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Role of JNK in mammary gland development and breast cancer

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Abstract

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 lumenal 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.

Introduction

The formation of epithelial organs requires the co-ordinated 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 cJun NH_2-terminal kinase (JNK) signaling pathway (1, 2). Thus, genetic analysis of *Drosophila* demonstrates 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 demonstrate 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

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epithelium (14, 15). These authors report that the drug SP600125 inhibits both JNK activity and lumenal 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 co-ordinated cell movements (12). In contrast, elongating mammary epithelial cell ducts form a multi-layer epithelium that moves co-ordinately 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 two 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). Since 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 lumenal clearance of epithelial cells. Moreover, studies of mammary carcinogenesis demonstrate 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 $KRas^{G12D}$ (23) (strain B6.129S4-*Krastm4Tyj*/J (Stock # 008179)), mice with conditional expression of *Trp53* (24) (strain B6.129P2-$Trp53^{tm1Brn}$/J (Stock # 008462)), mice expressing 4-hydroxy-tamoxifen-stimulated *Cre* (25) (strain B6;129-$Gt(ROSA)26Sor^{tm1(cre/ERT)Nat}$/J (Stock # 004847)), and *Villin-Cre* mice (26) (strain B6.SJL-Tg(*Vil-cre*)997Gum/J (Stock # 004586)) 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

Genotype analysis was performed by PCR using genomic DNA as the template. The $Jnk1^{+}$ (1.5 kb), $Jnk1^{LoxP}$ (1.1kb) and $Jnk1^{\Delta}$ (0.4kb) alleles were identified using the amplimers 5’-CCTCAGGAAGAAAGGGCTTATTTC-3’ and 5’-GAACCACTGTTCCAATTTCCATCC-3’. The wild-type *Jnk2* (400 bp) and knockout *Jnk2* (270 bp) alleles were identified using the amplimers 5’-GGAGCCCGATAGTATCGAGTTACC-3’, 5’-GTTAGACAATCCCAGAGGTTGTGTG-3’, and 5’-CCAGCTCATTCCTCCACTCATG-3’. The wild-type *Trp53* (288 bp) and $Trp53^{LoxP}$ (370

bp) alleles were identified using the amplimers 5'-AGCACATAGGAGGCAGAGAC-3' and 5'-CACAAAAACAGGTTAAACCCAG-3'. The *Trp53*$^{\Delta}$ (612 bp) allele was identified using the amplimers 5'-CACAAAAACAGGTTAAACCCAG-3' and 5'-GAAGACAGAAAAGGGGAGGG-3'. The wild-type *KRas* (285 bp), KRas^{G12D} (315 bp), and *LoxP-Stop-LoxP-KRas*^{G12D} (600 bp) alleles were identified using the amplimers 5'-GGGTAGGTGTTGGGATAGCTG-3' and 5'-TCCGAATTCAGTGACTACAGATGTACAGAG-3'. The *Rosa26* (600 bp) and *Rosa26-Cre*^{ERT} (300 bp) alleles were identified using the amplimers 5'-GCGAAGAGTTTGTCCTCAACC-3', 5'-GGAGCGGGAGAAATGGATATG-3', and 5'-AAAGTCGCTCTGAGTTGTTAT-3'. The *Villin-Cre* allele (450 bp) was detected using the amplimers 5'-TTACTGACCGTACACCAAATTTGCCTGC-3' and 5'-CCTGGCAGCGATCGCTATTTTCCATGAGTG-3'.

Cre-mediated recombination

Mice were treated with tamoxifen (Sigma, T5648) to activate *Cre*-mediated recombination in animals with an inducible Cre recombinase (strain B6;129-*Gt(ROSA)26Sor*$^{tm1(cre/ERT)Nat}$/J). The tamoxifen was dissolved in sunflower seed oil (10 mg/mL). Mice were treated by intraperitoneal injection with 1 mg tamoxifen each day for three consecutive days.

Mammary gland transplantation assays

Transplantation assays were performed using procedures described previously (27). Donor mice were euthanized and 1mm^3 fragments of the fourth inguinal mammary glands were removed aseptically and stored in DMEM on ice. Host mice (3 – 4 week old female nude mice) were anesthetized. The fourth 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 performed 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 *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} mice were viable. These mice express a 4-hydroxy-tamoxifen-stimulated *Cre* recombinase. We treated the *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} mice with tamoxifen, isolated mammary tissue, and prepared primary mammary epithelial cell cultures (Figure S1). Genotype analysis of genomic DNA prepared from mammary tissue and cultured epithelial cells demonstrated that tamoxifen caused ablation of the conditional *Jnk1* gene (Figure 1A). Immunoblot analysis confirmed that JNK protein was not detected in mammary tissues or epithelial cells of *Jnk1*$^{\Delta/\Delta}$ *Jnk2*$^{-/-}$ mice (Figure 1B).

