Dose-dependent induction of distinct phenotypic responses to Notch pathway activation in mammary epithelial cells

Marco Mazzonea,1, Laura M. Seldorsb, John Albeckc, Michael Overholtzerb, Sanja Sale*, Danielle L. Carrolld, Darshan Pandyaa, Yiling Lu*, Gordon B. Mills*, Jon C. Asterd, Spyros Artavanis-Tsakonas*, and Joan S. Bruggea,3

*Department of Cell Biology, Harvard Medical School, Boston, MA 02115; bCell Biology Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10065; cDepartment of Systems Biology, The University of Texas M. D. Anderson Cancer Center, Houston, TX 77030; and dDepartment of Pathology, Brigham and Women’s Hospital, Boston, MA 02115

Contributed by Joan S. Brugge, January 22, 2010 (sent for review December 3, 2009)

Aberrant activation of Notch receptors has been implicated in breast cancer; however, the mechanisms contributing to Notch-dependent transformation remain elusive because Notch displays dichotomous functional activities, promoting both proliferation and growth arrest. We investigated the cellular basis for the heterogeneous responses to Notch pathway activation in 3D cultures of MCF-10A mammary epithelial cells. Expression of a constitutively active Notch-1 intracellular domain (NICD) was found to induce two distinct types of 3D structures: large, hyperproliferative structures and small, growth-arrested structures with reduced cell-to-matrix adhesion. Interestingly, we found that these heterogeneous phenotypes reflect differences in Notch pathway activation levels; high Notch activity caused down-regulation of multiple matrix-adhesion genes and inhibition of proliferation, whereas low Notch activity maintained matrix adhesion and provoked a strong hyperproliferative response. Moreover, microarray analyses implicated NICD-induced p63 down-regulation in loss of matrix adhesion. In addition, a reverse-phase protein array-based analysis and subsequent loss-of-function studies identified STAT3 as a dominant downstream mediator of the NICD-induced outgrowth. These results indicate that the phenotypic responses to Notch are determined by the dose of pathway activation; and this dose affects the balance between growth-stimulative and growth-suppressive effects. This unique feature of Notch signaling provides insights into mechanisms that contribute to the dichotomous effects of Notch during development and tumorigenesis.

Notch family receptors control evolutionarily conserved intercellular signaling pathways that regulate interactions between physically adjacent cells (1). Notch receptors play a role in a variety of developmental processes by controlling many diverse processes including proliferation, differentiation, and apoptosis. The biological effects of Notch are mediated through interactions with plasma membrane-associated ligands expressed on adjacent cells. After ligand binding, Notch is subjected to proteolytic cleavages that release the Notch intracellular domain (NICD), which moves to the nucleus where it participates in transcriptional complexes to regulate Notch-dependent gene expression (3). Alterations of Notch pathway activity are associated with several human cancers (4). Most notably, alterations are linked to T-cell acute lymphoblastic leukemia, in which activating mutations within Notch-1 have been identified in >50% of tumors (5). Multiple lines of evidence implicate Notch receptors in breast cancer. Numb, a negative regulator of Notch pathway, is lost in >50% of human breast tumors through ubiquitination and proteasomal degradation, and its levels are inversely correlated with grade and proliferation rate (6). Furthermore, NICD is accumulated in a wide variety of human breast cancer cell lines, and elevated levels of Notch-1 and Jagged-1 mRNA in patients with breast cancer correlate with poor prognosis (7, 8). Within the murine mammary gland, transgenic expression of the activated form of Notch-1 or Notch-4 has been shown to provoke tumorigenesis (9, 10). In addition, both activated isoforms can transform normal murine and human mammary epithelial cells in soft-agar colony formation assays (11, 12).

The cellular mechanisms responsible for Notch-induced mammary tumorigenesis and dichotomous biological activities are not well understood. For this reason, we have examined the effects of NICD in 3D reconstituted basement membrane cultures of MCF-10A human mammary epithelial cells. Such cultures allow cells to organize into acinar structures that resemble the organization of mammary epithelial cells in vivo and consequently make it feasible to investigate morphogenetic activities of oncogenes that cannot be monitored in monolayer cultures (e.g., their ability to allow survival of cells in the luminal space, to disrupt cell–cell adhesion, their polarity, and other phenotypic effects) (13, 14). The most attractive aspect of these cultures is that it is possible to follow the fate of individual cells, because each acinus is generated by clonal outgrowth. Our studies revealed dramatic dose-dependent effects of Notch activation. Higher doses of Notch activity caused suppression of cell proliferation and clonal outgrowth, loss of ECM components and receptors, detachment of cells from ECM, and induction of cell-in-cell structures. Lower doses of Notch activity led to the generation of large colonies in soft agar and hyperproliferative acinar structures that maintain matrix adhesion. These results indicate that Notch pathway can induce heterogeneous phenotypes in mammary epithelial cells mainly through dose-dependent effects on matrix adhesion and cell proliferation.

