

# Notch1 mediates growth suppression of papillary and follicular thyroid cancer cells by histone deacetylase inhibitors

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## Abstract

**Notch1 is a multifunctional transmembrane receptor that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts. We have previously shown that Notch1 may function as a tumor suppressor and that histone deacetylase (HDAC) inhibitors can induce Notch1 expression in some endocrine cancers. Here, we showed that although there was minimal Notch1 expression in follicular thyroid cancer FTC236 and papillary thyroid cancer DRO cells, transfection of constitutive Notch1 plasmid into these cells led to growth inhibition, down-regulation of cyclin D1, and up-regulation of p21. Treatment of FTC236 cells with HDAC inhibitors valproic acid (1–4 mmol/L) or suberoyl bishydroxamic acid (10–30  $\mu$ mol/L) induced functional Notch1 protein expression and suppressed cell growth in a dose-dependent manner. Notch1 siRNA interference blocked the antiproliferative effect of HDAC inhibitors. Western blot analysis revealed the reduction of cyclin D1 and the increase of p21 in HDAC inhibitor-treated cells. These results indicate that HDAC inhibitors activate Notch1 signaling in thyroid cancer cells and lead to the suppression of proliferation by cell cycle arrest. Our findings provide the first documentation of the role of Notch1 signaling as a tumor suppressor in DRO and FTC236 cells, suggesting that Notch1 activation may be a potential therapeutic target for papillary and follicular thyroid cancers. [Mol Cancer Ther 2009;8(2):350–6]**

Received 6/21/08; revised 11/10/08; accepted 11/19/08; published OnlineFirst 02/03/2009.

**Grant support:** American Cancer Society Research Scholars grant 05-08301TBE; NIH grants CA117117 and CA109053; American College of Surgeons George H.A. Clowes, Jr., Memorial Research Career Development Award; Carcinoid Cancer Foundation Research grant, and the Society of Surgical Oncology Clinical Investigator Award.

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doi:10.1158/1535-7163.MCT-08-0585

## Introduction

Papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC) are the two most common thyroid cancers. They account for 90% to 95% of all thyroid malignancies and are most often seen in patients over 40 years of age (1). The tumor usually presents as an asymptomatic solitary intra-thyroid nodule. In patients with FTC, distant metastases at the time of diagnosis are reported in 11% to 20% of patients (2). FTC tends to metastasize hematogenously to lung and bone, whereas PTC is commonly metastatic to regional lymph nodes and lung. Synchronous lung metastasis in both types of cancers has been reported in ~20% of cases, with a mean age at presentation of >50 years (3).

Despite being classified collectively as well-differentiated thyroid carcinoma, PTC and FTC have distinct clinicopathologic features, biological behavior, and clinical outcome (4, 5). FTC is generally considered to be a more aggressive tumor than PTC and is associated with a worse prognosis. Patients with FTC often present with more advanced stage diseases and a higher incidence of distant metastases because of the propensity of vascular invasion. In contrast, lymph node metastasis is quite uncommon in FTC, with an average incidence of <10%. Thyroid cancer management has not changed substantially during the past decades. Treatment is based on total thyroidectomy, ablative doses of radioiodine, and suppressive treatment. The follow-up is based on measuring serum thyroglobulin and imaging with radioiodine scans.

Although the prognosis of patients with differentiated thyroid cancer is good, and most patients survive long disease-free intervals after appropriate thyroid surgery (6, 7) and, when necessary, radioiodine I<sup>131</sup> therapy (8), in a subset of patients, thyroid cells lack or lose the capacity to concentrate radioiodine over time. Lack of radioiodine uptake in thyroid cancer is usually associated with increased growth rate and larger tumor load. This is seen in ~50% of patients with distant metastases. Therefore, alternative strategies for the treatment of the metastatic papillary and FTCs are needed.

Notch1 is a multifunctional transmembrane receptor that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts (9–11). In human cancer cells, Notch1 acts as either a tumor suppressor or an oncogene with its role being dependent on its cellular context (12). The oncogenic role of Notch1 was identified in many types of cancer, including pancreatic cancer, colon cancer, non-small cell lung cancer, cervical cancer, renal cell carcinoma, and several lymphomas (13–15). However, we have shown the absence of active Notch1 in neuroendocrine tumors (NET), and that overexpression of Notch1 leads to tumor growth suppression (16). Moreover, the degree of growth inhibition is directly proportional to the amount of Notch1 present. Histone deacetylase (HDAC)

inhibitors are a class of potent antineoplastic agents that induce differentiation, growth arrest, and apoptosis of transformed cells. Very recently, we have shown that treatment of NET cells with the HDAC inhibitors valproic acid (VPA) and suberoyl bishydroxamic acid (SBHA) led to growth suppression associated with Notch1 activation (17–20). Moreover, a significant reduction in NET markers is mediated by Notch1 activation in HDAC inhibitor-treated cells. These findings indicate that activation of Notch1 signaling may have a therapeutic role in treating NETs.