To examine the effect of JNK-deficiency on primary mammary epithelial cells, we compared cultures of cells prepared from Control mice (*Cre*^{ERT}) and *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} 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 morphology of primary mammary epithelial cells (Figure 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 $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ 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 (Figure 2A). Moreover, cell cycle analysis by flow cytometry did not demonstrate a requirement of JNK for proliferation (Figure 2B). Indeed, $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ primary mammary epithelial cells incorporated significantly more BrdU than Control cells, indicating that JNK-deficiency may increase epithelial cell proliferation (Figure 2B). This conclusion was confirmed by measurement of cell proliferation (Figure 2C). Together, these data demonstrate 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* (Figure S2). Compound JNK-deficiency caused no detected defects in the proliferation of intestinal epithelial cells (Figure S2A) or colon tumor cells (Figure S2C,D). JNK-deficiency causes increased expression of p53 by MEF (20), but no increase in p53 expression was detected in JNK-deficient intestinal epithelial cells (Figure 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 is 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 (Cre^{ERT}) and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} 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 (Figure 3A). Moreover, assays using Boyden chambers with a Matrigel layer demonstrated that JNK-deficiency increased mammary epithelial cell invasion (Figure 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 (Figure 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 (Figure S2), consistent with a non-essential 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 (Cre^{ERT}) and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} mice. The organoids are formed by bilayer structures with lumenal mammary epithelial cells and basal myoepithelial cells that express smooth muscle actin

(30, 31). Branching morphogenesis is regulated by hormones/growth factors and by the interaction of the lumenal epithelial cells with basal myoepithelial cells and the extracellular matrix (29). Branching morphogenesis was detected in cultures of Control and $Jnk1^{\Delta/\Delta}Jnk2^{-/-}$ mammary organoids (Figure 4A). This observation indicated that JNK is not required for branching morphogenesis. However, quantitation of the branching indicated that JNK-deficient organoids exhibited significantly greater branching morphogenesis than Control organoids (Figure 4B). Together, these data demonstrate that JNK can influence mammary branching morphogenesis.

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 (Figure 3) and branching morphogenesis (Figure 4). We therefore examined the effect of JNK-deficiency on the expression of candidate genes that may influence these processes (Figure S3).

Matrix metalloproteases (MMPs) 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 (Figure 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 (TIMPs). Down-regulated 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 (Figure S3B). In contrast, the expression of two other genes that are implicated in branching morphogenesis (*Tgfβ1* and *Sprouty2*) was unaffected by JNK-deficiency (Figure 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 (Figure S3C). The decreased expression of integrin α5 protein was confirmed by immunofluorescence analysis (Figure 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 (Figures 3 & 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 fourth inguinal gland pair. Control (Cre^{ERT}) tissue was transplanted in one cleared mammary gland of a female nude mouse and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} tissue was transplanted in the contralateral gland of the same recipient mouse. Analysis of mammary gland development at 8 weeks following transplantation demonstrated that JNK-deficiency did not prevent the elaboration of mammary epithelial ducts (Figure 5A). However, JNK-deficiency caused an increase in the number of branches (Figure 5B,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 demonstrated the presence of terminal end buds (TEBs) in glands formed by Control and JNK-deficient tissue (Figure 5D). No differences in proliferating cells (PCNA-positive) or dying cells (TUNEL-positive) were detected between Control and JNK-deficient mammary glands (Figure S5). Sections of TEBs formed by transplantation of Control tissue demonstrated the presence of a lumenal space (Figure 5E). In contrast, this TEB lumenal space was partially filled with cells in glands formed by transplantation of JNK-deficient tissue (Figure 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 (Figure 5F).

Effect of JNK-deficiency on mammary tumorigenesis

JNK-deficiency influences the proliferation, motility, invasion activity, and branching morphogenesis *in vitro* (Figures 2 – 4) and mammary gland development *in vivo* (Figure 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 performed using donor tissue from Control mice ($KRas^{LSL\text{-}G12D/+}$ Cre^{ERT}) and mice with conditional expression of JNK ($KRas^{LSL\text{-}G12D/+}$ $Jnk1^{LoxP/LoxP}$ $Jnk2^{-/-}$ Cre^{ERT}). The recipient nude mice were transplanted with both Control tissue and JNK-deficient tissue in the fourth inguinal gland pair. The transplanted mice were treated with tamoxifen at two weeks post-surgery to induce expression of activated Ras ($KRas^{G12D}$) and to ablate the conditional *Jnk1* gene. No tumors were detected in these mice within 6 months of transplantation.

The $KRas^{G12D}$ oncogene may require a co-operating mutation to efficiently induce breast cancer (35). We therefore examined the effect of p53 loss-of-function on $KRas^{G12D}$ -induced breast cancer. Transplantation assays were performed using donor tissue from Control mice ($KRas^{LSL\text{-}G12D/+}$ $Trp53^{LoxP/LoxP}$ Cre^{ERT}) and mice with conditional expression of JNK ($KRas^{LSL\text{-}G12D/+}$ $Jnk1^{LoxP/LoxP}$ $Jnk2^{-/-}$ $Trp53^{LoxP/LoxP}$ Cre^{ERT}). These transplanted mice developed mammary carcinoma (Figure 6). Analysis of tissue sections demonstrated that both Control and JNK-deficient tumors were composed primarily of spindle-like cells (Figure 6A) that stained with an antibody to the proliferation marker PCNA (Figure 6B). Genotype analysis confirmed *Jnk1* gene disruption in tumors obtained from mammary glands transplanted with JNK-deficient tissue (Figure 6C). Kaplan-Meier analysis demonstrated that compound JNK-deficiency caused a significant increase in the number of mice with breast cancer (Figure 6D). The mean tumor volume at necropsy was 0.62 cm^3 ± 0.19 (mean ± SD; n = 7) and no significant difference between Control and JNK-deficient tumor volume was observed. The Control and JNK-deficient tumors were locally invasive (Figure 7A), but no metastasis was detected. Tumor sections stained for markers of basal-like (cytokeratin 5) and non-basal-like (cytokeratin 8) breast cancer demonstrated that the tumors obtained represented mixed origins (basal and non-basal). However, the JNK-defcient 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 $Jnk1^{-/-}$ $Jnk2^{-/-}$ 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 towards 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 physiological 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 *in vitro* (Figure 2). Moreover, transplantation assays demonstrated that JNK-deficient cells retained sufficient proliferative capacity to form a mammary gland *in vivo* (Figure 5). These data demonstrate 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 demonstrated that JNK is not required for proliferation (Figure 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 (Figure 3A). Similarly, JNK-deficiency caused increased invasion of mammary epithelial cells through a Matrigel layer (Figure 3B). Furthermore, JNK-deficiency did not prevent the formation of mammary epithelial cell ducts or the intestinal epithelium *in vivo* (Figures 5 & S2). Together, these data demonstrate 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 demonstrated that JNK is not required for the formation of a mammary gland in a virgin female mouse (Figure 5). However, developmental defects were detected. Thus, examination of terminal end buds (TEBs) at 2 weeks following transplantation demonstrated that lumenal cell clearance found in Control TEBs was incomplete in JNK-deficient TEBs (Figure 5E). Previous studies have established that lumenal 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 pro-apoptotic signaling by JNK (41, 42). Loss of JNK signaling may lead to defects in Bim/Bmf function and consequently failure of lumenal clearance (15). Nevertheless, it should be noted that the defect in TEB lumenal cell clearance caused by JNK-deficiency was partial (Figure 5E), suggesting the presence of redundant or compensatory mechanisms of lumenal cell clearance in the JNK-deficient mammary glands. This type of compensation has been noted in studies of Bim-deficient mammary glands (39).

