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OVER EXPRESSION OF THE NOTCH1 INTRACELLULAR DOMAIN (NICD) INHIBITS CELLULAR PROLIFERATION AND ALTERS THE NEUROENDOCRINE PHENOTYPE OF MEDULLARY THYROID CANCER CELLS

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Running Title: Notch1 signaling inhibits MTC growth

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The role of Notch1 as an oncogene or tumor suppressor appears to be cell-type specific. Medullary thyroid cancer (MTC) characteristically expresses the transcription factor achaete scute complex-like 1 (ASCL1) as well as high levels of the NE markers calcitonin and chromogranin A (CgA). In the present study, we show that the active Notch1 intracellular domain (NICD) is absent in human MTC tumor tissue samples and MTC-TT cells. To determine the effects of Notch1 expression, we created a doxycycline inducible NICD in MTC (TT-Notch). Treatment of TT-Notch cells with doxycycline led to dose-dependent induction of Notch1 protein with corresponding decreases in ASCL1 protein and NE hormones. ASCL1-promoter-reporter assay and Northern analysis revealed that ASCL1 reduction by Notch1 activation is predominantly by the silencing of transcription of ASCL1 gene. Over expression of ASCL1 in MTC cells indicated that CgA expression is tightly dependent upon the levels of ASCL1. This was further confirmed by experiments using siRNA against ASCL1 in which reduction in ASCL1 led to a reduction in both CgA and calcitonin. Further, we demonstrated that Notch1 signaling activation leads to phosphorylation of ERK1/2 but that the reduction in NE markers is independent of ERK1/2 activation. Activation of Notch1 resulted in significant MTC cell growth inhibition. Importantly, the reduction in MTC cellular growth was dependent on the level of Notch1 protein present. Moreover, no increase in growth with expression of ASCL1 in Notch1 activated cells was observed, indicating that the growth suppression observed by Notch1 activation is independent of ASCL1 reduction. Mechanistically, we show that MTC cell growth inhibition by Notch1 is mediated by cell cycle arrest associated with up regulation of p21.

Introduction

Medullary thyroid cancer (MTC) is a neuroendocrine tumor (NE) derived from the calcitonin-producing C-cells of the thyroid gland and accounts for 3-5% of cases of thyroid cancer (1;2). The only curative therapy for patients with MTC is surgical resection. Eighty percent of all MTCs are sporadic in nature and the remaining 20% are familial and caused by germline mutations in the RET proto-oncogene (2;3). While development of RET gene testing has allowed for early prophylactic thyroidectomy for patients with familial MTC, the majority of patients with sporadic MTC have persistent or recurrent disease after surgery, because the natural history of MTC is characterized by early metastasis. Understanding the molecular pathways, which control MTC and C-cell development and proliferation, is essential for the development of novel therapies for patients with advanced MTC.

Like other NE tumors, MTC cells secrete various hormones and NE markers such as calcitonin and chromogranin A (CgA) (4). In addition, MTC cells express high levels of achaete

scute complex like-1 (ASCL1, also known as achaete-scute homolog-1 or hASH1), an evolutionarily conserved basic helix-loop-helix transcription factor, which seems to be limited to NE tumors (5-8). Several recent studies suggest that ASCL1appears to be critical for C-cell development and may play an important role in MTC tumor growth and NE differentiation. Achaete-scute homolog-1 transgenic knockout mice failed to develop thyroid C cells which suggests that the achaete-scute homolog-1 is essential for normal C-cell development (9). Furthermore, raf-1 activation in human MTC cells also leads to significant reductions in ASCL1, CgA, calcitonin and growth (1;5;10). In addition, ASCL1 plays an important role in the development of other NE cells, such as adrenal chromaffin cells and pulmonary endocrine cells (11-13). These findings also indicate that pathway(s), which regulate ASCL1, may also regulate MTC growth and differentiation. During development, ASCL1 expression is tightly controlled by the Notch1 signaling pathway [see reviews (14;15)].

Notch1 is a multi-functional transmembrane receptor that regulates cellular differentiation in a variety of contexts. Recently, Notch1 has also been shown to play an essential role in the NE differentiation of the lung and gastrointestinal tract (16-19). In human cancer cells, Notch1 signaling engages in a dual role as either a tumor suppressor or an oncogene. Activation of the Notch1 signaling pathway has been shown to inhibit growth in prostate cancer, small cell lung cancer (SCLC), and pancreatic carcinoid, (8;20-22) and induce apoptosis in B-cells and other hematopoietic lineages *in vitro* (23). However, the degree of Notch1 activation required to achieve growth suppression is unclear since most of the studies utilized transient expression with very high levels of active Notch1.

In this report, we demonstrate the critical role of Notch1 in controlling both cellular proliferation and the NE phenotype in MTC cells. In the present study, we show that Notch1 protein (NICD) is undetectable in human MTC tumor samples and cell line. However, high levels of ASCL1 and CgA were found in the MTC cell line and tumor specimens. To determine the effects of Notch1 expression, we created a doxycycline inducible Notch1 intracellular domain (NICD) construct in MTC (TT-Notch). Using this doxycycline inducible *in vitro* model, we assessed the dose dependent effects of Notch1 on MTC cell growth. Expression of NICD resulted in significant inhibition of MTC cellular proliferation. Importantly, the degree of tumor cell growth inhibition was directly proportional to the amount of Notch1 protein present. Notably, Notch1 activation also caused reductions in the levels of calcitonin and CgA. Furthermore, we show for the first time by transient over expression of ASCL1 in MTC cells that CgA levels are directly correlated with ASCL1 protein levels. The fact that transfection of small interference RNA (siRNA) against ASCL1 in MTC cells resulted in a proportional reduction in CgA further confirms that the level of CgA is tightly regulated by ASCL1. Finally, we report that activation of Notch1 also leads to the phosphorylation of extracellular signal regulated kinase-1/2 (ERK1/2) in a dose dependent manner, but active ERK1/2 is not required for ASCL1 mediated suppression of NE markers.

Experimental Procedures

Human tissue samples Human MTC tumor samples and other control tumor samples were collected with the approval of the Institutional Review Board and Human Subjects Committee of the University of Wisconsin School of Medicine and Public Health. Tumor histology was verified by pathological review. Samples were snap frozen in liquid nitrogen and kept at -80º C for long-term storage. Tumor cell lysates were prepared from the frozen samples by grinding in liquid nitrogen. The powder was then lysed with lysis buffer as described (24). The lysates were analyzed by Western blot for Notch1, ASCL1 and CgA as described below.

Cell culture Human MTC cells (TT) were obtained from B.Nelkin (Johns Hopkins University, Balitmore, MD, USA) and maintained in RPMI 1640 (Life Technologies, Rockville, MD, USA) supplemented with 16% Fetal Bovine Serum (Sigma, St.Louis, MO, USA), 100 IU/ml penicillin and 100 µg/ml streptomycin (Life Technologies) in a humidified atmosphere of 5% CO_2 in air at 37°C (1;10). Doxycycline inducible cell lines were maintained similar to TT cells except that tetracycline free fetal bovine serum

(Clontech Laboratories, Palo Alto, CA, USA), 0.4 µg/ml of G418, and 0.4 µg/ml of hygromycin (Life Technologies) were used.

Doxycycline Inducible Notch1 The Tet-On expression system was obtained from Clontech Laboratories, A 2.3-kb BamH1-Xho I fragment containing the Notch1 intracellular domain (NICD; amino acids 1759-2556) from pTAN1-cDNA was subcloned into pRevTRE vector at the BamH1-Sal1 sites. The cloned construct, pRevTRE-Notch1, was confirmed by DNA sequencing. To create an inducible TT-Notch cell line, TT cells were first transfected with plasmid pRevTet-On (Clontech) containing Tet-responsive transcriptional activator using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) and selected in media containing 0.4 µg/ml G418 (Life Technologies). The resultant twelve G418-resistant, TT-Tet-on clones were screened for doxycycline-dependent inducibility of the reporter gene luciferase, by transient transfection of the pRevTRE-Luc vector into TT-Tet-on clones. A clone with low background, which reproducibly induced the luciferase gene over 60-fold, was maintained and used for transfection with the response plasmid containing the Notch1 (NICD) gene (pRevTRE-Notch1) and the empty vector (pRevTRE). TT-Tet-on cells were transfected with pRevTRE-Notch1 and empty vector, pRevTRE, using Lipofectamine 2000. Transfected cells were selected in 0.4 µg/ml hygromycin (Life Technologies). Resistant TT-Notch and TT-TRE (Vector alone) clones were treated with 1 µg/ml doxycycline for 48 hrs and screened for the presence of Notch1 protein by Western blot analysis.