In this study, we report that Notch1 is highly expressed in normal human thyroid tissue but markedly down-regulated in metastatic thyroid cancer specimens. Similarly, Notch1 signaling is minimal in metastatic PTC and FTC cells. Treatment of FTC cells with HDAC inhibitors VPA and SBHA resulted in a dose-dependent increase in Notch1 protein. Furthermore, a dose-dependent reduction in growth was also observed. Transfection of siRNA against Notch1 blocked the antiproliferative effect of HDAC inhibitors. These findings provide the first documentation of the role of Notch1 signaling as a tumor suppressor in thyroid cancer cells; suggesting that Notch1 activation may be a potential therapeutic target for PTCs and FTCs.

## Materials and Methods

### Cell Culture

DRO and FTC236 cells provided by Dr. Fiemu Nwariaku (UT Southwestern, Dallas, TX) were maintained in DMEM/Ham's F-12 (1:1; Invitrogen) medium supplemented with 10% FCS, 0.01 units/mL of thyroid-stimulating hormone, 10 µg/mL of insulin (Sigma-Aldrich), and 50 µg/mL of penicillin/streptomycin (Invitrogen). WI-38 cells, a human embryonic lung fibroblast, were maintained in minimal essential medium (Eagle) with 10% FCS.

### Notch1 Plasmid Transfection

A constitutive NICD plasmid (TAN1, kindly provided by Dr. Douglas W. Ball, Johns Hopkins University) or a vector plasmid LNCX1 as control was transiently transfected into DRO and FTC236 cells using LipofectAMINE 2000 (Invitrogen) as described previously (16). Next day, the cells were trypsinized, counted, and plated in equal amounts (5,000 cells/well) onto 24-well plates for a cell proliferation assay.

### Cell Proliferation Assay

Cell proliferation was measured by the methylthiazolyl-diphenyl-tetrazolium bromide (MTT; Sigma-Aldrich) rapid colorimetric assay as previously described (16). Briefly, 5,000 cells were seeded in quadruplicate on 24-well plates and incubated overnight under standard conditions to allow cell attachment. The cells were then treated with VPA (Sigma-Aldrich) in concentrations of 0 to 4 mmol/L or with SBHA (Sigma-Aldrich) in concentrations of 0 to 30 µmol/L and incubated for up to 6 days with changes in the medium every 2 days. The MTT assay was done by replacing the standard medium with 250 µL of serum-free medium containing MTT (0.5 mg/mL) and incubating at 37°C for 3 h. After incubation, 750 µL of DMSO (Fisher Scientific)

was added to each well and mixed thoroughly. The plates were then measured at 540 nm using a spectrophotometer (µQuant; Bio-Tek Instruments).

### Western Blot Analysis

Whole cell lysates were prepared as previously described (16). Total protein concentrations were determined using a bicinchoninic acid assay kit (Pierce Biotechnology). Denatured cellular extracts (30–50 µg) were resolved by SDS-PAGE, transferred onto nitrocellulose membranes (Bio-Rad), blocked in milk and incubated with the appropriate antibodies. The primary antibodies dilutions were 1:2,000 for Notch1 (Santa Cruz Biotechnology), cyclin D1, p21, p15, p27, and acetyl-histone H4 (AH4; Cell Signaling Technology); and 1:10,000 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Trevigen). Horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse secondary antibodies (Cell Signaling Technology) were used depending on the source of the primary antibody. For visualization of the protein signal, ImmunoStar (Bio-Rad Laboratories) or SuperSignal West Femto (Pierce Biotechnology) kits were used according to the instructions of the manufacturer.

