AP-1 Is a Key Regulator of Proinflammatory Cytokine TNFα-mediated Triple-negative Breast Cancer Progression*

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Triple-negative breast cancer (TNBC), a highly aggressive form of breast cancer with limited treatment options. Proinflammatory cytokines such as TNFα can facilitate tumor progression and metastasis. However, the mechanistic aspects of inflammation mediated TNBC progression remain unclear. Using ChIP-seq, we demonstrate that the cistrome for the AP-1 transcription factor c-Jun is comprised of 13,800 binding regions in TNFα-stimulated TNBC cells. In addition, we show that c-Jun regulates nearly a third of the TNFα-regulated transcriptome. Interestingly, high expression level of the c-Jun-regulated pro-invasion gene program is associated with poor clinical outcome in TNBCs. We further demonstrate that c-Jun drives TNFα-mediated increase of malignant characteristics of TNBC cells by transcriptional regulation of Ninj1. As exemplified by the CXC chemokine genes clustered on chromosome 4, we demonstrate that NF-κB might be a pioneer factor required for the regulation of TNFα-inducible inflammatory genes, whereas c-Jun has little effect. Together, our results uncover AP-1 as an important determinant for inflammation-induced cancer progression, rather than inflammatory response.

Triple-negative breast cancer (TNBC), which lacks expression of estrogen receptor (ER) and progesterone receptor (PR) as well as HER2 amplification, is more aggressive and has poorer survival than other types of breast cancer (1). TNBC is a clinically heterogeneous disease and accordingly, TNBC has recently been classified into five clusters, each harboring a dominant biological function/pathway (2). These are basal-like TNBC with DNA-repair deficiency and growth factor pathway expression, mesenchymal-like TNBC with epithelial-mesenchymal transition (EMT) and cancer stem cell features, immune-associated TNBC, luminal/apocrine TNBC with androgen-receptor (AR) overexpression, and HER2-enriched TNBC. Targeted therapies such as poly-ADP-ribose polymerase (PARP) inhibitors targeting DNA-repair deficiency, AR inhibitors and anti-EGFR therapies are currently under clinical evaluation. However, despite the ongoing development of novel targeted therapies for TNBC, there is a recognized need for additional approaches for each cluster and additionally the clinical success of the approaches above remains to be proven.

The AP-1 family of transcription factors consists of multiple Jun (c-Jun, JunB, and JunD) and Fos (c-Fos, FosB, Fra1, and Fra2) members. AP-1 regulates gene transcription through binding to 12-O tetra-decanoyl-phorbol-13 acetate (TPA)-responsive elements (TRE, 5′-TGAG/CTCA-3′) or cAMP response elements (CRE, 5′-TGA CGTCA-3′) via a basic leucine zipper (bZIP) domain, which is composed of a DNA-binding basic region and the leucine zipper dimerization region (3). AP-1 has been implicated in regulating various physiological and pathological cellular processes including proliferation, differentiation, growth, apoptosis, cell migration, and transformation (4). AP-1 inhibitors have therefore emerged as an attractive drug class for cancer therapy. AP-1 inhibition-specific retinoids such as SR11302 and MX781 have long been known to have antitumor effects in vitro and in vivo (5–8). In particular, the retinoid antagonist MX781 showed promising anticancer activities against advanced ER-negative breast cancer (5). The activation of AP-1 can be regulated by multiple mechanisms, including dimer composition, post-translational modifications and interactions with ancillary proteins (9). Our recent studies showed that AP-1 proteins, mainly Fra-1 and c-Jun, are highly expressed in TNBC compared with other types of breast cancer (10, 11), underscoring the importance of unraveling the AP-1 signaling pathway in TNBC.