JNK-deficiency caused altered branching morphogenesis. Transplantation assays demonstrated that JNK-deficiency caused increased branching of mammary ducts *in vivo*. (Figure 5B,F). This effect of JNK-deficiency to cause increased branching morphogenesis was also observed in FGF2-stimulated organoid cultures *in vitro* (Figure 4). The mechanism that accounts for increased branching morphogenesis is unclear, but it is established that this

process is regulated by hormones/growth factors and by the interaction of the lumenal epithelial cells with basal myoepithelial cells and the extracellular matrix (28, 29). One potential role of JNK is represented by expression of TIMP isoforms that inhibit matrix metalloproteases (MMPs). JNK-deficiency caused decreased expression of *Timp1/2/3* (Figure S3B). Decreased TIMP activity may lead to increased activity of MMP3 and MMP9 that function, in part, to promote branching morphogenesis (33, 34). This mechanism may contribute to the altered branching morphogenesis caused by JNK-deficiency.

JNK contributes to mammary tumor development

The effect of JNK-deficiency to perturb normal mammary gland development may be relevant to breast cancer. However, the contribution of JNK to breast cancer is unclear (1). 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 (Figure S2) with tissue-specific compound ablation of the *Jnk1* and *Jnk2* genes. These studies have demonstrated an essential role for JNK in *KRas*-induced lung cancer (46), but no required role for JNK in carcinogen-induced colon cancer (Figure 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 demonstrate that compound JNK-deficiency promotes tumor development in a *KRas/Trp53* mouse model of breast cancer. Further studies will be required to confirm the pro-tumorigenic effect of JNK-deficiency in a model of breast cancer that has direct relevance to human disease. Nevertheless, it is likely that pro-tumorigenic effects of JNK-deficiency reflect functional roles for JNK pathway gene (*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 pro-tumorigenic effects of JNK inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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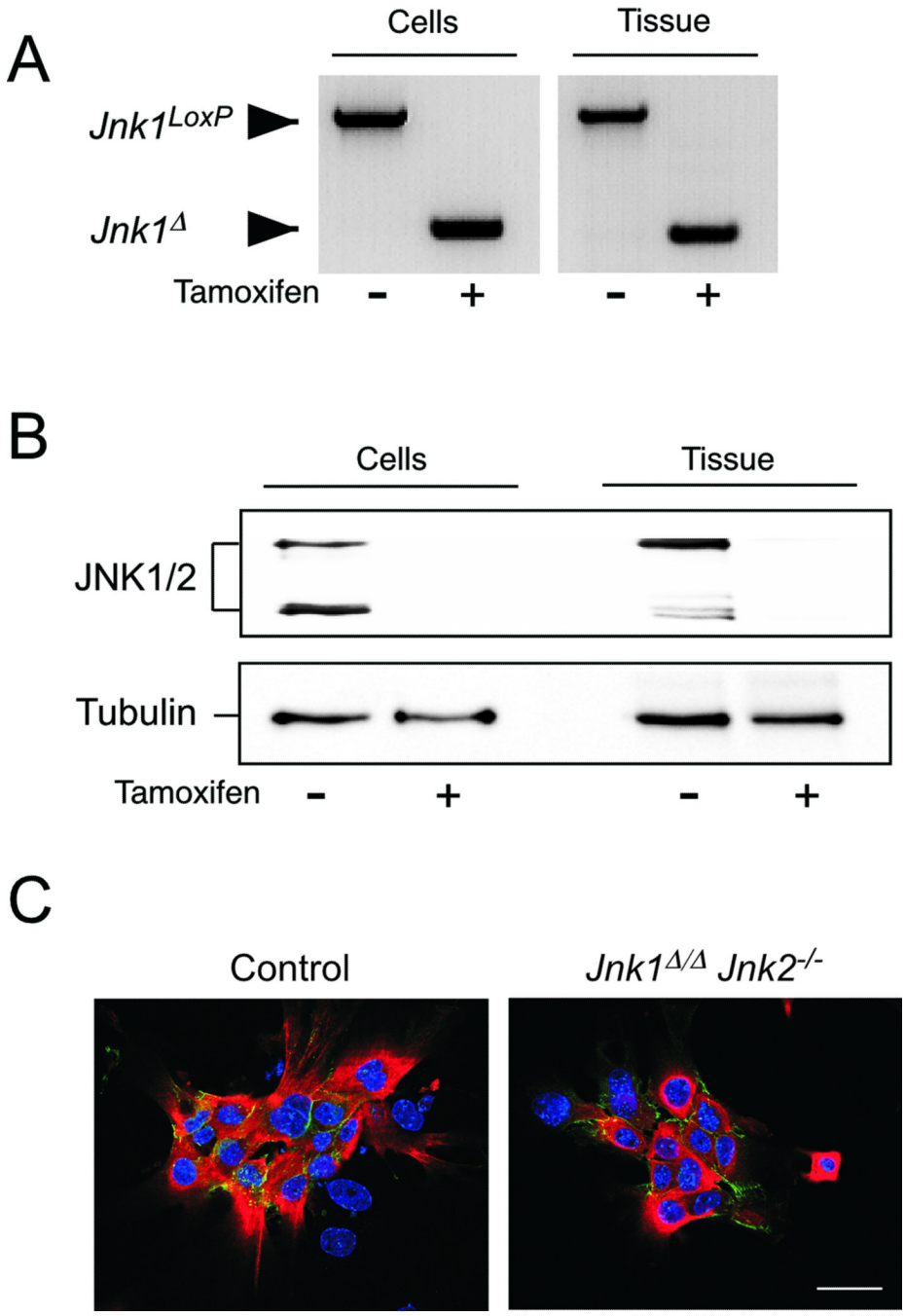

