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c-Jun N-terminal kinase promotes stem cell phenotype in triple-negative breast cancer through up-regulation of Notch1 via activation of c-Jun

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Abstract

c-Jun N-terminal kinase (JNK) plays a vital role in malignant transformation of different cancers, and JNK is highly activated in basal-like triple-negative breast cancer (TNBC). However, the roles of JNK in regulating cancer stem-like cell (CSC) phenotype and tumorigenesis in TNBC are not well defined. JNK is known to mediate many cellular events via activating c-Jun. Here, we found that JNK regulated c-Jun activation in TNBC cells and that JNK activation correlated with c-Jun activation in TNBC tumors. Furthermore, the expression level of c-Jun was significantly higher in TNBC tumors than in non-TNBC tumors, and high c-Jun mRNA level was associated with shorter disease-free survival of patients with TNBC. Thus, we hypothesized that the JNK/c-Jun signaling pathway contributes to TNBC tumorigenesis. We found that knockdown of JNK1 or JNK2 or

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

treatment with JNK-IN-8, an ATP-competitive irreversible pan-JNK inhibitor, significantly reduced cell proliferation, the ALDH1⁺ and CD44⁺/CD24⁻ CSC subpopulations, and mammosphere formation, indicating that JNK promotes CSC self-renewal and maintenance in TNBC. We further demonstrated that both JNK1 and JNK2 regulated *Notch1* transcription via activation of c-Jun and that the JNK/c-Jun signaling pathway promoted CSC phenotype through Notch1 signaling in TNBC. In a TNBC xenograft mouse model, JNK-IN-8 significantly suppressed tumor growth in a dose-dependent manner by inhibiting acquisition of the CSC phenotype. Taken together, our data demonstrate that JNK regulates TNBC tumorigenesis by promoting CSC phenotype through Notch1 signaling via activation of c-Jun and indicate that JNK/c-Jun/Notch1 signaling is a potential therapeutic target for TNBC.

Keywords

c-Jun N-terminal kinase; c-Jun; Notch1; cancer stem-like cells; triple-negative breast cancer

INTRODUCTION

Triple-negative breast cancer (TNBC), a subtype of breast cancer that is negative for estrogen receptor, progesterone receptor, and human epidermal growth factor receptor 2 (HER2), comprises 15–20% of breast cancers. Compared to other types of breast cancer, TNBC is more aggressive and has a worse prognosis because of its tendency to metastasize and recur¹. The major cause of death of patients with TNBC is recurrence (30–40% of cases), which might be due to the cancer stem-like cell (CSC) phenotype of TNBC cells^{2,3}. CSCs, a subpopulation of cancer cells, possess the capacity to self-renew and give rise to heterogeneous lineages that make up a tumor^{4,5}. CSCs play a critical role in tumor initiation, recurrence, and metastasis and are responsible for resistance to chemotherapy and radiotherapy^{5–8}.

c-Jun N-terminal kinase (JNK) has been reported to promote CSC self-renewal in glioma by enhancing expression of genes important for CSC self-renewal and maintenance⁹. JNK is a member of the MAPK family, which also includes ERK and p38 MAPKs. Three major JNK isoforms, JNK1, JNK2, and JNK3, have been identified. JNK1 and JNK2 are ubiquitously expressed, whereas JNK3 is expressed predominantly in the brain, heart, and testis¹⁰. JNK is activated through phosphorylation on Thr183 and Tyr185 residues by MAPK kinase MKK4/JNKK1 or MKK7/JNKK2¹¹. Once activated, JNK phosphorylates a large range of substrates, including transcription factors, such as c-Jun, ATF-2, Elk-1, p53, and c-Myc, and other molecules¹¹. JNK mediates many cellular events via activating c-Jun through phosphorylation on Ser63 or Ser73 residues. JNK has been implicated in proliferation, differentiation, apoptosis, stress response, and inflammation. JNK also plays a vital role in malignant transformation and metastasis in different cancers^{12,13}. JNK is highly activated in basal-like TNBC¹⁴. Reduced JNK activation is associated with better overall survival of patients with infiltrating ductal breast carcinoma¹⁵. However, the roles of JNK in regulating CSC phenotype and tumorigenesis in TNBC remain unknown.

In this study, we investigated the role of JNK signaling in regulating CSCs and tumorigenesis in TNBC. We found that JNK regulates TNBC tumorigenesis by promoting CSC self-renewal and maintenance through Notch1 signaling via activation of c-Jun. We also found that inhibition of JNK signaling by JNK-IN-8, an ATP-competitive irreversible pan-inhibitor of JNK that targets JNK1, JNK2, and JNK3 by binding to the cysteine 116 residue¹⁶, suppressed tumor growth in a TNBC xenograft mouse model by inhibiting acquisition of the CSC phenotype. Our findings indicate that the JNK signaling pathway is a potential therapeutic target for eliminating CSCs in TNBC.

