

NOTCH pathway inactivation promotes bladder cancer progression

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NOTCH signaling suppresses tumor growth and proliferation in several types of stratified epithelia. Here, we show that missense mutations in *NOTCH1* and *NOTCH2* found in human bladder cancers result in loss of function. In murine models, genetic ablation of the NOTCH pathway accelerated bladder tumorigenesis and promoted the formation of squamous cell carcinomas, with areas of mesenchymal features. Using bladder cancer cells, we determined that the NOTCH pathway stabilizes the epithelial phenotype through its effector HES1 and, consequently, loss of NOTCH activity favors the process of epithelial-mesenchymal transition. Evaluation of human bladder cancer samples revealed that tumors with low levels of HES1 present mesenchymal features and are more aggressive. Together, our results indicate that NOTCH serves as a tumor suppressor in the bladder and that loss of this pathway promotes mesenchymal and invasive features.

Introduction

NOTCH is a single-pass transmembrane receptor activated by interaction with transmembrane ligands of the DSL (Delta-like and Jagged) family present on the membrane of neighboring cells. There are 4 different NOTCH receptors (NOTCH1, -2, -3, and -4) and 5 ligands (JAGGED1 and -2 and DLL1, -3, and -4) in humans. Ligand binding to the extracellular domain of NOTCH induces a cascade of proteolytic cleavages ending with the processing by the γ -secretase complex and the release of the NOTCH intracellular domain. The NOTCH intracellular domain translocates to the nucleus, where it binds the transcription factor RBPJ, and the resulting complex activates the transcription of target genes, notably including transcriptional repressors of the HES and HEY family (1).

Alterations of the NOTCH pathway are frequent in multiple cancers. Paradoxically, these alterations can be grouped into two patterns with opposite functional effects, and each pattern is associated with specific tumor types (2). In particular, gain-of-function mutations of the NOTCH pathway are present in acute T cell lymphoblastic leukemia, chronic lymphocytic leukemia, and lung adenocarcinoma, indicating that the NOTCH pathway is oncogenic in these malignancies (3-5). On the contrary, loss-of-function mutations are observed in myeloid leukemia and in squamous cell carcinomas (SCCs) of different origins (head

and neck, lung, and skin), thereby implying that the NOTCH pathway plays a tumor-suppressive role in these cancer types (6-9). However, how the NOTCH pathway protects from squamous cancers is still incompletely understood.

Bladder cancer is a major health burden worldwide (10). The majority of bladder tumors arise in the urothelium, a specialized stratified epithelium, and a fraction of bladder cancers display squamous features, corresponding to a more aggressive form of bladder cancer (11). Taking into account that the NOTCH pathway is tumor suppressive in several types of SCCs, we hypothesized that this could also be the case in the urothelium.

Here, we demonstrate that patients with bladder cancer harbor loss-of-function mutations in both *NOTCH1* and *NOTCH2* receptors. In addition, genetic inactivation of the NOTCH pathway in the urinary bladders of mice by two different genetic means accelerates bladder cancer and promotes the formation of SCCs. Mechanistically, we show that loss of the NOTCH pathway promotes an epithelial-mesenchymal transition (EMT) in bladder cancer cells and this is partly mediated by loss of HES1. Finally, human bladder cancers with low levels of HES1 present mesenchymal features and have an aggressive phenotype.

Results

Loss-of-function mutations of NOTCH1 and NOTCH2 in bladder cancer. Genome-wide analyses of bladder cancer by others and us have identified mutations in NOTCH receptors (12-14); however, their functional meaning has remained unexplored. Missense mutations in the extracellular EGF repeats of NOTCH1 and NOTCH2 have been reported in skin, lung, and head-and-neck

