Role of JNK in mammary gland development and breast cancer

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Supplementary Materials and Methods

Mammary gland analysis

The fourth inguinal mammary gland was excised, spread on a glass slide and fixed (2 – 4 hrs.) in Carnoy's fixative (60% ethanol, 30% chloroform, 10% glacial acetic acid). The glands were rinsed with a series of 70%, 50% and 25% ethanol (15 mins each) and then water (5 mins), stained with carmine alum overnight, rinsed with 70%, 90% and 100% ethanol (15 mins each), and then twice with xylene (30 mins). The glands were mounted using Permount (Fisher Scientific) prior to examination. Sections (5 μm) prepared from the whole mounts following embedding in paraffin were counter-stained with light green (American MasterTech). Sections prepared from paraffin-embedded breast tumors were stained with H&E. Analysis was performed by a board-certified pathologist.

The proliferation marker PCNA was examined using sections blocked with the Endogenous Biotin Blocking Kit (Invitrogen), incubation with a mouse anti-PCNA antibody (Invitrogen), and detection using streptavidin conjugated to Alexa Fluor633 (Invitrogen). TUNEL assays were performed using an In Situ Cell Death Detection kit (Roche). Antibodies to cytokeratin 5 (Covance), cytokeratin 8 (Covance), and integrin α5 (Santa Cruz Biotechnology) were used to stain sections blocked with 5% normal goat serum. The sections were incubated with primary antibodies and immune complexes were detected with Alexa Fluor488 goat anti-rabbit IgG and Alexa Fluor546 goat anti-mouse IgG (Invitrogen). Sections were counterstained with DAPI and examined using a fluorescence microscope.

Primary mammary epithelial culture

Primary mouse mammary epithelial cells were prepared (1, 2). The inguinal and thoracic mammary glands of five 8 - 10 week old female virgin mice were removed, minced with two razor blades, and digested in DMEM/F12 supplemented with 2 mg/ml collagenase A (Roche), 100 units/ml hyaluronidase (Sigma, H3506), 100 units/ml penicillin/streptomycin (Invitrogen), and 60 units/ml nystatin (Sigma, N1638) for two hours at 37°C with gentle rotation in a final volume of 40 mL. The digested tissue was then centrifuged for 10 mins at 1,500 rpm and 10ml of the overlying layer of fat and organoids was taken and the remaining supernatant was discarded. The pellet was resuspended in 10 mL of DMEM/F12. The combined suspensions (20 mL) were centrifiged for 10 mins at 1,500 rpm and the pellet was resuspended in 4 mL DMEM/F12 plus 40 μl of DNase (2 units/mL) and shaken gently by hand (2-5 mins) at room temperature prior to the addition of 6 mL of DMEM/F12 and centrifugation for 10 mins at 1,500 rpm to collect mammary epithelial cells. Fibroblasts and other contaminating cells were separated from the mammary gland epithelial cells by differential centrifugation. The cell pellet was resuspended in 10 mL of DMEM/F12, placed in a centrifuge until 1,500 rpm and then immediately stopped. This procedure was repeated 6 times. The final cell pellet containing mammary epithelial cells was resuspended in complete medium [DMEM/F12 plus 1X insulin/transferrin/selenium (Sigma, I3146), 5 ng/ml EGF (Sigma, E4127), 50 μg/ml gentamycin (Sigma G1272), 1X Pen-Strep (Invitrogen), and

5% fetal bovine serum], filtered through 70 µm cell strainer (BD Falcon), and plated onto collagen-I coated T25 flasks (BD Biosciences).

Immunofluorescence analysis of primary mammary epithelial cell cultures

Cells grown on coverslips were washed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde (15 min), washed with PBS, and then incubated in blocking buffer (0.3% Triton / PBS / 5% normal goat serum) for 1 hr. at 25 °C. Incubation with primary antibodies (mouse anti-E-cadherin (Cell Signaling Technology) and rabbit anti-*pan*-cytokeratin (Sigma)) was performed in blocking buffer for 14 hr. at 4°C. The cells were washed and incubated with secondary antibody (AlexFluor-633 conjugated goat anti-mouse Ig or AlexaFluor-488-conjugated goat anti-rabbit (Invitrogen)) in blocking buffer (1 hr. at 25 °C). The coverslips were washed in PBS and mounted using VectaShield medium containing DAPI (Vector Labs.). Images were examined using a Leica TCS SP2 confocal microscope.