RESULTS

High c-Jun mRNA level is associated with shorter disease-free survival (DFS) of patients with TNBC, and c-Jun activation correlates with JNK activation. c-Jun, a transcription factor and a substrate of JNK, ERK, and p38, has been implicated in progression of different cancers^{17–19}. However, there has been no systematic analysis of association between expression and activation of c-Jun and clinical outcomes of patients with TNBC. We first compared protein expression levels of total c-Jun and phosphorylated c-Jun (pc-Jun) in primary tumors of patients with TNBC and non-TNBC using a public reverse-phase protein array (RPPA) dataset of The University of Texas MD Anderson Cancer Center²⁰. The expression level of total c-Jun was significantly higher in tumors of patients with TNBC than in tumors of patients with non-TNBC ($P < 2.2 \times 10^{-16}$; Figure 1a, left panel). The expression level of c-Jun phosphorylated at Ser73 was also higher in tumors of patients with TNBC than in tumors of patients with non-TNBC; however, the difference was not significant ($P = 0.063$; Figure 1a, right panel). Furthermore, the expression level of total c-Jun was significantly higher in tumors of patients with TNBC than in tumors of patients with estrogen receptor-negative and HER2-positive (HER2+) breast cancer ($P = 0.003$; Supplementary Figure 1a, left panel) or HER2-negative and estrogen receptor-positive (HR+) breast cancer ($P < 2.2 \times 10^{-16}$; Supplementary Figure 1a, left panel). The expression level of c-Jun phosphorylated at Ser73 was also higher in tumors of patients with TNBC than in tumors of patients with HR+ breast cancer ($P = 0.021$; Supplementary Figure 1a, right panel); however, no difference was observed between tumors of patients with TNBC and tumors of patients with HER2+ breast cancer ($P = 0.787$; Supplementary Figure 1a, right panel). We next examined the association between mRNA level of c-Jun and DFS of patients with primary TNBC using combined data from datasets previously published by Wang et al²¹ and Schmidt et al²². We found that high mRNA level of c-Jun correlated with shorter DFS of patients with primary TNBC ($P = 0.025$; Figure 1b). These results suggested that c-Jun may have clinical significance in TNBC.

Since c-Jun is a substrate of JNK, ERK, and p38, we next examined the association between activation of JNK or p38 and activation of c-Jun in primary tumors of patients with TNBC using the RPPA dataset. The association between ERK activation and c-Jun activation could not be examined because no data on ERK level were available. We found that expression level of c-Jun phosphorylated at Ser73 correlated with expression level of JNK phosphorylated at Thr183 ($r_{\text{Pearson}} = 0.645$; $P < 2.2 \times 10^{-16}$; Figure 1c) but not with expression level of phospho-p38 ($r_{\text{Pearson}} = -0.086$; $P = 0.334$; data not shown). Similar results were observed in all TNBC tumors, including primary and metastatic tumors (data not shown).

On the basis of the association of higher c-Jun mRNA level with reduced DFS and the correlation of c-Jun activation with JNK activation, we hypothesized that JNK/c-Jun signaling contributes to TNBC tumorigenesis.

Inhibition of JNK activity suppresses c-Jun phosphorylation and thereby inhibits cell growth

We first examined the role of JNK/c-Jun signaling in TNBC cell growth. We used HCC70, SUM149, and MDA-MB-231 TNBC cell lines, which express high levels of JNK and are tumorigenic and invasive. Because of limited expression of JNK3 in tissues¹⁰, we focused on JNK1 and JNK2. We found that inhibition of JNK activity using JNK-IN-8 in HCC70, SUM149, and MDA-MB-231 cells specifically suppressed c-Jun phosphorylation at both Ser63 and Ser73 but did not suppress phosphorylation of ATF-2 at Thr71 or Elk-1 at Ser383 (Figure 1d; Supplementary Figure 1b, left panel), indicating the targeting specificity of JNK-IN-8 toward JNK/c-Jun signaling. In addition, knockdown of JNK1 or JNK2 in HCC70 and SUM149 cells led to a dramatic reduction in phosphorylation of c-Jun and slight reductions in phosphorylation of ATF-2 and Elk-1 (Figure 1e). This finding was confirmed with a second set of siJNK1 and siJNK2 (Supplementary Figure 1b, right panel), indicating that JNK regulates c-Jun activation in TNBC cells. To confirm that JNK-IN-8 inhibited JNK activity toward c-Jun by specifically binding to the cysteine 116 residue on JNK, the JNK-IN-8 binding site, we overexpressed either MKK7-JNK1/2, a construct in which JNK is constitutively phosphorylated by MKK7 at Thr183, or MKK7-JNK1/2-C116S, a JNK1/2 mutant in which cysteine at 116 was replaced with serine, and then examined the effect of JNK-IN-8 on c-Jun phosphorylation. Compared to overexpression of the vector control or MKK7-JNK1/2, overexpression of MKK7-JNK1/2-C116S largely blocked the inhibitory effect of JNK-IN-8 on c-Jun phosphorylation at both Ser63 and Ser73 (Supplementary Figure 1c), suggesting that JNK-IN-8 inhibits JNK kinase activity toward c-Jun by specifically binding to the Cys116 residue on JNK.

