Aberrant Activation of Notch Signaling in Human Breast Cancer

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Abstract

A role for Notch signaling in human breast cancer has been suggested by both the development of adenocarcinomas in the murine mammary gland following pathway activation and the loss of Numb expression, a negative regulator of the Notch pathway, in a large proportion of breast carcinomas. However, it is not clear currently whether Notch signaling is frequently activated in breast tumors, and how it causes cellular transformation. Here, we show accumulation of the intracellular domain of Notch1 and hence increased Notch signaling in a wide variety of human breast carcinomas. In addition, we show that increased RBP-Jk-dependent Notch signaling is sufficient to transform normal breast epithelial cells and that the mechanism of transformation is most likely through the suppression of apoptosis. More significantly, we show that attenuation of Notch signaling reverts the transformed phenotype of human breast cancer cell lines, suggesting that inhibition of Notch signaling may be a therapeutic strategy for this disease.

Introduction

The lifetime risk of developing breast cancer is about one in nine for women with around 41,000 new cases being diagnosed in the United Kingdom each year. Until recently, it has also been the leading cause of cancer-related death in women. However, there has been a marked decline in mortality since 1989 due to increased screening, specialization of care, and the widespread adoption of tamoxifen treatment. Despite this, there are still just short of 13,000 deaths each year due to breast cancer, indicating that a better understanding of the disease is required to improve treatment.

The Notch pathway has been associated with several human cancers, including cervical and lung carcinoma, and neuroblastoma (1, 2). Most notably, it has been linked to T-cell acute lymphoblastic leukemia in T-ALL, where activating mutations within Notch1 have been identified in >50% of tumors (3). Furthermore, growth arrest occurs in several T-ALL-derived cell lines when Notch signaling is blocked, suggesting that modulating the Notch pathway may be an effective treatment strategy for this tumor type.

Within the murine mammary gland, aberrant activation of the Notch pathway leads to adenocarcinoma (4–8). In addition, overexpression of active forms of the Notch1 and Notch4 receptors transform both normal human and murine mammary epithelial cells (8–10). Despite this, the role Notch signaling plays in human breast cancer has received little attention. Initial hints of a role came from two studies showing increased expression of Notch1 protein in four breast tumors that overexpress H-ras (11) and expression of a truncated Notch4 mRNA encoding an active form in two breast cancer cell lines (9). A much clearer indication has come from two surveys examining Numb expression (12), a negative regulator of Notch pathway, and the mRNA levels of Notch receptors and their ligands (13) in breast carcinoma samples. Numb was lost in >50% of tumors due to ubiquitination and proteosomal degradation, and its levels were inversely correlated with grade and proliferation rate (12). Furthermore, the authors showed that colony formation was reduced by reintroducing Numb into epithelial cells derived from Numb-negative tumors. On the other hand, elevated levels of Notch1 and Jagged1 mRNA correlated with poor prognosis (13).

During normal mammalian development and homeostasis, Notch signaling plays an essential role in regulating cell fate, apoptosis, proliferation, and migration (14, 15). This is largely thought to be through the regulation of the expression of the Hes/Hey family of transcriptional repressors, although several other genes including p21 and cyclin D1 have been suggested to be direct targets (16, 17). The pathway is activated through the interaction of Notch receptors with Delta-like and Jagged ligands on neighboring cells (18). This leads to two proteolytic cleavages, which release the Notch intracellular domain (NICD) allowing it to enter the nucleus. Once within the nucleus, NICD interacts with RBP-Jk/VP16 and Mastermind to generate a large transcriptional activator complex (19).

The loss of Numb and elevation of Notch1 and Jagged1 mRNA levels in breast carcinomas has suggested that increased Notch signaling may occur in this tumor type. However, this is not necessarily the case as Numb only limits signaling through the pathway and does not alter its activation by ligands, whereas elevated mRNA levels does not necessarily equate to increased protein expression. Here, we provide direct evidence that Notch signaling is activated in a wide variety of human breast carcinomas as we observe the accumulation of NICD and the expression of known downstream target genes. More significantly, we show that attenuation of Notch signaling reverts the transformed phenotype of human breast cancer cell lines, suggesting that inhibition of Notch signaling may be a therapeutic strategy for this disease. Our data also indicate that the transformation of normal breast epithelial cells caused by increased RBP-Jk-dependent Notch signaling is due to the repression of apoptosis.