Figure 1. JNK-deficient mammary epithelial cells

A,B) *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} mice treated without or with tamoxifen *in vivo* were used to prepare mammary gland extracts and primary mammary gland epithelial cells. Genomic DNA was examined by PCR to detect *Jnk1*^{LoxP} and *Jnk1*$^{\Delta}$ alleles (A). The expression of JNK and α-Tubulin was examined by immunoblot analysis (B).

C) Primary cultures of *Cre*^{ERT} (Control) and *Jnk1*$^{\Delta/\Delta}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} mammary epithelial cells prepared from tamoxifen-treated mice were examined by immunofluorescence analysis by probing with antibodies to pan-cytokeratin (red) and E-cadherin (green). DNA was stained with DAPI (blue). Representative images are shown.

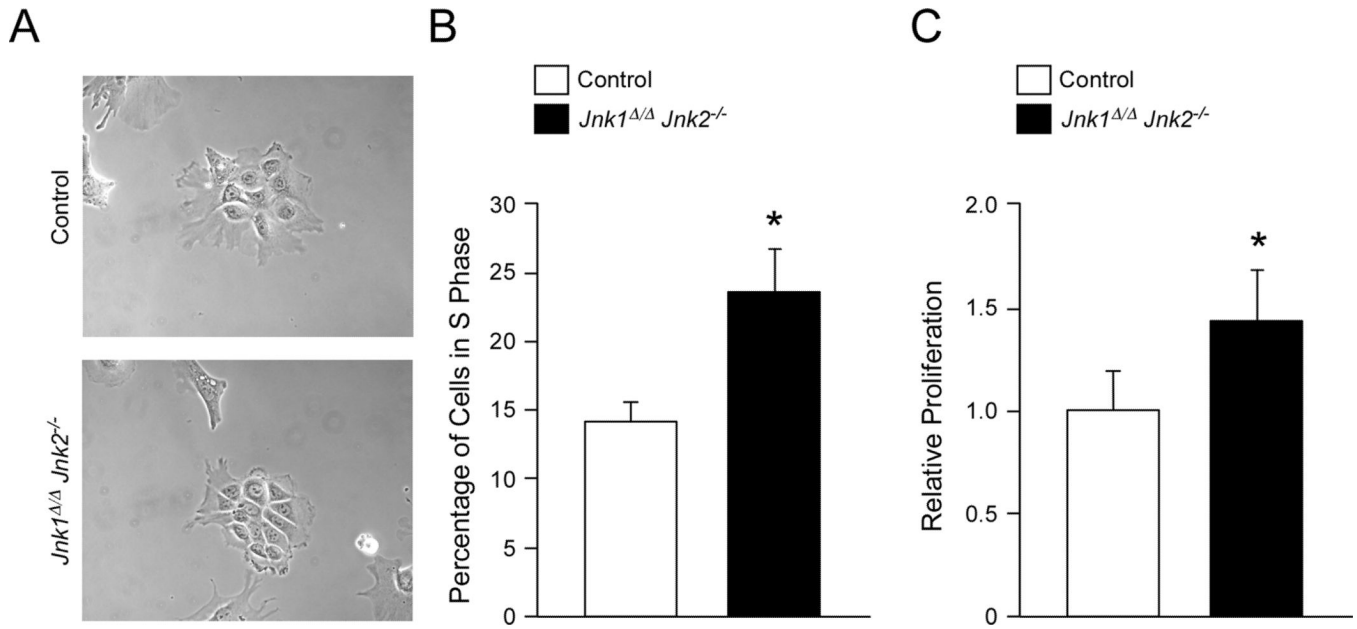

Figure 2. Effect of JNK-deficiency on mammary epithelial cell proliferation

A) Primary cultures of Cre^{ERT} (Control) and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} mammary epithelial cells prepared from tamoxifen-treated mice were examined by phase contrast microscopy. Representative images are shown.

B) The cells were pulse-labeled with BrdU and examined by flow cytometry. The number of BrdU positive cells (%) is presented (mean ± SD; n = 5). Significant differences between Control cells and JNK-deficient cells are indicated with an asterisk ($p < 0.05$).

C) Relative cell proliferation was measured using the WST-1 assay (mean ± SD; n = 3). Significant differences between Control cells and JNK-deficient cells are indicated with an asterisk ($p < 0.05$).

