Role of JNK in Mammary Gland Development and Breast Cancer

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Abstract

c-Jun NH2-terminal kinase (JNK) signaling has been implicated in the developmental morphogenesis of epithelial organs. In this study, we employed a compound deletion of the murine Jnk1 and Jnk2 genes in the mammary gland to evaluate the requirement for these ubiquitously expressed genes in breast development and tumorigenesis. JNK1/2 was not required for breast epithelial cell proliferation or motility. However, JNK1/2 deficiency caused increased branching morphogenesis and defects in the clearance of luminal epithelial cells. In the setting of breast cancer development, JNK1/2 deficiency significantly increased tumor formation. Together, these findings established that JNK signaling is required for normal mammary gland development and that it has a suppressive role in mammary tumorigenesis. Cancer Res; 72(2); 472–81. ©2011 AACR.

Introduction

The formation of epithelial organs requires the coordinated growth and movement of epithelial cell sheets. These developmental processes are critically regulated by many mechanisms, including cytokine and endocrine signal transduction pathways. One signaling pathway that has been implicated in epithelial organ morphogenesis is the c-Jun NH2-terminal kinase (JNK) signaling pathway (1, 2). Thus, genetic analysis of Drosophila shows that JNK is essential for the morphogenetic epithelial cell movements that occur during dorsal closure (3), thoracic closure (4), imaginal disc development (5), and formation of the egg dorsal appendages and micropyle (6). Studies of mammalian development show that JNK is required for closure of the optic fissure (7), eyelid closure (8, 9), and neural tube closure (10). Key molecular mechanisms that may underly these processes include a requirement of JNK for paxillin phosphorylation and epithelial cell motility (11) and a requirement of JNK for actin polymerization-dependent cell protrusions at the leading edge of the epithelial cell sheet (12). An understanding of the role of JNK in these developmental processes is important because the mechanisms may be relevant to both normal physiology and to disease states (1, 2).

The purpose of this study was to test whether JNK is required for mammary gland development (13). Indeed, JNK may play a critical role in morphogenesis of the breast epithelium (14, 15). These authors report that the drug SP600125 inhibits both JNK activity and luminal clearance of mammary epithelial cells (14, 15). However, SP600125 exhibits poor selectivity for JNK (16). It is therefore unclear whether JNK inhibition mediates the effects of SP600125 on morphogenesis of the breast epithelium. Moreover, detailed studies of breast epithelium development (17) indicate that this morphogenetic process differs substantially from other epithelial morphogenetic movements that are known to be JNK dependent (1, 2). Thus, JNK is required for shape changes in the cells that form the leading edge of the epithelial cell sheet prior to coordinated cell movements (12). In contrast, elongating mammary epithelial cell ducts form a multilayer epithelium that moves coordinately without extension of leading edge cells (17). Whether JNK contributes to this process during mammary gland development is unclear.

The JNK protein kinase in mammals is encoded by 2 ubiquitously expressed genes (Jnk1 and Jnk2) and by a third gene (Jnk3) that is selectively expressed in the brain (1). We have not detected developmental defects in mammary gland development in Jnk1−/− mice or Jnk2−/− mice (18). Because Jnk1 and Jnk2 display partially redundant functions (19, 20), we examined the effect of compound deficiency of Jnk1 plus Jnk2. Compound Jnk1−/−/Jnk2−/− mice die during embryonic development (10). We therefore employed a conditional gene ablation strategy. This experimental approach enabled examination of the role of JNK in primary cultures of mammary epithelial cells and mammary gland organoids in vitro. Furthermore, transplantation assays allowed analysis of the role of JNK in mammary gland development in vivo. We report that JNK is not required for mammary epithelial cell motility or formation of mammary epithelial cell ducts. However, JNK...
contributes to branching morphogenesis of the mammary epithelium and is required for normal luminal clearance of epithelial cells. Moreover, studies of mammary carcinogenesis show that JNK deficiency causes significantly increased breast cancer. Together, these observations indicate that JNK may play an important role in both mammary gland development and mammary carcinoma formation.