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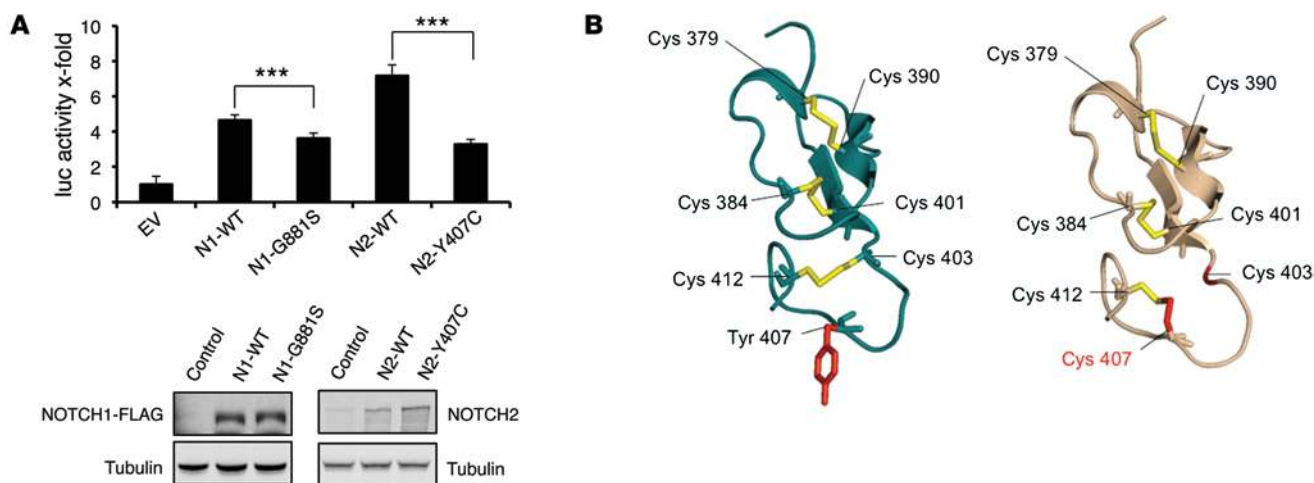


Figure 1. NOTCH1 and NOTCH2 missense mutations found in human bladder carcinomas lead to decreased NOTCH activity. (A) T24 cells were cotransfected with a plasmid carrying a luciferase reporter gene directed by an RBPJ-responder promoter and with constructs carrying the indicated versions of *NOTCH1* (N1) and *NOTCH2* (N2). Subsequently, they were exposed to HEK293T cells overexpressing the NOTCH ligand JAGGED1 to activate NOTCH signaling and, consequently, luciferase expression. Bars represent the average of 5 independent measurements of luciferase, and error bars represent SD. The levels of overexpression were assessed by immunoblot (tubulin and FLAG immunoblots from the left panel were performed in two different but equivalent membranes; see complete unedited blots in the supplemental material). *** $P < 0.001$, Student's t test. EV, empty vector. (B) Predicted structural effect of the NOTCH2-Y407C mutation is marked in red. Disulfide bridges predicted in WT and in NOTCH2-Y407C are marked in yellow.

SCCs (refs. 6–8 and Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI78185DS1). To examine the functional effect of human bladder cancer-associated mutations in NOTCH1 and NOTCH2, we chose the 2 mutations with the highest damaging prediction among those found by us in a previous exome-sequencing project (13), namely, NOTCH1-G881S and NOTCH2-Y407C (Supplemental Figure 1). The 2 mutant *NOTCH1* and *NOTCH2* cDNAs were overexpressed at similar levels in 2 different cell lines, T24 bladder cancer cells and HEK293T cells, and both triggered significantly reduced activity of an *RBPJ*-dependent luciferase reporter in comparison with their corresponding WT cDNAs (Figure 1A and Supplemental Figure 2). Interestingly, in silico modeling of the NOTCH2-Y407C

mutation predicted a change in the pattern of disulfide bridges in a region very close to the ligand interaction domain (ref. 15 and Figure 1B), and this could be the structural basis of its decreased activity. Together, these findings suggest that the NOTCH pathway plays a tumor-suppressive role in bladder cancer.

NOTCH pathway inactivation promotes bladder cancer in mice. To analyze the role of the NOTCH pathway in bladder carcinomas, we inactivated the pathway in the bladders of mice using two different strategies. First, we inactivated the two *Psen* paralogs, which encode the presenilin proteases essential for the activation of all the NOTCH receptors (16). In a complementary approach, we eliminated the *Rbpj* gene encoding the transcriptional factor critical for the canonical effects of the NOTCH pathway (17). More specifically,

