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Papillomavirus-Mediated Neoplastic Progression Is Associated with Reciprocal Changes in Jagged1 and Manic Fringe Expression Linked to Notch Activation†

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Infection by high-risk human papillomaviruses (HPV) and persistent expression of viral oncogenes E6 and E7 are causally linked to the development of cervical cancer. These oncogenes are necessary but insufficient for complete transformation of human epithelial cells in vivo. Intracellular Notch1 protein is detected in invasive cervical carcinomas (ICC), and truncated Notch1 alleles complement the function of E6/E7 in the transformation of human epithelial cells. Here we investigate potential mechanisms of Notch activation in a human cervical neoplasia. We have analyzed human cervical lesions and serial passages of an HPV type 16-positive human cervical low-grade lesion-derived cell line, W12, that shows abnormalities resembling those seen in cervical neoplastic progression in vivo. Late-passage, but not early-passage, W12 and progression of the majority of human high-grade cervical lesions to ICC showed upregulation of Notch ligand and Jagged1 and downregulation of Manic Fringe, a negative regulator of Jagged1-Notch1 signaling. Concomitantly, an increase in Notch/CSL (CBF1, Suppressor of Hairless, Lag1)-driven reporter activity and a decrease in Manic Fringe upstream regulatory region (MFng-URR)-driven reporter activity was observed in late-passage versus early passage W12. Analysis of the MFng-URR revealed that Notch signaling represses this gene through Hairy Enhancer of Split 1, a transcriptional target of the Notch pathway. Expression of Manic Fringe by a recombinant adenovirus, dominant-negative Jagged1, or small interfering RNA against Jagged1 inhibits the tumorigenicity of CaSki, an ICC-derived cell line that was previously shown to be susceptible to growth inhibition induced by antisense Notch1. We suggest that activation of Notch in cervical neoplasia is Jagged1 dependent and that its susceptibility to the influence of Manic Fringe is of therapeutic value.

Infection by oncogenically high-risk human papillomavirus (HPV) (such as HPV type 16 [HPV-16], HPV-18, and HPV-31) and continued expression of the viral E6 and E7 genes is causally linked to the progression of human cervical cancers, a major subset of neoplasia in women worldwide (60). While these genes are sufficient for immortalization of human keratinocytes, additional events are believed to be necessary for the progression of in vivo tumors (19, 61). The identity and role of secondary cellular events that may complement the functions of viral oncogenes is presently poorly understood. Recent studies have shown that Notch signaling complements the function of HPV oncogenes in in vitro transformation assays (27, 41, 55). Notch genes encode transmembrane receptors, which upon activation by ligands, are sequentially cleaved by proteases like tumor necrosis factor alpha-converting enzyme and presenilin-dependent γ-secretase (58). The cleaved intracellular Notch C-terminal fragment translocates to the nucleus and recruits CSL (CBF1, Suppressor of Hairless, Lag1) proteins to transcriptionally regulate target genes that encode for basic helix-loop-helix (bHLH) proteins (4). Hairy Enhancer of Split (e.g., HES1 and HES7) and HRY (Hairy-related transcriptional factor) are the few Notch-responsive bHLH family members that exhibit transcriptional repressor activity on target gene promoters containing N/E-box motifs (e.g., *neurogenin* and *MASH1*) (25). In addition to bHLH proteins, CSL-dependent Notch signaling has been shown to induce expression of cyclin D1, NF-κB2, and ErbB2 (37).

The suggestion that deregulated Notch signaling may be associated with human cervical cancer stems from the detection of intracellular forms of Notch1 protein in invasive tumors (59). The appearance of these intracellular forms in the transition of high-grade precursor lesions to invasive tumors suggests that activation of Notch signaling may contribute to the progression of HPV-associated cervical neoplasia (11). Truncated Notch1 alleles that generate activated forms of the Notch1 receptor (AcN1) also cooperate with HPV-16 E6 and E7 in the transformation of immortalized human keratinocytes (41) and primary keratinocytes (27). Generation of resistance to p53-induced apoptosis (36) and matrix withdrawal-induced apoptosis (anoikis) through the phosphatidylinositol 3-kinase–protein kinase B/Akt pathway have been suggested as the functional contribution of AcN1 in these cooperative transformation assays (41). Recent studies by Chakrabarti et al. (8) reveal that a variant of HPV-16 E6, L83V E6, prevalent in invasive

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cervical carcinomas, exhibits enhanced cooperation with AcN1 and E7 over the prototype E6 in transformation of immortalized human keratinocytes. Studies by Weijzen et al. have shown that inhibition of Notch1 expression by an antisense construct in a tumorigenic cell line derived from HPV-16-positive invasive cervical carcinoma, CaSki, resulted in the loss of the neoplastic phenotype in vitro and in vivo (55, 56).

In a subset of T-cell acute lymphoblastic leukemia, chromosomal rearrangement within the human Notch1 loci results in the expression of an oncogenic, truncated intracellular portion of Notch1 protein that constitutively activates this pathway (14). Such truncated Notch alleles generate tumors in a wide range of cancer models (3). In the absence of consistent chromosomal aberrations or mutations in Notch loci in cervical cancers, the detection of Notch ligands in these tumors (17) raises a potential role for ligand-dependent Notch activation in this context. Notch activation in cell fate determination occurs through interaction with ligands that are transmembrane proteins, which belong to the DSL family (Delta, Serrate/Jagged, Lag2) (44, 57). The human DSL family comprises two classes of ligands, Jagged (1 and 2) and Delta (1, 3, and 4) (28). Several studies have shown that the soluble extracellular portion of DSL ligands can act as an antagonist of endogenous ligand-induced Notch signaling (32, 54).

A diverse set of observations has suggested a role for DSL-dependent Notch signaling in regulating epidermal differentiation (29, 38, 52). In rodent epithelial cells, analogous to activated Notch1 alleles (7), Jagged1 has been shown to sustain transformation by adenovirus E1A (5). In addition to distinct spatiotemporal distribution of ligands, modulation of ligand-receptor specificity by Fringe is believed to underlie the basis of the ubiquitous use of Notch signaling in development and adult life (39). Fringe, a glycosyltransferase, identified first in *Drosophila melanogaster* and subsequently in vertebrates (Manic, Lunatic, and Radical), differentially glycosylates Notch receptors and DSL ligands (18). Fringe catalyzes the addition of β-1,3-*N*-acetylglucosamine to the preexisting *O*-fucose residues attached to the epidermal growth factor-like repeats of the Notch receptor and its ligands (34). The distinct expression pattern of Fringe genes in the mammalian epidermis also suggests a role for these glycosyltransferases in Notch-driven epidermopoiesis (51). During the dorsoventral boundary formation in the wing imaginal disk of *Drosophila*, Serrate-mediated Notch signaling is negatively regulated by Fringe (15). Negative modulation of Jagged1-mediated Notch signaling by mammalian Fringe genes has been reported in in vitro coculture assay systems that measure CSL-dependent Notch signaling (22). Recently, it has been shown that ligand-driven Notch signaling overcomes this negative influence by down-regulating Fringe expression via a feedback mechanism that involves HES family members (6).

In this study, we examined whether regulated changes in the expression of the Notch ligand, Jagged1, and its negative regulator, Manic Fringe (MFng), are linked to activation of the Notch pathway in the progression of HPV-associated cervical neoplasia. We show that Jagged1, but not Manic Fringe, is expressed in cervical carcinoma-derived CaSki cells. We demonstrate that neoplastic changes observed in the serial passage of isogenic lines of W12 cells, an HPV-16-positive low-grade squamous intraepithelial neoplasia (SIL)-derived cell line (45), are accompanied by upregulation of Jagged1 and downregulation of Manic Fringe expression. In parallel, we note a similar change in expression pattern of Jagged1 and Manic Fringe in the progression of a major proportion of high-grade precursor lesions to invasive tumors. Our results suggest that CSL-dependent Notch signaling downmodulates Manic Fringe expression. Finally, we show that inhibition of Jagged1-induced Notch signaling by a dominant-negative soluble form of Jagged1, by small interfering RNA (siRNA) against Jagged1, or by restoring Manic Fringe expression leads to the loss of the tumorigenic phenotype in CaSki cells.

MATERIALS AND METHODS

DNA constructs. The following plasmid cDNA expression constructs were used: pcDNA3-Neo (Invitrogen), pcDNA3-AcN1 (41) (subcloned from pCDNA-ICN1, gift of J. Aster), pcBOS-HA-Delta1 encoding full-length human Delta1 (gift of G. Weinmaster), pcDNA3-HES1 encoding mouse HES1 (subcloned from pCMV-HES1, gift of R. Kageyama), pcDNA3-MFng encoding full-length human Manic Fringe (subcloned from pBSK-MFng, gift of T. F. Vogt), pcDNA3-Sol hJag1 encoding the extracellular portion of human Jagged1, and pBS-Jagged1 encoding full-length rat Jagged1 (gift of T. Maciag).

Antibodies. Anti-extracellular Notch1 antibody (1:50; Neomarkers), anti-Jagged1 (2.5 μg/ml, sc-6011; Santa Cruz Biotech [SCB]), anti-Manic Fringe (2.5 μg/ml, sc-8238; SCB), anti-Notch1 antibody (1:50, sc-6014; SCB), anti-HES1 (2.5 μg/ml, sc-13844; SCB), anti-cytokerain10 (DAKO), and anti-cytokerain19 (DAKO) were used for immunohistochemical detection. Anti-Notch1 bTAN-20, anti-Notch2 C651.6DbHN, anti-Jagged1 TS1.15SH (from Developmental Studies Hybridoma Bank [DSHB]), antihemagglutinin (1:10, 12C5 monoclonal; American Type Culture Collection), anti-Jagged1 (N-19, sc-6012; SCB), anti-cleaved Notch1-specific antibody (Cell Signaling Technology), anti-β-actin (Sigma), and anti-green fluorescent protein (anti-GFP; Clontech) antibodies were used for immunoblotting.