To define the role of JNK in TNBC cell proliferation, we knocked down JNK in HCC70 and SUM149 cells and then examined the effects on cell proliferation. Compared to the scrambled siRNA (SCR) control, JNK1 or JNK2 knockdown (Figure 2a, left panel) significantly reduced proliferation (Supplementary Figure 2a, left panel) as analyzed using the CellTiter-Blue viability assay and significantly reduced anchorage-independent growth (Figure 2a, right panel) as analyzed using the soft agar assay. Compared to JNK1 knockdown, JNK2 knockdown reduced anchorage-independent growth by 41.40% ($P < 0.05$) more in HCC70 cells and 32.89% ($P < 0.05$) more in SUM149 cells. The inhibitory effects of JNK knockdown on anchorage-independent growth were further confirmed with the second set of siJNK1 and siJNK2 (Supplementary Figure 2a, right panel). JNK-IN-8 treatment also significantly reduced proliferation (Supplementary Figure 2b) and anchorage-independent growth (Figure 2b) in a dose-dependent manner. Similar results were observed in MDA-MB-231 cells (Supplementary Figure 2c). To elucidate the mechanisms of growth inhibition, we performed cell cycle distribution and apoptosis analyses. We found that JNK-IN-8 at low doses or for short periods suppressed cell proliferation by arresting the cell cycle at G2/M phase in HCC70 cells and at G1 phase in SUM149 and MDA-MB-231 cells, while JNK-IN-8 at high doses and for long periods suppressed cell proliferation by inducing

apoptosis in all three types of cells as indicated by increases in the sub-G1 fraction (Supplementary Table 1), in Annexin-V and 7-AAD staining (Supplementary Figure 3a), and in expression of cleaved caspase 3 and cleaved PARP (Supplementary Figure 3b). Moreover, overexpression of MKK7-JNK1/2-C116S largely blocked the inhibitory effect of JNK-IN-8 on cell proliferation (Supplementary Figure 2d), confirming that JNK-IN-8 inhibited cell proliferation by specifically targeting JNK through binding to the Cys116 residue on JNK. Together, these results suggested that JNK signaling is essential for TNBC cell growth.

JNK signaling is required for migration and invasion of TNBC cells

We next investigated the involvement of JNK signaling in migration and invasion, the two key processes of metastasis, in TNBC cells. Compared to the SCR control, JNK1 or JNK2 knockdown reduced migration and invasion in both HCC70 and SUM149 cells, with JNK2 knockdown having the greater impact (Figure 2c). Compared to JNK1 knockdown, JNK2 knockdown reduced migration and invasion by 25.85% ($P < 0.05$) and 29.77% ($P < 0.01$) more, respectively, in HCC70 cells and migration and invasion by 26.28% ($P < 0.001$) and 18.86% ($P < 0.0001$) more, respectively, in SUM149 cells. Inhibition of JNK signaling using JNK-IN-8 also inhibited migration and invasion (Figure 2d). Similar results were observed in MDA-MB-231 cells (Supplementary Figure 4a). These results suggested a possible role of JNK signaling in promoting migration and invasion of TNBC cells. Additionally, overexpression of MKK7-JNK1/2-C116S largely blocked the inhibitory effect of JNK-IN-8 on migration and invasion (Supplementary Figure 4b), confirming that JNK-IN-8 inhibits these cellular activities by specifically targeting JNK.

JNK signaling is required for CSC self-renewal and maintenance

JNK has been shown to promote self-renewal and maintenance of CSCs in glioma²³, and high JNK expression is seen in CSCs isolated from patient glioma cells²⁴. In hepatoma, high JNK1 activation is associated with overexpression of stem cell markers¹³. However, whether JNK regulates CSCs in TNBC remains unknown. To address this knowledge gap, we knocked down JNK and assessed the effects on CSC self-renewal and maintenance using the mammosphere formation assay, a standard assay to study stem cell properties *in vitro*²⁵. Compared to the SCR control, JNK1 or JNK2 knockdown significantly reduced mammosphere formation in both HCC70 and SUM149 cells, with JNK2 knockdown having a greater effect (Figure 3a). Compared to JNK1 knockdown, JNK2 knockdown reduced mammosphere formation by 40.64% ($P < 0.05$) more in HCC70 cells and 16.10% ($P < 0.05$) more in SUM149 cells. The inhibitory effects of JNK1 or JNK2 knockdown on mammosphere formation were confirmed with the second set of siJNK1 and siJNK2 (Supplementary Figure 5a). Inhibition of JNK signaling using JNK-IN-8 also significantly reduced mammosphere formation (Figure 3b) and suppressed formation of primary, secondary, and tertiary mammospheres (Figure 3c), indicating an essential role for JNK signaling in CSC self-renewal and maintenance. In addition, JNK1 or JNK2 knockdown or JNK-IN-8 treatment reduced the ALDH1⁺ (Figures 3d and e, respectively) and CD44⁺/CD24⁻ (Supplementary Figure 5b) subpopulations. Similar results were observed in MDA-MB-231 cells (Supplementary Figures 5b-d). Additionally, overexpression of MKK7-JNK1/2-C116S largely blocked the inhibitory effect of JNK-IN-8 on mammosphere

formation (Supplementary Figure 5e), confirming that JNK-IN-8 inhibits mammosphere formation by specifically targeting JNK. In contrast, compared to overexpression of the vector control or dominant-negative JNK (apf, a JNK mutant in which the dual phosphorylation motif Thr183-Pro-Tyr185 was replaced with Ala-Pro-Phe), overexpression of constitutively active MKK7-JNK1 or MKK7-JNK2 led to an increase in mammosphere formation (Supplementary Figure 6). Unexpectedly, in dominant-negative JNK2-overexpressing cells, an increase in mammosphere formation was observed (Supplementary Figure 6). This effect may have been due to incomplete inhibition of JNK2 kinase activity, an interpretation supported by the finding of Wojtaszek et al showing that expression of dominant-negative JNK2 in stable clones inhibited hypertonicity-induced JNK activation by 40–70%²⁶. Taken together, these results clearly demonstrate that both JNK1 and JNK2 promote CSC self-renewal and maintenance in TNBC, with JNK2 playing a dominant role.