Western blot analysis Cellular pellets were lysed in sample buffer as described (24). Total cellular protein concentrations were determined using bicinchoninic (BCA) assay kit (Pierce, Rockford, IL, USA). Cellular extracts (20-30µg) were boiled with equal amounts of loading dye (24) for 10min and separated on 10%SDS-PAGE. Proteins were transferred onto nitrocellulose membranes (Schleicher and Schuell, Keene, NH, USA) by electro blotting. Membranes were blocked in milk and proceed with primary and secondary antibodies as per the protocol described (24). The following primary antibody dilutions were used: Notch1 (1:500) HA probe (1:200); Santa Cruz Biotechnology, Santa Cruz, CA, USA), Mammalian achaete scute homolog 1(MASH1) to detect ASCL1 (1:1000; BD Pharmingen, San Diego, CA, USA), Hairy enhancer of split-1 (HES-1) (1:10000; a kind gift from T. Sudo, Toray Industries, Inc., Kanagawa, Japan), CgA (1:1000, Zymed Laboratories, San Francisco, CA, USA), pERK1/2, ERK1/2, p21, p27, CyclinD1, CDK4, pcdc2^{Tyr15} (1:1000, Cell Signaling Technology, Beverly, MA, USA) and G3PDH (1:10000, Trevigen, Gaithersburg, MD, USA). Primary antibody incubations were kept overnight at 4^0 C and then membranes were washed with wash buffer. Then the membranes were incubated with 1:500 dilutions of goat-anti-rabbit (for Notch1, HES-1, CgA and G3PDH) or goat anti-mouse secondary antibody (for MASH1) (Pierce) coupled with horseradish peroxidase. Membranes were washed in wash buffer and developed by Immunstar (Bio-Rad, Hercules, CA, USA) for HES1, CgA and G3PDH or Super West Femto chemiluminescence's substrate (Pierce) for MASH1, cell cycle inhibitors, and Notch1 according to the manufacturer's directions.

Notch1 functional analysis The plasmids containing either 4 copies of the wild type or mutant CBF-1 binding elements in pGL2pro (Promega, Madison, WI, USA) were obtained from Dr. Diane Hayward (Johns Hopkins University). TT-Notch cells were maintained in RPMI 1640 supplemented with 16% tetracycline free fetal bovine serum (Clontech), 0.4 µg/ml hygromycin, and 0.4 µg/ml G418. Overnight grown cells were cotransfected with 1µg of wild type or mutant CBF-1 plasmid, and 0.5 µg of plasmid containing a gene for ß-galactosidase driven by a CMV promoter (CMV-ß- gal plasmid) as an internal control for transfection efficiency. Next day cells were treated with or without doxycycline and incubated for 2 days to induce Notch1. Then the cells were lysed and the luciferase and ß-galactosidase assays were carried out as per the manufacturer's instruction (Promega) using a Luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI, USA) and plate reader respectively. Luciferase activity was determined relative to the expression calculated by wild type over mutant (25). Experiments were performed in triplicate at least twice.

Cell proliferation assay and Calcitonin measurement To measure proliferation rate, cells

were plated in triplicate in six well plates and MTT assay was performed as described (20;26). The cells were treated with or without doxycycline at various concentrations for indicated number of days. Experiments were performed at least twice. To determine the amount of calcitonin in cellular extracts, we utilized a calcitonin ELISA kit (Biosource International, CA, USA) as per the manufacturer's instructions. Calcitonin values were standardized and quantified relative to control cellular extracts. Samples were analyzed in triplicate.

Transfection, reporter assays and small interfering RNA experiments To determine the role of ASCL1 on the level of CgA, TT-Notch cells were transiently transfected with plasmid pCDNA 3.0 containing a CMV promoter derived ASCL1 gene fused with a hemaglutinin (HA) tag (a kind gift from Dr. D. Ball, Johns Hopkins University) using lipofectamine 2000 according to the manufacturer's directions. On the next day, transfected cells were treated with or without doxycycline for two days and then cell lysates were prepared and analyzed for the level of ASCL1 and CgA by Western analysis.

Luciferase assay for ASCL1 promoter *The* ASCL1 promoter DNA fragment (-7900/+37), containing the 5' flanking region of ASCL1 genomic DNA, including HES-1 binding site, was cloned into the luciferase reporter gene in pGL2 (Promega) (27). Transfection of the ASCL1 promoter DNA showed reduction in luciferase activity in the presence of HES-1 expression (27). To determine the effect of Notch1 activation on ASCL1 transcription, we transiently transfected the plasmid containing the luciferase gene under the promoter of ASCL1(27) into TT-Notch cells. Plasmid pGL2 control (positive control for luciferase) and pGL2basic (negative control) were also trasfected into TT-Notch cells as controls. In addition, the CMV-ß-gal plasmid was also co-transfected into TT-Notch cells to normalize the transfection. Next day, media was changed with or without doxycycline to activate Notch1 for two days. Then cell lysates were prepared and analyzed for luciferase activity as described (20).

ASCL1 siRNA To determine the role of ASCL1 on CgA, siRNA against ASCL1 (Santa Cruz - catalog # sc37692) and non-specific siRNA (Santa Cruz catalog # sc37007) were transfected into TT cells using lipofectamine 2000 as described by the manufacturer**.** After two days of incubation, cell lysates were prepared and analyzed for the levels of ASCL1 and CgA proteins by Western analysis.

Growth rescue experiment To determine the importance of ASCL1 in Notch1 mediated growth suppression, we carried out the MTT assay. Briefly, overnight grown TT-Notch cells were transfected with plasmid pCDNA 3.0 containing a CMV promoter derived ASCL1 gene fused with a hemaglutinin (HA) tag using lipofectamine 2000 according to the manufacturer's directions. Next day cells were trypsinized and equal number of cells were plated onto 24 well plates and incubated overnight. The following day, the cells were treated with or without doxycycline (1µg/mL) and incubated for 4 days. Then, MTT assay was carried out as described earlier.

Northern analysis To study the effect of Notch1 on ASCL1 transcription, TT-Notch cells were treated with doxycycline (0, 0.2, 0.5 and 1µg/mL) at indicated time points, and total RNA was isolated using RNA isolation kit (Qiagen, Inc., Valencia, CA). Ten µg of this RNA was separated on 1% formaldehyde denaturing agarose gel and transferred onto positively charged nylon membrane (AmbionInc.,Austin, TX). DNA probes using ASCL1 and G3PDH DNA were made using Random primer labeled kit (New England Biolabs, Inc., Beverly, MA). The probe was denatured and hybridized with the membrane using a standard protocol (24).

Statistical analysis ANOVA with Bonferroni post-Hoc testing (SPSS software version 10.0; SPSS Inc., Chicago, IL, USA) was utilized for statistical analysis. A *p* value of less than 0.05 was considered significant. Unless noted, data are represented as mean ± standard error.

Results

Expression of Notch1 components in MTC tumors. We have previously shown the presence of ASCL1 mRNA by Northern analysis in MTC, carcinoid, and pheochromocytoma cells (7). However, protein levels of ASCL1in human tumor samples have not been determined. Figure 1 shows significant amounts of ASCL1 protein present in six human MTC tumors [4 sporadic and two multiple endocrine neoplasia type 2A (MEN2A) patient] as well as in human MTC (TT) cells.

However, ASCL1 is not present in non-NE tumor tissues such as papillary thyroid cancer (PTC) samples (Figure 1). Furthermore, as expected, expression of the NE marker CgA is restricted to NE tumors (MTC).

Given the importance of Notch1 signaling and its role on ASCL1, we expected a lack of active Notch1 (NICD) protein in these NE tumors. As shown in Figure 1, MTC tumors lack NICD protein. In contrast, Notch1 is expressed in papillary thyroid cancer and pancreatic adenocarcinoma tissue. Since cleaved, active Notch1 is absent in MTC tumors and cell line, we hypothesized that over expression of active Notch1 (NICD) in MTC cells may down regulate ASCL1 and possibly affect cellular growth and/or differentiation. Thus we created a doxycycline inducible NICD (Notch1) system in TT cells.

Dose dependent functional Notch1 expression in TT-Notch cells. To determine the inducibility of NICD by doxycycline in TT-Notch cells, we carried out Western analysis after treatment with various concentrations of doxycycline. As shown in Figure 2A, there is no detectable Notch1 protein in TT-Notch cells in the absence of doxycycline. However treatment of TT-Notch cells with doxycycline led to an induction of Notch1 protein. Importantly, the amount of Notch1 protein is proportional to the dose of doxycycline treatment.

