Notch Activation Induces Akt Signaling via an Autocrine Loop to Prevent Apoptosis in Breast Epithelial Cells

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Abstract

The Notch pathway is aberrantly activated in a wide range of cancers, including breast carcinoma, and is required to maintain the transformed phenotype of many of these tumors. Notch signaling contributes to the transformed phenotype, in part, by preventing apoptosis in response to many different stimuli. However, it is unclear how Notch activation can lead to a general suppression of apoptosis. We show here that Notch signaling induced an autocrine signaling loop that activates Akt in breast epithelial cells. This activation of Akt was necessary for Notch-induced protection against apoptosis in the nontransformed breast epithelial cell line MCF10A. Moreover, inhibiting Notch signaling in breast cancer cells induced a decrease in Akt activity and an increase in sensitivity to apoptosis. Finally, the inhibition of ASK1 by Akt was responsible for the protection from apoptosis induced by DNA damage, as it prevented c-Jun NH2-terminal kinase-mediated phosphorylation and activation of p53. [Cancer Res 2009;69(12):5015–22]

Introduction

Becoming self-sufficient for growth signals is a key feature of transformed cells (1). This ability to grow independently of signals from other cell types can be acquired by either losing or lowering the requirement for ligands to activate growth factor receptors present on the cancer cell (1) or by activating an autocrine signaling loop through the secretion of the appropriate growth factors (2, 3).

The Notch signaling pathway regulates cell fate decisions, proliferation, and death. The Notch genes encode transmembrane receptors that are cleaved on binding of their ligands, leading to the release of the intracellular domain [Notch intracellular domain (NICD)], which translocates to the nucleus where it functions as a transcriptional coactivator. Notch signaling has been linked to a wide range of cancers and, in several cases, it has been shown to have a causal role in tumor development (4, 5). In T-cell acute lymphoblastic leukemia, activating mutations in Notch1 have been identified in >50% of all cases (6, 7). Notch signaling is also aberrantly activated in breast cancer (8–12) and changes in the pathway may prove to be useful prognostic markers. Elevated transcript levels for Notch1 and the ligand Jagged1 correlate with poor prognosis (9) and high NICD levels are associated with an increased risk of recurrence of ductal carcinomas in situ (DCIS) by 5 years (13). Finally, increased Notch signaling is required to maintain the transformed phenotype of breast cancer cell lines and the growth and survival of primary tumor cells (8, 10).

The contribution of Notch signaling to the transformed phenotype of cancer cells appears to be linked to the suppression of apoptosis (4, 5). We have shown that Notch activation in nontransformed breast epithelial cells induces a general resistance to apoptotic stimuli (10). Furthermore, inhibiting Notch signaling in cancer cells where it is constitutively active induces apoptosis or sensitization to apoptosis (14–16). Notch signaling has been shown to regulate apoptosis in different cancer cell lines through several different mechanisms, including the inhibition of p53, Foxo3a, or c-Jun NH2-terminal kinase (JNK) function, as well as the activation of Akt (17–21). A common aspect to all of these mechanisms is that they can be linked to the activation of Akt (22); for example, Foxo3a can be phosphorylated and inhibited by Akt (23). Also, activation of Akt has been shown to induce resistance to a wide range of apoptotic stimuli (22). Consequently, the general apoptotic resistance seen when Notch signaling is activated could be due to the induction of Akt function.

Here we show that Akt activation downstream of Notch signaling was necessary for resistance to apoptosis in the normal breast epithelial cell line MCF10A. On the other hand, inhibiting Notch signaling in breast cancer cells decreased Akt phosphorylation and increased sensitivity to apoptosis. In addition, we show that Akt prevented apoptosis in response to DNA damage by inhibiting ASK1. This inhibition of ASK1 led to a failure to induce JNK signaling and the subsequent activation of p53. Finally, we show that Notch-induced activation of Akt is due to the release of an autocrine factor into the culture medium.

