

Review Article

Notch in the development of thyroid C-cells and the treatment of medullary thyroid cancer

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Abstract: The Notch pathway plays an important role in the normal development of neuroendocrine cells, including the calcitonin producing C-cells of the thyroid. This effect has been elucidated to be mediated through modulation of Achaete-Scute Complex Like 1 (ASCL1), a transcription factor associated with poor prognosis in neuroendocrine cancer. Medullary thyroid cancer (MTC) is one of the neuroendocrine cancers derived from the thyroid C-cells. The Notch pathway has been shown to be inactive in MTC which may lead to altered expression of ASCL1. Artificial induction of Notch signaling in MTC cells can suppress ASCL1 expression, the cell growth as well as hormone secreting potential. Pharmacological activation of the Notch pathway also successfully suppresses the tumor growth in an animal model, which sheds light on the targeted therapy of Notch as a potential treatment for intractable MTC.

Key words: Notch, achaete-scute complex Like 1, medullary thyroid cancer

The Notch pathway has been the focus of research in a variety of malignancies since Notch was first functionally studied in human T-cell neoplasia. While classically described as an oncogene, in cancers of the diffuse neuroendocrine (NE) system, Notch has been documented as a potent tumor suppressor [1]. In addition to the role of this pathway in the malignant development of neuroendocrine tumors (NETs), the Notch pathway also act importantly in the embryological differentiation of these cell types [2, 3]. This review will focus on the role of the Notch pathway in the development of thyroid C-cells and medullary thyroid cancer (MTC), as well as the potential therapeutic exploitation of this pathway.

Overview of the Notch pathway

The Notch receptor is evolutionarily conserved from *Drosophila melanogaster*. In human cells, it has been widely accepted as a transmembrane receptor that regulates cellular survival, proliferation and differentiation [4-6]. The oncogenic role of Notch1 was first identified in hu-

man T-cell neoplasia. Later, it was shown that Notch1 is up regulated in many types of cancer, including pancreatic cancer, colon cancer, non-small cell lung cancer, breast cancer, renal cell carcinoma, glioma, and several lymphomas. Conversely, Notch1 signaling is very minimal or absent in prostate cancer and endocrine cancers such as small cell lung cancer (SCLC) and carcinoid. These apparent but paradoxical functions clearly indicate that the role of Notch1 signaling is dependent on its cellular context [1, 2].

The original Notch gene described in *D. melanogaster* encodes a 300kD single-pass transmembrane receptor [7]. While the basic structure of this molecule is conserved, four separate Notch isoforms are known to exist in humans, as opposed to just one in *D. melanogaster* [2]. Investigations into the Notch pathway, since its original identification, have shown that this full length fragment after translation is cleaved in the trans-Golgi by a furin like convertase and subsequently rejoined as a non-covalently linked heterodimer which is

