer tumorspheres. We propose that NF-κB promotes the function of TICs through several mechanisms. First, NF-κB may promote TIC self-renewal by stimulating EMT. Second, NF-κB promotes TICs by stimulating the expression of cytokines, such as IL-1β and IL-6. Notably, IL-6 has been implicated in the induction of EMT in breast cancer cells. Several reports support the finding that IL-6 promotes the function of TICs downstream of NF-κB. MCF10A cells transformed by Src gain the ability to form tumorspheres in a manner that depends on expression of IL-6. In addition, it was found that treatment of a claudin-low breast cancer cell line with IL-6 increased the proportion of TICs. That NF-κB upregulates the expression of many cytokines, and correspondingly can be activated downstream of these cytokines, indicates an important mechanism for sustaining NF-κB activation and promoting its TIC self-renewal properties in certain cancers. Additional roles for NF-κB in TICs likely include the upregulation of other key genes. Given the potential benefit of targeting TICs in breast cancer patients, the identification of NF-κB as a key regulator of TICs in the context of breast cancer provides new opportunities for targeting this population of cancer cells.
basal-like and claudin-low breast cancer cells, along with previous work studying NF-κB in Her2+ TICs, represents a significant opportunity for the development of more effective chemotherapeutics for breast cancer.

MATERIALS AND METHODS

Cell culture and reagents
SUM149, MDA-MB231, SUM159 and MCF10A cells were maintained as described (Supplementary Materials). Details regarding shRNA constructs and antibodies are also found in Supplementary Materials.

Tumorsphere formation assay
Cells growing adherently in serum-containing media were trypsinized with TrypLE Select (Invitrogen, Grand Island, NY, USA) to generate a single cell solution and then enumerated using a hemocytometer. Subsequently, 100 cells per well were plated in 3 ml of Mammocult media (Stem Cell Technologies, Vancouver, BC, Canada) on six-well low-adhesion plates (Corning, Corning, NY, USA). Cells were treated with compound A, TGFβ, cytokines or vehicle controls as described in the text. The number of tumorspheres formed per well were counted visually. Cell density is a critical parameter in the tumorsphere formation assay and cells may aggregate if cell density is too high.28 As such, we ensured that a disperse, low-density (100 cells in 3 ml of media) solution of cells was prepared for each tumorsphere formation assay to avoid cell aggregation. Furthermore, plates were not moved during the growth period to avoid cell aggregation. These precautions were taken to ensure the clonality of tumorspheres formed during this assay.28

Immunoblotting
See Supplementary Materials.

Fluorescence-activated cell sorting
Cell sorting was performed using a Beckman-Coulter (Dako, Carpinteria, CA, USA) CyAn and FlowJo software (TreeStar Inc., Ashland, OR, USA). See Supplementary Materials.

Figure 6. IL-1β and IL-6 stimulate the self-renewal of basal-like breast cancer cells downstream of NF-κB. (a) Real-time PCR showing expression of IL-1β, IL-6 or IL-8 in the bulk population of SUM149 cells stably expressing an empty vector or IκBα-SR. (b) ELISA analysis showing the abundance of IL-1β, IL-6 or IL-8 in the media of SUM149 cells stably expressing empty vector or IκBα-SR. (c) Quantification of tumorspheres formed by 100 SUM149 cells expressing empty vector or IκBα-SR, followed by treatment with IL-1β, IL-6, IL-8 or vehicle control.
Isolation of TICs

Cells were trypsinized using TrypLE Select (Invitrogen) and dissociated by incubation in Accutase (Invitrogen) for 15 min at 37 °C. The resulting cell solution was passed through a pre-separation filter (Miltenyi Biotec, Auburn, CA, USA) to generate a single cell suspension. Cells were then incubated with 100 μl of Dead Cell Removal microbeads (Miltenyi Biotec) per 10^7 cells for 15 min at room temperature. Subsequently, the cell and micro bead solution was resuspended in 20 ml of MACS buffer (Miltenyi Biotec) and passed through an LS column (Miltenyi Biotec) that was pre-moistened with MACS buffer and placed in a magnetized field. Live cells from the eluate were collected. Next, the resulting live cells were resuspended in 100 μl of MACS buffer per 10^7 cells and incubated with 75 μl of CD44 microbeads (Miltenyi Biotec) and 75 μl of FcR blocking reagent (Miltenyi Biotec) per 10^7 cells for 30 min at 4 °C. Subsequently, the cell and microbead solution was resuspended in 20 ml of MACS buffer (Miltenyi Biotec) passed through a LS column (Miltenyi Biotec) that was pre-moistened with MACS buffer and placed in a magnetic field. The CD44 + cells in the eluate were collected, the LS column was removed from the magnetized field and the CD44 + cells were collected from the column in 5 ml of MACS buffer.