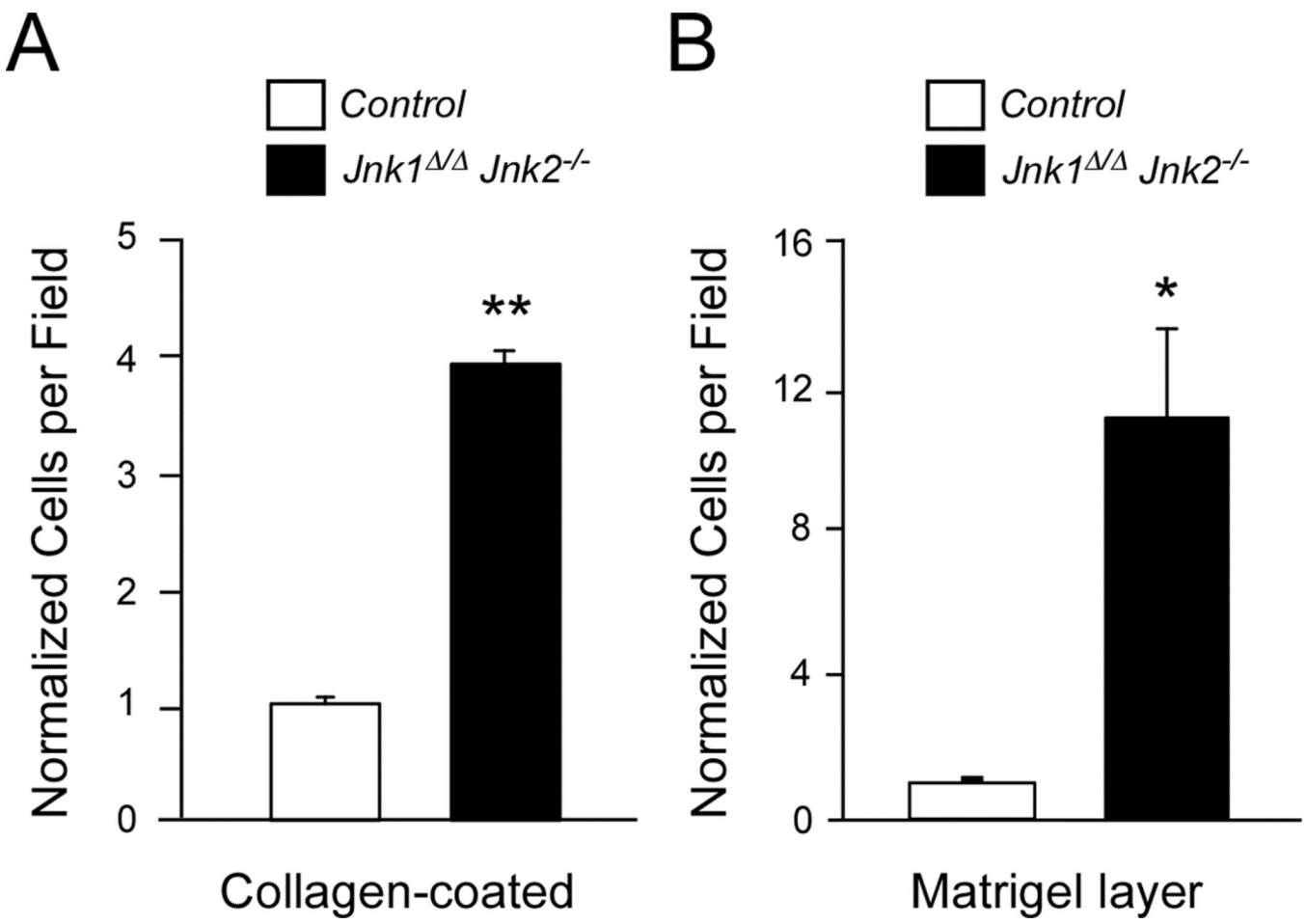

Figure 3. Effect of JNK-deficiency on mammary epithelial cell motility and invasion

A,B) Primary cultures of Cre^{ERT} (Control) and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} 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 (*, $p < 0.05$; **, $p < 0.001$).