Recent evidence indicates that proinflammatory signaling activated by inflammatory stimuli in the tumor microenvironment facilitates tumorigenesis (12). The inflammatory tumor microenvironment is largely orchestrated by various inflammatory cells, particularly tumor-associated macrophages, which secrete proinflammatory cytokines such as TNFα, IL-1, IL-6, and TGFβ (12). It is well recognized that TNFα signaling, via ligand binding to TNF receptors, induces a range of inflammatory genes through activation of the nuclear factor-κB (NF-κB) pathway (12). In addition, it is known that NF-κB activation plays critical roles in inflammation-induced tumor growth and metastasis (13). In contrast, much less is known about the nature of proinflammatory cytokine activation of AP-1 pathways.

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3 The abbreviations used are: TNBC, triple-negative breast cancer; ER, estrogen receptor; PR, progesterone receptor; EMT, epithelial-mesenchymal transition; TSS, transcriptional start site; GOBO, Gene Expression-Based Outcome for Breast Cancer Online.
Here, we define for the first time the inflammatory cistrome for the AP-1 transcription factor c-Jun and demonstrate that AP-1 activation is responsible for inflammation-induced malignant characteristics of TNBC cells, rather than the inflammatory response. We identify a set of c-Jun-regulated pro-invasion genes that are strongly associated with clinical outcomes in TNBCs. In particular, we characterize the Ninj1 gene, which is transcriptionally regulated by c-Jun to drive TNFα-mediated malignant characteristics of TNBC cells. Our study reveals a critical mechanism underlying inflammation-induced cancer progression and may have important implications for the development of targeted therapies for metastatic breast cancers.

**Experimental Procedures**

**Cell Lines and Reagents**—BT549 and Hs578T cells were obtained from the American Type Culture Collection. Human recombinant TNFα was purchased from Roche and was used at 10 ng/ml in all experiments.

**siRNA Transfection and Plasmids**—Nontargeting siRNA pool (D-001810-10-20) and c-Jun ON-TARGET plus SMARTpool (L-003268-0-0010) were purchased from Thermo Scientific. ON-TARGET plus NINJ1 siRNA pool (LQ-017671-00-0002), ON-TARGET plus RELA siRNA pool (LQ-003533-00-0002) and ON-TARGET plus NFKB1 siRNA pool (LQ-003520-00-0002) were purchased from Dharmacon. Transfection was conducted as described previously (14). The empty vector pCMV6-Entry and pCMV6-Ninj1 expression vector were purchased from Origene.

**Gene Expression Microarray Analysis**—BT549 cells were cultured to 50% confluency and transfected with control or c-Jun siRNA for 72 h, followed by treatment with or without TNFα for 6 h. Total RNA was extracted using the RNeasy Plus Mini Kit (Qiagen). Total RNA from three biological replicates was reverse transcribed, and cDNA was amplified using a TaqMan Gene Expression Master Mix kit (Thermo Scientific). Amplified cDNA was normalized to the expression of the c-Jun-regulated gene itself under untreated and TNFα-stimulated conditions.

**qPCR**—Total RNA was extracted, reverse transcribed, and subjected to real-time qPCR using gene-specific primers, as previously described (18). The expression of genes was normalized to the expression of β-glucuronidase and 36B4. Similar results were obtained for both reference genes, with only 36B4 normalized values presented.

**ChIP and ChIP-Seq**—ChIP and ChIP-Seq were performed as previously described (18, 19). The antibodies used were as follows: anti-c-Jun (H-79) and normal rabbit IgG from Santa Cruz Biotechnology, anti-p65 (ab7970) and anti-p50 (ab7971) from Abcam. ChIP-Seq data are deposited in GEO (accession number GSE71977).

**Western Blot Analysis**—Western blot analysis was performed as previously described (18). Antibodies used were anti-p65 (ab7970) and anti-p50 (ab7971) from Abcam, and anti-cleaved caspase-3 (Asp175) from Cell Signaling Technology.

**Statistical Analysis**—Data are presented as mean ± S.D. Comparisons were performed by two-tailed Student’s t test for single comparison or by one-way ANOVA followed by Bonferroni correction for multiple comparisons as appropriate. p values < 0.05 were considered as significant.