MITT

The MITT assays were performed as previously described using CellTiter cell viability reagent (Promega, Madison, WI, USA). See Supplementary Materials.

Enzyme-linked immunosorbent assay

ELISA kits (BD Biosciences, San Jose, CA, USA) were utilized according to the manufacturer’s instructions.

Quantitative real-time PCR

Real-time PCR was performed and analyzed as previously described using Taqman Gene Expression Assay primer-probe sets from Applied Biosystems (Grand Island, NY, USA).

 Xenograft tumor formation

Cells were trypsinized with TrypLE Select (Invitrogen), a single cell solution was generated and the cells were enumerated using a hemocytometer. Cell solutions were generated in 50:50 media:Matrigel (BD Biosciences) at concentrations of 10^6 or 10^5 cells/100 ml of CD44 microbeads (Miltenyi Biotec) and 75 μl of FcR blocking reagent (Miltenyi Biotec) per 10^7 cells for 30 min at 4 °C. Subsequently, the cell and microbead solution was resuspended in 20 ml of MACS buffer (Miltenyi Biotec) passed through a LS column (Miltenyi Biotec) that was pre-moistened with MACS buffer and placed in a magnetic field. The CD44 + cells in the eluate were collected, the LS column was removed from the magnetized field and the CD44 + cells were collected from the column in 5 ml of MACS buffer.

MITT

The MITT assays were performed as previously described using CellTiter cell viability reagent (Promega, Madison, WI, USA). See Supplementary Materials.

ABBRévIATIONS

EMT, epithelial-to-mesenchymal transition; ikB, inhibitor of kB; iKK, IkB kinase; IL, interleukin; NF-kB, nuclear factor-kB; TIC, tumor-initiating cell; TGFβ, transforming growth factor-β

conflicT of interest

The authors declare no conflict of interest.

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References


NF-kappaB MAY PROMOTE THE TUMOR-INITIATING CELL PHENOTYPE IN BREAST CANCER

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A breast tumor is composed of a heterogeneous population of cells, including a subset of cells termed tumor-initiating cells (TICs). These cells are particularly enriched in the basal and claudin-low subtypes of human breast cancer. TICs are characterized by robust self-renewal, elevated motility and invasiveness in vitro that translate to high metastatic potential in vivo, and radio- and chemoresistance. Thus, they may underlie a significant portion of the lethality of cancer and represent an important target for clinical intervention. Thus, it is important to understand signaling mechanisms that drive breast cancer-initiating cells.

The NF-kappaB (NF-kB) family of transcription factors is known to be involved in breast cancer. For example, we have shown that Her2, the EGFR family member associated with approximately 25% of breast cancer, is a potent activator of NF-kB to promote cancer-cell invasion. Others have shown that cells derived from murine mammary tumors in which NF-kB has been inactivated exhibit reduced self-renewal, and NF-kB is known to promote invasion and chemoresistance at least in some contexts in vitro. Mechanistically, NF-kB can mediate the epithelial-to-mesenchymal transition, the only cellular process yet known to promote the conversion of cells to TICs. We hypothesize that a distinct subset of NF-kB target genes promotes the TIC phenotype.

Our data support the hypothesis that NF-kB plays an important role in breast cancer TICs. Specifically, we have successfully isolated TICs (CD44+CD24-cells) from human breast cancer cell lines. Importantly, these cells more robustly express phosphorylated p65, a marker of NF-kB activity, than the bulk population. We also have successfully formed mammospheres from breast cancer cell lines in cell culture and observed that genetic inhibition of several components of NF-kB signaling results in reduced mammosphere formation. Finally, such inhibition of NF-kB results in a significant reduction of the percentage of TICs in the bulk population. Additional ongoing experimentation, both in vitro and in vivo, will address the contribution of the NF-kB pathway to the maintenance of breast cancer TICs.

In summary, our results suggest that NF-kB is integral to the TIC phenotype. Given the role TICs are thought to play in the initiation and progression of breast cancer, inhibition of NF-kB may be a beneficial therapeutic strategy and potentially improve clinical outcomes.

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