# NOTCH3 Signaling Pathway Plays Crucial Roles in the Proliferation of ErbB2-Negative Human Breast Cancer Cells

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## Abstract

**ErbB2-negative breast tumors represent a significant therapeutic hurdle because of a lack of effective molecular targets. Although NOTCH proteins are known to be involved in mammary tumorigenesis, the functional significance of these proteins in ErbB2-negative breast tumors is not clear. In the present study, we examined the expression of activated NOTCH receptors in human breast cancer cell lines, including ErbB2-negative and ErbB2-positive cell lines. Activated NOTCH1 and NOTCH3 proteins generated by γ-secretase were detected in most of the cell lines tested, and both proteins activated CSL-mediated transcription. Down-regulation of NOTCH1 by RNA interference had little or no suppressive effect on the proliferation of either ErbB2-positive or ErbB2-negative cell lines. In contrast, down-regulation of NOTCH3 significantly suppressed proliferation and promoted apoptosis of the ErbB2-negative tumor cell lines. Down-regulation of NOTCH3 did not have a significant effect on the ErbB2-positive tumor cell lines. Down-regulation of CSL also suppressed the proliferation of ErbB2-negative breast tumor cell lines, indicating that the NOTCH-CSL signaling axis is involved in cell proliferation. Finally, *NOTCH3* gene amplification was detected in a breast tumor cell line and one breast cancer tissue specimen even though the frequency of *NOTCH3* gene amplification was low (<1%). Taken together, these findings indicate that NOTCH3-mediated signaling rather than NOTCH1-mediated signaling plays an important role in the proliferation of ErbB2-negative breast tumor cells and that targeted suppression of this signaling pathway may be a promising strategy for the treatment of ErbB2-negative breast cancers.** [Cancer Res 2008;68(6):1881–8]

## Introduction

Breast cancer is the most common type of cancer in women. This disease often progresses to a malignant phenotype with highly invasive and metastatic growth properties (1). Continued research on the molecular mechanisms that underlie this disease has revealed that a receptor tyrosine kinase, ErbB2, is overexpressed in 20% to 30% of progressed breast tumors. This research resulted in the development of Herceptin, a humanized antibody against ErbB2. Although treatment of ErbB2-overexpressing breast tumors with Herceptin is one of the few examples of a successful molecular-based therapy for breast cancer (2, 3), its efficacy is limited because ErbB2-negative breast cancers do not respond to the medication. Therefore, an improved understanding of the molecular pathways involved in breast cancer development may facilitate the development of novel targeted molecular therapies.

NOTCH proteins belong to a family of conserved transmembrane receptors that play fundamental roles in cell fate decisions such as cell proliferation, differentiation, and apoptosis. In mammals, there are four NOTCH receptors (termed NOTCH1, NOTCH2, NOTCH3, and NOTCH4) and five ligands (termed Jagged1 and Jagged2 and Delta-like 1, Delta-like 3, and Delta-like 4). NOTCH signaling is activated through receptor-ligand interactions between neighboring cells resulting in successive proteolytic cleavages by tumor necrosis factor-α–converting enzyme and the γ-secretase complex. This processing releases the NOTCH intracellular domain (NICD), allowing it to enter the nucleus. Once within the nucleus, NICD binds to the CSL (also termed CBF-1 and RBP-Jκ) DNA-binding protein to generate a large transcriptional activator complex containing transcriptional coactivators of the mastermind-like (MAML) family. Formation of this complex results in the expression of various target genes, such as *Hes/Hey* family genes, which are involved in cell growth, differentiation, and survival (4).

There is increasing evidence supporting the association of one or more NOTCH pathways with breast cancer development (5, 6). Ectopic expression of active forms of NOTCH1 and NOTCH4 transforms both normal human and mouse mammary epithelial cells *in vitro* (7–9). Transgenic mice that overexpress the NICD of NOTCH1 or NOTCH3 in mammary glands develop mammary tumors (10), suggesting the transforming potentials of NOTCH1 and NOTCH3 *in vivo*. In human breast cancer, aberrant activation

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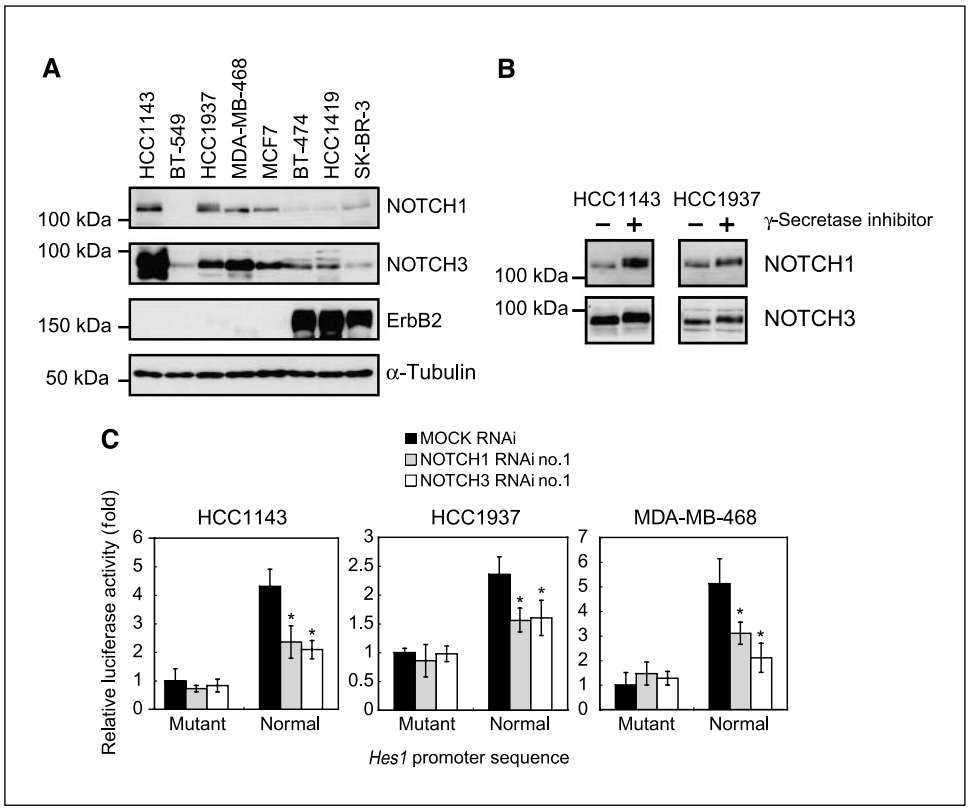