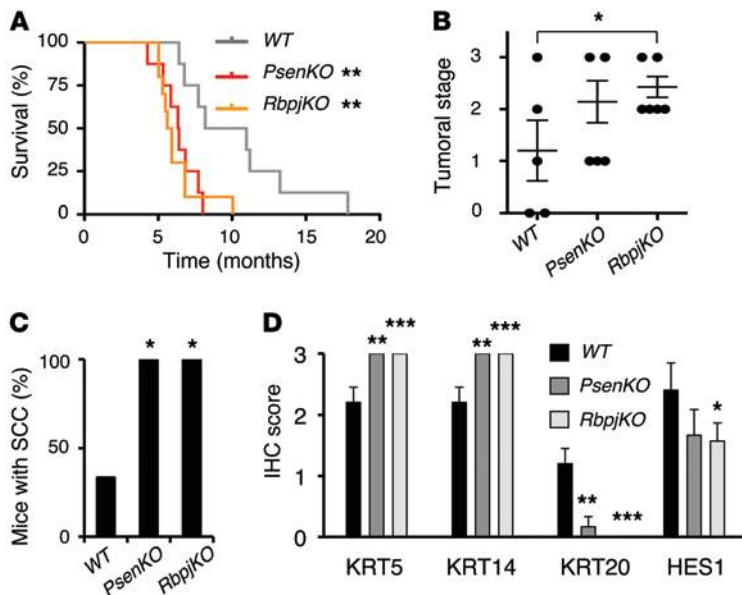


Figure 2. NOTCH inactivation promotes bladder cancer in mice. (A) Kaplan-Meier survival curves representing the life span of WT ($n = 8$), *PsenKO* ($n = 8$), and *RbpjKO* ($n = 10$) mice inoculated intravesically with adeno-Cre and, subsequently, given N-butyl-N-(4-hydroxybutyl)nitrosamine in the drinking water for 20 weeks. ** $P < 0.01$, log-rank test. (B) Bladder samples from WT ($n = 5$), *PsenKO* ($n = 7$), and *RbpjKO* ($n = 7$) mice were examined, and their lesions were graded. * $P < 0.05$, Student's t test. (C) Samples from the same mice as in B were histologically analyzed to determine the presence of SCC features. * $P < 0.05$, Fisher's exact test. (D) Immunohistochemical stainings of the same bladder samples as in B and C were scored for the expression of the indicated proteins. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student's t test.

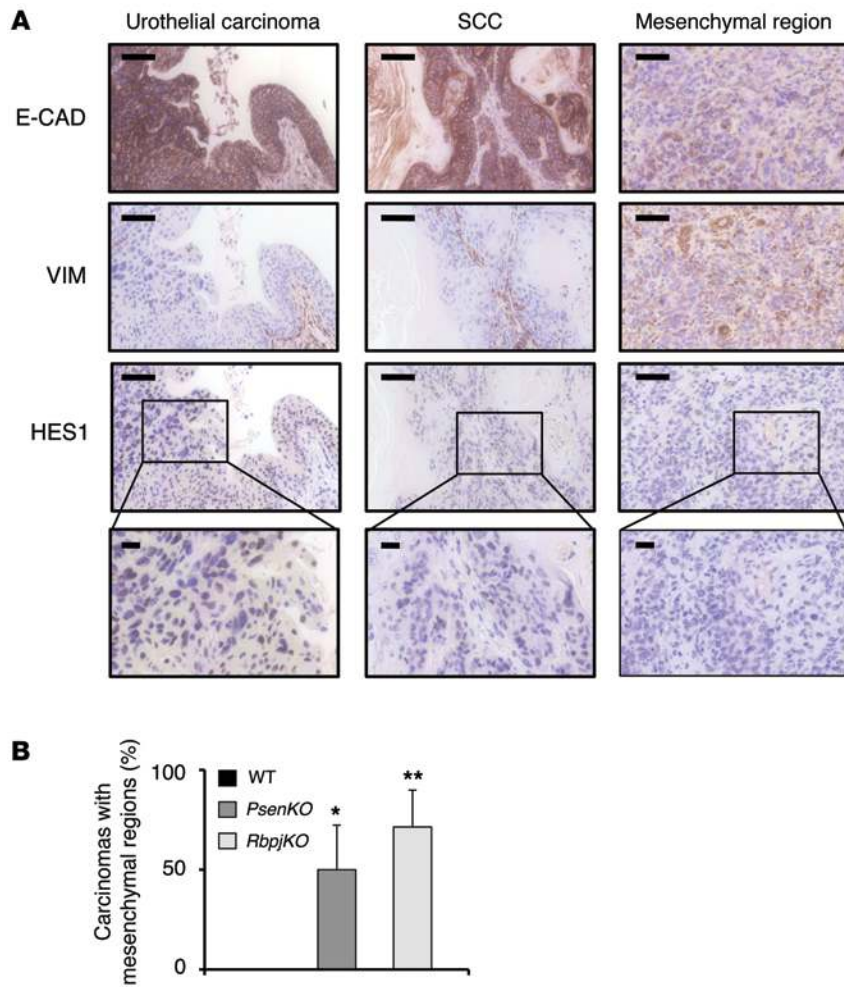


Figure 3. NOTCH inactivation induces the appearance of mesenchymal regions in SCCs. (A) Representative examples of IHC stainings against the indicated proteins on the indicated tumor subtypes and regions. Note the drastic decrease of HES1 and E-cadherin and the increase of vimentin in the mesenchymal region. Scale bars: 50 μ m (top 3 rows); 20 μ m (bottom row). (B) Quantification of the percentage of bladder carcinomas (WT, $n = 5$; *PsenKO*, $n = 7$; *RbpjKO*, $n = 7$) presenting a mesenchymal region in mice of the indicated genotypes, assessed by the loss of E-cadherin. * $P < 0.05$, ** $P < 0.01$, Student's t test.