Generation of cell lines and viruses. Cell lines were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS) unless mentioned otherwise. W12 keratinocytes were grown in Glasgow's modified Eagle's medium supplemented with FCS and epidermal growth factor in the presence of irradiated or mitomycin C-treated (20 μg/ml for 3 h; Sigma) murine 3T3 J2 fibroblast feeder cells, as previously described (10, 45). Culture conditions were the same for all passages of W12 (passages 9, 10, 16, 56, and 59) examined. All samples of W12 investigated were polyclonal. All transfections were carried out with Lipofectamine 2000 (GIBCO-BRL), as specified by the manufacturer, with QIAGEN purified plasmid DNA. The fibroblast feeder cells were removed by brief trypsinization prior to transfection of W12 cells. Normal human primary ectocervical keratinocytes (NCK) from passage 2 to 4 were grown in Glasgow's modified Eagle's medium supplemented with FCS, epidermal growth factor, cholera toxin, and bovine pituitary extract in the presence of irradiated murine 3T3 J2 fibroblast feeder cells, as described previously (46).

The recombinant adenoviruses expressing MFng (Ad-mFNG) were obtained by inserting the corresponding human cDNA into the BamHI-XhoI sites of pAdTrack-CMV-GFP and recombining them into the adenoviral backbone plasmid pAdEasy-1 in bacteria. Subsequent adenovirus generation, amplification, and infection were performed as described previously (20). Recombinant adenovirus-mediated Manic Fringe expression was independently confirmed by Northern blotting of total RNA isolated from 293 and CaSki cells infected with Ad-mFNG and Ad-GFP (data not shown).

Biopsy sample analysis. Formalin-fixed, paraffin-embedded cervical tissue samples from primary tumors were used (appropriate patient consents were obtained). The samples analyzed were taken from histologically proven vital parts of lesions. Histological evaluation comprising 46 squamous cervical carcinomas and 33 CIN IIIs of FIGO stages IB to IIA were undertaken. Expression of Notch1, Jagged1, HES1, and Manic Fringe transcripts were examined in cervical biopsy sections (3 to 4 μm) by mRNA in situ hybridization with fluorescein isothiocyanate (FITC)-labeled RNA probes and detected by using alkaline phosphatase-conjugated anti-FITC secondary antibody supplied by the RNA color kit (Amersham). The FITC-labeled antisense and sense riboprobes were in vitro transcribed by using SP6 or T7 primer and cDNA templates encoding the cytoplasmic portion of Notch1, full-length Jagged1, HES1, and Manic Fringe. The details of individual template vectors are mentioned above. The quality of staining was monitored in comparison with a sense strand-probed control. No signal in the entire section was scored as negative; two or many positive foci

distributed within the tumor or CIN III area were scored as positive. The number of positive cases over the total number of cases examined is represented as the detection frequency of the given transcript. Immunohistochemistry was performed on the indicated number of cervical biopsy sections to detect the levels of Notch1, Jagged1, and HES1 proteins by using the antibodies mentioned above. The intensity of the diaminobenizidine (DAB) precipitation was qualitatively graded as mild, moderate, or intense staining. To enable quantification, the number of positively stained cells out of 100 in 10 random fields (40× objective) were counted and represented as the percentage of positivity.

Raft cultures. Raft cultures were prepared as described previously (9). Briefly, 2×10^5 early passage (p9) and late-passage (p59) W12 cells and CaSki cells were seeded on collagen matrices containing DPC human fibroblast feeder cells and, when confluent, lifted to the air-liquid interface. After 12 days in culture, the organotypic rafts were fixed in 10% neutral buffered formalin, embedded in paraffin, and routinely stained with 1% hematoxylin and eosin. Immunohistochemistry was performed on organotypic raft sections to detect the levels of Notch1, Jagged1, HES1, and Manic Fringe proteins by using the antibodies mentioned above. The appropriate peroxidase-conjugated secondary antibodies (Molecular Probes) were used, and DAB precipitation was performed as previously reported (50).

Immunoblotting. For immunoblotting, 20 μg of total protein per cell lysate from mentioned cell lines was resolved by polyacrylamide gel electrophoresis, blotted, and probed with the primary antibodies mentioned at the appropriate dilutions as recommended by manufacturer. In the case of W12 cells, feeder cells were removed upon brief trypsin treatment, washed with phosphate-buffered saline, and lysed in 1× radioimmunoprecipitation assay buffer containing the appropriate protease inhibitors.

RNA interference against Jagged1. A 21mer oligonucleotide sequence (shown below in bold) targeting human Jagged1 from nucleotide 1392 to 1414 was selected by using the siRNA Selection Program from the Whitehead Institute for Biomedical Research. The 5′ to 3′ hairpin sense and antisense sequences are as follows: CCGGT**GGTATTCAGGACCCAACTGTG**CTCGAG**CACAGTTGGGTCCTGAATACC**TTTTTG and AATTCAAAAA**GGTATTCAGGACCCAACTGTG**CTCGAG**CACAGTTGGGTCCTGAATACC**A. The oligonucleotides were annealed and ligated into the AgeI-EcoR1 sites of the siRNA vector pLK01-puro (gift of Sheila Stewart) (47) to generate pLK01-puro sihJag1. The ability of this vector to block expression of Jagged1 was confirmed by transient transfection of 293T cells (data not shown).

Reporter assays. The CSL/Notch responsive promoter/reporter system contains a luciferase gene driven by the HES1 promoter that harbors two CSL binding sites (HES1-Luc) (gift of A. Israel) (44). Activation of Notch signaling results in transcriptional upregulation of this HES1-Luc reporter. Mutation in two of the CSL binding sites results in failure of this reporter from responding to Notch signaling (mutant HES1-Luc). Hence, the increase in reporter activity of HES1-Luc over mutant HES1-Luc represents a measure of CSL-dependent endogenous Notch signaling. Triplicate cultures of 2×10^5 cells were transfected with reporter construct (1 μg) alone or along with the plasmids mentioned. After 12 h, cells were either infected with recombinant adenovirus for 2 h (at a multiplicity of infection of 100) or treated with 20 μM presenilin-dependent γ-secretase inhibitor X (GI; Calbiochem). Cells were lysed after 48 to 72 h, and reporter activity was assayed by using a dual luciferase assay kit (Promega).

Soft agar colony formation assay. Soft agar colonies were generated as described previously (41) with CaSki cells under the mentioned conditions. Briefly, CaSki cells were treated with vehicle control (dimethyl sulfoxide [DMSO]) or 20 μM GI for 12 h or infected with the mentioned recombinant adenoviruses for 2 h at equal titers. Cells were trypsinized after 48 h of treatment or infection with recombinant adenoviruses; 10^4 cells were grown on soft agar for 21 days. Two hundred microliters of 1× DMEM containing 20 μM GI was added once every 3 days during the assay duration. Then colonies were counted from 10 to 30 random fields under ×10 magnification.

Tumor formation in nude mice. CaSki cells grown in 100-mm-diameter dishes were infected with Ad-GFP or Ad-mFNG as described above. After 48 h, cells were trypsinized, resuspended in ~200 μl of 1× DMEM, and injected subcutaneously in the flanks of nude mice (N:NIH-Swiss). Each mouse, aged between 6 and 8 weeks, was injected with 10^5 cells. After 21 days, the mice were sacrificed and the tumor volumes were calculated by measuring the mean diameters and using the formula $4/3\pi r^3$ (where r is the radius of the tumor). In experiments involving soluble Jagged1 and siRNA, CaSki cells grown in 100-mm-diameter dishes were transfected with the mentioned plasmids (mock vector, pcDNA3-Sol hJag1, pLK01-puro empty vector, pLK01-puro Si hJag1, or pLK01-puro Si GFP). After 48 h, cells were subjected to antibiotic selection. For selection, 1.8 μg of G418/ml was used for pcDNA3-based vectors and 0.5 μg of puromycin/ml was used for pLk01-puro-based vectors. Colonies resistant to antibiotic selection were pooled, amplified, and subsequently used for subcutaneous injections into mice as described above.

Manic Fringe promoter analysis. Analysis of the Manic Fringe gene from the human genomic DNA sequence (35), clone RP5-889J22 on chromosome 22q13.1, revealed the presence of a 5′ upstream regulatory sequence (nucleotide position −3500) with a TATA box (nucleotide position −10) following the predicted transcriptional start site (nucleotide position +1) and various transcriptional elements (identified by using TRANSFAC). The 5′ upstream regulatory sequence (nucleotide position −3500) from clone RP-889J22 (gift of Sanger Sequencing Centre) was subcloned into the promoterless pBasic-CAT reporter construct (pMfP-CAT−3.5kb/+1bp). The 5′ deletion constructs spanning the key transcriptional elements LhX2 (nucleotide position −650), HES1 (CACGCA/CTTGTG/CTTGTG) (nucleotide position −250), and glucocorticoid responsive element (GRE) (nucleotide position −30) were subcloned into the same reporter. Transcriptional analyses were performed by transfecting the reporter constructs into the cell lines mentioned. The chloramphenicol acetyltransferase (CAT) reporter activity was normalized to the cytomegalovirus (CMV) promoter-driven *Renilla* luciferase activity as described earlier (50). In experiments involving inhibition of ligand-induced Notch signaling, 20 μM GI (Calbiochem) was added 12 h posttransfection of the reporter constructs. Cells were lysed after 48 to 72 h, and reporter activity was assayed as described above. In experiments that involve RNA interference, the mentioned pLK01-puro constructs were cotransfected along with the mentioned Manic Fringe promoter-reporter. At 48 to 72 h posttransfection, the cells were lysed and reporter activity was assayed.