Materials and Methods

Mice
We have described Jnk2−/− mice (21) and mice with conditional expression of Jnk1 (22). Nude mice (strain NU/J, Stock # 002019), mice with conditional expression of KRas(G12D) (Ref. 23; strain B6.129P2-Tp53tm1(cre)/J, Stock # 008179), mice with conditional expression of Trp53 (Ref. 24; strain B6.129P2-LoxP-Stop-LoxP-Trp53tm4Tyj/J, Stock # 008179), and Villin-Cre mice (Ref. 26; strain B6.SJL-Tg(Vil-cre)997Gum/J, Stock # 004584) were obtained from the Jackson Laboratory. The mice used in this study were backcrossed to the C57BL/6 strain (Jackson Laboratories) and were housed in a facility accredited by the American Association for Laboratory Animal Care. The Institutional Animal Care and Use Committee of the University of Massachusetts approved all studies using animals.

Genotype analysis was carried out by PCR using genomic DNA as the template. The Jnk1+/− (1.5 kb), Jnk1α/α (1.1 kb), and Jnk1α (0.4 kb) alleles were identified using the amplifiers 5′-CCTCAGGAAAGGCTTATTTCC-3′ and 5′-GAACGACTGTTCCATTCATCC-3′. The wild-type Jnk2 (400 bp) and knockout Jnk2 (270 bp) alleles were identified using the amplifiers 5′-GGAGGCGGAGAGTAGTATCGAGTTACC-3′ and 5′-GTCGCTCATTCCTGAGGTGTTAT-3′. The wild-type Trp53 (288 bp) and Trp53α (370 bp) alleles were identified using the amplifiers 5′-AGCAATCCCAGAGGTTGTG-3′ and 5′-CCAGCTCATTCCTCTCACTCATG-3′. The wild-type Rosa26 (288 bp), KRasα (315 bp), and LoxP-Stop-LoxP-KRasα (600 bp) alleles were identified using the amplifiers 5′-GGTAGGTGGGATGATGCTG-3′ and 5′-TCCGAATCTCAGGATTCACTGAG-3′. The Rosa26 (600 bp) and Rosa26 (CreERT) (300 bp) alleles were identified using the amplifiers 5′-GGAGGGCGGAGAGTAGTATCGAGTTACC-3′, 5′-GGAGGGCGGAGAGTAGTATCGAGTTACC-3′, and 5′-AAAGTCGCTCATTCCTGAGGTGTTAT-3′. The Villin-Cre allele (450 bp) was detected using the amplifiers 5′-TTCTGAGGCTGTTCCCAATTTGGCCT-3′ and 5′-GTCGCTCATTCCTCTCACTGAG-3′.

Cre-mediated recombination
Mice were treated with tamoxifen (Sigma, T5648) to activate recombinase in animals with an inducible Cre recombinase (strain B6; 129-Gt(Rosa)26Sor(LacZ creERT2/2)Nat). The tamoxifen was dissolved in sunflower seed oil (10 mg/ml).

Mice were treated by intraperitoneal injection with 1 mg tamoxifen each day for 3 consecutive days.

Mammary gland transplantation assays

Transplantation assays were carried out using procedures described previously (27). Donor mice were euthanized and 1 mm3 fragments of the 4th inguinal mammary glands were removed aseptically and stored in Dulbecco’s modified Eagle medium on ice. Host mice (3- to 4-week-old female nude mice) were anesthetized. The 4th inguinal mammary gland on one side was excised, a small pocket was formed in the cleared fat pad proximal to the inguinal lymph node, and a fragment of donor mammary tissue was placed in the pocket. The same procedure was carried out on the contralateral side. Each host mouse was transplanted with both Control and JNK-deficient mammary tissue.

Results

Isolation of JNK-deficient mammary epithelial cells
The Jnk1 and Jnk2 genes are expressed in mammary epithelial cells. We employed a conditional gene ablation strategy to create mice with deficiency of JNK1 plus JNK2. We found that Jnk1flx/+lox/P Jnk2−/− CreERT mice were viable. These mice express a 4-hydroxy-tamoxifen-stimulated Cre recombinase. We treated the Jnk1flx/+lox/P Jnk2−/− CreERT mice with tamoxifen, isolated mammary tissue, and prepared primary mammary epithelial cell cultures (Supplementary Fig. S1). Genotype analysis of genomic DNA prepared from mammary tissue and cultured epithelial cells showed that tamoxifen caused ablation of the conditional Jnk1 gene (Fig. 1A). Immunoblot analysis confirmed that JNK protein was not detected in mammary tissues or epithelial cells of Jnk1flx/+lox/P Jnk2−/− mice (Fig. 1B).