### Luciferase Reporter Assay

Cells were transiently transfected with luciferase constructs as previously described (16). Wild-type (4xwt CBF1Luc; 2µg) CBF-1 luciferase reporter plasmids (a generous gift from Dr. Diane Hayward, Johns Hopkins University, Baltimore, MD) were cotransfected with cytomagalovirus β-galactosidase (CMV-β-gal; 0.5 µg; ref. 21). After transfection, cells were treated with VPA (2 mmol/L and 4 mmol/L), SBHA (20 µmol/L) or DMSO as control for 48 h. Cells were harvested and lysed, luciferase and β-galactosidase assays (Promega) were done in accordance with the manufacturer's instructions. Luciferase levels were measured using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory). Luciferase activity was expressed relative to β-galactosidase activity.

### Notch1 siRNA Interference Assay

To determine the effect of Notch1 on cellular proliferation, siRNA against Notch1 and nonspecific siRNA (Santa Cruz Biotechnology) were transfected into thyroid cancer cells using LipofectAMINE 2000 (Invitrogen) as described previously (18). The next day, the cells were trypsinized, counted, and plated in equal amounts (5,000 cells/well) onto 24-well plates. On the following day, the cells were treated with DMSO as control or VPA (4 mmol/L) or SBHA (30 µmol/L), MTT assay was done on days 2, 4, and 6.

### Human Tumor Analysis

Human normal thyroid tissues and thyroid cancer specimens were obtained through a human subjects institutional review board-approved protocol. Samples were processed for total protein as previously described (16). Total proteins were analyzed for Notch1 and GAPDH expression by Western analysis as described above.

### Statistical Analysis

ANOVA with Bonferroni post hoc testing was done using a statistical analysis software package (SPSS version 10.0, SPSS). *P* < 0.05 was considered significant.

## Results

### Expression of Notch1 in Human Normal Thyroid and Thyroid Cancer Specimens

Normal human thyroid tissue and thyroid cancer samples were analyzed for the active Notch1 (NICD) protein by Western blot. As shown in Fig. 1A, normal human thyroid tissues (*lanes 1 and 5*) have high levels of NICD protein (ratio of 1.0 when compared with GAPDH). Three primary thyroid cancers [two PTCs (*lanes 2 and 3*) and one FTC (*lane 6*)] had much lower levels of NICD. Interestingly, a PTC tumor isolated from a lymph node metastasis had virtually no NICD protein (*lane 4*). These data illustrate that human thyroid cancers have minimal to no detected Notch1 protein, whereas normal human thyroid tissue has abundant Notch1, supporting the idea that Notch may play a tumor-suppressive role in thyroid cancer.

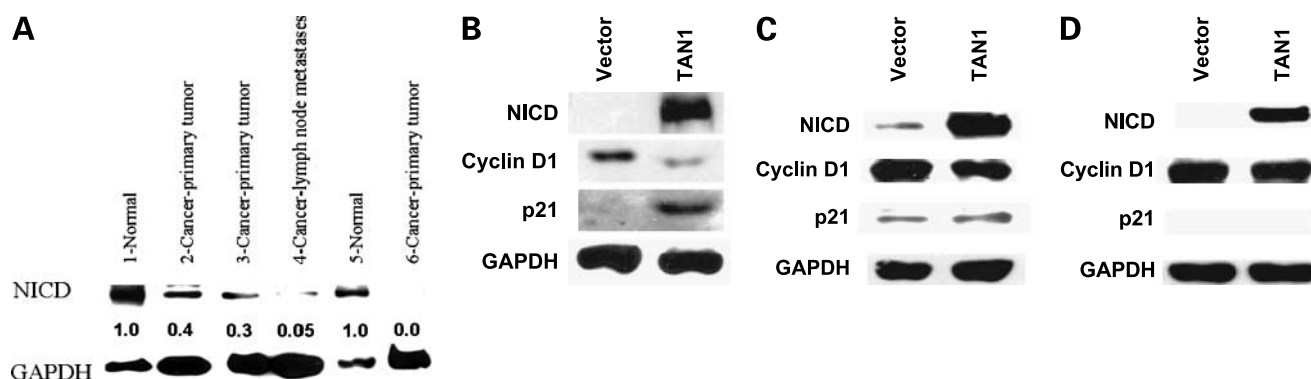
### Transfection of Thyroid Cancer Cells with the Notch1 Plasmid (TAN1) Lead to Notch1 Expression and Cell Growth Suppression