**Figure 1.** Expression of NOTCH receptors in human breast cancer cell lines. *A,* Western blot analysis of NICD of NOTCH1 and NOTCH3 and ErbB2 in eight human breast cancer cell lines. These cell lines are classified by ErbB2 protein expression status, ErbB2-negative cell lines (*HCC1143, BT-549, HCC1937, MDA-MB-468*, and *MCF7*) and ErbB2-positive cell lines (*BT-474, HCC1419*, and *SK-BR-3*). NICD of NOTCH1 or NOTCH3 is elevated in five of five or four of five ErbB2-negative cell lines, respectively. Expression of α-tubulin is shown as a loading control. *B,* Western blot analysis of NICD of NOTCH1 and NOTCH3 in ErbB2-negative HCC1143 and HCC1937 cells cultured in the presence or absence of γ-secretase inhibitor for 5 h. Treatment with γ-secretase inhibitor causes a slight shift of both bands to a higher molecular weight, indicating that these two bands represent the NICDs of NOTCH1 and NOTCH3. *C,* luciferase activity in ErbB2-negative HCC1143, HCC1937, and MDA-MB-468 cells cotransfected with a *Hes1* promoter luciferase construct normal or mutant CSL-binding sites and NOTCH1-, NOTCH3-, or mock siRNA. *Columns,* mean from experiments done in triplicate; *bars,* SD. *, $P < 0.05$ relative to mock-siRNA transfected samples.

of NOTCH1 has been observed (11), and elevated expression of the *NOTCH1* and *Jagged1* mRNAs correlates with poor prognosis (12). However, it is currently not known which, if any, of the NOTCH receptors are involved in the development of ErbB2-negative breast cancers.

In the present study, we investigated the significance of NOTCH signaling in ErbB2-negative human breast cancer cell lines. We found that RNA interference (RNAi)–mediated NOTCH3 knockdown inhibits cell proliferation and that the *NOTCH3* gene is amplified in a breast cancer specimen. These results suggest that NOTCH3 is crucial for ErbB2-negative breast tumor development and may therefore be a promising therapeutic target for these cancers.

## Materials and Methods

**Cell culture and transfections.** All cell lines were purchased from American Type Culture Collection and cultured according to the manufacturer's instructions. γ-Secretase inhibitor (GSI-12) was purchased from Calbiochem and dissolved in DMSO as a 50 mmol/L stock solution. All of the small interfering RNAs (siRNA; Human Stealth Select RNAi) were purchased from Invitrogen. Plasmid vector or siRNA was transfected into cells with LipofectAMINE 2000 (Invitrogen) according to the manufacturer's instructions.

**Western blot analysis.** Total cell protein was extracted in boiling SDS sample buffer [2% SDS, 50 mmol/L Tris-HCl (pH 6.8), 10% glycerol, 0.002% bromophenol blue, and 6% 2-mercaptoethanol]. Cell extracts were separated by SDS-PAGE, and the proteins were transferred to polyvinylidene difluoride membrane (Millipore). Western blots were blocked in TBS-T buffer [10 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, and 0.05% (v/v) Tween 20] containing 5% nonfat dry milk and probed with primary antibody in blocking buffer at 4°C overnight. Blots were washed with TBS-T buffer and incubated with secondary antibodies diluted in TBS-T at room temperature. Immunocomplexes were visualized with an enhanced chemiluminescence kit (GE Healthcare).

Primary antibodies were anti-NOTCH1, -NOTCH3, -NOTCH4, -ErbB2, -Erk1/2, and -Akt antibodies (Santa Cruz Biotechnology), anti-NOTCH2 and -Jagged2 antibodies (Orbigen), anti–α-tubulin antibody (Calbiochem), anti–phosphorylated Erk1/2, phosphorylated-Akt, Jagged1, and cyclin D1 antibodies (Cell Signaling Technologies), and anti-poly(ADP-ribose) polymerase (PARP) antibody (a kind gift from Dr. T Suzuki, Institute of Medical Science, University of Tokyo). All secondary antibodies were purchased from GE Healthcare.

**Proliferation assay.** Cells were plated at 20% to 30% confluency in 12-well plates and grown 8 h before siRNA transfection (day 0). On days 2, 4, and 6 after siRNA transfection, cells were trypsinized, and the viable cell number was counted with the Trypan blue exclusion method.

**Reporter constructs and luciferase assay.** The *Hes1* promoter-driven luciferase reporter construct was generated by insertion of the *Hes1* promoter (nucleotides −93 to −41) containing normal or mutated CSL-binding sites (CBS; ref. 13) into *Sph*I- and *Xba*I-digested 3xκB-luc luciferase vector (14). In this construct, the *Hes1* promoter sequence is located upstream of the firefly luciferase gene. The inserted sequences containing *Hes1* promoter is as follows: normal CBS, 5-CTCCCATTGGCTGAAAGTTACTGTGGGAAAGAAAGTTTGGGAAGTTTCACACG-3 and mutated CBS, 5-gcagCATTGGCTGAAAGTTACTGTGctgcAGAAAGTTTGGGAAGTgcagCACG-3. The CBS are underlined, and mutated regions are indicated by lower case letters.

For luciferase assays, cells were plated at 50% confluency in 24-well plates and grown overnight before siRNA transfection. Two days after siRNA transfection, the firefly luciferase reporter construct and the *Renilla* pRL-actin control plasmid were cotransfected into the cells. After 24 h of culture, the luciferase reporter activity was assayed with the Dual Luciferase Assay System (Promega).

**Processing and analysis of human breast cancer tissues.** H&E-stained sections of matched normal and breast tumor tissues were analyzed by a pathologist to confirm initial diagnosis, staging, and overall integrity of the tissue samples. Ten 10-μm-thick slices were cut from each frozen tissue

block. Genomic DNA was isolated in phenol/chloroform. The ethics committee of Dokkyo Medical University School approved the use of the tissue samples for DNA analyses, and written informed consent was obtained from all patients.

**Quantitative real-time PCR analysis for *NOTCH3* gene amplification.** Genomic DNA from each sample or cell line was analyzed for *NOTCH3* amplification with TaqMan technology on an ABI Prism 7900 Sequence Detection System (Applied Biosystems). The primer/probe set for the human *NOTCH3* gene was forward primer, 5-TGACCGTACTGGCGAGACT-3; reverse primer, 5-CCGCTTGGCTGCATCAG-3; and probe, 5-CTGGCTGCC-CGTTATG-3. The primer/probe set for the *RNaseP* gene (TaqMan RNaseP Control Reagents) was purchased from Applied Biosystems. The *RNaseP* gene was used as the reference non–amplified gene in tumor cell lines and tissues (15), and *NOTCH3* gene copy number was estimated by comparative CT (threshold cycle) method. With this method, samples with a normal copy number have a *NOTCH3/RNaseP* ratio of 1. Copy number values >2 SD of the mean value of normal breast tissues were designated as amplified *NOTCH3* gene copy. Tumors with *NOTCH3* levels within 2 SD of the mean of normal breast tissues were classified as nonamplified.

