Notch1-Mediated Tumor Suppression in Cervical Cancer with the Involvement of SST Signaling and Its Application in Enhanced SSTR-Targeted Therapeutics

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ABSTRACT

The role of Notch signaling in cervical cancer is seemingly controversial. To confirm the function of Notch signaling in this type of cancer, we established a stable Notch1-activated cervical cancer HeLa cell line. We found that Notch1 activation resulted in apoptosis, cell cycle arrest, and tumor suppression. At the molecular level, we found that a variety of genes associated with cyclic AMP, G protein-coupled receptor, and cancer signaling pathways contributed to Notch1-mediated tumor suppression. We observed that the expression of somatostatin (SST) was dramatically induced by Notch1 signaling activation, which was accompanied by enhanced expression of the cognate SST receptor subtype 1 (SSTR1) and SSTR2. Certain genes, such as tumor protein 63 (*TP63*, *p63*), were upregulated, whereas others, such as B-cell lymphoma 2 (*BCL-2*), *Myc*, *Akt*, and *STAT3*, were downregulated. Subsequently, knockdown of Notch1-in-

duced SST reversed Notch1-induced decrease of *BCL-2* and increase of *p63*, indicating that Notch1-induced tumor suppression may be partly through upregulating SST signaling. Our findings support a possible crosstalk between Notch signaling and SST signaling. Moreover, Notch-induced SSTR activation could enhance SSTR-targeted cancer chemotherapy. Valproic acid (VPA), a histone deacetylase inhibitor, suppressed cell growth and upregulated the expression of Notch1 and SSTR2. A combination therapy with VPA and the SSTR2-targeting cytotoxic conjugate CPT-SST strongly led to greater suppression, as compared to each alone. Our findings thus provide us with a promising clinical opportunity for enhanced cancer therapy using combinations of Notch1-activating agents and SSTR2-targeting agents. *The Oncologist* 2012;17:220–232

INTRODUCTION

The Notch gene family encodes single-pass, heterodimeric type I transmembrane receptors. Notch signaling is activated by the delta, serrate, lag-2 family of transmembrane ligands between two neighboring cells and involves cell–cell commu-

nication [1]. Notch signaling is highly conserved and plays a key role in the determination of cell fate, such as proliferation, differentiation, and apoptosis [1, 2]. Notch receptors and ligands are often aberrantly expressed in a variety of cancers. Notch signaling was found to be involved in the pathogenesis

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of many human cancers [3–6]. This signaling is highly cell and context specific [4, 6–8], and *Notch* can act as either a tumor suppressor or an oncogene [3, 9–11]. Notch signaling has been reported to play an oncogenic role in breast cancer [3, 8, 12], colorectal cancer [8, 12], ovarian cancer [3], pancreatic cancer [3, 8], melanoma [8], prostate cancer [13], leukemia, and lymphoma, etc. [8, 12, 14], but it plays a tumor-suppressive role in certain other cancers, such as cutaneous squamous cell carcinomas [15], pancreatic carcinoid (BON cells) [16, 17], skin cancer [3], small cell lung cancer [3], and medullary thyroid cancer [3, 18]. However, the opposite effect of Notch signaling has been observed in the same cancer cell lines [19–23]. Thus, *Notch* gene function in regulating cancer progression is unpredictable and Notch-mediated mechanisms remain unclear [9, 24, 25].

Cervical cancer is the second leading cause of cancer death in women worldwide. The high-risk human papillomavirus (HPV) is the most significant risk factor for developing cervical cancer [1, 22, 24]. However, HPV is a necessary, but not sufficient, cause for invasive cancer development [22, 26]. Other factors, such as Notch [27], nuclear factor (NF)- κ B [27], phosphatidylinositol 3-kinase catalytic subunit (PIK3CA) [28], cyclo-oxygenase (COX)-2 [29], R-Ras [30], and Ras homolog C (RhoC) [31], are also known to be involved. Furthermore, Notch signaling may have different roles during early and late stages of cervical cancer development [1]. Notch1 is upregulated in the early stages of cervical cancer [32–34]. However, Notch1 is reduced in late stages. Activated Notch1 signaling can suppress the growth of cervical cancer cells such as HeLa, SiHa, and CaSki cells [22]. Similar results were shown in HeLa cells [20, 21, 35]. However, the opposite effect of Notch signaling was observed in the same cancer cells. For example, in other cases, knockdown of Notch signaling resulted in growth arrest of HeLa and CaSki cells [19, 23]. Thus, the role of Notch signaling in cervical cancer development needs to be carefully evaluated. In our present study, we established a stable Notch1-activated cell line to confirm whether *Notch1* functions as either an oncogene or a tumor suppressor in cervical cancer and whether or not the signaling of G protein-coupled receptors (GPCRs) is involved.

GPCRs play important roles in modern drug development. G protein-coupled somatostatin (SST) receptors (SSTRs) have been found to be highly expressed in many cancer cells [36–39], and activated SST signaling has been applied to anticancer drug development [40–42]. Is there any connection between Notch signaling and SST signaling? Unfortunately, there are no reports and no evidence to prove the connection. High incidences of SSTR subtypes 1, 2, and 3 [43], Notch1, and its ligand jagged subtype 1 (JAG1) [2, 16, 27, 28] have been detected in human cervical cancer. SST signaling has been found to couple with multiple signal transduction pathways. Also, Notch signaling and SST signaling share certain common downstream pathways, such as the PI3K–Akt pathway [44, 45]. We hypothesized that there could be a correlation between Notch signaling and SST signaling in cervical cancer.

SST signaling has been shown to be significant in modern drug development. SST and its analogs act as endogenous inhibitory regulators of cellular functions such as cell proliferation and hormone release [46]. SST analogs have been applied in the treatment of certain cancers, especially endocrine tumors [47]. In particular, certain analogs have been applied as drug delivery vehicles for SSTR-targeted cancer therapy [40–42]. Therefore, a possible correlation between Notch signaling and SST signaling may open a new window for receptor-targeted drug development.

In our present study, a new cervical cancer HeLa cell line (HeLa-ICN1) with activated Notch1 signaling was established and used to investigate the effects of Notch signaling on tumor progression and SST signaling. We found that activated Notch1 signaling upregulated SST signaling and induced HeLa cell apoptosis, cell growth arrest, and tumor suppression. Furthermore, Notch1-mediated SST signaling was found to be involved in Notch-induced cell functions, suggesting that crosstalk exists between Notch signaling and SST signaling. The histone deacetylase (HDAC) inhibitor valproic acid (VPA), which activates Notch signaling in certain cancer cells, in combination therapy with the SSTR2-targeting cytotoxic CPT-SST conjugate was further investigated for treating tumors grown from cervical cancer cells.

MATERIALS AND METHODS

Plasmid Constructs and Virus Packaging

ICN1 (the active form of Notch1) was amplified by reverse transcription-polymerase chain reaction (RT-PCR) and inserted into retroviral vector carrying green fluorescent protein, pMSCV-GFP (pGFP). The new construct, named pICN1-GFP, and the vector, pGFP, were cotransfected with pVSV-G into a packaging cell line to achieve entire virus particles. These virus particles were measured for viral titers and then transduced into HeLa cells.

Cell Culture

Human cervical cancer HeLa cells from the American Type Culture Collection (Manassas, VA) and the newly established HeLa-GFP and HeLa-ICN1 cells were maintained in minimal essential medium with 10% fetal calf serum and were incubated at 37°C in a 5% CO₂ atmosphere.

RT-PCR, Real-Time PCR, and PCR Assay

Total RNA was isolated from tumor cells as described in the kit protocol (Invitrogen, Carlsbad, CA). The primers [22, 48, 49] and PCR conditions for RT-PCR are shown in Table 1.

