JOURNAL OF VIROLOGY, June 2005, p. 7889–7898 Vol. 79, No. 12
0022-538X/05/$08.00+0 doi:10.1128/JVI.79.12.7889–7898.2005

Complementation of Human Papillomavirus Type 16 E6 and E7 by Jagged1-Specific Notch1-Phosphatidylinositol 3-Kinase Signaling Involves Pleiotropic Oncogenic Functions Independent of CBF1;Su(H);Lag-1 Activation†

Karthikeyan Veeraraghavalu, Vanitha K. Subbaiah, Sweta Srivastava, Oishee Chakrabarti, Ruchi Syal, and Sudhir Krishna*

National Centre for Biological Sciences, TIFR, UAS-GKVK Campus, Bangalore 560065, India

Received 14 September 2004/Accepted 14 February 2005

We have analyzed the induction and role of phosphatidylinositol 3-kinase (PI3K) by Notch signaling in human papillomavirus (HPV)-derived cancers. Jagged1, in contrast to Delta1, is preferentially upregulated in human cervical tumors. Jagged1 and not Delta1 expression sustained in vivo tumors by HPV16 oncogenes in HaCaT cells. Further, Jagged1 expression correlates with the rapid induction of PI3K-mediated epithelial-mesenchymal transition in both HaCaT cells and a human cervical tumor-derived cell line, suggestive of Delta1;Serrate/Jagged;Lag2 ligand-specific roles. Microarray analysis and dominant-negatives reveal that Notch-PI3K oncogenic functions can be independent of CBF1;Su(H);Lag-1 activation and instead relies on Deltex1, an alternative Notch effector.

Human papillomavirus (HPV)-driven neoplasias exhibit features of DSL (Delta1;Serrate/Jagged;Lag2) ligand-mediated Notch activation (12, 41). Consistent with these observations, we have reported a functional role for the Notch ligand, Jagged1 (41). In contrast to earlier studies with DSL ligands, there is emerging evidence from diverse systems for distinct functions for members of the DSL ligand family (1, 16, 18). While there are clearly defined regions of expression and functions for DSL ligands in human epidermis (23, 30, 38), possible differences in their oncogenic role in the context of human neoplasia have not been analyzed.

In this study, we have compared the expression patterns and role of two key DSL ligands, Jagged1 and Delta1, in the progression of human cervical cancer, a papillomavirus-driven neoplasia (47). We have earlier shown that expression of activated forms of Notch1 receptor (AcN1) complement HPV-16 E6 and E7 and lead to transformation in vitro (29, 33) and tumor growth in vivo (8) in an immortalized human epithelial cell line, HaCaT (36). In this study, using similar assays (8), we have compared the tumorigenic potential of Jagged1 and Delta1 in HaCaT cells expressing HPV-16 E6 and E7. On detecting differences in the tumorigenic ability of these two ligands, we analyzed their ability to confer oncogenic properties like anoikis resistance and induction of epithelial-mesenchymal transition (EMT). We extended these observations to include an analysis of two HPV-16-positive invasive cervical tumor-derived cell lines, CaSki and SiHa, that show distinct differences in the expression pattern of Jagged1 and Delta1. Finally, we have investigated the nature of the Notch effector pathway downstream of Jagged1 that mediates pro-oncogenic functions.

There is a marked increase in the expression of Jagged1 in the progression of human high-grade cervical precursor lesions to invasive squamous cell carcinomas (SCC) (41). In contrast, in a major proportion of cervical intraepithelial neoplasia grade III (11 of 13) and invasive SCC cases (14 of 16), no detectable Delta1 transcripts were observed (Fig. 1). Correspondingly, immunohistochemistry revealed only a mild immunostaining for Delta1 protein in SCC (one of four) and CIN III (one of five) cases.

HaCaT cells endogenously express full-length Notch1 and Notch2 (6) and do not express detectable levels of Jagged1 or Delta1 (Fig. 2). Pooled stable transfectants of HaCaT cells expressing either Jagged1, Delta1 or mock-vector expressing neomycin resistance (Neo) was generated and expression levels were determined (Fig. 2A to F; supplemental Fig. 1A).

Expression of prototype E6 or an 83-amino-acid variant (L83V E6) prevalent in invasive cervical carcinoma (46) along with E7 and AcN1 in HaCaT cells resulted in a four- and ninefold increase in the number of colonies on soft agar, respectively (Table 1) (8). Analogous to AcN1 alone, expression of Jagged1 or Delta1 per se does not lead to any increase in colony formation in these soft agar assays (Table 1). Expression of E6 or L83V E6 along with E7 in HaCaT-Jagged1 cells generated a 3- and 7.5-fold increase, respectively, in colonies on soft agar. In contrast, HaCaT-Delta1 cells showed only a 1- and 1.8-fold increase in soft agar colonies on introduction of E7 and either E6 or L83 E6, respectively. In mammalian cell cultures, Manic Fringe has been shown to inhibit and enhance signaling by Jagged1 and Delta1, respectively (15). Consistent with our observations on the role of Manic Fringe in our recent study (41), expression of Manic Fringe inhibited the transforming potential in HaCaT-Jagged1 cells in soft agar assay (Table

* Corresponding author. Mailing address: National Centre for Biological Sciences, TIFR, UAS-GKVK Campus, Bangalore 560065, India. Phone: 011918012636421. Fax: 011918012636421. E-mail: skrishna@ncbs.res.in.

† Supplemental material for this article may be found at http://jvi.asm.org.