JNK regulates *Notch1* transcription via activating c-Jun

To dissect the molecular mechanisms underlying JNK signaling-mediated CSC regulation, we treated HCC70 and SUM149 cells with JNK-IN-8 for 48 h and then performed stem cell RT-PCR array analysis. We found that JNK inhibition led to down-regulation of stem cell-related molecules Notch1, FGF2, and cyclin D1 and up-regulation of stem cell-related molecules FZD1, FRAT1, HDAC1, and BMP3 at the mRNA level (Supplementary Table 2). Furthermore, Western blot analysis showed that of these molecules, Notch1 was the one whose expression at the protein level was most reduced following JNK-IN-8 treatment (Figure 4a), suggesting the involvement of Notch1 in JNK signaling-mediated CSC regulation. Next, we assessed whether JNK regulates Notch1 expression by examining the effects of JNK knockdown on Notch1 expression. We observed a reduction in Notch1 expression at the protein level following JNK1 or JNK2 knockdown in both HCC70 and SUM149 cells, with JNK2 knockdown having a greater effect (Figure 4b). The inhibitory effect of JNK knockdown on Notch1 expression was confirmed using the second set of siJNK1 and siJNK2 (Supplementary Figure 1b, right panel). As expected, a reduction in c-Jun phosphorylation at both Ser63 and Ser73 was also observed (Figure 4b; Supplementary Figure 1b, right panel), suggesting that JNK might regulate Notch1 expression via activating c-Jun. This interpretation was further supported by our finding that c-Jun knockdown reduced Notch1 expression at the protein level (Figure 4c). These results clearly showed that JNK regulates Notch1 expression via activation of c-Jun.

To address whether c-Jun regulates Notch1 expression at the transcriptional level, we examined Notch1 mRNA level using qRT-PCR at 96 h following c-Jun knockdown. A reduction in Notch1 mRNA expression was observed following c-Jun knockdown in both HCC70 and SUM149 cells (Figure 4d, left panel). Similar results were observed at 72 h following c-Jun knockdown (data not shown). To confirm that c-Jun regulates transcription of *Notch1*, we performed luciferase reporter assay using the HRP22069-PG04 vector, which contains the *Notch1* promoter and the coding sequences for both *Gaussia* luciferase and secreted alkaline phosphatase. Secreted alkaline phosphatase was used as a control for off-target effects of the expression plasmids and the transfection procedure. Compared to the SCR control, c-Jun knockdown significantly reduced luciferase activity in both HCC70 and SUM149 cells transfected with the HRP22069-PG04 vector (Figure 4d, right panel),

confirming that c-Jun regulates Notch1 expression at the transcriptional level. In addition, JNK-IN-8 treatment reduced expression of c-MYC, cyclin D1, and p21 (Figure 4a), downstream molecules in the Notch1 signaling pathway, indicating that Notch1 is the major stem cell signaling molecule downstream of JNK. Together, these results demonstrated that JNK regulates Notch1 expression at the transcriptional level via activating c-Jun.

JNK/c-Jun signaling promotes CSC self-renewal through Notch1 signaling

To assess whether JNK promotes TNBC cell growth and CSC self-renewal through Notch1 via activating c-Jun, we knocked down expression of c-Jun or Notch1 and examined the effects on TNBC cell growth and CSC self-renewal. Compared to the SCR control, c-Jun knockdown (Figure 5a) reduced cell proliferation (Figure 5b) and anchorage-independent growth (Figure 5c) in both HCC70 and SUM149 cells. Similar results were obtained following Notch1 knockdown (Figures 5a-c). Knockdown of c-Jun or Notch1 also reduced mammosphere formation (Figure 5d) and the ALDH1⁺ subpopulation (Figure 5e). In addition, Notch1 knockdown in cells overexpressing constitutively active MKK7-JNK1 or MKK7-JNK2 reduced mammosphere formation (Supplementary Figure 7a), confirming that JNK promotes CSC phenotype through Notch1 signaling. In contrast, overexpression of c-Jun or Notch1 intracellular domain (NICD1) largely blocked the inhibitory effects of JNK-IN-8 on mammosphere formation (Supplementary Figure 7b), confirming that JNK-IN-8 inhibits CSC self-renewal through inhibition of JNK/c-Jun/Notch1 signaling. Together, these results suggested that JNK/c-Jun signaling regulates CSCs in TNBC through Notch1 signaling.

JNK-IN-8 suppresses tumor growth in a TNBC xenograft mouse model through reducing CSC phenotype