As for real-time PCR, the primers were the same as described in Table 1 except for the SSTR1 primers (forward, 5' ATC TGC TGG ATG CCT TTC TAC G 3'; reverse, 5' CAG GTG CCA TTA CGG AAG ACG 3') and SSTR2 primers (forward, 5' GAG AAG AAG GTC ACC CGA ATG G 3'; reverse, 5' TTG TCC TGC TTA CTG TCA CTC CGC 3'). Real-time PCR assays were performed on a Bio-Rad iCycler (Bio-Rad, Hercules, CA). Assays were set up using the iScript™ cDNA synthesis kit and iQ™ SYBR® Green supermix (Bio-Rad).

Table 1. The major primer sequences for PCR amplification used in this study

Receptor	Primers	PCR conditions	PCR product (bp)	Reference or GeneBank No.
SST	F: 5' CCA ACCAGA CGG AGA ATG ATG C 3' R: 5' TTA GGG AAG AGA GAT GGG GTG TGG 3'	35 cycles (95°C, 40 seconds; 64°C, 30 seconds; 72°C, 30 seconds)	243	J00306
SSTR1	F: 5' AAA TGC GTC CCA GAA CGG GAC CT 3' R: 5' CAG GTT CTC AGG TTG GAA GTC TT 3'	35 cycles (95°C, 30 seconds; 65°C, 30 seconds; 72°C, 60 seconds)	993	[48]
SSTR2	F: 5' GAT GAT CAC CAT GGC TGT G 3' R: 5' CAG GCA TGA TCC CTC TTC 3'	35 cycles (95°C, 30 seconds; 55°C, 30 seconds; 72°C, 60 seconds)	892	[48]
SSTR3	F: 5' TCA TCT GCC TCT GCT ACC TG 3' R: 5' GAG CCC AAA GAA GGC AGG CT 3'	35 cycles (95°, 40 seconds; 65°C, 30 seconds; 72°C, 30 seconds)	221	[48]
SSTR4	F: 5' CAT GGT CGC TAT CCA GTG CA 3' R: 5' GTG AGA CAG AAG ACG CTG GTG AAC AT 3'	35 cycles (95°, 20 seconds; 60°C, 30 seconds; 72°C, 30 seconds)	276	[48]
SSTR5	F: 5' GCT CTT GGT GTT CGC GGA CGT 3' R: 5' CAG GTT GAC GAT GTT GAC GGT GAA G 3'	35 cycles (95°, 40 seconds; 65°C, 30 seconds; 72°C, 30 seconds)	298	[48]
Notch1 ^a	F: 5' GGC CAC CTG GGC CGG AGC TTC 3' R: 5' GCG ATC TGG GAC TGC ATG CTG 3'	35 cycles (95°C, 40 seconds; 65°, 30 seconds; 72°C, 30 seconds)	365	[22]
Notch2 ^a	F: 5' GGC CCC CTG CCC ACC ATG TAC 3' R: 5' CCC GCT GAC CTC CTC CAG C 3'	35 cycles (95°, 40 seconds; 65°C, 30 seconds; 72°C, 30 seconds)	343	[22]
Notch3 ^a	F: 5' TTC TTA GAT CTT GGG GGC CT 3' R: 5' GGA AGA AGG AGG TCC CAG AC 3'	35 cycles (95°, 40 seconds; 58°C, 30 seconds; 72°C, 30 seconds)	218	[49]
Notch4	F: 5' AGC AGA CAA ACT GCA GTG GA 3' R: 5' CTG TTG TCC TGG GCA TCT TT 3'	35 cycles (95°C, 40 seconds; 55°C, 30 seconds; 72°C, 30 seconds)	233	NM_004557
DLL1	F: 5' AGC ACG CAC CCT GCC ACA AT 3' R: 5' ACA GCC CAG CAG CAG CAT GA 3'	35 cycles (95°C, 60 seconds; 63.8°C, 30 seconds; 72°C, 60 seconds)	230	NM_005618
DLL3	F: 5' AAC AGC CCG GTG AAT GCC GA 3' R: 5' ACA CAA GCC GCC GTT GAA GCA 3'	35 cycles (95°, 60 seconds; 55°C, 30 seconds; 72°C, 60 seconds)	287	NG_008256
DLL4	F: 5' TGA TTC CTG CCG CCC AGC TT 3' R: 5' TGT AAC CGC AGT GGC GCC TT 3'	35 cycles (95°C, 60 seconds; 63.8°C, 30 seconds; 72°C, 60 seconds)	205	NM_019074
JAG1	F: 5' AAC GAC CGC AAC CGC ATC GT 3' R: 5' AAA GTG GGC AAC GCC CGT GT 3'	35 cycles (95°C, 60 seconds; 58.9°C, 30 seconds; 72°C, 60 seconds)	195	NM_000214
JAG2	F: 5' TGT GGT GCG GAT GGA AGC CT 3' R: 5' AAT GCA AGG TGA GGC GGG CA 3'	35 cycles (95°C, 60 seconds; 63.8°C, 30 seconds; 72°C, 60 seconds)	219	NM_002226

^aPrimer sequences are as described in the references; PCR conditions are optimized in our laboratory.

Abbreviations: DLL, delta-like; JAG, jagged; F, forward; PCR, polymerase chain reaction; R, reverse; SST, somatostatin; SSTR, SST receptor.

The cDNA synthesis was run for one cycle at 25°C for 5 minutes, 42°C for 30 minutes, and 85°C for 5 minutes and held at 4°C. PCRs were run in 20-μL reactions. Each 20-μL reaction contained 1 μL template (either cDNA or genomic or plasmid DNA), 10 μL iQ™ SYBR® Green supermix (Bio-Rad), and 1

μL of each of the corresponding forward and reverse primers. The PCR was further run under the following conditions: one cycle at 95°C for 5 minutes for initial denaturation and 40 cycles at 95°C for 30 seconds and 56°C (SSTR1) or 57°C (SSTR2) for 30 seconds for primer annealing and product

elongation. To further melt curve data collection and analysis, one cycle at 95°C for 1 minute and 55°C for 1 minute was run with an increase in the temperature in increments of 0.5°C from 55°C to 95°C. Experiments were finished by holding at 15°C. β -actin was used as an internal control. Results were calculated by applying the comparative C_T or $2^{-\Delta\Delta CT}$ method.

The PCR assay was performed as described in the kit instructions (SABiosciences Corporation, Frederick, MD). First, total RNA was isolated from HeLa cells by following the protocol of the Qiagen RNeasy® Mini Kit (Qiagen, Valencia, CA). For genomic DNA elimination, 5 μ g total RNA in 96-well plates, 2 μ L 5 $\times$ gDNA elimination buffer, and RNase-free water were added for a final volume of 10 μ L in a 0.5-mL tube. Genomic DNA elimination mixture was mixed gently, incubated at 42°C for 5 minutes, and put on ice immediately for at least 1 minute. Then, the RT cocktail was prepared by adding 4 μ L 5 $\times$ RT Buffer, 1 μ L primer and external control mix, 2 μ L RT enzyme mix, and RNase-free water for a final volume of 10 μ L. For the first-strand RT reaction, 10 μ L RT cocktail and 10 μ L Genomic DNA elimination mixture were mixed together and incubated at 42°C for exactly 15 minutes and the cDNA synthesis reaction was immediately stopped by heating at 95°C for 5 minutes. Ninety microliters double-distilled water (ddH₂O) was added to each 20 μ L of cDNA synthesis reaction. The real-time PCR was performed in a 96-well plate format. A total volume of 2,550 μ L of experimental cocktail was prepared by adding 1,275 μ L 2 $\times$ RT qPCR master mix (SABiosciences Corporation), 102 μ L diluted first-strand cDNA synthesis reaction, and 1,173 μ L ddH₂O. Into each well of the 96-well PCR assay plate was added 25 μ L experimental cocktail with an eight-channel pipettor. The real-time PCR detection was performed on a Bio-Rad CFX96 Real-Time System with the conditions of one cycle at 95°C for 10 minutes and 40 cycles at 95°C for 15 seconds and 60°C for 1 minute. Results were analyzed by applying the comparative C_T or $2^{-\Delta\Delta}$ method.