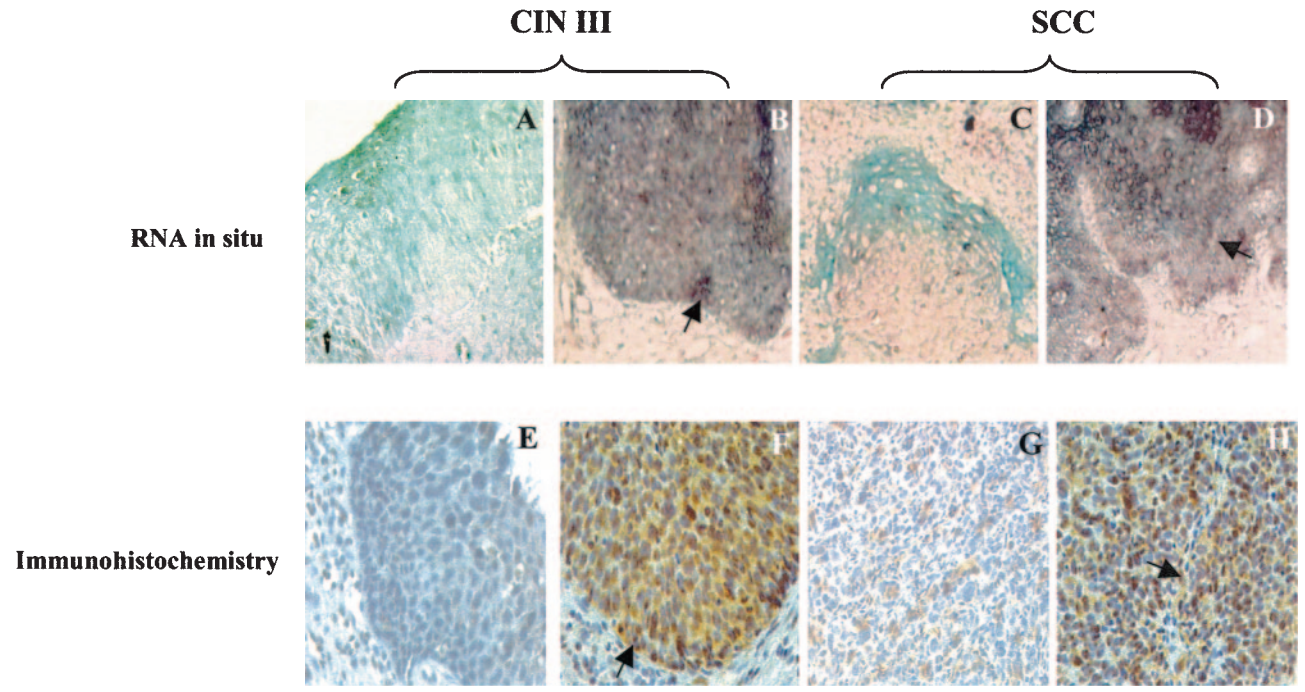

FIG. 1. Representative photomicrographs show expression of DSL ligands in CIN III and invasive squamous cell carcinoma of cervix (SCC) as determined by mRNA in situ hybridization (A to D) and immunohistochemistry (E to H); 11 out of 13 CIN III (A) and 14 out of 16 SCC (C) cases failed to show detectable Delta1 transcripts. (B) Detection of Delta1 transcripts in one of the CIN III cases. (D) Abundant expression of Jagged1 transcripts in SCC as reported earlier (41). Fluorescein isothiocyanate (FITC)-labeled mRNA probes and an alkaline phosphatase-conjugated anti-FITC antibody-based detection system were employed. Antisense staining is in purple (indicated by arrows) and the sections were counter stained with fast green. The FITC-labeled antisense and sense riboprobes were in vitro transcribed using SP6 or T7 primers from the cDNA templates encoding full-length Delta1 (pcDNA3-Delta1) and Jagged1 (pcDNA3-Jag1) (41). (E and G) Representative photomicrographs that show mild immunohistochemical staining of Delta1 (1:100; sc-9102; Santa Cruz Biotech) in CIN III and SCC cases, respectively. As a control for immunohistochemical staining, CIN III and SCC sections were stained for a proliferative marker protein, PCNA (Santa Cruz Biotech) (F and H, respectively). Arrows indicate areas of positive diaminobenzidine (DAB) staining. The counterstain is hematoxylin. Photomicrographs were taken under 40× magnification.

1). However, expression of Manic Fringe led to no increase in transformation in HaCaT-Delta1 cells (Table 1).

As in the in vitro assays (Table 1), expression of Jagged1 alone generates minimal tumors that are less than 10 mm^3 and do not show any features of progression (Fig. 2G). Consistent with the in vitro assays (Table 1), we observed that explants of HaCaT cells expressing Jagged1 along with E6 and E7 generated tumors greater than 90 mm^3 at 3 weeks (Fig. 2G) and were comparable to those generated by AcN1 in similar assays (8) in terms of volume (Fig. 2G) and morphology (data not shown). However, expression of E6 and E7 along with Delta1 generated lesions of less than 10 mm^3 (Fig. 2G). Coexpression of Manic Fringe inhibited Jagged1-mediated in vivo explant growth, generating tumors less than 30 mm^3 at 3 weeks. Nevertheless, Delta1 expressing cells showed no detectable change in the presence of Manic Fringe (Fig. 2G).

AcN1 has been shown to confer anoikis resistance in vitro (33) and promote growth of E6 and E7 expressing HaCaT cell explants in vivo through activation of phosphatidylinositol 3-kinase (PI3K)-protein kinase B (PKB)/Akt (8). We examined phosphorylated Akt (pAkt-Ser 473) levels as a measure of PI3K activation status in DSL-driven tumor explants. Tumors generated by Jagged1 along with E6 and E7 showed intense pAkt staining. However, expression of E6 and E7 alone or along with Delta1 failed to show any detectable pAkt. A representative photograph showing the pAkt levels is shown (supplemental Fig. 1B). These results suggest that the ability of Jagged1 to generate antiapoptotic signals through PI3K may account for the increased tumorogenic potential of the cells in vivo. Concomitantly, in in vitro assays, expression of Jagged1 and not Delta1 generated resistance to anoikis and induced phosphorylated forms of PKB/Akt (supplemental Fig. 2).

Recent studies by Zavadil and colleagues (45) revealed that Jagged1 expression is upregulated upon transforming growth factor (TGF)-β treatment in HaCaT cells and contributes to the induction of epithelial-to-mesenchymal transition (EMT), a key event that induces tumor cell motility and invasion. Further, overexpression of intracellular forms of Notch1 induces EMT accompanied by oncogenic transformation in immortalized endothelial cells (40). Activation of PI3 kinase-PKB/Akt signaling has been shown to promote EMT and invasion in several cell lines derived from carcinomas of breast and ovarian origin (5, 39). Characteristics associated with EMT include dissociation of cell-cell and cell-extracellular matrix contacts, acquisition of elongated cell morphology, rearrangement of the cytoskeleton, loss of epithelial markers (for example, E-cadherin and plakoglobin), expression of new intermediate filaments like vimentin and extracellular matrix proteins such as fibronectin that facilitate reduced intercellular adhesion and increased motility (39).