Materials and Methods

Plasmid constructs. cDNAs encoding the intracellular domain of human Notch1 and RBP-Jk/VP16 fusion protein were obtained from Dr. Martin Baron (University of Manchester) and Dr. Tatsuku Honjo (Kyoto University, Japan), respectively. cDNAs encoding murine Numb (Image clone 3991630), Puma (Image clone 6310857), and Noxa (Image clone 6517820) were obtained from the Medical Research Council Geneservice (Hinxton, Cambridge, United Kingdom). The full-length Numb cDNA was
recloned into pCDNA3.1(−) vector to permit selection of cell lines stably expressing the protein. The complete Puma and Noxa open reading frames were cloned by PCR into the pEYFP-C1 vector to generate cDNAs encoding YFP/Puma and YFP/Noxa fusion proteins.

Cell culture and transfection conditions. Four normal (MCF 10F, MCF 10A, MTSV1-7, HB4A) and eight tumorigenic human mammary epithelial cell lines (Hs578T, MDA-MB-468, MCF7, ZR75T, CAL51, MDA-MB-231, SK-BR-3, PMG-12) were obtained from Dr. Neal Anderson (University of Manchester). MCF 10A and MCF 10F cells were grown in a 1:1 mixture of Ham’s F12 medium and DMEM with 2 mmol/L l-glutamine. The medium was supplemented with 5% horse serum, 10 μg/mL insulin, 20 mg/mL epidermal growth factor (EGF), 500 ng/mL hydrocortisone, and 10 μg/mL cholera toxin. MTSV1-7 and HB4A cells were grown in DMEM with 2 mmol/L l-glutamine supplemented with 10% fetal bovine serum (FBS) and 5 μg/mL hydrocortisone. Hs578T, MDA-MB-468, MCF7, ZR75T, CAL51, MDA-MB-231, SK-BR-3, and PMG-12 cells were grown in DMEM with 2 mmol/L l-glutamine and 10% FBS. All cells were maintained in a humidified incubator at 37°C and 5% CO2. MCF 10A, MDA-MB-231, and MCF7 cells were transfected using the standard calcium phosphate coprecipitation method. Cell lines stably expressing BBR-Jc/VIP16, NCD, or Numb were selected by adding 600 μg/mL genetin (Invitrogen, San Diego, CA) to the normal medium.

Reverse transcription coupled PCR analysis. Cells were grown to near confluence in T-75 cm² flasks before cell lysis and RNA extraction using Stat-60 (Tel-Test) according to manufacturer’s instructions. cDNA was prepared from 5 μg total RNA using Superscript II reverse transcriptase (Invitrogen). Control reactions were done without Superscript II. Prepared cDNA was subsequently treated with RNase H (Invitrogen) to remove RNA/DNA hybrids.

The PCR primers that specifically recognize Notch1-Notch4, Delta-like1, Delta-like3, and Delta-like4, and Jagged1 and Jagged2 were designed using Vector NTI suite 7.0 sequence analysis software (sequences available on request). The specificity of the primers was confirmed using plasmid cocktails that encoded either all four Notch, three Delta-like, or two Jagged proteins and control cocktails that lacked a specific plasmid, which corresponded to the primer pair being analyzed. To determine expression of the genes, one tenth of the prepared cDNA was used in subsequent PCRs to generate a 1% normal serum, and the slides were incubated overnight at 4°C. Sections were then dehydrated and cleared in histoclear before staining (Sigma, St. Louis, MO). Immunoreactivity was detected using diaminobenzidine in PBS containing 1% normal serum), and washed again before incubation with Cy3-conjugated goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratory) and 0.1 mmol/L 4’,6-diamidino-2-phenylindole diluted in the same buffer for 1 hour. The cells were then washed again and mounted in DakoCytomation Fluorescent mounting medium. The percentage of cells exhibiting apoptosis, as judged by chromatin condensation, nuclear fragmentation, and cytochrome c release from mitochondria, was assessed using a Hamamatsu ORCA-ER digital camera on a Zeiss Axioplan2 microscope driven by Openlab image analysis software (Improvision, Lexington, MA).

Immunohistochemistry and tissue samples. Approval to remove normal and tumorigenic human breast tissues during reduction mammoplasty and from pathologic samples respectively was obtained by Dr. Rob Clarke from the Manchester Local Research Ethics Committees. Subsequently written informed consent was obtained from the women before surgery. These tissue samples were fixed in 10% neutral buffered formalin, embedded in paraffin, and sectioned on a microtome (Leica RM 2135). Tissue sections were mounted onto glass slides, de waxed in xylene, and rehydrated in graded alcohols. Endogenous peroxidases were quenched with 0.2% H2O2 in methanol for 10 minutes. To recover antigen sites, sections were incubated in citrate buffer under high pressure (Tefal Delicio) for 60 seconds. Non specific binding was blocked with 10% normal serum diluted in PBS; the serum used was from the same species as the secondary antibody was raised in. Primary antibodies were added in PBS containing 1% normal serum, and the slides were incubated overnight at 4°C. Sections were then washed in PBS, incubated with secondary antibody (diluted 1:100 in PBS containing 1% normal serum), and washed again before incubation with avidin-biotin complex Vectastain reagent (Vector Laboratories, Burlingame, CA). Immunoreactivity was detected using diaminobenzidine staining (Sigma, St. Louis, MO). Sections were counterstained with 5% Harris-modified hematoxlin to reveal morphology and washed in warm tap water. Samples were then dehydrated and cleared in histoclear before mounting. Controls were prepared for all sections without primary antibody.