Results

NICD Induces Heterogeneous Phenotypes in 3D Cultures of Mammary Epithelial Cells. To investigate the phenotypic changes induced by Notch pathway activation in mammary epithelial cells, we infected immortalized but nontumorigenic human MCF-10A cells with retroviral vectors that induce expression of either a drug-selectable marker alone (pBABE) or this marker together with constitutively active NICD. Stably infected MCF-10A cells overexpressing NICD marker alone (pBABE) or this marker together with constitutively active NICD were seeded in reconstituted basement membrane gels (Matrigel). As previously shown (15), MCF-10A cells grown in Matrigel form acinar-like 3D structures consisting of an outer layer of polarized, luminal cells that characterize normal mammary epithelial cells and acinar-like structures consisting of an outer layer of polarized, luminal cells that characterize normal mammary epithelial cells. Such structures allow cells to organize into acinar structures that resemble the organization of mammary epithelial cells in vivo and consequently make it feasible to investigate morphogenetic activities of oncogenes that cannot be monitored in monolayer cultures. Our studies revealed dramatic dose-dependent effects of Notch activation. Higher doses of Notch activity caused suppression of cell proliferation and clonal outgrowth, loss of ECM components and receptors, detachment of cells from ECM, and induction of cell-in-cell structures. Lower doses of Notch activity led to the generation of large colonies in soft agar and hyperproliferative acinar structures that maintain matrix adhesion. These results indicate that Notch pathway can induce heterogeneous phenotypes in mammary epithelial cells mainly through dose-dependent effects on matrix adhesion and cell proliferation.
with a basal deposition of basement membrane components such as laminins and collagens (see structures induced by pBABE in Fig. 1A). Strikingly, ≈10–20% of MCF-10A cells overexpressing NICD formed abnormal, noninvasive, hyperproliferative structures that were much larger than control structures (Fig. 1A and B). Formation of these structures was dependent on exogenous EGF, and EGF-deficient medium did not support proliferation of either pBABE- or NICD-infected cells (Fig. 1A, Insets), in contrast to the EGF-independent phenotypes of MCF-10A cells expressing ErbB2, IGF-1R, PIK3CA mutants, or V-12Ras (14). The majority of cells overexpressing NICD failed to initiate proliferation or formed small structures with a highly aberrant morphology (black arrows in Fig. 1A), indicating that NICD overexpression interferes with the outgrowth of most MCF-10A acinar structures. For the sake of reference, we term these growth-inhibited structures “abortive.”

Analysis of the NICD-induced structures at higher magnification by confocal microscopy using immunofluorescent markers that delineated cell membranes and nuclei revealed that many of the abortive structures were composed of large cells that contain internalized cells, reminiscent of previously described cell-in-cell structures (white arrows in Fig. 1A). Such structures are formed by the invasion of one cell into another by a process we previously referred to as “entosis” (16, 17). This process takes place following loss of matrix adhesion in a wide variety of normal and tumor cell lines in vitro and is commonly detected in nonadherent human tumor cells within fluid exudates (e.g., ascites, pleural fluid).

The detection of cell-in-cell structures in the 3D cultures suggested that NICD might induce loss of matrix adhesion. Indeed, NICD previously was found to cause detachment in both primary human mammary epithelial cells (18) and mouse mammary tumors (19). Accordingly, we found that MCF-10A cells transduced with a NICD retrovirus undergo a dramatic cell-detachment phenotype when replated shortly after infection and display cell-in-cell structures both in suspended and attached cells (Fig. 2A). In addition, NICD-infected cells also display reduced adhesion to

**Fig. 1.** NICD induces heterogeneous phenotypes in 3D cultures of mammary epithelial cells. (A) Control (pBABE) or NICD-overexpressing (NICD) MCF-10A cells were cultured in Matrigel for 2 weeks. The phase-contrast images show representative 3D structures. (Scale bars, 100 μm.) Inserts show the absence of 3D structures from cells cultured in absence of EGF. (Bottom panels) The fluorescent confocal microscopy images show staining for ß-catenin (red), K6/6 (green), and DAPI (blue). The arrows indicate representative abortive 3D structures. (Scale bar, 100 μm.) (B) (Top) Data represent the mean number of counted acinar 3D structures. Error bars show ± SD of three replicate samples from one representative experiment. Two fields were counted together per sample. *, P < 0.001 relative to control. (Middle) The mean acinar structure area data are represented as pixel² and were obtained using ImageJ software. Error bars show ± SD of three replicate samples from one representative experiment. At least five fields were counted for sample. *, P < 0.01 relative to control. (Bottom) Ratio of the number of acinar structures or abortive structures relative to the total number of 3D structures.