RESULTS

Expression analysis of Jagged1 and Manic Fringe in CaSki cells. Weijzen et al. recently reported that expression of Notch1 is necessary for maintenance of the neoplastic phenotype of CaSki cells (55). The expression status of Jagged1, Notch1, and Manic Fringe were evaluated in CaSki cells. A Western blot analysis revealed an intense expression of both Jagged1 and Notch1 in CaSki, but not in human primary NCK (Fig. 1A), suggesting that elevated expression of Notch pathway components may be associated with HPV-mediated transformation. Consistent with an increase in Jagged1 expression, a Northern blot analysis revealed that CaSki cells do not express Manic Fringe (Fig. 1B). These observations suggested that sustained activation of Notch signaling in CaSki cells may be Jagged1 dependent and that the reciprocal expression of Jagged1 and Manic Fringe are linked to the progression of cervical neoplasia. CaSki cells are derived from a human cervical tumor (33), and there are no corresponding isogenic cell lines representative of an earlier stage. Thus, to investigate changes in the Notch pathway during neoplastic progression, we used isogenic serial passages of W12 cells.

Reciprocal changes in the levels of Jagged1 and Manic Fringe expression in late versus early passage of W12 cells resemble the pattern observed with CaSki cells. The W12 cell line was derived from an HPV-16-positive cervical low-grade SIL (45). Early passages of W12 (up to p10) contain approximately 100 copies of only the episomal HPV-16 genome, and integration of these episomes occurs between passages 10 and 22. Isogenic late-passage W12 cells (p22 and above) contain only integrated HPV-16 (2). Previous studies have shown that episomal integration in sequential passages of W12 is accompanied by the acquisition of malignant features that are similar to those seen in human cervical neoplasia (1, 2). These features include enhanced colony-forming efficiency (CFE), increased resistance to differentiation, and a stepwise accumulation of cytogenetic abnormalities (21, 24).

The changes in expression levels of Notch pathway genes in

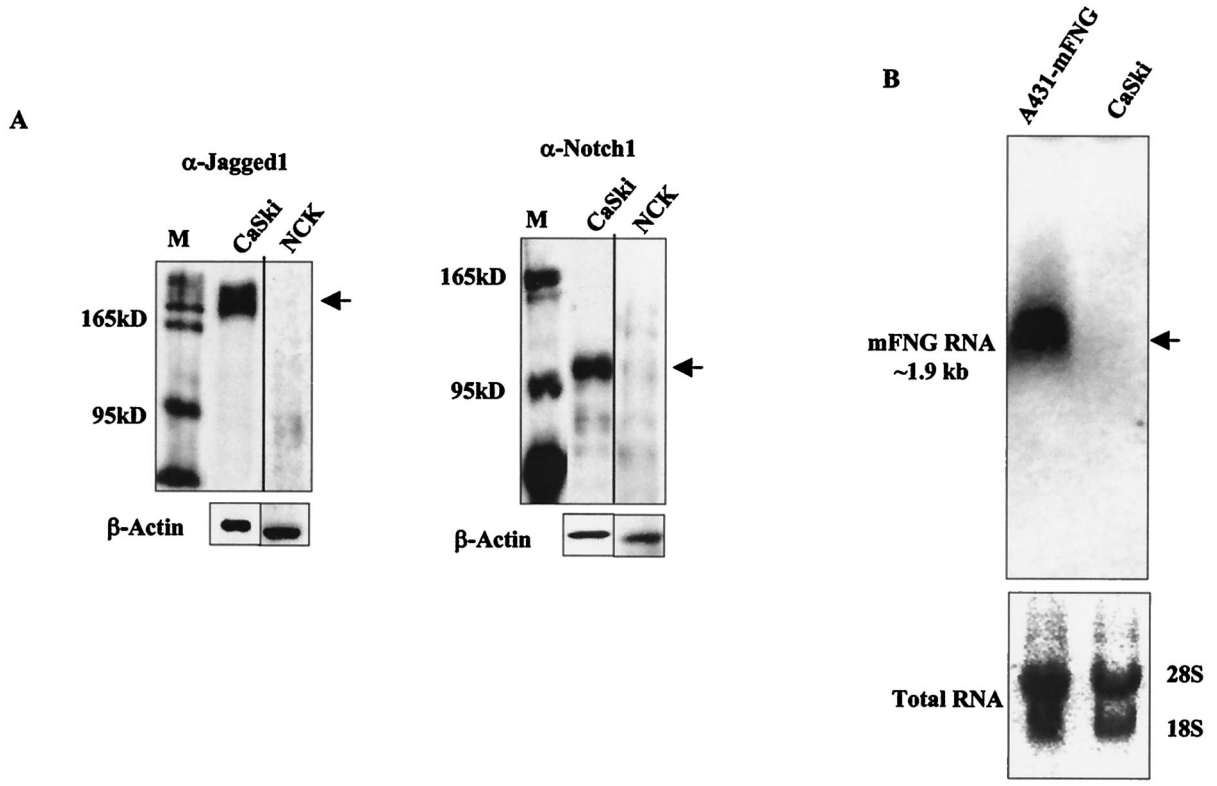

FIG. 1. Evaluation of Jagged1 and Manic Fringe expression in CaSki cells. (A) Total cell extracts from CaSki and NCK were analyzed by immunoblotting with anti-Jagged1 (TS1.15SH; DSHB) and anti-Notch1 (bTAN-20; DSHB) antibodies. Lane M, molecular mass marker with bands of indicated sizes. Jagged1 (~175-kDa band) and the cytoplasmic portion of Notch1 (~110-kDa band) are indicated by arrowheads. As a protein-loading control, the same blot was reprobed with anti-β-actin antibody. (B) Twenty-five micrograms of total RNA isolated from CaSki was subjected to Northern blot analysis with a ^{32}P-labeled Manic Fringe cDNA probe. Total RNA isolated from A431 cells stably expressing Manic Fringe (A431-mFNG) was loaded as a positive control. The 1.9-kb Manic Fringe transcript is indicated by an arrowhead. The lower panel shows 28S and 18S rRNA as loading controls.

early versus late-passage subpopulations of W12 cells were compared by immunoblotting. The episomal and integrated states of the HPV genome in the early and late-passage W12 cells used in this study have been analyzed in parallel (40). In comparison to early passage W12p10, W12p16, or W12p14, an increase in Jagged1 protein level was observed in late-passage W12p56 and W12p49 with feeders (Fig. 2A, panels 1 and 2). This result was consistently observed with two different sources of anti-Jagged1 antibodies, and the levels of expression were comparable to that detected in CaSki cell lysates. Likewise, a marked increase in Notch1 protein level was observed only in late-passage W12 cells (Fig. 2A, panel 3). Consistent with the detection of Jagged1 and Notch1 proteins, the late-passage W12p56 and W12p49 showed elevated levels of cleaved intracellular Notch1 protein (Fig. 2A, panel 4). Further, detection of activated Notch1 correlated with the appearance of HES1 protein in the late passages of W12 (Fig. 2A, panel 5). Compared to early passage W12p10 and W12p14, a marked reduction in the expression level of Manic Fringe was observed in late passage W12p56 and W12p49 (Fig. 2B). Manic Fringe expression was not detected in CaSki cells by Western blotting (Fig. 2B), consistent with the absence of Manic Fringe mRNA (Fig. 1B).

Collectively, immunoblotting revealed the upregulation of Jagged1, Notch1, and HES1 and the downregulation of Manic Fringe protein levels in late versus early passage of W12 cells, a feature that is exhibited by CaSki cells.

Reciprocal changes in levels of Jagged1 and Manic Fringe expression in late versus early passage of W12 cells correlate with features of neoplastic progression. The relationship between alterations in the expression of Notch pathway genes and progressive acquisition of neoplastic features like increased proliferation and resistance to differentiation was explored in an organotypic raft culture system generated by W12 cells. This organotypic raft system is an experimental model that closely resembles the in vivo HPV-infected cervical epithelial state (13). In this culture system, W12 keratinocytes give rise to a multilayered, stratified epithelial-like tissue, depending on the extent to which they retain the balance between proliferation and differentiation properties.

The changes in expression patterns of Notch pathway genes were compared between organotypic rafts generated by early passage W12p9 and late-passage W12p59 cells (Fig. 3). Mild but diffused Notch1 staining was observed in a few cells within the basal and suprabasal layers of early passage W12p9-generated rafts (Fig. 3A). However, the late-passage W12p59 showed intense Notch1 staining across all the layers of the raft section (Fig. 3B). Jagged1 expression was detected only in the membrane of terminally differentiating suprabasal layers of W12p9 rafts (Fig. 3D). The late-passage W12p59 showed in-