To determine the functionality of the Notch1 protein in TT cells, we utilized a well-described reporter system in which binding of Notch1 leads to high levels of luciferase expression (25). CBF-1/RBP-J kappa is the best-characterized downstream effector of Notch1 in mammals (25;28). Figure 2B shows that there is increases in the relative fold induction of luciferase activity due to the activation of Notch1. This increase in the luciferase activity is dependent on the amount of doxycycline treatment. However, in the absence of doxycycline there is a minimal amount of luciferase activity suggesting the lack of functional Notch1 activity (Figure 2B). Taken together, the results of the Western and Notch1 functional analysis clearly suggest that the Notch1 protein produced after doxycycline treatment in TT-Notch cells is functional and the activity is proportional to the amount of protein present.

Conservation of the Notch1 signaling pathway in MTC cells. To determine if the downstream targets of activated Notch1 are intact in MTC cells, Western blots were performed for Hairy Enhancer of Split-1 (HES-1) protein, an ASCL1 transcriptional repressor and an immediate downstream mediator of Notch1 signaling. In a dose-response assay, the TT-Notch cells treated with various concentration of doxycycline for 2 days showed an increase in the amount of HES-1 protein (Figure 3A). The increase in Notch1 expression resulted in a significant increase in the levels of HES-1 protein. However, the level of HES-1 protein plateaus after 0.5μg/mL doxycycline treatment. This is consistent with previous findings that HES-1 protein negatively regulates its expression by binding to its own promoter (29;30). HES-1 is also present in control cells at a minimal level and it has been shown that under normal conditions HES-6, another basic helix-loop-helix, protein binds to HES-1 and blocks the binding of HES-1 to the ASCL1 promoter (31;32). As shown in Figure 3A, HES-6 is expressed in TT cells and this could be the reason why we see ASCL1 expression in the presence of HES-1 protein in untreated cells. Importantly, the level of HES-6 is reduced after Notch1 activation. Activation of Notch1 has been shown to down regulate the expression of ASCL1 and its homologs in multiple organisms. Earlier studies have shown that ASCL1 expression is associated with NE markers (33). Furthermore, ASCL1 transcriptional repression due to Notch1-mediated HES-1 expression has been well characterized (27;34). In a dose-response assay, TT-Notch cells showed progressive reduction of ASCL1 and CgA proteins with increasing Notch1 protein (Figure 3A). As expected, treatment with various concentrations of doxycycline for 2 days, in TT-vector alone did not affect the expression level of ASCL1 and CgA (Figure 3B).

Notch1 signaling in MTC cells decreases calcitonin levels. We have shown that an increase in Notch1 protein leads to dose dependent reduction in CgA (Figure 3A). However, the most important bioactive hormone produced by MTC cells is calcitonin. Therefore, we were interested in the level of calcitonin after varying levels of Notch1 activation was achieved in MTC cells. As shown Figure 3C, induction of Notch1 resulted in a progressive reduction in calcitonin. The relative calcitonin reductions were 5% at 0.2μg/mL, 30% at 0.5 μg/mL, and 47% at 1.0μg/mL-doxycycline treatment (Figure 3C). TT-vector alone treated

with doxycycline did not show any reduction in calcitonin levels at all time points (data not shown).

Continuous requirement of Notch1 Induction. To determine the duration of induction of Notch1 by doxycycline, TT-Notch cells were treated with (0.5 and 1μg/ml) and without doxycycline for two days. The cells were then washed with PBS and grown in complete media without doxycycline for up to 6 days. Every two days, cell extracts were prepared and analyzed for the levels of Notch1, CgA, and ASCL1 proteins. As shown in Figure 3D, Notch1 protein is detectable only in the presence of doxycycline. Interestingly, when the media was changed to normal media without doxycycline, the Notch1 protein became undetectable as early as two days after withdrawal of the doxycycline. This result confirms that the induction of Notch1 is very tightly regulated and doxycycline is required all of the time. ASCL1 reduction also correlated with the presence of Notch1 protein. However, CgA reduction persisted up to 2 days after the withdrawal of doxycycline but the level increased to normal after day 2 (Fig3D).

Effect of the Notch1 signaling pathway in MTC cells. To determine the effects of long-term induction of the Notch1 signaling pathway, we treated the TT-Notch cells with 0, 0.2, 0.5, and 1μg/ml of doxycycline for up to 8 days. As can be seen in Figure 4, continuous doxycycline treatment persistently activated the Notch1 pathway in a dose dependent manner illustrated by the presence of Notch1 protein. Similarly, the level of HES-1 protein increased in Notch1 expressed cells compared to the untreated cells where Notch1 protein is absent. As mentioned earlier, HES-1 protein binds to its own promoter and regulates its expression. Therefore, we do not see a significant increase in HES-1 levels with increasing levels of Notch1 protein. Interestingly after 8 days of treatment with doxycycline, ASCL1 protein was barely detectable in TT-Notch cells. As shown in Figure 4, activation of the Notch1 pathway led to a significant dose dependent reduction in CgA. The reduction in CgA was evident at day 4 and pronounced at day 8.

Notch1 activation inhibits MTC cell growth. Activation of the Notch1 signaling pathway has been shown to inhibit growth in prostate cancer, SCLC, and carcinoid cells *in vitro* (8;20-22). To determine if Notch1 activation in MTC cells inhibits growth, we utilized cell counts. As shown in Figure 5A, there were no significant differences in growth rate between TT-Vector alone treated with or without doxycycline and TT-Notch cells without doxycycline treatment. However, doxycycline induced Notch1 expression led to inhibition of cellular growth. This suggests that persistent activation of Notch1 is required to inhibit the growth of MTC cells.

Notch1 dose-response inhibition of MTC cell growth. Inhibition of MTC cell growth required high levels of Notch1 protein. Furthermore, dose-response experiments showed a progressive decrease in ASCL1 and CgA. However, it has not previously been established if MTC proliferation is directly related to levels of Notch1 present. To determine the dose-response effects of Notch1 on MTC growth inhibition, we treated TT-Notch cells with varying concentrations of doxycycline as shown in the figure 5B, and measured cell viability by MTT. In the absence of doxycycline, TT-Notch cells had a high rate of cellular growth whereas in the presence of 0.1 μg/ml of doxycycline, there was modest growth suppression. Interestingly, increasing levels of doxycycline treatment led to proportional increases in growth suppression. Thus, the degree of MTC tumor cell growth inhibition is directly proportional to the amount of Notch1 protein present. Furthermore, as a control we also treated TT-vector alone cells with doxycycline at 0, 0.1,0.5 and 1μg/ml concentrations and did not see any reduction in cell growth (Fig 5C).

Notch1 inhibits growth by cell cycle arrest. It has been shown that transient activation of Notch1 in SCLC led to cell cycle arrest (22). The decrease in MTC cell growth by over expression of Notch1 may be due to decreased cell cycle transit or increased cell death. To determine the mechanism by which growth reduction occurs, we carried out Western analysis for various cell cycle regulatory proteins. As shown in Figure 6, the level of $p21^{waf1/cip1}$ is significantly and dose dependently up regulated in TT-Notch cells treated with different concentrations of doxycycline. However, there is no change in p21 level in TT-vector control cells treated with or without doxycycline. Interestingly, the level of $p27^{kip1}$ protein is reduced after Notch1 activation compared to the control. We also

analyzed the expression levels of cyclinD1 and Cdk4, which are involved in the regulation of the cell cycle. CyclinD1 levels increased in Notch1 activated cells, which supports the result of the reduction of p27 and increase of p21 proteins. However, there was no change in the level of Cdk4 protein. These results confirm the earlier report on Notch1 activation mediated cell cycle arrest in SCLC cells (22). Furthermore, we were interested in determining at which stage Notch1 activation was inducing cell cycle arrest. Phosphorylated cdc2 at position Tyr15 is associated with the regulation of cell cycle especially at G1/S-phase. Western analysis of Notch1 activated cells showed that there is an increase in the levels of phospho-cdc2^{Tyr15} with increasing concentration of doxycycline (Figure 6). Taken together, these data suggest that the growth inhibition could be due to cell cycle arrest and possibly at S-phase. This is consistent with the previously published result in SCLC that Notch1 mediated growth inhibition is due to cell cycle arrest.

Notch1 activates the ERK1/2 pathway but it is not essential for NE marker reduction. We, and others, have shown that activation of the Raf-1 pathway resulted in both a reduction in NE markers such as ASCL1 and CgA and growth of MTC-TT cells (5;10). In addition, Notch1 has been shown to activate ERK1/2 in SCLC cells (22). Since we see similar effects of NE marker reductions and growth inhibition with Notch1 activation in TT cells, we hypothesized that Notch1 associated NE marker reduction may be mediated by ERK1/2 activation. As shown in Figure 7A, activation of Notch1 led to phosphorylation of ERK1/2 in a dose dependent manner. However, when the phosphorylation of ERK1/2 was blocked by U0126, a potent inhibitor, there was no up regulation of ASCL1 and CgA in Notch1 activated cells (Figure 7B). As can be seen that the there is no change in the levels of total ERK1/2 proteins in UO treated cells (Figure 7B). This suggests that NE markers reduction is independent of ERK1/2 activation in Notch1 activated MTC cells.

