ENDO 2017 Annual Meeting, 2017.

Notch Pathway Inhibition Decreases Pituitary Tumor Growth and Increases Tumor Suppressor Gene Expression in GH3 Xenografted Nude/Nude Mice.

Zubeldia Brenner Lautaro, Santiago Rodriguez-Segui, Ana Maria Ornstein, Perrone Sofia, Freya Mertens, Hugo E J Vankelecom, Carolina Cristina y Becú Villalobos Damasia.

SAT-497

Notch Pathway Inhibition Decreases Pituitary Tumor Growth and Increases Tumor Suppressor Gene Expression in GH3 Xenografted Nude/Nude Mice.

Lautaro Zubeldia-Brenner[1], Santiago Rodriguez-Segui, PhD[2], Ana Maria Ornstein, Med Vet[1], Sofia Perrone, Msc[3], Freya Mertens[4], Hugo E J Vankelecom, PhD[4], S Carolina Cristina[3] and Damasia Becu-Villalobos, PhD[1],

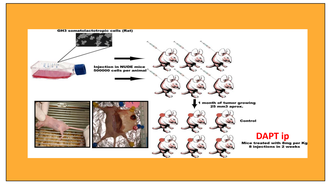
(1)Laboratory of Pituitary Regulation, Instituto de Biologia y Medicina Experimental CONICET, Buenos Aires, Argentina, (2)IFIBYME-UBA-CONICET, Buenos, (3)CIBA universidad del Noroeste de la Provincia de Buenos Aires, CITNOBA (UNNOBA-CONICET), Prov Buenos Aires, Argentina, (4)Department of Development and Regeneration, University of Leuven (KU Leuven), Leuven, Belgium. dbecu@dna.uba.ar

No conflicts of interest to disclose

Fundación René Barón

Introduction

Notch signaling pathway is involved in a wide group of processes including tumorigenesis and stem cell self-renovation. Nevertheless, its role in pituitary tumor generation has not been systematically addressed. Our principal goal was to evaluate if the pharmacological inhibition of Notch pathways alters somatolactotropic tumor growth, hormone secretion, and suppressor gene expression.



Methods

In vivo: 700000 GH3 somatolactotropic cells were injected subcutaneously in the flank of Nude/Nude mice; after 21 days of growth the tumors were treated ip with DAPT, a gamma secretase inhibitor which interferes with Notch activation (8 mg/kg of body weight, every three days). After three weeks of treatment the tumors were extracted, measured and frozen for protein and gene expression.
In vitro: 250000 GH3 rat somatolactotropic cells were cultured in adhesion in DMEM / F12 medium, supplemented by 10% fetal bovine serum, 1% glutamine and 1% penicillin / streptomycin, pH 7.3, and maintained at 37 °C and 5% CO2. After incubation in serum-free medium for 18–24 h cells were treated with DAPT (1, 5 or 10 uM, R&D Systems, Minneapolis, MN, USA) or vehicle. Aliquots of supernatant were collected for GH and prolactin measurements at 24 and 48 hours. To analyze gene and protein expression cells were detached and dissociated using trypsin (0.05%) with EDTA (0.02%; Life Technologies); MTS and scratch assay were also performed in additional cultures.

1) DAPT decreased GH3 xenograft tumor volume and hormone secretion

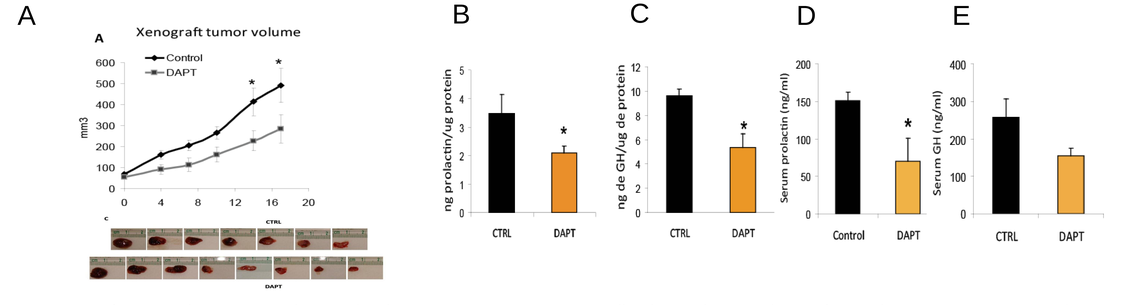


Figure 1: . A) Tumor volumes in animals treated with DAPT were in average 42% smaller than in the control group (tumoral mass + SE: 490 + 80,7 and 284+ 67,5 mm2, control and DAPT, n=12 y n=11 respectively). B-C) Moreover, the intratumoral prolactin and GH contents were significantly diminished, as were serum prolactin but not GH levels D-E) at the end of the treatment (n= 10-12). * $P \leq 0.05$, DAPT vs. control