Western Blot Analysis

The protocol was employed as described by the manufacturer (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Briefly, cells were harvested, resuspended in Radioimmunoprecipitation assay buffer with cocktail inhibitors, homogenized by passing through a 21-gauge needle, mixed with loading buffer containing fresh dithiothreitol, and heated for 5 minutes at 95°C. Supernatants were loaded to run on 8%–16% Tris-glycine gel after centrifugation at 10,000g. Protein was transferred from the gel to a nitrocellulose membrane, which was then blocked with 5% fat-free milk, washed, and incubated with Notch1 (sc-6014-R), SSTR2 (sc-25676), and antibodies (Santa Cruz Biotechnology, Inc.). The membrane was washed again and incubated with second antibody (Santa Cruz Biotechnology, Inc.). Films were developed according to the ECL™ system protocol (Amersham Biosciences, Little Chalfont, UK).

Enzyme-Linked Immunosorbent Assay

The SST concentration in media and inside cells was measured using enzyme-linked immunosorbent assay (ELISA) follow-

ing the kit instructions (Phoenix Pharmaceuticals, Burlingame, CA). Simply, 50 μ L of controls, prepared samples, and prepared peptide standards were added to each well of a 96-well plate. Twenty-five microliters rehydrated primary antibody was added to each well, and the plate was gently tapped to ensure thorough mixing then incubated overnight at 4°C. Twenty-five microliters rehydrated biotinylated peptide was added to each well except for the blanks. The plate was incubated for 90 minutes at room temperature. The contents of each well were discarded. The plate was washed with 350 μ L of assay buffer four times. Then, to each well was added 100 μ L streptavidin-horseradish peroxidase. The plate was incubated for 1 hour at room temperature, washed, and dried. One hundred microliters of prepared substrate solution was added to each well, mixed well, and incubated for 15–20 minutes. Fluorescence was measured using a VICTOR™ Reader (PerkinElmer, Boston, MA) at wavelengths of 325 nm and 420 nm (excitation and emission).

Fluorescence Polarization Cyclic AMP Assay

The fluorescence polarization cyclin AMP (cAMP) assay (FPA202) was done following the manufacturer's protocol (PerkinElmer, Boston, MA). Simply, 10 μ L of forskolin at different concentrations was added to each well. Then, to each well was added 10 μ L of cells suspended in stimulation mix (made fresh before use by mixing anti-cAMP antibody in stimulation buffer). Plates were incubated 30 minutes at 37°C. Twenty μ L detection mix (Fluo-cAMP stock diluted in detection buffer) was added and incubation was continued for 30 minutes at 37°C. Meanwhile, the standard curve and controls were done. cAMP was measured by a VICTOR™ Reader (PerkinElmer).

Cell Proliferation Assay (MTT)

The cell proliferation assay (Promega, Madison, WI) was performed as described previously [50]. Briefly, 50 μ L medium with or without tested compounds was added to 96-well plates. Another 50 μ L of HeLa cells (HeLa-GFP or HeLa-ICN1) (1×10^5 cells/mL) suspended in culture medium was dispensed into each well. The plates were incubated at 37°C for 3 days. Afterward, 15 μ L dye solution per well was added to plates and they were incubated at 37°C for 4 hours. Then, to each well was added 100 μ L solubilization solution and the plates were incubated at 37°C again until the contents in each well became a uniformly colored solution. The plates were measured at 570 nm by a VICTOR™ Reader (PerkinElmer).

Cell Colony Formation Assay

The assay was done as described elsewhere [51]. Two hundred HeLa-GFP or HeLa-ICN1 cells were added to each well of a six-well plate. The plates were continuously incubated for 7–8 days until cell colonies were visible. Colonies were fixed with 100% methanol at room temperature for 15 minutes and washed with phosphate-buffered saline (PBS). Colonies were further stained with 0.1% crystal violet at room temperature for 1 hour, rinsed with water, and photographed after being air dried.

Cell Cycle Analysis

Cell cycle analysis was done using flow cytometry. Cells (2×10^6) were harvested, washed with PBS, and fixed in 70% ethanol overnight at -20°C . After centrifugation (5 minutes, 200g), cells were resuspended in 5 mL PBS, incubated for 60 seconds, and centrifuged for 5 minutes at 200g. Cell pellets were suspended in 1 mL PBS with 0.1% Triton-100, 20 μg propidium iodide (Sigma p4864; Sigma-Aldrich, St. Louis, MO), and 200 μg DNase-free RNase A (Sigma R6513), kept for 1 hour at room temperature, and sent to Tulane Cancer Center for flow cytometry analysis.

In Vivo Tumor Growth and Treatment

After being harvested in the exponential growth phase and washed three times with ice-cold PBS, cervical cancer HeLa cells (4×10^6 cells/100 μL per mouse) were implanted s.c. into the flanks of nude mice (5–7 weeks of age upon arrival) (National Cancer Institute, Frederick, MD), as described previously [50]. For tumors grown from HeLa-GFP (control) cells and HeLa-ICN1 (Notch1 active) cells, all mice were monitored weekly. For tumors treated with VPA and the cytotoxic SST conjugate (CPT-SST), tumor-carrying mice were separated into four groups ($n = 7-8$) and treated using s.c. injection that was applied to the flank opposite the tumor. A control group was injected with PBS, and three groups were treated with the compounds. One group received 1 mg/kg CPT-SST and one group received 200 mg/kg VPA. The last group was treated with 200 mg/kg VPA in combination with 1 mg/kg CPT-SST. All mice were injected once a day each weekday for 21 days starting 11 days postimplantation. Tumor volumes were measured and body weights were taken once a week. The data were analyzed using GraphPad Prism 4.0 (San Diego, CA) and *t*-test statistical analysis.

RESULTS

Expression of SST and SSTRs and Notch Receptors and Ligands in Normal HeLa Cells

We first investigated the expression levels of Notch receptors and ligands in parental HeLa cells using RT-PCR. Our results showed that the receptors Notch1 and Notch2 and the ligand JAG1 are easily detectable, whereas there was low or no expression of Notch3, Notch4, delta-like 1 (DLL1), DLL2, DLL4, and JAG2 (Fig. 1A, 1B). SSTR2 and SSTR5 were highly expressed in HeLa cells, with a lower level of SSTR1 expression and no or trace amounts of SST, SSTR3, and SSTR4 (Fig. 1C, 1D).

Generation of HeLa Cells with Activated Notch1 Signaling

To determine the potential effects of activated Notch1 signaling on cell growth and the involvement of signaling pathways, HeLa cells were transduced with retroviruses expressing the intracellular domain of Notch1 (ICN1) with the bicistronic expression of GFP as well as retroviruses that express GFP as controls. The pair of transduced cells, HeLa-ICN1 (Notch1 ac-

tivated) and HeLa-GFP (control) were sorted by fluorescence-activated cell sorting (FACS) for GFP expression and confirmed for ICN1 protein expression by Western blot analysis (Fig. 1E).