Activation of Notch1 inhibits ASCL1 expression. Earlier, it has been shown that activated Notch1 degrades both endogenous and exogenous ASCL1 protein in SCLC cells (30). However, over-expression of HES-1 did not result in reduction of ASCL1 in SCLC cells (30). In contrast, over-expression of HES-1 in human pulmonary carcinoid cells significantly reduced ASCL1 protein (34). In the present study we found that Notch1 activation reduces ASCL1 protein in MTC cells. Therefore, we were interested in the mechanism of ASCL1 reduction by Notch1 activation. To determine this, we transiently transfected an ASCL1 promoter-luciferase reporter plasmid as described in methods. This plasmid has been shown to reduce luciferase activity in the presence of HES-1 expression in SCLC cells (27). Results from the luciferase reporter assay showed that the Notch1 activation resulted in a 30% decrease in luciferase activity in TT-Notch cells transfected with a plasmid containing the ASCL1 promoter (Figure 7C). This suggests that Notch1 signaling regulates ASCL1 transcription. To further confirm this, we performed Northern analysis for the status of the ASCL1 mRNA after various levels of Notch1 activation as indicated (Figure 7D). As shown in Fig.7D, the level of ASCL1 mRNA is reduced with increasing concentration of doxycycline treatment. Importantly at day 4, the level of ASCL1 mRNA is significantly reduced. Taken together, the ASCL1-promoter-Luciferase reporter experiment and Northern analysis indicated that the reduction in ASCL1 protein is predominantly due to transcriptional silencing of the ASCL1.

Effect of ASCL1 on CgA and calcitonin. Activation of either the Notch1 pathway or the Raf-1 pathway resulted in ASCL1 reduction and concomitant reduction in CgA and calcitonin, suggesting a tight correlation between ASCL1 and CgA and calcitonin levels. The finding that ASCL1 transgenic knockout mice failed to develop thyroid C cells suggests that ASCL1 is important for normal C-cell development (9). Similarly, blocking ASCL1 by antisense treatment in SCLC cells resulted in reduction of NE markers (33). However, there are no studies to date that illustrate a direct correlation between levels of ASCL1, CgA and other hormones. Our interest in the regulation of ASCL1 and NE hormones led us to explore the correlation between these two markers. To identify the relationship between ASCL1 and NE hormones, we transfected plasmid pCDNA3.0 containing HA epitope tag fused to the ASCL1 gene into TT-Notch cells. Western analysis of the cell lysates without doxycycline

treatment of over-expression of ASCL1 in TT-Notch cells resulted an overall increase in the level of CgA compared to no ASCL1 expression (compare lane 1 to lane 3 and 5; Figure 8A), suggesting that CgA expression is tightly dependent upon levels of ASCL1. However, doxycycline-treated ASCL1 over expressed TT-Notch cells (lane 4 and 6) showed reduction in CgA level compared to control cells (lane 3 and 5). Interestingly, Notch1 activated TT-Notch cell lysates containing exogenous ASCL1 protein did not show any reduction in exogenous ASCL1 as indicated by HA probe (Figure 8C). In contrast to this, Sriuranpong et al. reported that transient Notch1 expression using adenoviral vector led to a reduction in exogenous ASCL1 from the HA-ASCL1 vector (30) suggesting that Notch1 degrades ASCL1. However, this confirms our earlier results (Figure 7C and D) that ASCL1 reduction by Notch1 activation in MTC cells is predominantly at the transcriptional level. Furthermore, siRNA against ASCL1 resulted in CgA reduction (Figure 8A). As shown in Figure 8A, non-specific siRNA did not have any effect on either ASCL1 or CgA proteins. However, the decrease in the level of ASCL1 protein with increasing concentrations of ASCL1 siRNA resulted in a decrease in CgA in a dose dependent manner. Furthermore, to determine the level of calcitonin after ASCL1 depletion, we carried out a calcitonin ELISA using the ASCL1 depleted cell lysates from the siRNA treatment. As shown in Figure 8B, approximately a 50% reduction in the relative calcitonin level in ASCL1 siRNA treated group compared to both non specific siRNA and control groups. In conclusion, our results, for the first time, indicate that levels of NE hormones such as CgA and calcitonin are directly related to ASCL1 levels.

ASCL1 over expression in Notch1 activated TT cells is unable to rescue growth. Activation of the Notch1 pathway in TT cells led to a significant growth suppression and also a marked reduction in NE marker production. We have clearly shown that ASCL1 regulates hormone productions. However, it is not known whether ASCL1 is also involved in regulating the growth under Notch1 signaling activation. Therefore, to determine if the growth suppression by Notch1 activation is mediated by ASCL1, we carried out MTT cellular proliferation assay in ASCL1 over expressed TT-Notch cells with or without activation of Notch1 signaling by doxycycline treatment. Interestingly, transient expression of ASCL1 did not increase growth in Notch1 activated cells (Figure 8D). Importantly, Notch1 activated TT-Notch cells showed similar reduction in growth in the presence or absence of exogenous expression of ASCL1. Earlier, we have shown that the expression of ASCL1 protein from this plasmid is functional as indicated by the increase in CgA. Therefore, the result of this experiment indicates that the growth suppression by the activation of Notch1 signaling in TT cells is independent of ASCL1 reduction or growth is not regulated by ASCL1.

Discussion

Notch1 is a multi-functional transmembrane receptor that plays important roles in cellular differentiation, development, proliferation and survival (14;15;35). In Drosophila neural development, the most studied Notch1 signaling pathway, Notch1 maintains the neural progenitor stage and inhibits differentiation. Transgenic mice lacking the Notch1 ligand, delta-like gene-1 or the intracellular mediator RBP-Jkappa, led to an accelerated pancreatic endocrine differentiation with a specific increase in endocrine cells. As a result of this premature differentiation, the development of the pancreas is arrested due to the reduction in precursor cells. These findings clearly demonstrate that Notch1 signaling is required for the normal development of the pancreas (19). Similar to this study, HES-1 knock out mice also displayed severe pancreatic hypoplasia with an increase in endocrine cells (36). In a different study, using an achaete-scute homolog-1 (MASH1) knockout transgenic mouse model, mice died at birth due to the lack of development of thyroid C-cells (9). Taken together, these results suggest that components of the Notch1 signaling pathway tightly regulate the NE phenotype in the developing lung, thyroid, and GI tract. However, the role of Notch1 in cancer cells remains controversial yet interesting. Recent studies on Notch1 signaling in cancer biology have contributed to our understanding that Notch1 signaling can act either as a tumor suppressor (37;38) or as a tumor promoter (39), which suggests that the effects of Notch1 signaling are cellular context specific.

Transient expression of active Notch1 in small cell lung cancer, pancreatic carcinoid and prostate cancer cells inhibit cellular growth *in vitro*. Recently, we have shown that stable expression of estradiol-inducible active Notch1 (NICD) in pancreatic carcinoid, BON cells led to growth inhibition and a reduction of NE hormone production (20). However, the role of Notch1 signaling in MTC is not known. MTC derives from the calcitonin-producing thyroid C-cells and produces excess amounts of calcitonin. In addition, MTC expresses high levels of ASCL1 which seems to be limited to NE tumors (5;7;8). Various studies including MASH1 transgenic knockout mice revealed that ASCL1 plays an important role in the development of adrenal chromaffin cells and pulmonary endocrine cells (9;11-13). Several reports showed that the ASCL1 levels could be reduced by Notch1 mediated HES-1 protein activation (8;20;22;27). Despite the importance of the Notch1 signaling pathway in cell fate determination, the role of the Notch1 signaling pathway in MTC has, until now, not been described.

In this present study, we show that active Notch1 (NICD) is absent in MTC tumor cells and in human MTC tumor samples. These MTC tumors and cell lines, however, have high levels of ASCL1 and CgA. These findings are consistent with characteristics of other NE tumors. A dose-response active Notch1 induction by doxycycline led to an increase in functional Notch1 protein production, as measured by CBF-1 binding studies, resulting in activation of the Notch1 pathway. Furthermore, continuous Notch1 activation in TT-Notch cells inhibited tumor cell growth. Importantly, this growth reduction was dependent on the levels of Notch1 protein present. Our findings provide the first documentation of the role of Notch1 signaling as a tumor suppressor in MTC cells. We have also demonstrated that activation of Notch1 signaling in MTC reduces levels of calcitonin and CgA. Importantly, for the first time, we have shown in this study that ASCL1 tightly regulates CgA and calcitonin expression and possibly that ASCL1 is not involved in the regulation of growth.