we used mice carrying a Cre-excisable allele of *Psen1* and null for *Psen2* (*Psen1^{fl/fl} Psen2^{-/-}* mice; referred to herein as *PsenKO* mice) (18) or mice carrying a Cre-excisable allele of *Rbpj* (*Rbpj^{fl/fl}* mice; referred to herein as *RbpjKO* mice) (19). These mice, together with their corresponding WT littermates, were intravesically injected with adeno-Cre to induce the deletion of the excisable genes specifically in the bladder urothelium (20). To assess the efficiency of the Cre recombinase in the urothelium, we used *Rosa26::LSL-LacZ* mice carrying a silent *LacZ* reporter that becomes active upon Cre-mediated excision (21). As expected, PBS-treated *Rosa26::LSL-LacZ* mice were completely negative upon staining for LacZ (Supplemental Figure 3). In contrast, adeno-Cre-treated *Rosa26::LSL-LacZ* mice presented patches of the urothelium with intense LacZ staining that reached the urothelial basal layer, which is of relevance because bladder cancer-initiating cells reside in the basal layer of the bladder (ref. 22 and Supplemental Figure 3).

To evaluate the impact of NOTCH pathway inactivation on bladder cancer, *PsenKO*, *RbpjKO*, and WT mice were exposed for 20 weeks to the carcinogen N-butyl-N-(4-hydroxybutyl)nitrosamine, which is a commonly used experimental carcinogen with high selectivity for the urothelium (23, 24). As expected, all mice, regardless of their genotype, developed bladder tumors, and we only found tumors of urothelial origin. Interestingly, inactivation of the NOTCH pathway in *PsenKO* or *RbpjKO* mice resulted in a significant acceleration of blad-

der tumorigenesis and, thereby, shorter survival (Figure 2A). Tumors were classified according to current standard histological criteria (10), which include noninvasive tumors (stage 0), invasion of the underlying lamina propria (stage 1), invasion of the muscular layer (stage 2), and involvement of perivesical tissues (stage 3) (see examples in Supplemental Figure 4). Importantly, the inactivation of the NOTCH pathway was associated with the development of more invasive tumors (stages 2 and 3) (Figure 2B). This was further substantiated by the observation of bladder cancer infiltration into the prostates of *PsenKO* mice (2 of 4) and *RbpjKO* mice (2 of 5), which in some cases was perineural (Supplemental Figure 5). In contrast, none of the WT mice (0 of 4) had cancer infiltration into the prostate. The urothelial origin of the cancer cells infiltrating the prostate was confirmed by their positive staining for KRT14 (Supplemental Figure 5), which is a bona fide marker of stratified epithelia (25). Moreover, we confirmed that

intravesical adeno-Cre does not reach the prostate, as indicated by the absence of LacZ staining in the prostates of adeno-Cre-infected *Rosa26::LSL-LacZ* mice (Supplemental Figure 6).

Histologically, the *PsenKO* and *RbpjKO* tumors were diagnosed as SCCs (100%) (Figure 2C and Supplemental Figure 7). In contrast, in the case of WT mice, a fraction of tumors (33%) were SCCs and the rest were urothelial carcinomas (Figure 2C and Supplemental Figure 7). Supporting the above classification, overall quantification of immunohistochemical stainings indicated that, compared with WT tumors, *PsenKO* and *RbpjKO* tumors displayed a typical pattern of SCC tumors. In particular, they were high in keratins KRT5 and KRT14 and negative for keratin KRT20 (Figure 2D and Supplemental Figure 8). In addition, as expected, the overall levels of HES1 were reduced in *PsenKO* and *RbpjKO* tumors (Figure 2D).

We conclude that genetic inactivation of the NOTCH pathway in mice accelerates bladder carcinogenesis and favors the formation of highly invasive SCCs.