Cell proliferation

The incorporation of BrdU was examined by plating 500,000 cells in 60 mm dishes, incubation for 2 days, and treatment with 10 µM BrdU (16 h). The cells were harvested and processed for examination of BrdU incorporation by flow cytomtery (BrdU Flow Kit, BD Pharmingen). Briefly, the cells were fixed, permeabilized, incubated with DNase, and then incubated with an FITC-conjugated anti-BrdU antibody. The cells were stained with 7-Aminoactinomycin D prior to analysis by flow cytometry.

Cell proliferation was also examined using the WST-1 reagent (Roche). We plated 100,000 cells in 24-well dishes in triplicate. Absorbance at 450 nm was measured using a spectrophotometer after incubation for 1 h. The background in this assay was assessed by adding the WST-1 reagent to wells containing medium only. WST-1 measurements were made using cells cultured for 4 h and 48 h.

Boyden chamber assays

Mammary epithelial cells were trypsinized, washed in DMEM/F12, counted using a hemocytometer, and 150,000 cells in 0.5 mL DMEM/F12 (containing all supplements except serum) were plated in each Boyden chamber (24-well format) in triplicate. Invasion assays were performed using Matrigel-coated chambers (BD Matrigel Invasion Inserts, BD Biosciences). Cell migration assays were performed using chambers (BD Biocoat Control Inserts) coated with 15 µg/ml collagen I (BD Biosciences) overnight at 4°C and then blocked with 0.25% bovine serum albumin in DMEM/F12 (3). Complete medium (supplemented with 5% fetal bovine serum) was used as the chemoattractant in the lower chamber. The cells were incubated for 24 h. The cells that did not migrate (and therefore remained inside the upper chamber) were removed with a cotton swab. To determine the number of cells that migrated (attached to the underside of the membrane), the chambers were rinsed in PBS, fixed in ice-cold methanol (10 mins), and rinsed in two changes of PBS. The membranes were removed and mounted on a glass slide using VectaShield plus DAPI (Vector Labs). Cell nuclei were visualized using a Zeiss Axiovert fluorescence microscope. Images were acquired from four different fields

on each membrane and nuclei were counted using ImageJ software (http://rsb.info.nih.gov/ij).

Mammary organoid culture

Mammary organoid cultures were prepared (4, 5) from the inguinal and thoracic mammary glands of five 8 – 12 week old female virgin mice. The glands were removed, minced with two razor blades in parallel, and incubated with 50 mL of digestion medium (2 mg/mL collagenase (Sigma), 0.15% trypsin, 10% fetal calf serum, 1 µg/mL insulin, 0.5 µg/mL gentamycin (Invitrogen)) for 2 hours at 37°C with gentle rotation. The mixture was centrifuged (10 mins at 1,500 rpm), the supernatant removed, and the pellet was resuspended in 10 mL of DMEM/F12. This cell suspension was centrifuged (10 mins at 1,500 rpm), the supernatant removed, and the pellet resuspended in 4 mL of DMEM/F12 with 40U of DNAse. The suspension was gently shaken by hand (5 mins), and centrifuged (10 min at 1,500 rpm). The pellet was then resuspended in 10 mL of DMEM/F12 and centrifuged to 1,500 rpm and immediately stopped; this procedure was repeated four times to remove single cells. The resultant mammary organoids were resuspended in Matrigel (BD Biosciences) and plated in 8 chamber (50 µL per well) chamber slides (BD Biosciences). The Matrigel was allowed to set in a 37°C incubator (30mins) before addition of minimal medium (MM: DMEM/F12 plus 1X insulin/transferrin/selenium solution and 1X penicillin/streptomycin). Some wells were treated with 2.5 nM FGF2 (Sigma) one day after plating. The organoids were cultured for 7 days.