**Semiquantitative reverse transcription-PCR.** Cells were grown for 3 days after siRNA transfection. RNA was isolated with TRIzol (Invitrogen) according to the manufacturer's instructions. cDNA was prepared from 3 μg of total RNA with a Superscript III Kit (Invitrogen) according to the manufacturer's instructions. One-twentieth of the prepared cDNA was used in subsequent PCRs containing 0.5 μmol/L of each primer, 200 μmol/L of deoxynucleotide triphosphates, 1× Ex Taq buffer (TaKaRa), and 0.05 units/μL of Ex Taq DNA polymerase (TaKaRa). Primers for CSL were forward, 5-TTGGTGAGCGGCCTCCACCTAA-3; and reverse, 5-TTAG-GATACCACTGTGGCTGT-3. Primers for β-actin were forward, 5-ATGGAT-GATGATATCGCCGC-3; and reverse, 5-CTAGAAGCATTTGCGGTGGA-3. The PCR cycle was as follows: 30 cycles at 98°C for 10 s, 55°C for 30 s, 72°C for 90 s with a 1-min initial denaturation at 98°C, and a final 10-min elongation step at 72°C.

**Fluorescence *in situ* hybridization.** Fluorescence *in situ* hybridization (FISH) was performed essentially as described previously (16). Bacterial

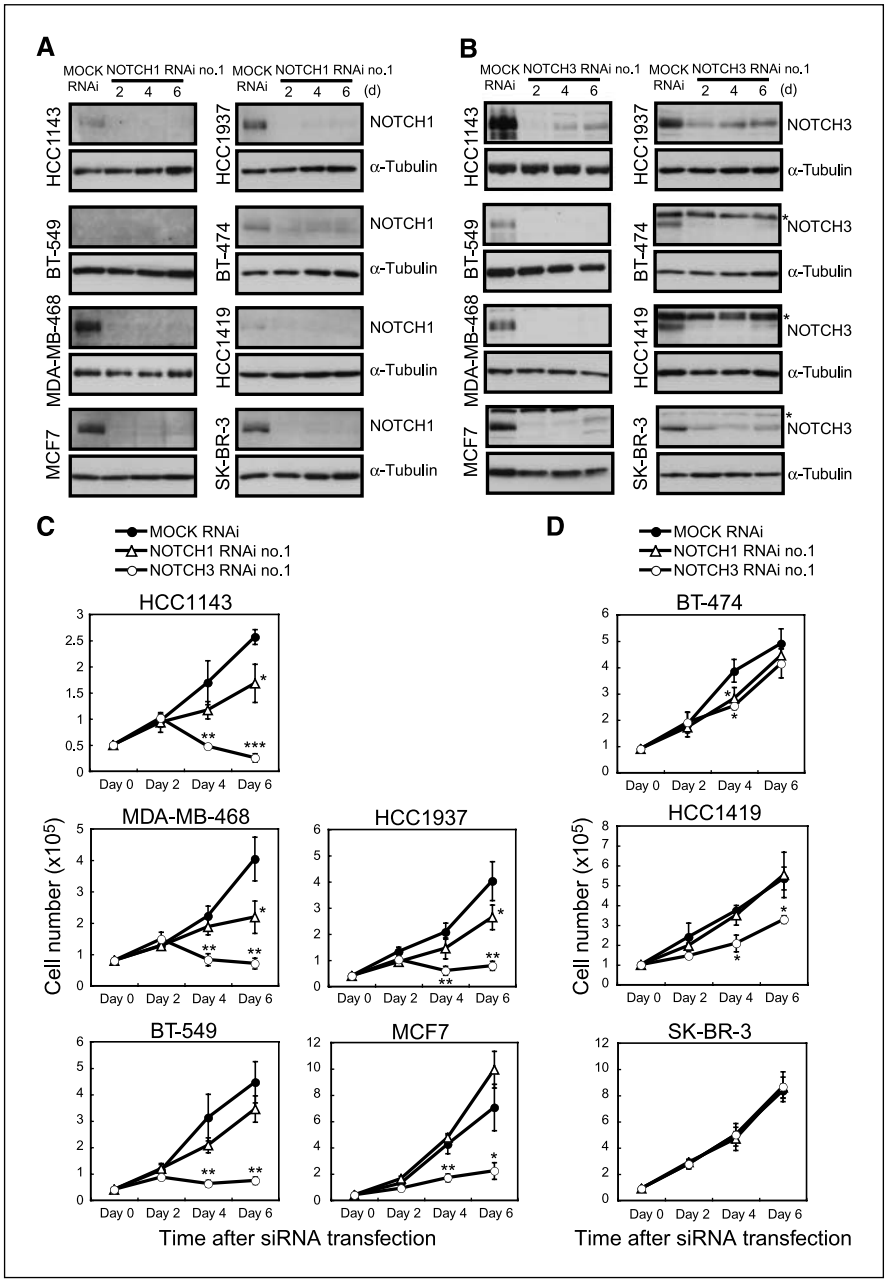


**Figure 2.** Effects of NOTCH1- and NOTCH3-RNAis on the proliferation of breast cancer cell lines. *A* and *B,* effect of NOTCH1- or NOTCH3-RNAi on protein levels in breast cancer cell lines. Western blot analysis shows significant reduction of the NOTCH1- and NOTCH3 proteins in NOTCH1 (*A*) and NOTCH3 (*B*) siRNA-transfected cells compared with the mock siRNA–transfected cells. Expression of α-tubulin is shown as a loading control. *, nonspecific bands. These nonspecific signals did not change after the transfection of siRNAs against NOTCH3. *C* and *D,* proliferation analysis of NOTCH1-, NOTCH3-, or mock siRNA–transfected ErbB2-negative or -positive cell lines. Although NOTCH1-RNAi causes a slight suppression of cell growth, NOTCH3-RNAi significantly reduces the proliferation of ErbB2-negative cells (*C*). In ErbB2-positive cells, neither NOTCH1- nor NOTCH3-RNAi caused a significant change in proliferation (*D*). *Points,* mean from experiments done in triplicate; *bars,* SD. *, $P < 0.05$; **, $P < 0.01$; and ***, $P < 0.001$ relative to mock siRNA–transfected samples.