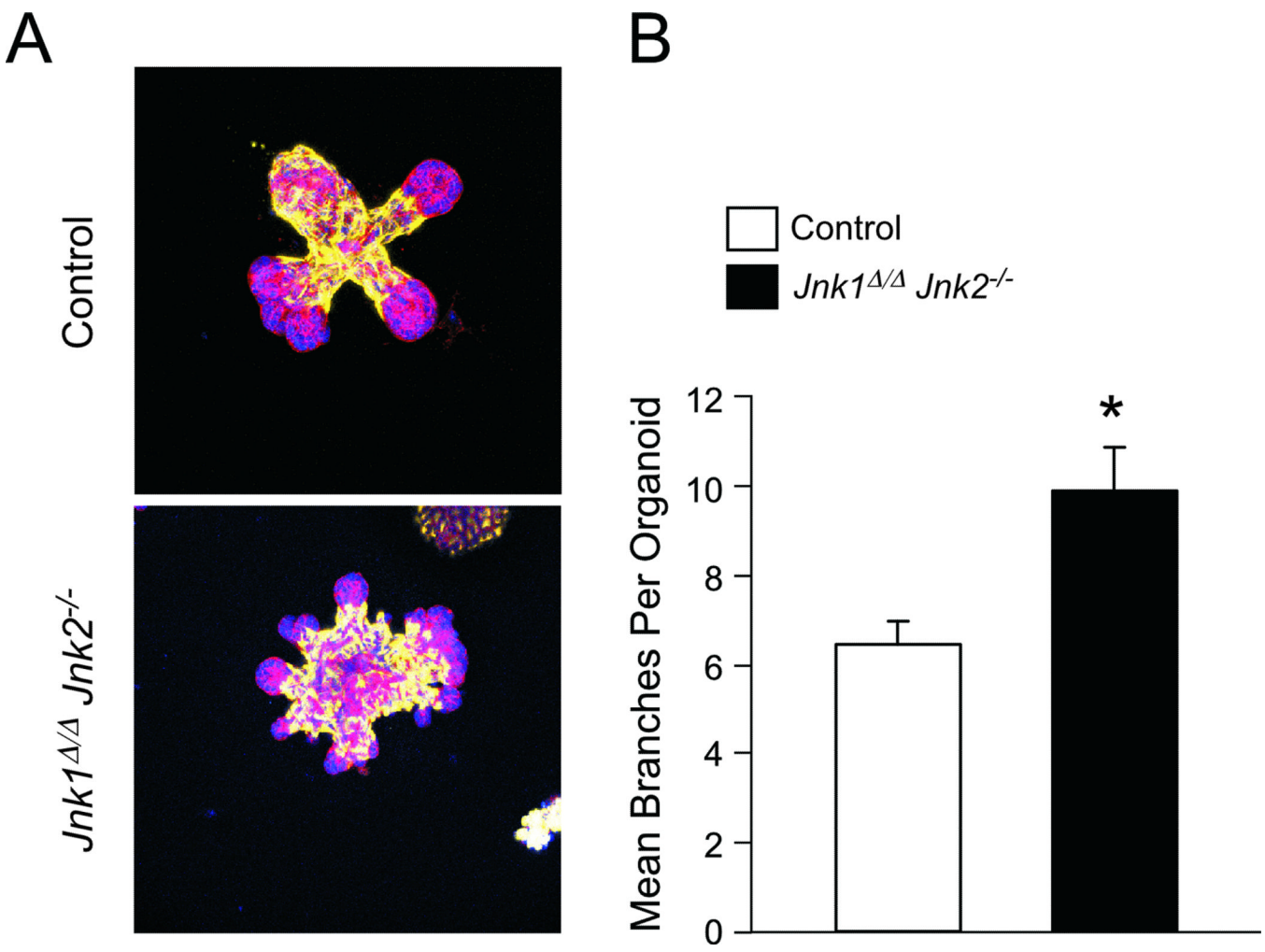

Figure 4. Effect of JNK-deficiency on mammary branching morphogenesis

A) Primary organoid Cre^{ERT} (Control) and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} cultures prepared 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 (*, $p < 0.01$).