Materials and Methods

Antibodies and Western blotting. Rabbit antibodies that recognize phospho-Thr183/Tyr185 JNK, phospho-Ser83 ASK1, total Akt, total glycogen synthase kinase (GSK)-3β, phospho-Ser473 AKT, total ASK1, phospho-Thr416 p53, phospho-Ser9 GSK-3β, SHP2, phospho-Ser23 c-Jun, cleaved caspase-3, and phospho-Ser166 MDM2 were from Cell Signaling Technology. Mouse antibodies that recognize p53, Noxa, FAK, MDM2, and PTEN were from Cell Signaling Technology, Abcam, BD Pharmingen, Santa Cruz Biotechnology, and Calbiochem, respectively. The goat antibody that recognizes INPP4B was from Santa Cruz Biotechnology. The mouse monoclonal antibody that recognizes tubulin has been described previously (24). All horseradish peroxidase-conjugated secondary antibodies were from Jackson ImmunoResearch. Western blotting was carried out as described previously (10).

Plasmid constructs. cDNAs encoding dominant-negative forms of Mastermind-like (dnMAML; ref. 25) and Akt (26), constitutively active forms of JNK (27) and Akt (28), and an NH2-terminally truncated form of ASK1 (lacking amino acids 1-649; ΔN-ASK1; ref. 29) were obtained from...
Dr. Gianluca Civenni (Friedrich Miescher Institute), Prof. Boudewijn Burgering (University Medical Centre Utrecht), Dr. Ulrike Rennefahrt (Universität Würzburg), Dr. Steven Anderson (University of Colorado), and Dr. Hidenori Ichijo (University of Tokyo), respectively. Lentiviral vectors for the inducible expression of NICD (p201-doubleTET and p199-N1IC-i2ESVzeo) were based on vectors from Wolfgang Hillen and Stephen Elledge and were a generous gift from Manfred Gessler (Universität of Würzburg). The NICD, RPB-Jκ/VP16, and Numb expression plasmids have been described previously (10).

**Cell culture conditions.** MCF10A cell lines, including those stably expressing NICD or the RBP-Jκ/VP16 fusion protein (MCF10A/NICD and MCF10A/RBP-Jκ), were maintained as described previously (10). A new MCF10A/NICD cell line was derived by lentiviral infection using the p199-NIIC-i2ESVzeo and p201-doubleTET plasmids. MCF10DCIS.com cells were grown in a 1:1 mixture of Ham’s F-12 medium and DMEM supplemented with 100 µg/mL penicillin, 100 units/mL streptomycin, 5% horse serum, and 2 mmol/L l-glutamine (Asterand; ref. 30). MCF7 cell lines were maintained as described previously (10). MCF7/Numb and MCF7/dnMAML cell lines were generated by selecting cells that stably carry the pcDNA3.1+Numb and pcDNA3.1(hygro)+dnMAML vectors, respectively.

For conditioned medium experiments, culture medium was recovered from cells that had been detached, washed twice in PBS, plated in serum-free medium, and cultured for 48 h. The conditioned medium was passed through a 45 µm filter to remove cellular debris before being added to MCF10A cells that had been starved by culturing for 48 h in serum-free medium.