However, the mechanism by which Notch1 causes the hormone reductions is not clear. Raf-1 activation in MTC cells also leads to significant reductions in CgA, calcitonin, and growth (1;5;10;40). Notch1 activates other signaling pathways such as the JAK-STAT pathway (41) or the Raf1-MEK-ERK1/2 pathway (5) that regulates NE hormone production in these tumors. However, the present study indicates that the reduction of NE hormone production is independent of ERK1/2 activation. However, we predicted that ASCL1 might regulate the expression of CgA and other hormones in MTC cells considering that ASCL1 is a basic helix-loop-helix transcription factor and exogenous expression of ASCL1 resulted in an increase in the level of CgA. Accordingly, loss of ASCL1 protein by ASCL1 siRNA transfection in MTC cells resulted in a reduction in CgA as well as calcitonin. This strongly indicates that the levels of CgA and calcitonin are regulated by ASCL1 protein. Interestingly, the fact that over expression of ASCL1 in TT-Notch cells led to an increase in CgA levels, further confirms that involvement of ASCL1 in the regulation of CgA. However, over expression of ASCL1 did not increase cellular proliferation in Notch1-activated TT cells indicating that Notch1-mediated growth suppression is independent of ASCL1 levels.

Although Notch1 signaling can act either as an oncogene or an anti-proliferator, the activation of Notch1 signaling in NE tumor cells leads to growth inhibition. Pancreatic adenocarcinoma cells have high levels of Notch1 expression and Notch1 is required for tumor growth (16;19;42). However, MTC and other NE tumor cells lack functional Notch1 (NICD) protein, and activation of Notch1 in these cells leads to a significant reduction in growth. While the growth suppressive effects of Notch1 activation have been well characterized in other tumor types, to our knowledge, this is the first report of Notch1 signaling inhibiting MTC tumor cell growth. Moreover, the findings of this study suggest that the strong inhibition of MTC cell growth by Notch1 signaling may be due to the alterations in cell cycle regulators, causing G1/S arrest. p21 is a universal inhibitor of cyclin dependent kinases and its expression is normally regulated by the p53 tumor suppressor protein. The observations of up regulation of p21, phospho-cdc2, and cyclinD1 suggest that growth inhibition of MTC cells by Notch1 expression could be due to cell cycle arrest.

In conclusion, this study illustrates that Notch1 inhibits growth and hormone production in MTC cells. Furthermore, the present study further emphasizes the critically important role of ASCL1 dependent hormone(s) regulations and ASCL1 independent growth regulation by the Notch1 signaling pathway in MTC tumors. Further research based on our findings may lead to the development of novel therapies for patients with MTC and possibly for other NE tumors based on activation of the Notch1 signaling pathway.

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Footnotes

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[1]The abbreviations used are: NICD, Notch1 intracellular Domain; NE, Neuroendocrine tumor; MTC, medullary thyroid cancer; PTC, papillary thyroid cancer; ASCL1, human achaete-scute homolog-1; ELISA, Enzyme linked immunosorbant assay; ANOVA, analysis of variance; CgA, chromogranin A; HES-1, hairy enhancer of split-1; HA, hemagglutinin; PBS, phosphate buffered saline; PA, pancreatic adenocarcinoma.

Figure legends

Figure 1. Expression patterns of Notch1 and neuroendocrine markers in various human medullary thyroid cancer (MTC) tumor samples and cell line. Western blot analysis of cleaved Notch1 protein in the human tumor samples of MTC, papillary thyroid cancer (PTC), pancreatic adenocarcinoma tumor (PA), and MTC-TT cells. Note the lack of cleaved Notch1 proteins in MTC tumors and TT cells. However Notch1 is present in non-NE tissues (PTC and PA). Interestingly, CgA and ASCL1 are present only in NE tumors and tumor cell line and are inversely related to Notch1 expression. Immunoblotting with an anti-G3PDH antibody confirms the relatively equal protein loading.

Figure 2. Doxycycline-induced Notch1 activation in MTC cells. (**A)**. TT-Notch cells treated with various concentrations of doxycycline as indicated in the figures showed increased levels of Notch1 protein. (**B)**. TT-Notch cells were transiently transfected with CBF-1 luciferase reporter constructs and then treated with various concentrations of doxycycline for 48 hours. Notch1 function was measured by the degree of CBF-1 binding utilizing a luciferase construct containing 4 CBF-1 binding sites (4xCBF1Luc) compared to background binding to a luciferase construct with mutated CBF-1 binding sites (4xmtCBF1Luc). The values represent the ratio of 4xCBF1Luc to 4xmtCBF1Luc expressed relative to TT-Notch control cells in the absence of doxycycline. Note that various concentrations of doxycycline treatment showed an increase in relative luciferase activity compared to the control (no doxycycline). Statistical analysis showed significant differences in luciferase fold induction ($p < 0.001$) in all groups.

Figure 3. Effects of Notch1 signaling on NE markers and its persistent requirement in MTC cells (**A)**. TT-Notch cells were treated with various concentrations of doxycycline, as indicated, for 2 days. Total cellular extracts were isolated and analyzed by Western blot for downstream mediators of Notch1 signaling. Increased Notch1 protein production by increasing concentration of doxycycline (Figure 1A) led to an increase in HES-1 protein. HES-6, a basic helix-loop-helix protein that binds to HES-1 and inhibits its activity, showed reduction with increasing amounts of Notch1 protein. HES-1 is a transcriptional suppressor of ASCL1 protein. TT-Notch control cells (no doxycycline) have large

amount of endogenous ASCL1. However, the ASCL1 level is reduced progressively with increasing amount of HES-1 protein. Similarly, CgA levels were reduced with increasing concentrations of doxycycline. **(B).** TT-vector (control) cells were treated with various concentrations of doxycycline, as indicated, for 2 days. Cellular extracts were analyzed for the presence of the levels of ASCL1 and CgA. Note that TT-vector cells treated with doxycycline showed no change in the levels of ASCL1 and CgA indicating that doxycycline alone does not have any effect on MTC cells. Immunoblotting with anti-G3PDH antibody confirms equal protein loading. **(C).** High levels of calcitonin are present in MTC cells in the absence of Notch1 activation [TT-Notch (C)]. However, activation of Notch1 in TT-Notch cells led to progressive decrease in relative calcitonin by 5% at 0.2, 30% at 0.5 and 47% at 1.0 µg/ml of doxycycline concentrations. **(D)**. TT-Notch cells were treated with 0.5 and 1µg/ml concentrations of doxycycline for 2 days. Then the media was removed and cells were washed with PBS and doxycycline free media was added for up to 6 days. Total cellular extracts were isolated and analyzed by western blot for the presence of downstream mediators of Notch1 signaling. Note that Notch1 (NICD) protein is present only in doxycycline treated cells indicating not only that constant doxycycline is required for the expression of NICD but also demonstrating the tight regulation of expression. Interestingly, ASCL1 expression is reduced when Notch1 is present. In contrast to this, CgA reduction is stable up to a 2day withdrawal of doxycycline but at later time points the level of CgA rebounds to levels equivalent to control cells.

Figure 4. Persistent activation of the Notch1 signaling pathway in TT-Notch cells by doxycycline treatment. TT-Notch and TT-vector cells were treated with various concentrations of doxycycline for 4 and 8 days and cellular extracts were prepared and analyzed for the activation of the Notch1 signaling pathway by Western blot. As predicted, the level of Notch1 protein is dependent on the amount of doxycycline at all time points. However, ASCL1 is barely detectable after 4 day in Notch1 activated cells. Interestingly, the NE marker CgA is reduced at day 4 and barely detectable by day 8. Immunoblotting with anti-G3PDH antibody confirms equal protein loading.

Figure 5. Notch1 signaling inhibits tumor cell proliferation. **(A)**. Growth assay by cell count. TT-vector and TT-Notch cells were treated with (Doxy) or without doxycycline (Control) at 1µg/ml for the indicated time periods. Viable cells were counted at each time point. TT-vector cells treated with or without doxycycline and TT-Notch cells in the absence of doxycycline did not have any reduction in cellular growth. Activation of Notch1 by doxycycline in TT-Notch cells reduced cell growth significantly at day 8 (58%) and day 10 (70%). **(B)**. Dose-response growth assay. TT-Notch cells were treated with various concentrations of doxycycline as indicated for 12 days and the MTT assay was carried out. At 0.1µg/ml doxycycline concentration, there was a slight growth reduction at 12 days. Interestingly there is a dramatic growth reduction of TT-Notch cells at 0.5 µg/ml and also in higher dose of doxycycline (0.8 and 1µg/ml) compared to 0.1µg/ml. At all time points the growth reductions were significant as compared to control (p <0.00001). **(C).** Control cells (TT-vector) treated with 0, 0.1, 0.5 and 1.0 µg/ml of doxycycline did not show growth reduction at 12 days. Thus, doxycycline did not have any effect on TT cell growth. At all time points there was no difference in proliferation compared to control.