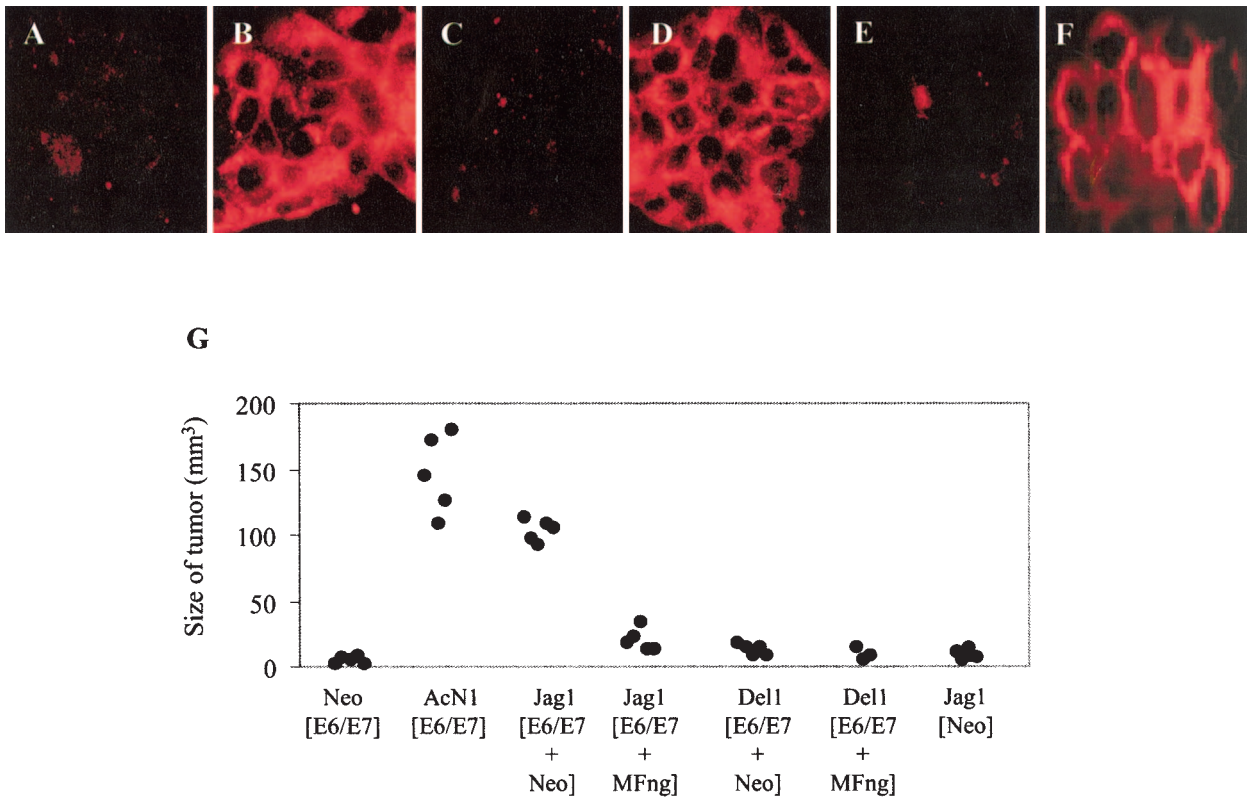

FIG. 2. HaCaT cells stably expressing Jagged1 and hemagglutinin (HA)-tagged Delta1 were stained using anti-Jagged1 (2.5 μg/ml, sc-6011; Santa Cruz Biotech) (B) and anti-Delta1 antibody (2.5 μg/ml, sc-9102; Santa Cruz Biotech) (D). Endogenous expression of Notch1 in HaCaT cells was detected using anti-Notch1 antibody (1:50, sc-6014; Santa Cruz Biotech) (F). A, C, and E are isotype control immunocytochemical staining. Photomicrographs were taken under 40× magnification. (G) HaCaT cells stably expressing mock vector (Neo), activated Notch1 (AcN1), Jagged1 (Jag1), or Delta1 were transiently transfected with the plasmid combinations (total of 10 μg) encoding either bicistronic HPV-16 E6 and E7 (E6/E7) {pMSIIref-E6/E7} (gift of M. Conrad-Stoppler and H. Stoppler), human Manic Fringe (MFng) (pcDNA3-MFng) or neomycin (Neo) (pcDNA3.0-Neo), and injected subcutaneously into nude mice. After 3 weeks, the mice were sacrificed, and tumor sizes were measured as described previously (8). The graph shows tumor volume after 3 weeks, and each dot represents a tumor from one mouse.

We assessed the motility of DSL-expressing HaCaT cells in an in vitro wound healing assay. In this assay, confluent monolayer of cells were "wounded" with a pipet tip, and the movement of the cells into the wounded area was monitored after 24 h (21, 26). Representative microphotographs of the wound-healing assay are shown in Fig. 3A. While HaCaT-Jagged1 exhibited 90% closure of the wounded nude area, HaCaT-Delta1 or control HaCaT-Neo covered only 20 to 30% of the nude region (Fig. 3C). Treatment with neutralizing α-Notch1 antibody targeted against the ligand-binding domain of Notch1 (11 to 12 EGF repeats), the pharmacological inhibitor of presenilin-dependent γ-secretase (GI) that blocks ligand-induced proteolytic processing of Notch1 (10), and the PI3K-specific chemical inhibitor LY294002 (LY) blocked the motility of HaCaT-Jagged1 cells in this assay (Fig. 3C). In addition, stable expression of the dominant negative soluble form of extracellular Jagged1 (sol hJag1) (25) or kinase dead dominant negative Akt (DN-Akt and HA-Akt K179 M) (33) also blocked the motility of HaCaT-Jagged1 cells in this assay (Fig. 3C). In these wound-healing assays, HaCaT cells expressing E6 and/or E7 failed to exhibit motility (Fig. 3C).

We then evaluated two human cervical tumor-derived cell

TABLE 1. Jagged1 cooperates with HPV-16 E6 or L83V E6 and E7 in transformation of HaCaT cells in soft agar colony formation assays[a]

Vector	*HaCaT-Neo*	*HaCaT-AcN1*	*HaCaT-Jagged1*		*HaCaT-Delta1*	
			Neo	MFng	Neo	MFng
Neo	4.6 ± 0.8	5 ± 0.5	5.3 ± 0.8	4 ± 0.5	4.3 ± 0.8	5 ± 0.5
E6 + E7	6 ± 0.5	**27.6 ± 0.8**	**18.3 ± 1.4**	***10 ± 1.5***	6.3 ± 0.8	5.6 ± 0.8
L83V E6 + E7	6 ± 0.57	**56.3 ± 3.7**	**45 ± 2.3**	***27.3 ± 2.3***	11.3 ± 0.88	13 ± 0.5

[a] The data represent mean ± standard error of the numbers of colonies generated by HaCaT cells expressing the mentioned combination of genes on soft agar assay in three independent experiments. The data were generated by microscopic counting of colonies in 20 random fields under a magnification of ×10 as described previously (33). HaCaT cells stably expressing mock vector (HaCaT Neo), activated Notch1 (HaCaT-AcN1), Jagged1 (HaCaT-Jagged1), and Delta1 (HaCaT-Delta1) are shown in boldface italic type, and transient infections of plasmids encoding E6, L83V E6 and E7, or mock neomycin resistance (Neo) alone or along with Manic Fringe (MFng) are shown in boldface roman type. Increases in colony numbers are shown in boldface roman type, and decreases in colony numbers are shown in boldface italic type.