Tumors with deficient NOTCH pathway display EMT features. Interestingly, all the *PsenKO* and *RbpjKO* SCCs presented regions of mesenchymal morphology that lacked E-cadherin and expressed vimentin (Figure 3A). These mesenchymal regions coexisted together with the predominant SCC component of *PsenKO* and *RbpjKO* tumors, characterized by high levels of E-cadherin and absence of vimentin (Figure 3A). Importantly, regions of mesenchy-

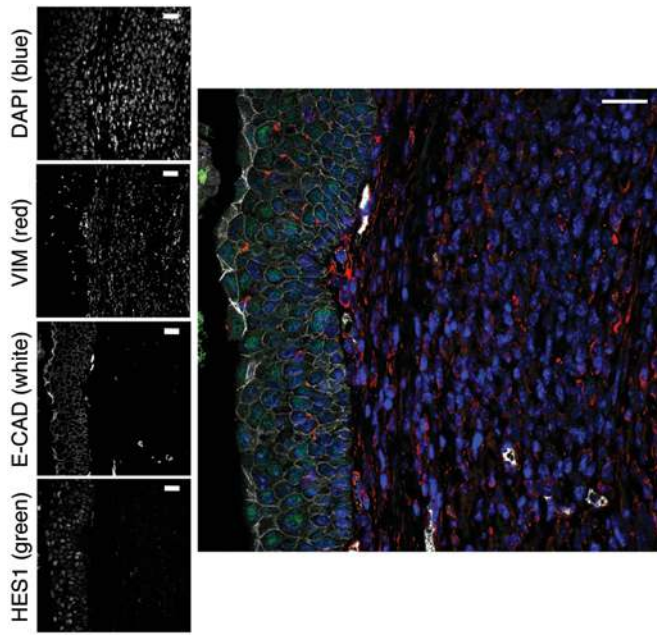


Figure 4. HES1 and E-cadherin colocalize in well-differentiated urothelium and are lost in vimentin-expressing mesenchymal regions. Immunofluorescence with the indicated antibodies was performed on a mesenchymal region from a *PsenKO* mouse, with adjacent remnants of well-differentiated urothelium (left) as an internal control for HES1 and E-cadherin expression. Scale bars: 25 μ m.

mal morphology were only found in *PsenKO* and *RbpjKO* tumors but not in WT tumors (Figure 3B). We, along with others, have implicated the transcriptional repressor HES1 as a relevant mediator of the NOTCH pathway in cancer (26–29). Remarkably, HES1 was clearly detected in the urothelial carcinomas present in WT mice, it was notably decreased in the SCCs, and it was essentially absent in the

mesenchymal regions of the SCCs (Figure 3A). To further support the relationship between HES1 and EMT, we performed immunofluorescence on paraffin sections of murine bladder carcinomas presenting a mesenchymal component. Interestingly, we observed that HES1 colocalizes with E-cadherin in the adjacent normal urothelium, but both were absent in the mesenchymal areas expressing vimentin (Figure 4). Together, these observations suggest that NOTCH pathway inactivation favors EMT in bladder SCCs.

To extend the above findings to human cancers, we performed data mining of the exome sequencing and RNA sequencing data for human bladder carcinomas available at The Cancer Genome Atlas (TCGA) (12). Our analyses indicated *NOTCH1* and *NOTCH2* mutant cancers clustered in a group defined by squamous molecular features (Fisher’s exact test, $P = 0.02$) and showed a tendency to have increased levels of the squamous markers *KRT5*, *KRT14*, and *TP63* (Supplemental Figure 9A). Of note, *NOTCH1* and *NOTCH2* mutant cancers showed a tendency toward a decrease in NOTCH targets (*HES1* and *HEY1*, Supplemental Figure 9B), which is in agreement with our previous functional characterization of NOTCH1 and NOTCH2 mutants (Figure 1 and Supplemental Figure 2). Interestingly, *NOTCH1* and *NOTCH2* mutant cancers expressed markedly lower levels of E-cadherin (*CDH1*) and higher levels of vimentin (*VIM*) (Supplemental Figure 9C). Analysis of the allele frequency for these missense mutations revealed that they were heterozygous (allele frequency lower than 0.5, with at least 60% of tumoral cells in the sample). This implies that just one mutant allele of *NOTCH1* or *NOTCH2* (with one WT allele still present) is sufficient to promote SCC bladder cancer. Collectively, these observations indicate that reduced NOTCH signaling favors squamous histology and the acquisition of mesenchymal features in murine and human bladder cancers.

NOTCH inactivation promotes EMT in a cell-autonomous manner. To explore the mechanism behind the association between NOTCH pathway inactivation and the acquisition of mesenchymal cellular