Figure 6. Notch1 signaling induces cell cycle arrest. TT-vector and TT-Notch cells were treated with doxycycline at 0, 0.2, 0.5 and 1µg/ml for the indicated period of times. Total cellular extracts were isolated and analyzed by Western blot using antibodies against p21, p27, cyclinD1, Cdk4, and phospho-cdc2^{Tyr15} proteins. Tumor suppressor protein, p21, and cyclinD1, were increased with increased concentrations of doxycycline whereas p27 is reduced. Cdk4 protein level was not changed with Notch1 expression. The level of phospho-cdc2^{Tyr15} was increased compared to control.

Figure 7. Notch1 signaling activates the ERK1/2 pathway but phosphorylated ERK1/2 is not required for NE marker reduction. **A.** TT-Notch cells were treated with doxycycline at 0, 0.2, 0.5 and 1µg/ml for

the indicated period of times. Total cellular extracts were isolated and analyzed by Western blot using antibody against phopho-ERK1/2 protein to determine the activation of the Raf-1/MEK/ERK1/2 pathway. A dose dependent increase in phosphorylated ERK1/2 was observed in Notch1 activated cells. **B.** TT-Notch cells were pre-treated with 10 μM U0126 for 45 minutes and then with doxycycline at 1μg/ml for 4 days. During the entire treatment period U0126 was present in the treatment groups. Total cellular extracts were isolated and analyzed by Western blot using antibodies against phopho-ERK1/2, regular ERK1/2, ASCL1, and CgA proteins to determine if Raf-1 pathway activation is required for NE marker reduction. Doxycycline treated cells showed an increase in phosphorylated ERK1/2 whereas similar treatment in the presence of U0126 showed the absence of phosphorylated ERK1/2, indicating that the Raf-1 pathway was successfully blocked by U0126. As expected, in Notch1 activated cells there was a reduction in NE markers such as ASCL1 and CgA. Interestingly, and perhaps surprisingly, inhibition of the ERK1/2 pathway did not increase ASCL1 and CgA to normal levels. Regular ERK1/2 was used as a loading control. **C. Notch1 signaling silences ASCL1 at the transcriptional level.** To determine if Notch1/HES-1 suppresses ASCL1 at the transcription level, we transfected a luciferase reporter plasmid pGL2 containing the ASCL1 promoter DNA fragment (-7900/+37) containing 5' flanking region of ASCL1 genomic DNA including HES-1 binding site, into TT-Notch cells. Luciferase and CMV-β-galactosidase activities were measured from cell lysates of control and Notch1 activated cells. Relative fold induction of luciferase activity was calculated after normalizing to β-galactosidase activity for Notch1 activated cells in relation to control cells. Notch1 activation lead to a 30% reduction in luciferase activity compared to control cells indicating that Notch1 signaling silences ASCL1 transcription. Plasmids pGL2-control and pGL2-basic served as positive and negative controls for luciferase activity, respectively. **D.** Total mRNA isolated from the Notch1 activated cells was separated on 1% formaldehyde denaturing gel. Northern analysis using ASCL1 probe showed reduction in ASCL1 mRNA after Notch1 activation. At day 4, the level of ASCL1 mRNA is significantly reduced. G3PDH probe showed equal amount of mRNA present in all the lanes.

Figure 8. Role of ASCL1 on the regulation of hormones and growth in TT cells. A. To determine the role of ASCL1 on CgA, ASCL1-HA plasmid (1 and 5μg DNA) was transfected into TT-Notch cells, which were then treated with or without doxycycline. Cellular extracts were isolated and analyzed by Western blot for the indicated proteins. As expected, Notch1 activation reduced ASCL1 protein levels. The presence of exogenously supplied ASCL1-HA protein was identified using HA antibody. CgA levels were reduced with Notch1 activation. However, exogenous ASCL1 protein increased the level of CgA in a dose dependent manner suggesting that ASCL1 might up-regulate CgA. Image quant software was used to quantify the band intensity and numbers were given below the CgA was normalized with G3PDH. As shown in the figure, there is an increase in CgA level fromTT-Notch cells to TT-Notch cells with ASCL1 plasmid (1>1.2>1.5 lanes 1,3 and 5 respectively). **B.** Small interfering ASCL1 RNA was transfected into TT cells and the cell lysates were analyzed for the level of ASCL1 and CgA proteins to further confirm that ASCL1 regulates CgA. With increasing concentrations of ASCL1 siRNA, there was a progressive reduction in ASCL1 protein. Importantly, reduction in ASCL1 resulted in a decrease in the level of CgA. **C.** To determine if silencing of ASCL1 leads to alteration in the level of calcitonin in TT cells, a calcitonin ELISA was performed using cell lysates from the control (C), non-specific siRNA (100nM) treated (NS), and ASCL1 siRNA (100nM) treated (ASCL1) cells. The results are shown as a relative calcitonin level compared to control treatment. As can be seen, there is almost 50% reduction in calcitonin level in ASCL1 siRNA treated cells indicating that ASCL1 also regulates calcitonin levels in TT cells. **D.** To determine the role of ASCL1 on cellular proliferation of TT cells, a growth rescue experiment by MTT assay was performed as described in methods. As seen in figure D, Notch1 activated TT-Notch cells (D) and TT-Notch cells with vector showed similar level of reduction in growth. Importantly, over expression of ASCL1 in Notch1-activated TT-Notch cells did not reverse Notch1-mediated growth inhibition.