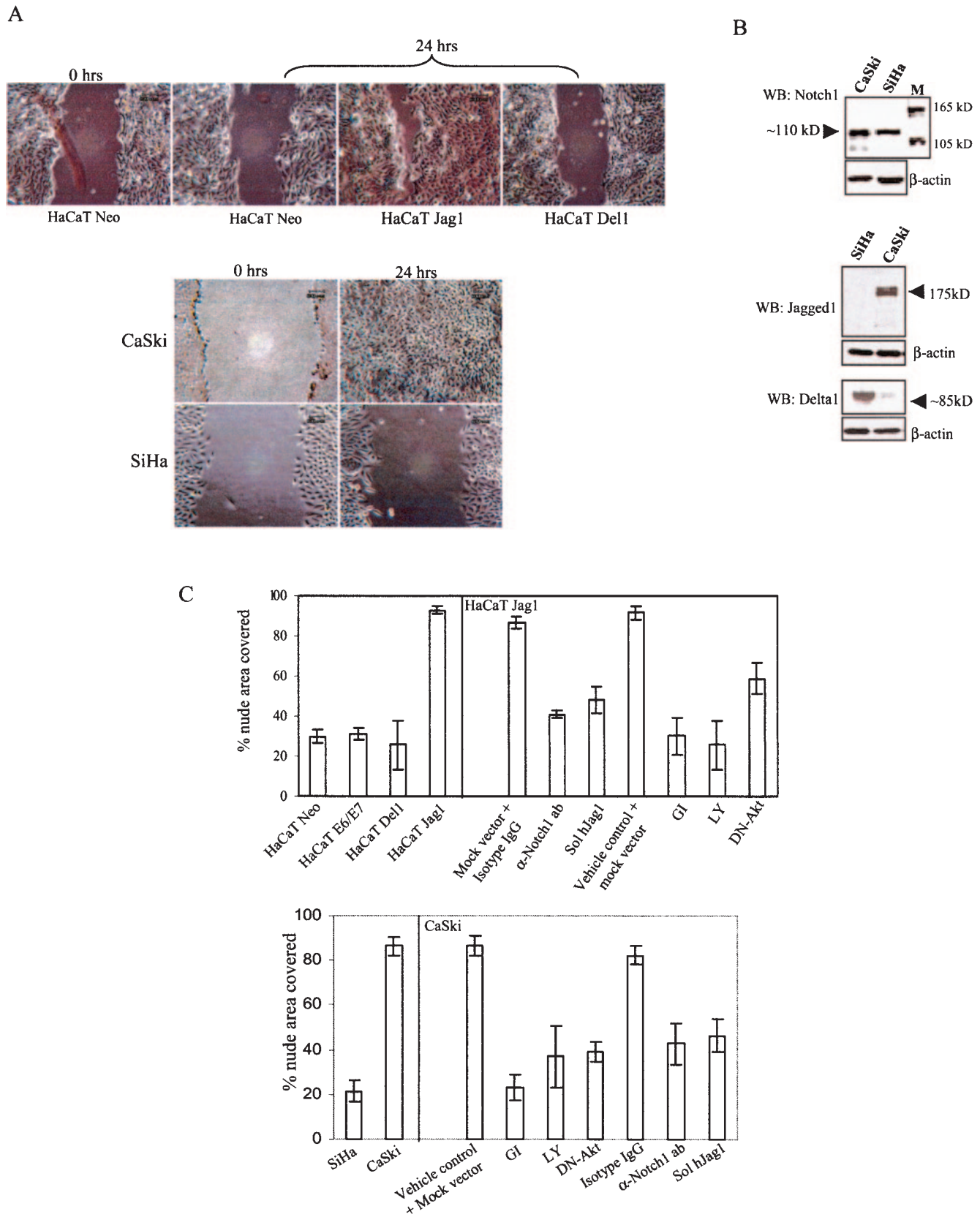

FIG. 3. (A) Representative photomicrographs (4× magnification) show the extent to which HaCaT-Neo, -Jagged1 (Jag1), and -Delta1 (Del1) cells exhibited motility in the presence of low serum (2% fetal calf serum) and covered the wounded nude area after 24 h in an in vitro wound-healing assay (26). The photomicrographs in the panel below show the extent to which CaSki and SiHa exhibit motility in a similar assay.

lines, CaSki and SiHa, as they express comparable levels of Notch1 but have high levels of Jagged1 and Delta1, respectively (Fig. 3B). In wound-healing assays, CaSki but not SiHa cells exhibited 90% closure of the wounded nude area in 24 h, and the motility was inhibited by LY, DN-Akt, GI, soluble Jagged1, and neutralizing anti-Notch1 antibody (Fig. 3C).

Using immunofluorescence staining, we confirmed that HaCaT-Jagged1 underwent EMT (Fig. 4). Features of EMT included downregulation of epithelial markers like E-cadherin and plakoglobin and upregulation of mesenchymal markers like fibronectin, and these were observed in HaCaT-Jagged1 cells (Fig. 4A). These features were not detected in HaCaT-Delta1 cells (data not shown). A Western blot analysis undertaken on these lysates show downregulation of E-cadherin and plakoglobin in HaCaT-Jagged1 but not in HaCaT-Neo cell lysates prepared 20 h post wounding (supplemental Fig. 3A). As observed with HaCaT-Jagged1 cells, downregulation of E-cadherin and plakoglobin, and upregulation of vimentin were observed by 24 h at the wound edge of migrating CaSki cells.

Representative microphotographs of immunofluorescence staining are shown in Fig. 4B. Consistent with the lack of motility in the wound-healing assay, SiHa cells did not show any alterations in the expression of EMT markers at 24 h (supplemental Fig. 3B). SiHa cells have been reported to undergo EMT in response to TGF-β (43). We examined the ability of these cells to undergo EMT over a longer duration in the wound response assay and found that only at 40 h, the cells at the wound edge show downregulation of plakoglobin and upregulation of fibronectin (supplemental Fig. 3B). Thus, the expression of Jagged1 and Delta1 correlated with the rapid induction of EMT in HaCaT and in two cervical tumor-derived cell lines. However, our analysis of human cervical tumor-derived lines is fairly limited and an examination of a larger panel of human cervical tumor derive lines would be instructive. These results also extend the oncogenic phenotypes that are mediated by PI3K in the context of Notch signaling in transformed human epithelial cells.

The canonical Notch signaling pathway involves ligand-receptor interactions, followed by successive proteolytic cleavages of Notch1 resulting in release of the intracellular domain of the receptor. This part of the receptor translocates to nucleus, binds and converts the transcription factor CBF1;Su(H); Lag-1 (CSL) from a repressor to transcriptional activator and regulate target genes (2). Studies based on Notch mutants that lack the key CSL-interacting domains have revealed contradictory results on the necessity of this signaling pathway in Notch-mediated transformation (4, 11, 17). We assessed the requirement of CSL-dependent Notch pathway in the generation of oncogenic functions like the induction of EMT and anoikis resistance.

Expression of dominant negative CSL (DN-CSL) (pCMV-DN-CBF1; gift of J. Aster) failed to abrogate both EMT-driven motility in HaCaT-Jagged1 (Fig. 5A) and CaSki cells (Fig. 5A). Further, DN-CSL does not inhibit PI3K-mediated phosphorylation of Akt in HaCaT-Jagged1 cells (Fig. 5B, compare lanes 2 and 3). In keeping with these results, the known CSL-dependent targets like Hes1 and cyclin D1 (34, 41) were upregulated in both Jagged1 and Delta1 expressing HaCaT cells (Fig. 5C). DN-CSL also failed to block Jagged1-driven anoikis resistance in HaCaT-Jagged1 and CaSki cells (supplemental Fig. 4A). The ability of DN-CSL to block CSL-dependent Notch signaling was confirmed using a Notch/CSL-promoter reporter assay system (supplemental Fig. 4B). These data suggest that CSL-activation can be independent of the processes that sustain PI3K activation in the context of dysregulated Notch signaling.

Following these observations we evaluated the involvement of Deltex1 (28, 31), in the induction of Jagged1-driven EMT and anoikis resistance (Fig. 5). Deltex has three distinct motifs, an N-terminal binding site for Notch; a proline-rich region proposed to be a potential SH3-domain-binding site; and a RING-H2 finger, involved in oligomerization (42). Like activated Notch signaling, ectopic expression of human or mouse Deltex1 in C2C12 cells suppressed myogenin expression under differentiation-inducing conditions (20, 42). In such overexpression assays, a mutant form of Deltex that lacked either the proline-rich domain or RING-H2 finger domain fail to oligomerize and therefore behaved as a dominant negative form, inhibiting activation of Notch signaling (27).

Interestingly, in wound-healing assays, expression of such a dominant negative form of Deltex1 (DN-Dtx1) blocked Jagged1-induced EMT in HaCaT-Jagged1 and CaSki cells (Fig. 5A). Concomitantly, we observed a dramatic reduction in PI3K activity, as measured by pAkt levels in HaCaT-Jagged1 cells expressing DN-Dtx1 (Fig. 5B, compare lanes 4 and 5). Further, coexpression of dominant-negative Deltex1 significantly reduced L83V E6 and E7-mediated transformation from seven- to fourfold in HaCaT-Jagged1 cells in soft agar colony formation assay (Fig. 5D). The extent of inhibition observed was comparable to that seen in cells treated with GI. In accordance with these observations, DN-Dtx1 increased the sensitivity of both HaCaT-Jagged1 and CaSki cells to anoikis (supplemental Fig. 4A). These results demonstrate a role for Deltex1 in Jagged1-driven PI3K activation and induction of pro-oncogenic features.

Jagged1 signaling has been suggested to have a transforma-

(B) Immunoblots reveal the analysis of CaSki and SiHa cell lysates for the expression of Notch1 (1:10; bTAN20; DSHB) (WB: Notch1), Jagged1 (1:10; TS1.15SH; DSHB) (WB: Jagged1), and Delta1 (1:300; sc-9102; Santa Cruz Biotech) (WB: Delta1). As a gel-loading control, the same blots were reprobed with β-actin antibody. M, molecular size markers. (C) Graphs represent the percent wounded nude area covered by the mentioned cell lines under different conditions. The % wound area covered was measured using a Nikon inverted microscope, and the associated Image-pro Plus software. Graph on the top and bottom show that HaCaT-Jagged1 and CaSki cells cover 90% of the wounded nude area by 24 h, respectively. Treatment with neutralizing anti-Notch1 antibody (α-Notch1 ab) (1:300; Neomarkers) or the inhibitor of presenilin-dependent γ-secretase (20 μM, GI; Calbiochem) or LY294002 (20 μM, LY; Calbiochem) or stable expression of plasmids encoding different cDNAs for soluble human Jagged1 (Sol hJag1 and pcDNA-sol Jag1) or dominant negative Akt (DN-Akt) resulted in significant inhibition of wound closure in both HaCaT-Jagged1 and CaSki cells ($P < 0.001$). Treatment with dimethyl sulfoxide (vehicle control) or mouse isotype immunoglobulin G or stable expression of mock vector were included as controls. The results shown represent the means ± standard deviation of the results from three independent experiments.