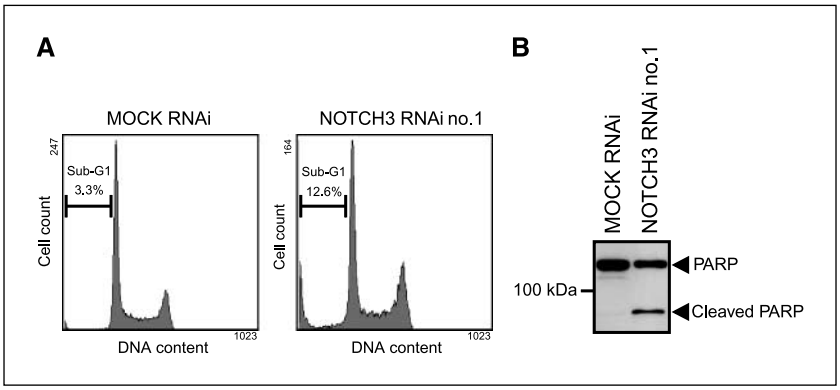


**Figure 3.** Effect of NOTCH3-RNAi on the apoptosis of ErbB2-negative breast cancer cells. *A,* accumulation of sub-$G_1$ cells after NOTCH3-RNAi transfection (12.6% vs. 3.3% with the mock-RNAi). HCC1143 cells were transfected with NOTCH3- or mock siRNA and harvested 4 d after transfection. Cell nuclei were stained with propidium iodide, and cellular DNA contents were measured with a flow cytometer. *B,* Western blot analysis to examine activation of the apoptotic signaling pathway. One substrate of executioner caspase (*PARP*) was cleaved in HCC1143 cells at 4 days after NOTCH3-siRNA transfection.

artificial chromosome (BAC) clone containing *NOTCH3* (CTD-2538G16) was obtained from Open Biosystems. The BAC DNA was labeled with a digoxigenin-11-dUTP by nick translation and the hybridization signals were visualized with a rhodamine-conjugated anti-digoxigenin antibody (Vector). FITC-labeled whole chromosome painting probe (Cambio) was used simultaneously to identify chromosome segments derived from human chromosome 19.

**Statistical analysis.** Student's *t* test was done for most data with Microsoft Excel:mac 2004 (Microsoft Corp.).

## Results

**Activation of NOTCH signaling pathways in human breast cancer cell lines.** To examine the activation of NOTCH signaling pathways in ErbB2-negative human breast cancer cells, we screened for activated forms of NOTCH proteins (NICD) in 35 human breast cancer cell lines by Western blot analysis with antibodies that recognize a region within each NICD. Almost all breast cancer cell lines expressed activated NOTCH1 and NOTCH3 at various levels (Supplemental Fig. S1). In contrast, NOTCH2 and NOTCH4 were undetectable in all cell lines examined (data not shown). The results of Western blot analysis for the eight cell lines that were used for further analyses are shown in Fig. 1*A*. We detected significant levels of NOTCH3 NICD in all eight cell lines, with particularly high levels of NOTCH3 NICD in HCC1143 cells. NOTCH1 NICD was detected in all cell lines other than BT-549 cells. γ-Secretase inhibitor treatment shifted both bands to a higher molecular weight, supporting the notion that these two bands represent the NICDs of NOTCH1 and NOTCH3 (Fig. 1*B*). These eight cell lines were classified on the basis of ErbB2 expression: ErbB2-negative cell lines (HCC1143, BT-549, HCC1937, MDA-MB-468, and MCF7) and ErbB2-positive cell lines (HCC1419, SK-BR-3, and BT-474).

Because activation of NOTCH signaling promotes CSL-mediated transcription of several target genes, including *Hes1*, a luciferase reporter construct driven by the *Hes1* promoter was used to confirm functional NOTCH1-CSL or NOTCH3-CSL signaling in the breast cancer cell lines. Although transfection of *Hes1* promoter vectors with mutated CBS showed low baseline luciferase levels, transfection of the constructs harboring the normal *Hes1* promoter showed significant luciferase activity in the cell lines expressing NOTCH1 and NOTCH3 at high levels (HCC1143, HCC1937, and MDA-MB-468; Fig. 1*C*). To verify the contributions of NOTCH1 and NOTCH3 to the luciferase activity, we performed RNAi-mediated knockdown of NOTCH1 or NOTCH3 in HCC1143, HCC1937, and MDA-MB-468 cells. The efficacy of RNAi is discussed in the next section. Down-regulation of NOTCH1 or NOTCH3 reduced *Hes1* promoter-driven luciferase activity (Fig. 1*C*). On the basis of these results, we concluded that both NOTCH1 and NOTCH3 signaling pathways are activated, resulting in CSL-mediated transcription in ErbB2-negative breast cancer cell lines.

**RNAi-mediated depletion of NOTCH3 protein inhibited proliferation and induced apoptosis of ErbB2-negative breast cancer cell lines.** To study the tumorigenic effects of NOTCH1 and NOTCH3 on ErbB2-negative breast cancer cell lines, we performed knockdown of NOTCH1 or NOTCH3 and evaluated the proliferation of these cell lines by counting cell numbers. The efficacy of RNAi was examined by Western blot analysis. More than 80% depletion of NOTCH1 or NOTCH3 proteins was observed in all eight cell lines at 2, 4, and 6 days after siRNA transfection (Fig. 2*A* and *B*). Because it was recently reported that aberrant NOTCH1 signaling was associated with breast cancer tumorigenicity (11), we first assessed the effect of NOTCH1-RNAi on cell proliferation. Depletion of NOTCH1 protein caused a slight reduction in the proliferation of three of five ErbB2-negative cell lines (HCC1143, HCC1937, and MDA-MB-468; Fig. 2*C*). However, the other ErbB2-negative cell lines and all ErbB2-positive cell lines did not respond to NOTCH1-RNAi (Fig. 2*C* and *D*), suggesting that the inhibitory effect of NOTCH1-RNAi on proliferation of the breast cancer cell lines was less effective. We next analyzed the proliferation of NOTCH3-RNAi–transfected cells. To our surprise, NOTCH3-RNAi drastically reduced the proliferation of all five ErbB2-negative cell lines (Fig. 2*C*). In contrast, NOTCH3-RNAi caused a slight reduction in the proliferation of only one of three ErbB2-positive cell lines (HCC1419; Fig. 2*D*). To exclude the possibility that the effects of NOTCH3-RNAi resulted from RNAi off-target effects, we confirmed that similar results could be obtained with different siRNAs (another siRNA, no. 2) against NOTCH3 (Supplemental Fig. S2*A*–*C*). To verify these results, we assessed the effects of NOTCH3-RNAi on the proliferation of six additional ErbB2-negative and six ErbB2-positive breast cancer cell lines (Supplemental Fig. S3*A*–*C*). Consistent with the above results, down-regulation of NOTCH3 significantly reduced the proliferation of five of six ErbB2-negative cell lines and slightly reduced the proliferation of one cell line (T47D). In contrast, down-regulation of NOTCH3 slightly suppressed the proliferation of three of six ErbB2-positive cell lines (UACC-893, HCC1954, and HCC202) and did not cause an apparent change in the proliferation of the rest of the cell lines (MDA-MB-361, MDA-MB-453, and ZR-75-30). Taken together, these results indicate that NOTCH3 plays an important role in the proliferation or survival of breast cancer cells, including ErbB2-negative cells and, to a lesser extent, ErbB2-positive cells.