**Figure 1.** Notch-induced Akt activation was necessary for resistance to apoptosis in MCF10A cells. A, Akt signaling was activated in MCF10A/RBP-Jκ and MCF10A/NICD cells. Whole cell lysates from MCF10A cells, MCF10A cells carrying the empty pcDNA3 (Vector), MCF10A/RBP-Jκ and MCF10A/NICD were analyzed by Western blotting for phosphorylation of Akt and its downstream targets. B, inhibiting Akt signaling sensitized MCF10A/RBP-Jκ and MCF10A/NICD cells to DNA damage-induced apoptosis. Whole cell lysates from MCF10A, MCF10A/RBP-Jκ, and MCF10A/NICD cells treated with 10 µmol/L SH6 and 100 µmol/L melphalan for 8 h as indicated were analyzed by Western blotting for Akt phosphorylation, Noxa expression, JNK phosphorylation, p53 accumulation, and caspase-3 cleavage. C, inhibiting Akt induced apoptosis in MCF10A cells regardless of RBP-Jκ-dependent Notch signaling. Parental, vector control, MCF10A/RBP-Jκ, and MCF10A/NICD cells were transiently transfected with either an empty vector or an expression plasmid encoding a dominant negative form of Akt. Transfected cells were identified by co-transfection of these plasmids with pEGFP-C1 (Promega). The percentage of GFP positive cells that were apoptotic was determined. Average of three independent experiments.
For coculture experiments, parental MCF10A were stained for 30 min with 5 μmol/L CellTracker Orange (Molecular Probes), and 2 x 10^4 of these stained cells were plated with an equal number of nonstained parental MCF10A or nonstained MCF10A/NICD cells. Twenty-four hours after seeding, cells were treated with melphalan to induce DNA damage. Cells were then analyzed for cell death as described below counting only CellTracker-stained cells.

**Transfection conditions.** Transient transfection of MCF7 and DCIS.com cells was carried out using Lipofectamine 2000 (Invitrogen). DNA (2 μg) was mixed in serum-free medium with 6 μL Lipofectamine 2000.

**Figure 2.** Notch inhibition decreased Akt phosphorylation and increased apoptosis sensitivity in breast cancer cells. A, Akt phosphorylation on ser473 was decreased in breast cancer cells when Notch signaling was inhibited. Whole cell lysates from MCF7 or DCIS.com cells transiently transfected with an empty pcDNA3.1 vector, or expression plasmide encoding Numb or dnMAML, were analyzed by Western blotting for Akt phosphorylation. B, inhibiting Notch signaling in breast cancer cell lines increased their sensitivity to melphalan-induced apoptosis. MCF7 and DCIS.com cells were treated with 1 μmol/L DAPT or 0.1% DMSO for 48 h before treatment with or without 25 μmol/L melphalan for 16 h. The percentage of apoptotic cells was determined. A two-way ANOVA showed that the DAPT-induced increase in cell death following melphalan treatment was significant (*, P < 0.05; **, P < 0.01). C, expressing Numb or dnMAML increased the sensitivity of breast cancer cell lines to melphalan-induced apoptosis. DCIS.com and MCF7 cells stably expressing Numb or dnMAML were treated with or without 25 μmol/L melphalan for 16 h and the percentage of apoptotic cells was determined. A two-way ANOVA showed that expression of NUMB or dnMAML induced a significant increase in apoptosis (*, P < 0.05; ***, P < 0.001). D, expression of a constitutively active form of Akt (Myr-Akt) blocked the increased sensitivity to melphalan following Notch inhibition. DCIS.com cells transiently transfected with an empty pcDNA3.1 vector or expression vector encoding MYR-AKT were treated with 1 μmol/L of DAPT or 0.1% DMSO for 48 h. Cells were then treated with or without 25 μmol/L melphalan for 8 h and the percentage of apoptotic cells was determined.
for 20 min at room temperature. Lipofectamine 2000/DNA complexes were added to cells dropwise in serum-free medium. This medium was removed and complete growth medium was added after 6 h.

Apoptosis analysis. Microscopic detection of apoptosis was carried out on both detached and adherent cells recovered after treatment. These cells were spun down onto slides using a Cytospin 2 (Shandon), fixed for 10 min with methanol/acetone (50:50, v/v) solution at -20°C, and stained with 1 μg/mL Hoechst 33342 for 15 min at 37°C. Stained cells were observed using a Hamamatsu ORCA-ER digital camera on a Zeiss Axiosplan2 microscope. Apoptotic cells were recognized due to their nuclear condensation and fragmentation and could be distinguished easily and reliably from necrotic cells. In addition, this method allowed the detection of all cells that had undergone apoptosis during the experiment. The percentage of apoptotic cells was calculated and presented with SE. Differences between samples were analyzed using two-way ANOVA followed by a Bonferroni post-test.