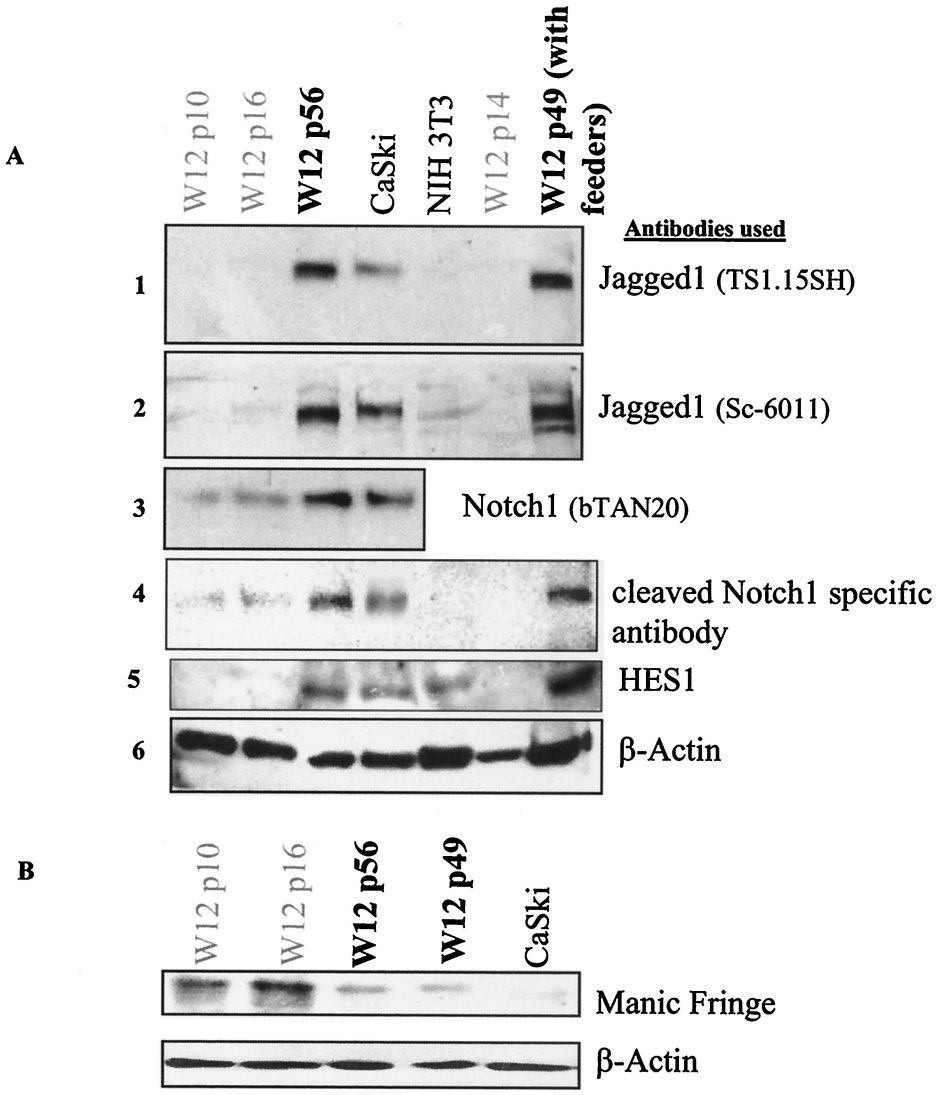

FIG. 2. Early versus late-passage W12 cells show alterations in expression of Jagged1 and Manic Fringe. (A) Total protein lysate from the cells mentioned were immunoblotted with the various antibodies indicated (panels 1 to 6). Lanes loaded with lysates from W12 late passages 49 and 56 are indicated in boldface type. An NIH 3T3 lysate was used as a negative control for Jagged1 expression. A CaSki lysate was loaded as a positive control for Notch1 and Jagged1 expression. The immunoblot of β-actin shows the protein loading control. (B) Total cell extracts from the cell lines mentioned were immunoblotted with anti-Manic Fringe antibody. As a protein-loading control, the same blot was reprobed with anti-β-actin antibody.

tense Jagged1 staining across all layers of the raft section (Fig. 3E). Consistent with these observations, an intense nuclear HES1 staining was detected in all layers of the raft generated by late-passage W12p59 (data not shown). In contrast to these observations, intense staining for Manic Fringe was observed in W12p9 rafts but not in W12p59 rafts (Fig. 3G and H). Interestingly, the above-mentioned features observed in late-passage W12p59 resemble the rafts generated by CaSki cells (Fig. 3C, F, and I).

We examined whether changes in the expression of Notch pathway genes were accompanied by progressive acquisition of neoplastic features like increased proliferation and enhanced resistance to differentiation in serial passages of W12 cells. We found that the late- versus early passage W12 cells cultured on organotypic rafts exhibited abnormal features resembling those seen in multistage progression of cervical carcinoma (30). The architecture of the raft tissue was analyzed by staining for keratin 19 (CK-19) and CK-10. CK-19, a marker for actively proliferating keratinocytes, is expressed in the basal layer of normal cervical epithelium (16). CK-10, a marker for differentiation stains the suprabasal layer of the epithelium (31).

Histomorphological analysis revealed that early passage W12p9 rafts generate a stratified epithelium (Fig. 4A). In W12p9 rafts, the suprabasal layer showed intense staining for CK-10 (Fig. 4D) while the basal layer of cells stained for CK-19 (Fig. 4G), suggesting that early passage W12p9 retains an intact balance of proliferation and differentiation properties. However, late-passage W12 cells gave rise to a moderately stratified epithelium with multiple dysplastic lesion-like out-

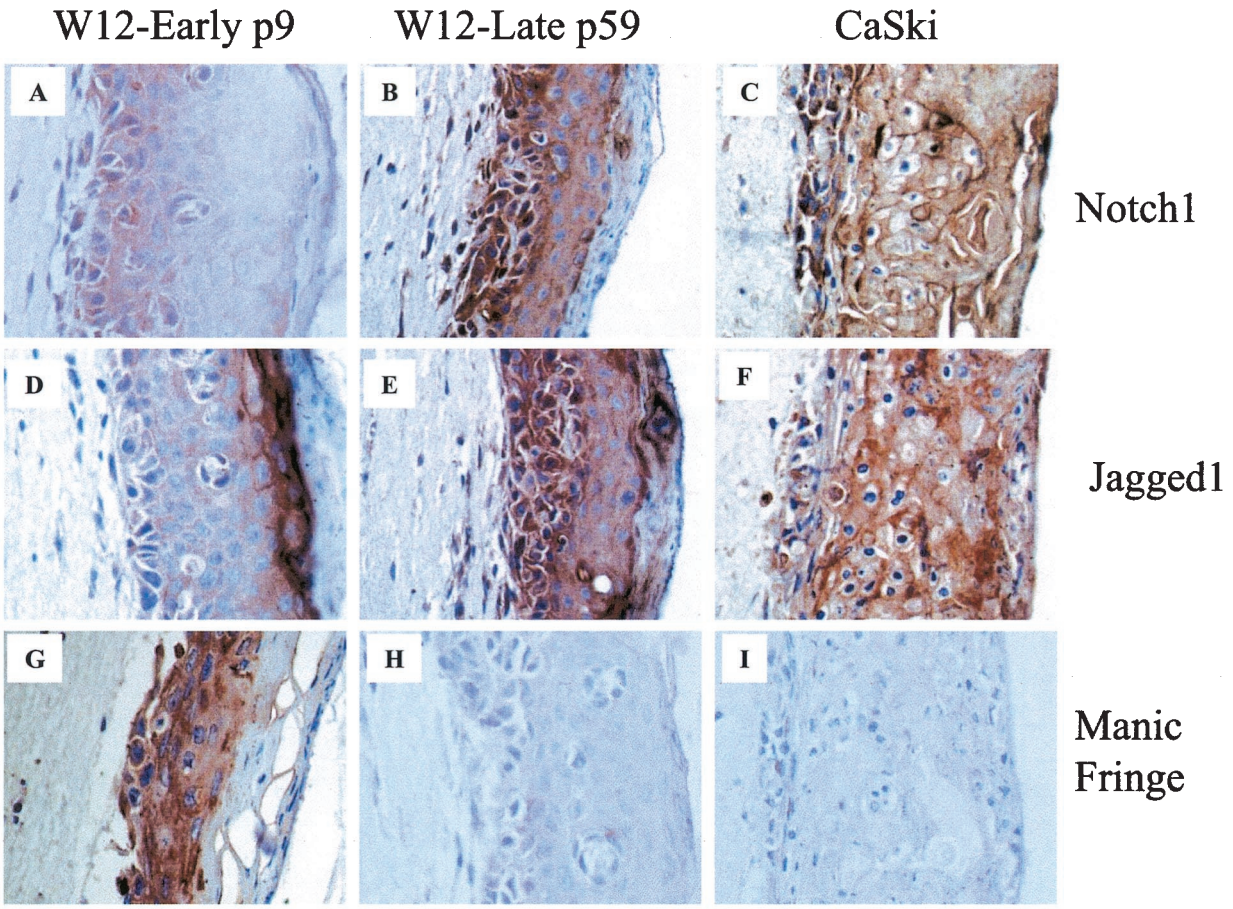

FIG. 3. Expression pattern of Jagged1 and Manic Fringe on organotypic raft cultures. Panels A, B, and C show immunohistochemical staining of Notch1 in organotypic rafts generated by W12p9, W12p59, and CaSki cells, respectively. Panels D, E, and F show expression of Jagged1 and panels G, H, and I show expression of Manic Fringe in rafts generated by W12p9, W12p59, and CaSki cells, respectively.

growths within the raft that stained negative for CK-10 (Fig. 4B and E). Further, intense staining for the proliferative marker, CK-19, was observed on all layers of raft (Fig. 4H). CaSki cells gave raise to rafts that were devoid of stratification and showed staining for CK-19 on all layers (Fig. 4C and I). This pattern was similar to the CK-19 staining observed in rafts generated by late-passage W12p59 (Fig. 4H). These results suggested that changes in the expression of Notch pathway genes were accompanied by progressive acquisition of neoplastic features in serial passages of W12 cells.

Enhanced Notch pathway activation in late-passage W12 cells. To examine whether a direct correlation existed between elevated expression of Jagged1, Notch1, HES1, and endogenous Notch activity, CSL-dependent reporter activity was measured in early and late-passage W12 cells by using a Notch/CSL-dependent promoter/reporter system. The difference in the reporter activity of HES1-Luc over that of mutant HES1-Luc was negligible in early passage W12p10 (Fig. 5A). A 4.5-fold increase in HES1-Luc reporter activity over mutant HES1-Luc was detected in late-passage W12p56, suggesting that the endogenous levels of CSL/Notch signaling are elevated in these cells. The role of Jagged1 in the enhanced CSL/Notch signaling in W12p56 was then examined. Expression of dominant-negative soluble extracellular Jagged1, si-hJagged1, Manic Fringe, or treatment with GI, which prevent ligand-induced proteolysis of Notch (12), resulted in marked inhibition of endogenous CSL/Notch-dependent HES1-Luc reporter activity (Fig. 5B). These results suggested that the observed increase in Jagged1 expression in late-passage W12 (Fig. 2 and 3) was responsible for elevated endogenous CSL-dependent Notch1 signaling in the neoplastic progression of W12 in vitro.