Figure 1

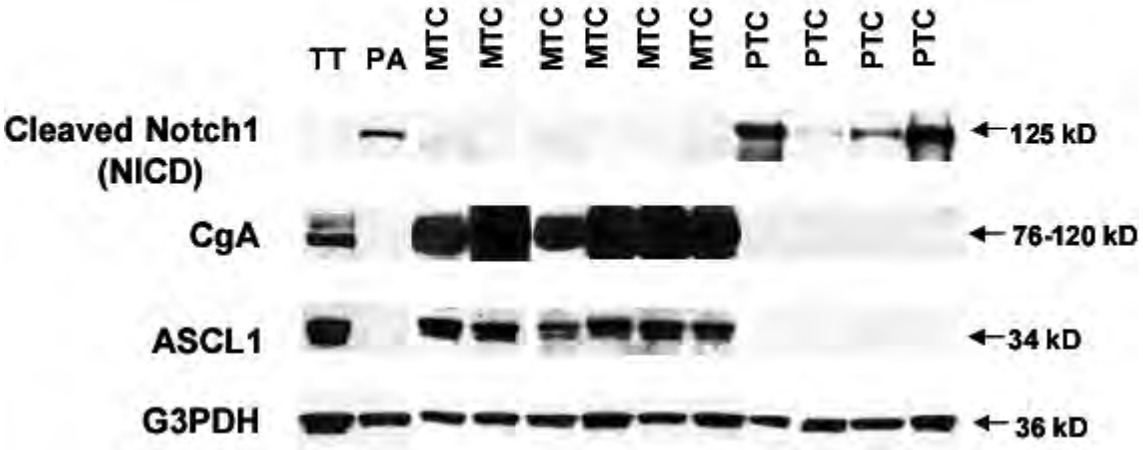

Figure 2

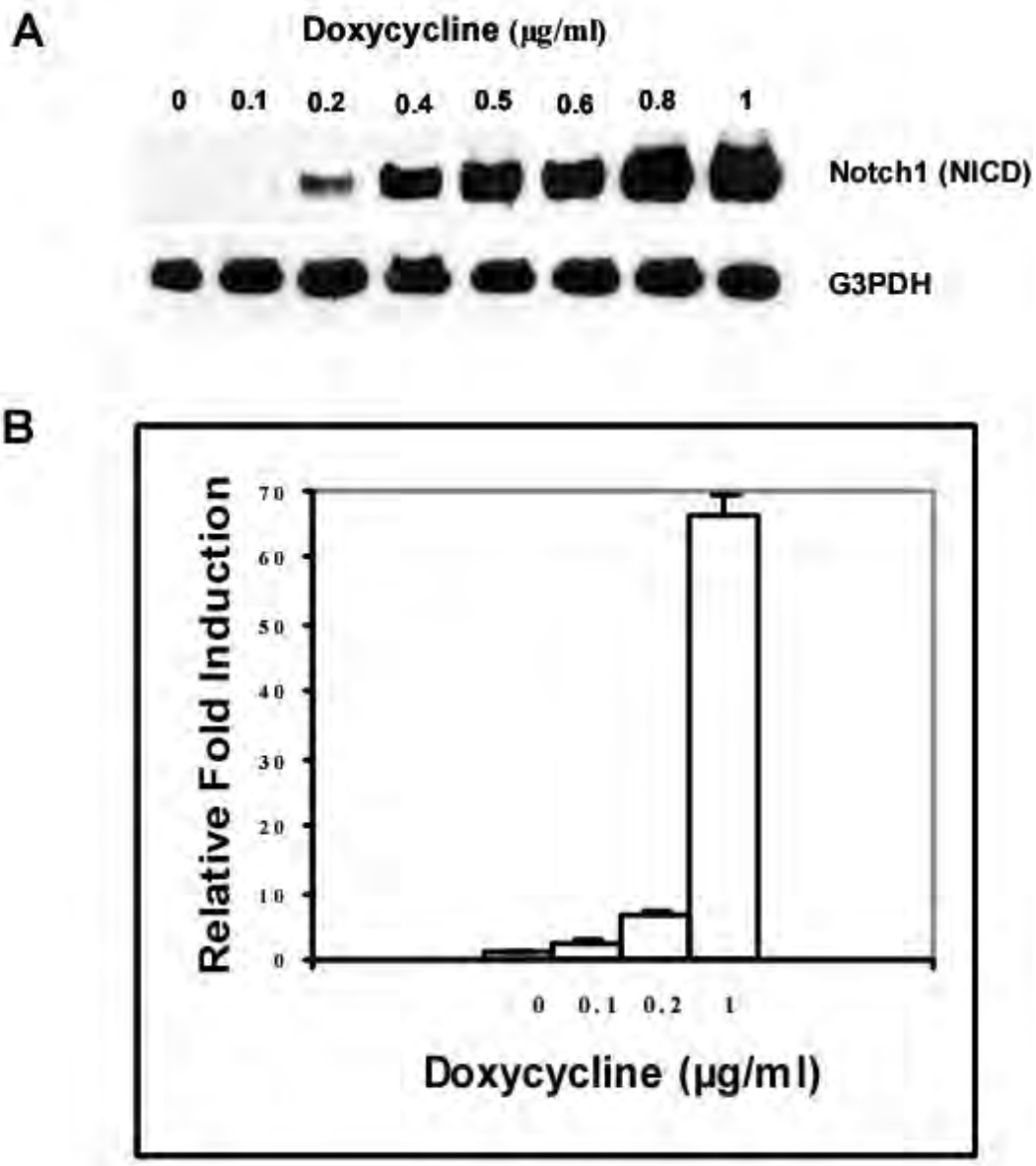

Figure 3

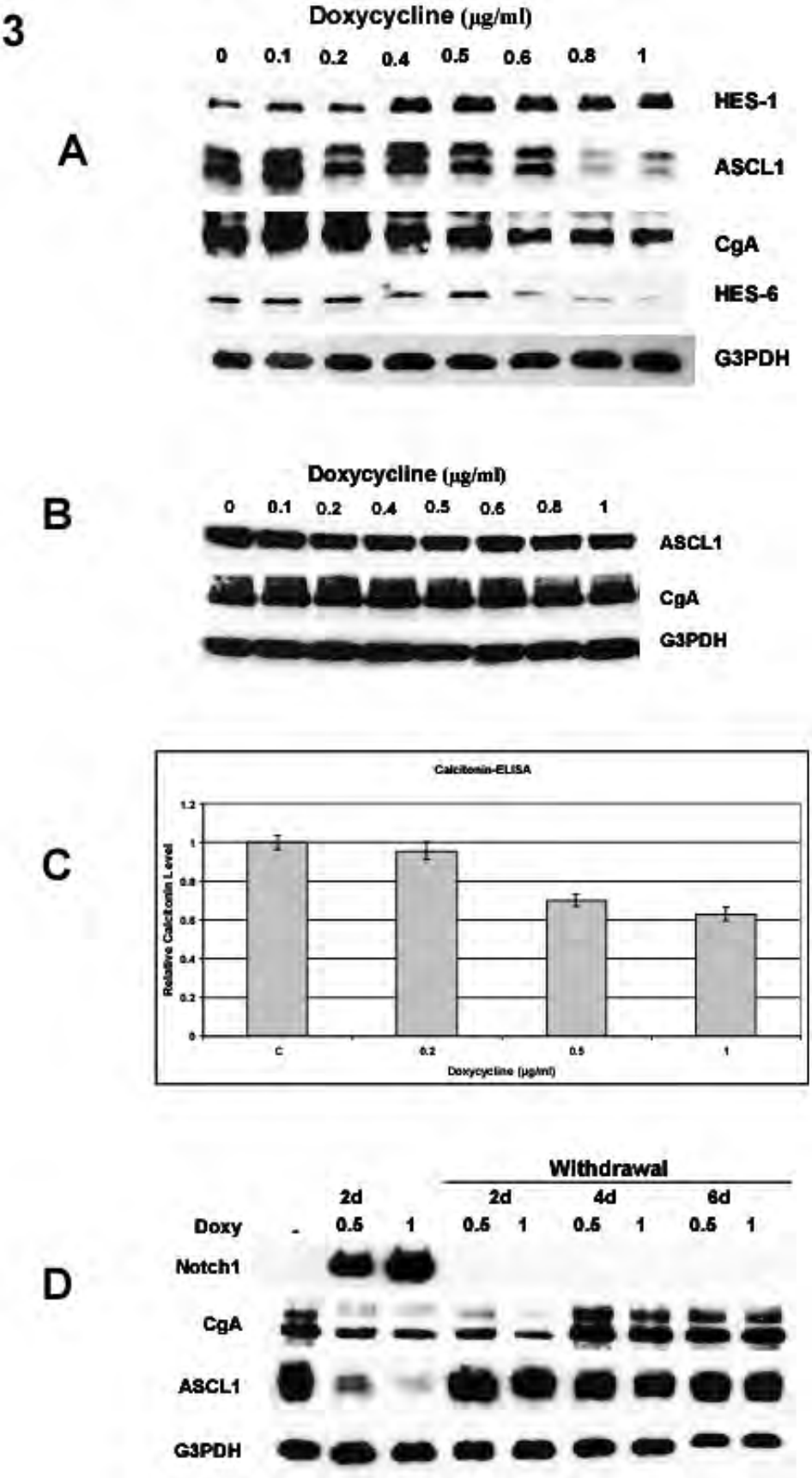

Figure 4