**Results**

**TNFα Signaling Generates a Unique AP-1 Cistrome in TNBC Cells**—We previously demonstrated that the inflammatory cytokine TNFα triggered EMT of TNBC cells through AP-1-induced ZEB2 up-regulation (14). However, the AP-1 signaling network responsive to TNFα remains largely unexplored. To begin to explore the AP-1 signaling network responsive to TNFα, we performed genome-wide mapping of c-Jun binding regions in BT549 TNBC cells treated with TNFα for 3 h. The resulting cistromes correspond to 4,570 and 13,800 binding sites in non-stimulated and TNFα-stimulated cells, respectively (Fig. 1A). Our analysis reveals that the de novo TNFα-induced c-Jun cistrome corresponds to 9230 binding regions. It has been described that c-Jun regulates its own expression through AP-1 sites in the promoter of the c-Jun gene (21). In line with this, a clear peak was identified in the promoter of the c-Jun gene itself under untreated and TNFα-stimulated conditions (Fig. 1B). De novo DNA motif search revealed several highly enriched motifs. As expected, the consensus AP-1 motif was the most enriched motif in c-Jun cistromes in the presence and absence of TNFα (Fig. 1C). Interestingly, the cistrome in the presence of TNFα was enriched for the NF-κB motif. Because TNFα stimulation could conceivably lead to activation of NF-κB (12), these findings suggest that c-Jun might bind sites occupied by NF-κB through mechanisms such as tethering. The overall distribution of c-Jun binding regions in relation to gene structures was very similar for non-stimulated and TNFα-stimulated cells, with most binding regions in intergenic and intronic parts of genes (Fig. 1D). To systematically address potential functional consequences of c-Jun binding regions, we used the genomics regions enrichment of annotation tool (GREAT), which is specifically suited to analyze ChIP-Seq data. The GREAT analysis suggests that TNFα-induced c-Jun DNA sites.
binding regulates apoptosis signaling pathways and oxidative stress responses (Fig. 1).

**AP-1 Modulates the TNFα-regulated Transcriptome in TNBC Cells**—We next investigated the role of c-Jun for TNFα-regulated gene expression programs. We suppressed c-Jun expression by RNA interference (11) and investigated the genome-wide effects on TNFα-mediated transcriptional regulation in BT549 cells. Fig. 2A shows that the expression of 1192 genes in control cells and 940 genes in c-Jun-depleted cells were activated or repressed (at least 1.5-fold), upon TNFα stimula-
tion (Fig. 2A). Specifically, treatment with TNFα resulted in activation of 684 genes (at least 1.5-fold) in control cells. Assigning the TNFα-up-regulated genes to functional categories, immune/defense/inflammatory responses were the most frequently represented GO classes, consistent with the known roles of TNFα. The majority (66%) of TNFα-regulated genes in control cells remained TNFα-responsive in c-Jun-depleted cells, but 409 genes were no longer responsive to TNFα upon c-Jun depletion. Furthermore, c-Jun depletion exposed a set of novel genes (157) subject to TNFα regulation. These results reveal that c-Jun controls more than one-third of the TNFα-regulated transcriptome.

Next, we focused on TNFα stimulation. We identified 616 genes, the expression of which were affected by decreased c-Jun expression in the presence of TNFα (Fig. 2B). To address which of the 616 genes that are direct AP-1 targets, we extracted the genes that included c-Jun binding regions within 20-kb upstream or downstream of a known transcriptional start site (TSS) in TNFα-stimulated cells. This revealed 204 direct c-Jun target genes in TNFα-stimulated cells (Fig. 2B). Using qPCR analysis, we confirmed changes in gene expression, as derived from microarray analysis, for 4 genes (EGR2, ZEB2, MMP9, and TNFAIP8) among the direct c-Jun target genes (Fig. 2C). Assigning the direct target genes to molecular and cellular functions, genes associated with regulation of cell proliferation, phosphorylation, cell adhesion, cell motion, intracellular signaling cascade, gene expression, apoptosis, and cellular homeostasis, among others, were highly enriched (Fig. 2D). Surprisingly, there was no enrichment for genes associated with inflammatory response. Taken together, these data imply that the main biological processes affected by c-Jun in TNFα-stimulated cells are cancer-related functions, controlling cancer progression, rather than inflammatory responses.
AP-1 Regulates TNFα-mediated Breast Cancer Progression