Reciprocal changes in Jagged1 and Manic Fringe expression in the progression of high-grade lesions to invasive tumors. Squamous cell carcinoma of cervix (SCC) fulfills the model for a classic multistage disease that evolves through well-defined noninvasive stages, which may be classified by using a two-tier system of low-grade SIL and high-grade SIL (HG-SIL). Our earlier observations showed that the cellular localization of Notch1 protein changes from membrane to nucleus during the progression of HG-SIL to invasive carcinoma (11), suggesting that activation of Notch1 occurs during this transition. This prompted us to evaluate whether the transition of HG-SIL to invasive carcinoma is accompanied by features of ligand-induced Notch activation like upregulation of Jagged1, full-length-Notch1, and HES1 and downmodulation of Manic Fringe. The expression of various Notch pathway genes were evaluated in high-grade cervical intraepithelial lesions (CIN III) and SCC cases by nonradioactive RNA in situ

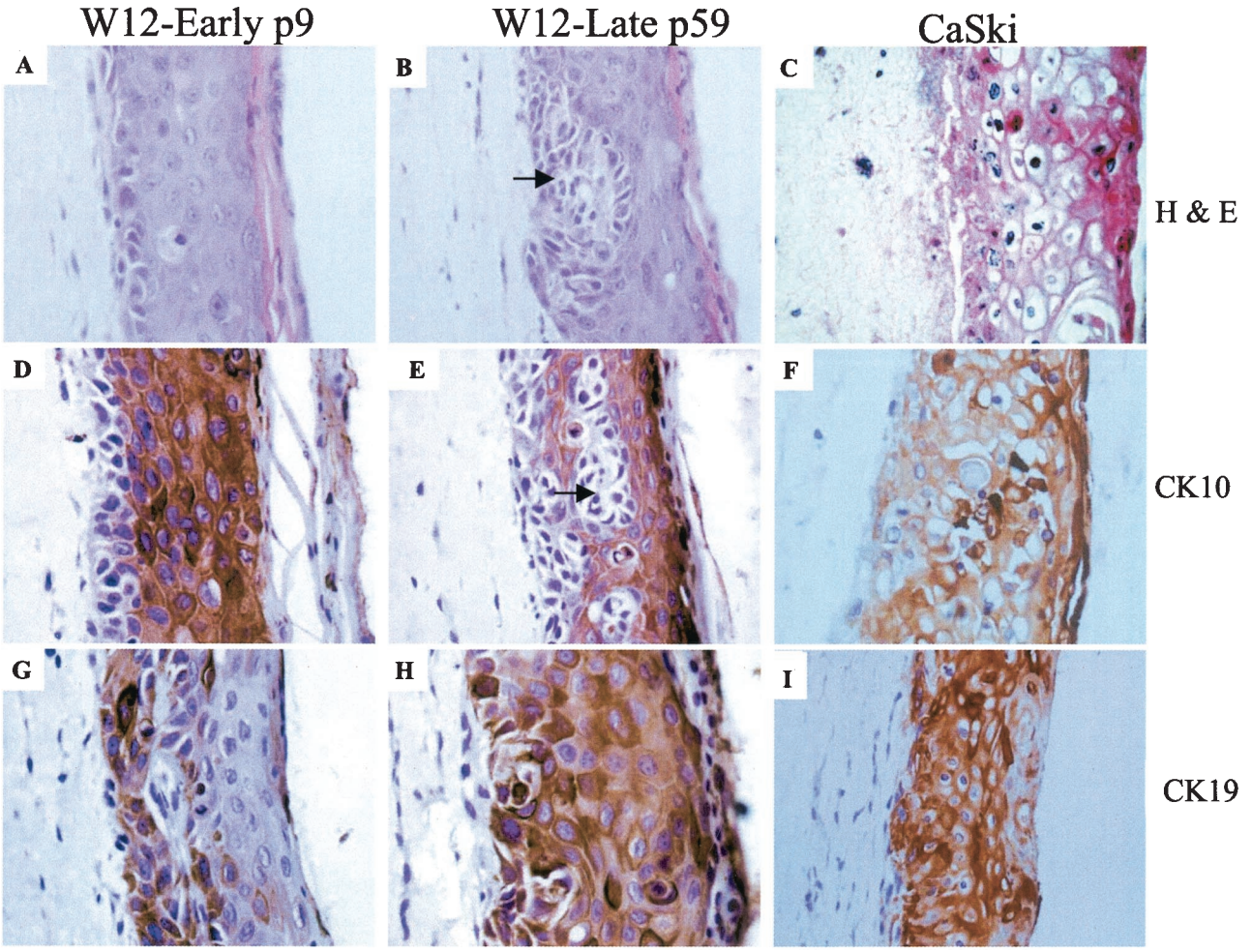

FIG. 4. Histomorphological characterization of organotypic raft cultures. Photographs show hematoxylin and eosin (H & E) histology of raft tissue generated by early passage W12p9 (A), late-passage W12p59 (B), and CaSki cells (C). Panels D, E, and F show immunohistochemical staining for CK-10 (CK10) in rafts generated by W12p9, W12p59, and CaSki cells, respectively. Panels G, H, and I show immunohistochemical staining for CK-19 (CK19) in rafts generated by W12p9, W12p59, and CaSki cells, respectively. Dysplastic pockets of cells in rafts generated by W12p59 are indicated by arrows.

hybridization. To confirm the validity of RNA in situ results, a few of the tissue specimens were subjected to immunohistochemical analysis. Representative microphotographs of RNA in situ and immunohistochemistry are shown in Fig. 6A to P.

In a major proportion of SCC cases (15 of 17), Jagged1 transcripts were uniformly detected across the field (Fig. 6B). However, Jagged1 transcripts were not detected in major proportion of CIN III cases (21 of 25) (Fig. 6A). Correspondingly, immunohistochemistry revealed an intense Jagged1 focal staining in 50 to 70% of tumor cells in SCC cases (Fig. 6D). In contrast, only a mild immunostaining was detected for Jagged1 in CIN III cases (Fig. 6C). In a major proportion of SCC cases (33 of 36), Notch1 transcripts (Fig. 6F) and full-length Notch1 protein were detected (Fig. 6H), whereas in CIN III cases, moderate positive staining of Notch1 transcript and protein was observed (Fig. 6G and E).

HES1 transcripts were detected in a major proportion of SCC cases (11 of 13) (Fig. 6J), whereas only a small proportion of CIN III cases (1 of 11) was positive for HES1 transcripts (Fig. 6I). In keeping with this result, while CIN III cases showed mild diffused immunostaining (Fig. 6K), an intense nuclear staining of HES1 protein was noticed in SCC cases (Fig. 6L). In the case of Manic Fringe transcripts, a major proportion of SCC cases was stained negative (32 of 37) (Fig. 6N) while a major proportion of CIN III cases (18 of 33) scored positive for Manic Fringe transcripts (Fig. 6M). Consistent with this finding, we observed immunohistochemical staining of Manic Fringe in CIN III cases but not in SCC cases (Fig. 6O and P, respectively). Both basal and suprabasal layers stained positive for Manic Fringe transcripts and protein (Fig. 6M and O, respectively).

An increase in detection frequency of Jagged1 transcripts from 16 to 88% (Fig. 6Q) strongly suggests that the transition of high-grade precursor lesions to invasive tumors is accompanied by the upregulation of Jagged1. Unlike Jagged1, no clear unequivocal change in the detection frequency of Delta1 transcripts was observed in the transition of high-grade precursor lesions to invasive tumors (data not shown). Consistent with the detection of Jagged1 and full-length Notch1 expression in SCC cases, an increase in detection frequency of HES1 from 9

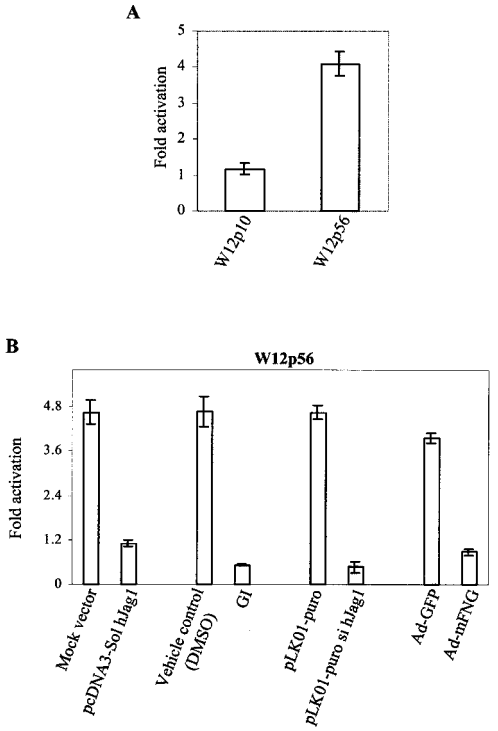

FIG. 5. Endogenous CSL-dependent Notch signaling in W12 cells. (A) W12p10 and W12p56 cells were transfected with either a HES1-Luc reporter (1 μg) containing two intact CSL binding sites or a mutant HES1-Luc reporter (1 μg) that lacked both of the CSL binding sites. The graph shows the increase in reporter activity of HES1-Luc over mutant HES1-Luc in W12p10 and W12p56 cells. (B) W12p56 cells were transfected with HES1-Luc reporter (1 μg) or mutant HES-Luc reporter (1 μg). The graph shows the increase in reporter activity of HES1-Luc over mutant HES1-Luc in W12p56 cells. In addition to the reporter constructs, cells were either cotransfected with 3 μg of mentioned plasmids (mock vector [pcDNA3-Neo], pcDNA3-Sol hJag1, pLK01-puro mock vector, or pLK01-Si hJag1) or treated with 20 μM GI or DMSO (vehicle control) or infected with recombinant adenovirus expressing GFP alone (Ad-GFP) or Manic Fringe (Ad-mFNG). The results shown represent the means ± standard errors of the means of the results from three independent transfection experiments. Transfection efficiency was normalized by using the dual luciferase assay system.

to 84% strengthens the notion that Notch receptor is activated during this transition. Further, a decrease in detection frequency of Manic Fringe transcripts from 43 to 13% suggests that Jagged1-induced Notch signaling is permissive during tumor progression. Reverse transcription-PCR analysis of a proportion of SCC cases for the expression of Notch pathway genes revealed a profile similar to the results obtained with mRNA in situ hybridization (data not shown). In 7 and 6 SCC cases (out of 9 cases), we detected Jagged1 and Notch1 transcripts, respectively. However, we were unable to detect Manic Fringe transcripts in these 9 SCC cases.