To examine the effect of JNK deficiency on primary mammary epithelial cells, we compared cultures of cells prepared from Control mice (CreERT) and Jnk1flx/+lox/P Jnk2−/− CreERT mice following treatment with tamoxifen. The epithelial cells were identified by immunofluorescence analysis by staining with antibodies to pan-cytokeratin and E-cadherin. We did not detect an effect of JNK deficiency on the proliferation of primary mammary epithelial cells (Fig. 1C).

Role of JNK in mammary epithelial cell proliferation

It is established that compound JNK deficiency in primary mouse embryonic fibroblasts (MEF) causes p53-dependent senescence (20, 22). Growth of Jnk1−/− Jnk2−/− MEF requires loss-of-function of the p53 pathway (22). We therefore anticipated that Jnk1flx/+ Jnk2−/− primary mammary epithelial cells would also exhibit reduced growth and senescence. However, the morphology of JNK-deficient epithelial cells was not typical of senescent cells (Fig. 2A). Moreover, cell-cycle analysis by flow cytometry did not show a requirement of JNK for proliferation (Fig. 2B). Indeed, Jnk1flx/+ Jnk2−/− primary mammary epithelial cells incorporated significantly more BrdU than Control cells, indicating that JNK deficiency may increase epithelial cell proliferation (Fig. 2B). This conclusion was confirmed by measurement of cell proliferation (Fig. 2C). Together, these data show that the effect of JNK deficiency on the proliferation...
of MEF markedly differs from mammary epithelial cells. Thus, JNK is required for MEF proliferation, but is not required for proliferation of mammary epithelial cells.

It is unclear whether the failure of JNK-deficient mammary epithelial cells to senesce reflects a specific role of JNK in mammary epithelial cells or whether this reflects a general role of JNK in other epithelial cells. To address this question, we examined the effect of JNK deficiency in intestinal epithelial cells using conditional gene ablation in vivo with Villin-Cre (Supplementary Fig. S2). Compound JNK deficiency caused no detected defects in the proliferation of intestinal epithelial cells (Supplementary Fig. S2A) or colon tumor cells (Supplementary Fig. S2C and S2D). JNK deficiency causes increased expression of p53 by MEF (20), but no increase in p53 expression was detected in JNK-deficient intestinal epithelial cells (Supplementary Fig. S2B). Moreover, ionizing radiation caused a similar increase in p53 expression by Control and JNK-deficient intestinal epithelial cells. Together, these data indicate that the p53-dependent senescence of JNK-deficient MEF (20, 22) reflects a particular role of JNK in MEF, but not other cell types (e.g., mammary and intestinal epithelial cells).

Role of JNK in mammary epithelial cell motility

It has been reported that JNK is required for actin polymerization-dependent cell protrusions at the leading edge of epithelial cell sheets during morphogenetic movements (12). It has also been reported that paxillin phosphorylation by JNK is essential for epithelial cell movement (11). Together, these data indicate that JNK is a critical cellular component that is required for cell motility. To test this hypothesis, we prepared cultures of mammary epithelial cells from Control mice (CreERT) and Jnk1<sup>D</sup>/D Jnk2<sup>C0</sup>/C0 CreERT mice. Comparison of Control and JNK-deficient primary mammary epithelial cells indicated that JNK is not required for cell motility. Indeed, studies using Boyden chambers coated with collagen showed...
that JNK deficiency increased mammary epithelial cell motility (Fig. 3A). Moreover, assays using Boyden chambers with a Matrigel layer showed that JNK deficiency increased mammary epithelial cell invasion (Fig. 3B). Together, these data do not support a critical role for JNK as a positive regulator of primary mammary epithelial cell motility. This finding contrasts with previous reports that JNK plays a key role in epithelial cell motility (2, 11). We therefore examined mice with JNK deficiency in the intestinal epithelium (Supplementary Fig. S2). Epithelial cells formed in intestinal crypts migrate on the surface of the villus to create the intestinal epithelium. Intestinal epithelium morphology was not disrupted by JNK deficiency (Supplementary Fig. S2). Consistent with a nonessential function of JNK in intestinal epithelial cell motility, together, these data indicate that JNK is not essential for epithelial cell motility.