TNFα/AP-1 Signaling Regulates Apoptosis and Cell Invasion and Determines Clinical Outcome of Breast Cancers—Since analysis of the c-Jun cistrome and transcriptome under conditions of TNFα stimulation suggested involvement of c-Jun in the regulation of expression of genes governing apoptosis and cell invasion, we next evaluated the effect of c-Jun depletion on cell apoptosis and invasion in TNBC cells in response to TNFα.

TNFα is a potent inducer of apoptosis in many tumor cell lines (22, 23). Consistently, we found that TNFα treatment induced apoptosis in BT549 cells, as judged by induction of the apoptotic marker cleaved caspase-3 (Fig. 3A). Importantly, c-Jun knockdown further sensitized BT549 cells to TNFα-induced apoptosis (Fig. 3A, compare lanes 4 and 2). These results were confirmed by an ELISA assay for apoptosis (Fig. 3A). The effect of c-Jun in sensitizing cells to TNFα-induced apoptosis was confirmed in an additional TNBC cell line, Hs578T (data not shown). To examine the effect of c-Jun on TNFα-induced invasion of TNBC cells, we performed transwell cell invasion assays for BT549 cells and observed that knockdown of c-Jun expression reduced TNFα-induced cell invasion (Fig. 3B). Our results show that c-Jun knockdown reduced the invasion ability of non-stimulated cells, which is consistent with our previous results (11). These results suggest that c-Jun potentiates TNFα-induced TNBC cell invasion.

We identified c-Jun direct target genes with known regulatory functions in apoptosis and cell invasion. Among 204 putative c-Jun direct target genes (Fig. 2B, 23 anti-apoptotic genes such as SNAI2 were identified to be down-regulated upon c-Jun knockdown, whereas 13 pro-apoptotic genes were identified to be up-regulated upon c-Jun knockdown (Fig. 3C). Moreover, 14 genes that promote cell invasion such as ZEB2 were down-regulated by c-Jun knockdown, whereas 5 genes that repress cell invasion were up-regulated by c-Jun knockdown. We further assessed whether the c-Jun-regulated pro-invasion gene set has a broader clinical significance using the Gene Expression-Based Outcome for Breast Cancer Online (GOBO) tool, expressing the outcomes in Kaplan-Meier survival plots. High expression levels of this pro-invasion gene set were shown to be associated with poor outcome in breast cancers, predominantly in basal tumors from 282 individuals (Fig. 3D). Collectively, these results indicate that the c-Jun-regulated gene expression program in response to TNFα has the potential to be used as a prognostic tool in breast cancer, especially TNBC.

TNFα/AP-1 Signaling Regulates Apoptosis and Cell Invasion via Ninj1—To determine the mechanisms how c-Jun affects TNFα-regulated cancer progression, i.e. reduced apoptosis and increased migration we focused on the c-Jun target gene, Nerve injury-induced protein 1 (Ninjurin1, Ninj1) which includes a TNFα-induced c-Jun binding site in close proximity to its TSS (Fig. 4A). Recently, it has been reported that lack of Ninj1 leads to growth suppression in colon cancer cells, implying that Ninj1 has potential oncogenic functions (24). However, its role in breast tumor growth and progression has not been studied. A highly conserved sequence with high homology to the AP-1 consensus motif was identified in the c-Jun binding region in close proximity to Ninj1 TSS (Fig. 4B). This region seems to be an active enhancer, marked by enrichment of H3K4me1, H3K4me3 and H3K27Ac (Fig. 4B). A ChIP-qPCR assay confirmed TNFα-induced c-Jun recruitment to the Ninj1 promoter (Fig. 4C). Furthermore, c-Jun knockdown resulted in reduced TNFα induction of Ninj1 mRNA expression (Fig. 4D). This finding was further validated in another TNBC cell line Hs578T (data not shown). These data demonstrate that AP-1 directly activates Ninj1 transcription.