Similar to findings in human thyroid cancer specimens, we observed that there was a minimal amount of active Notch1 in PTC and FTC cells. To determine the role of Notch1, PTC DRO cells and FTC236 cells were transfected with the constitutive Notch1 plasmid and strong expression of Notch1 protein was induced as expected. Transfection of the Notch1 plasmid led to down-regulation of cyclin D1 and up-regulation of p21 (Fig. 1B and C). In addition, WI-38 fibroblast cells were also transfected with the NICD plasmid. Although high levels of Notch1 were achieved, there was no change in cyclin D1 and p21 levels (Fig. 1D). To determine how cell growth was affected by the NICD plasmid transfection and subsequent alteration of cyclin D1 and p21, we did MTT assays. Overexpression of Notch1 in thyroid cancer cells FTC236 (Fig. 2A) resulted in a

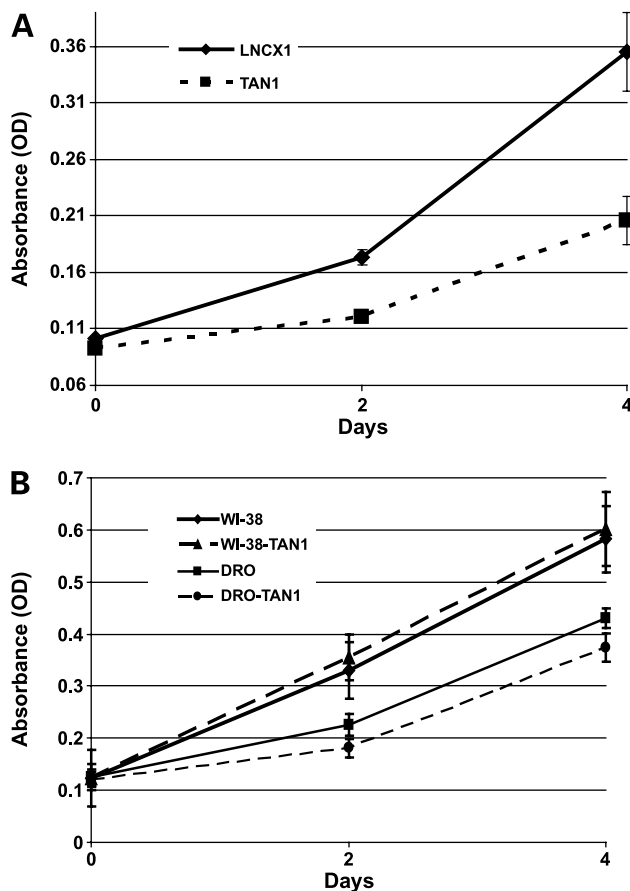
significant reduction in cellular proliferation, whereas DRO showed statistically significant marginal reduction with (Fig. 2B). Importantly, Notch1 overexpression had no effect on the growth of WI-38 (Fig. 2B), suggesting that the growth-suppressive effects of Notch1 are cell type-specific.

### VPA and SBHA Induce Active Notch1 Protein in Thyroid Cancer Cells

Based on our recent reports describing the ability of HDAC inhibitors to increase Notch1 protein levels in NET cells (17–20), we hypothesized that treatment of thyroid cancer cells with VPA and SBHA would also activate the Notch1 pathway. To assess the effects of VPA and SBHA on Notch1 protein expression, we performed Western blot analysis. As shown in Fig. 3A and B, both VPA and SBHA led to an increase of Notch1 in thyroid cancer cells. The levels of Notch1 increased with greater concentrations of VPA and SBHA in a dose-dependent manner. Interestingly, even at very low concentrations, VPA (1 mmol/L) and SBHA (10  $\mu$ mol/L) treatments led to increases in cleaved Notch1 protein. Consistent with their activity to induce Notch1 expression, VPA and SBHA inhibited HDAC and led to increased expression of AH4 in a dose-dependent manner (Fig. 3A and B). Although treatment with HDAC inhibitors resulted in an increase in the cleaved form of Notch1 by Western blotting, we wanted to determine if this protein was functional. Thus, we carried out a standard CBF-1 binding luciferase reporter assay. Active Notch1 binds with CBF-1 and other proteins to form a DNA-binding complex. This complex activates the transcription of target genes. As shown in Fig. 3C, 2 and 4 mmol/L of VPA treatment of thyroid cancer cells transfected with the luciferase construct resulted in a 2-fold and 4-fold increase in relative luciferase activity, respectively, whereas 20  $\mu$ mol/L of SBHA treatment of thyroid cancer cells resulted in a 6-fold increase over controls, indicating that