Activation of Notch1 Signaling Suppresses Cell Proliferation and Induces Apoptosis and Cell Cycle Arrest

We further evaluated the functions of Notch1 signaling in cervical cancer HeLa cells because of the fact that opposite effects of Notch1 signaling having been observed in the same cell lines [19–23]. We first determined if Notch1 signaling affects cell growth by performing in vitro cell proliferation assays. We found that activation of Notch1 signaling resulted in $>50\%$ inhibition (51.42%) of cell growth, compared with control cells (Fig. 2A). Meanwhile, cell colony formation assays showed that Notch1 signaling clearly reduced colony formation (Fig. 2B). Both HeLa-GFP and HeLa-ICN1 cells were further analyzed for apoptosis and cell cycling using FACS. The results in Table 2 and Figure 2 show that activated Notch1 signaling resulted in cell cycle arrest in the S phase, during which DNA damage often takes place (Table 2), and induced 16% cell apoptosis (Table 2 and Fig. 2C, 2D). Furthermore, the expression levels of cell proliferation, cell cycle, and apoptotic markers were assessed. For example, antiapoptotic BCL-2 and the proliferation marker proliferating cell nuclear antigen (PCNA) were downregulated but the tumor suppressor p63 and p21 were upregulated in HeLa-ICN1 cells (Table 3). Thus, Notch1 signaling plays a tumor suppressive role in cervical cancer HeLa cells.

Activation of Notch1 Signaling Suppresses Tumor Growth In Vivo

For the in vivo experiment, the results showed that activated Notch1 signaling (HeLa-ICN1) significantly suppressed HeLa tumor growth. At 34 days postimplantation, the tumor volume increased from $91.27 \text{ mm}^3 \pm 23.46 \text{ mm}^3$ to $1,445 \text{ mm}^3 \pm 534.6 \text{ mm}^3$ in the control group (HeLa-GFP) and from $88.56 \text{ mm}^3 \pm 11.05 \text{ mm}^3$ to $294.1 \text{ mm}^3 \pm 172.5 \text{ mm}^3$ in the HeLa-ICN1 group. The inhibitory rate associated with Notch1 activation was $>80\%$ (tumor weight, 86.18%; tumor volume, 84.82%) (Fig. 2E, 2F), indicating that Notch1 signaling is critical in regulating tumor suppression.

Notch1 Activation Stimulates Forskolin-Induced cAMP Accumulation

GPCRs have a huge family, including SSTRs. They are popularly investigated in modern drug research and development. cAMP is a critical second messenger of GPCRs. The cAMP assay was performed to determine whether or not the activation of Notch1 signaling affects cAMP accumulation in HeLa cells and to confirm further GPCR involvement. Forskolin, a receptor-independent cAMP activator that stimulates cAMP production via activating adenylate cyclase, was used for the investigation. Forskolin was found to stimulate cAMP production with a dose-dependent forskolin-stimulated increase in

Figure 1. Expression of SST receptors is mediated by Notch1 activation via RT-PCR, real-time PCR, ELISA, and Western blot assays. Notch1 activation has been demonstrated to upregulate the expression of SST, SSTR1, and SSTR2. RT-PCR was used to detect the expression of four Notch receptors (Lanes 1, 2, 3, and 4 show Notch1, Notch 2, Notch3, and Notch4, respectively) (A), five Notch ligands (Lanes 1, 2, 3, 4, and 5 show DLL1, DLL2, DLL4, JAG1, and JAG2, respectively) (B), five SSTRs (Lanes 1, 2, 3, 4, and 5 show SSTR1, SSTR2, SSTR3, SSTR4, and SSTR5, respectively) (C), and SST (Lanes 1 and 2 are HeLa-GFP and HeLa-ICN1 cells, respectively) (D) in cervical cancer HeLa cells. RT-PCR also showed the expression of Notch1-mediated SSTRs (Lane 1 and 2 show HeLa-GFP and HeLa-ICN1 cells, respectively) (H). Western blot assay showed high expression of the Notch1 active form ICN1 (anti-Notch1 antibody sc-6014-R) (E) and SSTR2 (anti-SSTR2 antibody sc-25676) (I) in the established stable HeLa cell line with activation of Notch1 signaling (HeLa-ICN1 cells) (Lane 1 and 2 show HeLa-GFP and HeLa-ICN1 cells, respectively). SST upregulation in Notch1-activated HeLa-ICN1 cells was demonstrated via real-time PCR at the mRNA level (F) and ELISA in medium (G).

Abbreviations: DLL, delta-like; ELISA, enzyme-linked immunosorbent assay; GFP, green fluorescent protein; JAG, jagged; PCR, polymerase chain reaction; RT-PCR, reverse transcription PCR; SST, somatostatin; SSTR, SST receptor.

both HeLa-ICN1 and HeLa-GFP cells. Treatment with forskolin at 1, 10, and 50 μ M resulted in the production of 0.005 pMol, 0.018 pMol, and 0.024 pMol cAMP per 10^3 cells in control HeLa cells and 0.05 pMol, 0.25 pMol, and 0.46 pMol cAMP per 10^3 cells in HeLa cells with activated Notch1 signaling (HeLa-ICN1), respectively. The cAMP production stimulated by forskolin in

HeLa-ICN1 cells was much higher than that in control HeLa-GFP cells (Fig. 3A). The cAMP concentration in HeLa-ICN1 cells was 10-, 14-, and 19-fold more than that in HeLa-GFP cells when treated with forskolin at 1, 10, and 50 μ M, respectively. Thus, the results support that activated Notch1 signaling may affect cAMP-associated signaling pathways.

Figure 2. Activation of Notch1 signaling induces apoptosis, antiproliferation, and antitumor growth. Cell proliferation assay (**A**) and cell colony formation assay (**B**) showed that Notch1 activation induces proliferation suppression (B1 and B2 are control HeLa-GFP and Notch1-activated HeLa-ICN1 cells, respectively). Flow cytometry assay showed that Notch1 activation (HeLa-ICN1) induced cell apoptosis (**D**), black arrow shows apoptosis) compared with control HeLa-GFP cells (**C**). The in vivo tumor growth assay further showed that Notch1 activation (HeLa-ICN1 cells) could induce suppression of cervical cancer HeLa cell tumor growth ((**E**), tumor volume; (**F**), tumor weight).

Abbreviation: GFP, green fluorescent protein.

Table 2. Cell cycle analysis on HeLa-ICN1 cells with Notch1 activation

Activity	HeLa-GFP (%)	HeLa-ICN1 (%)
Cell cycle progression		
G ₀ /G ₁	61.88 ± 3.13	49.67 ± 8.90
S	31.35 ± 2.43	41.05 ± 4.56
G ₂ /M	6.77 ± 0.93	9.27 ± 0.78
Apoptosis		16.84 ± 6.10
Proliferation	97.10 ± 1.35	53.96 ± 2.76

Establishment of a Gene Expression Profile in HeLa Cells with Activation of Notch1 Signaling

Based on the results above, we hypothesized that genes participating in the cAMP and GPCR signaling pathways might be involved in Notch1-mediated tumor suppression. In particular, we hypothesized that SST signaling may be involved because of the higher SST expression level observed in our initial investigation in HeLa-ICN1 cells. To test this hypothesis, we applied pathway-specific PCR assays to profile the expression of genes involved in cAMP/Ca²⁺ (PAHS-066A, SABsciences), GPCR (PAHS-071A) including SST and SSTR2, and cancer (PAHS-033A) signaling pathways.

We found that a panel of genes that are responsive to cAMP/Ca²⁺ and contain the cAMP response element, serum response element (SRE), or SRE-like enhancer sequences in their promoters were clearly mediated by Notch1 activation (Table 3). For example, some genes, such as *SST*, *FOS*, *COX-2*, *MMP2*, *MMP9*, *PIK3R1* (PI3K p85 α), *TWIST*, *THBS1*, *p21* (CDKN1A), *p27* (CDKN1B), and *p63*, were upregulated and some, such as *RB1*, *JunB*, *PCNA*, *STAT3*, *Akt* (*PKB*), and *MYC*, were downregulated (Table 3). Many of these genes are involved in regulating cell functions such as DNA repair, transcription, and the cell cycle. Furthermore, we found that some tested GPCR genes, such as *SSTR2*, *ADRB2*, and *AGTR2*, were obviously upregulated in HeLa-ICN1 cells. Plus, Notch1 activation affected various GPCR signaling-related pathways such as the mitogen-activated protein kinase (MAPK), PI3K/Akt1, protein kinase C, and Rho pathways and the Janus kinase–signal transducer and activator of transcription (JAK-STAT) cascade (PAHS-071A, SABsciences). Most of the signaling pathways mentioned above have been proven to be involved in SST–SSTR signaling transduction [43, 51, 52].