**Role of JNK in mammary branching morphogenesis**

Branching morphogenesis is an important aspect of mammary gland development (28, 29). This process can be studied in vitro using mammary organoid cultures in the presence of FGF2 (17). We prepared cultures of mammary organoids from Control mice (CreERT<sup>−/−</sup>) and Jnk1<sup>−/−</sup>/Jnk2<sup>−/−</sup> CreERT<sup>−/−</sup> mammary epithelial cells prepared from tamoxifen-treated mice were examined using Boyden chambers coated with Collagen I (A) or with a Matrigel layer (B). The relative number of cells that moved from the upper chamber to the lower chamber is presented (mean ± SD; n = 5). Significant differences between Control cells and JNK-deficient cells are indicated with an asterisk (*, P < 0.05; **, P < 0.001).

![Figure 3. Effect of JNK deficiency on mammary epithelial cell motility and invasion.](image)

**Effect of JNK deficiency on mammary epithelial cell gene expression**

It is established that the JNK signal transduction pathway can regulate gene expression (1). JNK-regulated gene expression may therefore account for the effect of JNK deficiency on epithelial cell motility and invasion (Fig. 3) and branching morphogenesis (Fig. 4). We therefore examined the effect of JNK deficiency on the expression of candidate genes that may influence these processes (Supplementary Fig. S3).

Matrix metalloproteases (MMP) play key roles in mammary gland development (28). Thus, ADAM17 induces shedding of the EGF receptor ligand amphiregulin that can induce expression of MMP2 (32). MMP2 (and its activator MMP14) can promote ductal elongation (33), and both MMP3 and MMP9 promote branching morphogenesis (33, 34). We found that JNK deficiency caused significantly decreased expression of Mmp2, Mmp9, and Mmp14 mRNA (Supplementary Fig. S3A). These changes do not account for the increased branching morphogenesis caused by JNK deficiency. MMPs and ADAM17 are negatively regulated by tissue inhibitors of metalloproteases (TIMP). Downregulated expression of TIMP1/2/3 could therefore increase MMP and/or ADAM17 activity and therefore influence branching morphogenesis (29). Indeed, a significant reduction in Timp1, Timp2, and Timp3 mRNA expression was caused by JNK deficiency (Supplementary Fig. S3B). In contrast, the expression of 2 other genes that are implicated in branching morphogenesis (Tgfβ1 and Sprouty2) was unaffected by JNK deficiency (Supplementary Fig. S3B). Together, these data suggest that decreased TIMP expression may contribute to increased branching morphogenesis caused by JNK deficiency.

The increased motility and invasion activity of JNK-deficient mammary epithelial cells detected in Boyden chamber assays may reflect altered integrin expression. We found decreased expression of α1, α5, α6, and β1 integrins and also decreased expression of the collagen receptor DDR1 in JNK-deficient mammary epithelial cells (Supplementary Fig. S3C). The decreased expression of integrin α5 protein was confirmed by immunofluorescence analysis (Supplementary Fig. S4). Previous studies have implicated integrin α2, integrin β1, and DDR1 in mammary gland development (29), but decreased expression of these proteins is not predicted to cause the increased motility, invasion, and branching morphogenesis caused by JNK deficiency (Figs. 3 and 4). The mechanism that accounts for increased motility and invasion by JNK-deficient mammary epithelial cells is therefore unclear.

**Effect of JNK deficiency on mammary gland development**

We employed transplantation assays to test the role of JNK in mammary gland development using the 4th inguinal gland pair. Control (CreERT<sup>−/−</sup>) tissue was transplanted in 1 cleared mammary gland of a female nude mouse and Jnk1<sup>−/−</sup>/Jnk2<sup>−/−</sup> CreERT<sup>−/−</sup> tissue was transplanted in the contralateral gland of the same recipient mouse. Analysis of mammary gland development at 8 weeks following transplantation showed that JNK
deficiency did not prevent the elaboration of mammary epithelial ducts (Fig. 5A). However, JNK deficiency caused an increase in the number of branches (Fig. 5B and C). These data indicate that JNK can influence mammary branching morphogenesis in vivo.