Collectively, these results indicate that preferential upregulation of Jagged1, full-length Notch1, and HES1 expression and downmodulation of Manic Fringe are hallmarks of the transition from high-grade precursor lesion to invasive carcinoma stage.

HES1 downmodulates Manic Fringe promoter activity. Expression data from clinical samples revealed that loss of Manic Fringe expression coincided with the appearance of HES1 in the transition of high-grade precursor lesions to SCC (Fig. 6). Likewise, the decline in Manic Fringe expression correlated with an increase in CSL-mediated Notch activity observed in early versus late-passage W12 cells (Fig. 5). To determine whether the observed increase in CSL-dependent Notch signaling was responsible for transcriptional downregulation of Manic Fringe expression, the upstream regulatory regions of the human Manic Fringe gene were analyzed. The genomic structure and mapping analyses of human Fringe genes have been reported earlier (35). Using transcriptional element search analysis (TRANSFAC), a region upstream of the Manic Fringe genomic coding sequence (at nucleotide position −3500) containing 3 putative HES1 binding sites was identified (Fig. 7A). These HES1 binding sites, CACGCA/CTTGTG/CTTGTG, were found at nucleotide position −250 upstream of the transcriptional start site (nucleotide position +1) of Manic Fringe and resembled the consensus N-box element CACNAG. HES1 has been shown to repress the activity of promoters containing N-box sequences upon direct binding (48). Detection of HES1 in late-passage W12p56 led us to postulate a negative regulatory role for HES1 on Manic Fringe expression. Therefore, the regulation of human Manic Fringe promoter activity in HPV-positive cell lines representative of early lesion and invasive carcinoma stages was evaluated.

A CAT reporter construct driven by the putative upstream region of the Manic Fringe gene, spanning the HES1 binding sites (pMfP-CAT), was generated. No detectable increase in the activity of this reporter over that of promoterless reporter (pBasic-CAT) was observed in late-passage W12p56 cells and in cell lines derived from human invasive cervical tumors, Caski, HeLa, and SiHa (Fig. 7B). In contrast, the same pMfp-CAT construct showed a sevenfold increase of the reporter activity in early passage W12p10 cells.

To investigate whether HES1 alone or AcN1-induced CSL signaling could modulate Manic Fringe promoter activity, promoter deletion constructs with or without HES1 interacting N-box sequences were generated (Fig. 7C). In W12p10 cells, expression of either HES1 or AcN1 downregulated the activity of pMfP-CAT (Fig. 7D). The reporter activity of promoter deletion constructs that retained intact HES1 binding sites (pMfΩ1P-CAT [nucleotide position −650] and pMfΩ2P-CAT [nucleotide position −250]) was inhibited upon HES1 overexpression. The 5′ promoter deletion construct that lacked HES1 binding sites (pMfΩ3P-CAT) retained promoter activity in the presence of HES1 in W12p10 cells. Apterous, a LIM-homeobox transcriptional factor, positively regulates *Drosophila* Fringe expression (26). LhX2, an ortholog of *Drosophila* Apterous (42), had a potential interacting site (CTTAGTTA AACAT) at nucleotide position −650 bp upstream of the predicted transcriptional start site of the Manic Fringe promoter. Coexpression of murine LhX2 in this assay failed to show any marked alteration in the reporter activity. These results support a role for HES1-specific negative regulation of the Manic Fringe promoter (Fig. 7D).

We next examined whether blocking endogenous Jagged1-mediated Notch signaling in CaSki cells would relieve the HES1-dependent repression of the Manic Fringe promoter reporter construct pMfP-CAT. Exogenous expression of dominant-negative soluble Jagged1 or inhibition of endogenous

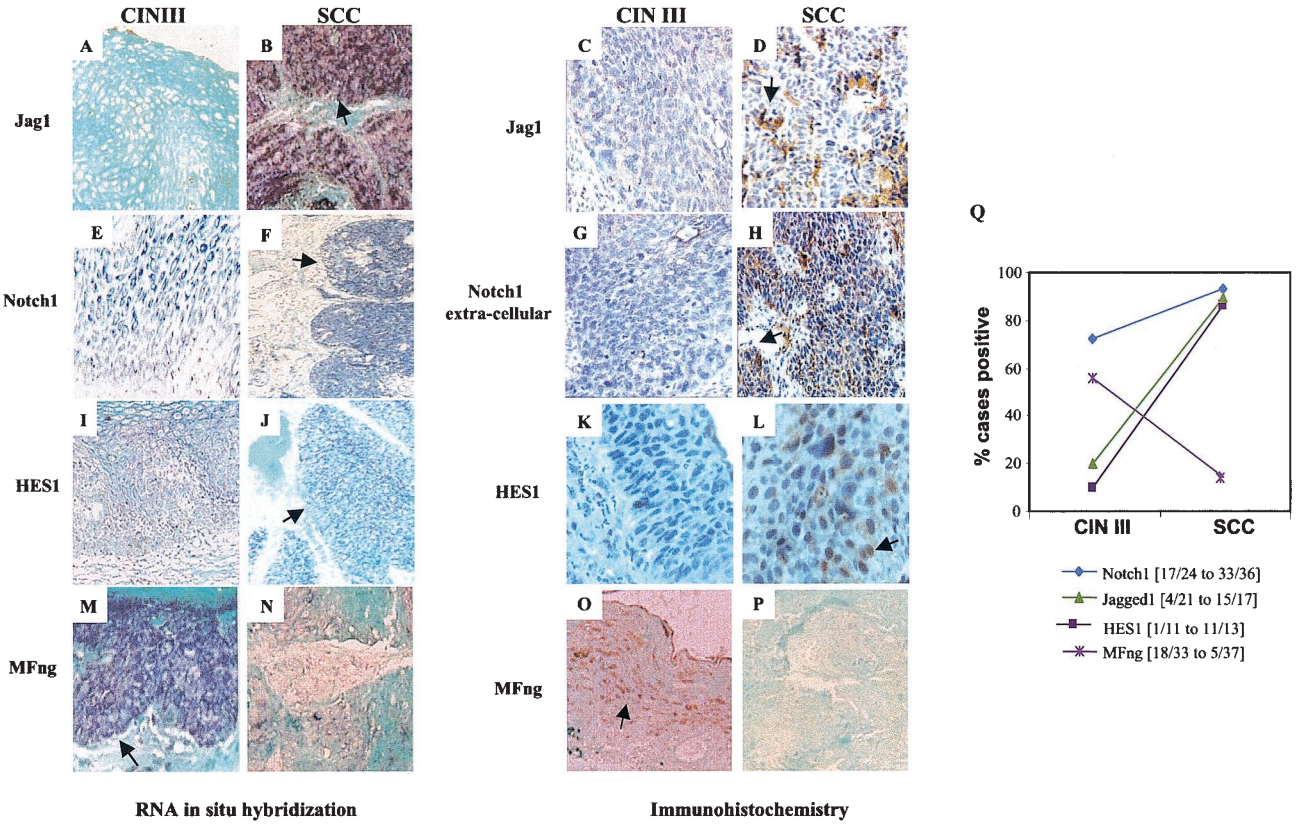

FIG. 6. Expression of Notch pathway genes in CIN III to SCC transition. (Left panel) Representative photomicrographs show expression of mentioned Notch pathway genes in CIN III and SCC cases as determined by mRNA in situ hybridization. (A and B) Jagged1 (Jag1); (E and F) Notch1; (I and J) HES1; (M and N) Manic Fringe (MFng). FITC-labeled RNA 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 counterstained with fast green. (Middle panel) Representative photomicrographs show immunohistochemical detection of mentioned Notch pathway genes in CIN III and SCC cases. (C and D) Jagged1 (Jag1); (G and H) full-length Notch1; (K and L) HES1; (O and P) Manic Fringe (MFng). Arrows indicate areas of positive DAB staining. The counterstain is either hematoxylin or fast green. Photomicrographs were taken under ×40 magnification. (Q) Graph shows the percentage of CIN III and SCC cases scored positive for the Notch1, Jagged1, Manic Fringe, and HES1 transcripts based on the results obtained from RNA in situ hybridization. The number of positive cases over the total number of cases analyzed is mentioned below the graph. The criteria employed for scoring samples positive or negative are discussed in Materials and Methods.

Jagged1 expression by sihJag1 resulted in seven- and ninefold increases in pMfP-CAT activity, respectively (Fig. 7E). Similarly, treatment with GI resulted in a ninefold increase in pMfP-CAT activity. Interestingly, expression of HES1 in cells treated with GI resulted in the reduction of pMfP-CAT activity from nine- to threefold. The 5′ promoter deletion construct that lacks HES1 binding sites (pMfΩ3P-CAT) exhibited eightfold promoter activity in CaSki cells. Blocking endogenous Jagged1-mediated Notch signaling in W12p56 cells by using similar reagents also relieved the HES1-dependent repression of the Manic Fringe promoter reporter construct pMfP-CAT (data not shown). On the whole, these results suggested that Jagged1-induced endogenous CSL/Notch signaling represses the Manic Fringe upstream regulatory sequence through HES1 binding of N-box elements. A direct role for HES1 in regulating the Manic Fringe upstream regulatory region (MFng-URR) was analyzed by an electrophoretic mobility shift assay with in vitro-translated HES1 protein and radiolabeled oligonucleotides spanning the N-box elements found on MFng-URR. HES1 protein bound oligonucleotides derived from the N-box sequences of MFng-URR (data not shown). Overall, these data showed that CSL-dependent Notch1 signaling through HES1 can downmodulate Manic Fringe promoter activity. These observations led us to examine whether inhibition of Jagged1-mediated Notch signaling by overexpression of Manic Fringe could eliminate neoplastic features in late-passage W12 and CaSki cells.