2) DAPT treatment decreased Notch pathway components in GH3 xenografts

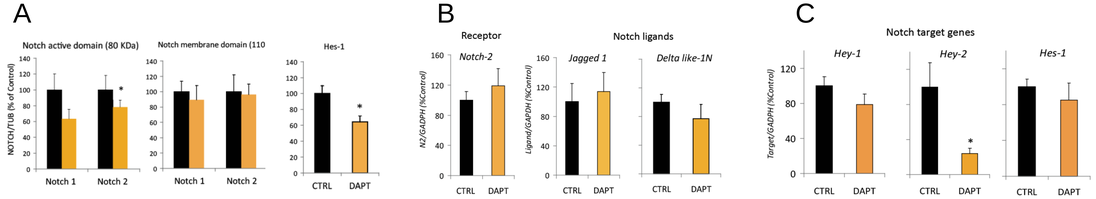
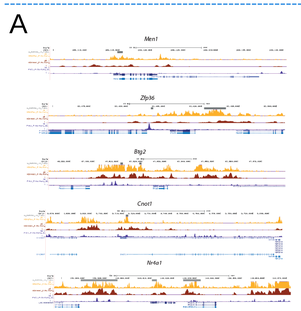


Figure 2: DAPT treatment decreased Notch 2 intracellular domain and the target gene Hes-1. A) The N2ICD (Notch intracellular active domain) product of cleavage of the receptor was significantly diminished in treated compared to non treated tumors, as was the transcription factor Hes-1 (a downstream effector of Notch (Western Blot). B) By Realtime qPCR no differences were found in Notch2, or Notch ligands mRNA levels. C) Of the canonical target genes mRNA expression of *Hey 2* was decreased by DAPT treatment. (n= 10-12). * $P \leq 0.05$, DAPT vs. control

3) Combined epigenomic and transcriptomic analysis in search for putative Notch targets. Two suppressors were increased by DAPT treatment



Notch Signaling	gene_id	gene	value Control	value PRL-GH Adenoma	log2(fold_change)	q_value	# of Active Regulatory Regions in GC cells,putatively bound by Notch1
Receptor	XLOC_023761	**NOTCH1**	9,2	1,0	-3,3	0,0012	0
Receptor	XLOC_001804	**NOTCH2**	6,4	0,4	-3,9	0,0012	2
Receptor	XLOC_011668	**NOTCH3**	14,0	6,0	-1,2	0,0391	0
Ligand	XLOC_019693	**DLL1**	7,2	1,2	-2,6	0,0081	0
Ligand	XLOC_011106	**DLL3**	3,3	2,8	-0,2	0,8830	0
Ligand	XLOC_007287	**DLL4**	6,2	2,2	-1,5	0,0697	0
Ligand	XLOC_014077	**JAG1**	8,2	5,4	-0,6	0,4125	0
Ligand	XLOC_007178	**JAG2**	43,5	8,8	-2,3	0,0012	0
Target	XLOC_018951	**HEY2**	4,8	0,6	-3,0	0,0187	0
Target	XLOC_015808	**HES1**	60,5	8,4	-2,8	0,0013	
Target	XLOC_001507	**HEYL**	16,4	4,6	-1,8	0,0053	0
Target suppressor	XLOC_001013	**BTG2**	258,3	10,3	-4,6	0,0012	3
Target suppressor	XLOC_008821	**CNOT1**	26,8	21,6	-0,3	0,7055	2
Target suppressor	XLOC_004992	**NR4A1**	1075,3	17,5	-5,9	0,0480	4
Target suppressor	XLOC_011102	**ZFP36**	249,4	34,0	-2,9	0,0567	1
Target suppressor	XLOC_004434	**MEN1**	22,5	18,7	-0,3	0,7073	2

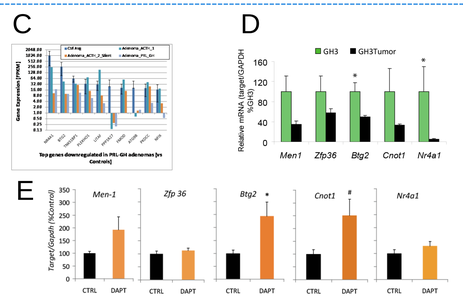


Figure 3: Finding putative Notch targets at the genomewide level: Epigenomics Combinations of histone modifications define active and inactive regions of the genome and Influence gene expression. In particular H3K4me1 + H3K27ac mark active regulatory regions of the genome (preferentially enriched in enhancers). We identified Notch targets using active regulatory regions (yellow and brown) in GC rat cells that overlap with Notch1 binding sites (grey boxes). As Notch1 ChIP-seq data was not available in GC cells we used Notch1 bound regions (T-ALL T-cell acute lymphoblastic leukemia) lifted over to the rat genome. Pit1 added additional information of tissue selective regulatory regions (blue curves). Five genes were selected using this bioinformatic approach