To test whether JNK may alter the early time course of mammary gland development, we examined mice at 2 weeks following transplantation. This analysis showed the presence of terminal end buds (TEB) in glands formed by Control and JNK-deficient tissue (Fig. 5D). Sections of TEBs formed by transplantation of Control tissue showed the presence of a lumenal space (Fig. 5E). In contrast, this TEB lumenal space was filled with cells in glands formed by transplantation of JNK-deficient tissue (Fig. 5E). These data indicate that JNK deficiency disrupts TEB morphology during mammary gland development. Moreover, JNK deficiency increased the number of branches detected at 2 weeks following transplantation (Fig. 5F).

**Effect of JNK deficiency on mammary tumorigenesis**

JNK deficiency influences the proliferation, motility, invasion activity, and branching morphogenesis in vitro (Figs. 2–4) and mammary gland development in vivo (Fig. 5). These changes indicate that JNK may influence mammary tumor development. To test this hypothesis, we examined the effect of mutational activation of the endogenous KRas gene in vivo.

Transplantation assays were carried out using donor tissue from tamoxifen-treated mice were examined by immunofluorescence microscopy by staining with an antibody to smooth muscle actin (SMA, green) and with phalloidin to stain F-actin (red). DNA was stained with DAPI (blue). Branching morphogenesis was initiated by treatment of the cultures with FGF2. The organoids were examined using a Leica TCS SP2 confocal microscope by acquiring 10 optical sections that were collapsed to a single image. Representative images are shown. B, the number of branches per organoid was measured (mean ± SD, n = 20). Significant differences between Control cells and JNK-deficient cells are indicated with an asterisk (*, P < 0.01).
JNK-deficient tumors were locally invasive (Fig. 7A), but no metastasis was detected. Tumor sections stained for markers of basal-like (cytokeratin 5) and nonbasal-like (cytokeratin 8) breast cancer showed that the tumors obtained represented mixed origins (basal and nonbasal) (Fig. 7B). However, the JNK-deficient tumors stained more efficiently for cytokeratin 5 than Control tumors, indicating that JNK deficiency may promote basal-like tumors in this KRas/Trp53 model of murine breast cancer.

Discussion

Mice with defects in JNK expression have provided insight into the biological function of the JNK signaling pathway (1, 36). However, the ubiquitously expressed Jnk1 and Jnk2 genes have partially redundant functions (19, 20) and compound mutant Jnk1<sup>−/−</sup> Jnk2<sup>−/−</sup> mice die during mid-embryogenesis (10). Studies of the effect of compound JNK deficiency have therefore largely focused on an analysis of MEF (20).

However, MEF are not representative of many differentiated cell types. Progress has been made toward the creation of mice with conditional and chemical genetic Jnk alleles (19, 22) that enable the analysis of cell types that are relevant to specific physiologic processes (37). The focus of this study was to employ conditional gene targeting to examine the role of JNK in mammary epithelial cells. We report that JNK contributes to mammary gland development. Importantly, the functions of JNK in mammary epithelial cells differ from those previously identified in MEF.

JNK is not essential for mammary epithelial cell proliferation or motility

Compound JNK deficiency in MEF causes increased p53 expression and senescence (20, 22). In contrast, JNK-deficient mammary epithelial cells did not exhibit a defect in proliferation <em>in vitro</em> (Fig. 2). Moreover, transplantation assays showed that JNK-deficient cells retained sufficient proliferative capacity to form a mammary gland <em>in vivo</em> (Fig. 5). These data show...
that JNK is not essential for proliferation of mammary epithelial cells. This conclusion may apply to other epithelial cells because studies of intestinal epithelial cells also showed that JNK is not required for proliferation (Supplementary Fig. S2). Thus, the effect of JNK deficiency to engage the p53-mediated senescence pathway may represent a specialized response of MEF to loss of JNK signaling.