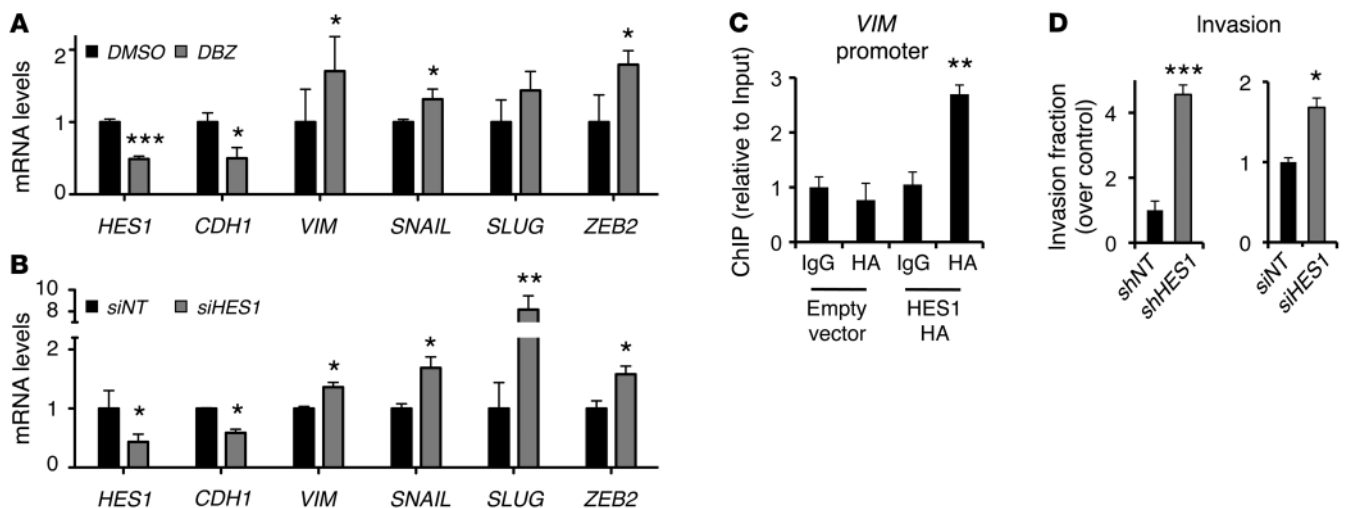


Figure 5. NOTCH inactivation promotes EMT in a cell-autonomous manner. Human urothelial carcinoma T24 cells were (A) treated with the γ -secretase inhibitor DBZ for 72 hours or (B) transfected with a nontargeting scrambled siRNA (*siNT*) or with *siHES1* for 48 hours, and the mRNA from the indicated genes was assayed by qRT-PCR. (C) ChIP using the indicated antibodies was performed on T24 cells infected with a HA-tagged version of HES1 or an empty vector and using PCR primers directed against the *VIM* promoter. (D) T24 cells infected with nontargeting control (*shNT*) or *shHES1* or transfected with *siNT* or *siHES1* were placed in invasion chambers, and invading cells were counted by confocal microscopy 20 hours later. For all panels, data correspond to the average of 3 independent experiments ($n = 3$). Error bars represent SEM. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, Student’s *t* test.

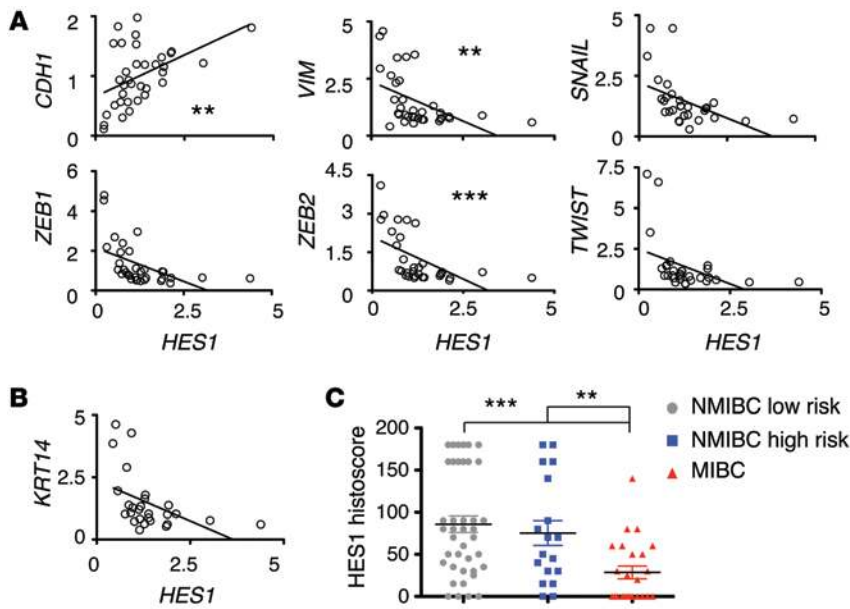


Figure 6. Low levels of HES1 correlate with EMT and invasiveness in human bladder cancers. (A) RNA samples from human bladder tumors were assayed for the indicated genes. The linear correlation of these genes with the corresponding expression of *HES1* (the paired number of cases was between 30 and 35) is shown. ** $P < 0.01$, *** $P < 0.001$, Spearman test. (B) Correlation analysis of *KRT14* and *HES1* RNA expression in 27 paired cases. (C) Paraffin sections from human bladder tumor samples were graded into the indicated categories and stained for HES1 expression. The individual HES1 expression levels for each grade, nonmuscle invasive bladder cancer (NMIBC) low risk ($n = 40$), NMIBC high risk ($n = 18$), and muscle invasive bladder cancer (MIBC; $n = 24$), are shown. ** $P < 0.01$, *** $P < 0.001$, Mann-Whitney test.