Results

Akt activation was necessary for Notch-confferred resistance to apoptosis in normal breast epithelial cells. We have shown previously that increased RBP-Jκ-dependent Notch signaling induces resistance to various apoptotic stimuli (10). We thus investigated the activation of general survival pathways such as Akt and extracellular signal-regulated kinase (ERK) signaling (22, 31). RBP-Jκ-dependent Notch signaling was activated in MCF10A cells as described previously (10). Although we found no change in ERK phosphorylation in MCF10A/NICD and MCF10A/RBP-Jκ cells (data not shown), Akt signaling was clearly activated as shown by Ser473 phosphorylation (Fig. 1A). The Akt targets GSK-3β, ASK1, and HDM2 were also phosphorylated on Ser9, Ser516, and Ser466, respectively (Fig. 1A).

We next showed that inhibiting Akt by pretreatment with SH6 efficiently inhibited Akt phosphorylation on Ser473 in MCF10A/NICD and MCF10A/RBP-Jκ cells (Fig. 1B, compare lanes 5 and 6 and lanes 9 and 10). This reduction in Akt signaling also restored the sensitivity of MCF10A/NICD and MCF10A/RBP-Jκ cells to melphalan-induced apoptosis leading to JNK phosphorylation, accumulation of the p53 target Noxa, and caspase-3 cleavage (Fig. 1B, compare lanes 3, 8, and 12). Similarly, we found that inhibiting Akt signaling by expressing a dominant-negative form of Akt induced apoptosis to a comparable extent in parental, vector control, MCF10A/NICD, and MCF10A/RBP-Jκ cells (Fig. 1C); the apparently more potent effects of a dominant-negative form of Akt than SH6 are most likely due to it actively antagonizing Akt signaling, causing a more profound drop in Akt function than SH6. Altogether, these data show that Notch-induced apoptosis resistance in MCF10A cells requires Akt function.

Notch inhibition decreased Akt signaling and sensitized breast cancer cells to apoptosis. The Notch pathway is constitutively active in most breast epithelial cancer cell lines (10, 15). Consequently, we looked to see whether Notch inhibition would reduce Akt function in breast cancer cell lines. We focused our analysis on the MCF7 and DCIS.com cell lines, which are both wild-type for p53 and thus are expected to respond to DNA damage in a similar manner as MCF10A cells. Pretreatment with DAPT, a γ-secretase inhibitor that prevents Notch cleavage and thus NICD production, induced a decrease of Akt phosphorylation in MCF7 and DCIS.com cell lines (Figs. 2A and 5A). Moreover, DAPT treatment induced an increase in sensitivity to melphalan treatment (Fig. 2B). We also inhibited Notch signaling by expressing Numb or dnMAML (25) in DCIS.com and MCF7 cells. Expression of either Numb or dnMAML induced a decrease in Akt phosphorylation (Fig. 2A) and a sensitization to melphalan treatment (Fig. 2C). To show that the increased sensitivity to melphalan-induced apoptosis was linked to reduced Akt activity, we expressed a constitutively active form of Akt (myr-Akt) in the presence or absence of melphalan and DAPT treatment. Expression of myr-Akt completely abrogated the increase in melphalan-induced cell death observed following DAPT treatment (Fig. 2D, compare columns 4 and 8).

Inhibition of ASK1/JNK signaling was responsible for Notch-confferred resistance to apoptosis in MCF10A cells. As Akt signaling can regulate p53 function through the phosphorylation of HDM2 and ASK1 (22), we next investigated the role of these downstream proteins in Notch-induced resistance to DNA damage. We used Nutlin-3 to disrupt the p53-HDM2 interaction and thus prevent p53 degradation. Although pretreatment with Nutlin-3 did induce a stabilization of p53, it failed to either restore Noxa

Figure 3. Inhibition of DNA damage-induced apoptosis was not mediated by HDM2. Inhibiting the HDM2/p53 interaction through Nutlin-3 treatment did not resensitize MCF10A/NICD and MCF10A/RBP-Jκ cells to melphalan-induced apoptosis. MCF10A, MCF10A/NICD, and MCF10A/RBP-Jκ cells were treated with 10 μmol/L Nutlin-3 or 0.1% DMSO for 1 h before treatment with or without 100 μmol/L melphalan for a further 16 h. The percentage of apoptotic cells was determined and whole cell lysates were analyzed by Western blotting for p53 accumulation, Noxa expression, and caspase-3 cleavage.