Primary antibodies used were as follows: anti-Notch1 (Rockland), anti-Notch3 (R&D Systems, Minneapolis, MN), anti-Jagged1 (Santa Cruz Biotechnology), anti-Jagged2 (Santa Cruz Biotechnology), and anti-Muc1 (Dako, Carpinteria, CA). Secondary antibodies used were obtained from Jackson Immunoresearch Laboratory (West Grove, PA) and DAKO.

Soft agar assays. A solution of 2.7% low melting Metaphor agarose (FMC, Rockland, ME) in distilled water was prepared, autoclaved and cooled in a water bath at 55°C for 2 hours. For the base layer, the 2.7% agar solution was mixed with the appropriate culture medium to reach a final concentration of 0.9%. One milliliter of this mixture was transferred to each 35-mm dish and allowed to cool and solidify for 1 hour. The cells were then washed to a final concentration of either 15,000/mL (MCF 10A) or 25,000/mL (MCF7, MDA-MB-231) and 0.3% agar. Two milliliters of the cell suspensions were layered on top of the base layer and allowed to cool and solidify for 1 hour. Plates were transferred to a humidified incubator at 37°C and 5% CO2 and were grown for 5 weeks (MCF 10A) or 2 weeks (MCF7, MDA-MB-231). The day before counting, colonies were stained with 1 mL/well of nitroblue tetrazolium (0.5 mg/mL NBT in PBS). Colonies were counted using an Olympus IMT-2 inverted microscope.

Drug treatment and apoptosis analysis. MCF 10A cells were treated for 4 hours with 0.1 μmol/L staurosporine (Calbiochem, La Jolla, CA) or 16 hours with 0.75 μmol/L melephanal (Sigma), 0.37 μmol/L mitoxantrone (Sigma), or 0.1 μmol/L thapsigargin (Sigma). Apoptosis was monitored by nuclear fragmentation and cytochrome c release from mitochondria. This method detects accumulated apoptosis over the time period of the experiment and consequently background levels will seem higher than with techniques that measure the rate of apoptosis at any one particular time point. After treatment with the different drugs, both attached and detached cells were harvested, spun down onto positively charged slides using a Cytopsin (Shandon), washed with PBS, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.5% Tween in PBS. Cells were then washed with PBS and incubated with anti–cytochrome c primary antibody (PharMingen, San Diego, CA) diluted in PBS containing 10% horse serum for 1 hour at room temperature. Following further washing, cells were incubated with Cy3-conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch Laboratory) and 0.1 μmol/L 4’,6-diamidino-2-phenylindole diluted in the same buffer for 1 hour. The cells were then washed again and mounted in DakoCytomation Fluorescent mounting medium. The percentage of cells exhibiting apoptosis, as judged by chromatin condensation, nuclear fragmentation, and cytochrome c release from mitochondria, was assessed using a Hamamatsu ORCA-ER digital camera on a Zeiss Axioplan2 microscope driven by Openlab image analysis software (Improvision, Lexington, MA).

Western blotting. Cells were washed twice in PBS, and total protein was extracted in boiling SDS lysis buffer [2% SDS, 30 mmol/L Tris-HCl (pH 7.4)]. Frozen tissue samples were diced with a clean razor blade before protein extraction in twice the volume of boiling SDS lysis buffer. Genomic DNA was sheared by passing the lysate through a 23-gauge syringe needle 10 times. Total protein concentrations were determined using the bicinchoninic acid protein assay kit (Pierce, Rockford, IL). Twenty micrograms of protein were resolved by SDS-PAGE, and the separated proteins were transferred to nitrocellulose membrane. The subsequent Western blots were blocked in TBS-T buffer [10 mmol/L Tris-HCl (pH 8), 150 mmol/L NaCl, 0.05% (v/v) Tween 20] containing 5% nonfat dry milk and were probed with the primary antibody in blocking buffer at 4°C overnight. Following washing in TBS-T buffer, the blots were incubated in secondary antibodies diluted in TBS-T at room temperature and developed after further washing with Super Signal West Pico and Femto Chemiluminescent substrates (Pierce).