We further analyzed how Ninj1 affects apoptosis and cell invasion in BT549 cells. We found that Ninj1 knockdown enhanced apoptosis under both untreated and TNFα-stimulated conditions, and further enhanced TNFα-induced apoptosis (Fig. 4E). The transwell cell invasion assay showed that Ninj1 knockdown greatly reduced the invasiveness of untreated and TNFα-treated BT549 cells (Fig. 4F). Together, these findings indicate that Ninj1 is a potential oncogene that increases malignant characteristics of TNBC cells, in agreement with previous findings in colon cancer cells (24). To determine the role of Ninj1 in apoptosis and invasion downstream of c-Jun, we examined whether simultaneous c-Jun knockdown and Ninj1 overexpression would rescue cells from the effects of c-Jun depletion. Increased apoptosis of BT549 cells upon c-Jun knockdown was reversed by overexpression of Ninj1 (Fig. 4G). This was further confirmed by an ELISA assay for apoptosis in BT549 cells (Fig. 4G) and Hs578T cells (data not shown). Furthermore, we found that reduced cell invasiveness upon c-Jun knockdown was reversed by overexpression of Ninj1 (Fig. 4H). These results demonstrate that TNFα/c-Jun signaling regulates apoptosis and cell invasion via Ninj1.

Identification of TNFα-induced AP-1 Binding Sites Flanking the CXC Chemokine Cluster on Chromosome 4—TNFα is well known as a central regulator of inflammation. Consistently, our transcriptome analysis identified 62 TNFα-induced inflammatory response genes. For example, TNFα treatment resulted in a striking induction of an array of CC and CXC chemokines involved in inflammatory cell recruitment (25), including Ccl2, Ccl5, Ccl7, and Ccl20, as well as Cxcl1, Cxcl12, Cxcl3, and Cxcl6. We identified a region which showed strong TNFα-induced c-Jun binding at the promoters of IL8, CXCL1, 2, and 3 (Fig. 5A, marked by solid boxes), within a genomic region spanning 0.4 MB on chromosome 4. Quantitative ChIP-qPCR experiments were performed to confirm TNFα-induced c-Jun recruitment to the promoters of IL8, CXCL1, 2, and 3 (Fig. 5B). We further analyzed whether the TNFα stimulated recruitment of c-Jun leads to changes in expression of the associated genes. Surprisingly, knockdown of c-Jun only slightly impaired TNFα-induced expression of IL8, CXCL1, 2, and 3 mRNAs (Fig. 5C), suggesting that c-Jun is not essential for chemokine gene expression.

NF-kB Regulation of the CXC Chemokine Cluster—Because NF-kB is an important regulator of proinflammatory gene expression, and we observed an enrichment of NF-kB motifs in the c-Jun cistrome upon treatment with TNFα, we hypothesized that NF-kB, rather than c-Jun, might be a master regulator of TNFα-inducible expression of chemokine genes. ChIP-qPCR analysis demonstrated that the NK-kB family members, p65 and p50, were bound to the CXC chemokine cluster and that this binding was enhanced upon TNFα treatment (Fig. 6A). The binding of p65 to these sites was more pronounced than p50 (Fig. 6A). Furthermore, siRNA-mediated knockdown of
p65 and p50 dramatically reduced the expression of IL8, CXCL1, 2, and 3 mRNAs in response to TNFα (Fig. 6, B and C). We further investigated the relationship between NF-κB and c-Jun recruitment to the promoters of IL8, CXCL1, 2, and 3 loci in the presence of TNFα. Knockdown of p65 and p50 prevented the recruitment of c-Jun to the promoters of IL8, CXCL1, 2, and 3 loci in the presence of TNFα.
3 (Fig. 6D). In contrast, knockdown of c-Jun increased rather than decreased the recruitment of p65 and p50 (Fig. 6E). The increased binding of NF-κB upon c-Jun knockdown could result from reduced competition between these factors for chromatin binding. Together, these findings indicate that NF-κB might be a pioneer factor for c-Jun recruitment to this