Immunofluorescence analysis of mammary organoids

Staining of mammary organoids was performed (4) using chamber slides (BD Biosciences) equilibrated with 25% (w/v) sucrose in PBS (1 h), fixed in cold 1:1 methanol:acetone overnight at -20^{o}C, and re-equilibrated in 25% (w/v) sucrose in PBS (1 h). The organoids were subsequently incubated (1 h) in blocking buffer (5% serum in PBS), a FITC-conjugated antibody to smooth muscle actin (SMA; Sigma) for 2 h at room temperature, rinsed three times with blocking buffer. AlexaFluor-546-conjugated-Phalloidin (Invitrogen) was used to stain F-actin. The coverslips were rinsed three times with blocking buffer (one wash with 300 nM DAPI), and then mounted with Prolong Gold (Invitrogen). The coverslips were examined using a Leica TCS SP2 confocal microscope.

Immunoblot analysis

Cell lysates were examined by probing with antibodies to ERK1/2 (Cell Signaling), JNK1/2 (BD Biosciences), p53 (Cell Signaling), and α-Tubulin (Sigma). Immune complexes were detected using enhanced chemiluminescence (NEN).

Analysis of mRNA

Total RNA was isolated from cell lines and real-time reverse transcription PCR was performed using TaqMan probes for *Ddr1* (Mm00432251_m1), *Ddr2*

(Mm00445615_m1), *Gapdh* (#4352339E), *Integrin alpha 1* (Mm01306371_m1), *Integrin alpha 2* (Mm00434371_m1), *Integrin alpha 5* (Mm00439797_m1), *Integrin alpha 6* (Mm00434375_m1), *Integrin beta 1* (Mm01253230_m1), *Mmp2* (Mm01253621_m1), *Mmp3* (Mm01168401_m1), *Mmp9* (Mm00442991_m1), *Mmp14* (Mm01318966_m1), *Adam17* (Mm01231071_m1), *Sprouty2* (Mm00442344_m1), *Timp1* (Mm00441818_m1), *Timp2* (Mm00441825_m1), *Timp3* (Mm00441827_m1), and *Tgfβ1* (Mm03024053_m1) (Applied Biosystems). The relative mRNA expression was calculated by normalizing to the amount of *Gapdh* mRNA in each sample.

Analysis of the intestinal epithelium

Mice were injected intraperitoneally with 100 mg/kg BrdU (Sigma) at 2.5 h prior to euthanasia. Immunohistochemistry on the small intestine and colon was performed using 5 µm paraffin sections treated with with Target Retrieval Solution (DAKO) and the Vectastain ABC kit (Vector Laboratories). Sections were stained with antibodies to JNK1/2 (Pharmingen), p53 (Cell Signaling), BrdU (BD Pharmingen), and phosphoSer^{63}-cJun (Cell Signaling).

Intestinal epithelial cells were isolated from the small intestine and colon. A longitudinal incision was made and feces were removed by repeated washing with PBS. The intestines (2–3 mm pieces) were incubated with Hanks' balanced salt solution (HBSS) supplemented with 30 mM EDTA (15 min at 37°C). The supernatant was aspirated, the epithelial cells were recovered by centrifugation, washed with ice-cold PBS, and flash frozen prior to biochemical analysis.

Colitis-associated colon cancer was examined using the azoxymethane/DSS model (6). Briefly, mice (6–8-week-old) were injected intraperitoneally with 12.5 mg/kg azoxymethane AOM (Sigma). After five days, 2.5% DSS (MW, 36-50 kDa; MP Biomedicals) was given in the drinking water for five days, followed by 16 days of regular water. This cycle was repeated twice (five days of 2.5% DSS and four days of 2% DSS) and mice were sacrificed ten days after the last cycle. Colons were removed, flushed with 1xPBS, fixed as “Swiss-rolls” in 10% formalin overnight and paraffin-embedded. Sections (5 µm) were cut stepwise (200 µm) through the entire block and stained with H&E.

Supplementary References

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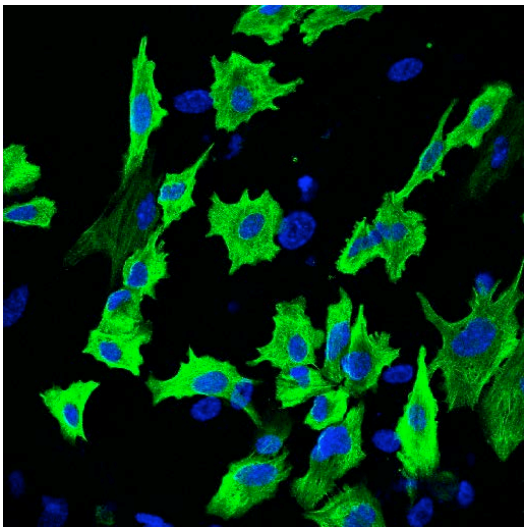

Figure S1. Primary cultures of mammary epithelial cells.