Primary antibodies used were as follows: anti-cleaved Notch1 (Rockland), anti-Numb (Abcam, Cambridge, MA), anti-Noxa (Abcam), anti-Puma (Novus Biologicals, Littleton, CO), anti-cleaved Caspase3 (Cell Signaling Technology, Beverly, MA), anti-Desmplains (kind gift of Prof. David Garrod, University of Manchester), anti-Hey1 (Santa Cruz Biotechnology), anti-E-cadherin (BD Biosciences, San Jose, CA), anti-tubulin (kind gift of Prof. Keith Gull, University of Oxford), anti-phospho-c-Jun NH2-terminal kinase (JNK) pY185pY185 (Biosource, Camarillo, CA), anti-phospho-p53 pT81 (Cell Signaling Technology, anti-Keratin18 (NeoMarkers, Fremont, CA), anti-Actin (Sigma), and anti-focal adhesion kinase.
Results

Notch receptors and DSL ligands expressed in the luminal epithelium of human breast. We initially sought to identify which Notch receptors and Delta-like and Jagged ligands are expressed in normal human breast tissue and where they are localized (Fig. 1). Reverse transcription-PCR analysis of the normal breast epithelial cell lines MCF 10F, HB4A, and MTSV1-7 showed expression of Notch1 and Notch3, Delta-like 4, and Jagged1 and Jagged2 (data not shown); the expression of these genes was also confirmed by Western blotting (data not shown). A similar expression profile was also seen in normal breast tissue obtained from reduction mammoplasty (Fig. 1A-C). The localization of Notch1 and Notch3 and Jagged1 and Jagged2 expression to the luminal epithelium in normal tissue was shown by immunohistochemistry (Fig. 1D). Together, these data show that Notch receptors and DSL ligands are expressed in the luminal epithelium of normal human breast tissue. In addition, their expression seems to colocalize, indicating that Notch signaling can occur.

Notch signaling transforms the normal breast epithelial cell line MCF 10A. Next, we examined whether increased Notch1 signaling could transform normal human breast epithelial cells. In doing so, we generated MCF 10A cell lines resistant to apoptosis induced by extracellular matrix withdrawal or anoikis. It also raised the possibility that the activation of RBP-Jδ–dependent signaling did indeed lead to cellular transformation (Fig. 2D).

Increased Notch signaling protects MCF 10A cells from drug-induced apoptosis. In addition to the growth of colonies, we also observed viable single MCF 10A/RBP-Jδ and MCF 10A/NICD cells in the soft agar (data not shown). This suggested to us that these cell lines were resistant to apoptosis induced by extracellular matrix withdrawal or anoikis. It also raised the possibility that the activation of RBP-Jδ–dependent signaling did indeed lead to cellular transformation (Fig. 2D).

Figure 1. Expression of Notch receptors and DSL ligands in the normal human breast. A–C, expression of Notch1 and Notch3 (lanes A2 and A4), Delta-like 4 (lane B4), and Jagged1 and Jagged2 (lanes C2 and C3) was detected in normal human breast tissue by reverse transcription-PCR (+RT, top). In contrast, primers specific to Notch2 (lane A3), Notch4 (lane A5), Delta-like 1 (lane B2), and Delta-like 3 (lane B3) genes failed to amplify a fragment, indicating that these genes were not expressed (+RT, bottom). Control reactions without reverse transcriptase were set up in parallel (−RT, bottom). N, Notch; Dil, Delta-like; Jag, Jagged. D, immunolocalization of Notch receptors and DSL ligands identified by reverse transcription-PCR analysis of normal breast epithelial cell lines and tissue samples. Sections of normal human breast tissue were stained with antibodies that recognize Notch1, Notch3, Jagged1, and Jagged2. All four proteins were observed in the lobular epithelium and not in surrounding adipose tissue (arrows). Adjacent sections were stained without primary anti-Notch1, anti-Notch3, anti-Jagged1, and anti-Jagged2 antibodies as controls. Samples were counterstained with haematoxylin to reveal morphology.
growth, the viable cells were visualized by staining with 0.33% NBT. A significantly larger number of colonies (growth in soft agar. Parental, vector control, MCF 10A/RBP-J lanes 1-4) to confirm equal loading. and reprobed with an antibody that recognizes focal adhesion kinase (FAK RBP-J) whereas MCF 10A/RBP-J and MCF 10A/NICD cells had an elongated fibroblast-like phenotype. C: RBP-Jn-dependent Notch signaling does not induce a change in cell fate from luminal to myoepithelium. Western blot analysis of E-cadherin and keratin 18 levels. Expression of keratin 18 (lanes 1-4) was unchanged by RBP-Jn-dependent Notch signaling. In contrast, overexpression of RBP-Jn (lane 3) or NICD (lane 4) reduced E-cadherin expression. Western blots were stripped and reprobed with an antibody that recognizes focal adhesion kinase (FAK) to confirm equal loading. D, activation of RBP-Jn-dependent Notch signaling leads to growth in soft agar. Parental, vector control, MCF 10A/RBP-Jn, and MCF 10A/NICD cells were plated in 0.3% agar at a density of 15,000 per well. After 6 weeks of growth, the viable cells were visualized by staining with 0.33% NBT. A significantly larger number of colonies (P < 0.0005) developed in wells containing MCF 10A/RBP-Jn and MCF 10A/NICD cells. Columns, average of six independent soft agar assays.