**Fig. 6.**

A. c-Jun genomic binding site

B. c-Jun peak at the Ninj1 promoter

C. Ninj1 ChIP-qPCR

D. Ninj1 qPCR

E. siRNA

F. No TNFα, TNFα

G. TNFα

H. Invasion assay
FIGURE 4. TNFα/AP-1 signaling regulates apoptosis and cell invasion via Ninj1. A, c-Jun binding peak at the promoter of the Ninj1 gene in TNFα-stimulated cells derived from ChIP-Seq. Black solid frames indicate positions of c-Jun binding peaks at the promoters of IL8, CXCL1, CXCL3, and CXCL2. At the top is shown a schematic representation of exons of the genes within a region spanning 500 kb on chromosome 4. B, ChIP-qPCR analysis confirms TNFα-induced recruitment of c-Jun to CXC chemokine genes. Data are shown as means with S.D. *** p < 0.001 compared with no TNFα (n = 3). C, mRNA levels of CXC chemokine genes were determined by qPCR in BT549 cells after transfection with control or c-Jun siRNA for 72 h, and followed by TNFα treatment for the indicated times. p values were generated by Student’s t test.

FIGURE 5. Identification of TNFα-induced AP-1 binding sites flanking the CXC chemokine cluster on chromosome 4. A, TNFα-induced c-Jun binding at chromosome 4 flanking the CXC chemokine gene cluster determined by ChIP-Seq. Black solid frames indicate positions of c-Jun binding peaks at the promoters of IL8, CXCL1, CXCL3, and CXCL2. At the top is shown a schematic representation of exons of the genes within a region spanning 500 kb on chromosome 4. B, ChIP-qPCR analysis confirms TNFα-induced recruitment of c-Jun to CXC chemokine genes. Data are shown as means with S.D. *** p < 0.001 compared with no TNFα (n = 3). C, mRNA levels of CXC chemokine genes were determined by qPCR in BT549 cells after transfection with control or c-Jun siRNA for 72 h, and followed by TNFα treatment for the indicated times. p values were generated by Student’s t test.
gene cluster and is required for the regulation of TNFα-inducible inflammatory genes, whereas c-Jun has little effect.

**Discussion**

Proinflammatory signaling in cancers activated by inflammatory stimuli such as TNFα in the tumor microenvironment facilitates both tumor development and metastatic progression (12). Although it has long been known that AP-1-mediated gene expression in response to inflammatory cytokines is important for the pathogenesis of a range of diseases including arthritis, septic shock, and inflammatory bowel disease (26), the role of AP-1-mediated proinflammatory signaling in tumor growth and progression remains largely unknown. In this study, we have defined molecular mechanisms that underlie TNFα/AP-1-mediated proinflammatory signaling, its functional consequences and potential clinical relevance in TNBCs. We demonstrate that the AP-1 transcription factor c-Jun is a mediator of TNFα-regulated transcriptional events in TNBC cells. High-level expression of c-Jun-regulated pro-invasion gene set is associated with poor outcome in breast cancer, especially TNBC. Importantly, we show that the main biological processes affected by c-Jun in the presence of TNFα in TNBC cells are cancer-related functions, controlling cancer progression, rather than inflammatory responses.