**Figure 1.** **A**, Western blot of human normal thyroid and thyroid cancer specimens for active, cleaved Notch1 (NICD). These levels were then quantitated by densitometry and compared with GAPDH. Although normal human thyroid tissue has abundant NICD, thyroid cancer specimens have significantly reduced levels of Notch1. *Lane 1*, normal thyroid; *lane 2*, papillary thyroid cancer-primary tumor; *lane 3*, papillary thyroid cancer-primary tumor; *lane 4*, papillary thyroid cancer from a lymph node metastasis; *lane 5*, normal thyroid; *lane 6*, FTC-primary tumor. FTC236 (**B**), DRO (**C**), and WI-38 (**D**) cells analyzed by Western blot for the expression of Notch1, cyclin D1, and p21 after transient transfection with a constitutive active Notch1 intracellular domain (NICD) plasmid. Although there was minimal Notch1 expression in the control cells, transfection of the Notch1 plasmid induced strong expression of Notch1 in all cells. However, down-regulation of cyclin D1 and up-regulation of p21 was only observed in the thyroid cancer cell lines (FTC236 and DRO) but not in WI-38 cells. Equal loading was confirmed with GAPDH.



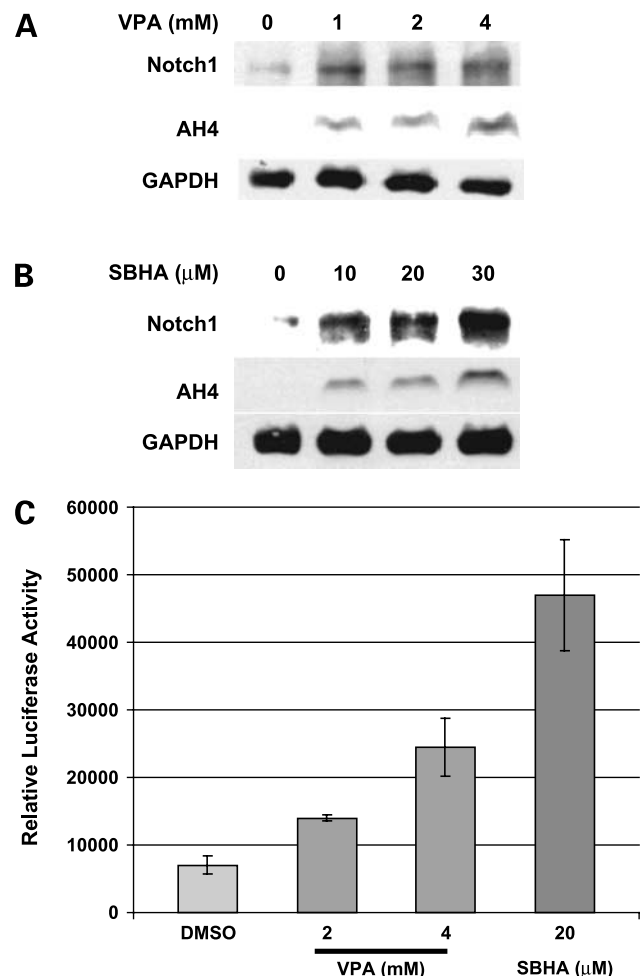
**Figure 2.** MTT cellular proliferation assays. Transient transfection of the NICD plasmid into FTC236 (A) and DRO (B) cells resulted in the suppression of cellular proliferation. There was no effect on WI-38 cellular growth (B).

the increase in CBF-1 binding was a result of the induction of active, cleaved Notch1 (NICD). This result is consistent with the amount of Notch1 produced by HDAC inhibitor treatment.

#### Notch1 Activation Suppresses the Growth of Thyroid Cancer Cells *In vitro*

HDAC inhibitor treatment has been shown to inhibit the growth of a variety of human cancer cells, including multiple myeloma (22), lymphoid cancers (23), malignant glioma (24), medulloblastoma (25, 26), neuroblastoma (27), endometrial cancer (28), cervical cancer (29), and ovarian cancer (30). Our previous reports showed that induction of Notch1 by HDAC inhibitors contributed to cell growth inhibition in medullary thyroid cancer (19, 20) and carcinoid cancer (17, 18). We used the MTT assay to measure cell viability after VPA and SBHA treatment in follicular-derived thyroid cancer cells. Thyroid cancer cells treated with VPA had a profound dose-dependent inhibition of growth (Fig. 4A). Statistically significant growth inhibition was also seen in thyroid cancer cells treated with SBHA (Fig. 4B;  $P < 0.05$ ).