JNK signaling has been implicated in the regulation of cell motility (2). The role of JNK may be mediated by JNK-dependent paxillin phosphorylation (11) and/or a requirement of JNK for actin polymerization-dependent cell protrusions (12). Nevertheless, we did not detect defects in mammary epithelial cell motility caused by JNK deficiency. Indeed, JNK deficiency caused an increase in mammary epithelial cell motility in Boyden chamber assays (Fig. 3A). Similarly, JNK deficiency caused increased invasion of mammary epithelial cells through a Matrigel layer (Fig. 3B). Furthermore, JNK deficiency did not prevent the formation of mammary epithelial cell ducts or the intestinal epithelium in vivo (Fig. 5 and Supplementary Fig. S2). Together, these data show that JNK is not essential for epithelial cell motility. This conclusion may reflect a redundant role of JNK for paxillin phosphorylation (11). Moreover, JNK-dependent leading edge cell protrusions may not be rate limiting for motility (e.g., roles of adhesion and rear-end detachment).

**JNK is required for normal mammary gland development**

Transplantation assays showed that JNK is not required for the formation of a mammary gland in a virgin female mouse (Fig. 5). However, developmental defects were detected. Thus, examination of TEBs at 2 weeks following transplantation showed that luminal cell clearance found in Control TEBs was incomplete in JNK-deficient TEBs (Fig. 5E). Previous studies have established that luminal clearance is caused by cell death, partially mediated by apoptosis (38), that involves the BH3-only proteins Bim (15, 39) and Bmf (40). Interestingly, Bim and Bmf are targets of proapoptotic signaling by JNK (41, 42). Loss of JNK signaling may lead to defects in Bim/Bmf function and consequently failure of luminal clearance (15). Nevertheless, it should be noted that the defect in TEB luminal cell clearance caused by JNK deficiency was partial (Fig. 5E), suggesting the presence of redundant or compensatory mechanisms of luminal cell clearance in the JNK-deficient mammary glands. This type of compensation has been noted in studies of Bim-deficient mammary glands (39).
Mutations in JNK pathway genes (Jnk1, Jnk2, Mkk4, and Mkk7) are detected in human cancers (43, 44), but it is unclear whether these mutations are causally related to tumorigenesis (45). Insight into the potential function of JNK has been obtained from murine studies of KRas-induced lung cancer (46), carcinogen-induced hepatocellular carcinoma (47), and colon cancer (Supplementary Fig. S2) with tissue-specific compound ablation of the Jnk1 and Jnk2 genes. These studies have shown an essential role for JNK in KRas-induced lung cancer (46), but no required role for JNK in carcinogen-induced colon cancer (Supplementary Fig. S2). In contrast, JNK plays a more complex role in hepatocellular carcinoma because JNK promotes an inflammatory microenvironment to support tumor development, but acts in hepatocytes to reduce tumor development (47). Together, these data indicate that, in individual tumor types, JNK may play no role in tumor development or may contribute (positively or negatively) to tumor pathology.

Studies using single gene ablation (Jnk1 or Jnk2) indicate that JNK deficiency can increase mammary carcinoma in the Trp53 BALB/c mouse model (18). Moreover, JNK2 deficiency shortens tumor latency and increases tumor multiplicity in a transgenic mouse model with expression of polyoma virus T antigen (48). These observations suggest that JNK may function to reduce tumor development. However, JNK1 and JNK2 exhibit partially redundant functions (20, 22). Consequently, single gene ablation (Jnk1 or Jnk2) may not provide insight into the effects of compound JNK deficiency (37, 47, 49). Here, we show that compound JNK deficiency promotes tumor development in a KRas/Trp53 mouse model of breast cancer (Fig. 6). Further studies will be required to confirm the protumorigenic effect of JNK deficiency in a model of breast cancer that has direct relevance to human disease. Nevertheless, it is likely that protumorigenic effects of JNK deficiency reflect functional roles for JNK pathway genes (Jnk1, Jnk2, Mkk4, and Mkk7) mutations that have been detected in human cancer (43, 44) and JNK-regulated genetic instability (48).

Small molecule inhibitors of JNK have been proposed to be useful for the treatment of metabolic and inflammatory disorders in humans (50). The design of such therapies should take into account the potential protumorigenic effects of JNK inhibition.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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