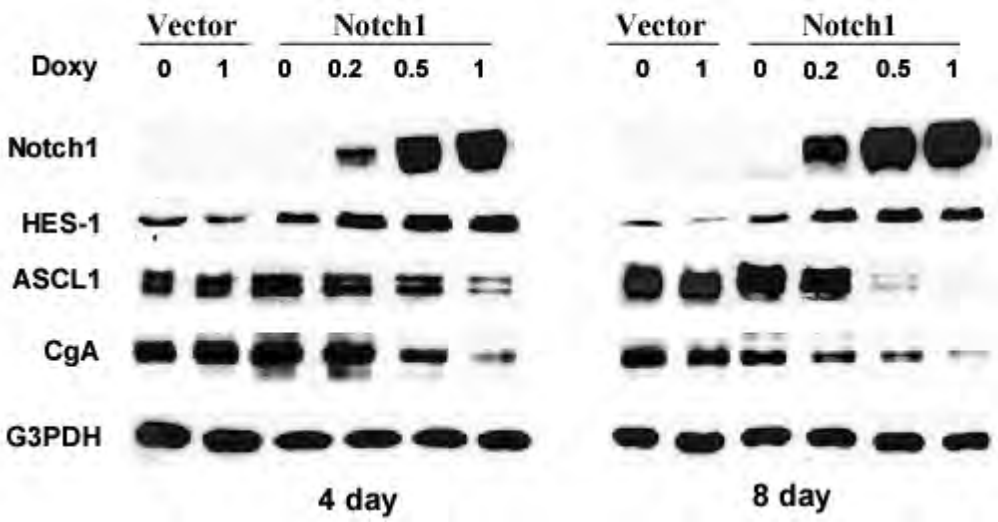

Figure 5

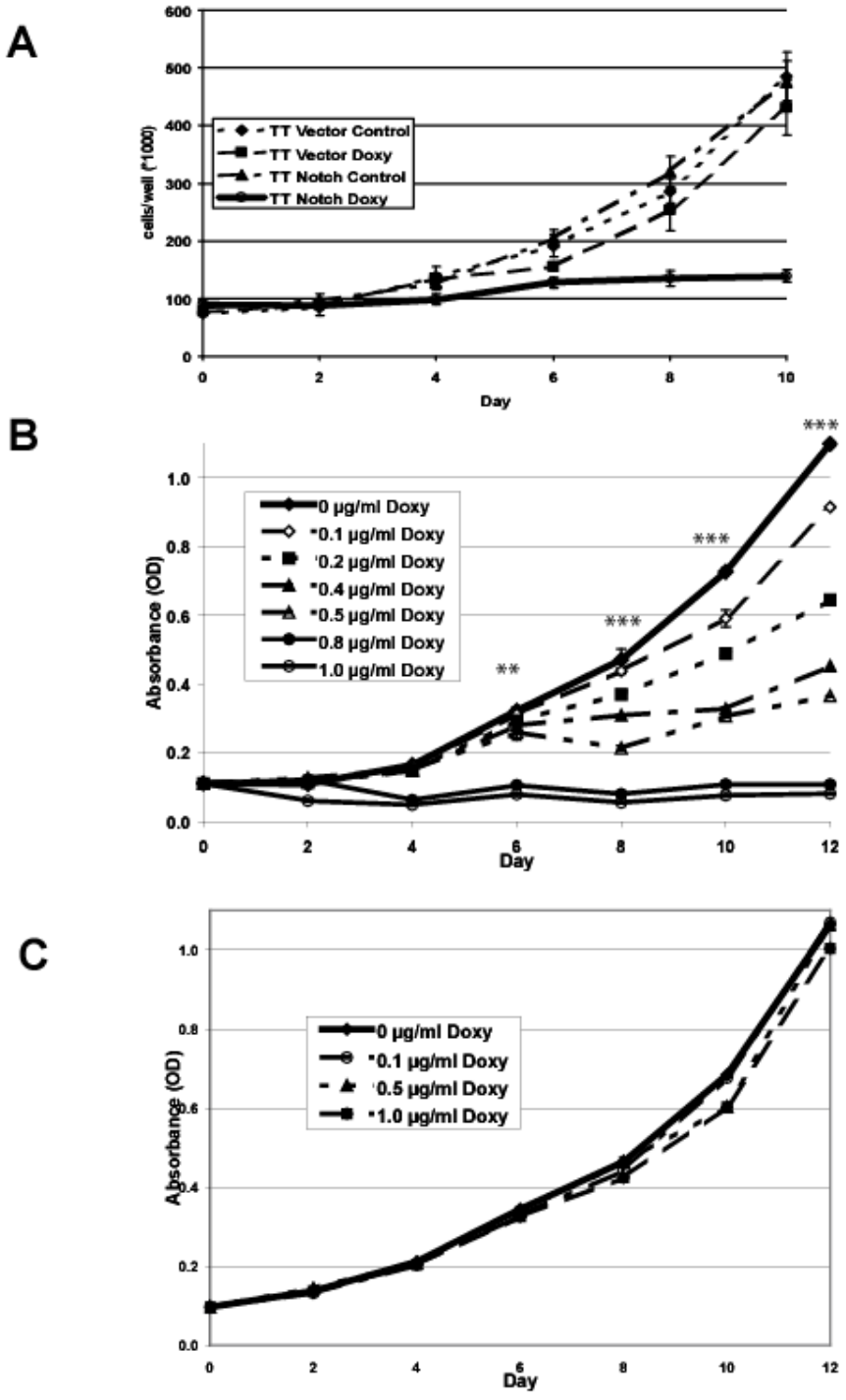

Figure 6

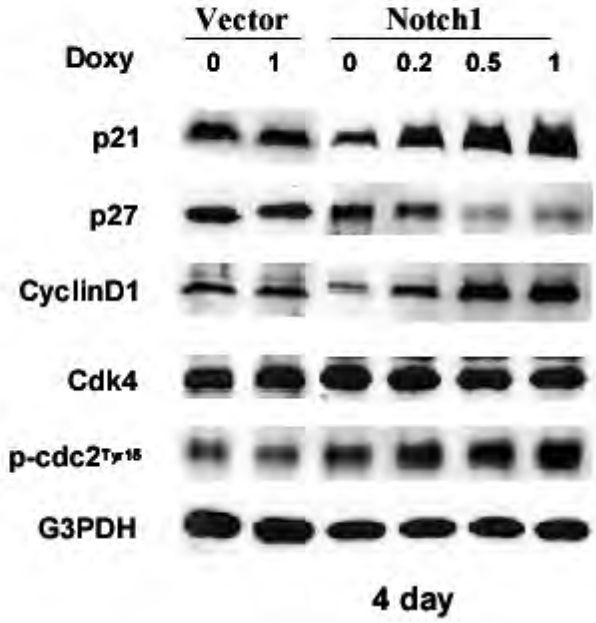

Figure 7

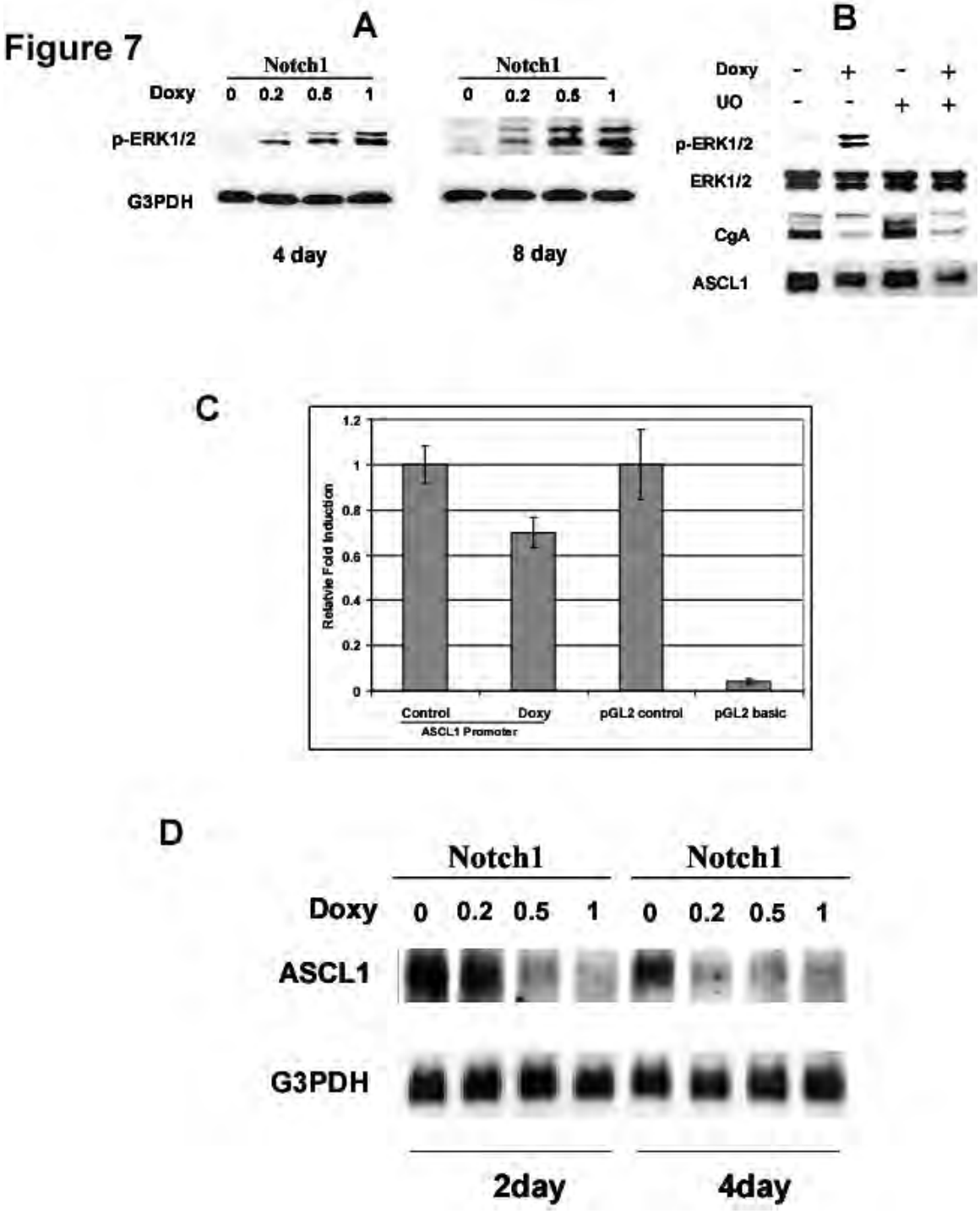

Figure 8