Inhibition of Jagged1/Notch signaling eliminates neoplastic features of CaSki cells. The role of Jagged1-dependent Notch signaling in the acquisition of neoplastic features, such as resistance to anoikis and CFE, was examined in early and late-passage W12 cells. Matrix withdrawn early passage W12p9 cells were sensitive to anoikis, whereas late-passage W12p56 cells were resistant to anoikis. Blocking Jagged1-mediated Notch signaling in late-passage W12p56 cells abolished resistance to anoikis (see Fig. S1 in the supplemental material). A progressive increase in the CFE was associated with the transition of early to late-passage monolayer cultures of W12 cells (2, 40). We observed a threefold increase in the CFE of W12p56 over that of W12p9. However, blocking Jagged1 function by exogenous expression of soluble Jagged1 or Manic Fringe failed to inhibit the enhanced CFE of late-passage

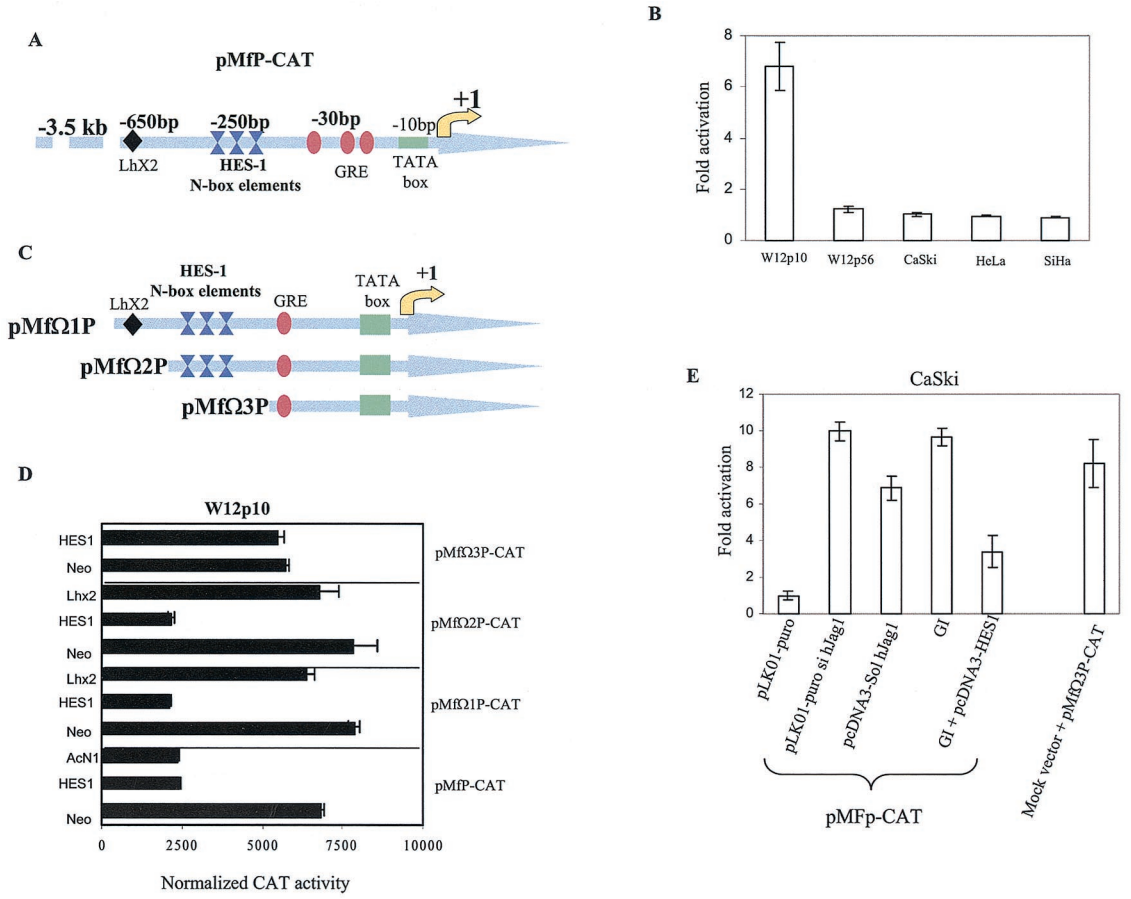

FIG. 7. HES1 negatively regulates Manic Fringe promoter activity. (A) Features of the 3.5-kb 5′ MFng-URR gene deduced from the human genomic DNA clone RP5-889J22 on chromosome 22q13.1. Sequence examination revealed a LhX2 binding element (nucleotide position −650), multiple HES1 interacting N-box elements (CACNAG) around nucleotide position −250, and multiple GREs at nucleotide position −30 upstream of the predicted transcriptional start site (nucleotide position +1). This 3.5-kb 5′ MFng-URR gene was cloned upstream of the CAT reporter gene (pMfP-CAT). (B) The promoterless CAT reporter (pBasic-CAT [1 μg]) and MFng-URR-driven CAT reporter (pMfP-CAT [1 μg]) constructs were transfected into W12p10, W12p56, HeLa, CaSki, and SiHa cell lines. The graph represents the increase in reporter activity of pMfP-CAT over pBasic-CAT. (C) Series of 5′ deletion MFng-URR-driven CAT reporter constructs lacking various transcriptional elements. pMfΩ1P-CAT retained the URR up to nucleotide position −650, spanning the LhX2, HES1, and GREs. pMfΩ2P-CAT retained the URR up to nucleotide position −250, spanning only the HES1 N-box and GREs. pMfΩ3P-CAT retained the URR up to nucleotide position −50, spanning only the GRE. (D) The graph represents the normalized CAT reporter activity of various Manic Fringe promoter deletion constructs in W12p10 cells cotransfected with 3 μg of pcDNA3-HES1 (HES1), pcDNA3-AcN1 (AcN1), pGLhX2, or mock vector (Neo). (E) CaSki cells were transfected with the pBasic-CAT (1 μg) or pMfP-CAT (1 μg) reporter construct. The graph shows the increase in reporter activity of pMfP-CAT over pBasic-CAT in CaSki cells. In addition to the reporter constructs, cells were either cotransfected with 3 μg of mentioned plasmids (pLK01-puro mock vector, pLK01-puro Si hJag1, pcDNA3-Sol hJag1, or pcDNA3-Hes1) or treated with 20 μM GI. The last bar represents the increase in reporter activity of pMfΩ3P-CAT over pBasic-CAT in CaSki cells. The results shown in panels B, D, and E represent the means ± standard errors of the means of the results from three independent transfection experiments. Transfection efficiency was normalized by using the dual luciferase assay system.

W12p56 (data not shown). Since late-passage W12 cells failed to recapitulate all the features of neoplastic transformation, the role of Jagged1-dependent Notch signaling in maintaining the tumorigenic potential of CaSki cells was evaluated. The Notch/CSL-dependent promoter/reporter system was used to ensure that the endogenous Jagged1-dependent CSL/Notch signaling was operative in CaSki cells. A 3-fold increase in HES1-Luc reporter activity over mutant HES1-Luc was observed in CaSki cells transfected with mock vector (Fig. 8A). Expression of dominant-negative soluble Jagged1 or treatment with GI resulted in the reduction of endogenous Notch/CSL-dependent HES1-Luc reporter activity to 1.02-fold (Fig. 8A). Similarly, blocking Jagged1 expression by using sihJag1 resulted in the reduction of CSL-dependent reporter activity from 3.9- to 1.1-fold. Further, restoring Manic Fringe expression in CaSki cells resulted in the reduction of CSL-dependent reporter activity from 2.7- to 1.7-fold, consistent with Jagged1-dependent endogenous CSL/Notch signaling in these cells.

Treatment of CaSki cells with GI (Fig. 8B) or infection with Ad-mFNG (Fig. 8C) resulted in three- and twofold reductions in the number of colonies grown on soft agar, respectively. CaSki cells expressing mock vector, soluble extracellular Jagged1, or sihJag1 or infected with Ad-GFP or Ad-mFNG were injected into immunodeficient BALB/c nude mice (Fig. 8D). Palpable tumors sized between 180 to 480 mm^3 were observed after 21 days in mice injected with untransfected CaSki cells