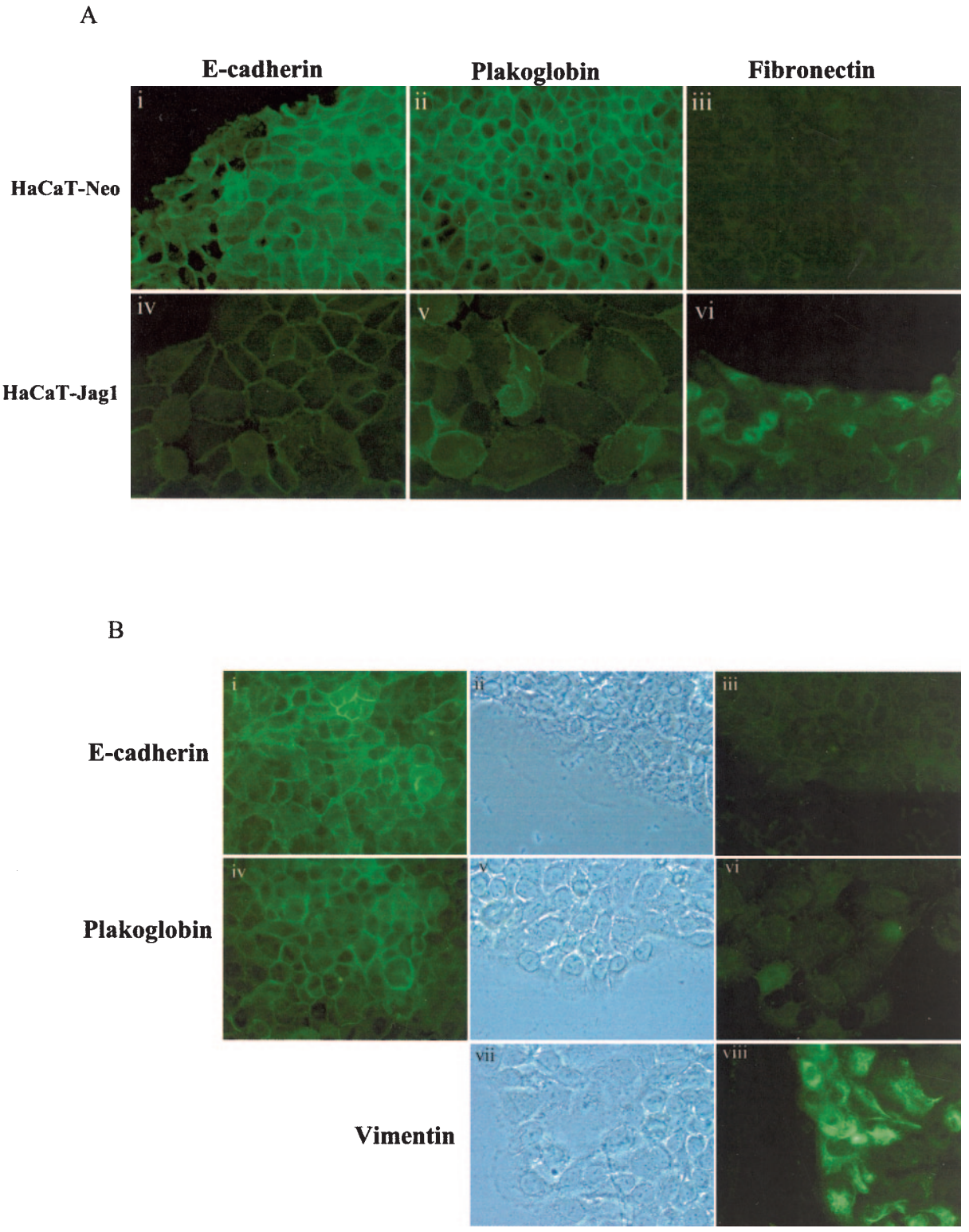

FIG. 4. Representative photomicrographs (40× magnification) show alterations in the expression of EMT markers in HaCaT-Jagged1 versus HaCaT-Neo cells (A) and CaSki cells (B). Immunofluorescence staining of E-cadherin (1:300; Calbiochem), plakoglobin (1:300; Sigma), and fibronectin (1:300; Sigma) is shown in HaCaT-Neo (A, i to iii) and HaCaT-Jagged1 (A, iv to vi) cells. (B) Immunofluorescence staining of E-cadherin (B, i) and plakoglobin (B, iv) in confluent monlolayer culture of CaSki cells are shown. Downregulation of E-cadherin (B, iii) and plakoglobin (B, vi), and upregulation of vimentin (B, viii) in CaSki cells at the wound healing edge are shown. B, ii, v, and vii represent bright-field images of the wound healing edge.