To investigate whether the growth-inhibitory effects of NOTCH3-RNAi are related to the induction of apoptosis, the effect of

NOTCH3-RNAi on apoptotic cell death of ErbB2-negative HCC1143 cells was examined with DNA/propidium iodide staining at 4 days after siRNA transfection. Whereas control siRNA-transfected cells showed a low basal sub-$G_1$ cell population (3.3%), NOTCH3-siRNA–transfected cells showed an increased proportion of sub-$G_1$ cells (12.6%; Fig. 3*A*). We also examined the activation of apoptotic signaling by Western blot analysis. One of the substrates of executioner caspase (PARP) was cleaved in HCC1143 cells at 4 days after NOTCH3-siRNA transfection (Fig. 3*B*). These results suggested that down-regulation of NOTCH3 induces the apoptosis of HCC1143 cells. These data suggest that the growth inhibition due to NOTCH3 down-regulation is attributable to an increase in apoptotic cell death.

**Knockdown of CSL or NOTCH3 ligands inhibited the proliferation of ErbB2-negative breast cancer cells.** We next examined the functional importance of CSL, a downstream effector of NOTCH signaling, on the proliferation of ErbB2-negative breast cancer cell lines (HCC1143 and HCC1937) using siRNA against CSL. Semiquantitative real-time PCR analyses revealed that expression of *CSL* was significantly reduced in CSL-siRNA–transfected cells (Fig. 4*A*). In the *Hes1* promoter-driven luciferase assay, we found that transfection of CSL-siRNA decreased the luciferase activity to a level similar to that in NOTCH3-RNAi–transfected cells (Fig. 4*B*). These results confirmed that RNAi-mediated CSL knockdown efficiently reduced CSL function. We counted cell numbers to assess the effect of CSL-RNAi on the proliferation of ErbB2-negative breast cancer cell lines and found that knockdown of CSL significantly reduced the proliferation of these cells (Fig. 4*C*). In contrast, CSL-RNAi caused little or no suppression of proliferation of ErbB2-positive breast tumor cell lines (BT-474 or SK-BR-3; Fig. 4*C*). The effect of CSL-RNAi on these cells was similar to that of NOTCH3-RNAi. We obtained similar results with different siRNAs against CSL (Supplemental Fig. S4*A*–*C*). Taken together, these results indicate