Figure 3: B-C) Differentially expressed genes in tumoral vs normal Human pituitary: Transcriptomics. RNAseq analysis. Samples used: 6 control samples (normal human pituitary, datasets obtained upon authorization from the restricted access dbGAP database); 3 pituitary tumor samples (ACTH, ACTH-silent, PRL-GH); Differentially expressed genes were defined according to tophat+cufflinks. In Table differences between Control and the PRL-GH adenoma are shown. In red, significant differences. Three of the 5 genes selected by epigenomic approach were downregulated in adenoma samples (*Btg2, Nr4a1, Zfp36*)

Figure 3: DAPT treatment increased the tumor suppressors *Btg2* and *Cnot1*. D) Comparative mRNA levels of *Men1, Zfp36, Btg2, Cnot1 and Nr4a1* in GH3 cells, and in xenografts resulting from GH3 inoculation (GH3 Tumor) .* P= 0.020. N 3 and 3. E) Effect of DAPT treatment on mRNA levels of Notch targets in excised tumors at the end of the treatment. * P≤ 0.01, and # P = 0.06; N 7 and 8, control and DAPT respectively.

4) DAPT decreases GH3 cell proliferation, migration and hormone secretion *in vitro*

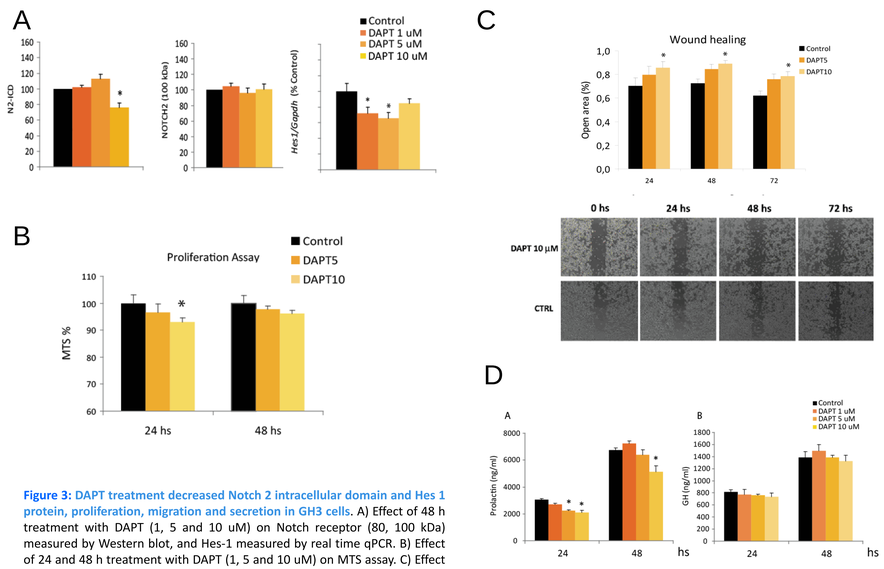


Figure 3: DAPT treatment decreased Notch 2 intracellular domain and Hes 1 protein, proliferation, migration and secretion in GH3 cells. A) Effect of 48 h treatment with DAPT (1, 5 and 10 uM) on Notch receptor (80, 100 Kda) measured by Western blot, and Hes-1 measured by real time qPCR. B) Effect of 24 and 48 h treatment with DAPT (1, 5 and 10 uM) on MTS assay. C) Effect of DAPT treatment on wound healing (scratch assay) measured as % open area. D) Effect of DAPT on GH and prolactin secretion. For all assays * P≤ 0.05 vs. control group, N= 4 independent cultures, of duplicate samples.

Conclusions:.

Our results provide strong evidence of a key role of the Notch pathway in GH3 somatolactotropic tumor proliferation in vitro and in vivo. DAPT inactivates secretase and therefore prevents the liberation of active Notch receptors. DAPT treatment decreased tumor volume in GH· xenografts, hormone secretion and Notch 2 and Hes1 expression. Furthermore, we demonstrate that this action is at least in part mediated by Notch system within GH· cells, as inferred from in vitro assays.

Furthermore, using epigenomics and transcriptomics analysis we detected serveral downstream targets of Notch, and revealed that two suprressors (Btg2 and Cnot1) were upregulated by DAPT treatment in vivo.

Therefore targeting the Notch system, and specific downstream effectors may emerge as possible additional therapies in resistant prolactinomas.