**Fig. 2.** NICD induces cell-to-matrix detachment. (A) Evidence of cell detachment and cell-in-cell structures in MCF-10A cells overexpressing NICD. (Upper) Representative phase-contrast images of MCF-10A cells stably infected with control (pBABE) or NICD-encoding retroviral vectors 48 h after replating. These cells also were seeded on day 0 in triplicate at 10 x 10⁴ per well in six-well plates in growth medium. Attached cells then were trypsinized and counted with a hemacytometer on days 3 and 5. Error bars show ± SD of three replicate samples from one representative experiment. *, P < 0.001 relative to control. (Lower) The confocal image shows representative cell-in-cell structures induced by NICD in suspension and stained for ß-catenin (red) and DAPI (blue). The phase-contrast image shows a representative cell-in-cell structure induced by NICD in attached cell monolayers. The percentage of similar structures also was determined by manual counting with a 100x objective. Error bars show ± SD of three replicate samples from one representative experiment. At least five fields were counted for sample. *, P < 0.005 relative to control. (Scale bars, 20 μm.) (B) Quantitation of the effect of NICD overexpression on cell adhesion to extracellular matrix. Cells infected with the indicated viral vectors were plated on dishes coated with mouse laminin-1 or basement membrane components for 24 h, and adherent cells were quantified following 1-h incubation with calcine acetoxymethyl ester at 37 °C. Fluorescence was measured at 485–520 nm. Values represent the mean ± SD of three replicate samples from one representative experiment. *, P < 0.001 relative to control. (C) Down-regulation of matrix-to-cell adhesion proteins by NICD. Lysates derived from MCF-10A cells infected with pBABE or NICD retroviruses were analyzed by Western blotting with the indicated antibodies. (D) The heat map shows down-regulation of integrins and ECM components by NICD at the transcriptional level. Gene-expression profiling of MCF-10A cells infected with pBABE (control) or NICD retroviruses was performed in triplicate. These data were analyzed as described in SI Material and Methods. (E) MCF-10A cells infected with the indicated retroviral vectors were analyzed by Western blotting with the indicated antibodies. (F) The same cell lines as in E were plated on dishes coated with mouse laminin-1 or basement membrane components for 1 h; then adherent cells were quantified as described above. Values represent the mean ± SD of three replicate samples from one representative experiment. *, P < 0.001 relative to control. (G) Comparative analysis of microarray data generated from analysis of control and NICD-expressing MCF-10A cells or from control and p63–down-regulated MCF-10A cells (20).
exogenous matrix proteins (e.g., mouse laminin-1 or reconstituted basement membrane components) (Fig. 2B). The cell-to-matrix detachment triggered by NICD overexpression is associated with a reduction in the levels of expression of integrin β1 (ITGB1) and integrin β4 (ITGB4) as well as the basement membrane component laminin γ2 (LAMC2), a subunit of laminin 332 (also referred to as “laminin 5”) (Fig. 2C). In addition, we found that mRNAs encoding multiple integrins and matrix proteins expressed in MCF-10A cells are significantly [false-discovery rate (FDR)-adjusted P < 0.01] down-regulated in cells expressing NICD (Fig. 2D and Dataset S1).

Of note, we also detected a marked reduction in expression levels of EGF receptor (EGFR) (Fig. 2C), an effect we previously reported as accompanying loss of integrin engagement in MCF-10A and other epithelial cells (20). Taken together, NICD-induced loss of matrix-to-cell adhesion, formation of cell-in-cell structures, and reduction in EGFR could contribute to suppress the outgrowth of acinar structures in 3D cultures of mammary epithelial cells.

**Inverse Regulation of Matrix Adhesion by Notch and p63.** The matrix-detachment phenotype induced by NICD strongly resembles the phenotype induced by p63 down-regulation that we reported previously (20). Notch has been shown to down-regulate p63 in keratinocytes (21, 22), raising the possibility that Notch may induce cell detachment in mammary epithelial cells through down-regulation of p63. We found that NICD expression induced down-regulation of p63 mRNA (fold change = −2.18; FDR-adjusted P = 1.85 × 10^−7; Dataset S1). Furthermore, NICD also suppresses p63 protein expression, correlating with ITGB4 and LAMC2 down-regulation (Fig. 2E). Conversely, overexpression of ΔNp63α, the predominant isoform expressed in MCF-10A and other epithelial cells, induces expression of ITGB4 and LAMC2 and increases matrix adhesion (Fig. 2E and F).

Because NICD overexpression in MCF-10A cells caused a loss of cell adhesion similar to that induced by down-regulation of p63 with an shRNA targeting the DNA binding domain of all six p63 isoforms (20), we looked for genes that were similarly regulated by NICD overexpression and p63 down-regulation in our microarray studies. The extent of overlap (232 genes) was significantly greater than expected by chance (P = 0.0034, Fisher’s exact test) (Fig. 2G and Dataset S2). Fifteen of these genes (including integrins ITGB2, ITGB4, ITGAV, ITGA10, ITGB1, and ITGB4 and laminins LAMC3 and LAMC2; Dataset S2) have been implicated in cell–matrix interactions based on GeneGO analysis. This value represents a highly significant enrichment in the Cell adhesion_Cell-matrix interaction GeneGO category (15 of 232 versus 166 of 13,106; P = 7.150−5). Thirty-six of the genes similarly regulated by NICD overexpression and p63 down-regulation showed the opposite effect in the microarrays of cells overexpressing ΔNp63α (20), further supporting an inverse relationship between genes regulated by NICD and ΔNp63α (Fig. S1 and Dataset S3). Seven of these genes have been implicated in cell–matrix adhesion, and eight are cytoskeletal proteins. Taken together, our data indicate that cell-to-matrix adhesion is inversely regulated by ΔNp63α and NICD and that ΔNp63α down-regulation is likely to contribute, at least in part, to NICD-induced matrix-to-cell detachment.