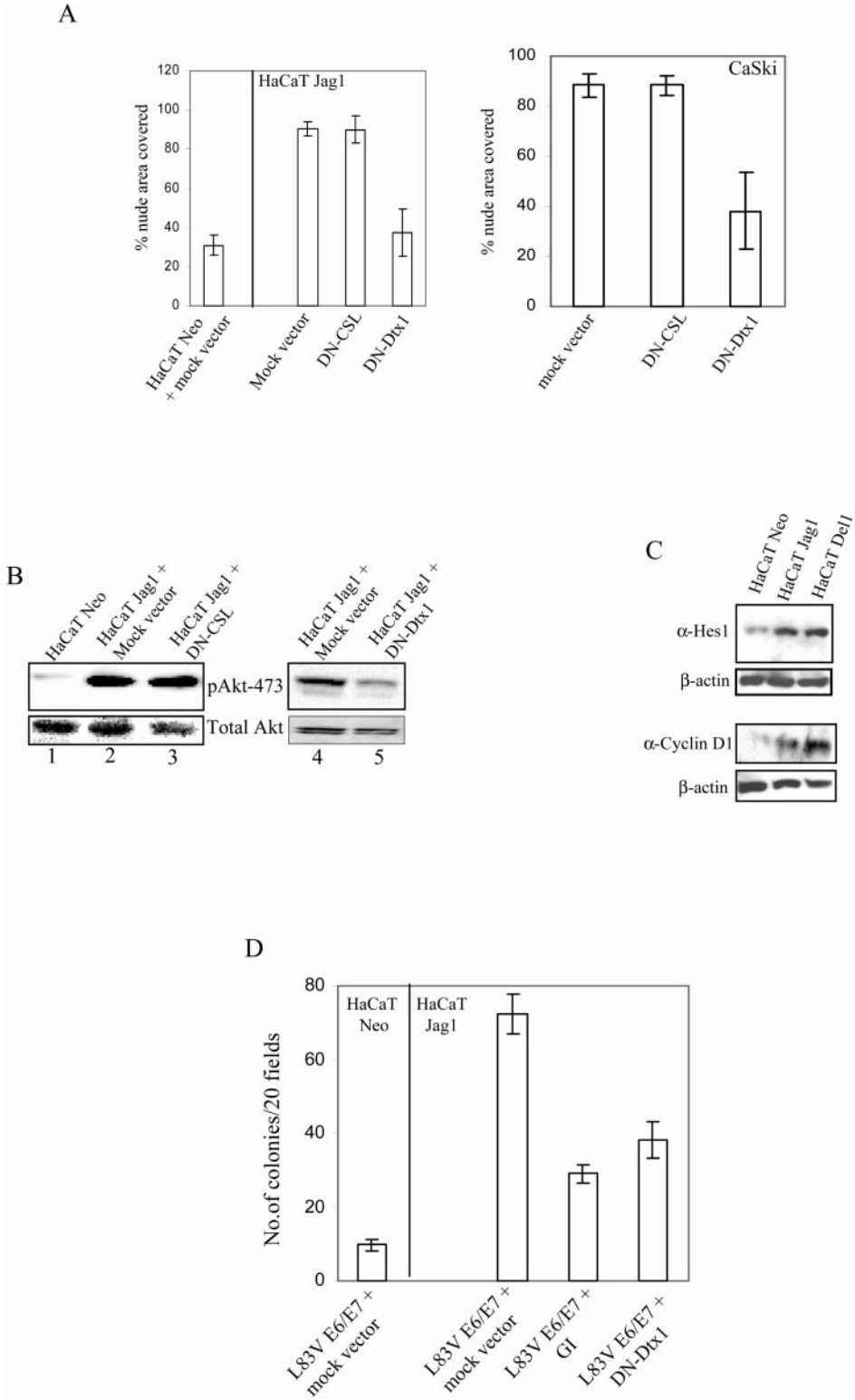

FIG. 5. (A) Graphs represent the percent wounded nude area covered in the presence of low serum (2% FCS) by the mentioned cell lines stably transfected with plasmids encoding different cDNAs (either mock vector or dominant negative CSL (DN-CSL and pcDNA3-DN CSL) or dominant negative Deltex1 (DN-Dtx1 and pEF-BOS-HA-hDxI-II). Graphs on the left and right show that mock vector-transfected (pcDNA3.0-Neo) HaCaT-Jagged1 and CaSki cells cover 90% of the wounded nude area by 24 h. Stable expression of DN-Dtx1 but not DN-CSL resulted in significant inhibition of wound closure in both HaCaT-Jagged1 and CaSki cells ($P < 0.001$). (B) Immunoblot on the top panel shows levels of phospho-PKB/Akt Ser473 (pAkt473) (1:1,000; Cell Signaling Technology) in HaCaT cells stably expressing mock vector or DN-CSL or DN-Dtx1. The same blot was reprobed to detect total Akt and is illustrated in the bottom panel. (C) Immunoblots show levels of Hes1 (1:300; sc-13844; Santa Cruz Biotech) and cyclin D1 (1:300; Santa Cruz Biotech). β-Actin levels show comparable protein loading across lanes. (D) Graph shows the number of colonies on soft agar generated by cell lines transfected with plasmids encoding L83V E6/E7 along with either mock vector or DN-Dtx1 or treated with GI (20 μM; Calbiochem). The data were generated by microscopic counting of colonies in 20 random fields under 10× magnification as described (33). The results shown in graphs A and D represent the means ± standard deviation from three independent experiments.

TABLE 2. DSL-specific regulation of gene expression in HaCaT cells.

Cells	Genes regulated	Fold change	Category
HaCaT-Jagged1	HES1	+2.09	Previously described as Notch targets
	Cyclin D1	+2.17	
Jagged1	Fibronectin	+3.4	
	Vitronectin	+2.78	Cell adhesion/motility/EMT
	Cathepsin K	+2.93	
	Early growth response 3	+2.1	
	PI3K-regulatory subunit	+1.96	
	ICAM-2, -5	+1.9	
	Cadherin-8, -11	−1.85	
	Catenin-α1	−2.17	
	Keratins-8, -15, -13	−2.2	
	Plakophilin-3, -4	−2.5	
	Discoidin domain receptor-1	−3.05	
	Epithelial membrane protein-2	−2.27	
	Rac/CD C42	−3.75	
	Claudin 1, 4	−2	
	Integrin cytoplasmic associated Protein-I	−15.15	
	Integrin-α3, -10, -L	−2.56	
HaCaT-Delta1	HES1	+2.26	Previously described as Notch targets
	Cyclin D1	+2.2	
Delta1	TNFR-1A	+2.77	
	Programmed cell death-7	+3.0	
	Apoptosis regulator	+1.89	
	Caspase recruitment domain-4	+2.1	
	Death-associated protein kinase-1	+2.5	
	RARα, -β	+2.4	

Summary of the results obtained from the microarray analysis of HaCaT cells stably expressing either Jagged1 or Delta1 against mock vector control for the expression of 9600 human sequence verified cDNA clones. A minimum of four experiments (including the reverse Cy3/Cy5 labeling) was performed with each sample to establish the relative fold differences in gene expression. A relative change in gene expression consistently either up or downregulated by 1.7 fold were scored and mentioned within brackets. Genes that have been previously reported to be the targets of Notch signaling along with those specifically involved in cell adhesion, motility and EMT process are categorized from HaCaT-Jagged1 gene expression profile. Genes that are specifically regulated in HaCaT-Delta1 cells are listed at the bottom of the table.

tion function in the context of adenoviral oncogenes that is dependent on the intracellular c-terminal PDZ (PSD-95/Dlg/Zo-1) ligand motif (3). We used a deletion construct of Jagged1 (pCMV-Jag1Δ^{PL}) (3) that lack c-terminal PDZ motif to show that in the context of HaCaT cells, this motif does not contribute to the induction of resistance to anoikis (data not shown). Further, expression of c-terminal PDZ motif mutant Jagged1 (ΔJagged1) supports both in vitro (supplemental Table 1) and in vivo tumor progression (supplemental Fig. 5A), and generates a wound-healing response analogous to the full-length Jagged1 construct (supplemental Fig. 5B).

The results presented so far led us to suggest that the expression of Jagged1 and Delta1 may correlate with different tumorigenic functions in the context of papillomavirus-mediated oncogenesis and Notch-PI3K-EMT induction can be independent of CSL-dependent Notch signaling. We examined the pattern of gene expression in HaCaT-Jagged1 and -Delta1 cells by cDNA microarray. Gene expression profiles of adherent HaCaT-Jagged1 and -Delta1 cells were scored in comparison to adherent HaCaT-Neo for the expression of 9,600 human genes that included known Notch targets like cyclin D1 and HES1.

The data analysis from experimental replicates revealed both DSL-specific and overlapping patterns of transcriptional response (Table 2). The complete data set with statistical analysis is provided (supplemental Table 2). Of the total 9,600 genes tested, 3.1% were differentially regulated in Jagged1-expressing cells and 3.6% in Delta1-expressing cells. Only 0.58% of genes were found commonly regulated in both Jagged1- and Delta1-expressing cells, which include upregulation of cyclin D1 and HES1 transcripts (Table 2). Genes encoding for cell-cell and cell-matrix interactions previously reported in the context of EMT were specifically regulated in HaCaT-Jagged1 cells (Table 2). These include upregulation of vitronectin, fibronectin, and early growth response gene 3 (Egr3), and downregulation of cadherins, catenin, keratins, and integrin subunits (19, 39). In addition, genes that are involved in regulating PI3K activity, like PI3K-regulatory subunit and intercellular adhesion molecules (ICAM), were upregulated in HaCaT-Jagged1 cells (32).

Immunoblotting was performed to validate the microarray gene expression results for Egr3 and ICAM2 (data not shown). In contrast, the transcriptional profile of HaCaT-Delta1 cells did not reveal changes in the expression status of genes involved in EMT. Instead, proapoptotic genes like TNFR-1A, apoptosis regulator, caspase recruitment domain-4, programmed cell death-7, death associated protein kinase-1 and epithelial differentiation associated genes like retinoic acid receptor-β (RARβ) and RARα were upregulated in HaCaT-Delta1 cells (Table 2).