To understand the molecular mechanisms underlying Notch1's activities as a tumor suppressor, a focused panel of genes associated with the cancer signaling pathway (PAHS-033A) was further analyzed using PCR assays. Many genes (covered in the gene panel of cancer pathway PAHS-033A) related to apoptosis, proliferation, the cell cycle, signal transduction, and transcription factors were upregulated (such as *integrin- α 1*, *integrin- α 4*, *MMP2*, *MMP9*, and *TWIST1*) or downregulated (such as *BCL-2*, *MAP2K*, *p105-Rb*, and *E6* and

E7) in HeLa-ICN1 besides those genes mentioned above (Table 3). Conclusively, Notch1-mediated tumor suppression may be mediated through the signaling pathway cascade involved with the components of the cAMP/Ca²⁺, GPCR, and cancer signaling pathways.

Confirmation of the Regulation of SST Signaling Via Notch1 Activation

Our data demonstrated that SST and SSTR2, at the mRNA level, were upregulated by activation of Notch1 signaling. Herein, individual RT-PCRs and real-time PCRs were applied to further investigate the expression of SST and all five SSTR subtypes in HeLa-ICN1 cells. The results from RT-PCR showed significant greater expression levels of SST, SSTR1, and SSTR2, with a slightly lower level of SSTR3 and no obvious difference in SSTR4 and SSTR5 (Fig. 1H) in HeLa-ICN1 cells. Further, through a more precise real-time PCR, we found similar higher expression levels of SST (Fig. 1D), SSTR1, and SSTR2 as in the RT-PCR. Activated Notch signaling dramatically activated SST transcription to >200-fold higher in HeLa-ICN1 cells than in HeLa-GFP cells (Fig. 1F), with higher expression levels of SSTR1 (16-fold) and SSTR2 (21-fold) and lower expression levels of SSTR3 (fourfold), SSTR4 (twofold), and SSTR5 (twofold) (Table 3). Besides, we also found that Notch1 activation could lead to higher expression levels of certain other GPCRs, such as all three bombesin (BN) receptors gastrin releasing peptide receptor (GRPR), neuromedin B receptor (NMBR), and bombesin-like receptor 3 (BRS3), and two of three vasoactive intestinal peptide receptors (VPAC1 and VPAC2) (data not shown).

Activated SST signaling has been shown to suppress cell proliferation and tumor growth [43, 46, 47], especially the SST analogs commonly used to treat endocrine tumors. Thus, SST and SSTR2 upregulation mediated via Notch1 activation may play a role in Notch-mediated tumor suppression. We further confirmed the expression of SST and SSTR2 in Notch1-activated HeLa-ICN1 cells via ELISA, Western blot analysis, and binding assays. For SST expression, we did ELISAs and found higher expression of SST at the protein level, the same trend as in the results via real-time PCR. The SST concentration was over twofold higher in the HeLa-ICN1 culture medium (657 pg/mL) than in the HeLa-GFP culture medium (311 pg/mL), but it was undetectable in cell lysates (Fig. 1G).

For SSTR2 expression, we found >70% higher SSTR2 expression at the protein level in HeLa-ICN1 cells than in HeLa-GFP cells via Western blot (Fig. 1I). Moreover, our further binding assays displayed a >10% greater SSTR density on HeLa-ICN1 cell surfaces than on HeLa-GFP cells (data not shown).

Involvement of SST Signaling in Notch1-Mediated Signaling Pathways

We demonstrated that Notch1 activation induced HeLa cell apoptosis, proliferation suppression, and cell cycle arrest and affected the expression of the relevant gene markers with downregulation of *BCL-2* and *PCNA* and upregulation of *p63* and *p21*. We wondered whether or not SST signaling is directly

Table 3. Effects of Notch1 signaling activation on the expression of certain genes

Symbol	Description	Fold difference (ICN:GFP)	Change
Upregulated genes			
<i>ANGPT1</i>	Angiopoietin 1	8.98 ± 2.78	↑
<i>ANGPT2</i>	Angiopoietin 2	7.25 ± 1.33	↑
<i>CDKN1A</i>	Cyclin-dependent kinase inhibitor 1A (p21)	7.36 ± 1.79	↑
<i>CDKN1B</i>	Cyclin-dependent kinase inhibitor 1A (p27)	2.75 ± 0.27	↑
<i>COX-2</i>	Cyclo-oxygenase 2	11.56 ± 1.58	↑
<i>COL1A1</i>	Collagen, type I, α1	11.61 ± 3.67	↑
<i>CTGF</i>	Connective tissue growth factor	3.64 ± 0.32	↑
<i>FOS</i>	V-fos FBJ murine osteosarcoma viral oncogene	3.90 ± 0.52	↑
<i>IL1R1</i>	Interleukin 1 receptor, type I	22.23 ± 1.26	↑
<i>ITGA1</i>	Integrin-α1	10.13 ± 1.40	↑
<i>ITGA4</i>	Integrin-α4	8.56 ± 0.40	↑
<i>ITGAV</i>	Integrin-αV	89.86 ± 37.95	↑
<i>ITGB3</i>	Integrin-β3	91.77 ± 64.86	↑
<i>MMP2</i>	Matrix metalloproteinase 2	49.33 ± 16.12	↑
<i>MMP9</i>	Matrix metalloproteinase 9	7.25 ± 1.33	↑
<i>MTSS1</i>	Metastasis suppressor 1	6.26 ± 1.68	↑
<i>P63</i>	Tumor suppressor	9.01 ± 0.51	↑
<i>PI3KR1</i>	Phosphatidylinositol 3-kinase p85α	9.30 ± 3.20	↑
<i>S100A4</i>	S100 calcium binding protein A4	4.82 ± 0.40	↑
<i>SOCS1</i>	Suppressor of cytokine signaling 1	3.6 ± 0.02	↑
<i>Serpine1</i>	Serpin peptidase inhibitor, clade E	27.43 ± 9.2	↑
<i>SST</i>	Somatostatin	202.5 ± 46	↑
<i>SSTR1</i>	Somatostatin receptor type I	15.89 ± 0.80	↑
<i>SSTR2</i>	Somatostatin receptor type II	20.77 ± 3.46	↑
<i>TWIST1</i>	Twist homolog 1 (<i>Drosophila</i>)	12.38 ± 3.44	↑
<i>THBS1</i>	Thrombospondin 1	73.77 ± 18.12	↑
Downregulated genes			
<i>AKT1</i>	AKT/protein kinase B	−4.06 ± 0.63	↓
<i>BCL-2</i>	B-cell chronic lymphocytic leukemia/lymphoma 2	−6.95 ± 1.30	↓
<i>E6/E7</i>	Human papillomavirus type 18 proteins E6 and E7	−10.38 ± 4.58	↓
<i>JUNB</i>	Jun B proto-oncogene (AP-1)	−3.88 ± 0.77	↓
<i>MAP2K1</i>	Mitogen-activated protein kinase kinase 1	−3.90 ± 1.68	↓
<i>MYC</i>	V-myc myelocytomatosis viral oncogene homolog	−3.61 ± 1.20	↓
<i>PCNA</i>	Proliferating cell nuclear antigen	−2.16 ± 0.02	↓
<i>STAT3</i>	Signal transducer and activator of transcription 3	−3.58 ± 0.88	↓
<i>SSTR3</i>	Somatostatin receptor type III	−3.78 ± 1.00	↓
<i>SSTR4</i>	Somatostatin receptor type IV	−1.90 ± 0.53	↓
<i>SSTR5</i>	Somatostatin receptor type V	−1.80 ± 0.30	↓