**NICD-Induced Hyperproliferative Response Requires STAT3 Activation.** To identify down-stream regulators of Notch pathway activation in mammary epithelial cells, we performed reverse-phase protein array (RPPA) analysis to probe the expression or phosphorylation state of 46 proteins, many of which are altered in oncogene- and growth factor-activated cells (23). Protein fractions were isolated from MCF-10A cells 48 h postinfection with pBABE or NICD retroviral vectors. Phosphorylation of T180/Y182 of p38 increased 2.18-fold, total Src levels increased 2.4-fold, and there also was a 2.4-fold increase in the level of cyclin D1. Of note, there was no increase in phosphorylation of proteins in either the PI3K-AKT or ERK pathway. Surprisingly, we found that the most prominent alteration in NICD-expressing cells was a more than 13-fold increase in phosphorylation (Tyr705) of STAT3 (Fig. 3A). This last finding was verified by Western blot analysis (Fig. 3 B and C).

To address whether JAK kinases are required for STAT3 phosphorylation or any NICD-induced phenotypes, we treated NICD-overexpressing cells with the pan-JAK inhibitor P6. P6 has been shown to have low toxicity in MCF-10A cells and to inhibit JAK/STAT3 signaling with higher sensitivity and specificity than the tyrophostin AG490 (24). P6 inhibited STAT3 phosphorylation as well as the outgrowth of NICD-induced hyperproliferative structures in reconstituted basement membrane gels (Fig. 3 B and D). Similar results were obtained by down-regulation of STAT3 using a previously characterized lentiviral vector, S3SH, encoding an shRNA targeting STAT3 (24) (Fig. 3 C–E). Interestingly, the inhibition of STAT3 phosphorylation by P6 or STAT3 shRNA down-regulation did not affect NICD-induced down-regulation of p63 and matrix...

![Fig. 3. NICD-induced hyperproliferative response requires STAT3 activation.](image-url)
adhesion proteins (Fig. 3 B and C). These data provide evidence that the JAK/STAT3 pathway is required for the NICD-induced outgrowth of acinar structures in 3D cultures but is not involved with the NICD-dependent effects on matrix-adhesion genes.

Dose-Dependent Regulation of the Heterogeneous Phenotypes Induced by Notch Pathway Activation. The heterogeneous phenotypic responses to NICD overexpression in 3D cultures could reflect quantitative differences in Notch pathway activation levels. To address this possibility, we used a bicistronic vector that coexpresses GFP and NICD to assess the level of NICD expression in the different types of NICD-induced 3D structures. Prior studies using this vector have shown that GFP expression correlates with levels of NICD expression (25). We found striking differences in the levels of GFP expression in the two different types of 3D structures induced by NICD (Fig. 4). In particular, the abortive structures contained significantly more intense GFP fluorescence than the large hyperproliferative structures, thus suggesting an inverse correlation between the level of NICD expression and the extent of clonal outgrowth. Quite notably, the abortive structures failed to show detectable levels by immunostaining for two matrix-adhesion proteins (ITGB1 and LAMC2) expressed in 3D cultures of MCF-10A cells, suggesting that high levels of NICD suppress the expression of these matrix-adhesion proteins (white arrows in Fig. 4). Consistent with this suggestion, the large hyperproliferative structures retained ITGB1 or LAMC2 expression (Fig. 4). These results indicate that high-dose NICD is associated with loss of matrix-adhesion proteins, formation of abortive structures, and limited outgrowth, whereas low-dose NICD is associated with maintenance of matrix proteins and hyperproliferation of 3D structures.

To address dose-dependent effects further, we induced Notch pathway activation at lower levels in the whole cell population using a weak gain-of-function variant of Notch-1, L1601P+ΔP, expressed from the same bicistronic internal ribosome entry site (IRES)-GFP vector described above. This oncogenic variant, originally found in human T-cell acute lymphoblastic leukemia, elicits a ligand-independent activation of the Notch receptor that is weaker than the activity associated with the expression of NICD (26). We corroborated this activity in 293T cells by examining a Notch-dependent luciferase reporter containing the transcription factor CSL binding site: L1601P+ΔP expressed 3-fold lower luciferase activity than NICD and about 2-fold higher luciferase activity than wild-type Notch-1 (Fig. S2A). In addition, we analyzed the gene-expression profiles induced 48 h after infection of MCF-10A cells with retroviral vectors expressing full-length Notch-1, L1601P+ΔP, or NICD (Fig. 5A). Although expression of each Notch-1 receptor variant was similar in all transduced cell populations (see NOTCH1 in Fig. 5A), we detected a dose-dependent induction of several Notch target genes (e.g., HEY1, HEY2) (Fig. 5A) as well as regulation of other relevant genes (e.g., integrins, ECM components, and STAT3 targets; Fig. S3). Full-length Notch-1 induced the weakest effect, with only four genes regulated (PLA2G2A, OLFM4, and NOTCH1 up-regulated; RAP1A down-regulated) at levels 1.5-fold or greater than control cells (FDR-adjusted P < 0.05). L1601P+ΔP induced an intermediate effect with 192 differentially expressed genes (147 up-regulated, 45 down-regulated) (Dataset S4), and NICD caused transcriptional changes in 1,790 genes (795 up-regulated, 995 down-regulated) (Dataset S1). Importantly, except for one gene, all genes induced by Notch-1 and L1601P+ΔP also were altered significantly by NICD (FDR-adjusted P < 0.05,
>1.35-fold), indicating that there are no unique gene programs induced by full-length or L1601P+ΔP Notch variants that are not also induced by NICD. Fig. S3 gives a more complete analysis of these array data.