Notch in regulating thyroid C-cells

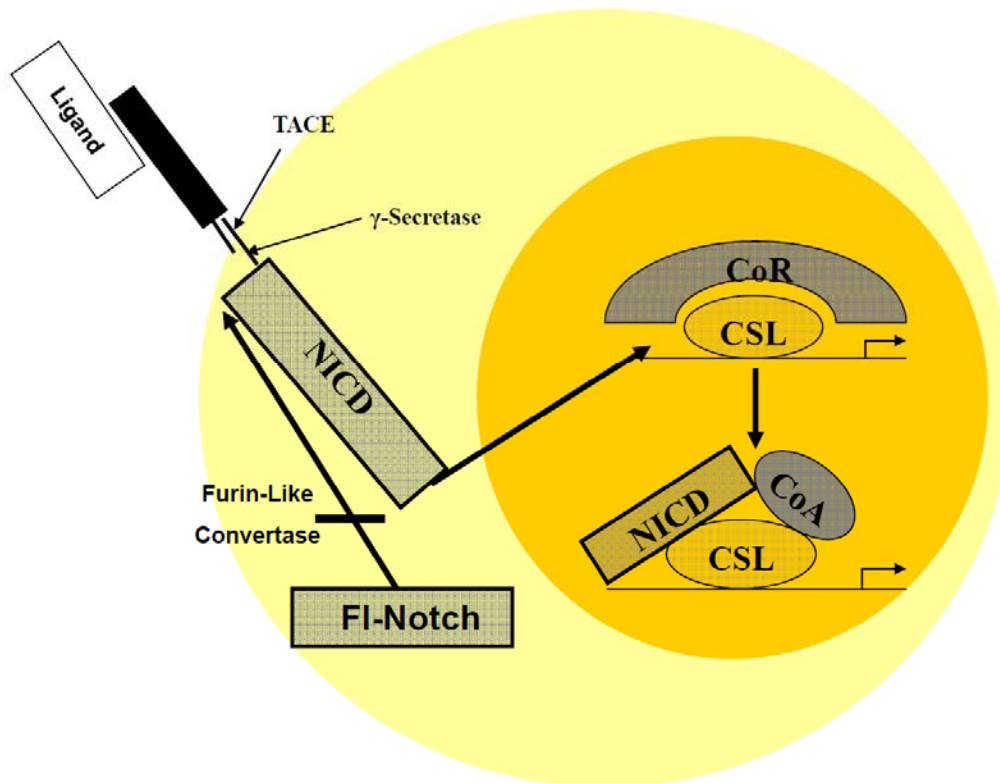


Figure 1. Full length Notch is translated and subsequently cleaved by a furin like convertase in the trans-Golgi network to create the mature, non-covalently bound, heterodimer present at the cell surface. When the extracellular portion of the heterodimeric receptor interacts with a ligand an initial proteolytic cleavage, mediated by TNF α Converting Enzyme (TACE), occurs. This initial cleavage event is followed by a second cleavage mediated by the γ -Secretase complex. These sequential cleavage events free Notch Intracellular Domain (NICD) to translocate to the nucleus. NICD is capable of removing the co-repressor complex (CoR) from the Centromere binding factor-1 – suppressor of Hairless – Lag (CSL) complex, replacing it with a co-Activating complex (CoA). Thus NICD turns the CSL element from a repressor to an activator. Downstream targets of this pathway include Hairy Enhancer of Split 1 and Achaete Scute Complex-Like 1. NICD is subsequently phosphorylated, ubiquitinated and degraded, reverting CSL back to a repressing element.

composed of both extra-cellular and intra-cellular domain. This heterodimer is predominately present at the cell surface though evidence exists for full length (i.e. non-cleaved) functional Notch receptors at the cell surface [7, 8]. A number of other processes including, glycosylation by the fringe family (lunatic, maniac and radical), interaction with the presenilin family and the metalloprotease, kuzbanian, have been described to regulate the expression of Notch receptors [6, 7].

The structure of the Notch transmembrane receptors includes an extracellular region with Epidermal Growth Factor-Like (EGF) repeats and cysteine rich Notch/Lin-12 (LN) repeats. The EGF sequences are important for ligand binding, while the LN repeats block signaling in the absence of a ligand. The cytoplasmic fragment contains a Regulation of Amino-Acid (RAM) domain, six ankryin repeats, two nuclear localization signals, a transcriptional transactivation domain and a proline-glutamine-serine-

threonine (PEST) rich sequence. The variation among Notch isoforms, from this basic structure, is primarily in the extracellular regions [2]. Both Notch 1 and 2 have 36 EGF repeats while 34 and 29 repeats are present in Notch 3 and 4 respectively. The cytoplasmic transactivation domain present in Notch 1 and 2 is absent in Notch 3 and 4 [2].

Notch pathway activation is triggered by the binding of the Notch receptor to one of a variety of ligands. In human, five ligands have been documented namely Dll-1,-3,-4 and jagged-1,-2. Using atomic force microscopy, it has been demonstrated that the interaction between the Notch ligands and the Notch receptor is exceptionally strong, lending support to the so called "lift-and-cut" hypothesis of Notch activation [8]. In this proposed mechanism, binding of a Notch ligand on an adjacent cell actually deforms the receptor and "lifts" the Notch receptor, allowing the first of two proteolytic events, namely the "cut," to occur. This first cleavage event is mediated by TNF α converting enzyme (TACE), a member of the disintegrin and metalloprotease (ADAM) family [6, 8]. This first cleavage removes the extracellular domain of the Notch heterodimer, leaving the membrane bound intracellular portion. It is within this transmembrane section that the next cleavage step occurs during which that Notch intracellular domain (NICD) is released mediated by the γ -secretase [2, 6-9]. It has been demonstrated that the γ -secretase mediated cleavage event can occur not only at the cell surface but also within trafficking endosomes, suggesting that endocytosis may play a role in the efficient activation of the Notch pathway. Additionally, subtle variations in the site of γ -secretase complex cleavage may impact the duration of Notch pathway signaling in the cellular environment [6, 8, 10].