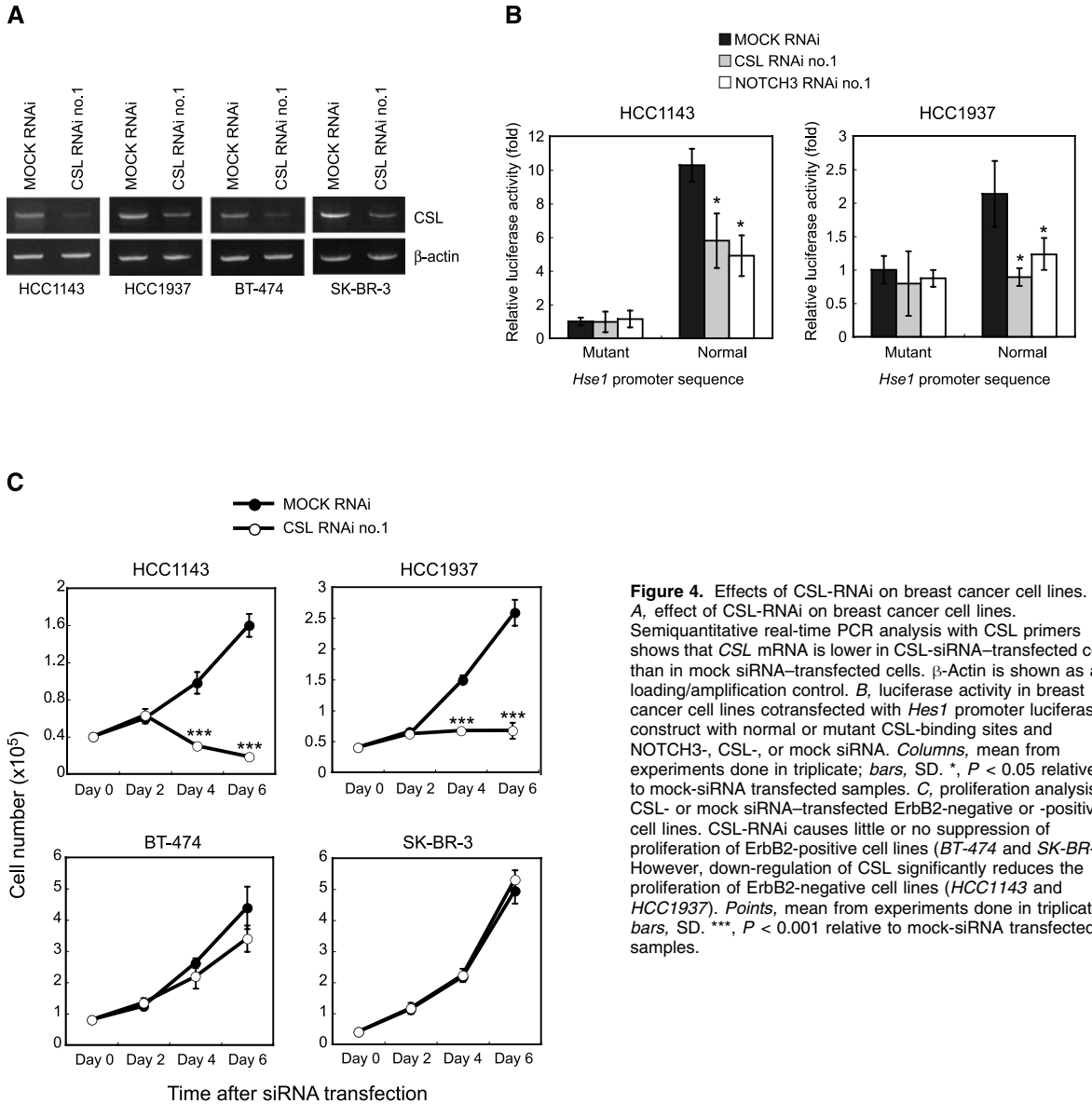


**Figure 4.** Effects of CSL-RNAi on breast cancer cell lines. *A,* effect of CSL-RNAi on breast cancer cell lines. Semiquantitative real-time PCR analysis with CSL primers shows that *CSL* mRNA is lower in CSL-siRNA–transfected cells than in mock siRNA–transfected cells. β-Actin is shown as a loading/amplification control. *B,* luciferase activity in breast cancer cell lines cotransfected with *Hes1* promoter luciferase construct with normal or mutant CSL-binding sites and NOTCH3-, CSL-, or mock siRNA. *Columns,* mean from experiments done in triplicate; *bars,* SD. *, $P < 0.05$ relative to mock-siRNA transfected samples. *C,* proliferation analysis of CSL- or mock siRNA–transfected ErbB2-negative or -positive cell lines. CSL-RNAi causes little or no suppression of proliferation of ErbB2-positive cell lines (*BT-474* and *SK-BR-3*). However, down-regulation of CSL significantly reduces the proliferation of ErbB2-negative cell lines (*HCC1143* and *HCC1937*). *Points,* mean from experiments done in triplicate; *bars,* SD. ***, $P < 0.001$ relative to mock-siRNA transfected samples.

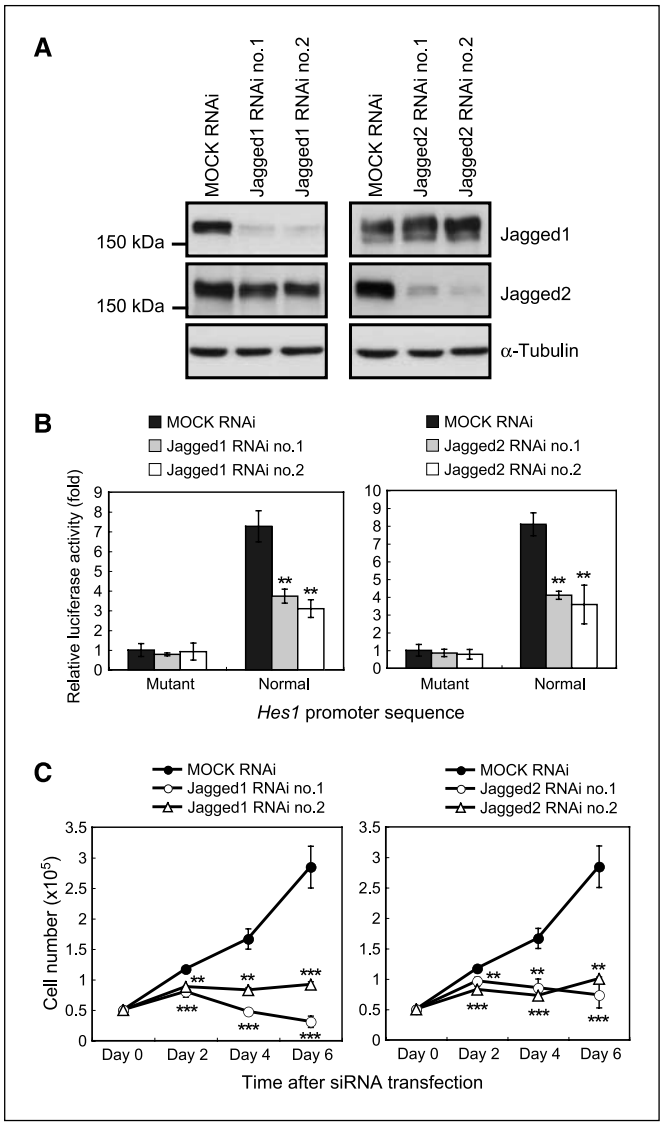


**Figure 5.** Effects of Jagged1- or Jagged2-RNAi on breast cancer cell lines. *A,* effect of Jagged1- and Jagged2-RNAis on ErbB2-negative HCC1143 cells. Western blot analysis shows that Jagged1 or Jagged2 protein levels in cells transfected with siRNAs directed against Jagged1 or Jagged2 are significantly lower than those in mock siRNA–transfected cells. The expression of α-tubulin is shown as a loading control. *B,* luciferase activity in HCC1143 cells cotransfected with *Hes1* promoter luciferase construct normal or mutant CSL-binding sites and Jagged1-, Jagged2-, or mock siRNAs. *Columns,* mean from experiments done in triplicate; *bars,* SD. **, $P < 0.01$ relative to mock-siRNA transfected samples. *C,* proliferation analysis of Jagged1-, Jagged2-, or mock siRNA–transfected HCC1143 cells. Down-regulation of Jagged1 or Jagged2 significantly reduces proliferation of these cells. *Points,* mean from experiments done in triplicate; *bars,* SD. **, $P < 0.01$; and ***, $P < 0.001$ relative to mock-siRNA transfected samples.

that NOTCH3-CSL signaling plays an important role in the proliferation of ErbB2-negative breast cancer cell lines.

To understand the molecular mechanisms of constitutive activation of NOTCH3-CSL signaling in ErbB2-negative breast cancer cell lines, we evaluated HCC1143 cells for the presence of NOTCH3 ligands by Western blot analysis and identified high-level expression of Jagged1 and Jagged2 in these cells (Fig. 5*A*). To examine whether these two ligands are involved in the activation of NOTCH3-CSL signaling in these cells, we depleted Jagged1 or Jagged2 proteins using siRNAs. The efficacy of each siRNA was verified by Western blot analysis (Fig. 5*A*). The *Hes1* promoter-driven luciferase assay showed that transfection of Jagged1- or Jagged2-siRNA decreased the luciferase activity to a level similar to that in NOTCH3-siRNA–transfected cells (Fig. 5*B*), suggesting that both Jagged1 and Jagged2 are involved in the steady-state activation of the NOTCH3-CSL signaling in HCC1143 cells. We next explored the functional significance of these ligands in the proliferation of HCC1143 cells. As shown in Fig. 5*C*, knockdown of Jagged1 or Jagged2 significantly reduced the proliferation of these cells. Identical results were obtained with other unrelated siRNAs against Jagged1 or Jagged2 (Fig. 5*A*–*C*), indicating that the effects of Jagged1- or Jagged2-RNAi did not result from RNAi off-target effects or cross-reactivity. Together, these results indicate that NOTCH3 and its ligands, Jagged1 and Jagged2, function as an important autocrine/juxtacrine loop in NOTCH3-CSL signal activation and play an important role in the growth of ErbB2-negative breast cancer cells.