Since suppression of JNK signaling using siRNA or JNK-IN-8 inhibited proliferation and anchorage-independent growth of TNBC cells and self-renewal of CSCs *in vitro*, we examined whether JNK-IN-8 treatment suppressed tumor growth using a SUM149 xenograft mouse model. Compared to the vehicle control, JNK-IN-8 significantly suppressed tumor growth in a dose-dependent manner (Figure 6a, $P < 0.01$ at 15 mg/kg, $P < 0.001$ at 30 mg/kg). This result suggested a vital role for JNK signaling in promoting tumorigenicity of TNBC cells. Tumor samples were collected on day 34 following JNK-IN-8 treatment and analyzed for expression of Ki-67 (proliferation marker), c-Jun, pc-Jun, Notch1, and ALDH1 by immunohistochemical staining (Figure 6b) and measurement of staining intensity (Figure 6c). No difference was observed between vehicle-treated and JNK-IN-8-treated tumors in expression of total c-Jun (data not shown). As expected, expression of Ki-67 ($P < 0.001$), c-Jun phosphorylated at Ser63 ($P < 0.05$), and c-Jun phosphorylated at Ser73 ($P < 0.01$) was significantly reduced in tumors from mice treated with JNK-IN-8 at 30 mg/kg compared to tumors from the vehicle-treated controls (Figures 6b and c). JNK-IN-8 treatment at 15 mg/kg also significantly reduced expression of Ki-67 ($P < 0.01$) and c-Jun phosphorylated at Ser73 ($P < 0.05$). These results suggest that JNK-IN-8 suppressed tumor growth by inhibiting JNK kinase activity toward c-Jun. In addition, expression of Notch1 ($P < 0.05$) and ALDH1 ($P < 0.01$) was significantly reduced in tumors from mice treated with JNK-IN-8 at 30 mg/kg compared to tumors from the vehicle-treated controls (Figures 6b and c), suggesting that JNK-IN-8 suppressed tumor growth by reducing CSC phenotype. Taken

together, our data demonstrate that JNK regulates TNBC tumorigenesis by promoting CSC phenotype through Notch1 signaling via activation of c-Jun and suggest that JNK/c-Jun/Notch1 signaling is a potential therapeutic target for TNBC.

DISCUSSION

JNK has been implicated in malignant transformation and metastasis in different cancers^{12, 13}. However, no studies to date have addressed the roles of JNK in regulating CSCs and tumorigenesis in TNBC. Here, we found that c-Jun was associated with clinical outcomes of TNBC patients and that c-Jun activation correlated with JNK activation in TNBC tumors. We also found that JNK promoted CSC self-renewal and maintenance in TNBC through up-regulation of Notch1 via activation of c-Jun. Suppression of JNK signaling inhibited CSC phenotype, leading to growth inhibition of TNBC cells both *in vitro* and *in vivo*. Taken together, our findings demonstrate that JNK/c-Jun/Notch1 signaling promotes CSC phenotype, which might contribute to tumor initiation and progression in TNBC (Figure 6d), and that targeting JNK signaling is a potential therapeutic strategy for TNBC.

JNK was originally discovered as a regulator of apoptosis in response to a variety of stress stimuli²⁷. In recent decades, emerging evidence has suggested that JNK also plays a role in proliferation and transformation of different cancers. JNK can potentiate or suppress tumorigenesis depending on the tumor type and the nature of the oncogenic event. We found that suppression of JNK signaling using JNK-IN-8 inhibited cell growth by arresting the cell cycle at G1 or G2/M phase in the case of exposure for short periods or at low doses and by inducing apoptosis in the case of exposure for long periods and at high doses. Furthermore, JNK-IN-8 suppressed tumorigenicity of TNBC cells in a xenograft mouse model. Consistent with our findings, studies by others also support a role for JNK signaling in promoting tumorigenesis, e.g., in lymphoma²⁸, colorectal cancer²⁹, and lung cancer³⁰. In breast cancer, JNK is highly activated in basal-like TNBC¹⁴; elevated JNK activation is associated with short survival of patients with infiltrating ductal breast carcinoma¹⁵. Furthermore, overexpression of a constitutively active JNK enhanced migration and invasion of breast cancer cells³¹. These findings suggest a pathological role for JNK signaling in different cancers. In contrast, Gozdecka et al showed that deletion of JNK promoted Ras-dependent cellular transformation and liver tumor formation through loss of ATF-2³². In our study, suppression of JNK activity slightly inhibited activation of ATF-2 or Elk-1 but dramatically inhibited activation of c-Jun. We also found that JNK promoted cellular transformation through up-regulation of Notch1 via activation of c-Jun. These differences between our findings and the findings of Gozdecka et al might be due to differences in the cell types used (human TNBC cell lines vs. Ras-transformed mouse embryonic fibroblasts) and tumor types studied (breast vs. liver). Interestingly, our study showed that JNK2 knockdown but not JNK1 knockdown largely suppressed activation of ATF-2, suggesting that JNK2 might promote CSC phenotype in TNBC through both ATF-2 and c-Jun, which form heterodimers and stimulate CRE-dependent transcription³³. This finding may explain why JNK2 knockdown had more substantial inhibitory effects than JNK1 knockdown on TNBC cellular activities and also suggests that JNK1 and JNK2 may regulate TNBC tumorigenesis through different mechanisms. Studies have shown that JNK1 and JNK2 have redundant but distinct functions. Both JNK1 and JNK2 have been shown to promote cell growth, which is

supported by the fact that intact mouse embryos with either *jnk1*^{-/-} or *jnk2*^{-/-} survive, whereas genetic disruption of both *Jnk1* and *Jnk2* alleles in mouse embryos is lethal³⁴. JNK1, but not JNK2, was essential for TNF α -induced proliferation and differentiation of myoblasts³⁵. Skin tumorigenesis was enhanced in *jnk1*^{-/-} mice but suppressed in *jnk2*^{-/-} mice³⁶. Thus, the functions of JNK isoforms depend on the cell type, nature of the stimulus, and involvement of other signaling pathways.