parental cells or the control cell line carrying the empty vector with any of the four compounds induced apoptosis (Fig. 3A). In contrast, apoptosis was not induced in MCF 10A/RBP-Jn and MCF 10A/ NICD cells following treatment with staurosporine, melphalan, or mitoxantrone (Fig. 3A). Apoptosis did, however, occur following thapsigargin treatment, showing that the apoptotic machinery downstream of the mitochondria is functional in these cell lines (Fig. 3A).

Melphalan and mitoxantrone treatment stimulates JNK signaling, which in turn induces p53 transcription through phosphorylation of Thr81 (Fig. 3B; data not shown). This leads to the expression of a number of different genes, including those that encode the proapoptotic BH3-only proteins Noxa and Puma (Fig. 3B; data not shown). On the other hand, JNK and p53 were not activated in MCF 10A/RBP-Jn and MCF 10A/NICD cells, leading to a failure to express Noxa and Puma (Fig. 3B; data not shown). We also found that Puma and Noxa were expressed in response to staurosporine treatment (data not shown). As Puma and Noxa are largely responsible for p53-induced apoptosis, the failure to express these proteins could explain the absence of apoptosis in MCF 10A/RBP-Jn and MCF 10A/NICD cells following melphalan or mitoxantrone treatment. This is likely to be the case as the deliberately overexpressed YFP-Noxa and YFP-Puma fusion proteins led to apoptosis in all four cell lines (Fig. 3C).

Together, these experiments indicate that the p53-mediated response to cellular damage is abolished in normal breast epithelial cells by increased Notch signaling. Furthermore, they suggest that the transformed phenotype observed in MCF 10A/RBP-Jn and MCF 10A/NICD cells may be due to the attenuation of several apoptotic mechanisms.

Notch signaling is activated in human breast cancer. Because Notch signaling can transform normal human breast epithelial cells, we next sought evidence of unregulated signaling in breast cancer. We initially surveyed a panel of normal and tumor cell lines. The tumor cell lines are derived from a wide range of breast cancers and include HER2/erbB2-positive, EGFR receptor (EGFR)-positive, p53-negative, and estrogen receptor (ER)-positive, and ER-negative cell lines. In all of the cancer cell lines, we observed a clear accumulation of NICD and loss of Numb in all the tumor samples (Fig. 4A); data not shown). To confirm that this increase in Notch signaling is within the luminal epithelium, we stained serial sections from fixed samples of the same tumors with antibodies that recognize Notch1 and the luminal epithelial marker Muc1.

Figure 2. Transformation of human mammary epithelial cells by RBP-Jn-dependent Notch signaling. A, Hey1 expression is induced in MCF 10A cells stably expressing RBP-Jn/VP16 or NICD. Western blot analysis of Hey1 levels in parental (lane 1), vector control (lane 2), and MCF 10A cells stably expressing RBP-Jn/VP16 (lane 3) or NICD (lane 4). B, activation of RBP-Jn-dependent Notch signaling induces morphologic transformation. Phase-contrast images of parental, vector control, MCF 10A/RBP-Jn, and MCF 10A/NICD cells in liquid culture. Vector control and parental MCF 10A cell lines exhibited an epithelial cobblestone-like morphology, whereas MCF 10A/RBP-Jn and MCF 10A/NICD cells had an elongated fibroblast-like phenotype. C, RBP-Jn-dependent Notch signaling does not induce a change in cell fate from luminal to myoepithelium. Western blot analysis of E-cadherin and keratin 18 levels. Expression of keratin 18 (lanes 1-4) was unchanged by RBP-Jn-dependent Notch signaling. In contrast, overexpression of RBP-Jn (lane 3) or NICD (lane 4) reduced E-cadherin expression. Western blots were stripped and reprobed with an antibody that recognizes focal adhesion kinase (FAK) to confirm equal loading. D, activation of RBP-Jn-dependent Notch signaling leads to growth in soft agar. Parental, vector control, MCF 10A/RBP-Jn, and MCF 10A/NICD cells were plated in 0.3% agar at a density of 15,000 per well. After 6 weeks of growth, the viable cells were visualized by staining with 0.33% NBT. A significantly larger number of colonies (P < 0.0005) developed in wells containing MCF 10A/RBP-Jn and MCF 10A/NICD cells. Columns, average of six independent soft agar assays.
Expression of Notch1 and Muc1 largely colocalized (Fig. 4C; data not shown).