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induction and cleavage of caspase-3 following melphalan treatment or to resensitize MCF10A/NICD and MCF10A/RBP-Jκ cells to DNA damage-induced apoptosis (Fig. 3). We thus conclude that phosphorylation of HDc2 by Akt in MCF10A cells in response to Notch signaling was not sufficient to confer resistance to DNA damage.

We next asked whether changes in ASK1 function downstream of Akt were required for the resistance to DNA damage-induced apoptosis. We found that inhibiting ASK1/JNK signaling prevented melphalan-induced apoptosis in MCF10A cells. MCF10A cells were treated overnight with 10 μmol/L SP600125 or 0.1% DMSO prior to treatment or not with 100 μmol/L melphalan for 8 h. Cells were re-treated with SP600125 every 2 h to maintain a strong inhibition of JNK or DMSO as a control. The percentage of apoptotic cells was determined and whole cell lysates were analyzed by Western blotting for c-jun, JNK and p53 phosphorylation, p53 accumulation, Noxa expression, and caspase-3 cleavage. A two-way ANOVA showed that the SP600125-induced decrease in apoptosis was significant (***, P < 0.001).

B, expressing a constitutively active form of JNK induced apoptosis in MCF10A/NICD and MCF10A/RBP-Jκ cells. Parental MCF10A, vector control MCF10A cells, MCF10A/NICD, and MCF10A/RBP-Jκ cells were transiently transfected with expression vectors encoding a JNK/MKK7 fusion protein which constitutively activates JNK signaling (CA-JNK) or a JNK/MKK7 fusion protein that carries point mutations within JNK and MKK7 rendering the protein inactive as a control. Transfected cells were identified by the co-transfection of these plasmids with pEGFP-C1 (Promega). The percentage of GFP positive cells that were apoptotic was determined in three independent experiments.

C, inhibiting Notch signaling increased the response of breast cancer cell lines to melphalan. DCIS.com cells transiently transfected with an empty pcDNA3.1 vector, or expression plasmids encoding Numb or dnMAML, were treated with or without 25 μmol/L melphalan. Similarly, DCIS.com cells pretreated for 48 h with 1 μmol/L DAPT were treated with or without 25 μmol/L melphalan. Finally, MCF7 cells carrying an empty pcDNA3.1 vector or stably expressing dnMAML were treated with melphalan. Whole cell lysates from these cell lines were analyzed by Western blotting for JNK and ASK1 phosphorylation. D, expressing a form of ASK1, which cannot be regulated by Akt phosphorylation (ΔN-ASK1), increased the sensitivity DCIS.com and MCF10A/NICD cells to melphalan-induced apoptosis. DCIS.com cells and MCF10A/NICD cells were transiently transfected with either an empty pcDNA3.1 vector or an expression plasmid encoding ΔN-ASK1. Cells were treated with or without 25 μmol/L melphalan 48 h later and the percentage of apoptotic cells was determined.

Figure 4. Suppression of the ASK1/JNK pathway by Akt mediates the Notch induced resistance to DNA damage-induced apoptosis in breast epithelial cells. A, inhibiting JNK signaling prevented melphalan-induced apoptosis in MCF10A cells. MCF10A cells were treated overnight with 10 μmol/L SP600125 or 0.1% DMSO prior to treatment or not with 100 μmol/L melphalan for 8 h. Cells were re-treated with SP600125 every 2 h to maintain a strong inhibition of JNK or DMSO as a control. The percentage of apoptotic cells was determined and whole cell lysates were analyzed by Western blotting for c-jun, JNK and p53 phosphorylation, p53 accumulation, Noxa expression, and caspase-3 cleavage. A two-way ANOVA showed that the SP600125-induced decrease in apoptosis was significant (***, P < 0.001).