Here, we show that JNK/c-Jun signaling regulated transcription of *Notch1*, which appeared to be responsible for maintaining CSC stemness in TNBC. Aberrantly activated Notch signaling has been implicated in initiation and progression of many different cancers³⁷⁻³⁹. Notch signaling is well known to regulate breast stem cell self-renewal and cell-fate determination^{40,41} and to be critical for maintaining CSC stemness in glioma²³. Inhibition of Notch signaling reduced CD133⁺ CSC subpopulations *in vitro* and brain tumor formation *in vivo*²³. Consistent with our findings, Yoon et al reported that JNK signaling acted through Notch2 to promote self-renewal of glioma CSCs²³. Harrison et al showed that breast CSC activity was governed through Notch4 signaling⁴². More importantly, Notch signaling was aberrantly activated in breast cancer cell lines and invasive primary breast tumor samples⁴². Our study demonstrates that JNK/c-Jun/Notch1 signaling governs self-renewal and maintenance of CSCs in TNBC. In contrast, Cantrell et al showed that JNK2 inhibited p53/Notch1-dependent cell differentiation in normal and cancer-derived mammary cells through down-regulation of *Notch1* transcription⁴³. This inconsistency might be due to differences in the cell types used (human TNBC cell lines *vs.* murine mammary cell lines), tumor types studied (human breast *vs.* murine polyoma middle T antigen mammary), and downstream molecules involved (c-Jun *vs.* p53).

Development of effective therapies for TNBC has been challenging because of the lack of well-defined therapeutic targets. It is believed that many cancers originate from CSCs and that CSCs are responsible for tumor recurrence and metastasis because of their capacity to self-renew and to give rise to heterogeneous lineages that comprise a tumor^{4,5}. The importance of CSCs in tumor initiation and metastasis has positioned CSCs as a potential target for cancer treatment. JNK signaling was essential for self-renewal and maintenance of glioma CSCs²³, and a high expression level of JNK was seen in CSCs isolated from patient glioma cells⁴⁴. Moreover, elevated JNK1 activation was associated with a poor prognosis in hepatoma and enhanced expression of stem cell markers¹³. JNK is highly activated in basal-like TNBC¹⁴. These findings along with ours suggest that controlling CSCs through targeting JNK signaling might overcome the problems of metastasis, recurrence, and resistance seen with current chemotherapies. Although our *in vivo* study showed that JNK inhibition reduced CSC phenotype, we recognize that further *in vivo* studies are needed to confirm that the tumor-initiating capacity of CSCs is dependent on JNKs. An appropriate experiment would be to examine the effects of stable knockdown of JNK1 or JNK2 on the tumor-initiating capacity of CSCs using a limiting-dilution xenotransplantation mouse model^{45,46}.

In conclusion, our findings demonstrate that JNK/c-Jun/Notch1 signaling plays an important role in TNBC tumorigenesis via regulating CSC phenotype and indicate that targeting JNK signaling has potential for TNBC treatment.

MATERIALS AND METHODS

Clinical analysis

Expression levels of proteins of interest in tumors were compared between patients with primary TNBC (n = 129), non-TNBC (n = 267), HER2+ breast cancer (n = 19), and HR+ breast cancer (n = 248) using a public RPPA dataset of MD Anderson Cancer Center. The association between mRNA levels of proteins and DFS of patients with TNBC (n = 79) was determined using combined data from datasets previously published by Wang et al ²¹ and Schmidt et al ²².

Cell culture and reagents

HCC70 and MDA-MB-231 cells were purchased from American Type Culture Collection and SUM149 cells from Asterand (Detroit, MI, USA). HCC70 cells were maintained in RPMI 1640 medium (Life Technologies Inc., Grand Island, NY, USA) and MDA-MB-231 cells in Dulbecco's modified Eagle's medium/F12 medium (Life Technologies Inc., Grand Island, NY, USA), both types of medium supplemented with 10% FBS and 1% antibiotic/antimycotic. SUM149 cells were maintained in Ham's F-12 medium (Life Technologies Inc.) supplemented with 5% FBS, 1% antibiotic/antimycotic, 5 µg/mL insulin, and 1 µg/mL hydrocortisone. All cell lines were validated by DNA typing at the MD Anderson Cancer Center Characterized Cell Line Core (Houston, Texas) and confirmed to be free of mycoplasma. JNK-IN-8 was provided by Dr. Kevin N. Dalby (The University of Texas at Austin, Austin, TX, USA) and Dr. Nathanael S. Gray (Harvard Medical School, Boston, MA, USA).

Mammosphere formation assay

To determine the impact of target gene knockdown on mammosphere formation, at 48 h after siRNA transfection, single-cell suspensions were prepared in TeSR-E8 medium (STEMCELL Technologies Inc., Vancouver, BC, Canada) supplemented with MammoCult proliferation supplements, 1% antibiotic/antimycotic, 4 µg/mL heparin, and 0.48 µg/mL hydrocortisone. The cells were then seeded in six-well ultra-low attachment plates (Corning Inc., Corning, NY, USA). To determine the impact of JNK inhibition on mammosphere formation, the cells were cultured with or without JNK-IN-8. After a 7-day incubation, mammospheres greater than 80 µm in diameter were counted using the GelCount system (Oxford Optronix Ltd., Milton Park, Abingdon, UK).