***NOTCH3* gene amplification in breast cancer.** An association between *NOTCH3* gene amplification and human cancers was recently established for ovarian cancer (17). Remarkably high expression of NOTCH3 protein in HCC1143 cells (Fig. 1*A*) prompted us to examine gene amplification of the *NOTCH3* gene in the cells. Southern blot and FISH analyses showed gene amplification of the *NOTCH3* gene (Supplemental Fig. S5*A*; Fig. 6*A*). The amplified region on the derivative chromosome 19 may consist of two blocks of amplicon (or two major amplicons) and single copy unit (Supplemental Fig. S5*B*). Quantitative real-time PCR was also done for the eight breast cancer cell lines to estimate *NOTCH3* DNA copy number. As shown in Fig. 6*B*, HCC1143 cells showed ~5-fold *NOTCH3* gene amplification compared with the other cell lines. The amplification concomitantly increased the *NOTCH3* mRNA level (Supplemental Fig. S5*C*).

We next examined whether *NOTCH3* gene amplification was present in tumor tissues from patients with breast cancer. DNA samples from 36 primary tumor tissues and matched normal breast tissues were analyzed by real-time PCR as described above. The distribution of relative gene copy of *NOTCH3* in normal and tumorous breast tissues is shown in Fig. 6*C*. When we used the upper limit of the 95% confidence interval (mean + 2 SD) for normal samples as cutoff, distinct *NOTCH3* gene amplification was detected in 1 of 36 breast cancer specimens. The *NOTCH3* mRNA level was also up-regulated in the tumorous tissue in which *NOTCH3* gene amplification was observed (Supplemental Fig. S5*D*). Expression data for estrogen receptor, progesterone receptor, and ErbB2 are tabulated together with data on individual patient and tumor characteristic (Supplementary Table S1). Eighty-two additional tumor specimens were tested for amplification; however, none showed significant amplification of *NOTCH3* (data not shown). These results indicate that *NOTCH3* gene amplification is rare in breast cancers.

## Discussion

In the present study, we found that NOTCH3 is expressed and plays crucial roles in the proliferation of ErbB2-negative breast cancer cell lines. We have also detected *NOTCH3* gene amplification in one breast cancer cell line and one breast cancer specimen, even though the frequency of *NOTCH3* gene amplification was low. Taken together with previous data indicating that NOTCH3 is expressed at high levels in human mammary stem/progenitor cells and that NOTCH signaling is crucial for the self-renewal of these cells (18, 19), our results suggest that NOTCH3 is one of the newly recognized "lineage-specific" oncogenes (20, 21) in breast cancer.

Lineage-specific oncogenes have the following properties: (*a*) crucial role(s) in normal lineage proliferation or survival during development, (*b*) persistent or deregulated expression in cancers of the associated lineages, (*c*) somatic genetic alterations in tumor subsets, (*d*) implicated in tumor survival or progression, and (*e*) functions as transcription factors rather than signaling proteins (20, 21). We believe that NOTCH3 may have all of the properties of lineage-specific oncogenes in breast cancer. We found that NOTCH3-RNAi suppresses proliferation in both NOTCH3 non–amplified and NOTCH3-amplified breast cancer cells, suggesting that NOTCH3 expression is required for the maintenance of breast cancer cells in a gene amplification–independent manner. Rare cases of *NOTCH3* amplification might facilitate the development of malignancy of breast cancer cells. Our present data, together with these findings, indicate that NOTCH3 has all of the properties of lineage-specific oncogenes in breast cancer. In addition, our work suggests that amplification of *ErbB2* may allow tumor cells to escape from NOTCH3 addiction. The model of "addiction shift" from NOTCH3 to ErbB2 should be tested.

Ectopic expression of NICD is known to enhance cell proliferation through nuclear factor κB (NFκB) or mitogen-activated protein kinase (MAPK)/Akt activation in various tumor cells (22–25). In human T-cell acute lymphoblastic leukemia and pancreatic cancer, constitutively active NOTCH1 activates the NFκB pathway and induces the transformation of T cells and pancreatic cells. Transgenic mice expressing a *lck* promoter–driven active form of NOTCH3 develop lymphoma via constitutive activation of the NFκB pathway and up-regulation of its target genes, such as cyclin D1. In melanoma cells, NOTCH1 signaling promotes cell progression by activating MAPK/Akt pathways. Therefore, we examined the possibility that NOTCH3 signaling is involved in NFκB or MAPK/Akt activation in ErbB2-negative breast cancer cell lines. Electrophoretic mobility shift assays identified high basal activation of NFκB in the ErbB2-negative HCC1143 cells (Supplemental Fig. S6*A*). However, NOTCH3-RNAi and γ-secretase inhibitor failed to reduce NFκB activity in these cells (Supplemental Fig. S6*A*). Expression of cyclin D1 also was not changed in NOTCH3-RNAi–transfected cells (Supplemental Fig. S6*B*). Western blot analysis with antibodies specific for the phosphorylated forms of Erk1/2 and Akt showed that depletion of NOTCH3 proteins induced up-regulation, rather than down-regulation, of phosphorylation of Erk1/2 and Akt through unknown mechanisms (Supplemental Fig. S6*C*). These findings suggest that the NFκB or MAPK/Akt pathways are not major downstream targets of NOTCH3 signaling in ErbB2-negative breast cancer cell lines.

Transgenic mice that overexpress an activated form of NOTCH1 or NOTCH3 in mammary glands develop mammary tumors, and therefore, it is believed that both NOTCH1 and NOTCH3 are involved in the tumorigenesis of mouse mammary epithelial cells (10). In the present study, we detected the activation of both the