Primary mammary epithelial cells were stained with an antibody to pan-cytokeratin (green). DNA was stained with DAPI (blue). A representative image is presented.

The cultures contained cytokeratin-positive epithelial cells. However, 11% of the cells were cytokeratin-negative and represent a contamination of the cultures with non-epithelial cells.

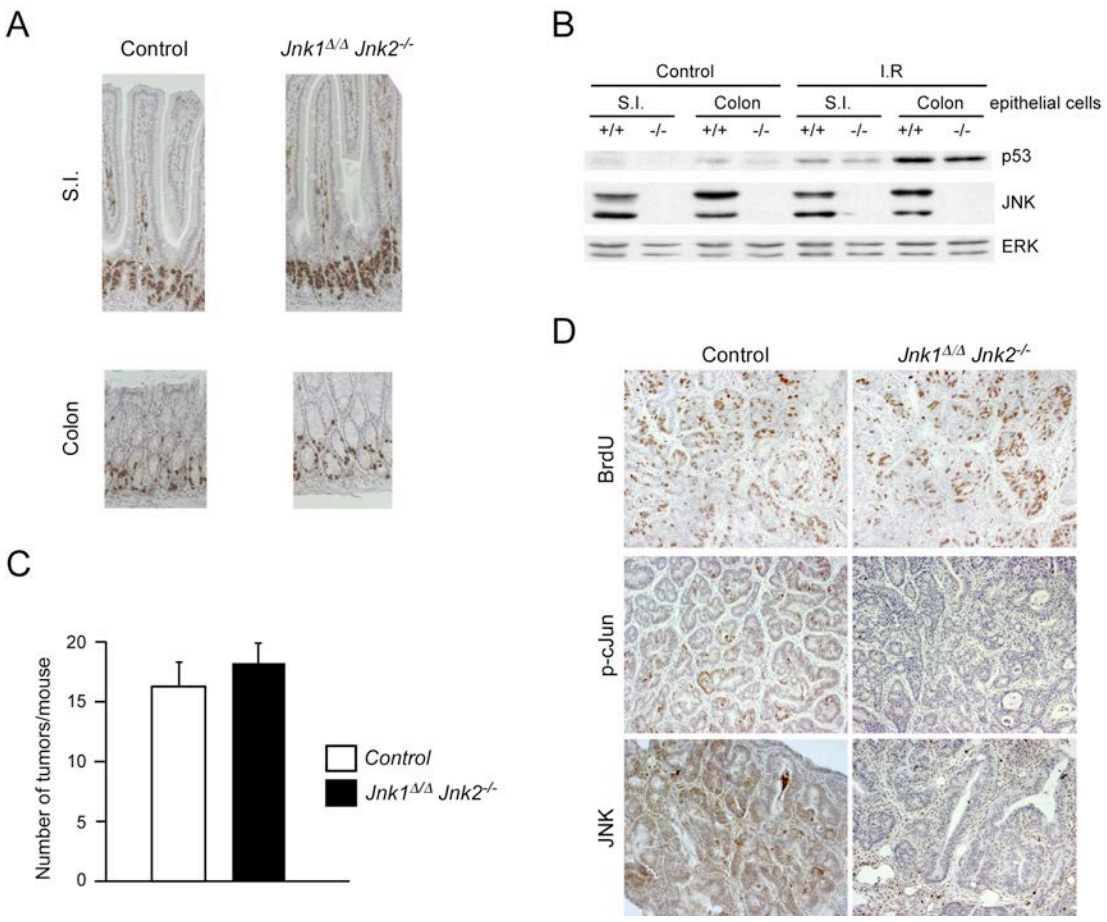

Figure S2. Effect of JNK-deficiency on the intestinal epithelium.

A) BrdU incorporation in the small intestine (S.I.) and colon was examined by immunohistochemistry. No significant differences were detected between control mice (*Villin-Cre*$^{+/-}$) and JNK-deficient mice (*Villin-Cre*$^{+/-}$ *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$).