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apoptosis. ASK1 is activated following DNA damage and stimulates JNK activity (32), which in turn phosphorylates and activates p53 (33). Akt can phosphorylate ASK1 on Ser^{83}, preventing its activation (34). As ASK1 was phosphorylated on Ser^{83} following Notch activation (Fig. 1A), we examined whether inhibition of ASK1/JNK signaling in MCF10A cells was sufficient to mimic Notch-induced apoptosis resistance. As expected, treatment with the JNK inhibitor SP600125 reduced phosphorylation of the JNK target c-Jun on Ser^{81} (Fig. 4A). Furthermore, inhibition of JNK prevented phosphorylation of p53 on Thr^{35}, p53 accumulation, and expression of Noxa in parental MCF10A cells (Fig. 4B). Moreover, JNK inhibition completely abrogated cell death induction (Fig. 4A). On the other hand, expression of a constitutively active form of JNK induced apoptosis in MCF10A/NICD and MCF10A/RBP-Jκ cells as readily as in parental MCF10A cells (Fig. 4B). This suggests that Notch signaling may also inhibit the ASK1/JNK pathway in breast cancer cell lines. Indeed, inhibiting Notch signaling in the breast cancer cell lines DCIS.com and MCF7, by expressing Numb or dnMAML, led to increased JNK phosphorylation following melphalan treatment (Fig. 4C). JNK phosphorylation was similarly increased in response to melphalan in DCIS.com cells treated with DAPT for 48 h (Fig. 4C). In keeping with this, ASK1 phosphorylation on Ser^{83} following melphalan treatment was reduced when Numb or dnMAML was expressed (Fig. 4C). Finally, restoring ASK1 signaling in DCIS.com or MCF10A/NICD by expressing a form of ASK1 that can no longer be regulated by Akt phosphorylation (∆N-ASK1) sensitized these cells to melphalan treatment (Fig. 4D). Altogether, these data show that inhibition of ASK1/JNK signaling was sufficient to mimic the resistance to DNA damage-induced apoptosis that occurs in response to Notch activation.

Akt activation in response to Notch signaling was due to the release of an autocrine factor. Akt activation by Notch has been described in several different systems (19, 20, 35, 36). In T-cell acute lymphoblastic leukemia, Notch has been shown to inhibit PTEN expression (19). However, we saw no changes in PTEN expression in MCF10A cells when Notch signaling was activated (Fig. 5A). Similarly, we saw no modification of PTEN expression when Notch signaling was inhibited following DAPT treatment of MCF7 cells (Fig. 5A). We thus looked at other phosphatases that regulate PIP_{3} levels, SHIP2 and INPP4B (37, 38). Again, activation of Notch signaling in MCF10A cells or inhibition of Notch signaling in MCF7 cells did not alter SHIP2 or INPP4B expression (Fig. 5A). This suggests that the activation of Akt following Notch signaling was not due to changes in the expression of these phosphatases.