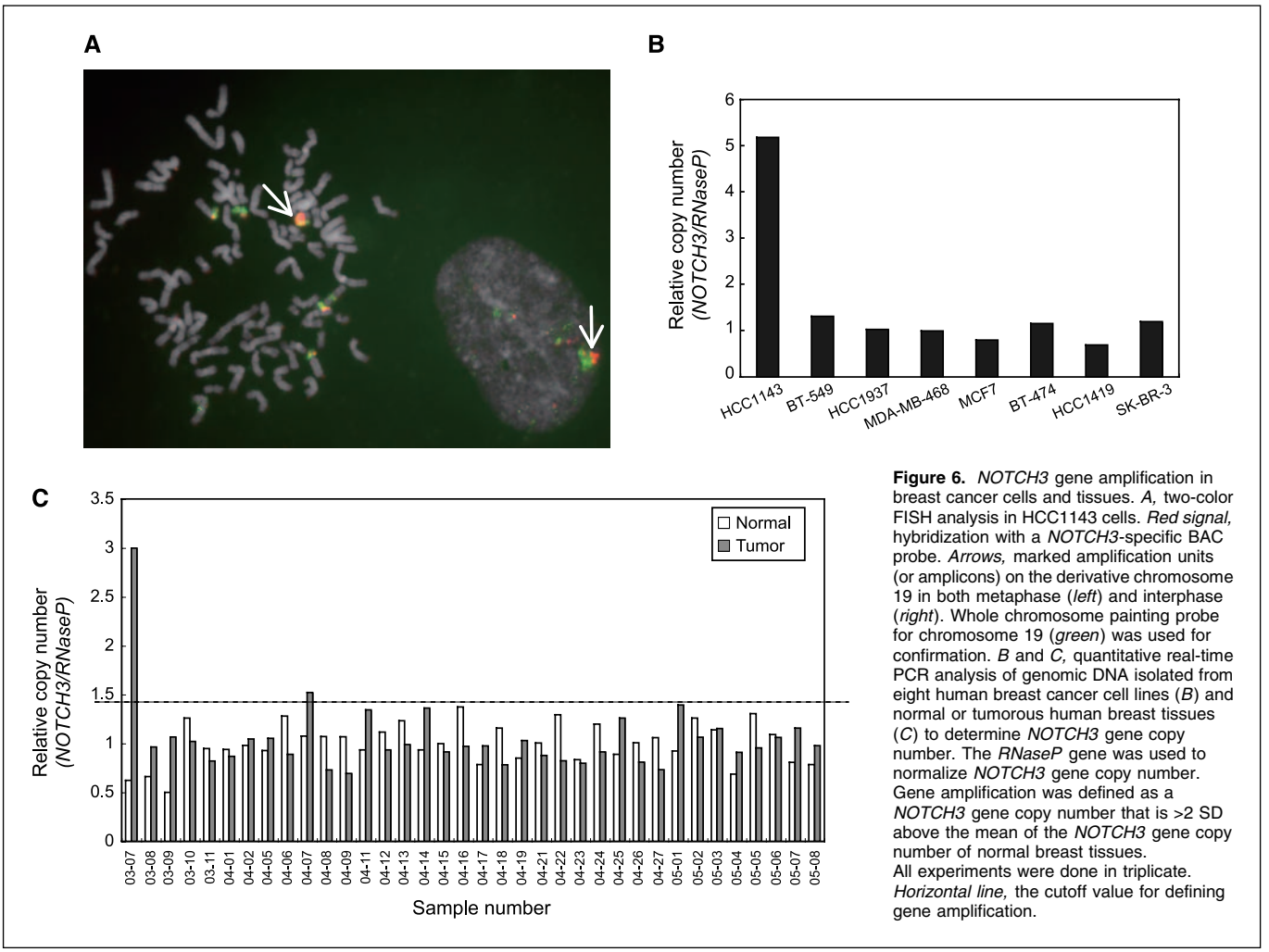


**Figure 6.** *NOTCH3* gene amplification in breast cancer cells and tissues. *A,* two-color FISH analysis in HCC1143 cells. *Red signal,* hybridization with a *NOTCH3*-specific BAC probe. *Arrows,* marked amplification units (or amplicons) on the derivative chromosome 19 in both metaphase (*left*) and interphase (*right*). Whole chromosome painting probe for chromosome 19 (*green*) was used for confirmation. *B* and *C,* quantitative real-time PCR analysis of genomic DNA isolated from eight human breast cancer cell lines (*B*) and normal or tumorous human breast tissues (*C*) to determine *NOTCH3* gene copy number. The *RNaseP* gene was used to normalize *NOTCH3* gene copy number. Gene amplification was defined as a *NOTCH3* gene copy number that is >2 SD above the mean of the *NOTCH3* gene copy number of normal breast tissues. All experiments were done in triplicate. *Horizontal line,* the cutoff value for defining gene amplification.

NOTCH1 and NOTCH3 signaling pathways in human ErbB2-negative breast cancer cell lines with a *Hes1* promoter-driven luciferase assay. However, knockdown experiments revealed that NOTCH3 plays a more important role than NOTCH1 in the proliferation of these cells. Although NOTCH1 and NOTCH3 are both members of the NOTCH family of proteins, there are structural differences (NOTCH3 lacks the transactivation domain present in NOTCH1) that are thought to underlie the functional difference between these proteins (26). We hypothesized that although *Hes1* is a common target of the NOTCH1 and NOTCH3 signaling pathways, there are target genes which are specific to each signaling pathway, and the differences in target genes may reflect the functional difference between NOTCH1 and NOTCH3 in the proliferation of ErbB2-negative breast tumor cells. Further comparative expression profiling analysis of NOTCH3-, NOTCH1-, and CSL-RNAi in ErbB2-negative breast cancer cells might improve our understanding of the molecular mechanisms that underlie our present findings of the anticancer effects of NOTCH3- and CSL-RNAi.

Targeted molecular therapies have been fruitful for subclasses of breast cancers. Hormone-targeted therapy such as tamoxifen is effective against estrogen receptor–positive tumors. ErbB2-positive breast cancers, which are often estrogen receptor–negative, respond to Herceptin, the humanized antibody against ErbB2. Recently, intensive studies have focused on estrogen receptor–negative, progesterone receptor–negative, and ErbB2-negative tumors because there are so few effective therapeutic targets. Our present results suggest that NOTCH3 rather than NOTCH1 may be an effective therapeutic target for ErbB2-negative breast cancers. For therapies that target specific molecules, specificity is a critical safety concern. In the present RNAi-mediated knockdown experiments, identical results were obtained when different siRNAs against NOTCH3 were tested, indicating that the growth inhibition and induction of apoptosis was specific to NOTCH3-RNAi and not due to off-target effects. The most important merit for NOTCH3 is that NOTCH3-deficient mice are viable and fertile and seem to develop normally, although they exhibit some arterial defects (27). In contrast, NOTCH1-deficient mice show early embryonic lethality (28–30), and mice with cell type–specific conditional knockouts of NOTCH1 exhibit defects in various cells, including T cells, neurons, and hair follicle cells (31–33). These results suggest that NOTCH1 function is important for the development and maintenance of various organs and that NOTCH1 inhibition may have severe toxic effects. γ-Secretase inhibitor, which inhibits signaling by all NOTCH family members, might also have undesirable side effects.

In conclusion, our results showed that proliferation of ErbB2-negative breast cancer cells is suppressed by NOTCH3- and CSL-RNAi. This novel finding contributes to our understanding of breast tumor pathogenesis and indicates that the NOTCH3-CSL signaling axis may be a therapeutic target for treating patients suffering from this type of breast cancer.

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