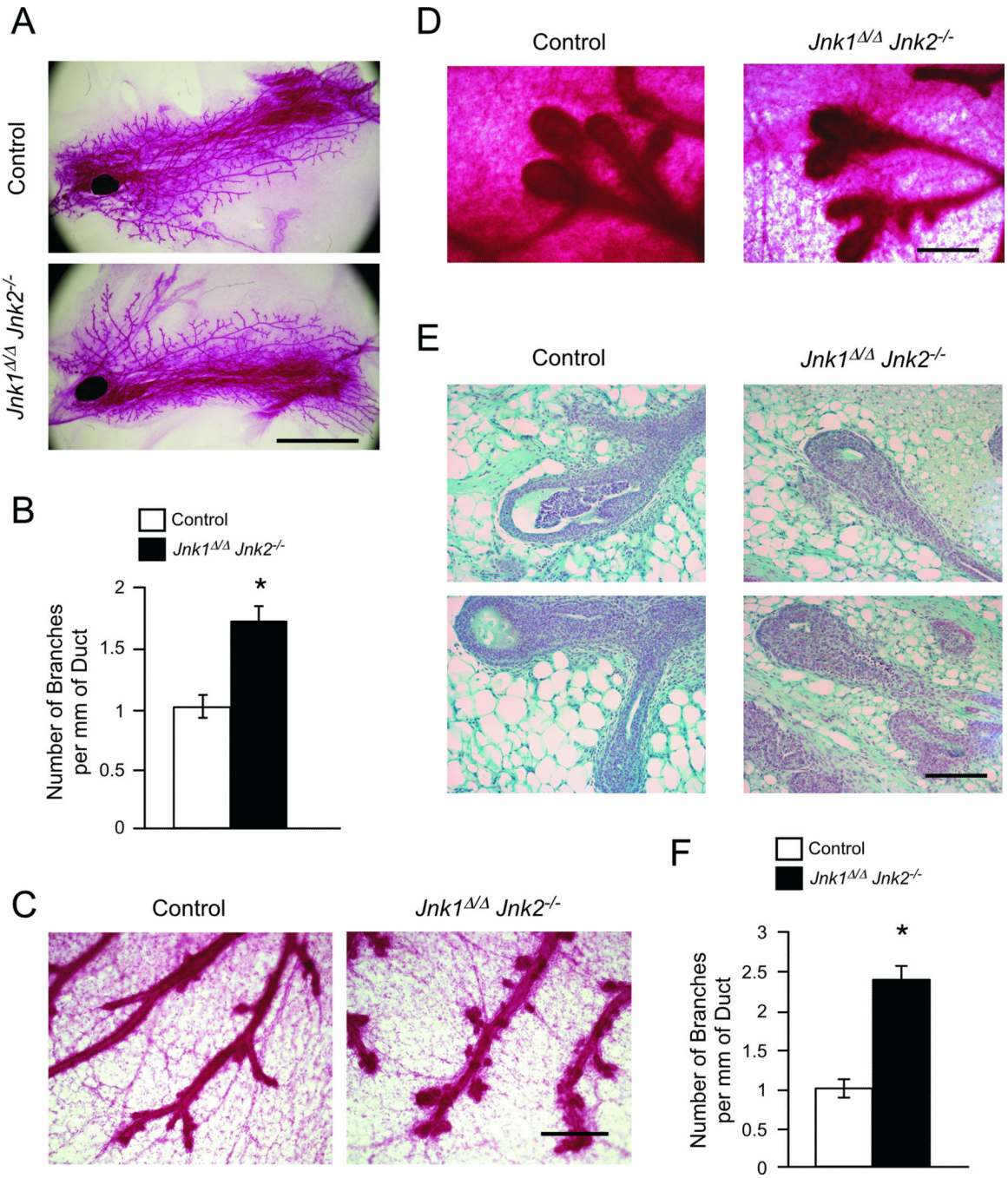

Figure 5. Effect of JNK-deficiency on mammary gland development

Transplantation assays were performed using mammary tissue from tamoxifen-treated female donor mice and female recipient nude mice. Control (Cre^{ERT}) tissue was transplanted in one cleared mammary gland and $Jnk1^{\Delta/\Delta}$ $Jnk2^{-/-}$ Cre^{ERT} tissue was transplanted in the contralateral gland of the same recipient mouse.

A–C) Whole mount mammary glands stained with carmine alum at 8 weeks following transplantation are shown (A,C). The number of branches per unit length (mm) of duct is presented as the mean ± SD; n = 3 (B). Statistically significant differences are indicated with an asterisk (*, $p < 0.05$). Scale bars: 5 mm (A); 200 μm (C).

D–F) Terminal end buds in whole mount mammary glands stained with carmine alum at 2 weeks following transplantation are shown (D). Stained sections of the end buds in whole mount mammary glands are presented (E). The number of branches per unit length (mm) of duct is presented as the mean ± SD; n = 3 (F). Statistically significant differences are indicated with an asterisk (*, $p < 0.05$). Scale bars: 200 μm (D); 100 μm (E).

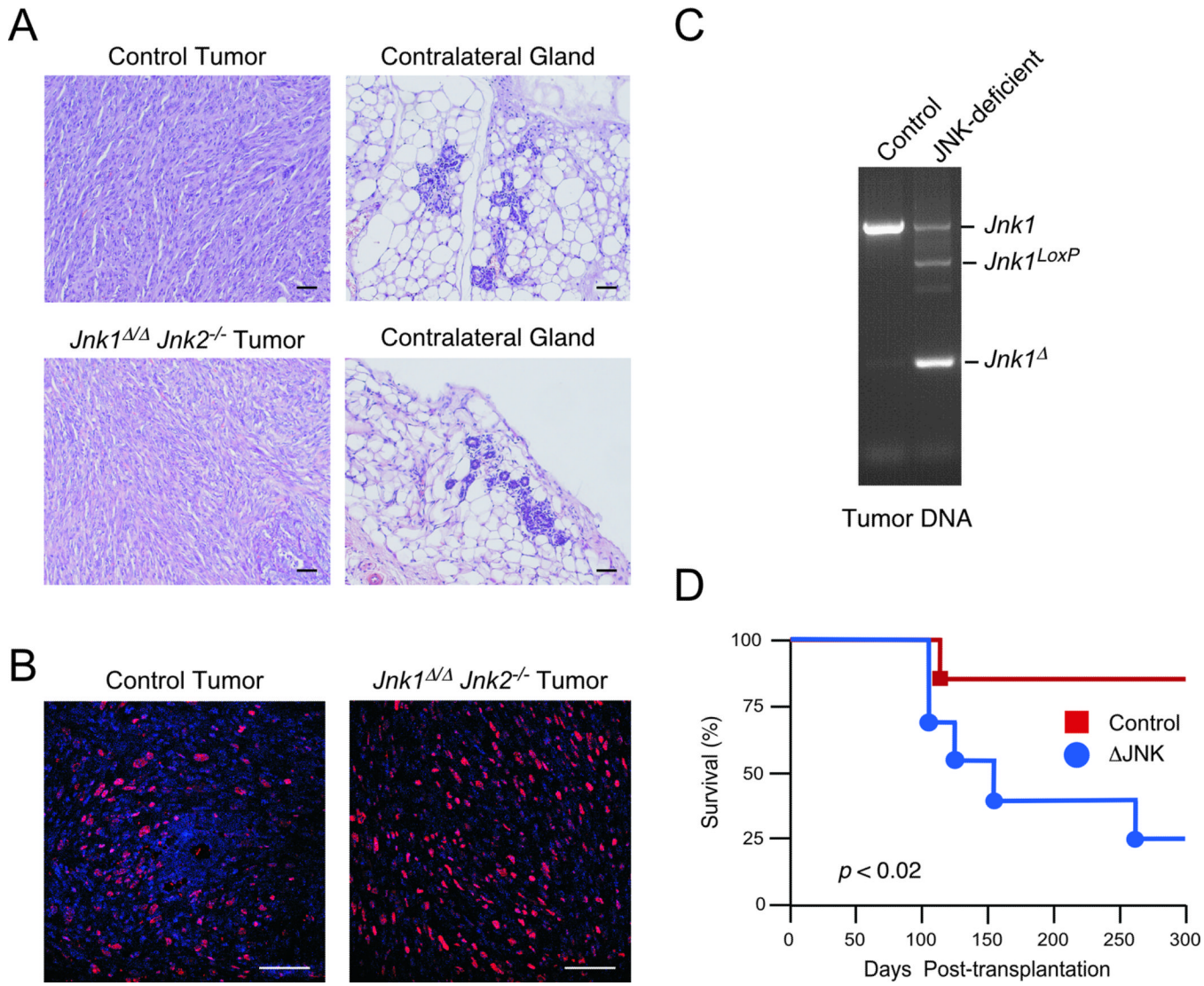

Figure 6. Effect of JNK-deficiency on mammary tumor development

Transplantation assays were performed using mammary tissue from female donor mice and female recipient nude mice. Control (*KRas*$^{LSL\text{-}G12D/+}$ *Trp53*$^{LoxP/LoxP}$ *Cre*^{ERT}) tissue was transplanted in one cleared mammary gland and *KRas*$^{LSL\text{-}G12D/+}$ *Trp53*$^{LoxP/LoxP}$ *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} tissue was transplanted in the contralateral gland of the same recipient mouse. The transplanted mice were treated with tamoxifen at two weeks post-surgery.