After the final cleavage by the γ -secretase complex, NICD is free to translocate to the nucleus and interact with targets, namely a transactivation complex known as CSL [(Centromere binding factor-1) – Suppressor of Hairless – Lag]. In the absence of NICD, the CSL complex represses transcription through a co-repressor complex containing a histone deacetylase [6, 8]. NICD interacts with this complex, displaces the co-repressor and forms the basis of a transcriptional activation complex that includes Mastermind, p300 histone acetyltransferase, and others. Thus NICD converts CSL from a transcrip-

tional repressor to a transcriptional activator [6, 8] **Figure 1**. In order to control aberrant signaling, NICD is rapidly phosphorylated, ubiquitinated and degraded [6]. This pathway has been described as the canonical Notch signaling pathway, to delineate it from a number of other Notch pathway interactions that have been described. We will limit our discussion to this pathway as it has been the most well characterized in medullary thyroid cancer [8].

A wide variety of Notch downstream targets have been identified, though only a limited number appear to be biologically significant in any given cell type at any given time. The nature of the restriction of targets is not currently well understood [6]. Among the better characterized targets in neuroendocrine cells is Hairy Enhancer of Split-1 (HES-1). This transcription factor subsequently controls the expression of a number of target genes, including Achaete Scute Complex-Like 1 (ASCL1) [3, 7, 8]. In addition to transcriptional control of ASCL1, Notch can mediate the direct proteasomal degradation of ASCL1 and HES-1 can inhibit the ability of ASCL1 to interact with DNA [11-15]. Thus the Notch pathway is an important regulator of ASCL1 expression and activity, the importance of this pathway in the normal and malignant development of thyroid C-cells, a kind of neuroendocrine cells, will be discussed shortly.

Normal development of thyroid C-cells

MTC is a NET derived from the calcitonin secreting C-cells of the thyroid gland. The C-cells share a common Amine Precursor Uptake and Decarboxylation (APUD) phenotype with NE cells in the bronchopulmonary and gastrointestinal systems as well as the adrenal chromaffin cells [3]. Studies on the embryological development of C-cells have shown that the basic Helix-Loop-Helix transcription factor, ASCL1, is highly expressed in the developing C-cells and is lost by the time these cells reach maturity [16]. A knockout ASCL1^{-/-} mouse was shown to have vastly reduced numbers of C-cells determined by staining for serotonin and calcitonin. Control adult C-cells, which did not express ASCL1, maintained their expression of serotonin and calcitonin. This suggests that ASCL1 is important for the growth and differentiation of C-cells, not necessarily the production of these hormones [16]. When the role of ASCL1 in the development of C-cells was further investigated, it turned out as

Notch in regulating thyroid C-cells

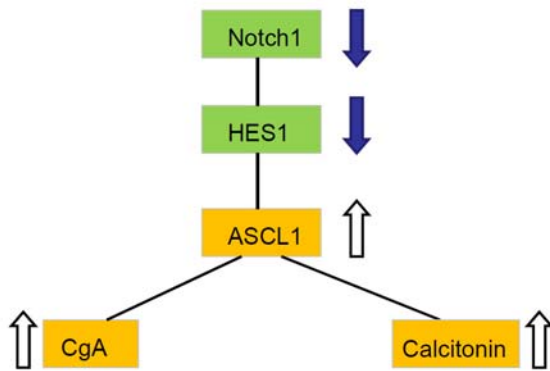


Figure 2. Aberrant Notch signaling in medullary thyroid cancer. Notch1 expression is down-regulated in medullary thyroid cancer, which results in down-regulation of HES1 and up-regulation of ASCL1. The CgA and calcitonin levels are both up-regulated during ASCL1 overexpression.

thought that this transcription factor supports the growth and survival of embryologic precursors by inhibiting apoptosis [17]. The essential finding, that ASCL1 is critical in the normal development of C-cells, has been replicated in other NE and neural cell types, suggesting that development of the neuroendocrine phenotype is dependent upon ASCL1 [3, 18-20].