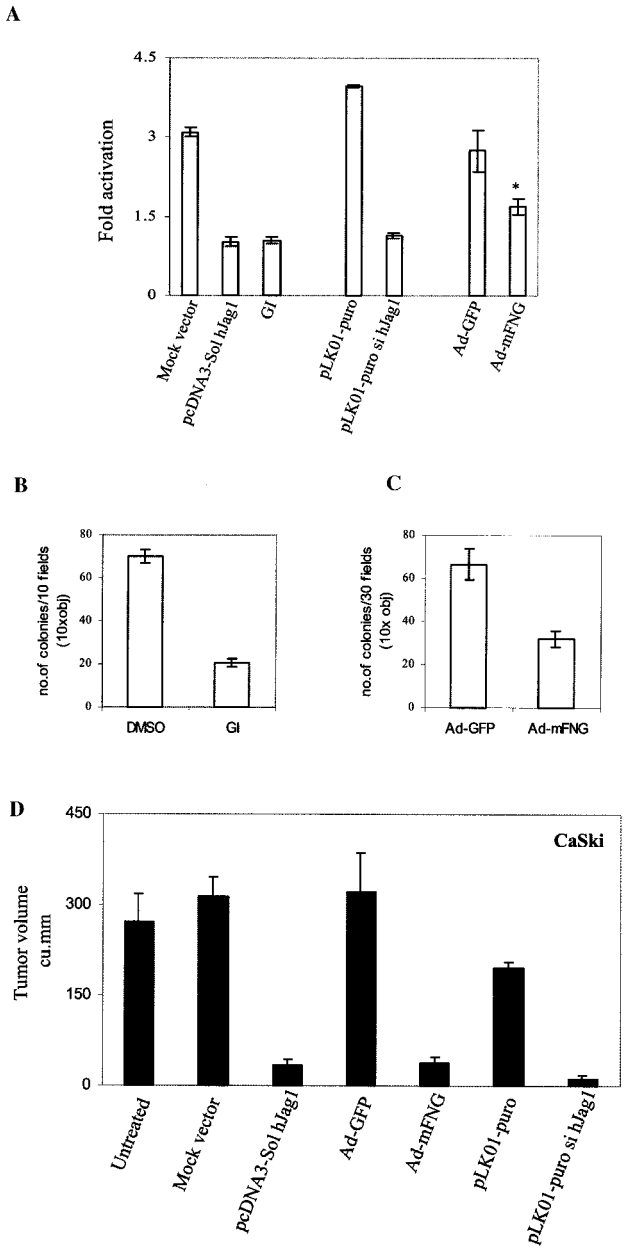

FIG. 8. Jagged1-induced Notch activity is necessary for the maintenance of tumorigenicity in CaSki cells. (A) CaSki cells were transfected with either HES1-Luc reporter (1 μg) or mutant HES1-Luc reporter (1 μg). The graph shows the increase in reporter activity of HES-Luc over mutant HES1-Luc in CaSki cells. In addition to the reporter constructs, cells were either cotransfected with 3 μg of the mentioned plasmids (mock vector [pcDNA3-Neo], pcDNA3-Sol hJag1, pLK01-puro mock vector, or pLK01-puro Si hJag1) or treated with 20 μM GI or infected with recombinant adenovirus expressing GFP alone (Ad-GFP) or Manic Fringe (Ad-mFNG). The results shown on the graph represent the means ± standard errors of the means of the results from three independent transfection experiments. Transfection efficiency was normalized by using the dual luciferase assay system. Student's *t* test was used to obtain statistical significance compared between cells infected with Ad-GFP and Ad-mFNG. *, $P < 0.05$. (B) The graph shows the number of colonies on soft agar generated by CaSki cells treated with vehicle control (DMSO) or 20 μM GI. The results shown on the graph represent the means ± standard errors of the means of the results from three independent experiments, and data were generated by counting colonies in 10 random fields under ×10 magnification. (C) The graph shows the number of colonies on soft agar generated by CaSki cells infected with Ad-GFP or Ad-mFNG. The results shown on the graph represent the means ± standard errors of the means of the results from three independent experiments, and data were generated by counting colonies in 30 random fields under ×10 magnification. (D) The graph shows tumor volumes in BALB/c nude mice generated by injection of either plain CaSki cells, CaSki cells transfected with mock vector (pcDNA3) or pcDNA3-Sol hJag1, CaSki cells infected with Ad-GFP or Ad-mFNG, and CaSki cells transfected with pLK01-puro or pLK01-si hJag1. Each bar represents the mean ± standard error of the mean of the tumor volume obtained at 3 weeks from five independent sets of experiments.

alone, expressing mock vector, or infected with Ad-GFP. In contrast, mice injected with CaSki cells expressing soluble Jagged1, sihJag1 construct, or Manic Fringe (Ad-mFNG) developed tumors that were consistently smaller (between 10 and 63 mm^3) under similar assay conditions. Expression of soluble Jagged1 in transfected and stably selected CaSki cells were confirmed by immunoblotting the conditioned media (see Fig. S2A in the supplemental material). Inhibition of Jagged1 expression in CaSki cells transfected and stably selected with siRNA against Jagged1 were confirmed by immunoblotting the total cell lysate with appropriate controls (see Fig. S2C in the supplemental material). Similarly, expression of Manic Fringe in Ad-mFNG infected CaSki cell lysates were confirmed by immunoblotting (see Fig. S2B in the supplemental material). The levels of Manic Fringe expression achieved by recombinant adenovirus were comparable to that observed in NCK and early passage W12p10 cells. Collectively, these results demonstrate that the constitutive endogenous CSL/Notch signaling detected in CaSki cells is primarily Jagged1 mediated and is necessary for the maintenance of neoplastic features.

DISCUSSION

In this study, we have investigated the expression and role of Jagged1 and Manic Fringe in the progression of HPV-associated cervical neoplasia. Following the detection of high levels of Jagged1 and a corresponding absence of Manic Fringe in the cervical tumor-derived cell line CaSki (Fig. 1), we analyzed both serial passages of W12 cells and human tumors. In early to late-passage W12 cells, along with accompanying features of neoplastic progression, we detected an increase and decrease in the levels of Jagged1 and Manic Fringe, respectively (Fig. 2, 3, and 4). In parallel, our analysis of clinical specimens revealed an increase in Jagged1 expression accompanied by a decrease in Manic Fringe levels in a major proportion of cases (Fig. 6). In addition, we detected an increase in the expression of HES1 levels in both late-passage W12 cells and invasive tumors, thus reinforcing the validity of this cell line as an in vitro cervical neoplasia progression model. Further, the elevated CSL-dependent promoter/reporter activity detected in late-passage W12 cells could be markedly reduced by blocking Jagged1-mediated Notch signaling (Fig. 5). The above observations led us to address the question of whether the activation of Notch signaling in a ligand-dependent manner could contribute to the downmodulation of Manic Fringe expression and hence sustain a negative feedback loop.

We evaluated the putative MFng-URR and detected bind-

ing elements for HES1, a bHLH family transcriptional repressor (Fig. 7). The reporter activity driven by the MFng-URR that contained HES1 binding elements was repressed in late-passage W12 and carcinoma-derived cell lines but not in early passage W12 cells (Fig. 7B). Further, deletion of HES1 binding sites within the MFng-URR or blocking Jagged1-mediated Notch activity rescued Manic Fringe promoter-driven reporter activity both in CaSki and late-passage W12 cells. These observations suggest that activation of Notch signaling, in a ligand-dependent manner, downregulates Manic Fringe expression through a CSL-dependent mechanism.

Finally, we found that inhibiting Jagged1 function by expressing soluble Jagged1, Manic Fringe, and siRNA against Jagged1 could block the tumorigenicity of CaSki cells in vivo. These results are consistent with a role for Notch signaling in maintaining the neoplastic phenotype of CaSki cells (55, 56). Consistent with the results obtained with CaSki cells, expression of Jagged1 generates resistance to anoikis and sustains tumor progression in xenograft explants of HaCaT cells expressing HPV-16 E6 and E7 oncogenes (K. Veeraraghavalu and S. Krishna, unpublished data). In contrast to the expression pattern of Jagged1, we are unable to detect a clear increase in the expression of the Notch ligand Delta1 (data not shown). Further, in HaCaT cells, expression of Jagged1, and not Delta1, sustains in vitro and in vivo neoplastic transformation in the presence of HPV-16 E6 and E7 oncogenes (Veeraraghavalu and Krishna, unpublished data). The increase in Notch activity observed in late-passage W12 correlates with Jagged1-dependent acquisition of resistance to anoikis (see Fig. S1 in the supplemental material) and thus supports previous observations linking the Notch pathway to prosurvival signals generated via the phosphatidylinositol 3-kinase–protein kinase B/Akt pathway (36, 41, 43). However, in spite of acquiring neoplastic properties like increased proliferation, anoikis resistance, and increased CFE, the late-passage W12 cells do not give rise to colonies on soft agar. It is presently unclear as to whether quantitative differences in activated Notch signaling or alternative pathways contribute to the differences between late-passage W12 and CaSki cells. The potential differences between late-passage W12 cells and CaSki are thus of enormous interest.

Truncations within Notch loci due to viral integration or chromosomal aberrations resulting in constitutive Notch pathway activation have been reported in the context of tumorigenesis (3). Thorland et al. have identified only a single HPV-16 DNA insertion within the Notch1 locus among 10 known gene loci in human primary cervical tumors (53). However, the potential functional consequence of this insertion has not been characterized. An independent study by Weijzen et al. showed that, in a nonepithelial cellular context like mouse and human primary fibroblasts, both key HPV oncogenes, E6 and E7, could upregulate Notch1 expression (55). Higher levels of E6 and E7 expression in the late- versus early passage W12 cells has been reported earlier (23). This increase in E6 and E7 may account for the elevated Notch1 expression in late-passage W12 cells observed in our study (Fig. 2). A similar increase in the expression of Notch1 in late-passage W12 has been recently reported by Lathion et al. (27). Weijzen et al. have shown that E6 and E7 expressing cells exhibit higher levels of presenilin1 and CSL-responsive reporter activity (55). However, upregulation of presenilin1 is not sufficient to activate Notch signaling in the absence of a ligand. Our results further strengthen the link between activation of Notch signaling and papillomavirus-associated cervical tumor progression.

Talora et al. have shown that overexpression of activated alleles of Notch1 with recombinant adenoviruses led to growth inhibition of high-risk HPV-positive cervical cancer-derived cells (49). However, a recent study performed by Lathion et al. showed that moderate levels of activated Notch1, as opposed to exaggerated levels, exhibit oncogenic properties in cooperation with HPV-16 E6 and E7 proteins (27). The observation that CaSki cells express detectable levels of endogenous Jagged1 and Notch1 (Fig. 1) and exhibit constitutive CSL/Notch activity (Fig. 8) itself suggests that levels of Notch signaling achieved by Jagged1 are not detrimental to HPV-16 harboring cervical carcinoma-derived cells. Further, we found that exogenous expression of Jagged1 in SiHa and HeLa cells resulted in elevated CSL-dependent Notch reporter activity but failed to induce growth arrest in a cell proliferation assay (data not shown). In addition, a recent report from our laboratory has shown that an E6 variant (83 amino acids) that has been linked to the progression of high-grade precursor lesions exhibited enhanced cooperation with activated Notch1 in the transformation of human epithelial cells (8). As all the cervical tumor-derived cell lines and invasive tumors do not show features of Jagged1-dependent signaling, we are currently attempting to understand the basis of this heterogeneity in cellular changes in cervical neoplasia.

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