Boldface type indicates that the expression of the genes is directly related to Notch1-mediated somatostatin signaling.

involved in these Notch-mediated cervical cancer cell functions. To confirm this, SST small interfering RNAs (siRNAs)

were transfected into stable Notch1-activated HeLa cells (HeLa-ICN1 cells). We found that SST expression was

Figure 3. cAMP production induced via activation of Notch1 signaling and the application of Notch1 activator VPA in suppressing tumor growth. **(A):** The cAMP assay showed that forskolin induced cAMP production in both HeLa-GFP and HeLa-ICN1 cells in a dose-dependent manner. However, Notch1 activation could extremely induce more cAMP production in HeLa-ICN1 cells than in HeLa-GFP cells. cAMP concentration in HeLa-ICN1 cells was 10-, 14-, and 19-fold more than that in HeLa-GFP cells when treated with forskolin at 1, 10, and 50 μM, respectively. **(B):** RT-PCR assay showed that the Notch1 activator VPA upregulated the expression of Notch1, SST, and SSTR2 in cervical cancer HeLa cells. **(C):** A cell proliferation assay showed that VPA and the SSTR2-specific cytotoxic SST conjugate CPT-SST alone suppressed cell growth in a dose-dependent manner, but combination treatment enhanced the suppression. Similar results were observed from an in vivo antitumor assay. The tumor inhibitory rates induced via VPA at a dose of 200 mg/kg and CPT-SST at a dose of 1 mg/kg were 46% and 57%, respectively, but the inhibitory effect of the combination treatment with VPA and CPT-SST was 85% **(D)**.

Abbreviations: cAMP, cyclic AMP; RT-PCR, reverse transcription polymerase chain reaction; SST, somatostatin; SSTR, SST receptor; VPA, valproic acid.

knocked down in a dose-dependent manner. Furthermore, SST knockdown reversed a Notch1-induced lower expression of BCL-2 and a Notch1-induced greater expression of p63, but it did not affect the expression of other genes such as *p21* (a Notch target gene) and *MAP2K1* (data not shown). These findings support the idea that SST signaling is involved in Notch1-mediated signaling pathways in suppressing cervical cancer cell growth and there is crosstalk between Notch signaling and SST signaling.

Application of SST Signaling Activation in Combination Cancer Therapy

Activated Notch1 signaling induces SSTR2 upregulation. We predicted enhanced in vivo antitumor ability by combining Notch1-mediated tumor suppression with Notch1-activating

SSTR2-targeting therapeutics. To confirm this, the small molecule VPA, a Notch1 signaling activator, was used for in vitro and in vivo experiments in combination with the SSTR2-targeting cytotoxic CPT-SST conjugate.

VPA-Mediated Expression of Notch and Certain GPCRs

We first investigated whether or not the Notch activator VPA activated Notch1 and SSTR2 in HeLa cells. VPA at serial doses of 0 mM, 1 mM, 4 mM, and 8 mM was used to treat HeLa cells. VPA indeed led to higher expression levels of Notch1 and SSTR2 via RT-PCR analysis (Fig. 3B). We further investigated the expression of other SSTR subtypes, BN receptors (GRPR, BRS3, and NMBR), and pituitary adenylate cyclase activating polypeptide receptors (PAC1, VPAC1, and VPAC2)

in VPA-treated HeLa cells. We found that SSTR3, SSTR4, PAC1, GRPR, and BRS3 were upregulated, whereas SSTR1 and VPAC1 were downregulated in HeLa cells (data not shown). The effect of VPA on SSTR1, SSTR3, and SSTR4 was different from that in HeLa-ICN1 cells. However, SSTR2 upregulation supports the idea that combination therapy with VPA and SSTR2-targeting agents could be applied in treating tumors.

VPA-Enhanced Antitumor Efficacy of SSTR2-Targeted CPT-SST Conjugate

In our previous study, the SSTR2-specific CPT-SST conjugate was demonstrated to potently inhibit the growth of various types of tumor [50]. VPA itself has been demonstrated to have antitumor efficacy [53] and upregulate SSTR2 in HeLa cells, as described above. Thus, we combined VPA with CPT-SST to test whether or not both together could enhance the antitumor efficacy over that seen with each alone.

We first investigated the effects of VPA on HeLa cell proliferation via in vitro MTT assay. We found that VPA itself suppressed cell proliferation in a dose-dependent manner (0–5 mM) (Fig. 3C). In addition, the SSTR2-specific cytotoxic SST conjugate CPT-SST made in our laboratories also induced growth arrest of HeLa cells in a dose-dependent manner (0–10 μ M). And it was observed that combination treatment with both VPA and CPT-SST could significantly enhance cell growth suppression (Fig. 3C).

Furthermore, we carried out an in vivo antitumor assay demonstrating that combination treatment with both VPA and CPT-SST strongly suppressed cervical cancer HeLa tumor growth to a greater extent than individual treatment. As shown in Figure 3D, the inhibitory effects from treatments of VPA at 200 mg/kg and CPT-SST at 1 mg/kg were 46% and 57%, respectively. However, inhibition from combination therapy with VPA at 200 mg/kg and CPT-SST at 1 mg/kg was 85%. The suppressive ability of combination therapy was better than that via VPA or CPT-SST alone (Fig. 3D), and these in vivo results suggest that VPA-mediated SSTR2 upregulation can increase the uptake and antitumor efficacy of SSTR2-targeting CPT-SST.

DISCUSSION

Most cervical cancers are highly correlated with high-risk HPV, especially HPV16 and HPV18. The oncogenes *E6* and *E7* of HPV16 and HPV18 are necessary for HPV-induced cervical cancer malignancy [22, 24, 26]. It is known that *E6* suppresses the tumor suppressor *p53* and *E7* targets another tumor suppressor, *p105-Rb*. The viral oncogenes *E6* and *E7* mediate upregulation of *Notch1* signaling. Notch signaling is necessary in cervical cancers and is upregulated at an early stage but downregulated in HPV-induced malignant cancers [32–34]. Talora and others found that Notch1 activation downregulated HPV *E6* and *E7* expression and resulted in specific growth suppression of HPV⁺ cervical cancer cells (such as HeLa and CaSki cells) but not HPV[−] cells [20–22]. In these cancer cells, Notch signaling acts as a tumor suppressor [22]. However, the opposite results were observed in the same cervical cancer cells, whereby *Notch1* seemingly acted as an oncogene. In Dr. Kast's laboratory, downregulation of Notch1 expression by

Notch1 antisense displayed significant inhibition of in vitro CaSki cell proliferation and even resulted in the loss of in vivo tumorigenicity [19]. Similarly, Notch1 knockdown by siRNA caused HeLa cell and tumor suppression [23]. The function of Notch1 signaling in the same cervical cancer cells is seemingly controversial. To ascertain whether or not *Notch1* acts as a tumor suppressor or an oncogene, we established a stable Notch1-overexpressing HeLa cell line (HeLa-ICN1) and found that activated Notch1 signaling significantly suppressed HeLa cell proliferation and tumor growth as well as induced cell apoptosis. Our findings support the idea that forced activated Notch1 signaling acts as a tumor suppressor in HeLa cells. Our previous findings have demonstrated that cells in culture may change or lose certain functions under multiple passages and in vitro conditions [42]. Thus, the different results observed above from different laboratories may possibly be a result of Notch dysfunction in cervical cancer or inadvertent change, such as a difference in cell age and change under identical conditions.