To compare the phenotypic alterations induced by the Notch-1 receptor variants, MCF-10A cells expressing wild-type Notch-1, L1601P+ΔP, and NICD were sorted by FACS, and cell populations expressing similar levels of GFP (see 2D panels in Fig. 5C) were isolated and analyzed for expression of relevant proteins, loss of adhesion, acinar formation, and growth in soft agar. Compared with NICD, L1601P+ΔP expression did not suppress endogenous p63, ITGB1, or LAMC2 significantly and induced a lower level of phospho-STAT3 (Fig. 5B). Unlike NICD, L1601P+ΔP expression did not cause cell detachment (see 2D panels in Fig. 5C), indicating that low-dose Notch activity is not sufficient to suppress matrix adhesion. Moreover, although there was a dose-dependent decrease in the proportion of acinar structures (see 3D panels in Fig. 5 C and D) as well as a dose-dependent increase in the proportion of growth-inhibited abortive structures induced by the three Notch-1 variants (Fig. 5D), the number of hyperproliferative structures did not correlate with the dose of Notch activity. In fact, overexpression of full-length Notch-1 caused only a small number of hyperproliferative 3D structures; however, quite remarkably, L1601P+ΔP induced a dramatic increase in such structures, 6-fold greater than the high-activity NICD variant (Fig. 5E). In addition, L1601P+ΔP showed a 10-fold greater activity than NICD in a more traditional transformation assay of colony formation in soft agar (Fig. S2B). These results further support the idea that lower levels of Notch pathway activation sustain matrix-to-cell adhesion and induce outgrowth of hyperproliferative structures and that high-dose Notch activity suppresses matrix adhesion and outgrowth.

To explore more specifically the dose-dependent effects of Notch on cell proliferation per se, we examined cell-cycle progression using time-lapse video microscopy of MCF-10A cells expressing the above-described Notch variants that induce different levels of pathway activation. Cell-cycle progression was monitored using a red fluorescent mCherry fusion protein of geminin, which is absent during the G1 phase and accumulates through the S, G2, and M phases of the cell cycle (27). Geminin levels drop at the metaphase–anaphase transition of mitosis when it is degraded by the anaphase-promoting complex. MCF-10A cells stably expressing mCherry-geminin were monitored for S phase reentry by time-lapse video microscopy from 24 to 72 h after infection with retroviral vectors expressing full-length Notch-1, L1601P+ΔP, or NICD (Video S1, Video S2, Video S3, and Video S4). Activation of Notch pathway at high dose by NICD suppressed reentry of cells into S phase (32% reentry relative to control), whereas expression of full-length Notch-1 or L1601P+ΔP had a more moderate effect (71% and 88% reentry, respectively, relative to control).

Taken together, our data indicate that high-dose NICD can affect cell growth directly and suggest that this inhibitory effect, in addition to loss of matrix adhesion, contributes to the suppression of clonal outgrowth induced by increased Notch activity in 3D cultures of MCF-10A cells (Fig. 5F).

Discussion
Our data show that constitutive activation of the Notch pathway induces distinct dose-dependent phenotypic responses. In particular, high levels of Notch pathway activation result in suppression of cell proliferation and clonal outgrowth, down-regulation of multiple matrix-adhesion molecules, dramatic loss of matrix adhesion, and formation of cell-in-cell structures, which we previously demonstrated is triggered by loss of ECM attachment to integrin receptors (16, 17). In contrast, lower levels of Notch-1 activity maintain matrix adhesion and induce a proliferative response associated with the outgrowth of acinar structures in 3D cultures and colonies in soft agar. Here, we propose a model to explain the dose-dependent heterogeneous responses to Notch pathway activation (Fig. 5F). At low doses, Notch-induced proliferative signals would dominate, and integrins expression would be retained; at higher doses, the death- and growth arrest/differentiation programs would dominate, down-regulating expression of p63, integrins, and matrix proteins, inhibiting outgrowth, and promoting luminal differentiation.

Our studies also highlight a major role for two pathways in the Notch-induced hyperproliferative response: the EGFR and JAK-STAT3 pathways. This idea is based on evidence that EGF-depleted medium failed to support proliferation of NICD-expressing MCF-10A cells and that inhibition of either JAK or STAT3 prevented outgrowth. The basis for the suppression of cell proliferation and clonal outgrowth by high-dose Notch activation probably is mediated by multiple effectors. (i) Loss of matrix adhesion could influence cell proliferation significantly, because epithelial cells require matrix adhesion for effective transduction of signals from growth factor receptor stimulation (28). (ii) The formation of cell-in-cell structures (through entosis) induced by loss of adhesion also would limit clonal outgrowth because internalized cells typically undergo lysosomal destruction (16, 17). (iii) The dramatic down-regulation of EGFR protein and mRNA expression by NICD also would limit the proliferative capacity of cells (Fig. 2C and Dataset S1). We have shown previously that EGFR is down-regulated dramatically following detachment from matrix (29); thus, loss of EGFR could be a secondary consequence of lack of integrin engagement. However, down-regulation of p63 by high-dose NICD also could contribute to EGFR repression, because loss of p63 has been shown to reduce EGFR mRNA expression (20). (iv) Multiple EGFR ligands (AREG, AREGB, HB-EGF; Dataset S1) also are down-regulated by NICD. (v) There is a dose-dependent induction of the cyclin CDK inhibitor p21, which previously was shown to be required for NICD-induced growth arrest in different types of epithelial cells (30,31) (Fig. S4A). Of note, we found that over-expression of NICD in p21−/− MCF-10A cells did not significantly rescue the suppression of clonal outgrowth in the 3D model employed here (Fig. S4B). Given the broad range of NICD-induced gene alterations affecting both cell proliferation and matrix adhesion (which is known to be required for cell proliferation in many contexts), it is likely that reversing the effects of NICD on any one gene will not alone rescue the suppression of clonal outgrowth.