RT-PCR stem array analysis

A human stem cell RT² Profiler PCR array (Qiagen Inc., Valencia, CA, USA) was used to evaluate the expression of 84 specific genes. Cells were treated with JNK-IN-8 (2.5 µM) for 48 h, and then total RNA was extracted from the cells using the miRNeasy mini kit (Qiagen Inc.) according to the manufacturer's instructions. cDNA was synthesized using the RT² First Strand Kit (Qiagen Inc.) and dispensed into the RT² Profiler PCR array. Each PCR array included five housekeeping genes (*B2M*, *HPRT1*, *RPL13A*, *GAPDH*, and *ACTB*), a control for genomic DNA contamination, a reverse transcription control, and a positive PCR control. The data were analyzed using Qiagen software (<http://www.sabiosciences.com/pcr/>

[arrayanalysis.php](#)). The criteria used to define differential expression of genes between each pair of classes tested were $P < 0.05$ and false discovery rate < 0.2 .

Quantitative RT-PCR

Cells were transfected with SCR (Sigma-Aldrich, St. Louis, MO, USA) or siRNA targeting c-Jun (Sigma-Aldrich). Seventy-two or 96 hours later, total RNA was extracted from the cells using the miRNeasy mini kit (Sigma-Aldrich) according to the manufacturer's instructions. One-step quantitative PCR reactions were performed using the SYBR green qPCR kit (Bio-Rad Laboratories, Hercules, CA, USA) with a pair of Notch1 primers [5'-ACAGCCTCAACGGGTA

CAA-3' (forward) and 5'-CACACGTAGCCACTGGTCAT-3' (reverse)] and a pair of human β -Actin primers [5'-GCGGGAAATCGTGCGTGACATT-3' (forward) and 5'-AGACAGTCTCCA

CTCACCAGGAAG-3' (reverse)]. The mRNA of human housekeeping gene β -Actin was used as a normalization control. The mRNA levels of Notch1 were first normalized to the mRNA levels of β -Actin, and then the fold induction of Notch1 mRNA was calculated on the basis of the Notch1 mRNA level in SCR-treated control cells.

Luciferase reporter assay

Cells were transfected with SCR or siRNA targeting c-Jun (Sigma-Aldrich) and then transfected 48 h later with HRPM22069-PG04 and NEG-PG04 expression vectors (Genecopoeia, Rockville, MD, USA). The HRPM22069-PG04 vector contains the *Notch1* promoter and the coding sequences for both *Gaussia* luciferase and secreted alkaline phosphatase. The NEG-PG04 vector was used as a negative control. At 48 h after transfection, the *Gaussia* luciferase and secreted alkaline phosphatase signals were measured using the Secrete-Pair dual luminescence assay kit (Genecopoeia) according to the manufacturer's instructions. To control for off-target effects of the expression plasmids or the transfection procedure, the *Gaussia* luciferase signal was normalized to that of secreted alkaline phosphatase.

TNBC xenograft mouse model

All animals were maintained and handled in accordance with the guidelines of the MD Anderson Institutional Animal Care and Use Committee. SUM149 (4×10^6) cells were implanted into one of the mammary fat pads of 4- to 6-week-old female athymic BALB/c nu/nu mice purchased from the Department of Experimental Radiation Oncology at MD Anderson. When tumor size reached 75–150 mm³, mice were randomly divided into three groups (10 mice/group) and treated with vehicle (0.9% NaCl, EtOH, and Tween 80 at a ratio of 880/20/100) or JNK-IN-8 at 15 or 30 mg/kg/day via intraperitoneal injections daily for 34 days. At the end of the study, tumor samples were collected and processed for immunohistochemical staining for expression of target proteins.

Statistics

All the data were presented as mean $\pm$ standard deviation except for the RPPA data, for which box-and-whisker plots were used to show the data structure. Differences in the

expression levels of proteins between two groups were analyzed using two-tailed Student's t test, and differences among multiple groups were analyzed using one-way analysis of variance. Tukey HSD tests were used for *post hoc* pairwise analysis. Association between expression levels of proteins was analyzed by Pearson correlation coefficient test. Correlation between mRNA levels of proteins and DFS of patients with TNBC was analyzed by the Kaplan-Meier method. Statistical significance of the *in vitro* results was assessed by two-tailed Student's t test. $P < 0.05$ was considered significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

c-Jun is associated with clinical outcomes of TNBC patients, and suppression of JNK activity inhibits c-Jun phosphorylation in TNBC cells. **(a)** Expression levels of c-Jun and c-Jun phosphorylated at Ser73 were higher in TNBC tumors than in non-TNBC tumors, as analyzed using an MD Anderson public RPPA dataset by two-tailed Student's *t* test. **(b)** In TNBC patients (*n* = 79), those with tumors expressing high mRNA level of c-Jun had shorter DFS. Median values were used as the cut-off values for high vs. low c-Jun mRNA expression levels. DFS was analyzed using datasets from Wang et al and Schmidt et al by the Kaplan-Meier method. **(c)** In TNBC tumors (*n* = 129), expression levels of c-Jun phosphorylated at Ser73 correlated with those of JNK phosphorylated at Thr183, as analyzed using the MD Anderson public RPPA dataset by Pearson correlation coefficient test. **(d-e)** JNK-IN-8 treatment **(d)** and JNK1 or JNK2 knockdown **(e)** inhibited c-Jun phosphorylation at Ser63 and Ser73 by JNK in TNBC cells as analyzed by Western blotting. β -Actin and α -tubulin were used as loading controls. Results in **(d)** and **(e)** are representative of three independent experiments.