In addition, Notch1 activation could regulate the expression of a panel of genes that are involved in various cancer-associated signaling pathways [20–22, 35]. As reported, activated Notch signaling suppressed c-Fos [20–22, 35], *E6* and *E7* [22, 35], NF- κ B, cyclin D1, BCL-2 [20], cyclin A1, cyclin E, and p105-Rb [21] expression and led to higher expression of p53 [21, 22, 35], p21 [22, 35], and phosphorylated extracellular signal-related kinase (pERK)1 and pERK2 [35]. We also found that activated Notch1 signaling downregulated the expression of *E6* and *E7*, p105-Rb, and BCL-2 in HeLa (HeLa-ICN1) cells, identical to that described above. We further observed that some genes, such as *PCNA*, *MYC*, *Akt1*, and *STAT3*, were downregulated. Others, such as *p21*, *p27*, *p63*, *MMP2*, *MMP9*, *MTSS1*, and *COX-2*, were upregulated. Many of these investigated genes are involved in cAMP/Ca²⁺, GPCR, and cancer signaling pathways. cAMP signaling and GPCR signaling may also be involved in Notch1-mediated signaling pathway cascades in a *E6*- and *E7*-dependent or -independent manner, although HPV *E6* and *E7* may be the major factors contributing to cell growth suppression induced by Notch activation. HPV *E6* and *E7* may not be the only way mediated by Notch1 signaling activation. Meanwhile, our findings showed that, most likely, SST activation is involved in Notch1-mediated cervical cancer cell apoptosis and growth arrest.

cAMP is commonly connected with GPCRs. Our cAMP assay showed that Notch1 activation could enhance forskolin-induced cAMP production. Also, it has been demonstrated that cAMP can activate SST transcription via activating phosphorylation of cAMP-response element binding protein that targets the SST promoter [54]. Remarkably, we further found that SST and its cognate GPCR members SSTR1 and SSTR2 were upregulated via activated Notch1 signaling. SST itself and activated SSTR signaling could suppress cell proliferation and tumor growth [47, 55, 56]. Plus, both Notch signaling and SST signaling couple and share certain common signaling pathways such as the MAPK and PI3K–Akt pathways [3, 9–11, 35, 47]. We also found that knockdown of Notch1-induced SST via SST siRNAs could reverse the Notch1-induced lower expression of antiapoptotic BCL-2 and greater expression of tu-

mor suppressive p63 in cervical cancer cells. This evidence indicates that a connection between Notch signaling and SST signaling and Notch1-mediated tumor suppression in cervical cancer is partly involved with novel SST signaling.

The treatment of cervical cancer is limited, with cytotoxic therapies and a poor prognosis for patients [39]. Thus, interest has increased in targeted therapeutics for this cancer type [39]. SSTR2-targeted cancer chemotherapy has been applied successfully to develop novel, safe, and efficacious drugs in treating advanced cancers [36, 40, 56]. In the present study, the finding that GPCRs such as SSTR2 and GRPR were activated by Notch1 signaling provides an opportunity for an enhanced receptor-targeted or multiple receptor subtype-targeted cancer therapy. The small molecule VPA has been used as an anticonvulsant drug [57, 58]. Also, VPA displays antitumor abilities [53]. VPA is under clinical investigation for the treatment of several cancers. We found that VPA activated the expression of Notch1 in HeLa cells and also, more importantly, activated the expression of SSTR2 and GRPR, both of which are major targets for receptor-targeted cancer therapy. We investigated the possibility of combination therapy with VPA and the SSTR2-specific CPT-SST. We found that the combination therapy could suppress much more tumor growth in cervical cancers than either single agent by itself. By taking advantage of VPA's dual functions (direct tumor-suppressive ability and SSTR2-activating ability), combination therapy with VPA and CPT-SST could also, to some extent, escape or reduce multi-drug resistance of cancer cells resulting from single high-dose drugs used during long-term treatment while increasing anti-

tumor activity. We also observed similar results with VPA and CPT-SST treating other cancers, such as small cell lung cancer, pancreatic carcinoid, and medullary thyroid cancer.

Our data indicate novel crosstalk between Notch signaling and SST signaling. Notch1 activation in certain cancers significantly displays tumor-suppressive activity. The small molecule HDAC inhibitor VPA acts as an activator of Notch signaling and SST signaling in these cancers and also is affordable and easily available; thus, VPA represents a potential promising therapeutic agent for these cancers. Importantly, receptor-targeted cancer therapy has been applied in modern drug research and development. Overall, our findings are of clinical significance and may provide a promising cancer therapeutic approach using signaling crosstalk within cancer cells.

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REFERENCES

1. Wu L, Griffin JD. Modulation of Notch signaling by mastermind-like (MAML) transcriptional co-activators and their involvement in tumorigenesis. *Semin Cancer Biol* 2004;14:348–356.
2. Wu L, Aster JC, Blacklow SC et al. MAML1, a human homologue of Drosophila mastermind, is a transcriptional co-activator for NOTCH receptors. *Nat Genet* 2000;26:484–489.
3. Kunnimalaiyaan M, Chen H. Tumor suppressor role of Notch-1 signaling in neuroendocrine tumors. *The Oncologist* 2007;12:535–542.
4. Allenspach EJ, Maillard I, Aster JC et al. Notch signaling in cancer. *Cancer Biol Ther* 2002;1:466–476.
5. Guan E, Wang J, Laborda J et al. T cell leukemia-associated human Notch/translocation-associated Notch homologue has I kappa B-like activity and physically interacts with nuclear factor-kappa B proteins in T cells. *J Exp Med* 1996;183:2025–2032.
6. Maillard I, Pear WS. Notch and cancer: Best to avoid the ups and downs. *Cancer Cell* 2003;3:203–205.
7. Weng AP, Aster JC. Multiple niches for Notch in cancer: Context is everything. *Curr Opin Genet Dev* 2004;14:48–54.
8. Dotto GP. Notch tumor suppressor function. *Oncogene* 2008;27:5115–5123.
9. Koch U, Radtke F. Notch and cancer: A double-edged sword. *Cell Mol Life Sci* 2007;64:2746–2762.
10. Leong KG, Karsan A. Recent insights into the role of Notch signaling in tumorigenesis. *Blood* 2006;107:2223–2233.
11. Sjölund J, Manetopoulos C, Stockhausen MT et al. The Notch pathway in cancer: Differentiation gone awry. *Eur J Cancer* 2005;41:2620–2629.
12. Bolós V, Blanco M, Medina V et al. Notch signalling in cancer stem cells. *Clin Transl Oncol* 2009;11:11–19.
13. Wang Z, Li Y, Banerjee S et al. Down-regulation of Notch-1 and Jagged-1 inhibits prostate cancer cell growth, migration and invasion, and induces apoptosis via inactivation of Akt, mTOR, and NF-κB signaling pathways. *J Cell Biochem* 2010;109:726–736.
14. Roy M, Pear WS, Aster JC. The multifaceted role of Notch in cancer. *Curr Opin Genet Dev* 2007;17:52–59.
15. Proweller A, Tu L, Lepore JJ et al. Impaired notch signaling promotes de novo squamous cell carcinoma formation. *Cancer Res* 2006;66:7438–7444.
16. Kunnimalaiyaan M, Traeger K, Chen H. Conservation of the Notch1 signaling pathway in gastrointestinal carcinoid cells. *Am J Physiol Gastrointest Liver Physiol* 2005;289:G636–G642.
17. Nakakura EK, Sriuranpong VR, Kunnimalaiyaan M et al. Regulation of neuroendocrine differentiation in gastrointestinal carcinoid tumor cells by Notch signaling. *J Clin Endocrinol Metab* 2005;90:4350–4356.
18. Kunnimalaiyaan M, Vaccaro AM, Ndiaye MA et al. Overexpression of the NOTCH1 intracellular domain inhibits cell proliferation and alters the neuroendocrine phenotype of medullary thyroid cancer cells. *J Biol Chem* 2006;281:39819–39830.
19. Weijzen S, Zlobin A, Braid M et al. HPV16 E6 and E7 oncoproteins regulate Notch-1 expression and cooperate to induce transformation. *J Cell Physiol* 2003;194:356–362.
20. Yao J, Duan L, Fan M et al. Notch1 induces cell cycle arrest and apoptosis in human cervical cancer cells: Involvement of nuclear factor kappa B inhibition. *Int J Gynecol Cancer* 2007;17:502–510.
21. Wang L, Qin H, Chen B et al. Overexpressed active Notch1 induces cell growth arrest of HeLa cervical carcinoma cells. *Int J Gynecol Cancer* 2007;17:1283–1292.
22. Talora C, Sgroi DC, Crum CP et al. Specific down-modulation of Notch1 signaling in cervical cancer cells is required for sustained HPV-E6/E7 expression and late steps of malignant transformation. *Genes Dev* 2002;16:2252–2263.
23. Yu H, Zhao X, Huang S et al. Blocking Notch1 signaling by RNA interference can induce growth inhibition in HeLa cells. *Int J Gynecol Cancer* 2007;17:511–516.
24. Dueñas-González A, Lizano M, Candelaria M