**Attenuation of Notch signaling reverts the transformed phenotype of human breast cancer cell lines.** The clear aberrant activation of Notch signaling in both breast cancer cell lines and tumor tissue samples suggests that it may play a significant role in tumor development. Consequently, inhibition of Notch signaling may revert the transformed phenotype of breast cancer cell lines. To test this, we overexpressed Numb, a negative regulator of the pathway (22), in the ER-positive cell line MCF7 and the metastatic cell line MDA-MB-231 (Fig. 5A; data not shown). In both cell lines, this prevented NICD accumulation (Fig. 5A; data not shown). In MCF7 cells, it also led to an accumulation of E-cadherin and a change in cell morphology so that the cells resemble normal breast epithelial cells (Fig. 5A and B). To determine whether the inhibition of Notch signaling had reverted the transformed phenotype of both cell lines, we plated them in soft agar. As expected, the parental cell line and control lines carrying empty vector formed multiple colonies, with nearly all cells forming one (Fig. 5C and D; data not shown). In contrast, <10% of MCF7/Numb and MDA-MB-231/Numb cells produced colonies (Fig. 5C and D; data not shown).

**Discussion**

We have shown that Notch1 and Notch3, Delta-like4, and Jagged1 and Jagged2 are coexpressed in the luminal epithelium of the lobules in the human breast, suggesting that Notch signaling can occur during normal development. In addition, we observed accumulation of NICD in a wide variety of human breast cancer cell lines and tissue samples, showing that the pathway is aberrantly activated in human breast cancer. Furthermore, it is likely that this activation of the Notch pathway is important in the...
Notch signaling is elevated in human breast cancer cell lines and tissue samples. A, Western blot analysis of NICD, Numb, and Hey1 levels in three normal (lanes 1-3) and eight tumorigenic human breast epithelial cell lines (lanes 4-11). Numb expression was undetectable in all eight cancer cell lines tested, whereas NICD and Hey1 were up-regulated. The blot was stripped and reprobed with an antibody that recognizes actin to confirm equal loading. B, Western blot analysis of NICD and Numb levels in two normal and nine breast cancer tissue samples. Protein lysates from two normal tissue samples (lanes A and B) were separated by SDS-PAGE with either seven ER and PR expressing tumors (tumors C1-C7; Table 1), seven ER expressing tumors that lack PR (tumors D1-D7, Table 1), or six tumors that overexpress ErbB2 or EGFR (tumors E1-E3, F1, and G1 and G2). Representative examples from these blots are shown along with the two normal samples. As with the cancer cell lines, Numb was down-regulated in all breast cancer patients, whereas NICD was up-regulated. The blots were stripped and reprobed with an antibody that recognizes epithelial marker Desmoplakin to confirm equal loading of the epithelial compartment. C, immunolocalisation of NICD, Numb, and Muc1 in a representative breast cancer tissue sample. Adjacent sections were stained with antibodies that recognise the NICD, Numb, and Muc1 antibodies (top). Samples were counterstained with hematoxylin to reveal morphology.

Figure 4. Notch signaling is elevated in human breast cancer cell lines and tissue samples. A, Western blot analysis of NICD, Numb, and Hey1 levels in three normal (lanes 1-3) and eight tumorigenic human breast epithelial cell lines (lanes 4-11). Numb expression was undetectable in all eight cancer cell lines tested, whereas NICD and Hey1 were up-regulated. The blot was stripped and reprobed with an antibody that recognizes actin to confirm equal loading. B, Western blot analysis of NICD and Numb levels in two normal and nine breast cancer tissue samples. Protein lysates from two normal tissue samples (lanes A and B) were separated by SDS-PAGE with either seven ER and PR expressing tumors (tumors C1-C7; Table 1), seven ER expressing tumors that lack PR (tumors D1-D7, Table 1), or six tumors that overexpress ErbB2 or EGFR (tumors E1-E3, F1, and G1 and G2). Representative examples from these blots are shown along with the two normal samples. As with the cancer cell lines, Numb was down-regulated in all breast cancer patients, whereas NICD was up-regulated. The blots were stripped and reprobed with an antibody that recognizes epithelial marker Desmoplakin to confirm equal loading of the epithelial compartment. C, immunolocalisation of NICD, Numb, and Muc1 in a representative breast cancer tissue sample. Adjacent sections were stained with antibodies that recognise the NICD, Numb, and Muc1 antibodies (top). Samples were counterstained with hematoxylin to reveal morphology.

etiology of breast cancer as blocking signaling through the pathway by overexpressing Numb reverts the tumorigenic phenotype of two very different breast cancer cell lines. Finally, our data suggest that increased RBP-Jκ–dependent Notch signaling transforms breast epithelial cells by preventing apoptosis in response to many different stimuli, including anoikis and p53-mediated, drug-induced apoptosis. Together, this suggests that targeting Notch signaling may represent a novel therapeutic strategy for the treatment of breast cancer.