Next, we did RNA interference assays, as described, to determine if HDAC inhibitor-mediated growth inhibition was dependent on Notch1 induction. Thyroid cancer cells were transiently transfected with siRNA against Notch1 or nonspecific siRNA. In the presence of nonspecific siRNA, VPA and SBHA treatment led to cellular growth inhibition as shown above. However, Notch1 siRNA blocked the antiproliferative effect of VPA and SBHA (Fig. 4C and D). These data strongly support the antiproliferative effect of Notch1 on thyroid cancer cells, and suggest that the growth inhibition seen with VPA and SBHA treatment were mediated by Notch1 signaling.



**Figure 3.** Expression of Notch1 and AH4 in thyroid cancer cells treated with HDAC inhibitors. There was a minimal level of Notch1 at baseline in thyroid cancer FTC236 cells. Treatment with VPA (A) and SBHA (B) led to the induction of cleaved Notch1 (NICD) protein in a dose-dependent manner. VPA and SBHA inhibit HDAC, resulting in the consistent expression of AH4 in a dose-dependent manner. Equal loading was confirmed with GAPDH. C, a luciferase assay done on FTC236 cells cotransfected with wild-type CBF-1-luciferase reporter plasmids and cytomegalovirus  $\beta$ -galactosidase plasmid. Luciferase activity was expressed relative to  $\beta$ -galactosidase activity. VPA treatment of FTC236 cells (2 and 4 mmol/L) resulted in a 2-fold and 4-fold increase in relative luciferase activity, respectively, whereas 20  $\mu$ mol/L of SBHA treatment of FTC236 cells resulted in a 6-fold greater relative luciferase activity. DMSO served as a control.

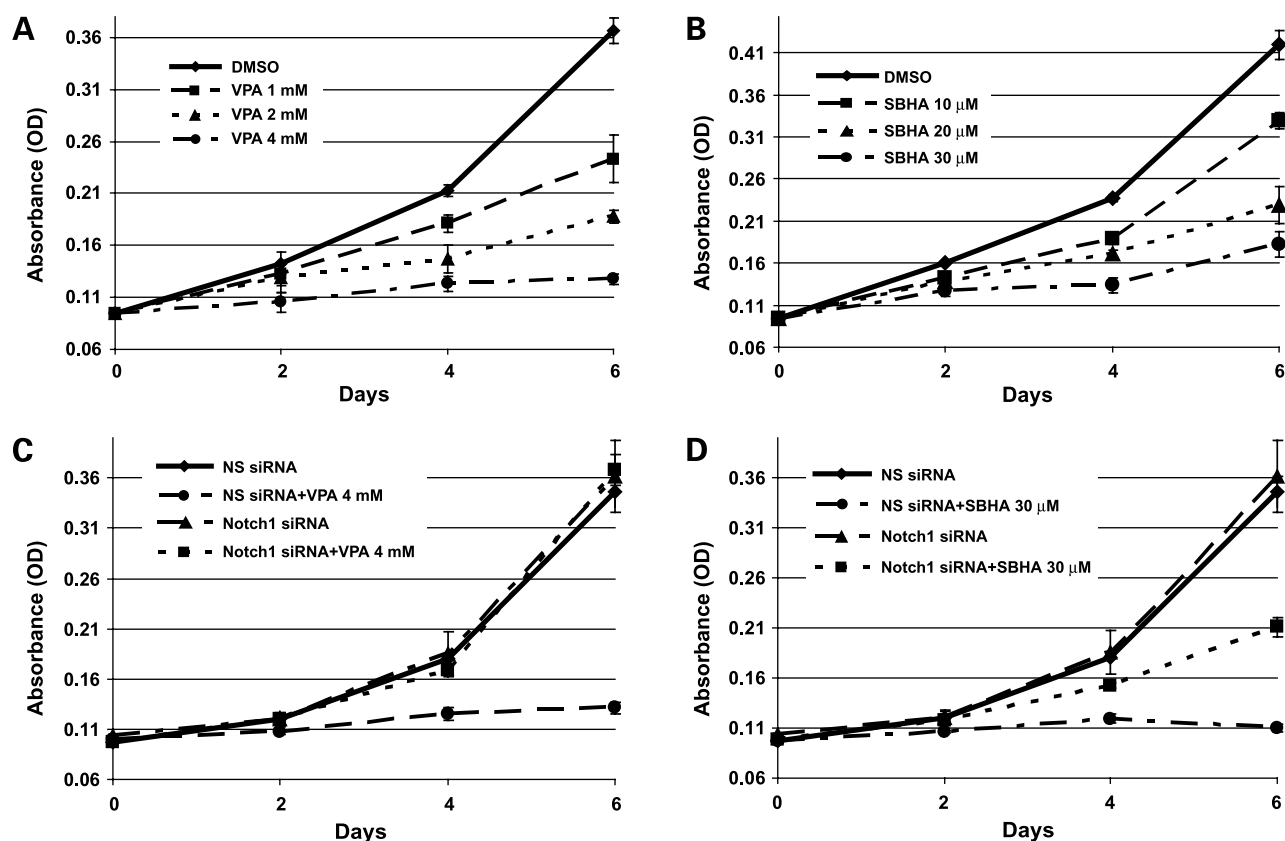
### Notch1 Activation Induces Thyroid Cancer Cell Growth Inhibition through Cell Cycle Arrest