B) S.I. and colonic epithelial cells were isolated and examined by immunoblot analysis by probing with antibodies to p53, JNK, and ERK. The effect of exposure of mice to ionizing radiation (800 Rads) prior to euthanasia at 270 mins post-irradiation. Control mice (*Villin-Cre*$^{+/-}$) and JNK-deficient mice (*Villin-Cre*$^{+/-}$ *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$) were compared.

C) Colitis-associated colon cancer was examined using the azoxymethane/DSS model. No statistically significant difference in the number of colon tumors was detected in control mice (*Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$) and mice lacking JNK in the intestinal epithelium (*Villin-Cre*$^{+/-}$ *Jnk1*$^{LoxP/LoxP}$ *Jnk2*$^{-/-}$). The data presented represent the mean ± SD (n ≥ 7).

D) Sections of control and JNK-deficient colon tumors were examined by immunohistochemistry using antibodies to BrdU, phosphoSer^{63}-cJun, and JNK.

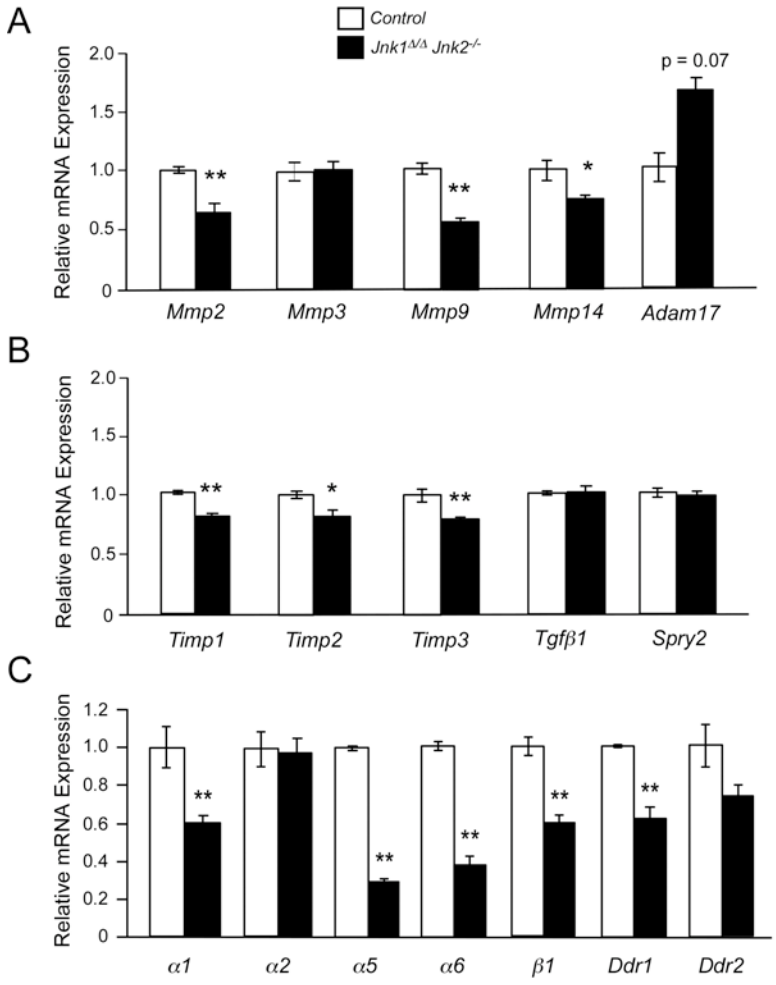

Figure S3. Effect of JNK-deficiency on mammary epithelial cell gene expression.

RNA was isolated from primary cultures of Control (*Cre*^{ERT}) and *Jnk1*$^{\Delta/\Delta}$ *Jnk2*$^{-/-}$ *Cre*^{ERT} mammary epithelial cells prepared from tamoxifen-treated mice. The expression of mRNA was examined by quantitative RT-PCR. The mRNA measurements were normalized to the amount of *Gapdh* mRNA detected in each sample. The data presented represent the mean ± SD (n = 5). Statistically significant differences between Control and JNK-deficient mammary epithelial cells are indicated with an asterisk (*, $p < 0.05$; **, $p < 0.001$).

A) The expression of *Mmp2/3/9/14* and *Adam17* mRNA.

B) The expression of *Tim1/2/3*, *Tgfβ1*, and *Spry2* mRNA.