et al. Epigenetics of cervical cancer. An overview and therapeutic perspectives. *Mol Cancer* 2005;4:38–61.

25. Malinowski DP. Multiple biomarkers in molecular oncology. I. Molecular diagnostics applications in cervical cancer detection. *Expert Rev Mol Diagn* 2007;7:117–131.
26. Perez-Plasencia C, Duenas-Gonzalez A, Alatorre-Tavera B. Second hit in cervical carcinogenesis process: Involvement of wnt/beta catenin pathway. *Int Arch Med* 2008;1:10–17.
27. Ramdass B, Maliekal TT, Lakshmi S et al. Co-expression of Notch1 and NF- κ B signaling pathway components in human cervical cancer progression. *Gynecologic Oncol* 2007;104:352–361.
28. Ma YY, Wei SJ, Lin YC et al. PIK3CA as an oncogene in cervical cancer. *Oncogene* 2000;19:2739–2744.
29. Kulkarni S, Rader JS, Zhang F et al. Cyclooxygenase-2 is overexpressed in human cervical cancer. *Clin Cancer Res* 2001;7:429–434.
30. Rincón-Arango H, Rosales R, Mora N et al. R-Ras promotes tumor growth of cervical epithelial cells. *Cancer* 2003;97:575–585.
31. Srivastava S, Ramdass B, Nagarajan S et al. Notch1 regulates the functional contribution of RhoC to cervical carcinoma progression. *Br J Cancer* 2010;102:196–205.
32. Zagouras P, Stifani S, Blaumueller CM et al. Alterations in Notch signaling in neoplastic lesions of the human cervix. *Proc Natl Acad Sci U S A* 1995;92:6414–6418.
33. Tewari KS, Taylor JA, Liao SY et al. Development and assessment of a general theory of cervical carcinogenesis utilizing a severe combined immunodeficiency murine-human xenograft model. *Gynecol Oncol* 2000;77:137–148.
34. Sun L, Zhan Q, Zhang W et al. Aberrant expression of Notch1 in cervical cancer. *Chinese J Clin Oncol* 2007;4:38–41.
35. Talora C, Cialfi S, Segatto O et al. Constitutively active Notch1 induces growth arrest of HPV-positive cervical cancer cells via separate signaling pathways. *Exp Cell Res* 2005;305:343–354.
36. Sun LC, Luo L, Mackey LV et al. Investigation of cancer cell lines for peptide receptor-targeted drug development. *J Drug Target* 2011;19:719–730.
37. Xiao D, Wang J, Hampton LL et al. The human gastrin-releasing peptide receptor gene structure, its tissue expression and promoter. *Gene* 2001;264:95–103.
38. Reubi JC, Läderach U, Waser B et al. Vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor subtypes in human tumors and their tissues of origin. *Cancer Res* 2000;60:3105–3112.
39. Reubi JC. Peptide receptors as molecular targets for cancer diagnosis and therapy. *Endocr Rev* 2003;24:389–427.
40. Engel JB, Schally AV, Halmos G et al. Targeted cytotoxic bombesin analog AN-215 effectively inhibits experimental human breast cancers with a low induction of multi-drug resistance proteins. *Endocr Relat Cancer* 2005;12:999–1009.
41. Sun LC, Coy DH. Somatostatin receptor-targeted anti-cancer therapy. *Curr Drug Deliv* 2011;8:2–10.
42. Sun L, Coy DH. Cytotoxic conjugates of peptide hormones for cancer chemotherapy. *Drugs Future* 2008;33:217–223.
43. Pyronnet S, Bousquet C, Najib S et al. Antitumor effects of somatostatin. *Mol Cell Endocrinol* 2008;286:230–237.
44. Calzavara E, Chiaramonte R, Cesana D et al. Reciprocal regulation of Notch and PI3K/Akt signalling in T-ALL cells in vitro. *J Cell Biochem* 2008;103:1405–1412.
45. Ghosh S, Choritz L, Geibel J et al. Somatostatin modulates PI3K-Akt, eNOS and NHE activity in the ciliary epithelium. *Mol Cell Endocrinol* 2006;253:63–75.
46. Reubi JC, Schaer JC, Laissue JA et al. Somatostatin receptors and their subtypes in human tumors and in peritumoral vessels. *Metabolism* 1996;45(suppl 1):39–41.
47. Grozinsky-Glasberg S, Shimon I, Korbonits M et al. Somatostatin analogues in the control of neuroendocrine tumours: Efficacy and mechanisms. *Endocr Relat Cancer* 2008;15:701–720.
48. Panetta R, Patel YC. Expression of mRNA for all five human somatostatin receptors (hSSTR1–5) in pituitary tumors. *Life Sci* 1995;56:333–342.
49. Bellavia D, Mecarozzi M, Campese AF et al. Notch3 and the Notch3-upregulated RNA-binding protein HuD regulate Ikaros alternative splicing. *EMBO J* 2007;26:1670–1680.
50. Sun LC, Mackey LV, Luo J et al. Targeted chemotherapy using a cytotoxic somatostatin conjugate to inhibit tumor growth and metastasis in nude mice. *Clin Med Oncol* 2008;2:491–499.
51. Patel YC. Somatostatin and its receptor family. *Front Neuroendocrinol* 1999;20:157–198.
52. Susini C, Buscail L. Rationale for the use of somatostatin analogs as antitumor agents. *Ann Oncol* 2006;17:1733–1742.
53. Greenblatt DY, Vaccaro AM, Jaskula-Sztul R et al. Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoma cancer cells. *The Oncologist* 2007;12:942–951.
54. Loeken MR. Effects of mutation of the CREB binding site of the somatostatin promoter on cyclic AMP responsiveness in CV-1 cells. *Gene Expr* 1993;3:253–264.
55. Du ZY, Qin RY, Xia W et al. Gene transfer of somatostatin receptor type 2 by intratumoral injection inhibits established pancreatic carcinoma xenografts. *World J Gastroenterol* 2005;11:516–520.
56. Celinski SA, Fisher WE, Amaya F et al. Somatostatin receptor gene transfer inhibits established pancreatic cancer xenografts. *J Surg Res* 2003;115:41–47.
57. Chateauvieux S, Morceau F, Dicato M et al. Molecular and therapeutic potential and toxicity of valproic acid. *J Biomed Biotechnol* 2010;2010 pii: 479364. Epub 2010 Jul 29.
58. Stefan H, Feuerstein TJ. Novel anticonvulsant drugs. *Pharmacol Ther* 2007;113:165–183.