The microarray analysis is consistent with the results presented in Fig. 3 and reinforces the role of CSL-independent Notch signaling in the activation of PI3K. Our results suggest that Deltex1, a positive regulator of Notch1 signaling (24, 28) in some contexts, can mediate PI3K activation. Presence of a potential SH3-binding site within Deltex1 suggests that this molecule may act as an adaptor for Notch to recruit SH3 domain containing nonreceptor signaling kinases like Src or

focal adhesion kinase (28). In support of this notion, recent work from Sade et al., reported the necessity of the Src kinase family member, p56lck, in the antiapoptotic signal generated by Notch via PI3K in lymphocytes (35). Taken together, these observations lead us to suggest that Deltex1 following DSL ligand-specific activation of Notch1 activates PI3K by the recruitment of signaling kinases.

The key observations that emerge from this study are that Jagged1 is preferentially upregulated in human cervical tumors and sustains tumor progression by HPV 16 oncogenes (Fig. 1). Consistent with these observations, expression of Jagged1 sustains transformation by HPV-16 E6-E7 oncogenes (Fig. 2) induces a rapid EMT response (Fig. 3), generates resistance to anoikis and leads to phosphorylation of Akt. We show that the induction of PI3K by Notch signaling can be independent of CSL and is linked to the function of Deltex1 (Fig. 5). We extend the observations linking Notch with EMT by showing that this is a PI3K-dependent phenotype.

Both SiHa and CaSki cells have been shown to have metastatic potential in vivo (7). In preliminary observations (data not shown), we find evidence of inefficient tumor formation in vivo by SiHa in comparison to CaSki at equivalent concentrations of these cells. A detailed analysis that also includes restoration of Jagged1 in SiHa cells and includes other cervical tumor-derived cell lines would offer potential insights into the possible context- and time-specific role of Jagged1 signaling in human cervical neoplasia. Talora et al. have suggested that at high levels of Notch1, there can be a tumor-suppressive mechanism that operates in cervical cancer cells (37). The work of Lathion et al. (22) addresses these issues in a comprehensive manner, and the results in this study along with other observations (8, 9, 29, 33, 41, 44) strengthen the link between the activation of Notch signaling and the progression of human cervical cancer.

We thank G. Weinmaster, T. F. Vogt, J. Aster, and K. Matsuno for providing the following cDNA expression constructs: Delta1, Manic Fringe, dominant negative CBF1, and dominant negative Deltex1, respectively. We specially thank A. Capobianco for early use of the C-terminal PDZ-binding motif mutant Jagged1 construct. We also thank the animal maintenance facility at NCBS, G. Mukherjee, for constant advice on histopathology and D. Subramanyam for important suggestions and helpful discussions during various stages of this study.

This work was principally supported by core funds from NCBS, TIFR, and Department of Biotechnology, India. K.V. and S.S. are recipients of a Kanwal Rekhi Development Fellowship from TIFR endowment funds and DBT postdoctoral award, respectively.

REFERENCES

1. **Amsen, D., N. J. M. Blander, G. R. Lee, K. Tanigaki, T. Honjo, and R. A. Flavell.** 2004. Instruction of distinct CD4 T helper cell fates by different notch ligands on antigen-presenting cells. Cell **117:**515–526.
2. **Artavanis-Tsakonas, S., M. D. Rand, and R. J. Lake.** 1999. Notch signalling: cell fate control and signal integration in development. Science **284:**770–776.
3. **Ascano, J. M., L. J. Beverly, and A. J. Capobianco.** 2003. The C-terminal PDZ ligand of JAGGED1 is essential for cellular transformation. J. Biol. Chem. **278:**8771–8779.
4. **Aster, J. C., L. Xu, F. G. Karnell, V. Patriub, J. C. Pui, and W. S. Pear.** 2000. Essential roles for ankyrin repeat and transctivation domains in induction of T-cell leukemia by notch1. Mol. Cell. Biol. **20:**7505–7515.
5. **Bakin, A. V., A. K. Tomlinson, N. A. Bhowmick, H. L. Moses, and C. L. Arteaga.** 2000. Phosphatidylinositol 3-kinase function is required for transforming growth factor beta-mediated epithelial to mesenchymal transition and cell migration. J. Biol. Chem. **275:**36803–36810.
6. **Blaumueller, C. M., H. Qi, P. Zagouras, and S. Artavanis-Tsakonas.** 1997. Intracellular cleavage of Notch leads to a heterodimeric receptor on the plasma membrane. Cell **90:**281–291.
7. **Cairns, R. A., and R. P. Hill.** 2004. A fluorescent orthotopic model of metastatic cervical carcinoma. Clin. Exp. Metastasis **21:**275–281.
8. **Chakrabarti, O., K. Veeraraghavalu, V. Tergaonkar, Y. Liu, E. Androphy, M. A. Stanley, and S. Krishna.** 2004. Human papillomavirus type 16 E6 amino acid 83 variants enhance E6-mediated MAPK signaling and differentially regulate tumorigenesis by Notch signaling and oncogenic Ras. J. Virol. **78:**5934–5945.
9. **Daniel, B., A. Rangarajan, G. Mukherjee, E. Vallikad, and S. Krishna.** 1997. The link between integration and expression of human papillomavirus type 16 genomes and cellular changes in the evolution of cervical intraepithelial neoplastic lesions. J. Gen. Virol. **78:**1095–1101.
10. **Doerfler, P., M. S. Shearman, and R. M. Perlmutter.** 2001. Presenilin-dependent gamma-secretase activity modulates thymocyte development. Proc. Natl. Acad. Sci. USA. **98:**9312–9317.
11. **Dumont, E., K. P. Fuchs, G. Bommer, B. Christoph, E. Kremmer, and B. Kempkes.** 2000. Neoplastic transformation by Notch is independent of transcriptional activation by RBP-J. signalling. Oncogene **19:**556–556.
12. **Gray, G. E., R. S. Mann, E. Mitsiadis, D. Henrique, M. L. Carcangiu, A. Banks, J. Leiman, D. Ward, D. Ish-Horowitz, and S. Artavanis-Tsakonas.** 1999. Human ligands of the Notch receptor. Am. J. Pathol. **154:**785–794.
13. **Grego-Bessa, J., J. Diez, L. A. Timmerman, and J. L. de la Pompa.** 2004. Notch and epithelial-mesenchyme transition in development and tumor progression: another turn of the screw. Cell Cycle **3:**718–721.
14. **Herwig, R., P. Aanstad, M. Clark, and H. Lehrach.** 2001. Statistical evaluation of differential expression on cDNA nylon arrays with replicated experiments. Nucleic Acids Res. **29:**E117.
15. **Hicks, C., S. H. Johnston, G. diSibio, A. Collazo, T. F. Vogt, and G. Weinmaster.** 2000. Fringe differentially modulates Jagged1 and Delta1 signalling through Notch1 and Notch2 receptors in mammalian cells. Nat. Cell Biol. **2:**515–520.
16. **Jaleco, A. C., H. Neves, E. Hooijberg, P. Gameiro, N. Clode, M. Haury, D. Henrique, and L. Parreira.** 2001. Differential effects of Notch ligands Delta-1 and Jagged-1 in human lymphoid differentiation. J. Exp. Med. **194:**991–1001.
17. **Jeffries, S., and A. J. Capobianco.** 2000. Neoplastic transformation by Notch requires nuclear localization. Mol. Cell. Biol. **20:**3928–3941.
18. **Jonsson, F., and E. Knust.** 1996. Distinct functions of the Drosophila genes Serrate and Delta revealed by ectopic expression during wing development. Dev. Genes Evol. **206:**91–101.
19. **Karavanova, I., S. Vainio, and I. Thesleff.** 1992. Transient and recurrent expression of the Egr-1 gene in epithelial and mesenchymal cells during tooth morphogenesis suggest involvement in tissue interactions and in determination of cell fate. Mech. Dev. **39:**41–50.
20. **Kishi, N., Z. Tang, Y. Maeda, A. Hirai, R. Mo, M. Ito, S. Suzuki, K. Nakao, T. Kinoshita, T. Kadesch, C. Hui, S. Artavanis-Tsakonas, H. Okano, and K. Matsuno.** 2001. Murine homologs of deltex define a novel gene family involved in vertebrate Notch signaling and neurogenesis. Int. J. Dev. Neurosci. **19:**21–35.
21. **Lampugnani, M. G.** 1999. Cell migration into a wounded area in vitro. Methods Mol. Biol. **96:**177–182.
22. **Lathion, S., J. Schaper, P. Beard, and K. Raj.** 2003. Notch1 can contribute to viral-induced transformation of primary human keratinocytes. Cancer Res. **63:**8687–8694.
23. **Lowell, S., P. Jones, I. Le Roux, J. Dunne, and F. M. Watt.** 2000. Stimulation of human epidermal differentiation by delta-notch signalling at the boundaries of stem cell clusters. Curr. Biol. **10:**491–500.
24. **Martinez Arias, A., V. Zecchini, and K. Brennan.** 2002. CSL-independent Notch signaling: a checkpoint in cell fate decisions during development? Curr. Opin. Genet. Dev. **12:**524–533.
25. **Masuya, M., N. Katayama, N. Hoshino, H. Nishikawa, S. Sakano, H. Araki, H. Mitani, H. Suzuki, H. Miyashita, K. Kobayashi, K. Nishii, N. Minami, and H. Shiku.** 2002. The soluble Notch ligand, Jagged-1, inhibits proliferation of CD34+ macrophage progenitors. Int. J. Hematol. **75:**269–276.
26. **Matsubayashi, Y., M. Ebisuya, S. Honjoh, and E. Nishida.** 2004. ERK activation propagates in epithelial cell sheets and regulates their migration during wound healing. Curr. Biol. **14:**731–735.
27. **Matsuno, K., D. Eastman, T. Mitsiades, A. M. Quinn, M. L. Carcanciu, P. Ordentlich, T. Kadesch, and S. Artavanis-Tsakonas.** 1998. Hum. deltex is a conserved regulator of Notch signalling. Nat. Genet. **19:**74–78.
28. **Matsuno, K., M. Ito, K. Hori, F. Miyashita, S. Suzuki, N. Kishi, S. Artavanis-Tsakonas, and H. Okano.** 2002. Involvement of a proline-rich motif and RING-H2 finger of Deltex in the regulation of Notch signaling. Development **129:**1049–1059.
29. **Nair, P., K. Somasundaram, and S. Krishna.** 2003. Activated Notch1 inhibits p53-induced apoptosis and sustains transformation by human papillomavirus type 16 E6 and E7 oncogenes through a phosphatidylinositol 3-kinase-protein kinase B/Akt-dependent pathway. J. Virol. **77:**7106–7112.
30. **Nickoloff, B. J., J. Z. Qin, V. Chaturvedi, M. F. Denning, B. Bonish, and L. Miele.** 2002. Jagged-1 mediated activation of notch signaling induces complete maturation of human keratinocytes through NF-kappaB and PPAR-gamma. Cell Death Differ. **9:**842–855.
31. **Ordentlich, P., A. Lin, C. P. Shen, C. Blaumueller, K. Matsuno, S. Artava-**