After establishing that Notch1 activation inhibits cellular proliferation in thyroid cancer cells, we were interested in determining the mechanism of action of this effect. We did Western blot analyses after 2 days of treatment with VPA to measure the effect of the drug on cell cycle-related gene expression. Treatment of thyroid cancer cells with VPA resulted in an increase in protein levels of the cyclin-dependent kinase inhibitors p21, p15, and p27 (Fig. 5A). The cell cycle promoter cyclin D1 was also suppressed by VPA.

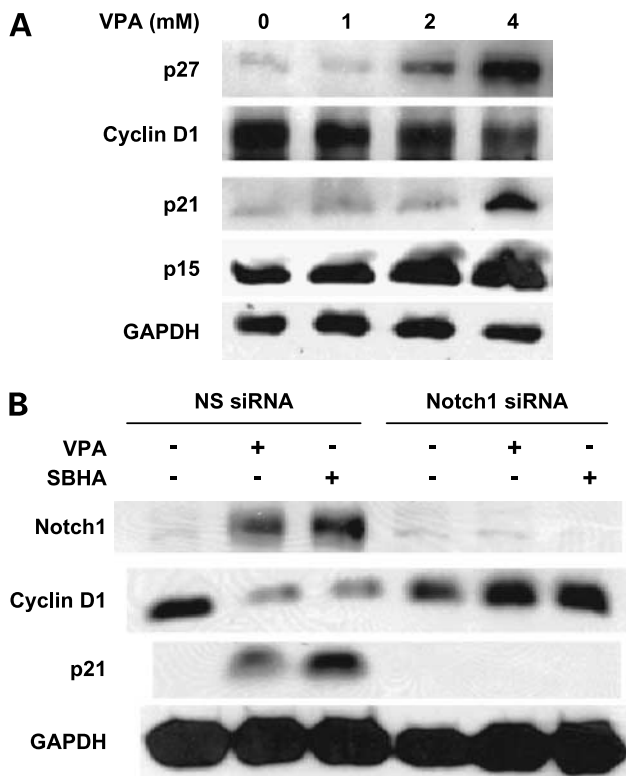
We next did siRNA interference assays to determine the effect of Notch1 blockage on the expressions of cell cycle-related genes. In the presence of nonspecific siRNA, VPA and SBHA treatment led to Notch1 expression as previously shown (Fig. 5B, lanes 2 and 3). Notch1 siRNA completely blocked Notch1 induction by VPA or SBHA (Fig. 5B, lanes 5 and 6). The down-regulation of cyclin D1 and up-regulation of p21 (Fig. 4B, lanes 2 and 3) induced by VPA and SBHA were also blocked in cells transfected with Notch1 siRNA (Fig. 5B, lanes 5 and 6). Again, these data strongly indicated that the cell cycle arrest effects of HDAC inhibitors were mediated by Notch1 signaling.

### Discussion

Follicular-derived thyroid cancers are usually sensitive to conventional therapy. The prognosis of most patients with differentiated thyroid cancer is very good (6, 7), and most patients survive long disease-free intervals after appropriate thyroid surgery, and when necessary, radioiodine  $I^{131}$  therapy (8). Unfortunately, this is not true for 30% of the cases, which develop toward dedifferentiation. The dedifferentiation process can lead to poorly differentiated thyroid cancer cells, which undergo a reduction in or disappearance of thyroid-stimulating hormone receptor and thyroglobulin expression, and therefore, lose their ability to capture iodine. Lack of radioiodine uptake in thyroid cancer is usually associated with increased growth rate and larger tumor load and is seen in ~50% of patients with distant metastases (31). The ability to capture iodine is clinically important because it is associated with the tumors' sensitivity to radioiodine therapy. In many patients with identifiable  $I^{131}$ -negative lesions, surgery and radiotherapy may not be feasible owing to inoperability, previous radiotherapy, or additional distant metastases. Meanwhile, conventional chemotherapy is often ineffective. Thus, alternative treatment options are needed.