To uncover AP-1-regulated genes in response to TNFα signaling, we report the first genome-wide map of c-Jun binding sites in TNFα-stimulated TNBC cells. We identified 13,800 c-Jun binding regions in the presence of TNFα, of which 9230 binding sites represented a de novo TNFα-induced c-Jun cis-act. The dominant binding motif was an AP-1 motif in both non-stimulated and TNFα-stimulated c-Jun cistromes, suggesting NF-κB as a pioneer factor providing points of chromatin access for the recruitment of AP-1 and other collaborating transcription factors.

It has been described that AP-1 plays a fundamental role in mediating tumor promotion induced by TNFα in mice through induction of a specific subset of AP-1 responsive genes (27), yet its target genes in human cells are not well characterized. To systematically address the role of c-Jun in TNFα-regulated gene expression programs, we used c-Jun knockdown experiments in BT549 cells. BT549 cells have been classified as basal-B, specifically as mesenchymal, claudin-low, and highly invasive (28). We showed that c-Jun controls around one-third of the TNFα-driven transcriptional events. Integration of transcriptome and cistrome data identified 204 direct c-Jun target genes in the presence of TNFα. Analyzing cellular functions of these genes based on gene ontology indicates that c-Jun, in this system, affects genes involved in cancer-related functions such as regulation of cell proliferation, cell adhesion, cell motility, and apoptosis. Taken together, these results suggest
that the biological processes affected by c-Jun can influence the progression of established breast cancer.

TNFα is a potent cytokine that induces apoptosis by several pathways. The most widely accepted pathway involves TNFR1 interacting with TRADD, which serves as a platform for recruitment of additional mediators, including FADD and TRAF2 (29). Caspase-8 is next recruited to this protein complex, and the active caspase-8 initiates a caspase cascade, which results in apoptosis. At the same time, TNFα signaling also activates the NF-κB survival pathway. NF-κB activation antagonizes TNFα-induced apoptosis by inducing the transcription of anti-apoptotic genes, including members of the Bcl-2 family (30). So far, the effect of AP-1 activation on TNFα-induced apoptosis in cancer cells has not been well characterized. The role of c-Jun in TNFα-induced apoptosis in mouse fibroblasts remains controversial. It was reported that c-Jun cooperates with NF-κB to prevent apoptosis induced by TNFα (31). Contrary to this is the observation that c-Jun does not affect TNFα-induced apoptosis (32). Our study showed for the first time that suppression of AP-1 signaling can potentiate TNFα-induced apoptosis in TNBC cells, which is analogous to the function of NF-κB in TNFα-induced apoptosis. TNFα-induced apoptosis has been previously observed in other breast cancer cell lines, including the estrogen receptor-positive MCF-7 (33) and HER2 overexpressing SK-BR-3 (23).

More and more evidence indicate that inflammation plays a critical role in tumor progression, including angiogenesis, invasion and metastasis (12). Indeed, many cancers arise from sites of infection, chronic irritation and inflammation (34). In this regard, recent studies have shown that proinflammatory signaling is associated with more aggressive breast cancers (35). More recent data suggest that, depending on the breast cancer subtypes, proinflammatory signaling can be associated with either good or poor clinical outcomes (36). We show here that, in highly invasive breast cancer cells, AP-1/proinflammatory signaling potently increases cell invasion. Consistent with this observation, we previously reported that TNFα/AP-1 signaling induces EMT in TNBC cells (14). Our results indicate a critical role for AP-1 signaling potentiates TNFα-driven tumor aggressiveness. Furthermore, we have identified a unique transcriptome, which consists of 14 target genes directly regulated by c-Jun upon proinflammatory signaling, that offers to be used as tool to predict the outcome of breast cancer, especially TNBC.