We have, however, noticed that MCF10A/NICD and MCF10A/RBP-Jκ cells can tolerate growth factor withdrawal (data not shown). We thus hypothesized that Akt activation could be due to the secretion of a growth factor that signals through an autocrine loop. To test this hypothesis, we starved MCF10A cells in serum-free and growth factor-free medium for 48 h and stimulated these cells with complete growth medium or serum-free and growth factor-free medium that had been conditioned by parental MCF10A cells or MCF10A/NICD cells. As expected, complete growth medium induced phosphorylation of Akt on Ser^{473} and phosphorylation of the Akt target GSK-3β, whereas conditioned medium from parental MCF10A cells did not (Fig. 5B). In contrast, conditioned medium from MCF10A/NICD cells induced robust Akt signaling (Fig. 5B). Furthermore, conditioned medium from DCIS.com cells stimulated phosphorylation of Akt on Ser^{473} in starved parental MCF10A cells (Fig. 5C), whereas conditioned medium from DCIS.com cells similarly increased in response to melphalan in DCIS.com cells. We thus hypothesized that Akt activation could be due to the secretion of a growth factor that signals through an autocrine loop. To test this hypothesis, we starved MCF10A cells in serum-free and growth factor-free medium for 48 h and stimulated these cells with complete growth medium or serum-free and growth factor-free medium that had been conditioned by parental MCF10A cells or MCF10A/NICD cells. As expected, complete growth medium induced phosphorylation of Akt on Ser^{473} and phosphorylation of the Akt target GSK-3β, whereas conditioned medium from parental MCF10A cells did not (Fig. 5B). In contrast, conditioned medium from MCF10A/NICD cells induced robust Akt signaling (Fig. 5B). Furthermore, conditioned medium from DCIS.com cells similarly increased in response to melphalan in DCIS.com cells. This suggests that Notch signaling inhibited the ASK1/PTEN signaling was sufficient to mimic the resistance to DNA damage-induced apoptosis that occurs in response to Notch activation. Akt activation by Notch has been described in several different systems (19, 20, 35, 36). In T-cell acute lymphoblastic leukemia, Notch has been shown to inhibit PTEN expression (19). However, we saw no changes in PTEN expression in MCF10A cells when Notch signaling was activated (Fig. 5A). Similarly, we saw no modification of PTEN expression when Notch signaling was inhibited following DAPT treatment of MCF7 cells (Fig. 5A). We thus looked at other phosphatases that regulate PIP_{3} levels, SHIP2 and INPP4B (37, 38). Again, activation of Notch signaling in MCF10A cells or inhibition of Notch signaling in MCF7 cells did not alter SHIP2 or INPP4B expression (Fig. 5A). This suggests that the activation of Akt following Notch signaling was not due to changes in the expression of these phosphatases.

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conditioned medium from DCIS.com cells treated with DAPT for 48 h was ineffective (Fig. 5C), suggesting that these cells also secrete a Notch-dependent factor that activates Akt. Finally, coculturing parental MCF10A cells with MCF10A/NICD cells protected parental cells from DNA damage-induced apoptosis (Fig. 5D). Together, these data show that activating Notch signaling caused breast epithelial cells to secrete a factor into the culture medium and protected MCF10A cells from apoptosis.

Discussion

Here, we have shown that Akt signaling was activated in MCF10A cells, which were transformed by RPB-Jκ-dependent Notch signaling. This activation of Akt led to the inhibition of ASK1 by phosphorylation on Ser83, which in turn inhibited JNK activation, phosphorylation of p53 on Thr81, and subsequent induction of apoptosis in response to DNA damage. Finally, the activation of Akt was due to an autocrine loop involving the release of a secreted factor, which may be a growth factor.

Akt activation by Notch signaling has been described in several different cellular models (19, 20, 39). However, the mechanism by which Akt is activated is not well understood. It has recently been shown that Notch signaling induces transcription of the epidermal growth factor (EGF) receptor in glioma cells (40). However, in MCF10A cells, expressing either NICD or the RPB-Jκ/VP16 fusion protein did not alter EGF receptor or HER-2 expression levels (data not shown). On the other hand, Notch signaling induces a c-Myc-and HES1-dependent down-regulation of PTEN expression in T-cell acute lymphoblastic leukemia, leading to an increase in Akt phosphorylation and activity (19). We did not see any changes in PTEN expression either when Notch signaling was activated in MCF10A cells or following DAPT treatment to inhibit Notch in breast cancer cells (Fig. 5A). Similarly, we saw no change in the expression of INPP4B and SHIP2, which can also regulate PIP3 levels and thus Akt activation (Fig. 5A; ref. 37, 38). It has also been shown that NICD can directly interact with p56lck and phosphatidylinositol 3-kinase in T cells to activate phosphatidylinositol 3-kinase and hence Akt (20). A similar mechanism is unlikely to play a role in breast epithelial cells, as we have shown that expression of the RPB-Jκ/VP16 fusion protein can activate Akt to the same extent as NICD (Fig. 1A).