RBP-Jκ–dependent Notch signaling is sufficient to transform breast epithelial cell lines. Several lines of evidence suggest that there is a second distinct intracellular signaling pathway, which requires Deltex function, downstream of the Notch receptors (20). However, the molecular mechanism of this pathway is not well understood. The pathway may be activated by the F3/Contactin family of GPI-linked ligands (23). Intracellularly, it has been suggested that signaling may occur through either sequestration of the transcriptional coactivator p300 (24) or inhibition of JNK (25).

Like the RBP-Jκ signaling cascade, this Deltex-dependent pathway can be activated by the overexpression of NICD (24, 25). Consequently, this raises the question of whether one or both of these pathways are required for cellular transformation by the overexpression of NICD. To address this question, deleted forms of the Notch1 and Notch4 intracellular domains have been overexpressed in normal mammary epithelial cell lines to determine the regions required for transformation (8, 9). However, these experiments have yielded conflicting results. For example, the transformation of HC11 cells by Notch1 requires a Notch molecule that can activate both Deltex-dependent and RBP-Jκ–dependent signaling (8). In contrast, overexpression of a deleted form of Notch4 protein that cannot apparently interact with RBP-Jκ and therefore may only activate Deltex-dependent signaling is sufficient to transform MCF 10A cells (9). However, recent experiments have indicated that a similarly deleted Notch1 protein can interact with RBP-Jκ in the presence of Mastermind, and that overexpression of this deleted form of Notch4 can weakly activate the RBP-Jκ–dependent Hes1 promoter (5, 19).

RBP-Jκ–dependent Notch signaling can be specifically activated by overexpressing a RBP-Jκ/VP16 fusion protein (21). We find that this mimics the effects of overexpressing NICD in MCF 10A cells (Figs. 2 and 3). In parallel experiments, we also attempted to generate a cell line in which only the Deltex-dependent Notch pathway was activated by overexpressing the Deltex protein (data not shown; refs. 24, 25). We were unable to isolate this cell line, but we could identify genetin-resistant individual cells. These cells contained a large number of lipid droplets, suggesting that they had undergone terminal differentiation. Together, this suggests that activation of RBP-Jκ–dependent Notch signaling on its own is sufficient to cause transformation. However, it should be noted that NICD can regulate signaling through a number of other signaling pathways, including the Wnt and transforming growth factor-β pathways (20, 26), and that this crosstalk may contribute to the transforming potential of the NICD protein.

Multiple apoptotic mechanisms are regulated by Notch signaling. Notch signaling has been shown to protect a wide range
of cell types from apoptosis, including glial, neural, endothelial cells, cervical keratinocytes, and leukocytes (27–34). Furthermore, Notch can protect these cells against diverse apoptotic stimuli. For example, expression of an active form of Notch1 in cervical keratinocytes protects them against anoikis and apoptosis induced by p53 overexpression (33), whereas its expression protects T cells, erythroleukemia, and endothelial cells from drug-induced apoptosis (27, 28, 30, 32). However, it is not clear that there is one single protective mechanism activated by which Notch signaling in these different systems. In cervical keratinocytes and T cells, overexpression of NICD leads to the activation of the phosphatidylinositol 3-kinase (PI3K)/Akt signaling pathway, which has been linked to several antiapoptotic mechanisms (32–34). These include phosphorylation and inactivation of the proapoptotic BH3-only protein Bad and phosphorylation of FOXO3, preventing it from inducing transcription of the BH3-only protein Bim (35). This activation of Akt may be mediated through the formation of a large protein complex consisting of a NICD, a Src family kinase and PI3K (32). In contrast, reduced JNK activity and up-regulation of the antiapoptotic proteins Bcl-2 and Bcl-xL are seen in erythroleukemia and endothelial cells when Notch signaling is increased (27, 28). The expression of Bcl-2 and Bcl-xL can directly prevent apoptosis by limiting the activity of the proapoptotic members of the Bcl-2 family (36). Reducing JNK signaling is also protective, as JNK can directly phosphorylate and regulate the activity of a number of proapoptotic Bcl-2 family members and p53 (37, 38). In the latter case, JNK phosphorylation increases p53 function.