nis-Tsakonas, and T. Kadesch. 1998. Notch inhibition of E47 supports the existence of a novel-signaling pathway. Mol. Cell. Biol. **18:**2230–2239.
32. **Perez, O. D., S. Kinoshita, Y. Hitoshi, D. G. Payan, T. Kitamura, G. P. Nolan, and J. B. Lorens.** 2002. Activation of the PKB/AKT pathway by ICAM-2. Immunity **16:**51–65.
33. **Rangarajan, A., R. Syal, S. Selvarajah, O. Chakrabarti, A. Sarin, and S. Krishna.** 2001. Activated Notch1 signaling cooperates with papillomavirus oncogenes in transformation and generates resistance to apoptosis on matrix withdrawal through PKB/Akt. Virology **286:**23–30.
34. **Ronchini, C., and A. J. Capobianco.** 2001. Induction of cyclin D1 transcription and CDK2 activity by Notch (ic): implication for cell cycle disruption in transformation by Notch (ic). Mol. Cell. Biol. **17:**5925–5934.
35. **Sade, H., S. Krishna, and A. Sarin.** 2004. The anti-apoptotic effect of Notch-1 requires p56lck-dependent, Akt/PKB-mediated signaling in T cells. J. Biol. Chem. **279:**2937–2944.
36. **Skobe, M., P. Rockwell, N. Goldstein, S. Vosseler, and N. E. Fusenig.** 1997. Halting angiogenesis suppresses carcinoma cell invasion. Nat. Med. **3:**1222–1227.
37. **Talora, C., D. C. Sgroi, C. P. Crum, and G. P. Dotto.** 2002. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. Genes Dev. **16:**2252–2263.
38. **Thelu, J., P. Rossio, and B. Favier.** 2002. Notch signalling is linked to epidermal cell differentiation level in basal cell carcinoma, psoriasis and wound healing. BMC Dermatol. **2:**7–17.
39. **Thiery, J. P.** 2002. Epithelial-mesenchymal transitions in tumour progression. Nat. Rev. Cancer **2:**442–454.
40. **Timmerman, L. A., J. Grego-Bessa, A. Raya, E. Bertran, J. M. Perez-Pomares, J. Diez, S. Aranda, S. Palomo, F. McCormick, J. C. Izpisua-Belmonte, and J. L. de la Pompa.** 2004. Notch promotes epithelial-mesenchymal transition during cardiac development and oncogenic transformation. Genes Dev. **18:**99–115.
41. **Veeraraghavalu, K., M. Pett, V. K. Rekha, P. Nair, A. Rangarajan, M. A. Stanley, and S. Krishna.** 2004. Papillomavirus-mediated neoplastic progression is associated with reciprocal changes in Jagged 1 and Manic Fringe expression linked to Notch activation. J. Virol. **78:**8687–8700.
42. **Yamamoto, N., S. Yamamoto, F. Inagaki, M. Kawaichi, A. Fukamizu, N. Kishi, K. Matsuno, K. Nakamura, G. Weinmaster, H. Okano, and M. Nakafuku.** 2001. Role of Deltex-1 as a transcriptional regulator downstream of the Notch receptor. J. Biol. Chem. **276:**45031–45040.
43. **Yi, J. Y., K. C. Hur, E. Lee, Y. J. Jin, C. L. Arteaga, and Y. S. Son.** 2002. TGFbeta1-mediated epithelial to mesenchymal transition is accompanied by invasion in the SiHa cell line. Eur. J. Cell Biol. **81:**457–468.
44. **Zagouras, P., S. Stifani, C. M. Blaumueller, M. L. Carcangiu, and S. Artavanis-Tsakonas.** 1995. Alterations in Notch signaling in neoplastic lesions of the human cervix. Proc. Natl. Acad. Sci. USA **92:**6414–6418.
45. **Zavadil, J., L. Cermak, N. Soto-Nieves, and E. P. Bottinger.** 2004. Integration of TGF-beta/Smad and Jagged1/Notch signalling in epithelial-to-mesenchymal transition. EMBO J. **23:**1155–1165.
46. **Zehbe, I., E. Wilander, H. Delius, and M. Tommasino.** 1998. Hum. papillomavirus 16 E6 variants are more prevalent in invasive cervical carcinoma than the prototype. Cancer Res. **58:**829–833.
47. **zur Hausen, H.** 2002. Papillomaviruses and cancer: From basic studies to clinical applications. Nat. Rev. Cancer **2:**342–350.