C) The expression of *integrin α1/2/5/6, integrin β1, and Ddr1/2* mRNA.

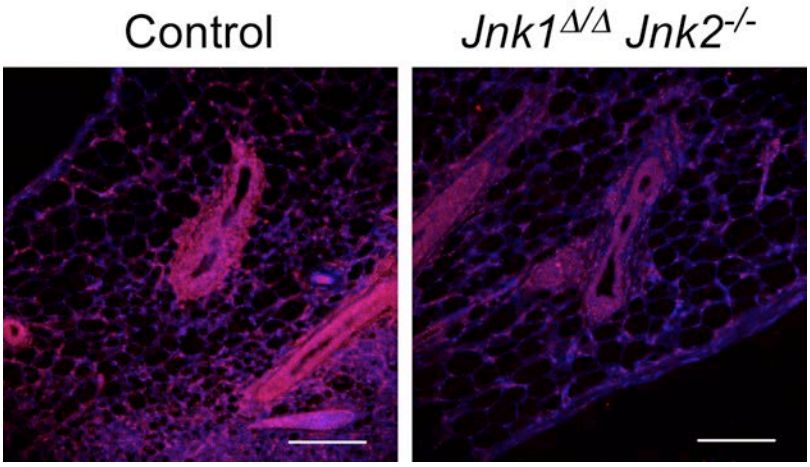

Figure S4. Characterization of integrin α5 expression in Control and JNK-deficient mammary glands.

Sections of Control and JNK-deficient mammary tissue from transplanted mice were stained with an antibody to integrin α5 (red). Nuclear DNA was stained with DAPI (blue). Scale bar = 150 μm.

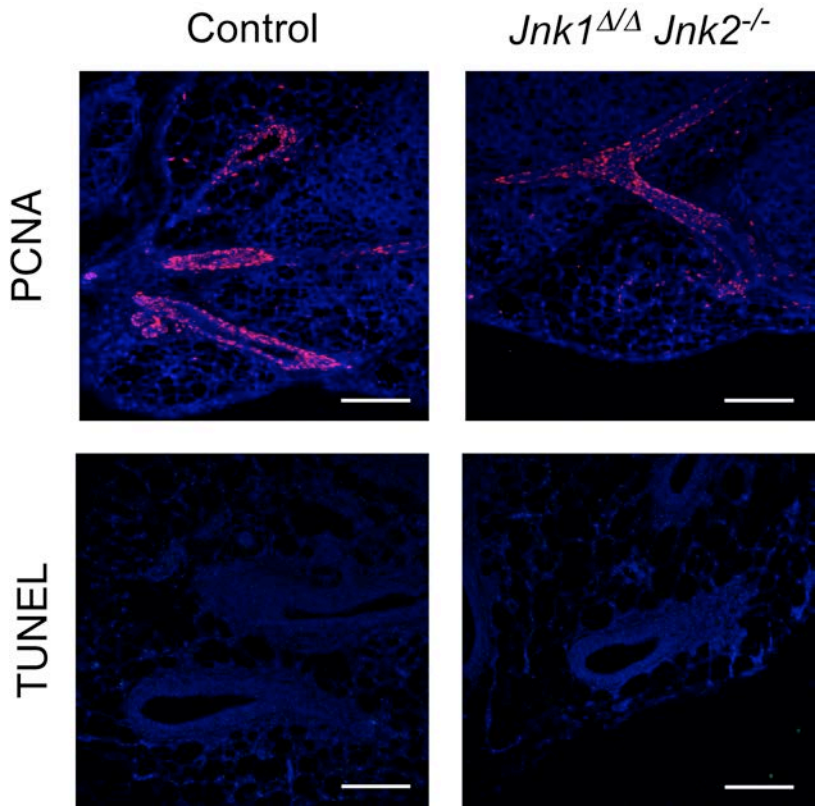

Figure S5. Characterization of Control and JNK-deficient mammary glands.

Sections of Control and JNK-deficient mammary tissue from transplanted mice were stained with an antibody to PCNA (red; upper panels) and by TUNEL assay (green; lower panels). Nuclear DNA was stained with DAPI (blue). Scale bar = 150 μm.

The Control and JNK-deficient tissue exhibited similar staining for the proliferation marker PCNA. No TUNEL staining of Control and JNK-deficient tissue was detected.