The results of our experiments suggest that in breast epithelial cells Notch signaling may protect them through one single mechanism. Melephalan and mitoxantrone are both DNA-damaging agents and, as expected, increased p53 activity, leading to the expression of Puma and Noxa (Fig. 3). We also observed up-regulation of Puma and Noxa following staurosporine but not thaspiarginin treatment (data not shown). This suggests that Notch signaling is able to regulate apoptosis that is specifically caused by Puma and Noxa expression but not other mechanisms. This possibility can also explain our failure to observe apoptosis in MCF 10A/RBP-Jc and MCF 10A/NICD cells plated in soft agar. Growth in soft agar induces apoptosis (or anoikis) due to the loss of survival signals from the surrounding extracellular matrix. This has been shown in both fibroblasts and squamous cell carcinoma cell lines to be due to the activation of p53, suggesting that anoikis, like apoptosis following DNA damage, is in part mediated by Puma and Noxa expression (39, 40).

**Aberrant Notch signaling in human breast cancer.** The accumulation of NICD in a wide range of breast cancers and breast cancer cell lines provides direct evidence that Notch signaling is activated in this tumor type (Fig. 4). The tumors analyzed include those which express ER and PR, respond well to tamoxifen treatment and have good clinical outcome, and tumors that lack ER, overexpress erbB2 or EGFR, and have poor prognosis. The data also suggest that Notch signaling may be activated at an early stage of tumor development as it occurs in such a wide range of tumors. This possibility is supported by the fact that we also found increased Notch signaling in the one ductal carcinoma in situ sample, a preinvasive form of breast cancer, in our collection of 20 tumor samples. Furthermore, we show that Notch may have a causative role in breast cancer as inhibition of the signaling pathway reverts the transformed phenotype of two very different breast cancer cell lines (Fig. 5).

### Table 1. Type, grade, and ER, PR, EGFR, and HER2/erbB2 expression of tumor samples analyzed

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Type</th>
<th>Grade</th>
<th>% ER+ nuclei</th>
<th>% PR+ nuclei</th>
<th>erbB2 expression</th>
<th>EGFR expression</th>
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<tr>
<td>C1</td>
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<td>77</td>
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<td>Ductal</td>
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<td>99</td>
<td>93</td>
<td>-</td>
<td>-</td>
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<tr>
<td>C3</td>
<td>Ductal</td>
<td>II</td>
<td>96</td>
<td>96</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C4</td>
<td>Ductal</td>
<td>III</td>
<td>73</td>
<td>90</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C5</td>
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<td>89</td>
<td>95</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
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<td>Lobular</td>
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<td>95</td>
<td>95</td>
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<tr>
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</tr>
<tr>
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<tr>
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<td></td>
<td>57</td>
<td>0</td>
<td>-</td>
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<tr>
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<tr>
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<td>15</td>
<td>+</td>
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<td>0</td>
<td>0</td>
<td>+</td>
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<tr>
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</tr>
<tr>
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<td>III</td>
<td>0</td>
<td>0</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

NOTE: Tumor type and grade were determined by histology. The % nuclei expressing the ER and PR, and the expression of HER2/erbB2 and EGFR were determined by immunohistochemistry. Abbreviation: DCIS, ductal carcinoma in situ.
Finally, the ability of Notch signaling to regulate several different apoptotic mechanisms makes its regulation an attractive possibility for cancer treatment. Many tumors become resistant to conventional chemotherapeutic drugs due to the inactivation of several apoptotic mechanisms present within normal cells. If these pathways can be reactivated, then the tumor cells can be made sensitive again to these drugs. However, the possibility of reactivating several different apoptotic mechanisms by regulating Notch signaling has a significant advantage.

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**Figure 5.** Inhibiting Notch signaling reverts the transformed phenotype of the human breast cancer cell line MCF7. A, Western blot analysis of Numb, NICD, and E-cadherin levels in parental (lane 1), vector control (lane 2), and MCF7 cells stably expressing Numb (lane 3). Overexpression of Numb led to a reduction in NICD levels and was accompanied by an up-regulation of E-cadherin. The blot was stripped and reprobed with antibody that recognizes focal adhesion kinase to confirm equal loading. B, phase-contrast images of parental, vector control, and MCF7/Numb cells in liquid culture. Parental and vector control cells displayed a spindle-like morphology, whereas MCF7/Numb cells had a more normal epithelial cobblestone-like phenotype. In addition, MCF7 cells expressing Numb grew in organized islands of cell in close contact. C and D, parental, vector control, and MCF7/Numb cells were seeded in 0.3% soft agar at a density of 50,000 per dish and left to grow for 10 days before staining with NBT for viable colonies. Overexpression of Numb dramatically reduced the number of viable colonies that grew in soft agar. Columns, average of six independent soft agar assays.

**References**

Notch Signaling in Breast Cancer


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