The understanding for the molecular mechanisms controlling the expression of ASCL1 in developing NE cells has been extended through study of developing pulmonary NE cells. Knockout of ASCL1 in the pulmonary epithelium reduces the number of NE cells, though the overall lung volume and number of Clara cells remains unchanged. This suggests that the observed changes in the pulmonary epithelium are not due to global hypoproliferation [19, 21]. It has been observed that HES-1 is broadly expressed in the developing pulmonary epithelium and that knockout of HES-1 results in the disordered growth of NE and non-NE epithelial cells. There is broad expression of ASCL1 in these HES-1^{-/-} mice, and a resulting 10 fold increase in NE cells with a concurrent decrease in the number of Clara cells and the overall lung mass [21]. Similarly, an increase in the number of gastrointestinal NE cells was seen in the HES-1 knockout mice [22]. Knockout of either Delta-Like Ligand 1 (Dll1), a Notch ligand, or RBP-jk, a component of the CSL complex, resulted in precocious NE differentiation in the developing pancreas [23].

These data, when taken in total, point to a critical role for ASCL1 in the development of NE tissue, including thyroid C-cells, and an important role for the Notch pathway in the determination of cell fate. It may be concluded that Notch signaling in the developing embryo is important in the NE vs. non-NE fate decision from a pool of common precursors. In this model, Notch expression shunts developing cells towards a non-NE fate, while absence of Notch signaling, and subsequently increased expression of ASCL1, drives precursors towards a NE fate [3].

The development of medullary thyroid cancer

NETs classically secrete a variety of bioactive hormones. As one special type of NETs, MTC is particularly known for over-secretion of calcitonin, chromogranin A (CgA), and carcinoembryologic antigen (CEA) [3, 24-27]. Derived from the C-cells of the thyroid, MTC accounts for just 3-5% of all thyroid cancer but up to 14% of thyroid cancer deaths [28, 29]. In addition, the 5-year survival for MTC is 83% which is worse than either papillary or follicular thyroid cancer (90-94%) [30, 31]. In addition to the morbidity associated with excessive hormone secretion and the increased mortality, the natural history of MTC is notable for early metastasis [32]. Thus, these facts make MTC a challenging malignancy to treat.

Sporadic MTC, as opposed to familial one, accounts for approximately 75% of all the cases. There are limited therapeutic options for these patients. Surgery is not often curative. Furthermore, standard chemotherapy and radiation have never shown a survival advantage [32-37]. A novel treatment strategy thus is greatly needed and the Notch pathway has been shown to be a potentially fruitful pathway for the treatment of unresectable MTC.

Targeting Notch as the treatment for medullary thyroid cancer

Recently, Kunnimalaiyaan et al. have shown that ASCL1 and CgA are highly expressed in this MTC by evaluating both human MTC tumor samples and the MTC cell line. In this study, it revealed that not only could knockdown of ASCL1 mRNA reduce the protein levels of ASCL1 but also the levels of CgA and the amount of calcitonin produced. Conversely, overexpression of

Notch in regulating thyroid C-cells

ASCL1 resulted in the increased expression of CgA [38]. The role of ASCL1 in the malignant development of NETs was extended with an overexpression study in murine pulmonary epithelium. It was found that overexpression of ASCL1, along with SVT Large T antigen, to overcome the G1/S checkpoint, was sufficient to induce a malignancy with NE features [39]. These data suggest that ASCL1 has an important role in regulating the malignant phenotype of NETs in general and MTC in particular.

Given the inhibitory role of the Notch on ASCL1 in developing C-cells, the role of Notch1 signaling in MTC was then investigated. A lack of full length and active Notch1 protein was observed in human MTC samples and the MTC cell line at baseline. Stable expression of NICD in MTC cell line resulted in the increase in both NICD and HES-1 proteins. Along with this activation of the Notch1 pathway, a decrease in the levels of ASCL1 and calcitonin was noted as well as a progressive inhibition of growth through the induction of cell cycle arrest [38]. These data indicate that the Notch1 signaling pathway is intact in MTC cells and that induction of Notch1 could inhibit the growth of MTC while simultaneously suppress NE hormone production. The latter is more clinically essential since activation of the Notch1 pathway seems promising in treating the debilitating symptoms associated with the neuroendocrine malignancies.