Together, these observations suggest that Akt signaling is activated in response to Notch signaling by a novel mechanism in breast epithelial cells. Furthermore, it is likely to be through the transcription of a Notch target gene, as the effects of expressing NICD and an active form of RPB-Jκ were indistinguishable (Fig. 1A). Although the identity of the target gene is currently not clear, our results suggest that it is either a secreted factor or a protein required for the secretion of the appropriate factor, as serum-free and growth factor-free conditioned medium from MCF10A/NICD cells was sufficient to activate Akt signaling in parental MCF10A cells (Fig. 5B). Furthermore, parental MCF10A cells cocultured with NICD-expressing MCF10A cells were protected from apoptosis (Fig. 5D).

To elucidate the molecular mechanism underlying the resistance to DNA damage-induced apoptosis and the failure to activate p53 (10), we determined which downstream targets of Akt were required. In particular, changes in ASK1/JNK signaling and HDMD activity were examined, which have both been shown to regulate p53 function downstream of Akt (33, 41). HDMD2 was phosphorylated on Ser166 in response to Notch signaling (Fig. 1A). However, inhibiting the interaction between HDMD2 and p53 by treating with Nutlin-3 was not sufficient to restore the sensitivity of MCF10A/NICD and MCF10A/RRP-Jκ cells to DNA damage-induced apoptosis (Fig. 3).

In contrast, the inhibition of JNK signaling does appear to be significant (Fig. 4B; ref. 10). Inhibiting JNK signaling in parental MCF10A cells was sufficient to mimic the Notch-induced apoptosis resistance (Fig. 4A), whereas expressing a constitutively active form of JNK induced apoptosis in MCF10A/NICD and MCF10A/RRP-Jκ cells (Fig. 4B). Furthermore, JNK signaling was activated more strongly in DCIS.com and MCF7 cells in response to melphalan treatment when Notch signaling was inhibited (Fig. 4C).

Notch signaling has been shown to inhibit JNK function in both Drosophila and mammalian cells (42, 43). Within mammalian cells, NICD inhibits JNK by interfering with the scaffold function of JNK-interacting protein 1 (17). However, we suspect that this is not the only way Notch can limit JNK signaling, as we found that expressing the RPB-Jκ/VP16 fusion protein inhibited JNK as potently as NICD (Fig. 1B). We describe here an alternative mechanism whereby Notch signaling inhibits JNK function through the induction of Akt signaling, which, in turn, phosphorylates and inhibits upstream kinases of the JNK pathway such as ASK1 (Figs. 1A and 4C). In keeping with this, we found that expression of a NH2-terminally truncated ASK1 molecule, which lacks the Akt phosphorylation site at Ser83, restores the sensitivity of cells in which Notch signaling is activated, DCIS.com and MCF10A/NICD, to DNA damage (Fig. 4D).

Notch signaling has also been shown to regulate p53 signaling by several other mechanisms (18, 35, 44, 45). Mungamuri and colleagues showed that Notch inhibited p53 function through the activation of mTORC1 and eIF4E (35). However, rapamycin did not sensitize MCF10A/NICD to apoptosis, suggesting that this mechanism does not play a role in these cells (data not shown). NICD has also been observed to directly interact with p53, inhibiting its phosphorylation and subsequent activation (18). As expression of RPB-Jκ/VP16 in MCF10A cells was as effective as expression of NICD, we again suspect that this mechanism does not contribute significantly to the regulation of p53 function in these cells.

We therefore conclude that, in breast epithelial cells, Notch activation causes a general apoptosis resistance through the activation of Akt and specifically inhibits p53-mediated apoptosis in response to DNA damage through an Akt-dependent inhibition of JNK. Finally, as Notch is now considered as an attractive target for cancer therapy (5), the results presented here may help with the design of treatment strategies that can be combined with Notch pathway inhibitors to improve therapy and reduce unwanted side effects.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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