Figure 2.

JNK is essential for growth, migration, and invasion of TNBC cells. (a-b) JNK1 or JNK2 knockdown (a) or JNK-IN-8 treatment (b) reduced anchorage-independent growth of HCC70 and SUM149 cells as analyzed by soft agar assay. α -Tubulin was used as a loading control. (c-d) JNK1 or JNK2 knockdown (c) or JNK-IN-8 treatment (d) suppressed migration and invasion of HCC70 and SUM149 cells. In c, cells were treated with siJNK1 or siJNK2 and 48 h later assayed for migration and invasion. In d, cells were pre-treated with JNK-IN-8 for 2 h and then assayed for migration and invasion in the presence of JNK-IN-8.

$P < 0.05$; * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. Images were taken at 20x magnifications. Results are representative of three independent experiments.

Figure 3.

JNK is required for self-renewal and maintenance of CSCs. (a-b) JNK1 or JNK2 knockdown (a) or JNK-IN-8 treatment (b) inhibited mammosphere formation in HCC70 and SUM149 cells as analyzed by mammosphere formation assay. (c) JNK-IN-8 treatment at 0.5 μ M in HCC70 cells and at 1 μ M in SUM149 cells inhibited primary (P0), secondary (P1), and tertiary (P2) mammosphere formation. (d-e) JNK1 or JNK2 knockdown (d) or JNK-IN-8 treatment (e) reduced ALDH1⁺ subpopulations in HCC70 and SUM149 cells. Cells were treated with siJNK1, siJNK2, or JNK-IN-8 and subjected 48 h later to flow cytometry

analysis. DEAB, diethylaminobenzaldehyde, an ALDH inhibitor, used as a negative control. SSC-A, side scatter-area. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. Results are representative of three independent experiments.

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Figure 4.

JNK regulates Notch1 expression via activation of c-Jun. (a-c) JNK-IN-8 treatment (a), JNK1 or JNK2 knockdown (b), or c-Jun knockdown (c) reduced expression of Notch1 and c-Jun phosphorylation at Ser63 and Ser73 as analyzed by Western blotting. In a, JNK-IN-8 treatment also reduced expression of c-MYC, cyclin D1, and p21. Cells were treated with JNK-IN-8 for 48 h or with siJNK1, siJNK2, or sic-Jun for 72 h and then subjected to Western blotting. α-Tubulin was used as a loading control. (d) c-Jun knockdown reduced Notch1 mRNA expression (left panel) as analyzed by qRT-PCR and luciferase activity of the *Notch1* promoter (right panel) as analyzed by luciferase reporter assay. * $P < 0.01$; ** $P < 0.001$; *** $P < 0.0001$. Results are representative of three independent experiments.

Figure 5.

c-Jun and Notch1 are required for CSC self-renewal. (a-e) c-Jun or Notch1 knockdown (a) reduced proliferation (b) as analyzed by CellTiter-Blue viability assay, anchorage-independent growth (c) as analyzed by soft agar assay, mammosphere formation (d) as analyzed by mammosphere formation assay, and ALDH1⁺ subpopulations (e) as analyzed by flow cytometry in HCC70 and SUM149 cells. α -Tubulin was used as a loading control. SSC-A, side scatter-area. # $P < 0.05$; * $P < 0.01$; ** $P < 0.001$. Results are representative of three independent experiments. DEAB, diethylaminobenzaldehyde, an ALDH inhibitor, used as a negative control.

Figure 6.

JNK inhibitor suppressed tumor growth in a SUM149 xenograft mouse model and schematic of JNK/c-Jun/Notch1 signaling-mediated regulation of CSCs in TNBC. **(a)** JNK-IN-8 suppressed tumor growth in a SUM149 xenograft mouse model. SUM149 cells (4×10^6) were injected into one of the mammary fat pads of nu/nu mice. When the tumors were about 75–150 mm³, the mice were treated with JNK-IN-8 for 34 days. **(b)** Immunohistochemical staining showing expression levels of Ki-67, c-Jun phosphorylated at Ser63 and Ser73, Notch1, and ALDH1 in tumors from vehicle-treated and JNK-IN-8-treated mice. Images were taken at 20x magnifications. H&E, hematoxylin-eosin. **(c)** Measurement of intensity of immunohistochemical staining of Ki-67, c-Jun phosphorylated at Ser63 and Ser73, Notch1, and ALDH1 in tumors from vehicle-treated and JNK-IN-8-treated mice. In **a** and **c**, #*P* < 0.05; **P* < 0.01; ***P* < 0.001. **(d)** Schematic of JNK/c-Jun/Notch1 signaling-mediated regulation of CSCs in TNBC. The JNK signaling cascade is initiated by external stimuli, e.g., inflammatory cytokines, growth factors, or stress. Once the signaling cascade is initiated, activated MAPK kinase MKK4 or MKK7 activates JNK by phosphorylation on Thr183 and Tyr185, and activated JNK translocates into the nucleus, where it phosphorylates

c-Jun on Ser63 and Ser73. Activated c-Jun binds to the promoter of *Notch1* and promotes *Notch1* transcription, leading to CSC self-renewal and subsequent tumor growth. Treatment with JNK inhibitors could effectively inhibit tumorigenesis by suppressing CSC phenotype.

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