Interestingly, high-dose NICD did not prevent activation of the JAK-STAT pathway. STAT3 phosphorylation correlated positively with Notch activation levels, showing increased phosphorylation at elevated levels of Notch pathway activation (Fig. 5F), as expected. NICD-mediated up-regulation of p63 in NICD-expressing cells was constitutively activated in more than 50% of primary breast tumors and tumor-derived cell lines. Recently, it has been shown that direct protein–protein interactions can coordinate cross-talk between the Notch-Hes and JAK-STAT pathways (32). Our data indicate that the JAK-STAT pathway is required for outgrowth of hyperproliferative structures induced by NICD but is not involved with the NICD-dependent effects on matrix adhesion (Fig. 3). Thus, the death-destruction program is independent of the JAK-STAT3 pathway, and the outgrowth failure at high-dose NICD does not involve suppression of STAT3 activation.

Recently, Notch-1 also was shown to promote commitment of mouse mammary stem cells (MaSCs) exclusively along the luminal lineage (33), and Notch-3 was shown to be required for differentiation of bipotent progenitor cells into luminal progenitors (34). Differentiation into luminal cells is associated with loss of p63 and reduction in expression of basal integrins and matrix proteins. Thus, the p63 down-regulation induced at high dose in NICD-expressing MCF-10A cells also would be consistent with a Notch-dependent luminal differentiation program. Interestingly, our analysis of NICD-regulated genes in MaSC-enriched, luminal progenitor, and mature luminal cell gene sets (35) suggests that Notch suppression of p63 may be responsible for the down-regulation of multiple integrins and matrix proteins in luminal progenitor cells, because these cells are distinguished from MaSCs by low levels of p63 and NICD-regulated matrix-adhesion proteins (Fig. S5). High-dose NICD also could collaborate with other differentiation factors to suppress pro-
...liferation during terminal differentiation in vivo. It is possible that Notch limits expansion of MaSCs, because knockdown of the canonical Notch effector cerebromedial binding protein 1 (CBF-1) in the MaSC-enriched population was reported to elevate stem cell activity in vivo as well as the formation of aberrant end buds in mice; and inhibition of Notch signaling using a gamma-secretase inhibitor increased the size of MaSC-enriched colonies in vitro significantly (31). In another study, basal cell proliferation was elevated during pregnancy in mice with targeted CBF-1 disruption (36). These results suggest a role for endogenous Notch signaling in restricting MaSC expansion. Although Notch-1 may not be the active Notch receptor in these cells, NICD overexpression may have promiscuous activities on other Notch family targets.

In summary, our data have revealed mechanisms whereby phenotypic responses to Notch are influenced by dose-dependent effects on cell adhesion and growth in mammary epithelial cells. Dose-dependent effects of Notch have been observed in other contexts as well. Studies in *Drosophila melanogaster* have revealed that cells expressing different levels of Notch can regulate distinct cell-cycle mediators, suggesting that a dose-dependent mechanism could contribute to growth regulation in this organism (37). In addition, in cervical cells, high-dose NICD suppresses expression of the human papilloma virus oncogene E6 and E7, whereas at moderate doses NICD collaborates with E6 and E7 to transform primary cells (38). Notch control of both proliferation and differentiation also resembles the dichotomous dose-dependent activities of Myc on these two phenotypes in epidermal cells (39). It is likely that the level and duration of Notch activation within the mammary gland differentially affects mammary cell growth and adhesion. In addition, our data also suggest that during the evolution of epithelial tumors triggered by Notch pathway activation, there would be a selection for cells expressing lower levels of Notch pathway activation because these cells would have the greatest proliferative potential. It is clearly of great interest to determine which factors affect the level of Notch pathway function and to explore further the basis for dose-dependent effects during distinct phases of development and tumorigenesis.

**Materials and Methods**

Details of the procedures and microarray analysis are described in *S*Text. Assays were conducted with three replicate samples in at least two independent experiments. Statistical analyses were performed using unpaired Student’s *t* test. Raw and processed microarray data can be accessed at the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo; GSE20285 and GSE20286).

**ACKNOWLEDGMENTS.** We thank Qiang Sun for providing an image of NICD-induced invading cells, Christopher Winter, Heike Keilhack, Pamela Carroll, and Stephan Kraus for helpful comments on the project, Jackie Bromberg (Memorial Sloan-Kettering Cancer Center, New York) for generously providing the Stats3 shRNA vector, and & Perrin Lloyd for helpful comments on the manuscript and members of the Brugge laboratory for fruitful discussions. In addition we are grateful for a gift from Lee Jeans through the Entertainment Industry Foundation (J.S.B.). This work also was supported by Grants R01 CA098402 and R37 NS26084 from the Leukemia and Lymphoma Society (to A.T.S.-T.); Grants P01 CA119070 to (I.C.A.), P01 CA099031 (to G.B.M.), and P01 CA105134 (to J.S.B.) from the National Cancer Institute; Grant P30CA16672 from Cancer Center Support Grant to (G.B.M.); and a grant from Merck Research Laboratories Boston (to J.S.B.). M.M. was a recipient of the L. Fontana and M. Lionello fellowship from the Associazione Italiana per la Ricerca sul Cancro. J.A. is supported by a multidisciplinary postdoctoral fellowship from the U.S. Army Breast Cancer Research Program.