The involvement of Ninj1 in mediating the effects of AP-1 on apoptosis and cell invasion is intriguing. Ninj1 is a small adhesion molecule that is involved in cell-cell interactions (37). It is broadly expressed not only in the injured nervous system but in all tissues of epithelial origin (37). Recent evidence indicates that Ninj1 has oncogenic potential. In line with this, up-regulation of Ninj1 expression was observed in hepatocellular carcinoma (38) and acute lymphoblastic leukemia (39). Cho et al. reported that knockdown of Ninj1 suppresses cell proliferation and enhances apoptosis in human RKO colon cancer cells (24). They further demonstrated that silencing of Ninj1 increases p53 expression through modulating p53 mRNA translation in RKO cells that express wild-type p53. Here, we provide evidence that Ninj1 silencing enhances TNFα-induced apoptosis and inhibits cell invasion in TNBC cells, supporting that Ninj1 acts as a tumor promoter.

TNFα is best known as a central regulator of inflammation. Consistently, our transcriptome analysis identified 62 TNFα-induced inflammatory response genes. For example, TNFα stimulation resulted in a striking induction of an array of CC and CXC chemokines involved in inflammatory cell recruitment (25), including Ccl2, Ccl5, Ccl7, and Ccl20, as well as Cxcl1, Cxcl2, Cxcl13, and Cxcl6. TNFα triggers activation of both NF-κB and AP-1 signaling pathways. While the critical role of the transcription factor NF-κB for the TNFα-induced inflammatory response is well established, the role of AP-1 signaling in cytokine/chemokine expression is not fully explored. Here, we identify a TNFα-inducible c-Jun binding region flanking a cluster of four highly TNFα-regulated chemokine genes. This region has been found to harbor p65 binding sites induced by interleukin-1 (IL-1) in KB cells (40). By quantitative ChIP-PCR, we confirmed the binding of both AP-1 and p65 at this CXC locus. However, our c-Jun and NF-κB knockdown experiments reveal that c-Jun is not an essential component governing chemokine gene expression, whereas NF-κB appears to be an important regulator. Accordingly, there was no enrichment for genes associated with inflammatory response in the c-Jun direct target transcriptome in the presence of TNFα (Fig. 2D). However, few c-Jun target genes, such as TNFAIP8L2 (41) and MMP9 (42) have been shown to be involved in the inflammatory response. Of relevance to our reported data, NF-κB activation was essential for induction of IL-6 and IL-8 mRNA expression in rheumatoid arthritis synovial fibroblasts, whereas c-Jun has no effect (43). On the contrary, induction of IL8 by TNFα plus interferon γ has been suggested to be mediated through NF-κB in cooperation with AP-1 (44). It is therefore possible that c-Jun may contribute to the expression of IL-8 in other cell types or when cells are subject to alternative stimulation. Mechanistically, we speculate that NF-κB might be a pioneer transcription factor that functions to maintain an open chromatin architecture, because its absence diminishes c-Jun binding. In contrast, the absence of c-Jun did not affect, but rather increased NF-κB binding, which may be due to reduced competition between these two factors for chromatin binding. Indeed, recent studies have suggested a role of NF-κB as a pioneer factor that promotes an open chromatin in response to proinflammatory signaling (36, 45). The role of AP-1 binding but no activation at this CXC chemokine locus remains to be determined. It is possible that the AP-1 exerts further functions such as regulating the kinetics of TNFα-mediated gene expression or mediating long-range chromatin interactions.

In conclusion, our results revealed a novel mechanism by which the inflammatory microenvironment stimulates breast cancer progression. We propose that inhibition of AP-1 function may have therapeutic potential in aggressive breast cancer.

**Author Contributions**—Y. Q. and C. Z. designed experiments. Y. Q., H. H., and C. Z. performed experiments. C. Z., Y. Q., P. J., and I. S. analyzed data. Y. Q., H. H., and C. Z. interpreted the results of experiments. Y. Q., C. Z., and K. D. W. drafted the manuscript. All authors reviewed the results and approved the final version of the manuscript.
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