features, we treated the human bladder carcinoma cell line T24 with the γ -secretase inhibitor dibenzazepine (DBZ) (Figure 5A). DBZ treatment induced downregulation of *HES1* and, concomitantly, an increase in *VIM* and a decrease in *CDH1*, thus supporting the notion that NOTCH inhibition induces EMT in human bladder cancer.

To directly test the impact of reduced *HES1* levels on EMT, we transfected T24 cells with an siRNA against *HES1* (*siHES1*, Figure 5B) or infected these cells with a lentivirus expressing an shRNA against *HES1* (*shHES1*, Supplemental Figure 10) (of note, *siHES1* and *shHES1* target different sites of *HES1* mRNA). Interestingly, both *siHES1* and *shHES1* induced a decrease in *CDH1* expression and an increase in *VIM* expression (Figure 5B and Supplemental Figure 10), thus recapitulating our above observations in NOTCH-deficient mice and human bladder carcinomas. EMT is a complex process promoted by several master regulators, including transcription factors from the Zeb, Twist and Snail families (30). Interestingly, in addition to *VIM* upregulation, *HES1* downregulation also promoted an increase in *SNAIL*, *SLUG*, and *ZEB2* expression when T24 cells were treated with DBZ (Figure 5A) or with *siHES1* (Figure 5B).

HES1 is a well-known transcriptional repressor (26–28), and, based on this, we wondered whether it could bind to any of the EMT genes upregulated by *HES1* inhibition (Figure 5, A and B). To test this, we performed a ChIP assay using a HA-tagged version of *HES1* (31) and analyzed putative binding sites of *HES1* in the proximal promoter regions (up to 5 kb upstream of the transcription initiation site) of *VIM*, *SNAIL*, *SLUG*, *ZEB1*, *ZEB2*, and *TWIST*. This approach is limited because relevant *HES1* binding sites could be distant from the proximal promoter regions. In spite of this, we could detect a clear binding of HA-*HES1* to the *VIM* promoter (Figure 5C) but not to the proximal promoters of the other tested genes (data not shown).

At a functional level, EMT is associated with an increase in cell invasion (30), and, therefore, we examined this cellular property in T24 cells treated with *shHES1* or *siHES1*. For this, we performed Matrigel invasion chamber assays, and we observed increased invasion in *shHES1*- and *siHES1*-treated T24 cells compared with cells treated with a nontargeting control (Figure 5D).

We conclude that the NOTCH pathway, through *HES1*, contributes to the stabilization of the epithelial phenotype of bladder cancer cells.

Low HES1 expression correlates with aggressive cancer features. To further explore the possible association between *HES1* and EMT, we examined two independent sets of human primary bladder cancer samples. One set consisted of RNA samples that were analyzed by cDNA microarrays and quantitative RT-PCR (qRT-PCR), and the other set consisted of paraffin blocks analyzed by immunohistochemistry. Using microarray-derived mRNA data (32), we observed a positive correlation between the levels of *HES1* and *CDH1* and a remarkable inverse correlation between *HES1* expression and EMT effectors, including *VIM* (Figure 6A). These observations were validated using qRT-PCR on a subset of the above RNA samples (Supplemental Figure 11). We also found an inverse correlation between *HES1* and *KRT14* mRNA levels (Figure 6B) and protein levels detected by immunohistochemistry (Supplemental Figure 12), which supports our previous analysis on the bladder carcinoma tumor set from TCGA (Supplemental Figure 9). Moreover, immunohistochemical analyses indicated that *HES1* expression was significantly lower in the muscle invasive bladder carcinomas (MIBC) compared with that in nonmuscle invasive tumors (Figure 6C and Supplemental Figure 13). Finally, an independent analysis of Oncomine-deposited data (33–35) confirmed these clinical findings (Supplemental Figure 14). These observations indicate that low levels of *HES1* associate with mesenchymal features and invasive properties in human bladder carcinomas.