**Figure 4.** An MTT assay was used to measure cell viability after VPA and SBHA treatment of FTC236 cells. Cells treated with VPA had a profound dose-dependent inhibition of growth (A). Statistically significant growth inhibition was also seen in FTC cells treated with SBHA (B). After FTC236 cells were transiently transfected with siRNA against Notch1 or nonspecific siRNA, VPA (C) and SBHA (D) treatment led to cell growth inhibition in the presence of nonspecific siRNA. Although in the presence of Notch1 siRNA, the antiproliferative effects of VPA and SBHA were significantly blocked.



**Figure 5.** Effect of Notch1 activation on the expression of cell cycle-related regulators. **A**, treatment of FTC236 cells with VPA resulted in a dose-dependent increase of the cyclin-dependent kinase inhibitors p21, p15, and p27. The cell cycle promoter cyclin D1 was also suppressed by VPA. **B**, after FTC236 cells were transiently transfected with siRNA against Notch1 or nonspecific siRNA, VPA and SBHA treatment led to the up-regulation of p21 and down-regulation of cyclin D1 in the presence of nonspecific siRNA (lanes 2 and 3). However, in the presence of Notch1 siRNA, the changes induced by VPA and SBHA were significantly blocked (lanes 5 and 6). The RNA interference assay confirmed that the growth inhibition of FTC236 cells by VPA and SBHA was a result of activation of Notch1 signaling. Equal loading was confirmed by GAPDH.

Notch1 is a multifunctional transmembrane receptor that regulates cellular differentiation, development, proliferation, and survival in a variety of contexts. However, the role of Notch1 in cancer cells remains controversial. Recent studies show that Notch1 signaling can act as either a tumor suppressor or as a tumor promoter, transient expression of active Notch1 in small cell lung cancer, pancreatic carcinoid, and prostate cancer cells inhibits cell growth *in vitro* (see review in ref. 12). We have shown that stable expression of Notch1 (NICD) in pancreatic carcinoid BON and TT cells (16, 32) leads to growth inhibition and reduction in neuroendocrine hormone production. However, the role of Notch1 signaling in follicular-derived thyroid cancer cells has, until now, not been described.

In this study, we have shown that active Notch1 (NICD) is minimal in thyroid cancer cells. NICD induction by VPA and SBHA led to a dose-responsive increase in functional Notch1 protein production as measured by Western blot and CBF1 binding studies, resulting in activation of the

Notch1 pathway. Furthermore, continuous Notch1 activation in thyroid cancer cells inhibited tumor cell growth. Notably, this growth reduction was dependent on the levels of Notch1 protein present. RNA interference experiments confirmed that these effects of VPA and SBHA on cell proliferation are mediated by Notch1 signaling. Our findings provide the first documentation of the role of Notch1 signaling as a tumor suppressor in thyroid cancer cells.

Moreover, the findings of this study suggest that the strong inhibition of thyroid cancer cell growth by Notch1 activation may be due to alterations in cell cycle-related gene expression. p21 is a universal inhibitor of cyclin-dependent kinases. The observations of up-regulation of p21 and down-regulation of cyclin D1 following the activation of Notch1 suggest that growth inhibition of FTC cells may be due to cell cycle arrest.

HDAC inhibitors comprise a diverse group of structurally heterogeneous compounds that exert antineoplastic effects in a variety of cancers *in vitro* and *in vivo* including multiple myeloma, lymphoid cancer, malignant glioma, neuroblastoma, cervical and ovarian cancer, and melanoma. Our group has shown that VPA or SBHA inhibits a variety of NETs, including medullary thyroid cancer (19, 20) and gastrointestinal and lung carcinoid cancer (17, 18) through the induction of Notch1. To our knowledge, this article is the first report indicating the activation of Notch1 by HDAC inhibitors and cell growth suppression in follicular-derived thyroid cancer cells. Based on these findings, a clinical trial will be initiated at our institution to evaluate the effectiveness of activating Notch1 signaling by HDAC inhibitors for the treatment of patients with advanced and poorly differentiated thyroid cancer.

## Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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