---

Supporting Information

Mazzone et al. 10.1073/pnas.1000896107

SI Materials and Methods

Cell Culture, Vectors, and Virus Production. Cells from a human mammary epithelial cell line (MCF-10A) were cultured as described previously (1). The pBABE-based retroviral vectors encoding the Notch-1 intracellular domain (NICD), p63 isoform (ΔNp63α), as well as the murine stem cell virus (MSCV)-internal ribosome entry site (IRES)-GFP–based retroviral vectors encoding the Notch-1 variants (Notch-1, L1601P+ΔP, NICD) have been described previously (2–4). Vascular stomatitis virus G-pseudotyped retroviral vectors were generated and employed to infect MCF-10A cell lines according to standard procedures (5). S3SH, the shRNA-lentivirus targeting STAT3, has been characterized previously (6) and was generated according to a standard protocol.

3D Acinar Morphogenesis Assays. Cells were cultured in growth factor-reduced reconstituted basement membrane (Matrigel; BD Biosciences) and processed, where indicated, for indirect immunofluorescence/confocal microscopy as described previously (3).

Microarray and Statistical Analysis. Total RNA was isolated 48 h after retroviral infection and subjected to reverse transcription, labeling, and hybridization to hgu133plus2 gene chip arrays (Affymetrix). The experiment was performed in triplicate. Background correction, normalization, and differential expression were assessed using the RMA algorithm and Limma packages in BioConductor software (www.bioconductor.org). The criteria for selecting differentially expressed genes were false discovery rate (FDR)-corrected P < 0.05 and a fold change > 1.5. Gene-expression heat maps were generated with the Java TreeView program 1.1.1 (Sun Microsystems). To identify the overlap between Notch-induced transcriptional changes and previously published p63 expression profiles (6), biological enrichment analysis was performed using the RMA categories in the NICD-specific signature versus 60% for the NICD-specific signature. This difference suggests that transcriptional repression is dominant over transcriptional activation at high-dose Notch activation. In addition, GeneGO Pathway Map analysis revealed that the two gene-sets were enriched in different classes of genes (Fig. S3A). Consistent with the observed phenotypic responses, the most significantly enriched categories in the NICD-specific signature were cell adhesion-
related, highlighting the strong influence of NICD on cell-to-matrix adhesion. Most notably, the integrins and ECM components down-regulated by NICD (Fig. 2D) were not significantly affected by L1601PΔP, further supporting a dose-dependent down-regulation of matrix-adhesion genes induced by Notch activation (Fig. S3B). In contrast, the most significantly enriched class for the L1601PΔP and NICD signature was the developmental Notch signaling pathway GeneGO category (P = 1.8 × 10^{-5}) (Fig. S4). In addition, we also found that 15 of the 33 cancer-related STAT3 target genes, recently reviewed in ref. 10, are significantly regulated by NICD (FDR-adjusted, P < 0.05) (Fig. S3C). Of these genes, CCND1, BCL2L1, and IL1B also are statistically altered (FDR-adjusted P < 0.05) by L1601PΔP. None of these STAT3 target genes are regulated by the full-length Notch-1. Analysis of the gene-expression profiles of the Notch variants did not reveal any obvious explanation for the high-dose suppression of cell proliferation observed in 3D cultures of MCF-10A cells, because we detected an increase in expression of genes that both positively and negatively regulate proliferation in NICD-expressing samples compared with L1601PΔP (Dataset S4 and Dataset S5).

Cell Cycle Progression Analysis Based on Time-Lapse Video Microscopy. A red fluorescent live-cell S-phase reporter was constructed by PCR amplification of bases 1–330 of human geminin from pCDNA3-mAG-hGeminin (a kind gift from Atsushi Miyawaki, Brain Science Institute, Hirosawa, Japan) (11) and was cloned in frame at the 3’ end mCherry in pMSCV-puro. The red fluorescent protein (RFP)-geminin cassette then was cloned downstream of nuclear localization sequence (NLS)-cyan fluorescent protein (CFP) (a marker for cell tracking) and IRES in a pMSCV-based retroviral vector lacking a selection marker. MCF-10A cells stably expressing the NLS-CFP/RFP-geminin combination were plated in 96-well plates with optically clear bottoms (Corning #3603). Cells were infected for 24 h with the indicated viruses. The medium then was replaced with microscopy-optimized growth medium (identical to normal MCF-10A growth medium but containing 2% horse serum and no phenol red), and time-lapse fluorescence imaging was conducted at 20-min intervals for the following 48 h using a 10x objective on a Nikon TE2000E microscope equipped with an environmental control chamber. Proliferation was scored by counting the percentage of cells undergoing division in the interval between 48 and 60 h postinfection (to allow sufficient expression of the transduced proteins) that reentered S-phase (as indicated by induction of the RFP-geminin reporter) within 12 h after division.