Discussion

The NOTCH pathway can exert both oncogenic and tumor-suppressive effects. In general, NOTCH signaling is tumor suppressive in cancers of squamous histology, as is the case with esophageal, skin, and lung SCCs (2, 6–8, 36). Since the urinary bladder is a stratified epithelium that can give rise to SCCs, we reasoned that the NOTCH pathway could be tumor suppressive in this tissue. Interestingly, although some observations

have suggested that the NOTCH pathway could be oncogenic in bladder cancer (37, 38), there is substantial evidence indicating a tumor-suppressive role. In particular, a high fraction of bladder carcinomas present deletion of the long arm of chromosome 9q, where *NOTCH1* is located (39), and low levels of *NOTCH1* and *JAGGED1* are associated with short survival in bladder cancer patients (40). Even more, a genome-wide association study in patients with bladder cancer found a significant association with a SNP in a region near to *JAGGED1*, and carriers of this SNP showed a trend to have lower *JAGGED1* expression, again suggesting a tumor-suppressive role for the NOTCH pathway (41). Following on these observations, our analyses of *NOTCH* mutations from bladder cancer patients, mouse genetic models, cell-based assays, and human cancer samples provide solid evidence that the canonical NOTCH pathway plays a relevant tumor-suppressive role in bladder cancer. Importantly, as in other types of cancers, loss of function of the NOTCH pathway in the bladder is associated with SCCs.

Previous studies on mouse and human keratinocytes have proposed a mechanism for the tumor-suppressive activity of the NOTCH pathway in skin that relies in part on its role in terminal differentiation (42, 43). Specifically, in the absence of NOTCH signaling, keratinocytes do not fully differentiate and remain in a “stem-like” phenotype. In fact, in the esophagus, it has been demonstrated that lack of NOTCH signaling induces mutant clones that expand in a process known as field cancerization and that are at the origin of esophageal SCCs (44). A similar process could operate in the case of bladder cancer formation.

In our current study, we have found that inactivation of the NOTCH pathway induces EMT in squamous bladder cancer cells. Our mechanistic analyses imply that NOTCH signaling, through HES1, stabilizes epithelial features and prevents EMT in a cell-autonomous manner. We cannot exclude that other targets of the NOTCH pathway, like HEY1, could also contribute to the observed effects described here. In other cancer cellular contexts unrelated to stratified epithelia, such as prostate, breast, pancreas, lung adenocarcinoma, and others, the NOTCH pathway induces, rather than represses, EMT (45). Our data suggest that the repression of EMT could be a general tumor-suppressive mechanism of the NOTCH pathway in stratified epithelia. This, again, reflects the dual behavior of the NOTCH pathway, depending on the cellular type.

We and others have described antitumoral effects of pharmacological NOTCH inhibitors in preclinical models of T cell lymphoblastic leukemia, lung adenocarcinoma, pancreatic adenocarcinoma, and breast and intestinal tumors (46–50). Our data raise a note of caution on broad inhibition of the NOTCH pathway in the clinic, as this could concomitantly increase the incidence of tumors originated in stratified epithelia, such as bladder.

Methods

Further information can be found in the Supplemental Methods.

Animal experimentation. Mice were housed at the specific pathogen-free barrier area of the CNIO. Mice were observed on a daily basis and sacrificed when they showed overt signs of morbidity or tumors in accordance with the *Guidelines for Humane Endpoints for Animals Used in Biomedical Research*.

Microarray analysis. RNA was purified as described above and analyzed with an Agilent 2100 Bioanalyzer. Samples showing RNA integrity number above 8 were selected for microarray analysis. Genome-wide transcriptome experiments were performed using the Affymetrix HuGene-1_0-st-v1 microarray at the Genomics Facility of the Cancer Research Center (Salamanca, Spain) using standard procedures. Data sets have been deposited in GEO (accession no. GSE38264). The other two analyses described in Supplemental Figure 14 were performed using previously described available data sets (33–35).

Statistics. Unless otherwise specified, data are presented as mean \pm SEM. Two-tailed Student's *t* test was carried out to assess the significance of expression levels in qRT-PCR, luciferase assay, and mouse IHC data. Correlation of protein or RNA expression data in human samples was evaluated using the Spearman correlation coefficient. Survival curves were tested by the log-rank test. The percentage of squamous tumors between groups and the clustering of human bladder tumors were evaluated using Fisher's exact test. HES1 protein levels in human bladder tumors were tested using the Mann-Whitney test. *P* values lower than 0.05 were considered significant.

Study approval. All animal procedures were performed according to protocols approved by the CNIO-Instituto de Salud Carlos III (CNIO-ISCIII) Ethics Committee for Research and Animal Welfare. In the case of human samples, written informed consent was obtained from all patients, and the study was approved by the Ethical Committee for Clinical Research of each of the participating hospitals, namely, the University Hospital “12 de Octubre” and the hospitals participating in the consortium EPICURO (51).

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