Stockhausen et al. described the induction of Notch1 signaling in neuroblastoma, a pediatric NET, with Valproic Acid (VPA), a branched chain fatty acid that has long been in clinical use for neuropsychiatric disorders [40]. VPA is known as a histone deacetylase inhibitor (HDACi). Other HDACi compounds include suberoyl bis-hydroxamic acid (SBHA) and Trichostatin A (TsA). While VPA has a long clinical record in humans, SBHA and TsA are still in pre-clinical investigation.

Treatment of MTC cells *in vitro* with the HDACi compounds, VPA and SBHA, induced the expression of full length Notch1 and NICD. This is highly suggestive of Notch1 signaling pathway induction by HDACi [41, 42]. The similar finding was further confirmed in carcinoid cells, a related NET. Greenblatt et al. also showed that this induction resulted in increased activity at the CSL enhancer site. This elucidates that the VPA mediated induction of Notch1 results in

physiologically relevant Notch signaling [43]. Thus, these data clearly suggest that the HDACi compounds can activate physiologically relevant levels of Notch1 protein and then trigger the downstream targets.

Inducing Notch1 signaling with VPA, TsA or SBHA additionally resulted in the progressive inhibition of MTC cell growth. In contrast to the cell cycle arrest induced in the MTC cells with Notch stable expression, the HDACi compounds are able to induce apoptosis in MTC, as evidence by the cleavage of Poly (ADP) ribose polymerase (PARP) which is a marker of the final product in common apoptotic pathway [41, 42]. Ning et al. studied effect of HDACi compounds on MTC using a subcutaneous xenograft nude mouse model and found that MTC tumor growth was significantly suppressed in the treatment group. The similar finding was reported in another study using a carcinoid xenograft model [43, 44]. These data suggest that the induction of Notch1 signaling observed *in vitro* can be replicated *in vivo*.

Treatment with HDACi compounds resulted in the inhibition of ASCL1 and CgA protein levels just like what has been observed in the Notch stable overexpression system. These results are consistent with the known role of Notch in the suppression of ASCL1. They also support ASCL1 as a regulator of CgA in MTC and the ability of ASCL1 to suppress apoptosis in the developing C-cell [2, 17, 38]. Except for the reduction in CgA, the level of calcitonin was suppressed in MTC cells after treatment with VPA [41]. Abrogation of the Notch1 with specific siRNA reversed the effects of the HDACi compounds on ASCL1, CgA and apoptosis, suggesting a critical role for the Notch1 pathway in HDACi treatments as opposed to non-specific drug effects [41, 42]. It was observed from subcutaneous xenograft tumors that treatment with HDACi compounds resulted in the reduction of ASCL1 protein levels, suggesting a similar mechanism *in vivo* as was observed *in vitro* [44-46]. These data suggest that the HDACi compounds are able to induce Notch1 signaling *in vitro* and *in vivo*. In addition, the drug effects on cellular proliferation and NE hormone secretion are mediated through the Notch1 pathway.

Based on the proven efficacy of activating the Notch1 pathway with HDACi compounds together with the drug safety of VPA in humans, a

Phase II clinical trial is in development. It will provide valuable information for treating the MTC together with other unresectable NE tumors.

Summary

MTC is a special variety of neuroendocrine tumors derived from thyroid C-cells. Neuroendocrine cells including C-cells share a common APUD phenotype and are phenotypically marked by the expression of various bioactive hormones in the adult tissue and the expression of ASCL1 in the developing tissue. In the development of NE cells, the Notch / HES-1 / ASCL1 pathway plays a critical regulatory role as Notch pathway activation may be able to shunt precursor cells away from a NE fate.

In addition to being an important mediator of the differentiation and maturation of NE tissue, ASCL1 is highly expressed in MTC and a number of other NE malignancies. Over-expression of ASCL1 along with SVT large-T antigen can induce a malignancy with NE features, suggesting a central role for ASCL1 in NE oncogenesis. The Notch pathway is capable of suppressing the proliferation of MTC cells as well as reducing the amount of ASCL1 expressed by these cells. This finding can be replicated both *in vitro* and *in vivo* using pharmacologic activators of the Notch1 pathway.

Future directions will include the mechanism of Notch silencing in MTC, the role of additional Notch isoforms as well as the mechanism of pharmacological Notch1 induction. Additionally, a clinical trial is in development with VPA, the first pharmacologic activator of Notch1 to be examined in a clinical setting.

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Notch in regulating thyroid C-cells

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