A) Sections of transplanted breast mammary glands were stained with H&E. Scale bar = 50 μm.

B) Sections of breast tumors were stained with an antibody to PCNA (red). DNA was stained with DAPI. Scale bar = 75 μm.

C) Genomic DNA isolated from Control and JNK-deficient breast tumors was examined by PCR using amplimers to detect the wild-type *Jnk1* allele (1.5 kb), the *Jnk1*^{LoxP} allele (1.1 kb), and the ablated allele Δ*Jnk1* (0.4kb).

D) Kaplan-Meier analysis of tumor-free survival of transplanted mice (n = 8). The development of JNK-deficient tumors was significantly more rapid than Control tumors ($p < 0.02$; Log-rank test).

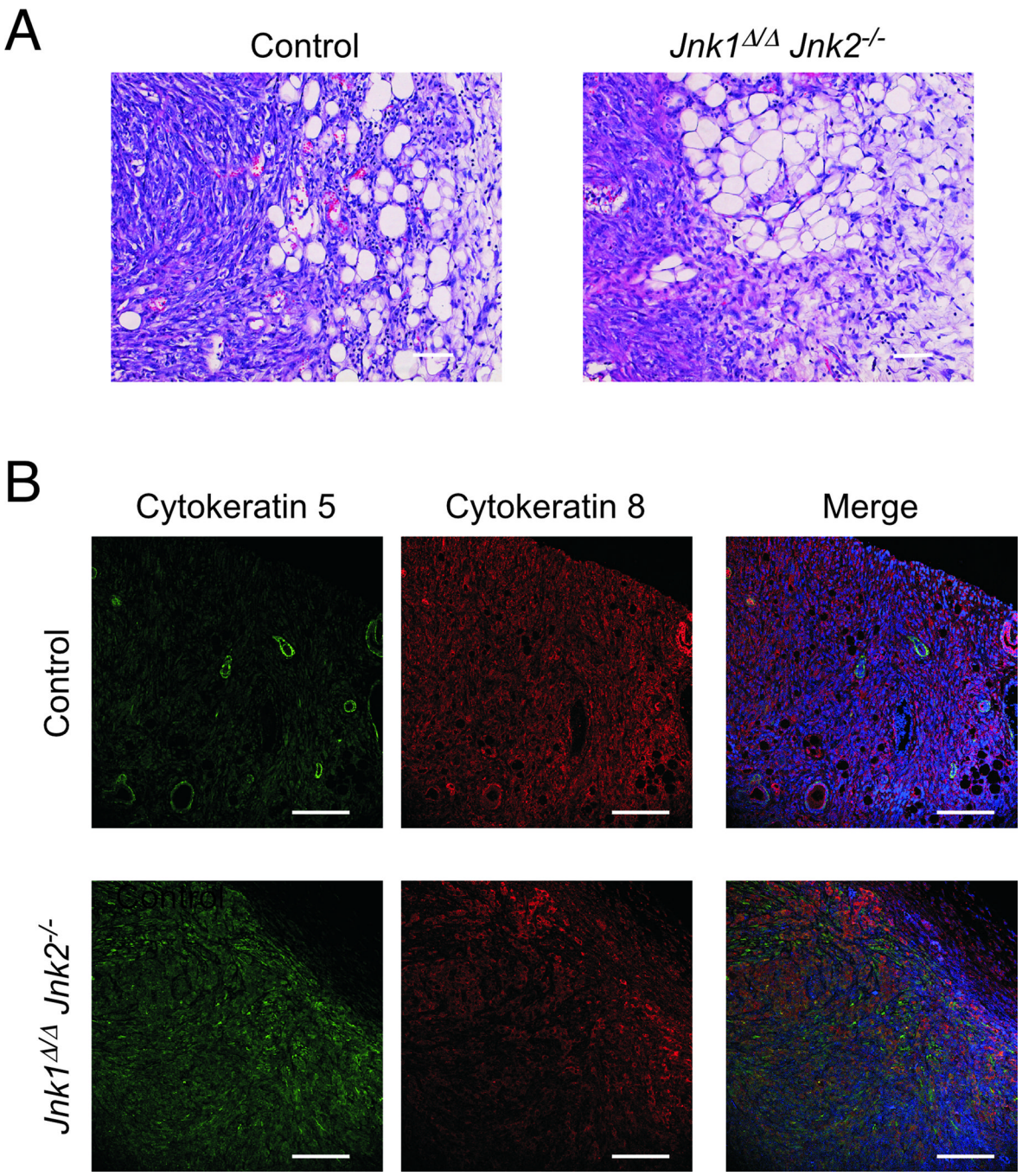

Figure 7. JNK-deficiency causes increased basal-like mammary carcinogenesis

Transplantation assays were performed using Control and JNK-deficient tissue (Figure 6).

A) Sections showing the periphery of Control and JNK-deficient mammary tumors were stained with H&E. Scale bar = 150 μm.

B) Sections of Control and JNK-deficient mammary tumors were stained with an antibody to cytokeratin 5 (green) and cytokeratin 8 (red). DNA was stained with DAPI. Scale bar = 150 μm.