Fig. S1. Genes that show the opposite effect in NICD and ΔNp63α-overexpressing cells. Thirty-six genes similarly regulated by NICD and shRNA-p63 show the opposite effect in ΔNp63α-overexpressing cells. Black arrows indicate the cell adhesion-related genes. Blue arrows indicate cytoskeletal proteins.
Fig. S2. Additional analysis of the Notch-1 receptor variants. (A) 293T cells were transfected in triplicate with a control MSCV-based plasmid or plasmids encoding various forms of human Notch-1 (10 ng/well), the pGL2-CSL(4×)-luciferase reporter plasmid (250 ng/well) and a Renilla luciferase internal control plasmid (5 ng/well). Firefly luciferase activity was normalized to Renilla luciferase activity in cell lysates prepared 48 h after transfection. (B) Cells infected with the retroviral vectors encoding the indicated Notch variants were plated in soft agar and grown for 3 weeks. Data were obtained as the mean number of colonies per six-well culture of 100,000 cells. Error bars show ± SD of three replicate samples from one representative experiment.
Fig. S3. Dose-dependent induction of distinct gene-expression programs regulated by Notch pathway activation. (A) GeneGO functional enrichment analysis of the NICD-specific (black) and L1601P+ΔP and NICD (gray) gene signatures. The dashed line designates the threshold for statistical significance (P = 0.05). The enrichment score is defined as $-\log(P$ value). Consistent with the observed phenotypic responses, the most significantly enriched categories in the NICD-specific gene signature were cell adhesion-related, highlighting the strong influence of NICD on cell-to-matrix adhesion. Of note, the GeneGO category N-acylethanolamines, phospholipase A2 pathway also is enriched significantly; however, this pathway may not be relevant to dose-dependent phenotypes because PLA2G2A is constitutively induced by full-length Notch-1, L1601P+ΔP, and NICD. (B) The heat map shows the average fold-change relative to pBABE controls of a set of integrins and ECM proteins expressed in MCF-10A cells (see also Fig. 2D). ITGB4 also is significantly regulated by L1601P+ΔP (FDR-adjusted, P < 0.05). None of these integrins and ECM proteins is significantly affected by full-length Notch-1. (C) The heat map shows that 15 of the 33 cancer-related STAT3 target genes, recently reviewed in ref. 1, are significantly regulated by NICD (FDR-adjusted, P < 0.05). Of these genes, CCND1, BCL2L1, and IL1B are also statistically significant in the L160P+ΔP and NICD data set (FDR-adjusted, P < 0.05). None is regulated by the full-length Notch-1 data set.

Fig. S4. (A) Dose-dependent induction of the CDK inhibitor p21 in MCF-10A cells by NICD. The Western blots shown in Fig. 5B were analyzed for expression of p21. (B) Control and p21−knock-out (p21KO) MCF-10A cells were infected with pBABE or NICD retrovirus and cultured in Matrigel for 2 weeks. The phase-contrast images show representative 3D structures. Of note, the knock-out of p21 did not rescue the suppression of clonal outgrowth induced by NICD in 3D. The same cell lines also were analyzed by Western blots with the indicated antibodies. The p21KO cells were the kind gift of Ben Ho Park (The Johns Hopkins University, Baltimore, MD) and were previously characterized (1).


Fig. S5. Expression of NICD-regulated integrin and ECM components in normal human mammary subpopulations. Gene-expression profiles of normal human mammary cell subpopulations were derived from fluorescence-activated cell sorting of cell suspensions from reduction mammoplasties, as recently published (1). Subpopulations were defined as mammary stem cell (MaSC)-enriched (CD49fhi, EpCAM−), luminal progenitor (CD49f+, EpCAM+), and mature luminal (CD49f−, EpCAM+). Data downloaded from the Gene Expression Omnibus (GSE16997, http://www.ncbi.nlm.nih.gov/geo/) were normalized, background-corrected with offset 16, quantile-normalized, log2-transformed, and median centered using BioConductor software.

Video S1. Time-lapse imaging of MCF-10A cells expressing mCherry-geminin following infection with control-GFP. MCF-10A cells were engineered to express NLS-tagged CFP and mCherry-geminin stably. Cells were infected with control IRES-GFP virus. Beginning 1 day after infection, cells were imaged at 20-min intervals for 48 h. The NLS-CFP signal was processed in ImageJ using an edge-detection filter to create a marker of the nuclear perimeter (blue). The mCherry-geminin signal is shown in red, and the GFP signal in green.

Video S2. Time-lapse imaging of MCF-10A cells expressing mCherry-geminin following infection with NICD. This movie was prepared identically to Video S1 but using NICD-IRES-GFP virus.

Video S3. Time-lapse imaging of MCF-10A cells expressing mCherry-geminin following infection with Notch-1. This movie was prepared identically to Video S1 but using Notch-1-IRES-GFP virus.
Video S4. Time-lapse imaging of MCF-10A cells expressing mCherry-geminin following infection with L1601P+ΔP. This movie was prepared identically to Video S1 but using L1601P+ΔP-IRES-GFP virus.

Video S4.

Other Supporting Information Files

Dataset S1 (XLS)
Dataset S2 (XLS)
Dataset S3 (XLS)
Dataset S4 (XLS)
Dataset S5 (XLS)